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Evaluating Ambulatory Drug Information Services through Physician's Perspective: A Mixed Method Study for Quality Improvement

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ABSTRACT

Aim/Background: Drug-Information (DI) Services are essential in ambulatory care, as it aid patient treatment decisions. In a collaborative care environment, physicians are integral members of the care team, contribute invaluable insights drawn from their first-hand experience with medication management and understanding of patient requirements. Their perspectives help to identify gaps and ensures evolving clinical practice needs. By leveraging their expertise, DI services can be improved to better support collaborative teams, resulting in more efficient care processes. **Materials and Methods:** Drug-information services delivered by pharmacist at ambulatory setting was evaluated on monthly-basis for period of 6-months. The physicians evaluated this service with specially-designed evaluation tool, which had eight-items with 5-point likert scale for response. Overall experience rating was recorded on numerical-scale (1-10). The responses of each items were evaluated quantitatively. Further, qualitative inputs was obtained in tool was subjected for manual thematic analysis. **Results:** When the Drug-information services was evaluated from a physician's perspective, it was found that there is a need for effective communication and sharing. As its related item received an average score of 4.23 on a five-point scale. The overall average rating was 9.38. Additionally, thematic analysis indicates that the Drug information services delivered by pharmacists were perceived positively and express satisfaction. They also suggested to expand these services across all Outpatient settings. **Conclusion:** Ambulatory pharmacist-led Drug information services had created positive image on all stakeholders. Still, there is room for improvement in disseminating the drug information during the practice, which can be addressed by skill development programs.

Keywords: Ambulatory care, Collaboration, Drug information, Physician's perception.

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INTRODUCTION

Drug Information (DI) plays a crucial role in healthcare by delivering written and verbal guidance regarding drugs and drug therapy in direct response to inquiries from health-care providers, committees, organizations, patients, or the general public.¹ The Drug Information Service (DIS) is a dedicated service led by trained pharmacy professionals who deliver precise, unbiased, and factual information primarily addressing patient-oriented issues encountered by health-care teams. The purpose of these services is to ensure that health-care professionals and patients receive accurate, authoritative, and current information that they can rely on to make informed decisions for optimizing the therapeutic outcomes.^{1,2} Furthermore, the World Health

Organization (WHO) recognizes centers of drug information as a key element of national programs that encourage the rational medication use.² Despite its significance, DIS in India are still in their early stages, with only a limited number of centers currently addressing these needs across the country. This is due to lack of workforce and infrastructure, interest or awareness among stakeholders.¹

In context of Medication use safety

Despite the growing array of available medications and the introduction of diverse treatment combinations, physicians are increasingly constrained for time, making it difficult for them to stay updated on drug information. This has created an urgent need for independent and unbiased drug information to enhance patient care. In India, the irrational use of drugs is prevalent, contributing to serious issues such as adverse events, drug interactions, antibiotic resistance, and other Drug-Related Problems (DRPs).^{2,3} Outpatient medications which are frequently prescribed by multiple physicians, have a significantly higher



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risk of drug-related problems, such as drug-drug interactions and unintentional medication regimen changes.^{4,5} Moreover, Medication errors in outpatient settings are a significant concern, as most of which occur during the prescribing process.⁶ Hence, it is imperative to deliver relevant, current, and easily accessible drug information to healthcare professionals.^{7,8} In this context, DIS play a crucial role in identifying and preventing ADRs and medication errors, while also advocating for the rational use of medications.²

In context of Pharmaceutical care

Drug Information (DI) services is a fundamental aspect of the pharmaceutical care services that clinical pharmacists must deliver.⁹ In the current healthcare environment, these services are essential due to the increasing patient load, the prevalence of co-morbid conditions, and many more concerns.³ The provision of DI services assists practitioners in rationalizing therapy for better patient care by ensuring the credibility and appropriateness of information sourced from various platforms. As abundance of online literature and access for information exists, it is crucial to evaluate it critically. Pharmacists assist physicians by evaluating these resources and educating them about medications.⁸ Further, carrying additional check on prescriptions.¹⁰

To improve patient outcomes, the collaboration between physicians and pharmacists is vital, as they have complementary and supportive roles.¹¹ Physicians appreciate and endorse these collaborative patient care services, recognizing their importance.¹² Pharmacists are responsible for ensuring high-quality, evidence-based, relevant drug information and being attuned to the needs of physicians. The goal of this study is to evaluate pharmacists' drug information services from the perspective of physicians, thereby promoting effective collaboration and enhancing overall patient care.

MATERIALS AND METHODS

Study design: Retrospective observational study.

Study setting: General medicine outpatient setting of a tertiary care hospital.

Study duration: Six months.

Context

The ambulatory pharmacist had recently implemented drug information services in the outpatient setting. These services were specifically designed to address the needs and concerns of both collaborating physicians and their patients. This collaborative approach ensured that all stakeholders received relevant and up-to-date information, which ultimately improved patient care and treatment outcomes. The pharmacist worked directly with the outpatient care team to deliver drug information services. Both written and verbal communication methods were utilized

and adapted during this process. To maintain complete records, each inquiry was carefully addressed and documented using standardized forms.

Quality assessment

A monthly evaluation was conducted to assess the effectiveness of Drug Information (DI) services from the perspective of physicians. This assessment used a mixed-method approach aimed at improving the drug information services offered to meet the evolving needs of the medical team.

Quantitative data analysis

During the quality assessment, physicians were permitted to randomly select and review any documented drug information service that had been delivered. They evaluated the drug information using a specially-designed evaluation tool, which comprised eight items rated on a 5-point Likert scale, ranging from "strongly agree" to "strongly disagree." Additionally, an overall experience rating was collected on a numerical scale from 0 to 10. This evaluation tool has demonstrated reliability, with a Cronbach's alpha value of 0.74, exceeding the generally accepted criterion of 0.7. The responses for each item were recorded and quantitatively analyzed using descriptive statistics, with support from SPSS software version 20.

Qualitative data analysis

The Evaluation tool included an open-ended section for physicians to provide suggestions or improvements regarding the implemented drug information service. Qualitative insights were obtained from this section and were subjected to manual thematic analysis. This process involved several critical steps, including data familiarization, initial code generation, theme identification, and summarization of the findings from the responses. The feedback gathered from the evaluation team was organized and screened for data familiarization, leading to the generation of initial codes. These codes included categories such as satisfaction, improved compliance, knowledge enhancement, positive impact, and suggestions for expansion. The coded data was then reviewed to identify and develop themes. Throughout this process, codes and themes were continuously evaluated to summarize the key findings effectively.

RESULTS

Nature of drug information services provided at practice site

A total of 153 drug information inquiries were received, among which 21.5% ($n=33$) were from physicians and rest from the ($n=120$, 78.5%) patients. Physician's mainly inquired regarding the drug identification and composition ($n=8$, 24%) followed by drug use and indication ($n=6$, 18%) and safety and efficacy ($n=6$, 18%) related queries during the OPD hours (Figure 1). All drug

information queries was addressed immediately i.e., within half an hour of inquiry by using primary and secondary resources by the pharmacist. Furthermore, it is observed that physicians preferred verbal communication and face-to-face interactions for drug information during the ambulatory care. The nature of drug information inquiries varied among senior and junior physicians. Junior resident doctors mainly inquired regarding the drug identification, composition and its indication whereas senior physician enquired regarding the safety and efficacy profile, advanced pharmacology. In our study, 54% of inquirers were made by junior resident doctors.

Whereas patients mainly needed the drug information related to drug use and its indication ($n=32$, 26.6%) followed by drug identification and composition ($n=23$, 19.1%) and costs ($n=21$, 17.5%) (Figure 2). These drug information queries were addressed using primary and secondary resources through verbal mode of communication by face to face interaction ($n=73$, 60.84%) and ($n=47$, 39.16%) by telephonic intervention. Around 87% ($n=104$) of drug information queries were answered immediately within half an hour and rest of queries ($n=16$, 13%) were answered within 1-2 hr.

Drug information services-Quality assessment by understanding physician's perspective

Quantitative assessment

A team of nine doctors, including senior and junior resident doctors, was assigned at random to evaluate the quality of drug information services on a monthly basis. The evaluation team is made up of two or three people, with one senior member or someone with minimum 5 years practice experience serving as a mandatory member. The average age of evaluating team was 31.6 ± 4.3 years (Table 1). When quality of ambulatory drug information service was evaluated, it is found that all the items in the tool had received good score i.e., above 4 score with average overall rating of 9.38 out of 10. The average scores of evaluating tool items has represented in Table 2. Two items (Q5, Q6) of the

tool has received rating of 4.23 each respectively. i.e., it indicate that need for communication skill improvement.

Qualitative assessment

During the evaluation of insights provided by physicians regarding implemented drug information services at the practice site qualitatively by using thematic analysis. Following findings has been observed. The thematic analysis identifies three key themes regarding the implementation of ambulatory drug information services. First, physicians expressed satisfaction with these services. Second, ambulatory drug information services in outpatient care were well received. Finally, there is a strong suggestion to implement these services across all Outpatient Departments (OPDs) (Table 3).

DISCUSSION

In our study patients contributed to majority of drug information inquiries, followed by healthcare professionals which is contrasting to the Shankpal A study's, where only 23.8% came from patients, followed rest are from various healthcare providers such as clinical pharmacist interns (15.1%), Physicians and DMOs (12%), nurses (11.5%), clinical researchers (4.4%), clinical pharmacists (3.9%) and others (3.9%).² This difference observed may be due to the nature and type of care and contextual settings. These findings also suggest that there is greater need for drug information services for both the stakeholders involved in the patient care. Additionally, in our study approximately 54% of the queries were from junior doctors. These findings were parallel to the study conducted by Seidel P, which indicates that among primary care physicians, drug information service utilization was more commonly observed among those who are younger and have junior doctor status.¹³

Vinterflod C *et al.*, (2018) conducted a study in Sweden exploring physicians' perspectives on clinical pharmacy services. The findings revealed that physicians highly valued their collaboration with pharmacists, recognizing them as drug experts whose advice was clinically relevant and beneficial. This underscores the

Table 1: Ambulatory drug information service- evaluating team demographic characteristics.

Evaluators	Age group (in years)	Gender	Years working as physician	no. of discussions with the ambulatory pharmacist
P1	40-45	Male	13	25+
P2	30-35	Male	5.5	10-25
P3	25-30	Female	3	25+
P4	25-30	Male	2.4	10-25
P5	25-30	Female	4.1	25+
P6	30-35	Male	6	25+
P7	25-30	Female	1.5	10-25
P8	25-30	Male	2.5	10-25
P9	25-30	Female	1.5	10-25

critical need for fostering strong interprofessional collaboration and partnerships to successfully implement and optimize clinical pharmacy services.¹¹ Our study also highlight the importance of teamwork between healthcare professionals to enhance patient care and achieve better therapeutic outcomes.

Amin P *et al.*, conducted a study to understand providers' perspectives on clinical pharmacy services, and their findings indicate that primary care providers are highly satisfied with and benefit from clinical pharmacy services. Providers identified drug information and disease-focused management as valuable pharmacy services. Providers advocated for clinical pharmacists to expand their role with providers and integrate into primary care teams.¹² These findings were similar with our study findings obtained through thematic analysis, which indicate that care

providers are extremely satisfied with implemented ambulatory drug information services and want these services to be expanded to other outpatient settings.

The majority of DIS was provided through web based resource in study conducted by Behera SK *et al.*, and the study conducted by Rajanandh *et al.*, reveals that more than half of inquiries were addressed through verbal mode, and among which one-fourth of the queries were regarding dosage and indication.^{1,14} These findings are similar to our study. Only a small percentage of the references used by DIC in Behera's SKs study came from textbooks; the majority came from online sources.¹ Because they are convenient and easy to access, especially in the context of ambulatory care, primary and secondary web-based resources were also used in our study.

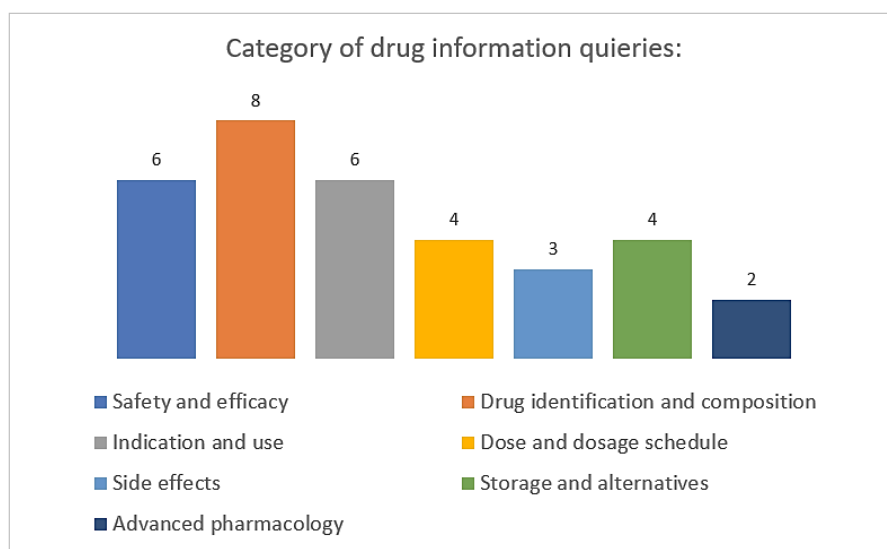


Figure 1: Category of drug information inquiries requested during ambulatory care by physicians.

Table 2: Quality assessment of Ambulatory drug information services.

Sl. No.	Questions	Average rating	Percentage
1	Does ambulatory pharmacist displayed professionalism while providing drug information services?	4.69	93.8%
2	Does ambulatory pharmacist provided the drug information service on time?	4.69	93.8%
3	Do you believe that the resources used as a reference are good enough for resolving drug information query?	4.53	90.6%
4	Do you believe that answer provided were appropriate for given drug information query?	4.46	89.2%
5	Do you think that given drug information query was communicated effectively?	4.23	84.6%
6	Do you believe that ambulatory pharmacist-led drug information service saved time in OPDs?	4.23	84.6%
7	Do you believe that ambulatory pharmacist provided drug information service was useful while prescribing treatment?	4.53	90.6%
8	Are you satisfied with the efforts made by the ambulatory pharmacist to answer queries at the practice site?	4.53	90.6%

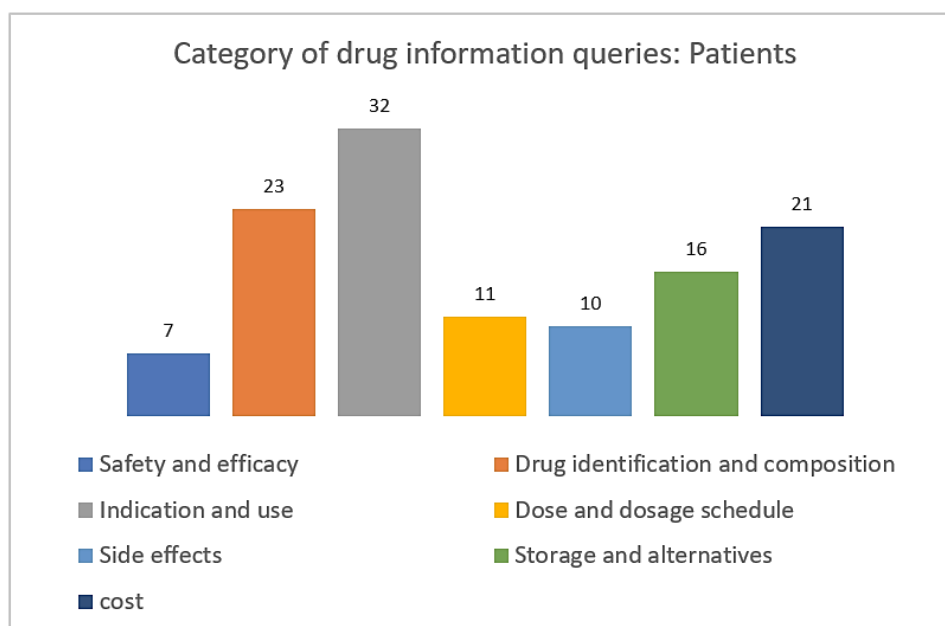


Figure 2: Category of drug information inquiries requested during ambulatory care by physicians.

Table 3: Thematic analysis of physician's insights regarding ambulatory drug information services.

Themes	Key Insights	Supporting quotes
High satisfaction	Services were perceived positively and appreciated during OPD hours.	"Satisfied with services, This initiative helped Us during OPD Hours." (P4)
Positive Perception	Services offered clear guidance, support, or explanations to patients regarding their treatments	"It help in reducing patients' dilemmas."(P1) "It helped patients a lot." (P3)
Need for Service Expansion	Services were valued and its potential was identified	"Implement these kinds of services in all OPD." (P6, P8)

A study by Shankpal revealed that drug information seekers required immediate responses, with 10 respondents (2.6%) needing answers right away, while 60 respondents (15.7%) expected responses within 30 min.² This highlights the vital role of Drug Information Services (DIS) provided by clinical pharmacists in ensuring prompt patient care. These findings align with our research findings. Furthermore, In Shankpal A study- healthcare professionals rated the services as follows: 48% received a rating of "very good," 24% were rated as "good," 18% were deemed "satisfactory," and 10% selected "none."² In our study, all aspects of DI service, i.e., every item of tool has a score of over 80%, with overall score exceeded 90%. These

findings indicate that the success of these services depends on the stakeholders (i.e., Pharmacist) involved in delivering them.

The implementation of drug information services in ambulatory settings is more necessary, according to the thematic analysis of our study. The results are consistent with a study by Shankpal A. that found that while 14% of people were content with their options and did not feel the need for a DIC, 86% of the healthcare community felt that a DIC service was necessary in the hospital.² According to Behera SK's research, one possible explanation for the less inquiries is may be due to (a) the recently created DIC. (b) Lack of awareness among health-care professionals about DIS and DIC; these results were comparable to those of our study.² This finding suggests to create awareness among healthcare providers and involve them to interprofessional activities.

CONCLUSION

Drug information services are essential in ambulatory care. Pharmacist-led drug information services in ambulatory settings have fostered a positive perception among all stakeholders. However, there is still room for improvement in sharing drug information during practice, which could be enhanced through skill development programs.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

DI: Drug Information; **DIS:** Drug Information Service; **WHO:** World Health Organization; **DRP-** Drug-Related Problem; **SPSS:** Statistical Package for Social Sciences; **OPD:** Outpatient Department; **DMO:** Duty Medical Officer; **DIC:** Drug Information Center.

ETHICAL APPROVAL AND CONSENT TO PARTICIPATE

The institutional ethical clearance was obtained prior initiating the study. Reference number: JSSMC/IEC /110523/ 17 NCT /2023-24.

SUMMARY

Pharmacist-led ambulatory drug information services are critical for outpatient care. These services are essential for making treatment decisions for patients, reducing medication errors at the prescribing level, and ensuring efficient outpatient care workflows. Upholding the integrity and quality of these services requires a thorough understanding of physicians' needs as well as proactive engagement with them. As a result, regular assessments are required for continuous quality improvement and long-term sustainability. Furthermore, Pharmacist training and skills play a vital role in effective implementation of these services.

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Evaluating the Correlation between HbA_{1c} and Severity of Diabetic Retinopathy by Assessing the Risk Factors for Elevated HbA_{1c} Levels in Tertiary Care Hospital

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ABSTRACT

Background: Diabetic Retinopathy (DR) is a primary cause of blindness worldwide and a chronic condition that threatens vision. It affects approximately 10% of diabetic patients. To impede the development and course of DR, it is critical to manage its risk factors effectively. **Objectives:** This study's main goal was to evaluate the relationship between patients' HbA_{1c} levels and the degree of diabetic retinopathy at a tertiary care hospital. Finding the risk variables linked to higher HbA_{1c} levels in DR patients was the secondary goal. **Materials and Methods:** This study was an observational prospective study involving patients who fulfilled the requirements for inclusion and exclusion. Data collection was done using a patient profile form and interviews. The study subjects were inpatients from the general medicine department who were diagnosed with diabetes and other comorbidities. The data collected was compiled and subjected to statistical analysis to identify trends and correlations. **Results:** The study included 150 participants with Type 2 Diabetes Mellitus (T2DM). The data showed a higher prevalence of T2DM in the 40-70 age group, with men being more prone to diabetes than women. Smokers were also more prevalent. A significant 95.3% of participants were affected by DR, indicating a high prevalence in the study group. The analysis revealed a gradient in HbA_{1c} levels, linking poor glycemic control to DR progression. However, statistical analysis showed no significant variations in Hemoglobin A1c levels across the stages of DR. **Conclusion:** According to the results diabetes, alcoholism, hypertension, and elevated cholesterol were more common in the DR group. Managing these factors can help delay DR progression. Despite no significant changes in HbA_{1c} across DR stages, it remains a reliable predictor for DR with high diagnostic performance, including strong sensitivity and specificity.

Keywords: Diabetic Retinopathy, HbA_{1c}, Diabetes Mellitus, Lipid Profile, Hypertension, Cholesterol.

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INTRODUCTION

The World Health Organization (WHO) defines diabetes as a chronic metabolic disease characterized by elevated blood glucose (blood sugar) levels. Over time, diabetes can cause serious harm to the heart, blood vessels, eyes, kidneys, and nerves. Usually affecting adults, type 2 diabetes is the most common type and is caused by the body's inability or resistance to produce enough insulin. Over the past three decades, type 2 diabetes has become far more prevalent worldwide, irrespective of wealth. There are several types of Diabetes Mellitus (DM), including:

- Type 1,
- Type 2,
- Gestational Diabetes,
- Neonatal Diabetes,
- secondary diabetes resulting from steroid use or endocrine problems.¹

Diabetes is linked to a number of issues that impact different organ systems, such as the heart, kidneys, nerves, and eyes. Diabetes-Related Retinopathy (DR) is one of these side effects that most commonly causes vision impairment and blindness in working-age individuals.²

In around 10% of people with diabetes, Diabetic Retinopathy (DR) is a microvascular disease with potentially fatal consequences. As a result, DR is quite concerning clinically. As an increase in



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microvascular consequences is caused by DM morbidity. DR prevalence is therefore expected to increase to 5.4% by 2025.³

To slow the onset and progression of DR, it is essential to recognize and control the risk factors. Several dangers the duration of blood pressure, serum creatinine, glucose levels, diabetes, and lipid profile are among the risk factors for DR that have been identified.⁴

Diabetic retinopathy is categorized as either Non-Proliferative Diabetic Retinopathy (NPDR) or Proliferative Diabetic Retinopathy (PDR) according to its severity and stage NPDR is additional classifications as extremely mild, mild, moderate, and severe.⁴ Microvascular anomalies, including retinal hemorrhages, microaneurysms, and intraretinal microvascular anomalies, are what define Non-Proliferative Diabetic Retinal Disease (NPDR). On the other hand, PDR is defined by the development of aberrant blood vessels on the surface of the retina, which, if untreated, can cause a significant loss of vision. Previous research has indicated that High blood levels of Triglycerides (TG), Total Cholesterol (TC), Low-Density Lipoprotein Cholesterol (LDL-C), and High-Density Lipoprotein Cholesterol (HDL-C) are known as dyslipidemia, and they may be a risk factor for the onset of diabetic macular edema and the advancement of DR.⁵

A blood test called a Hemoglobin A_{1c} (HbA_{1c}) test determines your average blood sugar (glucose) level over the previous two to three months. One form of sugar found in your blood that is derived from the food you eat is glucose. Glucose is used as fuel by your cells.⁶

Red Blood Cells (RBC) consist of the protein hemoglobin, which binds to blood glucose. More of your hemoglobin will become coated with glucose as your blood glucose levels rise. The A1C test measures the proportion of glucose-coated hemoglobin in your red blood cells.⁷

An A1C test can be used to assess your average blood sugar during the last three months because:

- Hemoglobin and blood sugar are attached for as far as red blood cells are viable.
- The lifespan of RBC is three months.⁷

An HbA_{1c} test result must be lower than 5.7% in order to be classified regular or in the non-diabetic group.⁸ Anyone with a HbA_{1c} score between 5.7% and 6.4% is considered to have prediabetes, while those with a value of 6.5% or higher are diagnosed with diabetes. HbA_{1c} measures the average blood sugar levels over the past two to three months. Poorer glycemic management and extended exposure to hyperglycemia are indicated by higher HbA_{1c} values. Extended high blood sugar levels cause endothelial dysfunction, which damages blood vessels all over the body, including the retina.⁴

Increased retinal blood flow can result from high blood glucose levels and inadequate glycemic management, putting shear stress on the retinal arteries. Elevated HbA_{1c} values, which suggest poor glycemic management, are linked to worse treatment outcomes and decreased treatment efficacy in DR patients.⁴

Retaining vision and delaying the progression of Diabetic Retinopathy (DR) require achieving and maintaining adequate glycemic control through medication adherence and lifestyle adjustments. A HbA_{1c} of less than 6.9% of total hemoglobin is considered normal.⁸

This study's goal was to ascertain whether Hemoglobin A1c levels and DR are related. This would allow for the prediction of the disease's progression and the implementation of early treatments.

MATERIALS AND METHODS

This chapter deals with description of different steps taken by the investigators for the study. It includes research approach, setting, and sampling, sampling techniques, tools for data collection, and plan for data collection.

Research Approach

Materials

Study design

An observational study.

Study Period

6 months.

Study Site

Department of General Medicine, Department of Ophthalmology of MVJ Medical College and Research Hospital.

Study Population

In patients who were diagnosed with DIABETES.

Inclusion Criteria

- Patients diagnosed with Type 2 DM.
- Patients of either sex above the age of 21 years.
- Patients willing to participate in the study.

Exclusion Criteria

- Patients with vision loss.
- Pregnant and lactating women.
- Patients on long term steroids.
- Patients with dense cataracts and corneal opacities.

Sample size calculation

The numbers to be included in the study was calculated using the following formula for the sample size estimation with quantitative outcome variables.

$$\text{Sample Size } (n) = \frac{2(SD)^2 \left(\left(\frac{Z\alpha}{2} + Z\beta \right)^2 \right)}{d^2}$$

Where,

SD=The standard deviation obtained from previous studies.

Study Sample Size

150 patients.

Instruments used

Patient profile form and patient interview.

Informed Consent Form

The Informed Consent Form was prepared in step with ICH-GCP guidelines and was approved by the Ethical committee. Informed Consent was taken from the College authorities. The Informed Consent Form is provided in Annexure-2.

Human Ethical Clearance

Human ethical clearance was obtained from the Institutional Ethics Committee of Krupanidhi College of Pharmacy for carrying out the research work with Ethical Clearance Number [REF: MVJMC&RH/IEC-123/2024]. Ethical Clearance Certificate is provided in Annexure-1.

Statistical analysis

All Descriptive data will be represented using Mean, Median, Standard deviation and Ranges.

Methods

- An observational study was conducted in the general medicine and ophthalmology ward of MVJ Medical College and Research Hospital, Hoskote.
- The study was conducted on gaining approval from the institutional human ethical committee, MVJ Medical College and Research Hospital, Hoskote.
- All patients who meet the study criteria were enrolled in to the study after taking the informed consent from the patients and also but using the lab investigations from their records.
- The demographic, HbA_{1c} value and stage of diabetic retinopathy were documented.
- All the data were analysed using descriptive statistics.

Analysis of data

Statistical analyses were performed using MX-Excel. Choice of descriptive statistical method was based on distribution normality as determined through normal probability plot.

RESULTS

Descriptive Analysis

Sociodemographic profile scale

Age, Height, Weight Distribution

Among the 150 patients with Type 2 diabetes mellitus, the median age was 59.5 years, with half of the participants ranging in age from 49.75 to 70 years. The median height was 164 cm, ranging from 157 to 170 cm, and the median weight was 64.8 kg, with an interquartile range of 57.25 to 73.85 kg. The median BMI was 24.05, indicating that most participants were within the normal weight range.

Gender distribution

Males constituted majority of the population with being 56.7% of the group, while females made up 43.3%.

Distribution based on financial status

Financially, 98.7% of the participants reported being stable, with only 1.3% indicating poor financial status Distribution based on family history.

51.3% of participants reported a family history of relevant conditions, while 48.7% did not.

Distribution based on lifestyle factors

The study data reveals distinct patterns in alcohol consumption and smoking habits among participants. Approximately 24.7% of the participants ($n=37$) reported consuming alcohol, while the majority, 75.3% ($n=113$), were non-drinkers. In contrast, smoking was more prevalent, with 68% of the participants ($n=102$) identifying as smokers, leaving 32% ($n=48$) as non-smokers.

Distribution based on co-morbidity

The results highlight the prevalence of various comorbidities in the study population. A significant majority, 72.7% ($n=109$), have hypertension. 27.3% ($n=41$) have artery disease, 26% ($n=39$) have renal impairment, 38.7% ($n=58$) have elevated cholesterol and 16.7% ($n=25$) have thyroid disorders. Thyroid issues are less common in this population compared to the other comorbidities.

Distribution based on management of diabetes

In the study cohort, Metformin is used by 50% of participants, making it the most prevalent medication. Other medications show much lower usage rates: Glimistart, Glibenclamide, and several newer agents like Dapagliflozin and Tenueligliptin are each used by only 0.7% of participants. Insulin is used by 4%, Glycomet and

Insugen by 8.7%, Vildagliptin and Mixtard by 5.3%, Glimepiride by 12%, and Voglibose by 3.3%. The usage of medications such as Glimison, Adglim, Voggam and Zometin is very minimal, with each being used by less than 1% of the cohort. This reflects a varied but generally low utilization of many diabetes medications outside of Metformin.

Data on Diabetic Retinopathy

The data on DR shows that 95.3% ($n=104$) of the population has been diagnosed with DR, while only 30.7% ($n=46$) do not have the condition. Based on this, most participants are affected by DR, highlighting a high prevalence of the condition in the study population

Based on Clinical Variables

The results regarding the duration of diabetes diagnosis, retinal examination through funduscopy, and HbA_{1c} values provide insights into the participants' clinical conditions. The median duration of diabetes is 7 years, with an Interquartile Range (IQR)

Table 1: Clinical variables.

Variables	Interquartile range
Duration of diabetes diagnosis (IQR)	7 (3-15)
Funduscopy, n (%)	
Yes	135 (90)
No	15 (10)
Normal fundus, n (%)	
Yes	31 (20.7)
No	119 (79.3)
HbA _{1c} value	9.35 (7.70-10.40)

of 3 to 15 years. This indicates that half of the participants have had diabetes for between 3 and 15 years, reflecting a mix of both relatively recent and long-term diagnoses.

A large majority, 90% ($n=135$), underwent funduscopy, a key procedure for assessing the retina for diabetic complications. Of those who underwent funduscopy, only 20.7% ($n=31$) had a normal retinal examination, while 79.3% ($n=119$ participants) showed abnormalities.

The median HbA_{1c} value is 9.35%, with an IQR of 7.70 to 10.40. This indicates poor blood sugar control, as values above 6.5% are diagnostic of diabetes, and values above 9% are associated with an increased risk of complications, including diabetic retinopathy (Table 1).

Based on Prevalence of Grades of Diabetic Retinopathy

The distribution of DR stages within the study population reveals varying degrees of retinal damage. Among the participants, 20% are in the mild Non-Proliferative Diabetic Retinopathy (NPDR) stage, characterized by early signs of retinopathy with minimal retinal damage. A larger proportion, 30% are in the moderate NPDR stage. Severe NPDR, which indicates advanced retinal damage present in 16% of the population. The most advanced stage, Proliferative Diabetic Retinopathy (PDR), is present in 2.7%, where abnormal blood vessel growth in the retina can lead to severe vision loss or blindness (Figure 1).

Relationship Between Grades of Dr And HbA_{1c}

The HbA_{1c} levels across different stages of DR in the study cohort show a distinct pattern of glycemic control. For the overall

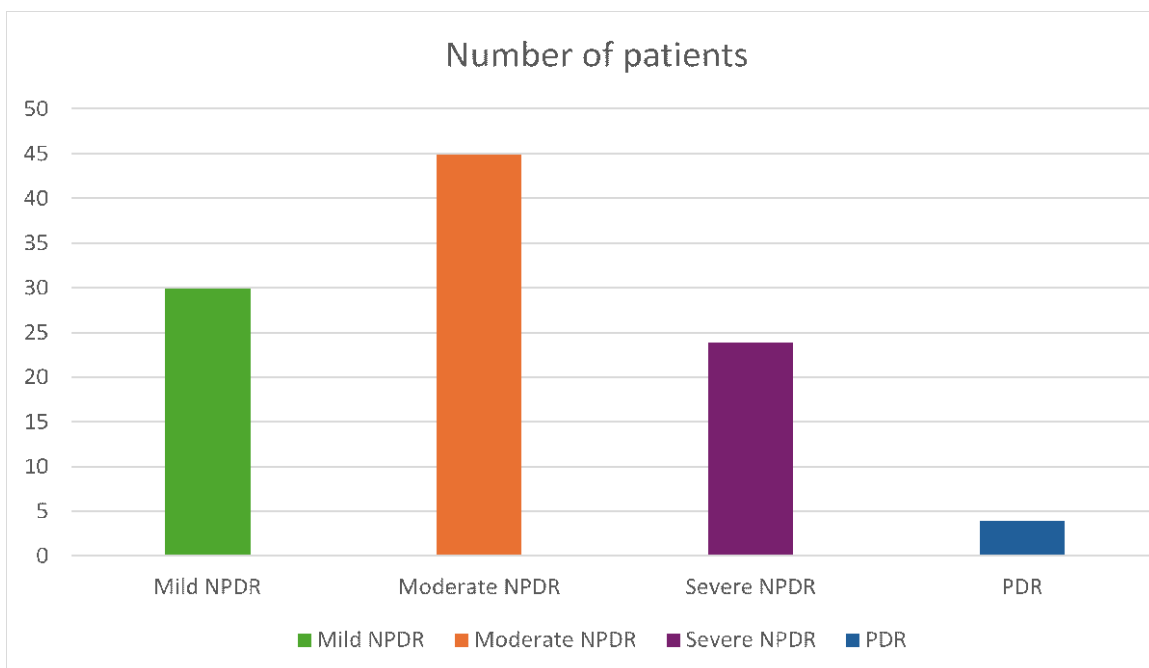


Figure 1: Distribution of DR grades among patients with diabetic retinopathy.

cohort, the median HbA_{1c} value is 9.35%, with an Interquartile Range (IQR) of 7.7 to 10.40%. Participants with mild NPDR have a median HbA_{1c} of 8.7% (IQR 7.7 to 9.4%), reflecting relatively better glycemic control compared to those in more advanced stages. In contrast, individuals with moderate NPDR exhibit a median HbA_{1c} of 10% (IQR 9.15 to 10.95%), suggesting poorer glycemic control. Those with severe NPDR have the highest median HbA_{1c} at 12% (IQR 10.40 to 13%), highlighting very poor glycemic management associated with significant retinal damage. For participants with PDR, the median HbA_{1c} is 10.30% (IQR 8.12 to 13.0%). This gradient in HbA_{1c} levels underscores the link between poor glycemic control and the progression of diabetic retinopathy (Table 2).

Comparison of various clinical, laboratory parameters and co-morbidities distribution between subjects with and without DR

The comparison between subjects with and without Diabetic Retinopathy (DR) reveals several significant associations. Family history of diabetes, alcohol consumption, hypertension, and elevated cholesterol are significantly more prevalent among those with DR, with *p*-values of 0.019, 0.028, 0.003, and 0.035, respectively. Conversely, gender, smoking, artery disease, renal impairment, and thyroid disorders do not show significant differences between the two groups (Table 3).

Table 2: Relationship between grades of DR and HbA_{1c}.

Variables	HbA _{1c} (IQR)
Overall cohort	9.35 (7.7-10.40)
Mild	8.7 (7.7-9.4)
Moderate	10 (10.95-9.15)
Severe	12 (10.40-13)
PDR	10.30 (8.12-13.0)

Table 3: Comparison of Various Clinical, Laboratory Parameters and comorbidities distribution Between Subjects with and Without Diabetic Retinopathy.

Variables	Diabetic retinopathy, n (%)		<i>p</i> -value
	Present (<i>n</i> =104)	Absent (<i>n</i> =46)	
Gender			0.460
Male	61 (71.8)	24 (28.2)	
Female	43 (66.2)	22 (33.8)	
Family history	60 (77.9)	17 (22.1)	0.019
Smoking	32 (66.7)	16 (33.3)	0.627
Drinking	31 (83.8)	6 (16.2)	0.028
Hypertension	83 (79.8)	26 (23.9)	0.003
Artery disease	29 (70.7)	12 (29.3)	0.820
Renal impairment	29 (74.4)	10 (25.6)	0.429
Cholesterol	46 (44.2)	12 (20.7)	0.035
Thyroid	18 (17.3)	7 (28)	0.751

Variation of HbA_{1c} between various stages of DR

Most patients with DR are categorized in the highest HbA_{1c} group, with 76.8% of overall DR patients having HbA_{1c} level 3. In the mild stage of NPDR, 96.3% of patients are also in this highest HbA_{1c} group, with very few patients in the lower HbA_{1c} groups. Similarly, in the moderate stage of NPDR, 95.6% of patients fall into the highest HbA_{1c} group, with minimal representation in the lower groups. All patients in the severe stage of NPDR, as well as those with PDR, are in the highest HbA_{1c} group (100%). However, statistical analysis reveals that the *p*-values for the different stages of NPDR and PDR are all greater than 0.05. This indicates that there are no statistically significant differences in HbA_{1c} levels across these stages of DR, suggesting that the variation in HbA_{1c} levels does not significantly change with the progression of DR stages.

Receiver Operating Characteristic (ROC) Analysis for HbA_{1c} for DR prediction

The ROC analysis for HbA_{1c} as a predictor of DR shows an AUC of 86.1%, highlighting its strong ability to differentiate between patients with and without DR. The test has a sensitivity of 80% and specificity of 78.6%. The cutoff value of >8.45% signifies an elevated risk for DR. Overall, HbA_{1c} is an effective marker for predicting DR, demonstrating robust performance with a high AUC and reasonable sensitivity and specificity (Figure 2 and Table 4).

DISCUSSION

Diabetic Retinopathy (DR) is a progressive eye condition linked to DM, particularly characterized by damage to the retinal tiny blood vessels. Its development is influenced by factors like hypertension, hyperlipidaemia, dysregulated hormones, growth factors, and prolonged hyperglycaemia, all contributing to oxidative stress, inflammation, and neurovascular damage.

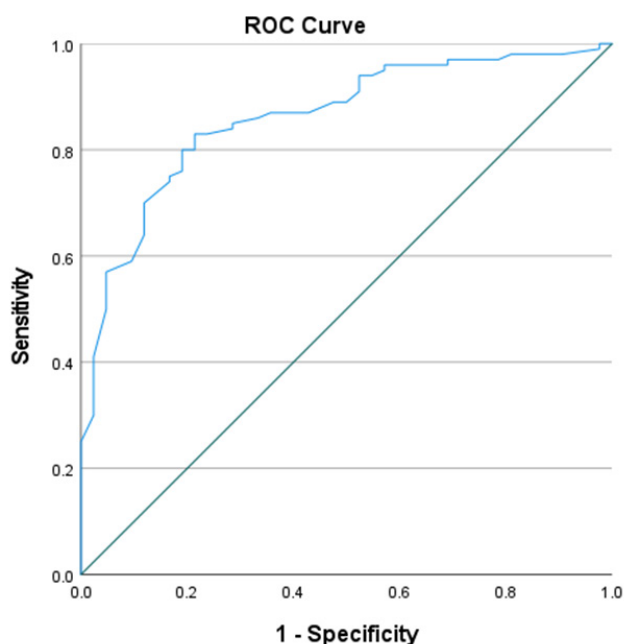


Figure 2: ROC Curve.

Table 4: Receiver Operating Characteristic (ROC) analysis for HbA_{1c} showing strong predictive capability for diabetic retinopathy.

AUC	Sensitivity	Specificity	Cut off
86.1	80	78.6	>8.45

Among the risk factors, hyperglycaemia plays a significant role in DR progression by stimulating the buildup of Reactive Oxygen Species (ROS) and inflammatory mediators, which causes microglial cells to become activated.

In this study, the majority of DR patients were aged between 40 and 70, with a median age of 60. Most were male, with a median height of 164 cm and weight of 64.8 kg. DR severity varied, with 20% of cases having mild (NPDR), 30% moderate NPDR, and 16% severe NPDR. A clear association was found between the duration of diabetes and DR severity, as longer diabetes duration increases the risk and severity of DR, aligning with previous studies indicating a higher prevalence of DR after 10 to 20 years of diabetes.

Our study showed a notable rise in DR cases as the duration of diabetes increased, with higher HbA_{1c} levels correlating with more severe DR. Most DR patients (76.8%) had a HbA_{1c} level greater than 6.5%. Although the difference in HbA_{1c} levels between various stages of DR was not statistically significant ($p>0.05$), a trend toward worsening retinopathy with higher HbA_{1c} was observed. These findings suggest that chronic high blood sugar contributes significantly to the development of DR.

A higher chance of developing DR is also linked to a number of risk factors for Type 2 Diabetes Mellitus (T2DM), such as poor blood sugar control (high HbA_{1c}), hypertension, obesity,

high cholesterol, smoking, and kidney disease. These elements emphasize the significance of regulating blood pressure, cholesterol, and blood sugar levels to avoid or postpone the beginning of DR by contributing to vascular damage, inflammation, and insufficient retinal circulation.

Our findings are consistent with research by Singh A *et al.*,⁹ which identified correlations between elevated serum triglycerides, systolic blood pressure, and HbA_{1c} with DR. Our study also highlighted hypertension and elevated cholesterol as significant factors in DR progression. A risk analysis revealed that a family history of diabetes, alcohol consumption, hypertension, and high cholesterol were more prevalent in DR patients, with significant p -values (0.019, 0.028, 0.003, and 0.035, respectively).

Furthermore, our research supports the findings of Sabanayagam *et al.*,¹⁰ They revealed that the optimal HbA_{1c} cut-off points for detecting mild to moderate DR were 6.6% and 7.0%. Our study indicated an Area Under the Curve (AUC) of 86.1%, with a sensitivity of 80% and a specificity of 78.6%. A higher risk of DR was linked to an HbA_{1c} level above 8.45%.

In summary, this study highlights how crucial it is to manage risk variables associated with diabetes, including blood pressure, cholesterol, and blood glucose, in order to avoid or postpone diabetic retinopathy. In order to reduce the risk of vision loss, diabetic patients must have proactive monitoring and care, as evidenced by the close association between these parameters and the severity of DR.

CONCLUSION

Since 95.3% of the group had (T2DM), the study emphasizes the severe burden of Diabetic Retinopathy (DR) in these patients. Poor glycemic control was identified as the primary risk variable for diabetic retinopathy disease and elevated HbA_{1c} levels were prevalent, with a median HbA_{1c} of 9.35%. It is possible that poor glycemic control may not always predict disease progression, even though it is associated to the onset of DR. All stages of DR were related with elevated HbA_{1c} values, suggesting that inadequate glycemic management is a major contributing factor to the illness. An AUC of 86.1% in ROC analysis demonstrated that HbA_{1c} was a good predictor of DR; however, no significant variations in HbA_{1c} were detected between different stages of DR, indicating that it may not be a reliable indicator of disease progression.

Further supporting the importance of various risk factors in the advancement of DR is the study's finding of substantial connections between DR and comorbidities such hypertension, high cholesterol, and a genealogy of diabetes. Smoking and other illnesses i.e. vascular disease, renal impairment, and thyroid disorders did not exhibit a significant link with DR, despite alcohol use showing such an association. These results highlight the vital necessity of all-encompassing management plans that

address comorbidities and concentrate on glycemic control in order to lower the onset of diabetic retinopathy. Fundoscopy is one of the routine screenings that is necessary for early detection and treatment. Future studies should look into new biomarkers and variables that, in addition to HbA_{1c} levels, could be more accurately used to predict the course of DR.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

DR: Diabetic Retinopathy; **NPDR:** Non-Proliferative Diabetic Retinopathy; **PDR:** Proliferative Diabetic Retinopathy; **HbA_{1c}:** Hemoglobin A_{1c}; **T2DM:** Type 2 Diabetes Mellitus; **ROC:** Receiver Operating Characteristic; **AUC:** Area under Curve; **BMI:** Body Mass Index; **SD:** Standard Deviation; **IQR:** Interquartile Range.

SUMMARY

At a tertiary care facility, this study assesses the relationship between HbA_{1c} levels and the degree of Diabetic Retinopathy (DR) in patients with Type 2 Diabetes Mellitus (T2DM). Most of the 150 patients had poor glycaemic control and were diagnosed

with DR in one of its stages. HbA_{1c} levels continued to be a strong predictor of DR occurrence with high sensitivity and specificity, despite the fact that they did not differ significantly across DR phases. Significant correlations between DR and other risk factors, including high blood pressure, high cholesterol, alcohol use, and family history, were also found in the study. In order to slow the advancement of DR and protect vision, these results highlight the significance of early screening and thorough care of diabetes and its related comorbidities.

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QbD Approach in Formulation and Evaluation of Alcaftadine Ophthalmic *in situ* Gel

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ABSTRACT

Background: *In situ* gels are advanced formulations that undergo sol-to-gel transition upon exposure to physiological conditions like temperature, pH, or ions. **Aim:** The main aim of the research was to implement the Quality by Design (QbD) concept in preparation and assessment of Alcaftadine ocular *in situ* gel to improve the residence time, bioavailability and reduce the frequency of administration. **Materials and Methods:** A Custom design was employed to optimize the formulation, focusing on two critical components Poloxamer 407 and 188, to investigate their influence on Critical Quality Attributes (CQAs) like viscosity, gelation temperature, gelation time, and drug content. Nine formulations were developed and optimized. The optimal formulation was further subjected to ocular irritation, *in vitro* release study, *ex vivo* permeation study, and stability testing. **Results:** Out of nine formulations, the optimized formulation (F7) showed a viscosity of 74.5 (cps), gelation temperature at 36°C, gelation time in 10.2 sec, and 98.72% drug content. Each formulation was evaluated in triplicate ($n=3$). Statistical analysis was performed on the collected data using a zero-intercept multiple regression model. The fit was good ($R^2=0.80$, $p=0.1068$) for gelation temperature, ($R^2=0.82$, $p=0.0893$) for gelation time, and ($R^2=0.85$, $p=0.0596$) for drug content, indicating reliable prediction. *In vitro* release studies followed the Hixson-Crowell model ($R^2=0.9908$) with a release of 99.67%. *Ex vivo* permeation studies revealed a flux of 2.361 and permeability coefficient of 5.904 cm/hr. Draize irritancy test suggested non-irritancy. Stability studies suggested storing optimal formulation at $5\pm3^\circ\text{C}$. **Conclusion:** The research concluded that Alcaftadine can be formulated into an ocular *in situ* gel using a robust formulation optimization approach within the QbD framework.

Keywords: Alcaftadine, Ocular *in situ* gel, Quality by Design, Custom Design, Poloxamer 188 and 407.

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INTRODUCTION

Conventional ophthalmic preparations are the most challenging for ocular drug delivery. Although over 90% of commercial ophthalmic products are eye drops due to patient compliance and wide acceptance, they have certain drawbacks like rapid pre-corneal loss, frequent installation, nasolacrimal discharge, blurred vision and lack of controlled release.¹

Attaining maximum therapeutic action of the drug at the targeted area is a major challenge in ocular administration, due to which various ocular preparations, including gels, ointments, and ocular inserts, were formulated to maximize drug retention time on the ocular surface after being administered. These formulations have been shown to extend the duration of corneal contact.

However, ointments and inserts were not widely accepted due to non-adherence to treatment.²

In situ gels have recently emerged as a significant advancement in the field of ophthalmic formulations. These are aqueous solutions that contain physiologically sensitive polymers, like temperature, ion, and pH-sensitive polymers that transform into viscous gel upon instillation. As this system undergoes instant gelation upon administration as a drop and remains for several hours on the ocular surface, patient compliance could be enhanced by minimizing the administration frequency.³

Alcaftadine is an antihistaminic drug used to treat ocular pruritus associated with allergic conjunctivitis.⁴ The currently marketed eye drops have certain disadvantages such as mild burning, stinging, runny, sore throat, and headache. Hence, the Alcaftadine ocular *in situ* gel was formulated using thermo-sensitive polymers, such as Poloxamers. Poloxamers being amphiphilic undergoes gelation at body temperature ($35-37^\circ\text{C}$) when the concentration of poloxamers is above 15% (w/w) which is the optimal concentration for preparation of *in situ* gel for ocular application.⁵



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The manufacturing processes with the application of the QbD technique ensures predetermined product requirements through formulation design and development. The most effective strategy involves identifying how the process and variables of the formulation influence product quality and optimizing parameters in accordance with final requirement.⁶

As there is lack of literature available on the application of QbD in the formulation of Alcaftadine ophthalmic *in situ* gel, this research aims to address this gap by developing and optimizing thermosensitive *in situ* gel of Alcaftadine using QbD framework to enhance bioavailability, reduce administration frequency and improve patient compliance.

MATERIALS AND METHODS

Materials

Alcaftadine was obtained from Dr. Reddy's Laboratories, Hyderabad, as a gift sample. Hydroxy propyl β -cyclodextrin purchased from Innovative Chemicals, Mumbai. Poloxamer 407 and Poloxamer 188 were obtained from Sigma-Aldrich Chemicals Pvt. Ltd., Mumbai and Benzalkonium Chloride was obtained from SD Fine Chem Limited, Bengaluru.

Methods

Pre-formulation studies

Determination of partition coefficient

10 mg of drug was added into 50 mL of volumetric flask containing 25 mL of aqueous solution (pH 7.4 simulated tear fluid) and 25 mL of n-octanol (organic solvent). The flask was shaken to equilibrate the sample between the two phases for 24 hr using a rotary shaker and was allowed to stand for 30 min in a separating funnel to obtain two separate phases. Drug content was measured in both phases at 254 nm using UV spectroscopy and the partition coefficient was calculated using the formula:

$$k = \frac{\text{Conc. of drug in organic phase}}{\text{Conc. of drug in aqueous phase}}$$

Differential Scanning Calorimetric (DSC)

Thermal properties of alcaftadine and physical interaction between drug and excipients were analyzed using a DSC. The characteristic endothermic peak was observed by heating a sample of 2-4 mg in a tightly closed, aluminum pans with a flat base at a scanning rate of 100 °C/min from 250°C to 340°C under nitrogen at the flow rate of 20 mL/min.

Determination of solubility

The solubility of drugs was determined by adding an excess drug to 2 mL of solvents such as water, methanol, and STF pH 7.4 and then mixing in a vortex shaker. At room temperature, the supersaturated solution was mixed for 48 hr. The centrifugation

of the sample was done over 15 min with a speed of 5000 rpm. After filtering and mixing with suitable solvents, the resulting supernatant was analyzed through a UV spectrophotometer at 254 nm.

Phase solubility studies

The phase solubility studies were carried out to find out the ideal concentration of HP- β -CD required to improve the solubility of the drug. Excess of Alcaftadine was added to 2 mL STF (pH 7.4) in Eppendorf tube containing HP- β -CD of 0.5, 1, 2, 5, and 10%. The electromagnetic stirrer was used to mix the solutions at 200 rpm for 24 hr at 25°C. The Centrifugation of the sample was done over 15 min with a speed of 5000 rpm. After filtering and mixing with STF, the resulting supernatant was analyzed through a UV spectrophotometer at 254 nm.

Quality by Design (QbD) Approach

Drug approval criteria and standards for the Quality by Design (QbD) process have been made mandatory to ensure predefined product specifications through the design for the development of formulations and manufacturing processes.

Quality Target Product Profile (QTPP)

The QTPP of the formulation was defined on basis of the functional features and a survey of literature. This provides a thorough description of the product that emphasizes its quality characteristics.⁷ Table 1 lists the QTPP elements along with a justification for each QTPP element.

Table 1: QTPP elements with justification.

QTPP	Target	Justification
Dosage form	Ophthalmic	To eradicate the local inflammation.
Dosage type	<i>In situ</i> gel	Increase contact time between drug and tissue.
Dosage strength	2.5 mg/mL	To attain maximum therapeutic action.
Route of administration	Ophthalmic	Applied topically to the specific area.
Packaging	Low-density polyethylene bottle with polypropylene cap	Acts as a barrier against light and temperature.
Drug Release	Release of target amount for 24 hr	To ensure the sustained release of the product.
Stability	25-30°C	To assess the degradation pattern of the formulation by monitoring product quality.

Table 2: CQAs elements with justification.

Quality Attributes of a product		Target	CQA	Justification
Physical attributes	Color	Patient acceptance	No	Since clarity and appearance can reduce visual discomfort and can lead to poor patient compliance, it is essential for ocular preparation.
	Appearance			
Viscosity		Liquid should be flow-able	Yes	The dropper is used for the installation.
pH		6-7.4	Yes	To avoid irritation
Gelling time		Instant gelation at 36°C	Yes	To avoid the drainage of the formulation, and improving the contact time.
Gelation temperature		30-37°C	Yes	Undergoes transition from liquid into gel at physiological temperature
Clarity		Clear	Yes	Improve patient compliance
Drug content		Greater than 90%	Yes	To show the required therapeutic effect.
Permeability		Corneal penetration	Yes	The higher the permeability, the greater the bioavailability of the drug
Sterility		Absence of microorganisms	Yes	Sterility of formulation is required to prevent the growth of microorganisms.

Critical Quality Attributes (CQA)

Physical appearance does not have much impact on the end product quality, but should be considered for ensuring patient acceptance. Other elements of CQAs are pH, viscosity, gelation time, gelation temperature, and *in vitro* drug release studies, which directly impact the quality of the final product that is required to be controlled in every step of formulation to maintain safety, efficacy and potency.⁸ The Critical factors associated with the CMA and CPP were screened and their justification is given in Table 2.

Quality Risk Management (QRM)

In ocular *in situ* gel formulation, risk assessment aims to identify the hazards, and minimize the level of its risk through control measures.⁹ The main objective of the QRM is to analyze the influence of material attributes and process parameters on CQAs by using tools such as the Ishikawa diagram, Risk estimation matrix, Heat map, and Pareto chart. Ishikawa diagram: illustrates how the quality attributes of drugs are affected by causes and sub-causes. The Risk Estimation Matrix (REM) was used to determine the effect of CQA vs QTPP and CQA vs CPP by giving the Risk Priority Number (RPN 1: low, 3: Medium, and 9: High risk). The total RPN for CQA vs CPP was represented by a heatmap.¹⁰

Design of Experiment (DoE)-Custom design

Using Custom design, we can choose the number of runs that corresponds to the cost of the experimental condition, and it is possible to determine which effects are desired to be estimated. Additionally, custom designs were used for the construction of split-plot and related designs by supporting variables that are

very difficult or impossible to change.¹¹ The attributes that have an impact on the outcomes of the final product were screened using QTPP elements, CQAs, and risk assessment. The critical material attributes and critical quality attributes, along with their goals and limits have been mentioned in Table 3.

Preparation of temperature-sensitive ocular *in situ* gel

The polymer solution was prepared using the cold technique. Poloxamer 407 was dissolved in distilled water, followed by Poloxamer 188, with continuous stirring. It was then hydrated in a refrigerator at 2-8°C overnight.¹² In a separate beaker, required quantity of HP- β -CD (10%) was dissolved in distilled water and then drug (2.5 mg/mL) was added and stirred using a magnetic stirrer. This drug solution was combined with the hydrated polymer solution under agitation. Sodium chloride (0.2%) and Benzalkonium chloride (0.01%) were added as tonicity agents and preservatives.¹³ Totally nine different formulations were formulated by altering the % of Poloxamer 407 and Poloxamer 188, as listed in Table 4.

Checking the CQAs of ocular *in situ* gelling system

The Critical Quality Attributes for the ocular *in situ* gel was selected based on their safety, efficacy and patient compliance of the formulation. These parameters were identified as high priority attributes using QRM tools like Ishikawa diagram, risk estimation matrix. As these CQAs directly influence gelling behavior, ocular comfort, and drug bioavailability of the formulation, which are critical for product therapeutic performance and patient acceptability, they were selected for evaluation and optimization using a Design of Experiment (DoE).

Table 3: Variables and limits of Custom design (Categorical).

Independent Variables			
Variables	Lower limit	Mid value	Upper limit
Poloxamer 407 (%w/v)	13	14	15
Poloxamer 188 (%w/v)	26	28	30
Dependent Variables			
Responses	Goal	Lower limit	Upper limit
Gelation temperature °C	Match target	32	37
Gelation time in seconds	Minimize	10	30
Drug content (%)	Maximize	90	100

Table 4: Gelation temperature, Gelation time and capacity, Drug content of various formulations.

Formulation	Selected Factors		Average (±) SD			Gelling capacity
	Poloxamer407(%w/v)	Poloxamer 188(%w/v)	Gelation temperature	Gelation time	Drug content	
F1	13	30	37.6±0.3	12.3±0.360	96.5±0.360	++
F2	14	26	42.1±0.264	23.1±0.264	95.8±0.264	+
F3	15	28	37.5±0.360	12.6±0.3	98.73±0.127	++
F4	14	30	37.41±0.200	19.1±0.655	96.5±0.3	++
F5	13	28	43.8±0.1	18.5±0.435	95.5±0.264	++
F6	13	26	39.16±0.16	13.5±0.3	94.6±0.360	++
F7	15	30	36.0±0.458	10.2±0.1	98.72±0.236	+++
F8	15	26	39.4±0.4	15.3±0.2	98.5±0.4	++
F9	14	28	44.3±0.360	18.9±0.1	98.43±0.153	++

Gelation temperature

Gelation temperature ensures appropriate sol-to-gel transition at ocular surface temperature (32-37°C) for maintaining a sustained release of the drug, prolonging retention time, improving bioavailability.

Method

Two, 10 mL glass tubes were taken and placed in a water bath. A quantity of 1 g formulation was present in one, and the other contained 1 mL water. The tube with water had a thermometer inserted into it. As the water bath temperature gradually increased, the formulation gel transitioned from a solution to a non-flowing state upon tube inversion and was recorded as (T₁). Subsequently, the water bath temperature was decreased, and (T₂) was noted when the gel resumed flow. Gelation temperature was determined by taking the mean of T₁ and T₂.¹⁴

Gelation capacity and Time

To measure the time required for the solution to form a gel upon contact with tear fluid. A rapid gelation time (≤30 sec) reduces precorneal drainage and enhance drug retention.

Method

Gelling capacity was measured by adding 100 µL of the formulation into the glass tube filled with 2 mL STF (pH 7.4) kept at 37°C. Time required for the solution to form a gel and the time for dissolving the formed gel were recorded.

Gelation capacity was noted with the following observations:

- + Gelation occurred after a few minutes and the gel dissolved quickly.
- ++ Immediate gelation, stable for few hours (2-4 hr).
- +++ Immediate gelation, persisted for extended period of time (>6 hr).¹⁵

Drug content

Drug content ensures uniform distribution of the drug and consistent therapeutic effect.

Method

For measuring drug content, 1 mL of the *in situ* gel was added into a 100 mL volumetric flask, and volume was adjusted to the required level with freshly prepared STF. The UV spectrophotometer was used to measure absorbance at 254 nm.¹⁶

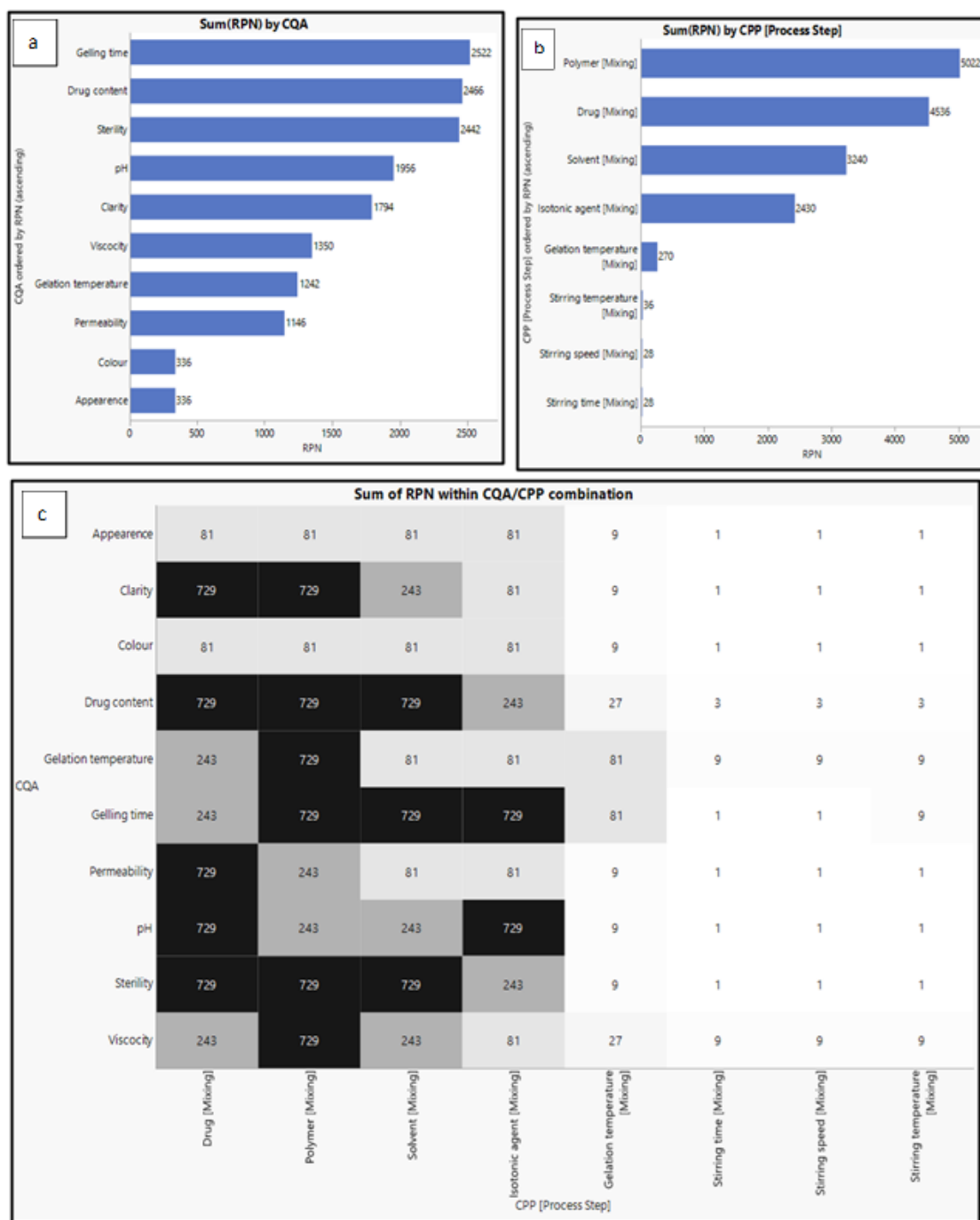


Figure 1: a: CQA Pareto charts, b: CPP Pareto charts, c: CQA vs CPP heatmap.

Rheology study

Viscosity is essential as it plays dual role where the lower viscosity before gelation ensure easy instillation, the higher viscosity after gelation prolongs the residence time.

Method

To measure the viscosity of the formulation, it was introduced into the viscometer with a spindle number 61 and rate of shear was progressively raised between 10 to 200 rpm. Spindle number 61 was selected due to its suitability for low to moderate viscosity

fluid before gelation and also ensures accurate rheological measurements of the developed formulation across wide shear rate range. Before and after gelation, the formulated *in situ* gel's viscosity was measured. The gelation was induced by the addition of STF in a ratio of 40:7 at a temperature of 37°C.¹⁷

Model fit of selected responses of *in situ* gel formulation

The measure used to characterize the relationship between a response and a variable is called model fitting. After

characterization, the selected responses for each of the nine formulations were integrated into the design and were subjected to examination to determine the statistical significance of model fit. Statistical significance can be achieved by variables with a *p*-value less than 0.05.

Statistical optimization of selected responses of *in situ* gel formulation

To optimize the independent variables integrated into the design and to determine whether the goal and desirability were met, the prediction profiler was generated for each response. After the models have been developed and validated, the area within the variable contour plot was evaluated to determine the optimal design space.

Characterization of Optimized Formulation

Visual Appearance and Clarity

The visual appearance and clarity of the formulation were assessed by comparing it against both white and black backgrounds under fluorescent light, for identifying existence of any particulate matter.¹⁸

Determination of pH

The stability and extent of drug solubility in the formulation were influenced by pH. The formulation was designed carefully with respect to pH to avoid patient irritation upon instillation and to ensure the stability of the formulation. For measuring the pH of the formulation, a digital pH meter was used.¹⁹

In vitro Release Study

A Franz diffusion cell with a cellulose acetate dialysis membrane was used to assess the cumulative release of drug of the optimized formulation. The donor chamber was loaded with 1 mL of the preparation, and 50 mL of STF was added to the receptor chamber, with a temperature of 37°C and stirred at 100 rpm using a magnetic stirrer. The study was performed for 24 hr. At regular intervals, a sample of 1 mL was extracted and substituted with 1 mL of STF. The total drug released from the formulation was measured using Spectrophotometric assay at 254nm.²⁰

Kinetics study

Various kinetic models were applied to the *in vitro* drug release data of the *in situ* gel to interpret the drug release mechanism.²¹

Ex vivo/Corneal Permeability Studies

The permeability study was performed using modified Franz diffusion. A goat corneal membrane preserved in normal saline and immersed in STF (pH 7.4) for 30 min was placed between the receptor and the donor chamber. The donor chamber was loaded with 1 mL of the preparation, and 50 mL of STF was added to the receptor chamber, with a temperature of 37°C and stirred at

100 rpm using a magnetic stirrer. The study was performed for 24 hr. At regular intervals, a sample of 1 mL was withdrawn and substituted with 1 mL of STF. UV-visible spectroscopy was used at 254nm to analyze the % of drug permeated through the corneal membrane.²²

Ocular Irritation Study

The Draize irritancy test was performed using albino New Zealand rabbits of both sexes (1.5 to 2 kg) to determine the likelihood of ocular irritation in the optimized formulation. The right eye received a 25 µL dose of the formulation, with the left eye serving as a control, received 50 µL 0.9% NaCl. The right eye was observed using the split lens and photographed at time intervals: 1, 2, 4, 24, 48, 72 hr, and one week, for any changes in the conjunctiva, iris, cornea, and then compared with the left eye. Routine inspections were conducted to identify any indications of redness, swelling, and secretion.²³ The potential for eye irritation assigns scores based on four grades given below:

- 0-Absence of redness, inflammation, or increased tear flow.
- 1-Mild redness and inflammation with minimal tear flow.
- 2-Moderate redness and inflammation with increased tear flow.
- 3-Severe redness and inflammation with increased tear flow.

Stability studies

The final optimized formulation (F7) underwent stability testing as per ICH Q1A (R2) and US FDA stability guidelines for industry Q1A (R2). The formulation vials were stored in a stability chamber for accelerated stability study (25°C±2°C/60% RH±5% RH) and long-term stability study (5°C±3°C). The drug used was thermally stable, however, the storage conditions for Poloxamer 407 and Poloxamer 188 vary between 2-8°C. Therefore, the stability protocol for "drug products intended for storage in a refrigerator" was followed. Visual appearance, pH, clarity, viscosity, gelling capability, and drug content of the samples were observed²⁴ at 0, 1, 2, and 3 months for accelerated and long-term stability study due to lack of time.

RESULTS AND DISCUSSION

Risk assessment

The risks associated with the formulation were analyzed by identifying the causes and sub-causes. As per the REM, the CQA such as the gelation time, gelation temperature, viscosity, and drug content were considered to be high-risk elements and they were summed and represented by a heatmap shown in Figure 1 which indicates the rank of the gelation time (RPN=729), gelation

temperature (RPN=729), and viscosity (RPN=729) having much impact on the drug and polymer.

Pre-formulation studies

Determination of Partition coefficient

Using STF and n-octanol system, the partition coefficient of Alcaftadine was found to be 1.756 ± 0.090 which indicates moderate lipophilicity, suggesting drug can effectively permeate corneal epithelium while maintaining adequate aqueous solubility.

Differential Scanning Calorimetric (DSC)

The thermal behavior of alcaftadine was studied using DSC. The pure drug showed distinct endothermic peak at 226.9°C

indicating its crystalline nature. In the drug-polymer mixture the peak shifted to 237.51°C indicating drug-polymer interactions and reduced crystallinity, indicating enhanced solubility and dispersion within the formulation. The additional peak at 59.69°C was likely due to melting transition of the polymeric excipients used, which has melting point range 50-60°C, indicating their presence in physical mixture (Figure 2). There were no unexpected peaks observed, suggesting the absence of major chemical compatibility and indicating that drug remains stable within polymeric matrix.

Solubility Analysis

The determined solubility of Alcaftadine in different solvents such as water, STF at pH 7.4, and methanol was limited to 0.071, 0.078, and 0.099 mg/mL, respectively.

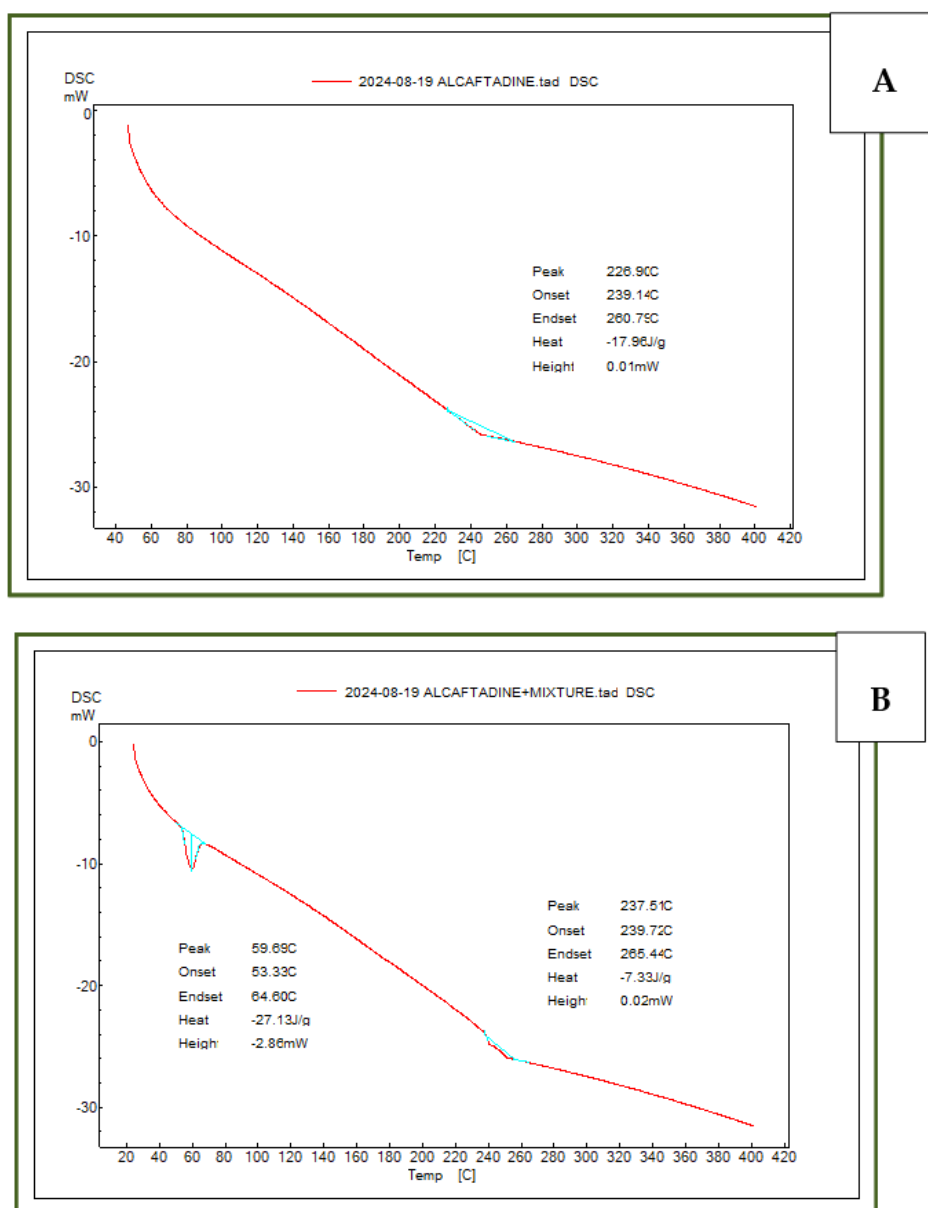


Figure 2: DSC of a: Drug and b: Physical mixture of drug and polymer.

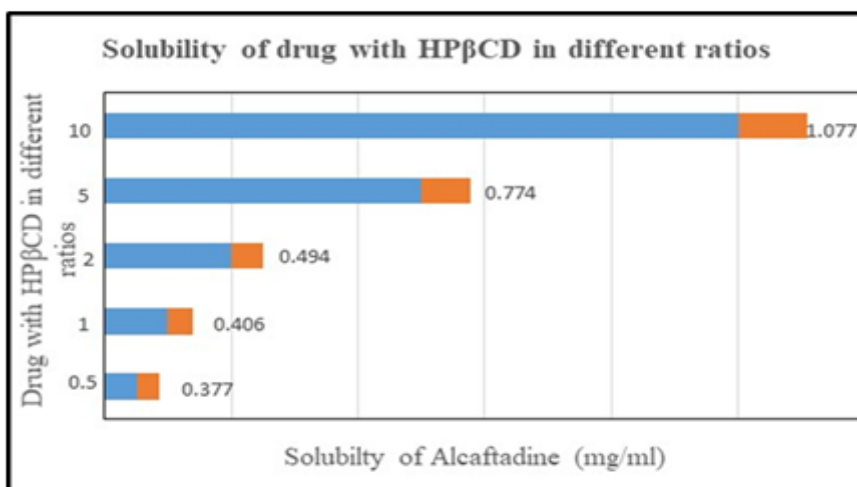


Figure 3: Solubility of drug with HPβCD in different ratios.

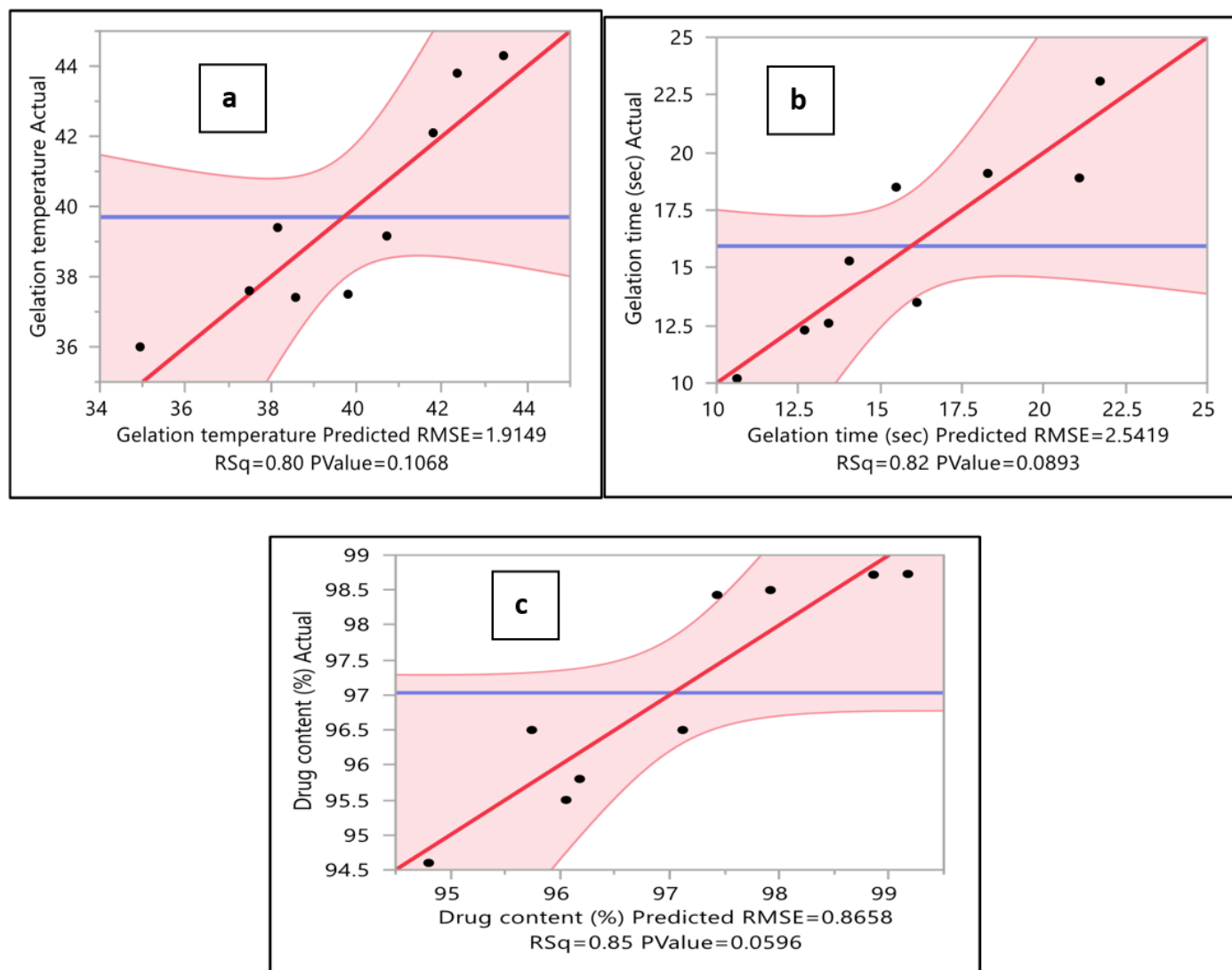


Figure 4: Actual versus Predicted plot of selected responses a: Gelation temperature, b: Gelation time and c: Drug content.

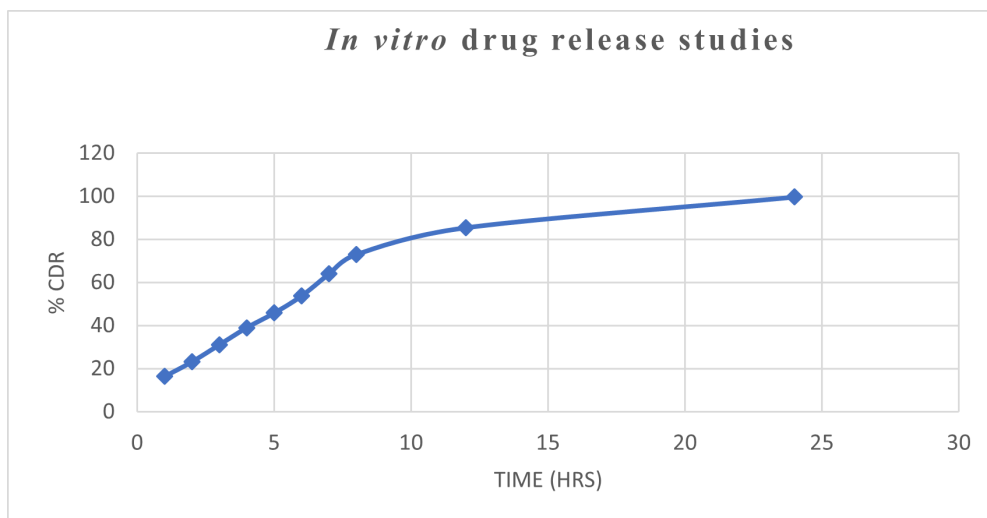


Figure 5: Graphical representation of *in vitro* drug release of Optimized formulation.

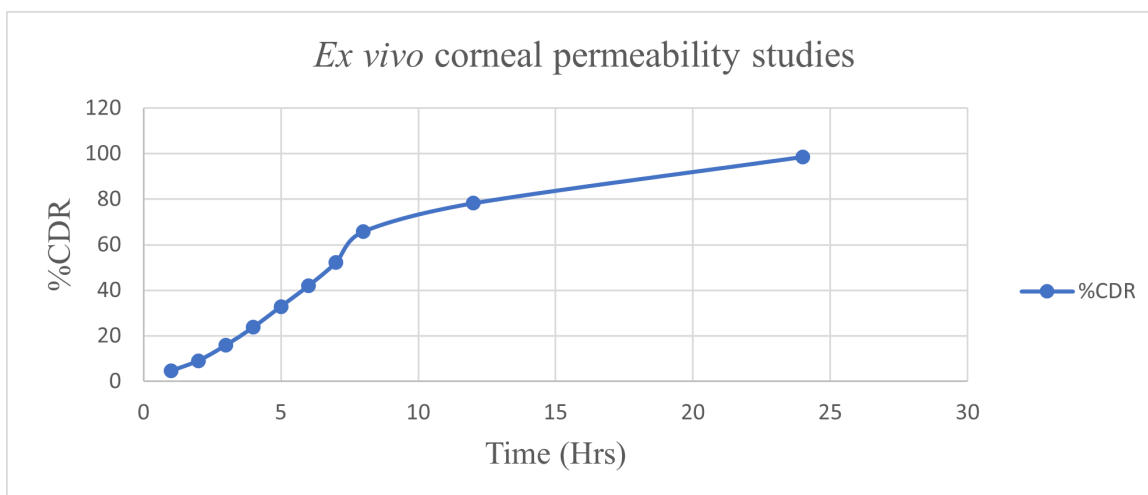


Figure 6: Graphical representation of *ex vivo* permeation studies.

Table 5: Ocular irritation score card for optimized formulation (Right eye) and 0.9% NaCl solution (left eye).

Duration	Parameters					
	Optimized formulation (Right eye)			0.9% NaCl solution (left eye)		
	Redness	swelling	Tear drainage	Redness	swelling	Tear drainage
1 hr	1	0	2	0	0	0
2 hr	1	0	1	0	0	0
4 hr	0	0	1	0	0	0
24 hr	0	0	0	0	0	0
48 hr	0	0	0	0	0	0
72 hr	0	0	0	0	0	0
1 week	0	0	0	0	0	0

Phase solubility studies

Solubility of Alcaftadine was gradually increased by increasing the HP β CD concentration. At the maximum concentration of HP β CD (10%), Alcaftadine solubility was 1.077 mg/mL as shown in Figure 3, confirming HP β CD utility as a solubilizing agent in the formulation for improving aqueous solubility.

Formulation of ophthalmic *in situ* gel by Custom Design

In total, 9 runs as shown in Table 4 were generated by the JMP® 14.0.0 software based on the limits assigned to each factor, according to which formulations were developed and analyzed.

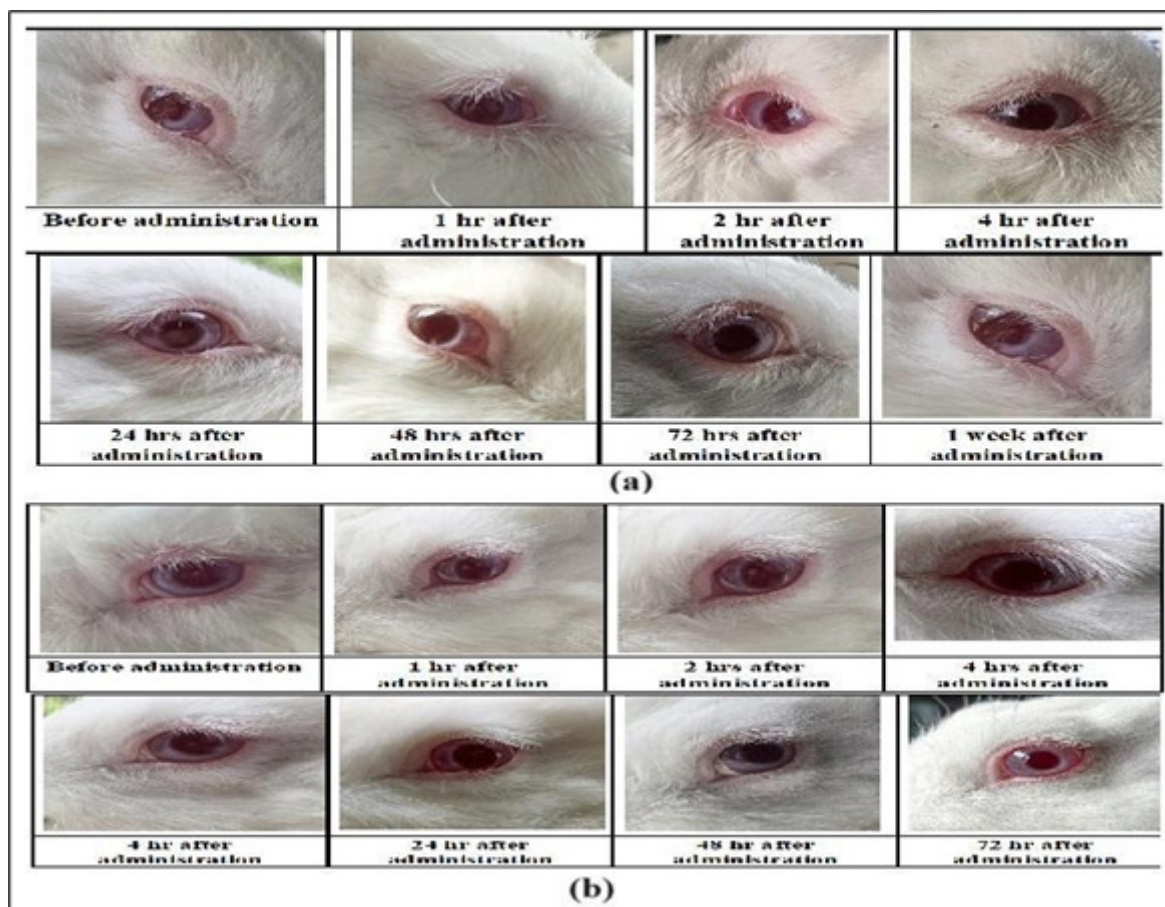


Figure 7: Ocular irritation study of a: right eye administered with optimized Alcaftadine formulation and b: left eye administered with 0.9% NaCl solution.

Table 6: Long-Term Stability data at (5 $\pm$ 3 $^{\circ}$ C) and Accelerated Stability data at (25 $\pm$ 2 $^{\circ}$ C) of optimized formulation for a period of 3 months.

Evaluation parameters	Long-Term Stability data				Accelerated Stability data			
	0 day	30 days	60 days	90 days	0 day	30 days	60 days	90 days
Appearance	Clear	Clear	Clear	Clear	Clear	Clear	Clear	Clear
Clarity	Free flowing liquid	Free flowing liquid	Free flowing liquid	Free flowing liquid	Free flowing liquid	Free flowing liquid	Free flowing liquid	Free flowing liquid
pH	6.5	6.0	7.2	6.9	7.2	6.9	7.0	6.5
Viscosity(cps)	74.6	71.8	72.3	70.3	68.9	70.6	71.3	73.4
Gelation Time (sec)	9.7	10.4	10.6	9.8	10.2	9.5	10.7	10.1
Gelation temperature ($^{\circ}$ C)	36.5	37.5	36	36.5	36	37	36.5	37.4
Drug content (%)	98.01	98.63	99.12	98.88	99.72	99.57	99.32	99.83

Checking the CQA's of ocular *in situ* gelling system

Gelation temperature

The gelation temperature of all the formulations were found between 36°C to 44.3°C. The optimized formulation F7 exhibits gelation of 36°C, within the ideal physiological range for ocular application which enhance retention time and minimize drug loss. The data is summarized in Table 4.

Gelation time and gelation capacity

The Gelation time of all the formulations were observed between 10.2 sec to 23.1 sec and gelling capacity between + to +++ as shown in Table 4. The optimized formulation F7 showed the fastest gelation of 10.2 sec and better gelling capacity of +++, indicating immediate gelation, stable for extended duration, reduced drainage and enhanced residence time.

Drug content

The percentage of drug content was determined for all the formulations as shown in Table 4 and the values were observed between 94.6 to 98.73%, with optimized formulation F7 achieving 98.72%, confirming uniform distribution and minimal drug loss during formulation.

Rheology study

Viscosity measurements of the optimized formulation were taken using the viscometer with a spindle number 61 before and after gelation at a shear rate of 10-200 rpm. Spindle number 61 was selected due to its suitability for low to moderate viscosity fluid before gelation and also ensures accurate rheological measurements of the developed formulation across wide shear rate range. The results were found to be 74.5 before gelation, allowing easy instillation and 283.6 (cps) after gelation. This shift ensures prolonged ocular retention and minimize drug drainage.

Model fit of selected responses of optimized alcaftadine formulation

To ensure the model fit, data collected for nine formulations was incorporated into the design. Statistical analysis was performed on the collected data through the use of a zero-intercept multiple regression model. The $R^2=0.80$ and $p=0.1068$ for response gelation temperature (°C), $R^2=0.82$ and $p=0.0893$ for response gelation time in seconds, and $R^2=0.85$ and $p=0.0596$ for Response drug content (%) was observed and presented in Figure 4. The results showed reasonably good model fit, although p -value were slightly above the ($p<0.05$) threshold, they indicated borderline significance, suggesting a reliable model with minimal experimental deviation. The minor deviations from significance may be attributed to experimental runs ($n=9$). The small % difference between predicted and actual values further supports model adequacy. Furthermore, all experimental values, were

within the pharmaceutical acceptable ranges supporting practical relevance and robustness of the formulation.

Statistical optimization of the selected response of Alcaftadine ISG Formulation

The prediction profiler provides a comprehensive representation of the variability that exists within the extreme values by displaying a continuous correlation between numerous factors and multiple responses. Based on the results obtained from evaluation parameters, F7 was selected as optimized formulation. The maximum global desirability value is 94.41% which confirms the success of the optimization approach; hence the desired goal was achieved for all the 3 responses. The % difference was obtained by subtracting the experimental and predicted data. Since the data did not vary too much, this was considered to be the best formulation illustrating the model's validity.

Characterization of optimal ocular *in situ* gelling system

Visual Appearance and Clarity

All the formulations were evaluated for visual appearance and clarity by comparing it against both white and black background under fluorescent light. All (F1-F9) formulation were observed to be free flowing and clear liquid solution.

pH

The pH of the formulations was found between 6.1 to 7.2 which is within the physiological range of tears, between 5 to 7.4, thus ensuring non-irritancy of the formulation.

In vitro drug release study

The percentage of drug release over 24 hr was found to be 99.67%, as in Figure 5 indicating sustained release which is ideal for prolonged ocular action and reduced dosing frequency.

Release Kinetic studies

Various kinetic models were applied to the drug release data of the *in situ* gel to interpret the drug release mechanism. Fit accuracy was assessed by R^2 value. The Hixson-Crowell model showed a high correlation ($R^2=0.9908$) as compared to the zero-order ($R^2=0.3507$), First-order ($R^2=0.9812$), and Higuchi ($R^2=0.9283$) models indicating surface area-dependent sustained release via gel erosion. To determine the n value, the data obtained was fitted into the Korsmeyer-Peppas model. As per Korsmeyer-Peppas, the mechanism was super case II as the value of n (0.520) was less than 1.

Ex vivo Corneal Permeability studies

The goat cornea was used as a model for the *ex vivo* corneal permeation analysis. 98.56% of drug release was obtained by using the optimized Alcaftadine formulation over a period of 24

hr with a flux of 2.361 and permeability coefficient of 5.904 cm/hr demonstrating enhanced corneal penetration. The results shown in Figure 6.

Ocular irritation study

The results summarized in Figure 7 and Table 5, indicated no observable clinical signs, such as conjunctival redness, edema, congestion, or discharge, in the rabbit's eye, which suggests the formulation was non-irritant, making it safe for ocular use.

Stability studies

The three months stability studies at both accelerated ($25\pm3^{\circ}\text{C}$) and long term ($5\pm3^{\circ}\text{C}$) conditions were conducted for optimized Alcaftadine formulation. Table 6 shows that the formulation were stable concerning their properties at $5\pm3^{\circ}\text{C}$ and $25\pm3^{\circ}\text{C}$, after evaluating their physical appearance, drug content, gelling capacity, viscosity, and pH. It is confirmed that the formulation should be stored at $5\pm3^{\circ}\text{C}$ by the stability tests, thereby achieving a result that is within the limit. In the results, no parameter exhibits wider variations. Thus, the formulation demonstrated stability at refrigeration temperatures. Although long term studies usually require 12 months, only a 3 months study was done due to time constraints.

CONCLUSION

QbD approach was utilized in developing an Alcaftadine ocular *in situ* gel formulation to manage allergic conjunctivitis. The main aim of the research was to enhance precorneal residence time, improve ocular bioavailability thereby reducing the frequency of administration and ensure patient acceptability. Initially, QTPP was listed and CQAs for *in situ* gel were identified such as drug content, gelling time, and gelation temperature. The Risk table, Pareto charts, and Heatmap are risk assessment tools used to analyse the risk associated with the Material Attributes and Process Parameters which influence the CQAs of the finished product. Pre-formulation studies such as partition coefficient, DSC, and solubility Studies were conducted and the outcomes indicated satisfactory results. Thermosensitive polymers such as Poloxamer 407 and 188 were identified as critical variables that have an impact on CQAs. A custom design was selected as the Design of Experiments (DoE) to optimize design space and achieve maximum desirability for individual responses. The optimized formulation was evaluated for viscosity, drug release kinetics, corneal permeability, eye irritation, stability studies and ensures promising quality characteristics.

ACKNOWLEDGEMENT

The authors are thankful to Dr. Reddy's Laboratories, Hyderabad, India for providing Alcaftadine.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

API: Active Pharmaceutical Ingredient; **CDR:** Cumulative Drug Release; **CQA:** Critical Quality Attributes; **DOE:** Design of Experiment; **FTIR:** Fourier Transform Infrared Spectroscopy; **HP β CD:** Hydroxy propyl Beta Cyclodextrin; **ISG:** *In situ* Gel; **QbD:** Quality by Design; **REM:** Risk Estimation Matrix; **STF:** Simulated Tear Fluid.

ETHICAL APPROVAL

This study received ethical approval for the use of animals, under the approval number KCP/IAEC/PCOL/QA/148/April 2024.

SUMMARY

This study applies a Quality by Design (QbD) framework to develop a thermosensitive ophthalmic *in situ* gel of Alcaftadine. A custom design was used to evaluate the influence of Poloxamer 407 and 188 on Critical Quality Attributes (CQAs) such as gelation temperature, gelation time, and drug content. The optimized Formulation (F7) exhibited ideal gelation at 36°C within 10.2 sec, viscosity of 74.5 cps, and drug content of 98.72%. The maximum global desirability value is 94.41% which confirms the success of the optimization approach. *In vitro* release followed the Hixson-Crowell model with 99.67% drug release. *Ex vivo* permeation showed a flux of 2.361 and a permeability coefficient of 5.904 cm/hr. The Draize test confirmed non-irritancy and stability was maintained at $5\pm3^{\circ}\text{C}$. The study demonstrates successful QbD-based formulation of a stable and effective Alcaftadine *in situ* gel for ocular delivery.

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Quality by Design Assisted Formulation Development of Tapentadol Hydrochloride-Loaded Pharmacosomal Transdermal Gel

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ABSTRACT

Objectives: The objective of the present study was to develop a tapentadol hydrochloride-loaded pharmacosomal Transdermal Gel (TPG) through Quality by Design. Pharmacosomes function as potential carriers to intensify the transdermal permeation of drugs. Hence, it overcomes the problems associated with oral tapentadol, such as drug dependence, abuse, and misuse. **Materials and Methods:** The QbD strategy was framed as per ICH Q8, including the quality target product profile, critical quality attributes, critical material attributes, risk assessment studies, and experimental design. A pharmacosome formulation was developed and optimized by constructing a central composite design. All nine runs of the pharmacosome experiments were prepared and characterized using the ethanol injection method. The optimized pharmacosomes were converted into a pharmacosomal gel using Carbopol 934P and characterized for pH, viscosity, spreadability, as well as *in vitro* drug release, *ex vivo* permeation, and stability profiles. **Results:** Pharmacosomes were optimized using a numerical global desirability function (0.85). The *in vitro* release and *ex vivo* permeation of TPG were found to be 83.5% and 83.2%, respectively. Stability studies demonstrated the potency of the formulation, and the shelf life of TPG was predicted to be 25.980 months. **Conclusion:** The results evidence the potential of Pharmacosomal transdermal delivery as a novel, effective, and alternative to conventional oral administration, offering improved permeation, reduced systemic side effects, and minimized risks of drug abuse and withdrawal symptoms.

Keywords: Tapentadol HCl, Pharmacosomes, Central Composite Design, Drug Abuse, Quality by Design (QbD).

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INTRODUCTION

According to the Center for Disease Control and Prevention (CDC) 2023, 8.5% have high-impact chronic pain and about 24.3 (%) of adults experience chronic pain.¹ Pain can be classified as acute (broken bones, surgery, or childbirth), chronic (arthritis, diabetes, cancer, or back pain), nociceptive (tissue damage), neuropathic (nerve damage), and psychogenic (caused by either tissue or nerve damage).²

Tapentadol hydrochloride (TPL) serves as a centrally acting opioid analgesic that is effective in treating moderate-to-severe pain.³ Tapentadol Hydrochloride is available in oral tablet strengths of 50 mg, 75 mg, and 100 mg, with a maximum daily dose of 600-700 mg administered in divided doses every 4-6 hr.⁴

Tapentadol hydrochloride is categorized as a BCS class I drug with a half-life ($t_{1/2}$) of 4 hr and oral bioavailability of 32% after a single dose administration. It takes 25-30 hr to reach a steady-state plasma concentration.⁵ The oral route of tapentadol requires frequent dosing because of its poor bioavailability and short half-life. Also, it causes poor patient compliance and leads to drug abuse, misuse, tolerance, and withdrawal symptoms.⁶ To address the challenges associated with oral administration of Tapentadol, it was formulated into a transdermal gel utilizing pharmacosomes as a carrier system.

Pharmacosomes are the amphiphilic complexes formed by lipid drugs that contain an active hydrogen atom. These drugs bind to lipids through covalent bonds, electrostatic interactions, or hydrogen bonding. Pharmacosomes are spherical drug particles directly attached to the lipids and can exist as superfine vesicular, micellar, or hexagonal aggregates, depending on the chemical structure of the drug-lipid complex. The amphiphilic nature of pharmacosomes helps reduce the interfacial tension and enhances mesomorphic activity at higher concentrations. This reduction in interfacial tension leads to an increased contact area,



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thereby improving drug bioavailability.⁷ The pharmacosomes have unique properties like small size, amphiphilicity, active drug loading, high entrapment efficiency, and stability.⁸

Hence, pharmacosomes were selected as carriers for the transdermal delivery of Tapentadol, as they can help overcome challenges such as skin permeation of the drug.⁹

The main objective of the study was to develop a pharmacosomal transdermal delivery system for Tapentadol as a novel and effective alternative to conventional oral administration. This system aims to improve drug permeation, reduce systemic side effects, and minimize the risks of drug abuse and withdrawal symptoms, thereby decreasing the abuse potential of oral Tapentadol hydrochloride.

MATERIALS AND METHODS

Materials

Tapentadol Hydrochloride (TPL) was obtained from Symed Labs Hyderabad. Soya lecithin, Ethanol, Chloroform, and Carbopol 934P were purchased by SD Fine Chem, Pvt. Ltd., Triethanolamine was procured from Merck Limited (Mumbai, India).

Methods

Quality by Design (QbD) approach

Quality by Design (QbD) is a methodology approach for formulation development. According to ICH Q8 guidelines, which emphasize product and process understanding and process control. QbD provides a robust understanding of the product performance, which is essential for the Quality Target Product Profile (QTPP) framework, identifying Critical Quality Attributes (CQAs), and determining Critical Material Attributes (CMAs) and Critical Process Parameters (CPPs).¹⁰

Quality Target Product Profile (QTPP) Framework

The Quality Target Product Profile (QTPP) provides a detailed specification of a product that emphasizes its quality characteristics to ensure product performance, safety, and efficacy.¹¹ The QTPP was designed for a transdermal pharmacosome gel, including strength, dosage form, route of administration, container closure system, and stability. Table 1 lists the QTPP elements for the TPL transdermal gel and their justifications.

Critical Quality Attributes (CQAs)

The Critical Quality Attributes (CQAs) are those affecting the performance of the products.¹² The CQAs related to transdermal pharmacosome gels include particle size, drug entrapment, assay, impurity level, viscosity, diffusion, and permeation flux. Table 2 lists the potential CQAs affecting the performance of the pharmacosomal gel of the and their justifications.

Quality Risk Assessment (RA)

A comprehensive risk assessment was performed to determine the factors influencing the quality of the final product. Critical Process Parameters (CPPs) and Critical Material Attributes (CMAs) were identified through the Ishikawa diagram, risk estimation matrix, and determining Risk Priority Number (RPN). The Ishikawa diagram is a qualitative tool that helps in understanding the interactions and critical factors that impact the drug product performance,¹³ as presented in Figure 1. The RPN score was calculated to get the clarity to screen out the potential factors against responses. The RPN score was studied through the pareto charts based on that the factors and the responses were screened. The RPN score was estimated using the QbD add-ins version of JMP 18.0.2. The RPN was assessed and predicted for the selected factors against the responses. The variables are assigned according to their risk priority, like low, medium, and high levels, with 1,3, and 9, respectively as shown in Figure 2.

Design of Experiment - Central Composite Design (CCD)

Central Composite Design (CCD) was been selected for the optimization of pharmacosomes. Based on the risk assessment study, The factors and the responses were selected. the factors were soya lecithin and temperature, and the responses were % Entrapment efficiency, vesicle size (nm), % *in vitro* drug release. The factors were assigned at two levels, and response targets were assigned. The soya lecithin was selected as a factor because it helps in forming stable vesicles, improves drug encapsulation, and modulates release. The temperature governs the gelation, viscosity, and drug release kinetics. The selected factors, their levels, and responses were represented in Tables 3 and 4, respectively. The CCD helps in estimating the nonlinearity of responses in the given data set, maximizing information in a minimum experimental trial, operating the formulation with a minimum number of experiments, and minimizing the cost of the experiment, The CCD was constructed.¹⁴ The CCD predicted nine runs of experiments, and it was represented in Table 5.

Preparation of the Transdermal Pharmacosome Gel

Preparation of the pharmacosomes

The drug and phospholipid were taken in a beaker and dissolved in ether to form the drug lipid complex. In another beaker, the water was heated till it reached 55-60°C. The drug lipid complex was then loaded into a syringe with a fine-gauge needle and injected slowly into the hot aqueous medium to form vesicles.¹⁵

Characterization of pharmacosomes

Entrapment Efficiency

1 g of pharmacosomes was accurately measured and put in an Eppendorf tube, and diluted with saline phosphate buffer (pH 7.4). Centrifugation was performed for 1 hr at 10,000

rpm at 4°C. Further, the supernatant layer was separated, and a suitable dilution was made with the saline phosphate buffer (pH 7.4), and the drug concentration was estimated by the UV Spectrophotometer at 214 nm.¹⁶ The measurements were done in triplicate.

Vesicle Size

To determine vesicle size, 100 mg of pharmacosomes was accurately weighed and diluted with distilled water. Then, it was placed in a sonicator for 15 min to ensure uniform distribution of the particles, and then the size of the particle and its distribution were determined using the dynamic light scattering technique (Malvern zetasizer).¹⁷ The measurements were done in triplicate.

In vitro Release study

The *in vitro* release study was performed using the Franz diffusion cell apparatus, which consists of two compartments, i.e. donor compartment and the receptor compartment. The dialysis membrane (Himedia-70 mm) was placed between the two compartments, and the pharmacosomes were placed on the top of the donor compartment. The phosphate buffer 7.4 was filled in the receptor compartment and maintained at temperature of 37°C for approximately 24 hr. Then 1 of the mL sample was withdrawn from the receptor compartment through the sampling port at specified time intervals of (30 min, 1, 2, 3, 4, 5, 6, 23, and 24 hr), and replaced with the freshly prepared buffer medium and then the samples were analysed using UV spectrophotometer at 214 nm.¹⁸ The measurements were done in triplicate.

Model Fit

All nine formulations of pharmacosomes were evaluated, and the data were input into the CCD and analysed for model fit. The

data was analysed statistically using a multiple regression model to determine the importance of the factors (soya lecithin and temperature) and responses (vesicle size (nm), % entrapment efficiency, and % *in vitro* drug release). The actual vs. predicted plot was analysed for the model fit. Finally, the optimization of the formulation was done through numerical optimization using through prediction profiler. The optimized formulation was then validated.

Preparation of Pharmacosomes Gel

The 1% w/v Carbopol 934P was added little by little to the distilled water under continuous stirring for up to 1 h. To the formed gel, 5 mL of the pharmacosomes were dispersed, and then triethanolamine was added dropwise to adjust the pH. The neutralization causes the Carbopol to swell and form a stiff and clear gel encapsulating the pharmacosomes.¹⁹

Evaluation of Tapentadol-loaded Transdermal Pharmacosome Gel

pH

1.0 g of gel was weighed accurately, and the gel was transferred to a 100 mL beaker containing distilled water. The pH of the TPG was measured using the digital pH meter. The pH was measured three times, and the average was calculated.²⁰

Viscosity

The viscosity of prepared gels was measured using a Brookfield viscometer using spindle No: 4 at a controlled temperature of 25±2°C at 50 rpm for gel. The selection of spindle and rpm was done based on the trial-and-error method. The gel was filled in a jar, and the spindle was lowered perpendicularly with care to

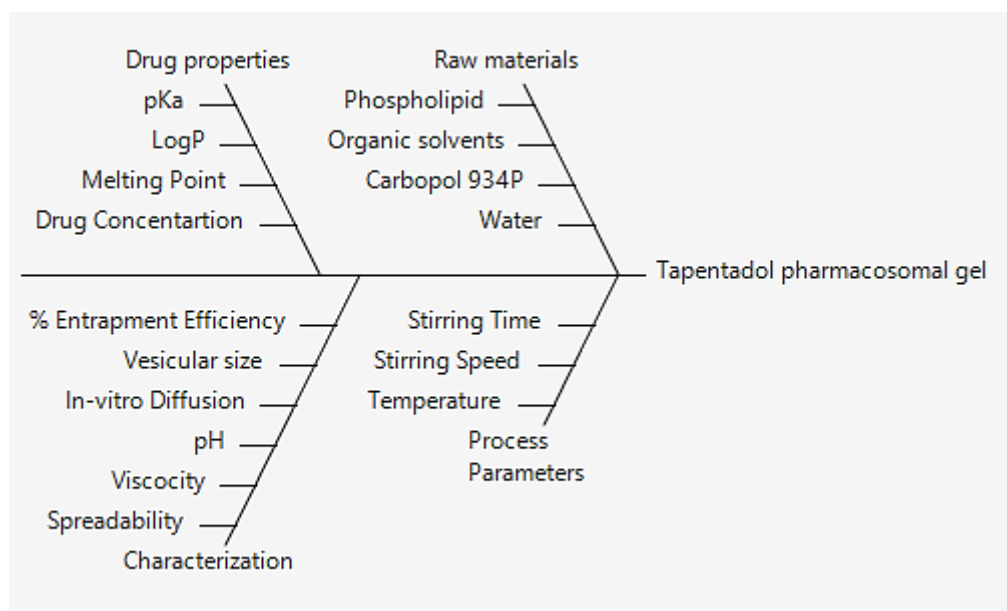


Figure 1: Cause and Effect Diagram.

Table 1: QTPP of the Tapentadol HCl Transdermal Pharmacosomes Gel.

QTPP elements	Target	Justifications
Dosage form	Gel	Ease of administration.
Dosage type	Sustained release transdermal gel	Improvement in bioavailability.
Dosage strength	64 mg	Desired therapeutic activity.
Route of administration	Topical	Skin targeted without systemic side effects.
Stability	At least 3 months shelf life at room temperature	Affect the product quality.
Container closure system	Triple laminated Aluminium child-resistant pouch	Preservation of product throughout the shelf life.
pH	Between 5 to 8	Compatible with skin.
Spread ability	Medium	Uniformly spreading on the skin.
Gel strength	Medium	Polymer concentration that determines gel strength.
Pharmacokinetic parameters	T_{max} , C_{max} , Area under the curve	Ensures the safety and efficacy of the product.

avoid the contact between the spindle and the bottom of the jar the viscosity of the gel was noted.²¹

Spreadability

The spreadability of TPG was measured using glass slides (20×20 cm). 1 g of the TPG gel was placed between the two slides, and a weight of 200 g was placed on the upper plate to compress and to obtain a uniform thickness of the gel. A measured quantity of weight 100 g was placed in the pan attached to the pulley with the help of the hook to the upper slide. The time required for the separation of the slides was calculated in seconds using the formula:

$$S=M.L/T$$

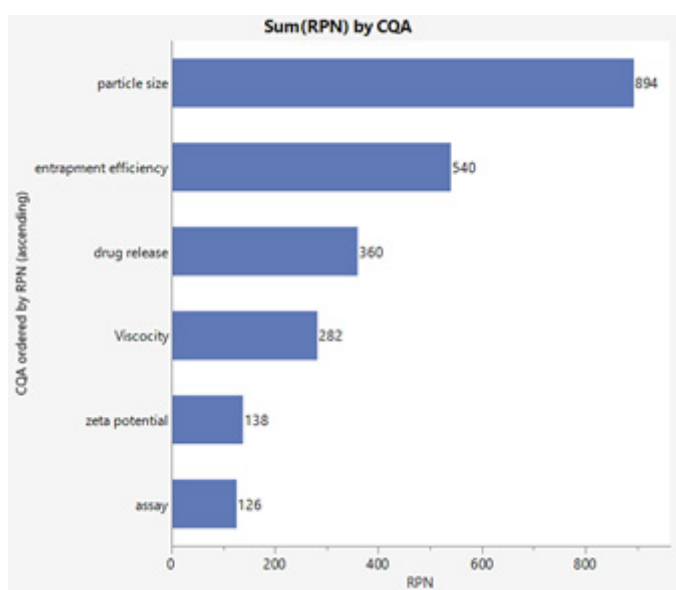
Where M=weight attached to the upper slide.

L=glass slide length.

T=slide separation time.²²

In vitro Release study

The *in vitro* release study was performed using the Franz diffusion cell apparatus, which consists of two compartments, i.e. donor compartment and the receptor compartment. The dialysis membrane (Himedia-70 mm) was placed between the two compartments, and the TPG was placed on the top of the donor compartment. The phosphate buffer 7.4 was filled in the receptor compartment and was maintained at a temperature of 37°C for approximately 24 hr. Then 1 of the mL sample was withdrawn from the receptor compartment through the sampling port at specified time intervals of (30 min, 1, 2, 3, 4, 5, 6, 23, and 24 hr), and replaced with the freshly prepared buffer medium and then the samples were analysed using UV spectrophotometer at 214 nm.²³

**Figure 2:** Risk Priority Number (RPN).

Ex vivo drug permeation studies on porcine skin

The *ex vivo* drug permeation study was performed using the Franz diffusion cell apparatus, which consists of two compartments, i.e. donor compartment and the receptor compartment. The porcine skin was placed between the two compartments, and the TPG was placed on the top of the donor compartment. The phosphate buffer of pH 7.4 was filled in the receptor compartment and was maintained at a temperature of 37°C for approximately 24 hr. Then 1 of the mL sample was withdrawn through the sampling port from the receptor compartment at specified time intervals of (30 min, 1, 2, 3, 4, 5, 6, 23, and 24 hr), and replaced with the freshly prepared buffer medium and then the samples were analyzed using UV spectrophotometer at 214 nm.²¹

Table 2: Critical Quality Attributes of Drug Product.

Quality Attributes of Drug Product	Target	CQA	Justification
Particle size	Nano meter range (50- 200 nm)	Yes	Particle size will significantly affect the permeation across the skin.
Drug entrapment	80-100%	Yes	The higher value of % drug entrapment will provide maximum permeation flux and skin retention.
Assay	100 w/w label claims total impurities on the drug	Yes	Assay affects safety and efficacy. Formulation and process variables can affect the assay; therefore, this is considered a CQA of the drug product.
Impurity level	Product to be quantified after 3 months of stability study and at least comparable to RP in the same conditions. Known impurities: NMT 0.2% and Unknown impurities: NMT 0.20%	Yes	Degradation products affect safety and efficacy. Formulation and process variables can affect the formulation of the degradation product; therefore, this is considered as a CQA of the drug product. Limits defined are based on international guidelines and consider the maximum daily dose intake.
Viscosity	Medium	Yes	The viscosity has an impact on the rate of drug release, hence should be medium.
Diffusion	Sustain release that can meet NLT 80% in 24 hr	Yes	Diffusion is critical to ensure the therapeutic effect.
Permeation flux	4-8 mg/cm ² /h	Yes	Higher permeation flux is indicative of higher cumulative amount of drug.

Kinetics Study

To understand the drug release from the TPG, the *in vitro* release data were fitted into multiple kinetic models. The correlation between the data and each model was studied.²⁴

Stability Study

Following the ICH Q1 guidelines, the 3 batches of TPG were prepared and subjected to the stability study for 9 months. The TPG was subjected to a temperature of 25°C±2°C and a Relative Humidity (RH) of 60±5%. The formulation was periodically analyzed for appearance, pH, and drug content at intervals of 0, 3, 6, and 9 months. Based on the assay of the formulation shelf life of the TPG was predicted using Minitab® version 19.²⁵

RESULTS AND DISCUSSION

Quality by Design for Tapentadol Pharmacosomal Transdermal Gel

Quality by Design

The QbD was employed to investigate the TPG formulation's QTPP and CQAs. The factors that have the most influential effects on the formulation quality are considered. The CQAs of pharmacosomes were entrapment efficiency, particle size, and % release of the drug. The risk assessment done through the Ishikawa diagram and the RPN helped in identifying and screening the significant factors that affect drug performance. The formulation variables were selected as soya lecithin and temperature based on the RPN score. CCD was constructed to evaluate the relationship

Table 3: Variables and their Limits in Central Composite Design.

Variables	Lower limit	Upper limit
Soya lecithin (mg)	159.8	319.6
Temperature °C	55	60

between the selected factors and responses mathematically. Finally, the design space was studied through the numerical optimization method.

Responses of Pharmacosomes

Entrapment Efficiency

The entrapment efficiency of the drug into the pharmacosomes states the total amount of drug that remains in the vesicle. The higher the entrapment efficiency lower the dose of drug that can be administered. The soya lecithin forms a lipid bilayer around the drug molecule, and the formed bilayer leads to increased encapsulation. The formation of the bilayer increases the entrapment of the drug and prevents it from degradation. The entrapment efficiency for all nine formulations ranges from 72.4% to 90.6% as shown in Table 5. The results showed that higher entrapment efficiency was found in the formulation containing 239.7 mg of soya lecithin. The increase in the soya lecithin concentration can lead to increased lipid accumulation, which leads to insufficient space for the entrapment of the drug. The increase in temperature can enhance their incorporation into lipids, improving entrapment efficiency and also leading to improved diffusion. The increased temperature above the optimal

level affects the formation of the bilayer structure, destabilizes the drug or carrier, and causes reduced entrapment efficiency. So, the temperature had to be optimized to maximize the entrapment efficiency.

Vesicle Size

The vesicle size of the pharmacosomes influences the drug's ability to penetrate through the skin. Soya lecithin will play an important role in the formation of vesicles. Higher concentrations of soya lecithin generally led to larger vesicles due to the availability of more lecithin molecules to form the bilayer. The vesicle size of all nine formulations ranges from 111.4 to 183 nm Table 5. The smaller vesicle size can enhance skin penetration, cellular uptake, and improve therapeutic efficiency. Higher temperatures generally lead to an increase in vesicle size. This is because the lipid bilayer becomes more fluid at higher temperatures, allowing the lipid layer to expand. There is an optimal temperature range of 55-60°C, above this temperature they transition to the liquid crystalline phase, and below it remains as a non-bilayer gel phase. The consistent temperature should be maintained to obtain the desired vesicle size and to maintain the stability over time.²⁶

Table 4: Responses in the Central Composite Design.

Responses	Goal	Lower limit	Upper limit
Entrapment Efficiency (%)	Maximize	70	95
Vesicle size (nm)	Match target	100	200
% <i>In vitro</i> drug release	Match target	50	80

In vitro Drug Release Studies

Soya lecithin significantly increases the skin permeability of drugs, which is critical for transdermal delivery. The pharmacosomes act as a potential carrier to penetrate the drug through the skin layers and increase the transdermal delivery of the drug. The *in vitro* release study for all nine formulation ranges from 53.07% to 78.37%. Thus, indicates that the pharmacosomes can easily penetrate through the skin by interacting with the lipid layers of the stratum corneum. The low drug release was seen in the formulation containing 319.6 mg of soya lecithin had the low drug release because the increased concentration of the soya lecithin can cluster the lipid bilayer and decrease the drug release. Elevated temperatures can enhance drug solubility and diffusivity, leading to accelerated release rates, Hence, the variable chosen facilitates drug diffusion Table 5.

Model Fit of Selected Response of Pharmacosomes

The R^2 and p values for the responses of entrapment efficiency, vesicle size, and *in vitro* release were 0.84 and 0.0925, 0.99873 and <0.0001, 0.99783 and <0.0001, respectively. The results indicate that it was a good model fit and a strong correlation between the actual and the predicted plot (Figure 3).

Statistical Optimization of Pharmacosomes

The numerical optimization of the formulation was done through the global desirability function value of 0.85, as shown in Figure 4. The optimized formulation predicted by the CCD had 245.47 mg of soya lecithin and a temperature of 56.60°C. This combination of the selected factors was predicted to achieve the desirable responses of vesicle size, % entrapment efficiency, and % *in vitro* drug release.

Table 5: Evaluation of Entrapment Efficiency, vesicle size, and % *in vitro* drug release for the Tapentadol HCl Formulations.

Runs of experiment	Soya lecithin (mg)	Temperature (°C)	% entrapment efficiency	Vesicular size (nm)	% <i>in vitro</i> drug release
1	159.8	55	86.6±0.15	131.3±0.97	76.425±0.07
2	319.6	60	82.3±0.20	183±1	64.020±0.06
3	159.8	57.5	86.8±0.05	146.8±0.90	74.579±0.12
4	239.7	60	85.02±0.11	120.6±0.89	58.067±0.03
5	239.7	55	90.6±0.18	111.4±0.73	78.374±0.15
6	319.6	55	72.4±0.15	166±0.85	69.064±0.09
7	239.7	57.5	84.4±0.05	136.8±0.94	56.802±0.20
8	319.6	57.5	74.09±0.02	174±0.53	53.077±0.14
9	159.8	60	88.8±0.1	126.3±0.88	66.034±0.02

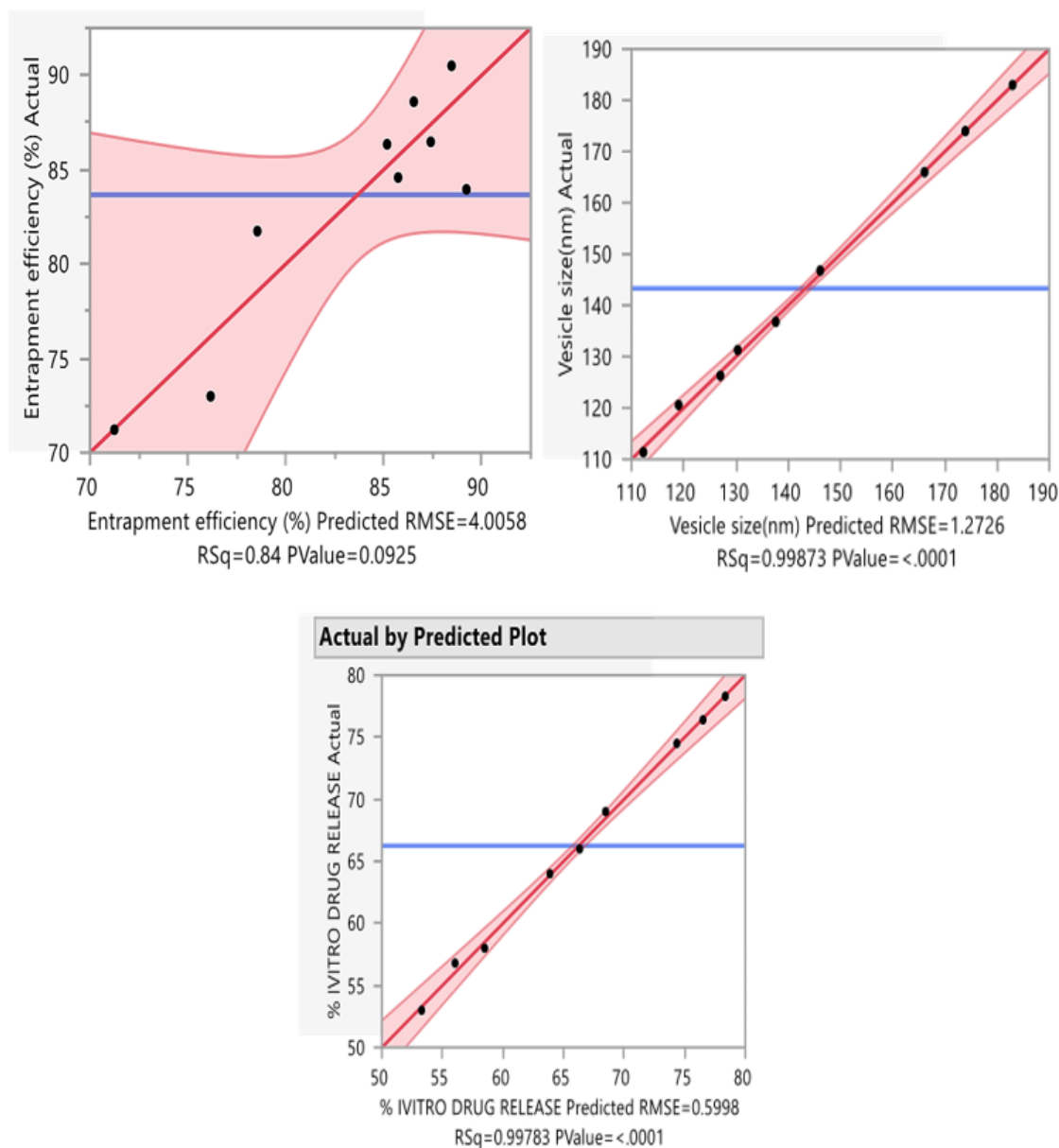


Figure 3: Actual vs Predicted Plot for Selected Responses.

Characterization of TPG

pH

In the transdermal drug delivery system, the pH plays an important role. The pH of the TPG gel was found to be 6.8, which was close to the physiological pH of the skin. So, it provides an easy penetration through the skin layers and making it suitable for the transdermal application. Thus, provides good compatibility, easy penetration, reduced skin irritation, and maintains good compatibility with the skin as shown in Table 6.

Viscosity

The Carbopol 934P had a significant role in maintaining the viscosity of the gel; the higher the concentration of Carbopol 934P, the more viscous of gel. The Carbopol 934P helps in increasing

the residence time of the TPG on the skin layer by maintaining the viscosity of the gel. The viscosity of the TPG was found to be 8,679 cps, providing a semisolid consistency and ensuring that the gel can adhere well to the skin and have prolonged contact at the application site, and enhances the residence time, which was critical for the controlled release, as shown in Table 6.

Spreadability

The coverage of the skin area and duration of the gel on the skin are assessed using the gel's spreadability. The increased spreadability of the gel over the skin surface leads to increased absorption. The spreadability of the TPG was found to be 13.75 g/cm/sec and 6.9 cm spreaded at 60 sec. The results ensure that the gel can spread over the skin properly at a minimal time and ultimately leads to increased absorption as shown in the Table 6.

In vitro Release for TPG

The drug release from the TPG was dependent on the Carbopol 934P, and was a choice of polymer for the transdermal delivery. The Carbopol 934 regulates the drug diffusion through the skin and provides controlled release. The *in vitro* drug release of the optimized TPG was found to be 89.7%, which indicates the gel effectively delivers the drug through the skin barriers and provides maximum therapeutic effect, and the results are shown in Table 6.

Kinetics Study

The drug release data were fitted to multiple kinetic models (Zero-order, First-order, Higuchi, and Korsmeyer-Peppas),

Table 6: Evaluation of the Optimized Tapentadol Hydrochloride Gel.

Parameters	Experimental report
Colour	White
Appearance	Smooth, homogeneous, viscous, and transparent
pH	6.8
Viscosity (cP)	8,679
Spread ability (gm.cm/sec)	13.75 gm.cm/sec and 6.9 cm spreaded at 60 sec
Pure drug diffusion	25.38%
<i>In vitro</i> drug release (hours)	89.7%±0.25
<i>Ex vivo</i> permeation (hours)	83.2%±0.48

and the highest correlation coefficient ($R^2=0.921$) was observed for the Zero-order model. This indicates a constant rate of drug release. The zero-order kinetics observed may be attributed to the controlled release behaviour imparted by the pharmacosomes-lipid matrix and the diffusion-modulating properties of the Carbopol gel. These factors together facilitate a steady drug release rate over 24 hr, as shown in Figure 5.

Ex vivo permeation study for the pharmacosomes gel

The *ex vivo* permeation study of the pharmacosomal transdermal gel demonstrated a cumulative drug permeation of 83.2% over 24 hr. This enhanced permeation may be attributed to the lipidic nature of pharmacosomes, which improves drug solubility and facilitates better interaction with skin lipids. Additionally, the use of Carbopol as a gel base ensures uniform distribution and prolonged contact with the skin surface, further promoting effective drug penetration. The studies showed excellent permeability and ensured that the optimized TPG can easily

Table 7: Stability Study of Optimized Formulation.

Time in month	Appearance			pH			Drug content (%)		
	B1	B2	B3	B1	B2	B3	B1	B2	B3
0	✓	✓	✓	6.9	6.9	6.8	98.54	98.50	98.34
3	✓	✓	✓	6.8	6.7	6.7	98.30	98.20	98.26
6	✓	✓	✓	6.8	6.8	6.5	98.25	97.96	98.05
9	✓	✓	✓	6.6	6.6	6.5	98.01	97.90	97.09

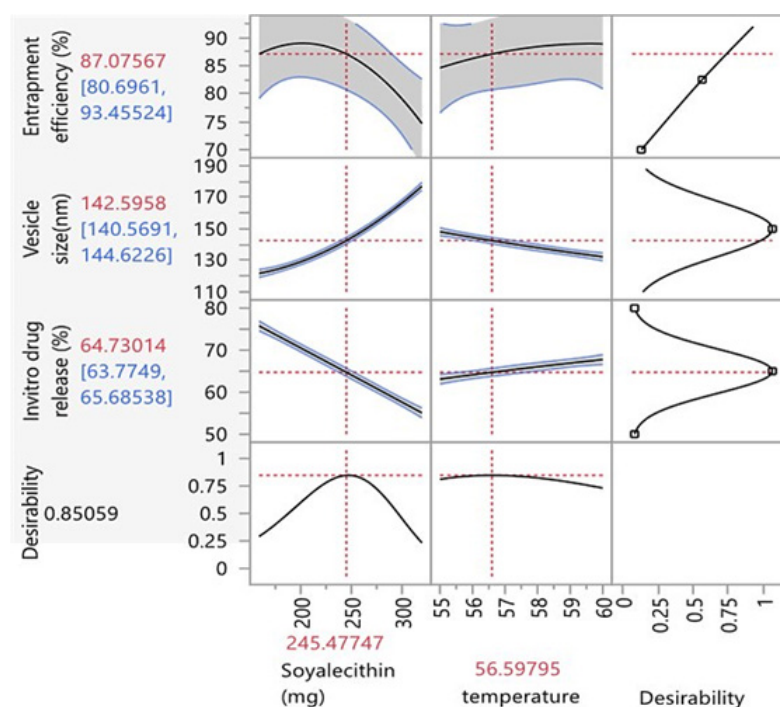


Figure 4: Prediction Profiler for Optimized Formulation.

penetrate the skin barriers. The *ex vivo* permeation studies of the optimized TPG formulation are shown in Table 6 and Figure 6.

Stability Study

The stability studies of the TPG formulation were evaluated for the parameters such as appearance, pH, and % drug content at different time intervals 0, 3, 6, and 9 months. The investigated parameters showed no significant changes, no leakage, and no degradation in the formulation, and it was determined to be physically and chemically stable. The results are shown in Table 7.

Prediction of shelf life

Shelf-life is defined as the period during which there is 95% confidence that at least 50% of the response remains within the specified limits. The shelf-life of the TPG was estimated using Minitab® software version 19 for three different batches. The batch factor was considered as a fixed parameter and shelf-life was determined individually for each batch represented in Table 8. The overall shelf-life would be the shortest estimated shelf-life among all three batches, which was found to be 25.980 months. The estimated shelf-life plot for all batches is presented in Figure 7.

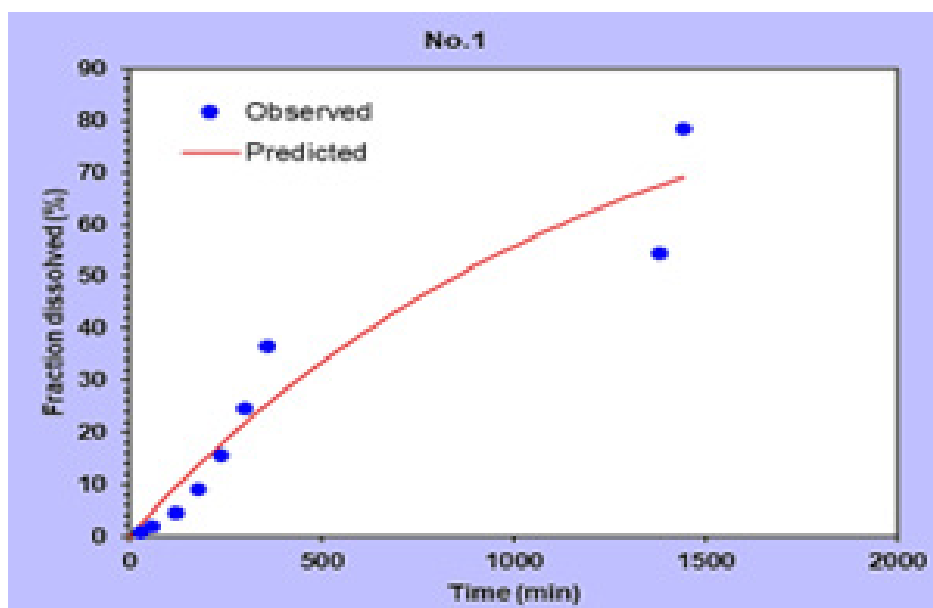


Figure 5: Zero order release kinetics of TPG.

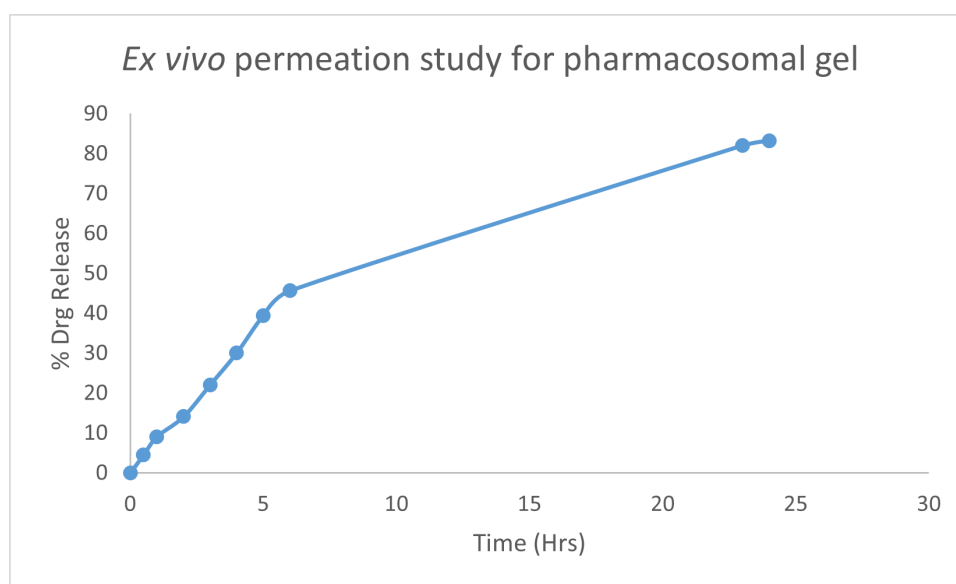


Figure 6: *Ex vivo* Permeation Study of TPG.

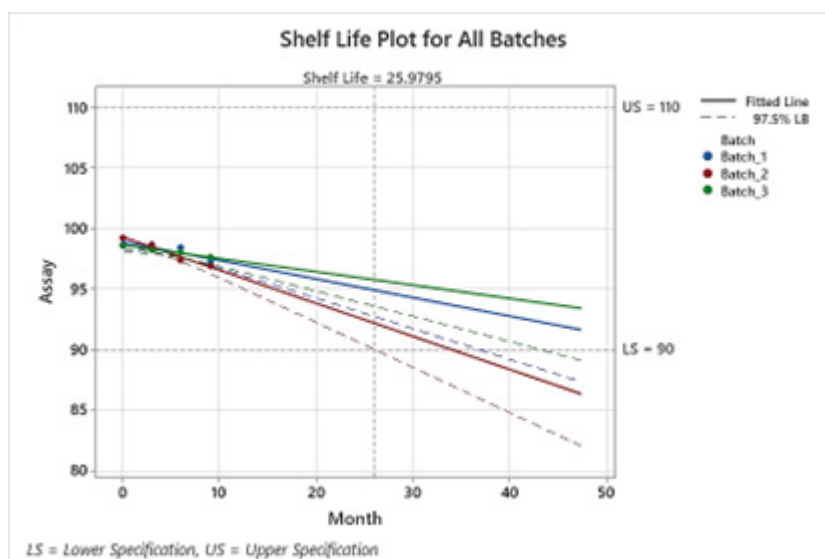


Figure 7: Overall Shelf Life of Tapentadol-Loaded Pharmacosomal Gel.

Table 8: Shelf-life estimation table.

Batch	Shelf Life
Batch 1	36.771
Batch 2	25.990
Batch 3	43.079
Batch 4	25.980

CONCLUSION

This study successfully developed and optimized a Tapentadol hydrochloride-loaded pharmacosomal transdermal gel using a Quality by Design (QbD) approach. Central composite design enabled systematic evaluation of formulation variables, identifying soya lecithin concentration and temperature as critical factors influencing vesicle size, entrapment efficiency, and drug release behavior. Pharmacosomes were prepared using the ethanol injection method and optimized through a numerical method based on a global desirability function, then incorporated into a Carbopol-based gel matrix. The *in vitro* drug release followed a zero-order kinetic model, indicating a controlled and sustained release profile. *Ex vivo* permeation studies demonstrated efficient transdermal delivery with a cumulative drug permeation of 83.2% over 24 hr evidences excellent permeability. Stability studies conducted as per ICH guidelines confirmed the physical and chemical stability of the formulation over nine months, with a predicted shelf life of 25.98 months. These results support the potential of pharmacosomal transdermal delivery as a promising alternative to oral Tapentadol administration, offering improved skin permeation, reduced systemic side effects, and minimized risk of drug abuse and withdrawal symptoms. This formulation provides a scientifically sound platform for further research in transdermal pain management and controlled drug delivery systems.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

CQA: Critical Quality Attributes; **CDC:** Centers for Disease Control and Prevention; **CCD:** Central Composite Design; **QbD:** Quality by Design; **QRM:** Quality Risk Management, **QTPP:** Quality Target Product Profile; **REM:** Risk Estimation Matrix; **SD:** Standard Deviation; **TPL:** Tapentadol hydrochloride; **TPG:** Tapentadol hydrochloride-loaded Pharmacosomal Transdermal Gel; **UV:** Ultra visible; **%CDR:** Percentage Cumulative Drug Release.

SUMMARY

The main purpose of this study was to develop Tapentadol hydrochloride transdermal pharmacosomes gel by using QbD approach. Because of the fluctuation in plasma concentration, side effects, less half-life, drug abuse, drug tolerance and withdrawal symptoms of the oral tapentadol hydrochloride. The pharmacosomes has been selected as a carrier because of its enhanced transdermal permeation. In this study central composite design has been employed in identifying the CQAs and the risk assessment was done using the Ishikawa and the risk estimation matrix (REM). The design had nine formulations. For all these batches the evaluation tests like Vesicle size (nm), % *in vitro* drug release, and % entrapment efficiency were performed. The data from all the nine formulation was interpreted and the optimized pharmacosomes formulation was successfully converted to

pharmacosomal gel using carbapol 934P and validated through a prediction profiler. Finally, the formulation was characterized.

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Formulation and Development of *Garcinia cambogia* Floating Tablet by Pelletization Technique

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ABSTRACT

Aim/Background: *Garcinia cambogia*, derived from *Garcinia gummi-gutta*, is a natural source of Hydroxycitric Acid (HCA), a bioactive compound known for its anti-obesity effects and inhibition of lipid biosynthesis. However, HCA exhibits pH-dependent instability, particularly degrading under alkaline conditions. This research aimed to formulate gastroretentive floating tablets using *Garcinia cambogia* pellets to enhance the stability and bioavailability of HCA in the acidic environment of the stomach. **Materials and Methods:** *Garcinia cambogia* extract was processed into pellets via hot-melt extrusion followed by spheronization to retain the structural integrity of HCA and minimize its degradation. These pellets were then incorporated into floating tablets using direct compression. Key excipients included Sodium Bicarbonate and Citric Acid (to generate effervescence), Microcrystalline Cellulose 101 (as a diluent), and Hydroxypropyl Methylcellulose K100 (as a release modifier). The formulated tablets were evaluated for physical characteristics such as hardness, floating lag time, total floating duration, and *in vitro* drug release. **Results:** The optimized batch demonstrated robust floating behavior, with a floating lag time of 73 sec and a total buoyancy duration exceeding 24 hr. *In vitro* dissolution testing showed a sustained release profile, with a cumulative drug release of 101.34% over 8 hr. The formulation successfully protected HCA in acidic media and ensured prolonged gastric retention. **Conclusion:** The study confirms that gastroretentive floating tablets of *Garcinia cambogia*, developed through pellet-based systems, effectively stabilize Hydroxycitric Acid and facilitate its sustained release. This approach improves HCA's therapeutic efficacy and patient compliance, highlighting its potential in advanced gastroretentive drug delivery systems.

Keywords: *Garcinia cambogia* Extract, Hydroxycitric Acid, Extrusion-Spheronization, Floating Pellets, Gastroretentive Drug Delivery System.

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INTRODUCTION

Oral dosage forms have undergone substantial advancements in the last four decades, primarily attributable to their therapeutic benefits, which encompass ease of administration, improved patient adherence, and versatile formulation alternatives. Nonetheless, the efficacy of controlled drug delivery is frequently constrained by variable drug absorption, which is affected by individual physiological parameters such as gastrointestinal transit time and the duration of drug retention within the stomach.

Systems of Gastroretentive Drug Delivery (GRDDS) have been engineered to mitigate these difficulties by facilitating an extended presence of the pharmacological agent within the

gastric environment. Such systems are especially advantageous for therapeutics exhibiting a limited absorption spectrum, those necessitating targeted action in particular segments of the GI tract, compounds that undergo degradation in the intestinal milieu, and substances characterized by inadequate solubility in intestinal fluids.¹

Among the different types of GRDDS, Floating Drug Delivery Systems (FDDS) are a well-established approach that ensures buoyancy in gastric fluids. Effervescent FDDS are commonly used, wherein gas is generated through a reaction between carbonates or bicarbonates and gastric acid, which gets trapped in polymers, ensuring floatation. FDDS are effective in achieving sustained drug release and improving oral bioavailability.¹⁻³

Both single-unit and multiparticulate gastroretentive formulations have been devised, with multiparticulate systems being favored due to their capacity to mitigate variability in drug bioavailability and diminish the likelihood of dose dumping. *Garcinia* acid (HCA), a derivative of citric acid present in flora such as *Garcinia atroviridis*, *Garcinia cambogia*, and *Garcinia*



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indica, is extensively promoted as a weight reduction adjunct. An enzyme essential to citrate metabolism and fatty acid production, ATP-citrate lyase, is competitively inhibited by HCA.⁴⁻⁷

HCA is a carboxylic acid with hydroxyl groups, making it susceptible to degradation in basic environments through deprotonation, decarboxylation, or hydrolysis. In contrast, the acidic pH of the stomach enhances its solubility and bioavailability, making it essential to maintain an acidic to neutral pH in formulations to preserve its stability. Given these characteristics, *Garcinia cambogia* is well-suited for gastroretentive drug delivery.^{8,9}

Multiparticulate formulations, such as pellets, present numerous advantages compared to single-unit dosage forms, including a diminished likelihood of drug degradation and a more consistent distribution of the active pharmaceutical ingredient. Consequently, the primary aim of this investigation is to engineer gastroretentive floating pellets containing *Garcinia cambogia* extract utilizing the extrusion-spheronization methodology. While sodium bicarbonate (NaHCO_3) served as a gas-producing agent, Ethyl Cellulose (EC) and Hydroxypropyl Methylcellulose (HPMC) were employed as release-delaying Agents.¹⁰⁻¹³

MATERIALS AND METHODS

Materials

Garcinia cambogia 60% Extract, HPMC K100, MCC 101, Ethyl Cellulose, Citric Acid, Sodium Bicarbonate, Sodium Starch Glycolate, Magnesium Stearate, Aerosil were provided by OmniActive Health Technologies, Thane, India.

HPMC K100M functions as a hydrophilic matrix former and swelling agent that, upon exposure to gastric fluids, forms a gel layer around the pellets, promoting buoyancy, sustained drug release, and structural integrity during spheronization and gastric transit. MCC 101, a widely used binder and filler, improves spheronization, enhances mechanical strength, and regulates pellet density, making it essential for forming robust, uniform pellets in floating systems. Ethyl cellulose, a hydrophobic polymer, coats the pellets to control drug release by slowing fluid penetration while also aiding in buoyancy by preventing excess water absorption. Ethanol serves as a solvent and granulating agent, enabling the uniform dispersion of ethyl cellulose and other excipients. Citric acid acts as a pH modulator and reacts with sodium bicarbonate in the acidic gastric environment to release carbon dioxide, which becomes trapped in the gel matrix formed by HPMC, thereby reducing pellet density and ensuring prolonged gastric retention through floatation.

Methods

Characterization of *Garcinia cambogia* 60% Extract

Garcinia cambogia 60% Extract was evaluated for its by HPLC methods with Chromatographic Condition: Column- 250 x 4.6

mm, 5 μm ODS (Purosphere® star or equivalent), Column Temp.- 25°C, Flow rate- 1.0 mL/min, Wavelength- 215 nm, Elution type- Isocratic.

Diluent- 30% Ortho phosphoric acid: Water (1:9), Run time- 16 min and Retention Time- (-) Hydroxycitric acid (5.0 min $\pm$ 10%). The Mobile Phase was used as 0.05 M Potassium dihydrogen orthophosphate. In the process of sample preparation, a precise quantity of the sample was measured and placed into a 50 mL dry volumetric flask, after which 35 mL of diluent were added. To guarantee full dissolution of the ingredients, the resultant mixture was sonicated for 10 min. Additional diluent was then added to make up the volume. The solution was first filtered through a 0.45 μm membrane filter before being used for analysis.

Drug-excipient compatibility study by DSC

One crucial technique for determining whether medications and excipients in *Garcinia cambogia* floating pellet formulations are compatible is Differential Scanning Calorimetry (DSC). The investigation entails a comprehensive analysis of the thermal properties of the pure pharmaceutical compound, distinct excipients, and their 1:1 physical amalgamation. Samples of pellets, weighing between 2 to 5 mg, are meticulously placed in aluminium pans and subsequently subjected to DSC analysis within a nitrogen environment at a controlled heating rate of 10°C/min. across a temperature spectrum extending from 30°C to 300°C. The generated thermograms are systematically compared to identify any potential interactions, which may be evidenced by alterations in melting points, emergence of new peaks, disappearance of existing peaks, or variations in enthalpic measurements. In the absence of significant alterations, the drug and excipients are deemed to exhibit compatibility.

Formulation of GCE Pellets

The extrusion-spheronization technique was employed to formulate floating pellets of *Garcinia cambogia* extract using ethyl cellulose and HPMC K100 as release-retardant polymers. First, a binding solution was made by dissolving ethyl cellulose in ethanol. A homogeneous powder blend was prepared by accurately weighing and mixing *Garcinia cambogia* extract, HPMC K100, MCC 101, citric acid, and sodium bicarbonate, followed by sieving through a #50 mesh. To create a wet mass with the right consistency, the ethyl cellulose solution was progressively introduced to the powder mixture. The cylindrical extrudates created by processing this wet mass via an extruder with a 1 mm opening were then promptly moved to a spheronizer, where they were rotated for 5-10 min at 1000-1500 rpm to create homogenous spherical pellets. To guarantee homogeneity, the resultant pellets were sieved through a #20 mesh screen. They were then dried in a hot air oven set between 40 and 50°C until the required moisture content was reached. For later analysis and manufacture into floating tablets, the final dried pellets were kept in an airtight container.^{3,14,15}

Experimental Design

In this approach, two factors were assessed at three levels each, and experimental trials were carried out utilizing all nine potential combinations with Design Expert Software. In this study, the concentrations of HPMC K100 M (A) and sodium bicarbonate (B) were selected as independent variables. The disintegration time and the floating lag time were selected as the dependent variables. A statistical model with interactive and polynomial terms was used to evaluate the response.

$$Y = b_0 + b_1 \cdot A + b_2 \cdot B + b_{12} \cdot AB + b_{11} \cdot A^2 + b_{22} \cdot B^2$$

The dependent variable in this instance is Y, the arithmetic mean response is b_0 , and the estimated coefficients for factors A and B are b_1 and b_2 , respectively. The major effect (A and B) represents a change in one factor at a time from a low to a high value. The interaction term (AB) shows responses to simultaneous changes in two components. The polynomial terms (A^2 , B^2) used to analyze nonlinearity are included in Tables 1 and 2. Nine formulations with varying quantities of components were developed, as shown in Table 3. The dependent variables are Y1 (disintegration time) and Y2 (floating lag time, in seconds).¹⁵

Formulation of Floating Tablets

The tablets were then prepared by Direct Compression. After passing through sieve number 40, sodium starch glycolate, magnesium stearate, MCC 101, GCE Pellets, and aerosil were all carefully measured. The components were ground in a geometric pattern using a mortar and pestle for 10 min. Tablets were then made by compressing the mixture. As shown in Table 4, the ingredients were taken.^{11,12}

Evaluation Parameters of Pellets

Particle Size Distribution

The sieve method is a straightforward technique used to determine the particle size distribution of a dry sample. In this method, a set of sieves with decreasing mesh sizes-such as sieve numbers 16, 18, 20, and 24-are stacked with the coarsest (No. 16) on top and the finest (No. 24) at the bottom, followed by a pan to collect finer particles. A known weight of the sample is poured into the top sieve and the entire stack is shaken either manually or using a mechanical shaker for about 10-15 min. After shaking, the material retained on each sieve is carefully weighed. The percentage of material retained on each sieve is then calculated to determine how the particles are distributed across different sizes. This information is used to assess the material's grading and suitability for specific applications.

Angle of Repose

To assess the flow characteristics of the formulation, the angle of repose for *Garcinia cambogia* floating pellets was computed using the funnel method. To make a conical pile, carefully weighed pellets were placed in a funnel and let drop freely onto a flat surface. Following measurements of the cone's height (h) and radius (r), the angle of repose (θ) was calculated using the following formula:

$$\text{Angle of repose } (\theta) = \frac{h}{r}$$

The angle of repose provides insight into the flow characteristics of the pellets, with lower values indicating better flowability, which is essential for efficient processing and tableting.

Table 1: Variables in 3² factorial designs.

Independent Variables	Levels		
	-1	0	1
HPMC K100 M (A)	5%	7.5%	10%
Sodium Bicarbonate(B)	12.5%	15%	17.5%

Table 2: Factorial design of pellet batches.

Batch no.	A	B	Actual Values(mg)	
F1	-1	-1	5	12.5
F2	-1	+1	5	17.5
F3	+1	-1	10	12.5
F4	+1	+1	10	17.5
F5	0	-1	7.5	12.5
F6	+1	0	10	15
F7	0	+1	7.5	17.5
F8	-1	0	5	15
F9	0	0	7.5	15

Table 3: Composition of Factorial batches of GCE Floating Pellets.

Ingredients	F1(gm)	F2(gm)	F3(gm)	F4(gm)	F5(gm)	F6(gm)	F7(gm)	F8(gm)	F9(gm)
<i>G. Cambogia</i> Extract	50	50	50	50	50	50	50	50	50
HPMC K100M	5	5	10	10	7.5	10	7.5	5	7.5
MCC 101	10	10	10	10	10	10	10	10	10
Ethyl Cellulose	10	10	10	10	10	10	10	10	10
Ethanol	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.
Citric acid	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5
Sodium Bicarbonate	12.5	17.5	12.5	17.5	12.5	15	17.5	15	15
Total	100 g	100 g	100 g	100 g	100 g	100 g	100 g	100 g	100 g

Drug Content Uniformity

A precisely weighed quantity of 500 mg crushed pellets was diluted in 100 mL of 0.1N HCl, sonicated, filtered, and put through an examination using High-Performance Liquid Chromatography (HPLC) to determine the drug's concentration. The drug content was expressed as a percentage of the total amount of drug in the formulation.

In vitro Floating Study

Using 900 mL of 0.1N HCl at $37 \pm 0.5^\circ\text{C}$ and USP dissolving equipment II, floating behavior was evaluated. The floating lag time, or the time it took for the pellets to start floating, and the total floating duration were visually recorded after a specific weight of pellets was added to the medium.

Evaluation of Tablets

Weight Variation Test

Using a digital balance, twenty tablets were chosen at random and weighed separately. To guarantee uniformity, the average weight and percentage deviation from the mean were computed.

Hardness Test

A Monsanto hardness tester was used to evaluate the tablets' mechanical strength. The kg/cm^2 force needed to shatter the tablet was noted.

Thickness and Diameter

The thickness and diameter of the tablets were measured with a Vernier caliper to ensure consistent tablet measurements.

Friability Test

Tablet friability was evaluated using a Roche friabilator. For 4 min, a weighted sample of tablets was rotated at 25 rpm, and the weight loss % was noted. Less than 1% was regarded as an acceptable friability value.

Table 4: Tablet Composition of GCE Floating Pellets.

Ingredients	For 1 Tablet	For 25 Tablets
GCE Pellets	833 mg (Drug 250 mg)	20.825 g (Drug 6.25 g)
MCC 101	150 mg	3.750 g
Sodium Starch Glycolate	10 mg	0.250 g
Magnesium Stearate	6 mg	0.150 g
Aerosil	1 mg	0.025 g
Total	1000 mg	25 g

Drug Content Uniformity

A weighed amount of crushed *Garcinia cambogia extract* pills were diluted in 100 mL of 0.1N HCl, sonicated, filtered, and then exposed to HPLC analysis in order to determine the consistency of the drug content.

Disintegration Test

The USP Disintegration Apparatus (Basket Rack Assembly) is used in the test, which mimics the conditions of gastric fluid in a 0.1N HCl solution (pH 1.2). In order to replicate the physiological conditions of the stomach, the medium's temperature is kept at $37 \pm 0.5^\circ\text{C}$.

In vitro Drug Release Study

In 0.1N HCl at $37 \pm 0.5^\circ\text{C}$, dissolution testing was carried out at 50 rpm using a USP class II dissolving apparatus. Samples were taken out at predetermined intervals, filtered, and subjected to HPLC analysis. To determine whether there were any differences in the drug release characteristics, the release profile was contrasted with floating pellets.

Release Kinetic Study

To identify the drug release mechanism and best-fit model, the dissolution data were fitted into several kinetic models, including zero-order, first-order, Higuchi, and Korsmeyer-Peppas models.

RESULTS AND DISCUSSION

This study aimed to develop and assess gastroretentive floating tablets of *Garcinia cambogia* using a palletization approach to produce sustained drug release and prolonged stomach retention. Floating Drug Delivery Systems (FDDS) offer a practical way to improve drug absorption by keeping stomach contents buoyant, and the potential of GRDDS to increase bioavailability by prolonging drug retention in the stomach has been a topic of much interest in recent years, particularly for medications with a limited window for absorption.

Characterization of *Garcinia cambogia* 60% Extract

(-)-Hydroxycitric Acid (HCA) in *Garcinia cambogia* 60% extract was quantitatively evaluated under isocratic conditions using High-Performance Liquid Chromatography (HPLC). The separation was carried out at 25°C using a mobile phase of 0.05 M potassium dihydrogen orthophosphate flowing at 1.0 mL/min

on a 250×4.6 mm, 5 µm ODS (Purosphere® star or equivalent) column. The analysis took place at 215 nm and lasted 16 min.

Following a precise weighing and dissolution in a 30% ortho phosphoric acid: water (1:9) diluent, the extract sample was sonicated for 10 min, filtered through a 0.45 µm membrane, and then introduced into the HPLC apparatus. The resulting chromatogram (Figure 1) displayed a distinct peak for (-)-Hydroxycitric acid at 5.0 min (±10%) retention time, characterized by its sharp and symmetrical shape. A stable baseline throughout the analysis confirmed the absence of significant matrix interference. The strong absorbance at 215 nm indicated robust (-)-Hydroxycitric acid response under these conditions, demonstrating the method's sensitivity and specificity. The use of isocratic elution and straightforward sample preparation ensures method robustness and reproducibility for routine (-)-Hydroxycitric acid analysis in *Garcinia cambogia* extracts. This USP validated HPLC method offers a dependable approach for quality control and standardization of herbal formulations containing *Garcinia cambogia*.¹⁶

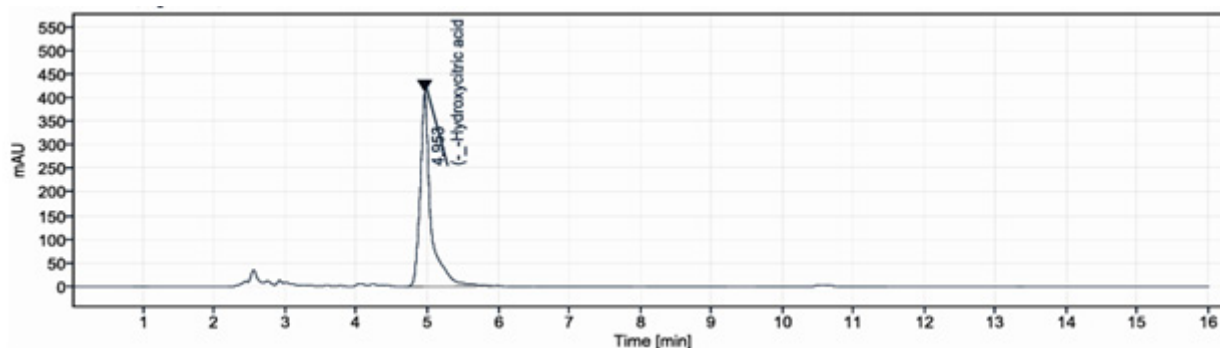


Figure 1: Chromatogram of *Garcinia Cambogia* Extract.

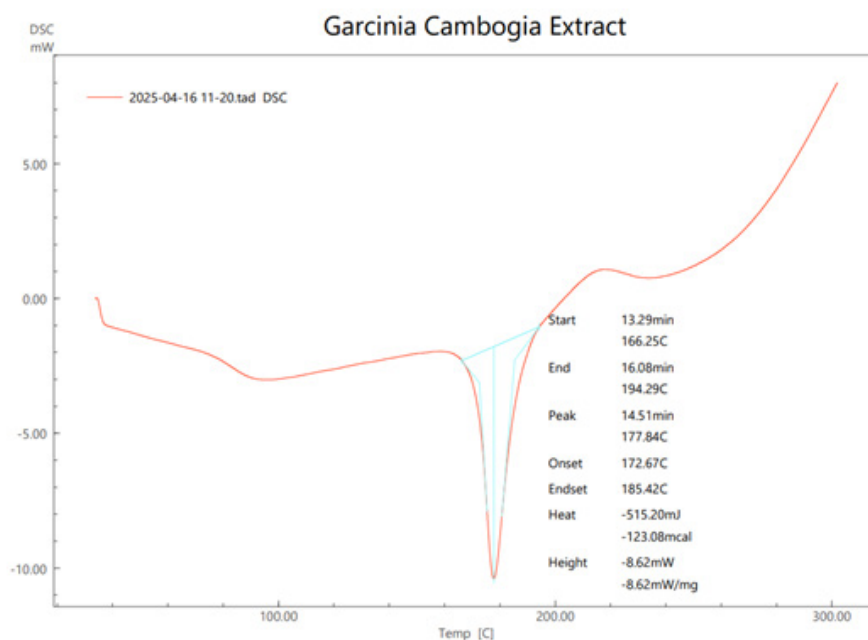


Figure 2: DSC of *Garcinia cambogia* Extract.

Drug-excipient compatibility study

The thermal behavior of the pure drug and its mixture with excipients was analyzed using Differential Scanning Calorimetry (DSC). The melting point of *Garcinia cambogia* extract showed at 176-178°C. The endothermic peak of Extract was observed from 166.25-194.29 °C with peak at 177.84°C (Figure 2). In the DSC thermogram of the pellets, a slight shift in the melting

endothermic peak was observed at 180.20°C (171.16-185.19°C) (Figure 3). The comparison is shown in Figure 4.

The drug's combination with excipients may be the cause of these tiny changes in peak position and broadening, which could marginally lower the purity of the constituent parts. However, the absence of significant changes in the thermal profile suggests that no strong interactions or incompatibilities exist between the drug and the selected polymers. Thus, it was concluded that *Garcinia*

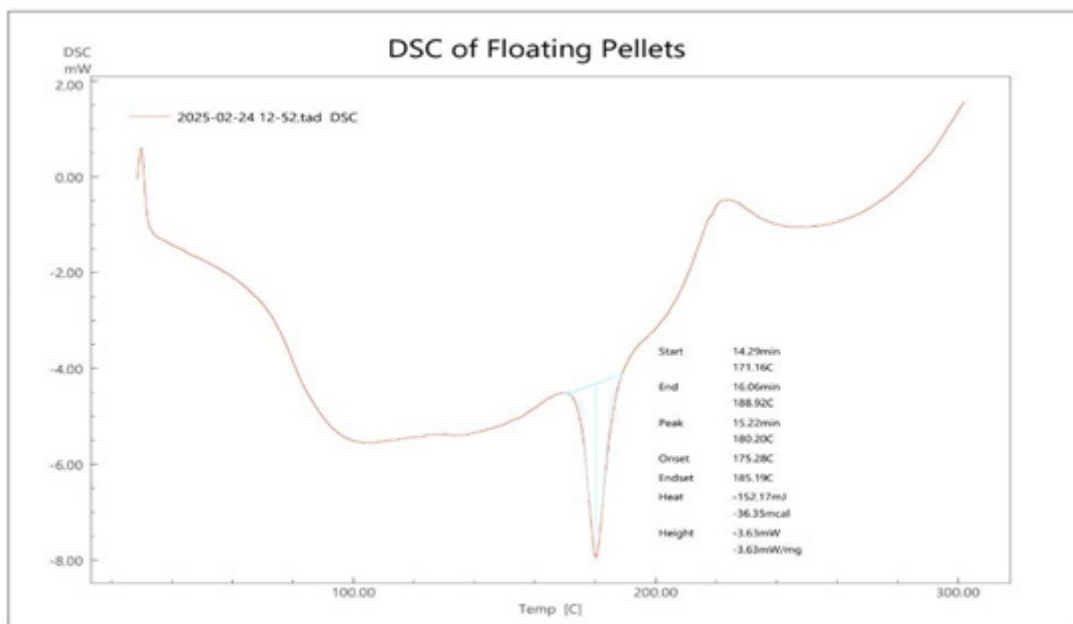


Figure 3: DSC of Pellets.

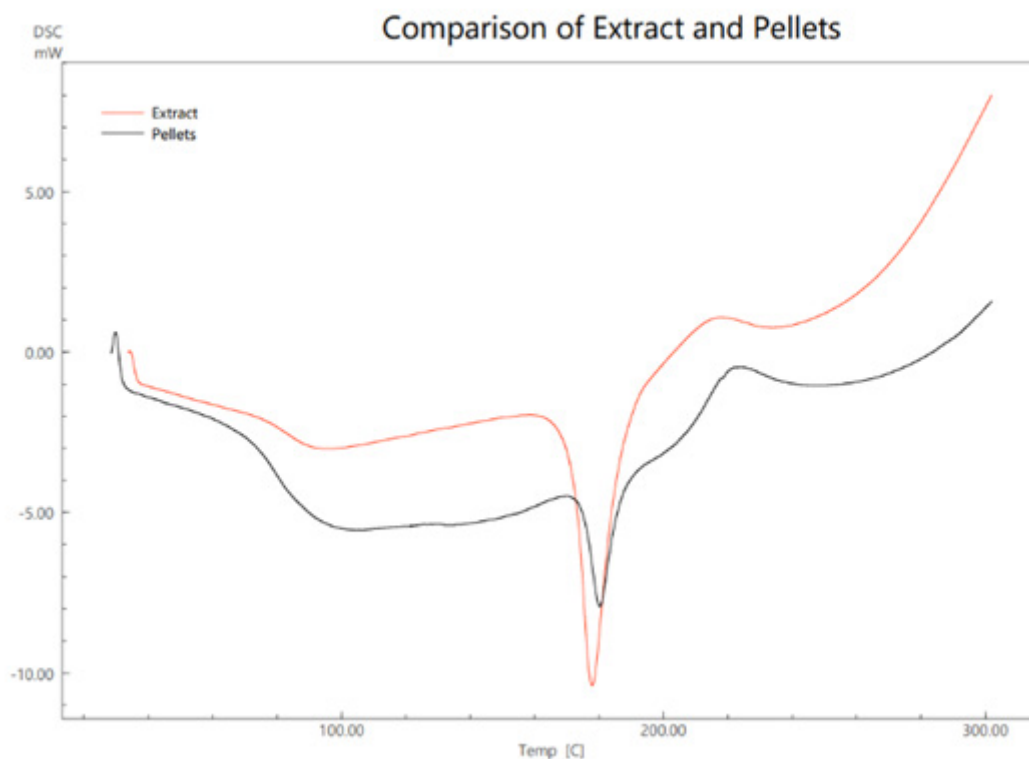


Figure 4: Comparison of Extract and Pellets.

cambogia extract and the excipients used in this formulation were compatible.

Evaluation of *Garcinia cambogia* floating pellets

By adding sodium bicarbonate as a gas-forming agent and Hydroxypropyl Methylcellulose (HPMC K100) and ethyl cellulose as release-delaying agents, the extrusion-spheronization method successfully produced the floating pellets. The Produced Formulations demonstrated an average particle size ranging from 0.998 mm to 1.050 mm (Table 5), which is comparable to other reported multiparticulate systems designed for gastric retention.

Previous studies have also shown that smaller pellet size enhances uniform gastric distribution, thereby reducing inter-individual variability in drug absorption. Also, the F9 batch showed perfectly Spherical and strong pellets. Other batches either had irregular shapes or were very brittle.

The floating behavior study revealed that the developed formulations exhibited floating lag times between 68.24 and 111.47 sec, with a total floating duration exceeding 12 hr. These findings are in line with earlier research on herbal extract formulations that are gastroretentive, where sodium bicarbonate was essential for producing gas and maintaining buoyancy.

Table 5: Particle Size Distribution of Floating Pellets.

Batch no.	% Retained (%)				Mean (mm)
	#16	#18	#20	#25	
F1	6±1.13	45±1.58	28±0.50	21±0.95	1.005±0.048
F2	4±1.10	46±1.31	29±0.78	21±1.02	0.998±0.028
F3	3±1.81	49±1.84	31±0.63	17±0.56	1.007±0.057
F4	7±1.94	42±1.30	29±1.54	22±0.97	1.002±0.022
F5	8±0.81	42±1.82	26±0.54	24±1.51	1.003±0.046
F6	5±0.75	54±1.82	28±0.65	13±1.13	1.033±0.029
F7	7±1.70	52±1.95	35±0.97	6±1.54	1.050±0.041
F8	6±0.72	53±0.64	34±0.78	7±1.02	1.046±0.026
F9	7±1.13	49±0.34	34±0.71	10±0.80	1.039±0.039

Table 6: Evaluation of GCE Floating Pellets.

Batch no.	Particle Size Distribution(mm)	Angle of Repose	Drug Content Uniformity (%)	Floating Lag Time (secs)
F1	1.005±0.048	26.4±0.51°	99.14±0.091	90.15±3.45
F2	0.998±0.028	24.5±0.2°	99.35±0.122	75.64±1.32
F3	1.007±0.057	27.2±0.36°	99.65±0.036	111.47±1.67
F4	1.002±0.022	28.8±0.25°	99.24±0.053	84.78±3.24
F5	1.003±0.046	24.8±0.11°	98.89±0.015	78.87±4.33
F6	1.033±0.029	26.4±0.36°	99.74±0.034	96.24±5.14
F7	1.050±0.041	25.1±0.57°	99.98±0.054	68.24±3.11
F8	1.046±0.026	24.3±0.34°	99.47±0.056	77.35±4.33
F9	1.039±0.039	24.4±0.24°	99.83±0.08	73.33±4.55

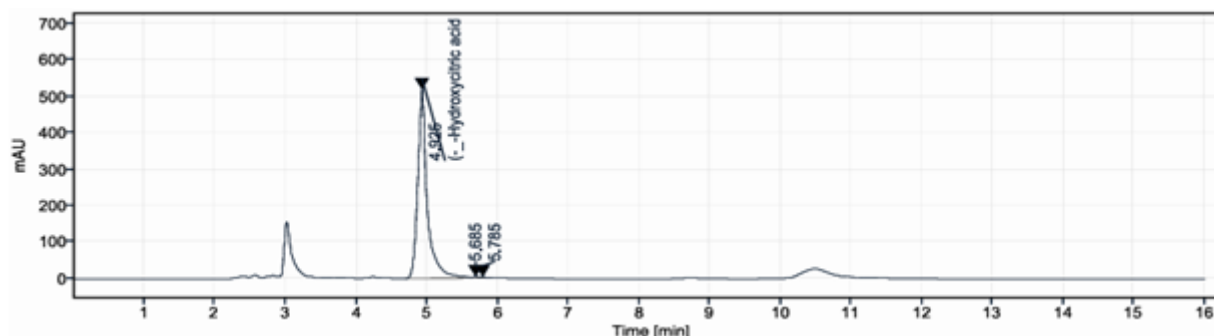


Figure 5: Chromatogram of Floating Pellets.

Similar studies have reported that increasing sodium bicarbonate concentration leads to faster floating initiation due to enhanced CO₂ generation, which aligns with the findings of this study.¹⁶ The drug content from pellets was analyzed by HPLC for which one of reference chromatogram for F9 pellets is shown in Figure 5. The drug content was found to be in the range 98.89 to 99.98%. Table 6 represents the evaluation of the pellets.

Evaluation of *Garcinia cambogia* Tablets

The produced formulations showed sustained drug release, with the drug release percentage ranging from 89.9% to 96.6% over 6 hr, according to the *in vitro* drug release research (Figures 6 and 7). The release kinetics followed a first-order model (Figure 8), indicating that the drug release was concentration-dependent. This conduct is in line with previously documented floating formulations of bioactive plant extracts in which the release rate was controlled by polymeric matrices like ethyl cellulose and HPMC. The swelling index study also confirmed that the tablet matrix gradually absorbed gastric fluid, contributing to the sustained release effect.

The factorial design analysis confirmed that both HPMC K100 and sodium bicarbonate significantly influenced floating lag time and disintegration time (Table 7). The model was significant ($p < 0.05$), according to statistical analysis, which supported the validity of the experimental results. Higher concentrations of HPMC

K100 led to prolonged drug release, while increased sodium bicarbonate levels improved buoyancy but slightly reduced disintegration time due to enhanced effervescence. By applying factorial design tablet formulation F9 showed desirability value 1 and therefore it is selected as an optimized formulation of tablet (Table 8). ANOVA for Floating lag time and Disintegration time was studied (Tables 9 and 10).

$$\text{Floating Lag Time (Y1)} = 71.78 + 8.23 \cdot A - 8.64 \cdot B - 3.04 \cdot AB + 15.79 \cdot A^2 + 2.55 \cdot B^2$$

$$\text{Disintegration Time (Y2)} = 123.07 + 8.442 \cdot A + 8.363 \cdot B + 1.85 \cdot AB + 0.215 \cdot A^2 + 3.57 \cdot B^2$$

The study has many limitations in spite of these encouraging findings. The complex environment of the real stomach may not have been accurately replicated by the simulated gastric settings used for the *in vitro* floating and drug release tests. Individual physiological differences, food consumption, and digestive tract movement are some of the variables that could affect the formulation's *in vivo* performance. Furthermore, additional pharmacokinetic research is required to confirm the formulation's bioavailability and therapeutic efficacy in humans, even if it demonstrated better retention and release characteristics.

Future research should focus on conducting *in vivo* studies to evaluate the gastric retention time and absorption profile of

Table 7: Evaluation of Floating Pellets.

Batch no.	Disintegration Time (secs)	% Drug Release (1 hr)	% Drug Release (3 hr)	% Drug Release (6 hr)
F1	111.99±2.14	30.1±1.09	68.8±1.44	92.5±1.37
F2	124.57±3.16	32.7±1.3	62.5±1.11	95.1±0.83
F3	125.24±3.53	30.4±0.93	68.1±0.85	93.5±1.04
F4	145.22±3.27	25.1±0.76	63.4±1.24	95.6±1.02
F5	118.03±1.24	26.3±0.52	68±0.57	96±0.66
F6	131.86±2.33	31.2±1.05	66.7±0.86	96.6±1.34
F7	135.65±2.14	26.4±0.79	66.7±0.92	93.2±1.11
F8	115.11±3.74	32.5±0.63	64.8±1.44	89.9±0.99
F9	122.67±3.86	30.1±0.29	66.7±0.85	93.01±0.82

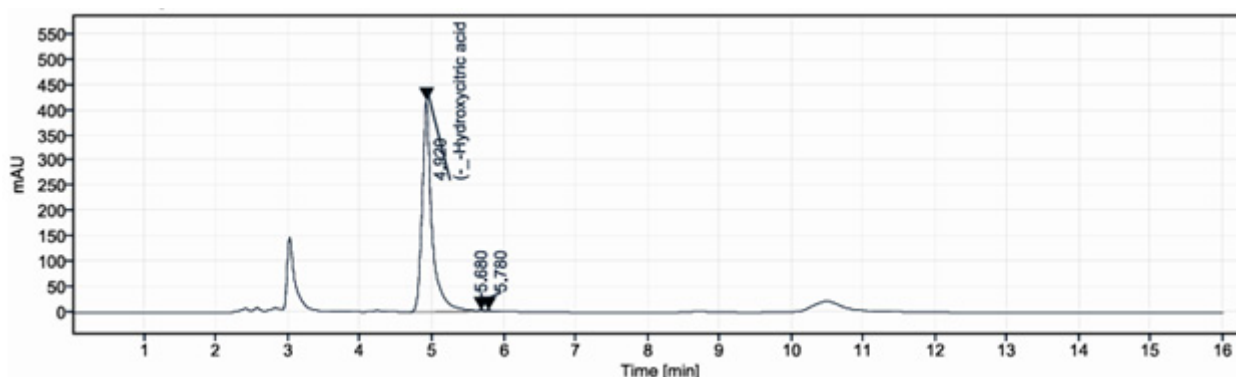


Figure 6: Chromatogram of Tablet.

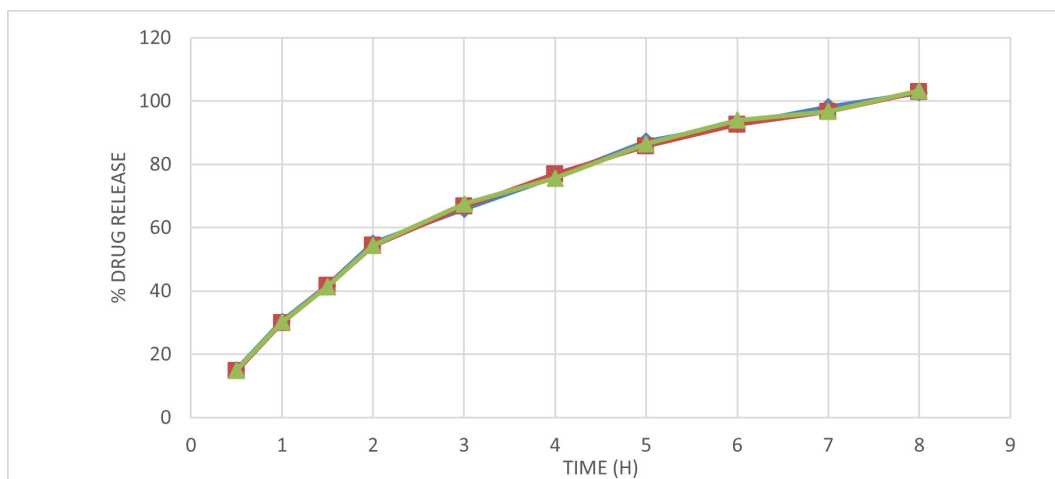


Figure 7: % Drug Release for Optimized F9 Batch Floating Tablets.

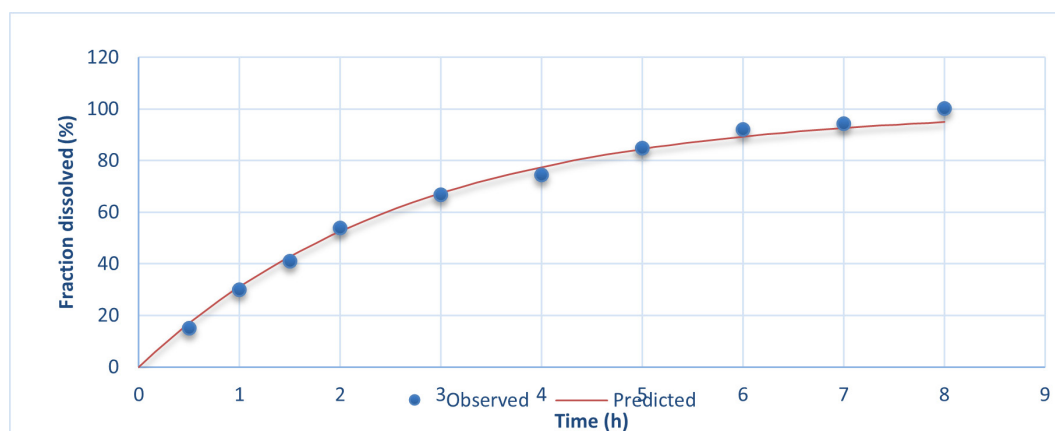


Figure 8: Drug Release Kinetic (First Order Kinetics).

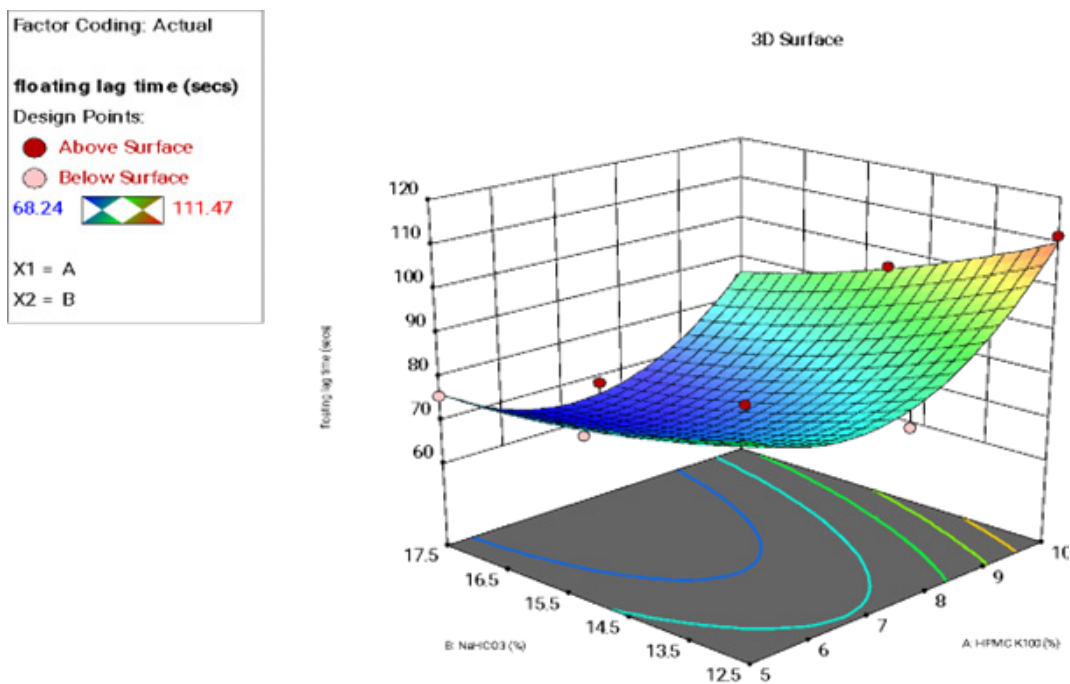


Figure 9: 3D Surface graph for Floating Lag Time.

Table 8: Evaluation of Optimized F9 Batch Floating Tablets.

Parameters	Values
Avg. Weight	1000.89±0.016 mg
Hardness	4.77±0.419kg/cm ²
Length	18 mm
Diameter	5 mm
Thickness	4 mm
Friability	0.36%
Floating Lag Time	73±4.55 sec
Disintegration Test	122.67±3.86 sec

Table 9: Optimization Table.

	Intercept	A	B	AB	A ²	B ²
Floating lag time	71.7789	8.225	-8.63833	-3.045	15.7917	2.55167
<i>p</i> -values		0.0130	0.0113	0.2061	0.0097	0.4109
Disintegration time	123.07	8.44167	8.36333	1.85	0.215	3.57
<i>p</i> -values		<0.0001	<0.0001	0.0074	0.6302	0.0030

Table 10: ANOVA for Floating Lag Time.

Source	Sum of Squares	d _f	Mean Square	F-value	<i>p</i> -value	
Model	1402.49	5	280.50	19.56	0.0170	Significant
A-HPMC K100	405.90	1	405.90	28.31	0.0130	
B-NaHCO ₃	447.72	1	447.72	31.23	0.0113	
AB	37.09	1	37.09	2.59	0.2061	
A ²	498.75	1	498.75	34.79	0.0097	
B ²	13.02	1	13.02	0.9083	0.4109	
Residual	43.01	3	14.34			
Cor Total	1445.50	8				

Table 11: ANOVA for Disintegration Time.

Source	Sum of Squares	d _f	Mean Square	F-value	<i>p</i> -value	
Model	886.51	5	177.30	547.31	0.0001	Significant
A-HPMC K100	427.57	1	427.57	1319.84	<0.0001	
B-NaHCO ₃	419.67	1	419.67	1295.46	<0.0001	
AB	13.69	1	13.69	42.26	0.0074	
A ²	0.0924	1	0.0924	0.2854	0.6302	
B ²	25.49	1	25.49	78.68	0.0030	
Residual	0.9719	3	0.3240			
Cor Total	887.49	8				

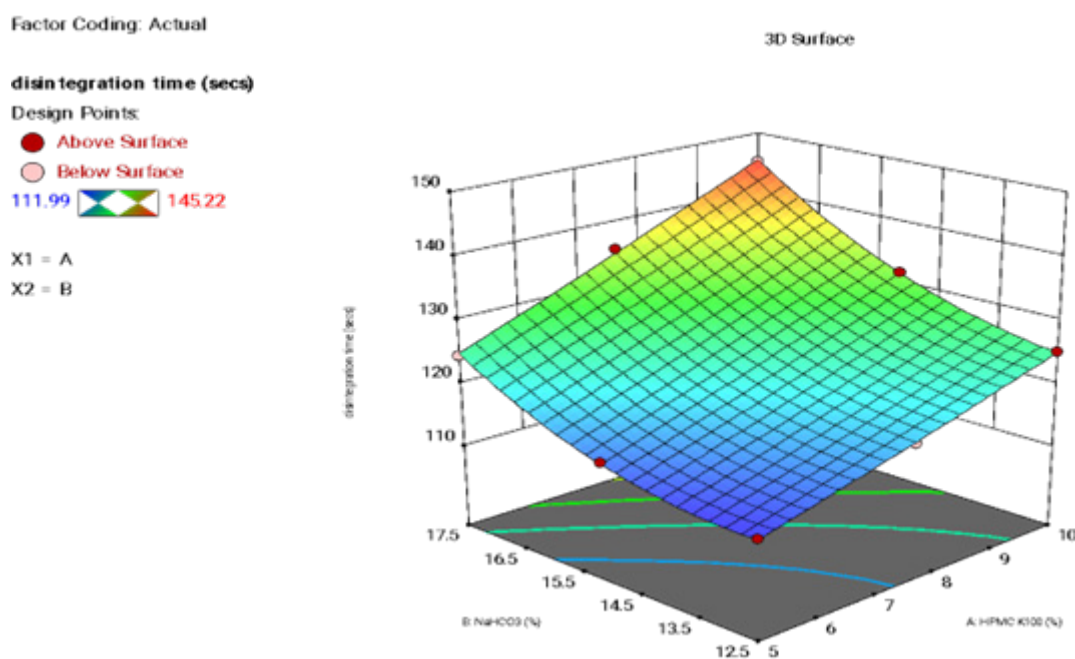


Figure 10: 3D Surface graph for Disintegration Time.

the developed formulation. Additionally, exploring alternative natural polymers or mucoadhesive agents may further enhance drug retention and improve patient compliance. Advanced imaging techniques, such as gamma scintigraphy, could also be employed to track the movement and behavior of floating tablets in the gastrointestinal tract.

CONCLUSION

The present study successfully formulated and developed gastroretentive floating tablets of *Garcinia cambogia* using the pelletization technique. The findings demonstrated that Hydroxy Propyl Methyl Cellulose (HPMC K100) and ethyl cellulose effectively controlled drug release, while sodium bicarbonate ensured buoyancy by generating gas for floatation. The factorial design analysis confirmed that both the concentration of HPMC K100 and sodium bicarbonate significantly influenced floating lag time (Figure 9) and disintegration time (Figure 10).

The optimized formulation exhibited prolonged gastric retention, improved drug release kinetics, and desirable mechanical properties, making it a promising candidate for controlled delivery of *Garcinia cambogia* extract (Table 11). The *in vitro* floating and dissolution studies indicated sustained drug release, following first-order kinetics, which aligns with the desired therapeutic effect. Furthermore, drug-excipient compatibility studies confirmed stability without significant interactions.

In conclusion, the developed gastroretentive floating pellets and tablets provide an effective approach to enhance the bioavailability of *Garcinia cambogia* by ensuring prolonged gastric retention. These findings support further *in vivo* evaluation and potential

clinical application of this formulation for weight management and other therapeutic benefits.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

GIT: Gastrointestinal Tract; **GRDDS:** Gastroretentive Drug Delivery Systems; **HCA:** Hydroxycitric Acid; **HCl:** Hydrochloric Acid; **HPLC:** High-Performance Liquid Chromatography; **HPMC:** Hydroxypropyl Methylcellulose; **MCC:** Microcrystalline Cellulose.

SUMMARY

In summary, the developed gastroretentive floating tablet of *Garcinia cambogia* demonstrated promising results in terms of buoyancy, sustained drug release, and formulation stability. With further *in vivo* validation, this approach could serve as a potential strategy for improving the bioavailability and therapeutic efficacy of *Garcinia cambogia* in weight management and related health applications.

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Bergenin Protects against Stroke by Modulating Neuroinflammatory Pathways in the Brain: An *in silico* Approach

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ABSTRACT

Aim/Background: Ischemic stroke is a leading cause of mortality and disability globally, primarily caused by cerebral artery occlusion that triggers complex pathological cascades. Existing interventions like thrombolysis with rt-PA or thrombectomy are limited by narrow therapeutic windows and significant risks. Thus, there is an urgent need for adjunctive neuroprotective strategies. Bergenin, a phytopharmaceutical from *Bergenia* species, is known for its antioxidant, anti-inflammatory, and neuroprotective properties, but its exact mechanisms in stroke remain underexplored. **Materials and Methods:** This study employed systems biology and network pharmacology approaches to identify bergenin's molecular targets and mechanisms in stroke therapy. *In silico* target prediction (PharmMapper, ChemMapper, SwissTargetPrediction) identified 59 potential targets. Stroke-associated genes ($n=4561$) were retrieved from DisGeNET, GeneCards, and OMIM. Twenty overlapping targets were analyzed via Gene Ontology (GO) and KEGG enrichment. PPI network and compound-target-pathway mapping identified 10 hub proteins. Molecular docking and Molecular Dynamic (MD) simulations evaluated binding affinity and stability. **Results:** Docking revealed strong binding of bergenin to GSK3 β , CDK2, MMP9, and MAPK14. MD simulations confirmed complex stability with GSK3 β and MMP9. KEGG analysis implicated MAPK, PI3K-Akt, and IL-17 pathways in its neuroprotective mechanism. **Conclusion:** Bergenin likely mitigates stroke-induced neurotoxicity by targeting neuroinflammatory signaling pathways. This study provides a scientific foundation for future preclinical and clinical research on bergenin as a multitargeted neuroprotective agent in ischemic stroke.

Keywords: Stroke, Bergenin, Network pharmacology, Molecular docking, Dynamic simulation, MMP9, GSK3 β , Neuroinflammation.

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INTRODUCTION

Stroke is the leading cerebrovascular disease, characterized by high mortality, morbidity, and incidence. As per the World Stroke Organization (2022), stroke claims 6.5 million lives annually, equating to twenty every minute. It is classified into ischemic and hemorrhagic types, with ischemic strokes comprising 62% of cases.¹ These arise from a sudden blockage of blood flow in a cerebral artery, leading to oxygen and glucose deprivation, subsequent neuronal damage, and neurological deficits. Stroke imposes a heavy socioeconomic burden, leaving patients with severe disabilities and long-term physical, psychological, and social consequences. It is medical emergency and current treatments, such as thrombolytic therapy using rt-PA and

surgical thrombectomy, are limited by strict time windows, contraindications, and risks like hemorrhagic transformation, leaving a critical need for alternative or adjunctive neuroprotective therapies.²

Emerging evidence highlights the potential of phytopharmaceuticals for preventive and supportive therapy to reduce disease burden and speed up post stroke recovery. Owing to complex pathophysiology of stroke, novel compounds capable of safely targeting multiple ischemic pathways such as excitotoxicity, oxidative stress, neuroinflammation, and neuronal cell death are urgently required to develop effective and holistic stroke therapies. Plants are an incredible reservoir of promising secondary metabolites and byproducts with diverse biological activities. Among these, bergenin has garnered significant attention in the 21st century pharmacological research. This bioactive compound is widely distributed across various plant genera within the Euphorbiaceae, Saxifragaceae, and Myrsinaceae families. *Bergenia* species have been extensively used in Asian



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traditional medicine, including Ayurveda, Unani, and various folk remedies since 7th century. They have been traditionally employed to treat conditions such as lithiasis, dysuria, polyuria, dysentery, muscular pain, bronchitis, menorrhagia, gonorrhea, and syphilis.³ In many cases, bergenin is the key active compound responsible for the therapeutic properties of its plant sources. It possesses diverse pharmacological properties, including antioxidant, anti-inflammatory, antiplatelet, antihyperlipidemic, antiapoptotic, and neuroprotective activities.⁴ Our screening for novel anti-ischemic agent highlights bergenin's potential as a neuroprotective candidate against cerebral ischemia-reperfusion injury, attributed to its multi-targeted therapeutic approach. However, there is no comprehensive scientific evidence for diverse therapeutic roles and underlying mechanisms of action.

Network pharmacology sprang up as a cornerstone research tool in modern drug discovery, providing a multidimensional view of "drug-target-gene-pathway" interactions. Rooted in systems biology and multi-directional pharmacology, it offers a comprehensive framework to investigate the holistic mechanisms of action of traditional medicines, including polyherbal formulations. Additionally, it enables the exploration of potential therapeutic targets and the underlying mechanisms of single multitarget compounds for treating complex diseases. However, validating the results of network pharmacology analysis by virtual and/or experimental methods is crucial to confirm the efficacy of predicted drug-target interactions. Given the high cost and time-intensive nature of *in vivo* and *in vitro* studies, *in silico* computational techniques, such as molecular docking and dynamics simulation, have gained prominence as efficient and reliable alternatives.⁵ These techniques facilitate the screening of predicted targets by enabling the detailed analysis of binding affinities and molecular interactions between drug candidate and amino acid residues within the active sites of target proteins.

The present study aimed to determine the key targets and pathways involved in bergenin's neuroprotective effects against ischemic stroke and provide a robust scientific basis for future pharmacological research at molecular level.

MATERIALS AND METHODS

ADME analysis of bergenin

Qikprop module of Schrodinger suite, Maestro v13.0 (D.E. Shaw Research, NY 2021) was utilized to predict ADME properties of bergenin.

Identification of Biological Targets of Bergenin

To identify potential targets of bergenin, three established web servers with distinct algorithms were employed. 1) PharmMapper applies pharmacophore mapping across databases like TargetBank, DrugBank, BindingDB, and PDTD.⁶ Filters were set

to "Human protein targets" (max: 1,000), and UniProt IDs were retrieved. 2) ChemMapper utilizes SHAFTS-based 3D similarity matching, integrating ~300,000 annotated structures and over 3 million compounds from major bioactivity databases (DrugBank, BindingDB, ChEMBL, PDB).⁷ Human-specific targets with a similarity threshold of 1.2 were selected, and duplicates removed. 3) SwissTargetPrediction combines 2D/3D similarity against ~3,70,000 bioactive molecules across 3,000 proteins.⁸ The top 100 human targets were extracted. Overlapping targets from all three servers were visualized via a Venn diagram to determine the most probable biological targets of bergenin. Corresponding gene symbols were retrieved using UniProtKB's ID mapping tool.

Collection of stroke disease targets

Three bioinformatic platforms were utilized to gather known stroke-related targets using suitable filters and specific keywords 'cerebral ischemia' and 'ischemic stroke'. 1) Disease Gene Network (DisGeNet) is a publicly accessible database with gene-disease associations.⁹ 2) Online Mendelian Inheritance in Man (OMIM), a comprehensive compendium of human genes and Mendelian disorders, provided data on over 16,000 genes.¹⁰ Entrez IDs obtained were converted to UniProt IDs. 3) GeneCards, integrating data from ~150 sources, provided detailed information on annotated and predicted human genes.¹¹ GeneCards IDs were converted to UniProt IDs. A consolidated list was prepared by removing duplicates.

Acquisition of therapeutic targets of bergenin against stroke

A Venn diagram intersected bergenin's biological targets with known stroke targets, identifying common targets which may be the key therapeutic targets.

Construction of Protein-Protein Interaction (PPI) network

A PPI network was developed for key therapeutic stroke targets of bergenin in STRING web portal¹² (medium confidence level=0.4) for human proteins. The constructed network was further analyzed using Cytoscape 3.9.1, and hub nodes exhibiting connectivity above the average degree were selected for docking studies.

Gene Ontology (GO) enrichment/ KEGG pathway enrichment analysis

DAVID bioinformatics database facilitates gene function annotation and enrichment analysis.¹³ GO analysis identified enriched categories, including Biological Processes (BP), Cellular Components (CC), and Molecular Functions (MF). KEGG pathway analysis explored gene interactions or gene products within biological pathways, revealing functional pathways associated with bergenin's therapeutic targets for stroke.

Construction of 'bergenin-target-pathway' network

Cytoscape 3.9.1 was used to build a compound-target-pathway network,¹⁴ linking bergenin with its key therapeutic stroke targets and KEGG pathways. CytoHubba plugin analyzes network topological features, such as degrees and centrality measures, to reveal the potential complex molecular mechanisms underlying the antiischemic properties of bergenin. Hub targets with degrees above the average were prioritized for docking studies.

Molecular Docking

Key hub proteins, identified via STRING PPI and bergenin-stroke target-pathway networks, were docked with bergenin and their standard ligands using GLIDE-XP (Schrödinger Maestro 13.0). Protein structures were sourced from RCSB-PDB and refined with the OPLS-2005 force field via Protein Preparation Wizard. Selection criteria included human, non-mutant proteins with co-crystallized non-peptide ligands and resolution <2.5 Å. Bergenin's 2D structure was converted to 3D and optimized using LigPrep. Receptor grids were generated around co-crystal ligands or predicted active sites via Sitemap. Docking scores <-5.0 kcal/mol were considered favorable; <-7.0 indicated strong binding.¹⁵

Molecular dynamics simulation

Selected ligand-protein complexes were subjected to 100 ns Molecular Dynamics using DESMOND (Schrödinger). Systems were built in a $10 \times 10 \times 10$ Å orthorhombic box with TIP3P water, neutralized with Na^+/Cl^- ions, and energy-minimized using OPLS-2005. Simulations were run under the NPT ensemble (300 K, 1 bar), with Nose-Hoover thermostat and Martyna-Tobias-Klein barostat (1 ns relaxation), using the RESPA integrator. System equilibration preceded simulation. Trajectories were analyzed for root mean square deviation (RMSD), root mean square fluctuation (RMSF), secondary structural elements (SSE), protein-ligand contact profiles, and interaction types/subtypes. Ligand-specific parameters included torsion angles, radius of gyration (rGyr), intra-molecular hydrogen bonds (intraHB), Molecular Surface Area (MolSA), Solvent-Accessible Surface Area (SASA), and Polar Surface Area (PSA).

RESULTS

ADME analysis of bergenin

QikProp analysis predicted bergenin's physicochemical and ADME properties to be within acceptable ranges (Table 1). It showed zero Lipinski's rule violations and medium oral absorption, supporting its potential as a drug-like candidate.

Identification of biological targets of bergenin

PharmMapper, ChemMapper, and SwissTargetPrediction identified 326, 504, and 106 human protein targets for bergenin, respectively. After deduplication, 59 overlapping targets were identified as its most probable biological targets.

Collection of stroke disease targets

To identify potential stroke-related therapeutic targets, three databases-DisGeNET, OMIM, and GeneCards were queried, yielding 116, 1,081, and 4,085 genes, respectively. After removing duplicates, a total of 4,561 unique stroke-associated genes were compiled for further analysis.

Acquisition of therapeutic targets of bergenin against stroke

A Venn diagram was constructed to intersect bergenin's 59 predicted targets with the 4,561 stroke-related genes, revealing 20 common targets (Figure 1A). Their UniProt IDs were mapped via DisGeNET to obtain gene and protein names. These were further analyzed through network and enrichment studies to explore their functional roles and molecular pathways in stroke therapy.

Construction of PPI Network

The PPI network of the 20 therapeutic targets comprised 20 nodes and 51 edges, with edge thickness indicating interaction strength (Figure 1B). Cytoscape analysis identified seven hub proteins-MMP9, EGFR, PTPN1, STAT1, PGR, MAPK14, and KDR-based on degree values exceeding the average of 5.1. These were selected for molecular docking studies.

Gene Ontology enrichment/KEGG pathway enrichment analysis

GO enrichment revealed 13 BP, 10 CC, and 15 MF ($p \leq 0.01$) among the 20 genes. Bar and line plots display GO terms by gene percentage and $-\log_{10}P$, respectively (Figures 1C-E). KEGG analysis identified 17 significant pathways ($p \leq 0.01$), visualized in a bubble chart showing pathway names, rich factor, gene count and $-\log_{10}P$ (Figure 2A).

Construction of 'Bergenin-target-pathway' network

A network connecting bergenin, its 20 stroke-related targets, and 17 KEGG pathways was constructed (Figure 2B). Using the CytoHubba plugin, centrality analysis identified seven key hub targets- EGFR, GSK3 β , CDK2, MMP9, MAPK14, MET, and STAT1 (degree ≥ 6.5) for docking analysis.

Molecular docking

To validate network-based findings, molecular docking was performed using GLIDE-XP. Bergenin and respective standard drugs were docked against ten non-redundant hub proteins: MMP9, EGFR, PTPN1, STAT1, PGR, MAPK14, KDR, GSK3 β , CDK2, and MET. Docking details including target genes, PDB IDs, docking scores, respective standard drugs used, binding energies, interaction types, and key residues, are summarized in Table 2.

Bergenin showed favorable binding (≤ -5 kcal/mol) with most targets, except PTPN1 and STAT1, and strong affinity (≤ -7 kcal/

mol) with PGR, GSK3 β , and CDK2. Notably, GSK3 β and EGFR exhibited stronger binding affinity with bergenin than their standard ligands. Figures 3A-J depict the 3D binding poses. Based on interaction strength and neuroprotective relevance, bergenin-GSK3 β and bergenin-MMP9 complexes were selected for further simulation studies.

Molecular dynamics simulation

To assess complex stability, 100 ns MD simulations were conducted for bergenin with GSK3 β and MMP9.

Bergenin in complex with GSK3 β

RMSD averaged ~ 2 Å for the apo protein and ~ 6.5 Å for the complex, indicating minimal structural fluctuations and stable binding over time (Figure 4A). RMSF analysis showed high flexibility (>5 Å) in ALA 382, GLN 295, PRO 294, and PHE 293, none of which were binding-site residues, thus not affecting stability. The complex maintained 23.66% α -helices and 9.28% β -sheets (total SSE: 32.94%).

Hydrogen bonds were formed with ILE 62, LYS 85, ASP 133, VAL 135, PRO 136, TYR 140, LYS 183, GLN 185, ASN 186, and ASP 200; hydrophobic contacts with PHE 67, ASN 70, ALA 83, and LEU 188. Water-bridges and polar interactions involved above 20 residues including ARG 141 and ASP 200. The complex maintained ~ 8 contacts consistently (max: 12), with PHE 67 contributing 59% of the interaction time and ILE 62 contributing 43%. Ligand properties included average rGyr: 3.48 Å, MolSA: ~ 270 Å², SASA: 100 Å², and PSA: ~ 270 Å².

Bergenin in complex with MMP9

RMSD for the protein averaged ~ 2 Å and ~ 2.5 Å for the complex, suggesting good conformational stability (Figure 4C). RMSF exceeded 5 Å for PHE 110 and GLU 111, both involved in binding, possibly affecting local interaction strength. Other flexible residues (e.g., GLY 213-215) were non-critical to binding. SSEs comprised 21.25% α -helices and 14.81% β -strands (total: 36.16%).

Key interactions included hydrogen bonds with GLY 186, GLU 402, and TYR 423; hydrophobic contacts with PHE 110, LEU 187/188, and HIS 401; and water-bridges with residues like GLU 111, ASP 182, and TYR 423 (Figure 4D). Ionic interactions, especially zinc-mediated, were consistently observed with HIS 401, HIS 405, and HIS 411 (100% contact time). GLU 402 maintained 40% interaction time. Ligand parameters were similar to the GSK3 β complex (rGyr: 3.48 Å; MolSA: ~ 270 Å²; SASA: 100 Å²; PSA: ~ 270 Å²).

DISCUSSION

Ischemic stroke resulting from reduced blood flow to a brain region due to the blockade of supplying artery is a medical emergency with high mortality and morbidity.¹ Treatment with

surgical thrombectomy and/or thrombolytics suit a very few patients owing to their association with severe complications like haemorrhagic transformation, contraindications, and adverse effects.² Though recanalization of the blocked artery is crucial for survival of brain tissue, it turns out to be detrimental with reperfusion associated neurotoxicity.^{2,16} Extensive scientific research indicates supportive holistic neuroprotection offered by phytopharmaceuticals in order to combat reperfusion injury. Several phytochemicals have demonstrated potential in mitigating ischemic damage in preclinical studies; however, only a handful have successfully progressed to clinical application.¹⁷ The search for novel neuroprotective moieties remains the need of the hour. Owing to the complex pathophysiology of cerebral ischemia-reperfusion injury, a compound that simultaneously targets multiple pathophysiological processes, rather than focusing on a single pathway, is more likely to offer effective neuroprotection.¹⁶ One such phytochemical is bergenin, a secondary metabolite derived from the *Bergenia* genus, known for its diverse biological activities and pharmacological properties. Recent research has provided strong molecular-level evidence supporting the neuroprotective potential of bergenin through various signaling pathways. Bergenin has been shown to enhance the Reelin signaling pathway in Alzheimer's disease models, reducing amyloid-beta aggregation, oxidative stress, neuroinflammation, and apoptosis, thus improving spatial memory.¹⁸ Additionally, bergenin activates the PPAR- γ pathway and inhibits NF- κ B signaling in models of high glucose-induced neuronal damage, offering protection against diabetic neuropathy by reducing inflammation and oxidative stress.¹⁹ In an hemorrhage model, bergenin was found to activate the Nrf2/HO-1 pathway, bolstering antioxidant defenses and promoting

Table 1: ADME analysis of bergenin.

Property/ Descriptor	Value	Acceptable range
mol MW (g/mol)	328.275	130 to 725
donor HB	5	0 to 6
accept HB	12.05	2 to 20
QPlogPo/w	-1.564	-2 to 6.5
Rule of five	0	Maximum 4
QPlogS	-1.711	-6.5 to 0.5
QPPCaco (nm/sec)	39.273	<25 poor and >500 great
Human oral absorption	2	Low, 2-Medium and 3- High
QPlogBB	-2.031	-3 to 1.2
QPPMDCK (nm/sec)	14.953	<25 poor and >500 great

mol MW- molecular weight, donor HB- hydrogen bond donors, accept HB- hydrogen bond acceptors, QPlogPo/w- log octanol/water partition coefficient, QPlogS- aqueous solubility, QPlogBB- brain/blood partition coefficient, QPPCaco-2- Caco-2 (Cancer coli-2) cell permeability and QPPMDCK- apparent MDCK (Madin-Darby Canine Kidney) cell permeability.

neuronal survival.²⁰ Bergenin activates the PI3K/Akt pathway in Parkinson's disease models, promoting neuronal survival and mitigating dopaminergic neuron loss.²¹ Furthermore, in a study by Zhang *et al.*, in an ischemic stroke model, bergenin upregulates the Sirt1/FOXO3a pathway, contributing to cellular stress response and functional recovery.²² This laid important groundwork and formed the basis for our present study which succeeded in expanding the mechanistic understanding of bergenin by elucidating its capacity to modulate interconnected, temporally dynamic neurovascular pathways critical to stroke

progression and recovery. These findings collectively highlight bergenin's broad neuroprotective actions, supporting its potential as a therapeutic agent for neurodegenerative diseases and stroke.

Notably, study by Oliveira *et al.*, provides valuable insights into the safety profile and dosing of bergenin. In their research, a single high oral dose of 2,000 mg/kg of bergenin was administered to mice, and over a 14-day observation period, no toxic signs were observed. The mice exhibited normal behavior and physiological functions, indicating a favorable safety profile. Additionally, the

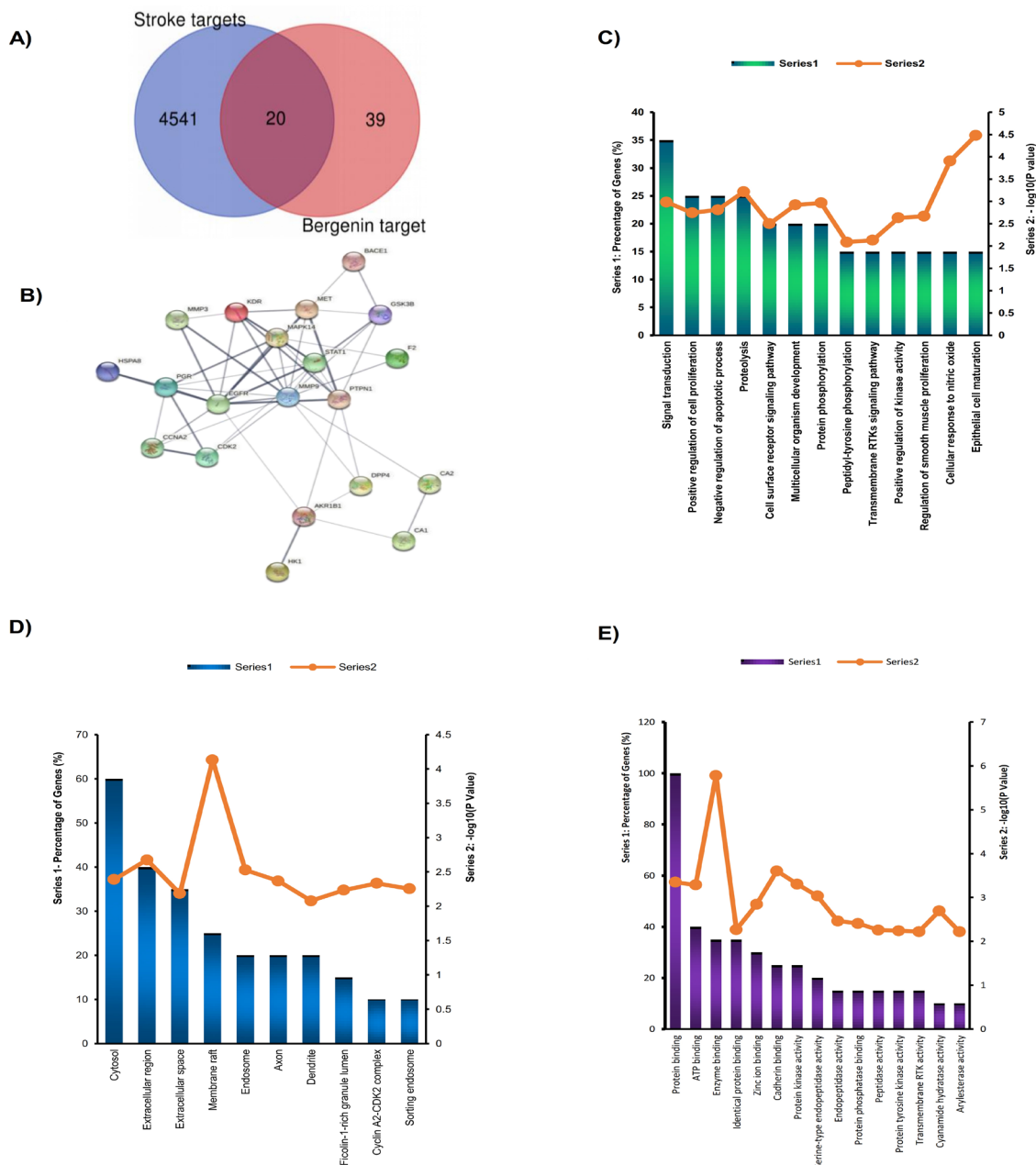


Figure 1: STRING PPI and GO functional enrichment analysis of therapeutic target genes of bergenin: A) Venn diagram depicting intersection of stroke targets and bergenin targets, B) STRING PPI network, C) GO analysis-Biological Processes, D) Cellular Components, and E) Molecular Functions.

Table 2: Molecular docking studies.

	Gene (PDB Id)	Bergenin		Respective standard drug Docking score (kcal/mol)	No. and type of interactions	Amino acid residues involved
		Docking score (kcal/mol)	Glide energy (kcal/mol)			
1	MMP9 (1GKC)	-5.48	-41.03	-7.4 (SB-3CT)	2 H-bonds	GLY 186 and GLU 402
2	EGFR (4JQC)	-7.56	-40.92	-6.06 (Erlotinib)	4 H-bonds	LYS 721, MET 769, THR 766 and ASP 831
3	PTPN1 (1PXH)	-4.01	-28.23	-5.28 (Dephostatatin)	4 H-bonds	ASP 48, LYS 116 and LYS 120
4	STAT1 (1YVL)	-4.59	-34.43	-4.68 (Fludarabine)	5 H-bonds 1 Pi-cation	ARG 331, THR 327, GLU 559, LYS 566 and LYS 567
5	PGR (1E3K)	-8.48	-45.78	-11.8 (Mifepristone)	2 H-bonds 1 Pi-Pi stacking	ASN 719, GLN 725 and PHE 778
6	MAPK14 (3FLY)	-5.71	-41.53	-6.81 (VX-702)	4 H-bonds 1 H-bond with H ₂ O	ALA 34, ASP 112, SER 154, ASN 155 and ASP 168
7	KDR (3VO3)	-5.88	-44.05	-6.85 (Cabozantinib)	2 H-bonds	GLU 885 and ILE 1044
8	GSK3 β (5K5N)	-7.47	-39.59	-6.91 (TWS119)	3 H-bonds	ILE 62 and VAL 135
9	CDK2 (6GUE)	-7.06	-48.45	-7.91 (GW8510)	3 H-bonds	LEU 83, ASP 86 and ASP 145
10	MET (3F66)	-6.15	-41.05	-9.54 (PF-02341066)	3 H-bonds 1 Pi-Pi stacking 1 Pi-cation	ILE 1084, LYS 1110, ASP 1164, ARG 1208 and TYR 1230

MMP9-matrix metallo-proteinase9, EGFR- epidermal growth factor receptor, PTPN1- protein tyrosin phosphatase 1B , STAT1- signal transducer and activator of transcription 1, PGR- progesterone receptor, MAPK14- mitogen activated protein kinase 14, KDR- vascular endothelial growth factor receptor 2, GSK3 β - glycogen synthase kinase 3 beta, CDK2- cyclin dependent kinase 2 and MET- hepatocyte growth factor receptor.

study evaluated the anti-inflammatory efficacy of bergenin at a dose of 25 mg/kg, further supporting its therapeutic potential at this lower dose.²³

The present study employed a systematic approach leveraging a suite of renowned web based and software tools to uncover the intricate relationships between bergenin-targets-pathways. QikProp analysis indicated that bergenin exhibits medium human oral absorption and compliance with the Lipinski rule without any violations, implying favorable drug like properties (Table 1). From the venn diagram between 59 potential biological targets of bergenin and the 4,561 potential therapeutic targets for stroke, 20 overlapping target genes were identified (Figure 1A). The STRING PPI network analysis identified seven key hub target proteins. KEGG enrichment analysis highlighted 17 pathways (Figure 2A), which were used to construct a 'bergenin-target-pathway' network, revealing seven hub target proteins. All

hub target proteins from both networks were further validated through molecular docking (Figures 3A-J). Eight out of ten hub proteins- MMP9, GSK3 β , CDK2, MAPK14, EGFR, KDR, MET and PGR (Table 2) showed good binding affinity (<-5 kcal/mol). It is notable here that, all these targets are an integral part of the top enriched biological processes from GO analysis which include; signal transduction, positive regulation of cell proliferation, negative regulation of the apoptotic process, proteolysis, and cell surface receptor signaling pathways. Also, they were implicated in the most stroke relevant enriched KEGG pathways like MAPK signaling, PI3K-Akt signaling and IL-17 signaling pathway.

Stroke often presents with sudden neurological impairments caused by disrupted cerebral blood flow, which initiates a cascade of events including oxidative stress, neuroinflammation, apoptosis, and significant disruption of the Blood-Brain Barrier (BBB), contributing to neurotoxicity. Bergenin may

help counter these effects by targeting key proteins such as MMP9, which worsens stroke outcomes by compromising the BBB and promoting inflammation. MMP-9 activation is also linked to tPA-induced hemorrhagic transformation post the 3-hr therapeutic window, suggesting that MMP-9 inhibitors could enhance tPA's effectiveness of ischemic stroke treatment. In a rat embolic focal ischemia model, minocycline when given along with tPA successfully widened the therapeutic window. Compounds such as minocycline, atorvastatin, melatonin, and emerging agents like SB-3CT have shown MMP-9 inhibition, though selectivity remains a challenge.²⁴ Bergenin significantly suppressed levels of MMP-9 and alleviated streptozocin-induced diabetic retinopathy in rats²⁵ and inhibited the human cervical cancer cell growth by decreasing MMP-9 expression.²⁶ Our computational analysis suggests bergenin as a potential MMP-9 inhibitor and a promising scaffold for drug design (Figures 3A, 4C and 4D). Bergenin also appears to inhibit GSK3 β and CDK2, both associated with inflammation and programmed cell death, supporting neuronal survival. Docking studies (Table 2, Figure 3H) showed strong GSK3 β binding (-7.47 kcal/mol) compared to TWS119 (-6.91 kcal/mol), supporting its therapeutic potential. GSK3 β regulates oxidative stress pathways via Nrf2 and CREB/AP1. TWS119 protected against hemorrhage when given post rtPA treatment in an MCAO rat model and improved neurological outcomes, reduced cerebral edema, infarct volume, and BBB permeability.²⁷ While several direct and indirect GSK3 β inhibitors have shown anti-ischemic potential in preclinical models, their precise mechanisms remain unclear. Recent studies have shown that bergenin can downregulate GSK3 β levels and

inhibit ferritinophagy-mediated ferroptosis in cisplatin-induced acute kidney injury through the GSK3 β /Nrf2/PPAR γ pathway.²⁸ Further insights into bergenin's interaction with the active site residues of GSK3 β and MMP9 based on dynamics simulation data, are discussed later in this section. While CDKs promote cell cycle progression, Osuga *et al.*, found that their activation signals neuronal death after ischemia, suggesting CDK inhibitors as potential stroke therapeutics.²⁹ Roscovitine, a CDK2 inhibitor, effectively reduced brain lesions in both focal and global ischemic rat models.³⁰ CDK2 inhibitors, primarily used in cancer treatment, remain in preclinical stages for stroke, requiring further studies on safety and efficacy. Additionally, bergenin modulates MAPK14 (p38), a key mediator of inflammation, oxidative stress, BBB disruption, and apoptosis. Research indicates that inhibiting or modulating the expression of MAPK14 (p38) can help reduce tissue damage following a stroke.³¹ Xiang *et al.*, found that bergenin pretreatment impedes hepatic ischemia reperfusion injury in mice and ascribed it to its antiinflammatory and antiapoptotic properties by decreasing the levels of related proteins including phosphorylated MAPK 14.³² Additionally, the other hub targets with good affinity were growth factor receptors- EGFR, KDR, MET and a steroid receptor PGR (Table 2). These factors exhibit neurotrophic properties and are crucial in post-stroke recovery, contributing to protective mechanisms such as neurogenesis, angiogenesis, and synaptic plasticity, all of which are essential for brain repair and remodeling.³³⁻³⁵ Bergenin's influence on KDR may enhance angiogenesis and tissue regeneration, while modulation of EGFR and MET could facilitate neuroprotection and recovery. Its association with PGR

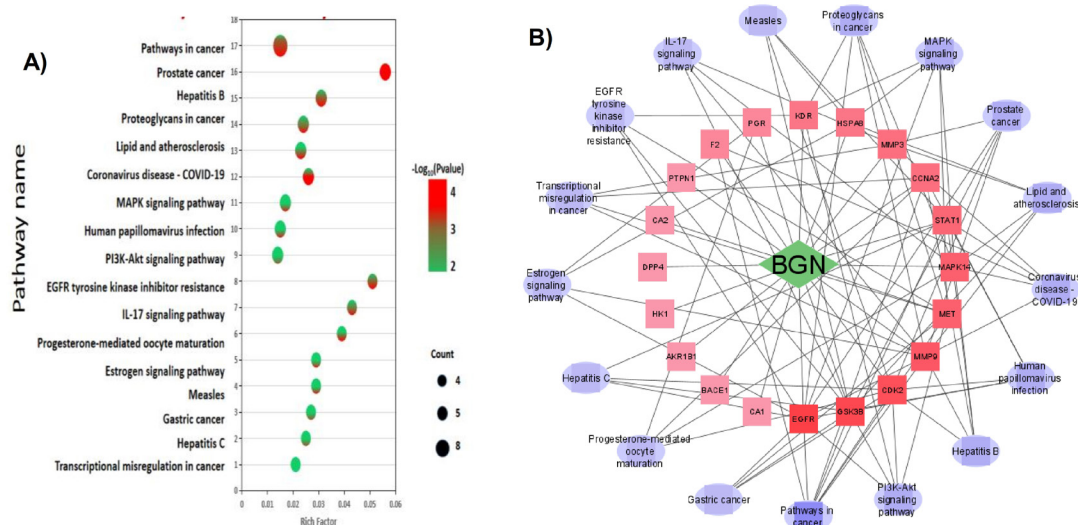


Figure 2: KEGG pathway enrichment analysis report and 'Bergenin-target-pathway' network: A) KEGG Bubble chart: X axis- rich factor, Y axis- pathway name, bubble size- target gene count and bubble color- green to red indicates lower to higher significance, adjusted $-\log_{10}(p\text{-value})$. B) Cytoscape network: Green diamond at center- bergenin, red squares- key stroke targets and violet ellipses- KEGG pathways, color shading from lighter to darker represents increasing node degree value.

indicates potential influence on progesterone mediated pathways and may also offer anti-inflammatory and anti-apoptotic benefits. Collectively, these mechanisms suggest bergenin's potential in alleviating post-stroke symptoms like cognitive and motor deficits through its antioxidant, anti-inflammatory, and neuroprotective properties.

KEGG pathway enrichment analysis indicated the following 17 pathways for bergenin's stroke therapy- pathways in cancer, prostate cancer, hepatitis B, proteoglycans in cancer, lipid and atherosclerosis, coronavirus disease- COVID-19, MAPK signaling pathway, human papillomavirus infection, PI3K-Akt pathway, EGFR tyrosine kinase inhibitor resistance, IL-17

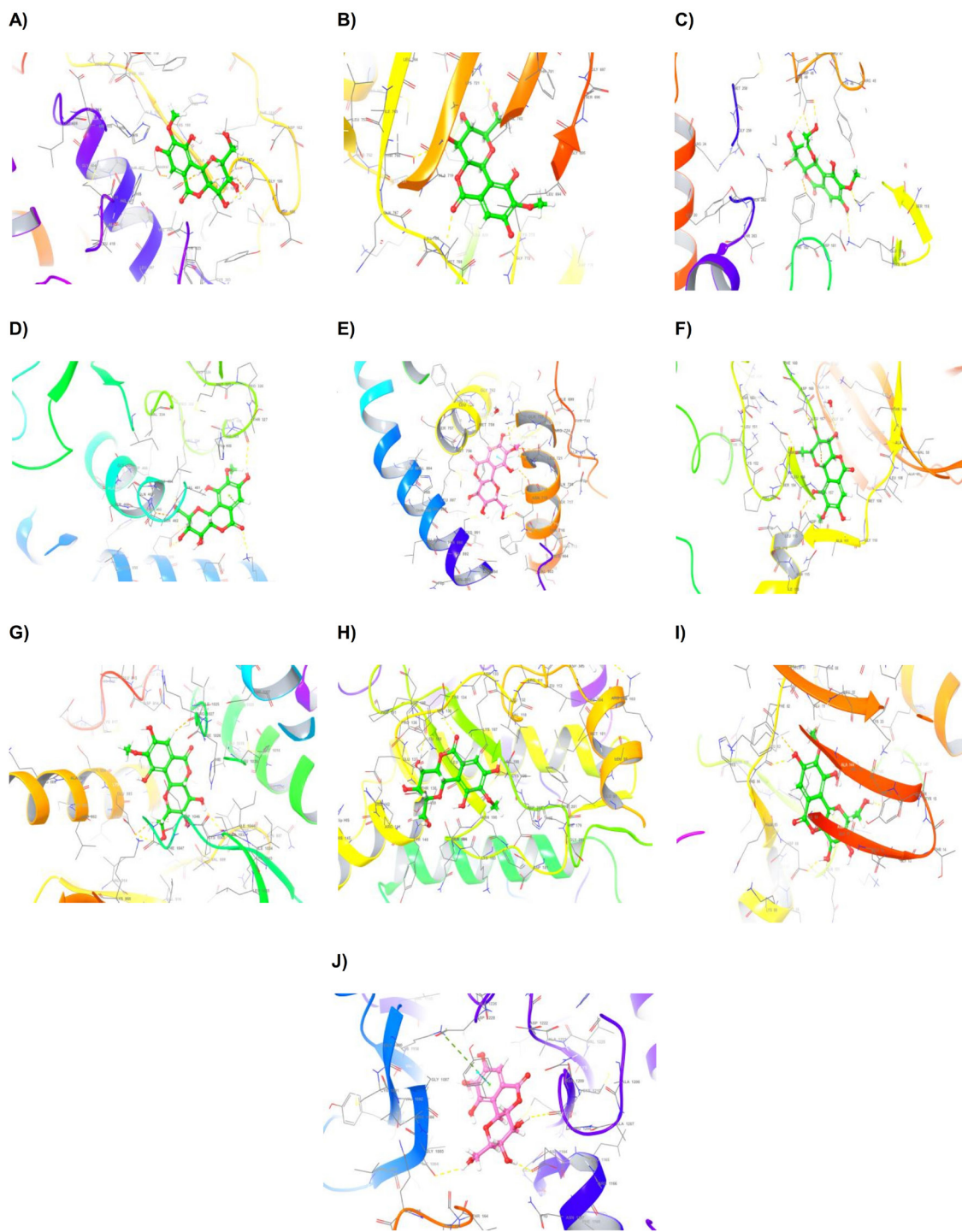


Figure 3: 3D docking poses of 10 hub target proteins with bergenin. A) MMP9, B) EGFR, C) PTPN1, D) STAT1, E) PGR, F) MAPK14, G) KDR, H) GSK3 β , I) CDK2 and J) MET.

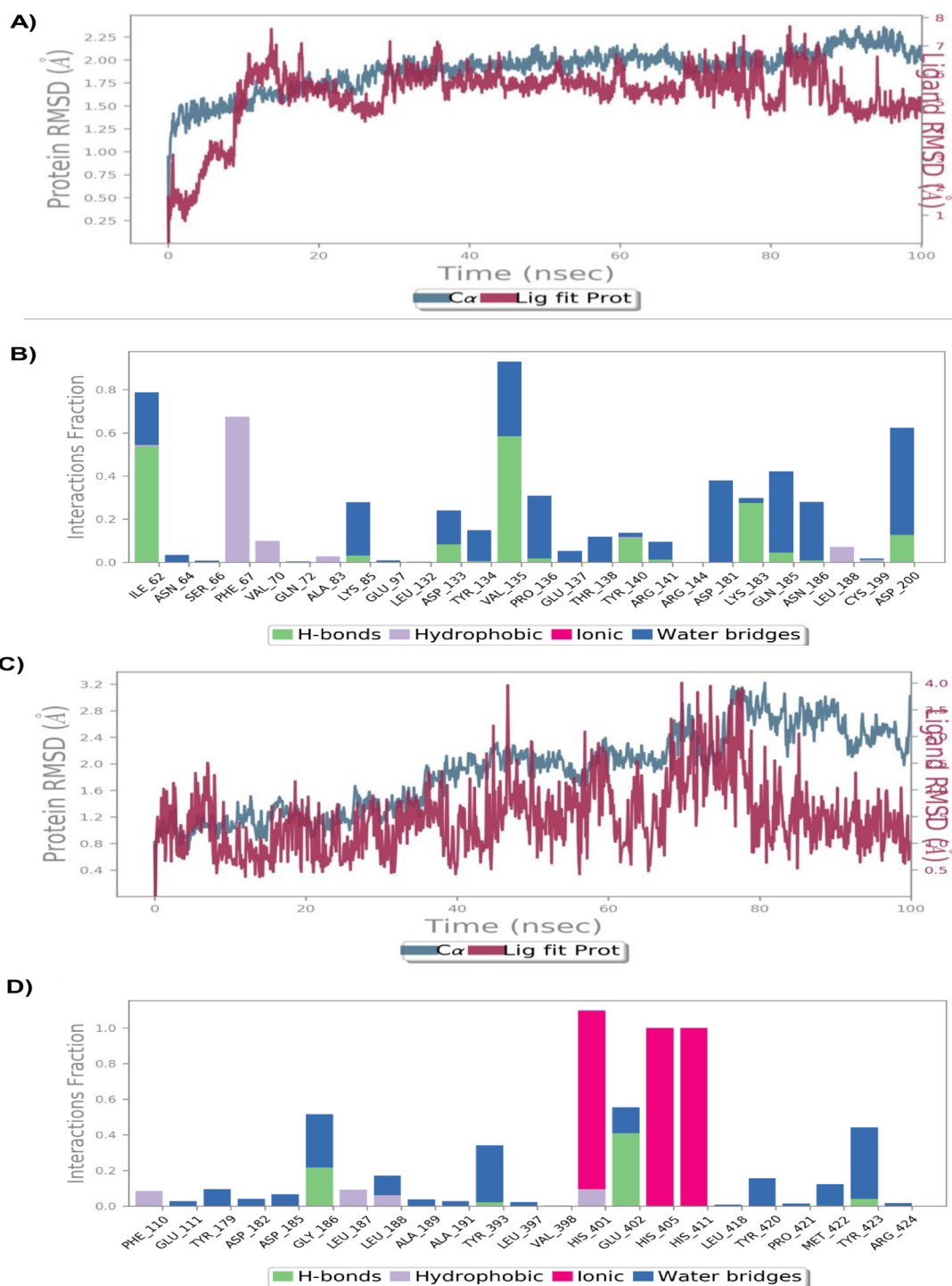


Figure 4: Molecular dynamics simulation outputs: A) RMSD graph of bergenin-GSK3β, B) Protein-ligand interaction fractions histogram of bergenin-GSK3β, C) RMSD graph of bergenin-MMP9 and D) Protein-ligand interaction fractions histogram of bergenin-MMP9.

signaling pathway, progesterone-mediated oocyte maturation, estrogen signaling pathway, measles, gastric cancer, hepatitis C and transcriptional misregulation in cancer (Figure 2A). Stroke is a complex, multifactorial condition marked by a series of interconnected biological disturbances, including endothelial dysfunction, chronic inflammation, oxidative damage, and

disrupted immune responses. These mechanisms not only drive acute neuronal injury during ischemia but also hinder the brain's ability to repair and regenerate over time. Interestingly, many molecular pathways active in stroke are also involved in other pathological conditions like cancer, infectious diseases, and metabolic syndromes. For instance, the signaling mechanisms

that regulate cancer cell proliferation, survival, and angiogenesis are similarly activated during the brain's recovery phase after stroke.³⁶ Likewise, disruptions in lipid metabolism and the development of atherosclerosis play central roles in ischemic stroke by fostering plaque buildup, clot formation, and vascular blockages.³⁷ Infections such as COVID-19 can aggravate stroke risk by inducing systemic inflammation, vascular damage, and blood clotting abnormalities.³⁸ Critical signaling cascades-such as MAPK,³¹ PI3K-Akt³⁹ and EGFR³³-regulate inflammation, neuronal survival, and tissue repair in post-stroke conditions. Additionally, hormonal systems, particularly those mediated by estrogen and progesterone, influence inflammation and provide neuroprotection.³⁵ Following a stroke, aberrant transcriptional activity involving stress-related regulators like NF- κ B and p53 can further exacerbate cell death and contribute to fibrotic changes in brain tissue.³⁹ These overlapping mechanisms highlight the importance of broad-spectrum therapeutic strategies, such as bergenin, which can modulate multiple targets involved in stroke's complex pathophysiology, offering a promising approach to mitigate both acute and chronic damage.

Moreover, KEGG pathway analysis suggested that bergenin's neuroprotective effects in stroke may be primarily, though not exclusively, linked to its ability to regulate MAPK, PI3K-Akt, and IL-17 signaling pathways- key regulators of immune-inflammatory responses to stressors like ROS and DAMPs. MAPK pathway activation, typically downstream of TLR4 signaling, drives phosphorylation events that modulate cell survival, inflammation, and apoptosis.³⁹ Bergenin showed strong binding affinity with MAPK14 and other related proteins like KDR, MET and EGFR in this pathway and has been reported to reduce phosphorylated MAPK14 levels in various models.³² In ischemic stroke, DAMPs released from necrotic neurons cross the compromised BBB, triggering rapid activation and infiltration of immune cells. Th17 and γ δT cells exacerbate injury via IL-17 production, which activates NF- κ B and MAPK/AP-1 pathways, promoting inflammatory cascades.⁴⁰ Bergenin demonstrated stable interactions with key IL-17 pathway proteins (MAPK14, MMP3, MMP9, GSK3 β) and is known to repress Th17 differentiation and neutrophilic inflammation via CDK1-APC/C-Cdh1 signaling through PPAR γ activation.⁴¹ The PI3K-Akt pathway, central to neuroprotection, modulates cell survival, apoptosis, and vascular integrity via branches like PI3K-Akt/Nrf2 (antioxidative), PI3K-Akt/mTOR (anti-autophagy), and PI3K-Akt/NF- κ B (anti-inflammatory in neurons, pro-inflammatory in microglia).³⁹ Bergenin was shown to activate this pathway in models of Parkinson's disease²¹ and targeted key proteins of this pathway such as GSK3 β , CDK2, KDR, MET, and EGFR in our study. Altogether, our systems biology approach identified novel targets (MMP9, GSK3 β , MAPK14, CDK2) and key signaling pathways (MAPK, IL-17, PI3K-Akt) that may underlie bergenin's neuroprotective effects,

primarily by modulating immune-driven neuroinflammation in ischemic stroke.

Molecular dynamics simulation studies were conducted to assess the interaction stability, flexibility, and binding behavior of bergenin with GSK3 β and MMP9, offering insights into its inhibitory potential. Analysis of simulation outputs (Figure 4) demonstrated stable bergenin-protein complexes. GSK3 β , a serine/threonine kinase, remains constitutively active under basal conditions through autophosphorylation at Thr216. Its inhibition, often mediated by phosphorylation at Ser997 via the Akt pathway, activates downstream neuroprotective mechanisms like the Nrf2 pathway.³⁹ The enzyme contains a functional ATP-binding site formed by residues such as Lys85 and Glu97 (forming a salt bridge), and a regulatory activation loop spanning from the DFG to APE motifs. Key catalytic residues like Asp133, Tyr134, Val135, and Cys199 play essential roles in stabilizing inhibitors within the ATP pocket.⁴¹ Bergenin was found to interact with several of these critical residues. Hydrogen bonding was observed with Lys85, Asp133, Val135, and Asp200, while water bridge interactions involved additional residues including Glu97, Tyr134, and Cys199 (Figure 4B). Interaction persistence data showed that Val135 was involved throughout the simulation, with Phe67 and Ile62 contributing significantly as well, highlighting bergenin's strong affinity for the polar region of GSK3 β , a key determinant for effective inhibition. Similarly, bergenin formed a stable complex with MMP9, a zinc-dependent protease implicated in blood-brain barrier disruption through degradation of extracellular matrix proteins. MMP9's catalytic core features a zinc ion coordinated by His401, His405, and His411, with Glu402 contributing to its enzymatic activity.⁴² Bergenin maintained consistent ionic interactions with the zinc-coordinating histidines (Figure 4D) and formed a hydrogen bond with Glu402, lasting nearly 40% of the simulation time suggesting effective engagement with the enzyme's active site. Overall, the simulation data support bergenin's potential as an inhibitor of GSK3 β and MMP9, with strong and stable binding at critical active site residues, positioning it as a promising candidate for further drug development.

The pathogenesis of stroke is intricate and involves a convergence of multiple pathological processes including oxidative damage, inflammation within the nervous system, excitotoxicity, compromise of the BBB, programmed cell death, abnormal angiogenesis, vascular restructuring, and delayed neurodegeneration. Due to this complexity, treatments aimed at a single molecular target often prove insufficient, prompting growing interest in multi-target therapeutic strategies. Here, bergenin has emerged as a promising candidate due to its ability to engage with various critical molecular targets implicated in stroke. In the acute stage (within hours to days), it may help preserve BBB integrity through MMP9 inhibition, dampen inflammation via MAPK14 and EGFR, and reduce apoptosis through modulation

of GSK3 β and CDK2. During the subacute phase (days to weeks), bergenin may enhance neuronal survival via MET and PGR, regulate angiogenesis through KDR, and influence glial activity through EGFR and MAPK14. In the longer-term recovery phase, activation of MET and PGR by bergenin could support neuronal regeneration and functional recovery, presenting it as a versatile neuroprotective agent. Thus, bergenin's predicted multi-target activity matches stroke's complex, time-evolving pathology remarkably well, however dosing and timing will be crucial.

Current study succeeded in identifying the unexplored targets and molecular mechanisms of bergenin's neuroprotection against ischemic stroke, further creating a solid scientific background using *in silico* approaches like network pharmacology and computational methods for future *in vitro* and *in vivo* research at molecular level.

CONCLUSION

Ischemic stroke is a major global cause of death and long-term disability. Conventional treatments, such as thrombolytic agents and mechanical thrombectomy, face significant limitations, including a restricted therapeutic window, contraindications, and an increased risk of hemorrhagic transformation. These challenges underscore the need for holistic neuroprotective therapies as alternative or adjunctive treatment strategies. Emphasizing the potential of phytopharmaceuticals in neuroprotection, this research was conducted to explore or identify therapeutic targets and elucidate the underlying mechanisms of bergenin's neuroprotective effects against stroke through a systematic and computational approach. Network pharmacology and molecular docking studies identified MMP9, GSK3 β , MAPK14, and CDK2 as key therapeutic targets of bergenin in stroke. KEGG categories like cancer signaling, viral response, MAPK, PI3K-Akt, EGFR resistance, IL-17, and transcriptional misregulation all map directly onto stroke-relevant mechanisms. Thus, multi-pathway modulation, as seen with bergenin's predicted targets, offers a biologically coherent and therapeutically promising strategy against the complex cascade of stroke injury and recovery. Further, molecular dynamic simulations confirmed the stability of interactions of bergenin with MMP9 and GSK3 β . These findings suggest that bergenin's multitargeted neuroprotective potential in mitigating ischemic damage might be majorly by modulating neuroinflammatory pathways and promoting recovery. Although computational findings are promising, additional *in vitro* and *in vivo* studies are required for validation. This study lays a solid groundwork for future pharmacological research, highlighting bergenin's potential as a promising therapeutic option for stroke treatment.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

Akt: Proteinkinase B; **API:** Activator Protein1; **APC/C:** Anaphase-promoting complex/ Cyclosome; **BindingDB:** Binding Database; **Cdh1:** Cadherin 1; **CDK1:** Cyclin dependent kinase 1; **ChEMBL:** Chemical database of bioactive molecules with drug-like properties; **CREB:** Cyclic adenosine monophosphate response element binding protein; **DAVID:** Database for Annotation, Visualization; and Integrated Discovery, **FOXO3a:** Forkhead box class O 3a; **GLIDE-XP:** Gap, Location, Image quality, Density; en-face TR morphology- Extra Precision; **IL-17:** Interleukin 17; **IL-1 β :** Interleukin-1-beta; **IL-6:** Interleukin 6, **MMPs:** Matrix metalloproteases, **PDB:** Protein Data Bank, **PDTD:** Potential Drug Target Database; **PI3K:** Phosphatidylinositol 3-kinase; **PPAR γ :** Peroxisome Proliferator-Activated Receptor Gamma; **SHAFTS:** SHape-FeaTure Similarity; **Sirt1:** Silent information regulator sirtuin 1; **STAT3:** Signal transducer and activator of transcription 3; **STRING:** Search Tool for Retrieval of Interacting Genes/Proteins; **TNF α :** Tumor necrosis factor- alpha; **UniProt:** Universal Protein Resource; **UniProtKB:** Universal Protein Knowledgebase.

SUMMARY

Ischemic stroke remains a leading cause of death and disability worldwide, with current treatments limited by narrow therapeutic windows and risks such as hemorrhagic transformation. This study explored bergenin's neuroprotective potential through a systematic computational approach. Network pharmacology and molecular docking identified MMP9, GSK3 β , MAPK14, and CDK2 as key therapeutic targets, implicating critical pathways like MAPK signaling, PI3K-Akt signaling, and IL-17 signaling in stroke pathology. Molecular dynamic simulations further confirmed bergenin's stable interactions with MMP9 and GSK3 β . Bergenin is showing the right kind of multitarget profile for a complex disease like stroke but we need real-time validation studies, target engagement proofs, and carefully staged intervention models to move it from "promising" to "practical."

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Protective Effect of *Viburnum opulus* Hydroalcoholic Extract on Cisplatin Induced Nephrotoxicity

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ABSTRACT

Introduction: Acute Kidney Injury (AKI) is a severe disorder often caused by nephrotoxic agents like cisplatin, which induces oxidative stress, inflammation, and renal damage. *Viburnum opulus* is a medicinal plant recognized for its antioxidant and anti-inflammatory properties has shown potential in mitigating oxidative stress-related disorders. **Objectives:** The main purpose of this study was to measure the nephroprotective benefits of *Viburnum opulus* Ethanolic extract (VOE) against rats' cisplatin-induced AKI. The specific objectives included phytochemical analysis of VOE, assessment of its *in vitro* antioxidant activity and evaluation of its nephroprotective effects through renal function markers, oxidative stress parameters, and histopathological examination. **Materials and Methods:** VOE was prepared and subjected to qualitative and quantitative phytochemical screening. Its *in vitro* antioxidant potential was assessed using DPPH, ABTS and reducing power assays. For the *in vivo* study, five groups of rats were used: control, cisplatin-induced AKI, positive control (Silymarin 50 mg/kg) and two VOE pre-treatment groups (200 and 400 mg/kg p.o.). VOE was administered orally before cisplatin induction (7 mg/kg via i.p.) and nephrotoxicity was measured by measurement serum creatinine, BUN, urea and uric acid levels, along with oxidative stress markers in kidney tissues. Histopathological examination was performed to assess renal tissue damage. **Results:** Phytochemical analysis revealed a rich profile of phenolics (169.7±4.66 mg GAE/g), flavonoids (139.9±1.58 mg QE/g), and other bioactive compounds. VOE exhibited strong *in vitro* antioxidant activity, as demonstrated by DPPH, ABTS and reducing power assays. *In vivo* studies showed that VOE pre-treatment significantly attenuated cisplatin-induced nephrotoxicity by improving renal function markers, reducing oxidative stress, and preserving kidney histology. **Conclusion:** The findings suggest that VOE possesses significant nephroprotective potential against cisplatin-induced AKI, likely due to its antioxidant and bioactive phytochemical constituents. This study demonstrates the capacity of VOE as a natural therapeutic agent for preventing cisplatin-induced renal damage, warranting further research to explore its precise mechanisms and clinical applicability.

Keywords: *Viburnum opulus*, Nephroprotective, Cisplatin, Nephrotoxicity, Oxidative stress, Phytochemicals.

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INTRODUCTION

Cisplatin, a key chemotherapeutic drug that is used for the treatment of numbers of solid tumors like testicular, bladder, ovarian, neck and head and lung cancers, poses a serious clinical issue because of its dose-limiting nephrotoxicity.^{1,2} This adverse effect, manifesting as acute kidney injury, is a major concern, potentially leading to chronic kidney disease and impacting patient survival and quality of life. Cisplatin-induced AKI is typified by a precipitous drop in the glomerular filtering rate, as seen by increased BUN and serum creatinine levels, electrolyte abnormalities and oliguria.³ The severity of cisplatin-induced

nephrotoxicity varies among patients, influenced by factors such as age, pre-existing renal conditions, and concomitant medications.

The processes behind cisplatin-induced nephrotoxicity are complicated, including a number of interrelated events. Because renal tubular cells have high metabolic activity and express specialized transporters, cisplatin preferentially accumulates in these cells, especially in the proximal tubules. Inside the cells, cisplatin undergoes aquation, forming reactive species that interact with various cellular components, triggering a cascade of damaging processes. One of main mechanisms is formation of ROS, which results in oxidative stress, a condition when creation of ROS and the antioxidant defense mechanism of cells are out of balance.⁴ Excessive ROS inflict damage on cellular macromolecules, including lipids, proteins, and DNA, disrupting cellular function and integrity.³ Lipid peroxidation, a consequence



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of ROS-induced damage to cell membranes, further compromises cellular function. DNA damage, another critical consequence of cisplatin's reactivity, may trigger apoptosis and cell cycle arrest, which aids in the demise of renal tubular cells.¹

In addition to oxidative stress, cisplatin-induced nephrotoxicity involves inflammation, a complicated biological reaction when inflammatory mediators including cytokines and chemokines are activated. These mediators recruit inflammatory cells to the kidneys, exacerbating tissue damage. Furthermore, cisplatin can directly activate inflammatory pathways, further contributing to the inflammatory response in the kidneys.² The pathophysiology of cisplatin-induced nephrotoxicity is significantly influenced by the interaction of oxidative stress, inflammation and apoptosis.³

Current nephroprotective strategies, such as hydration and the use of diuretics, have been employed to mitigate cisplatin-induced kidney damage; however, their efficacy is often limited and accompanied by side effects such as dehydration, electrolyte imbalances, and diminished renal blood flow.^{4,5} Therefore, there is an urgent need for safer and more effective alternatives to protect the kidneys during cisplatin therapy. Due to the limitations of current strategies for preventing and managing cisplatin-induced kidney damage, there is a pressing need for effective and safe nephroprotective agents. Natural remedies made from plant and other living things have shown great promise in reducing the nephrotoxicity caused by drugs.⁶ The plant *Viburnum opulus* L., sometimes referred to as European cranberrybush or guelder-rose, has an extensive record of traditional medicinal usage.⁷ Traditional medicine has utilized the fruit, bark, and leaves of the *V. opulus* plant for its alleged medicinal properties. The possible nephroprotective qualities of *V. opulus* preparations have recently been the focus of research.^{8,9} Studies have revealed that *V. opulus* contains a diverse array of bioactive compounds with flavonoids, tannins and iridoids, which have cytoprotective, anti-inflammatory, and antioxidant properties.^{7,10} These properties suggest that *V. opulus* extracts may offer protection against kidney damage caused by cisplatin by reducing inflammation, oxidative stress and renal cell death.

In a rat model, this work attempts to examine the nephroprotective efficacy of *Viburnum opulus* extract against cisplatin-induced nephrotoxicity. We hypothesize that *V. opulus* extract will reduce cisplatin-induced renal dysfunction, as proved by improved serum creatinine and BUN levels, reduced oxidative stress markers, and histopathological preservation of renal tissue. This research will add to the increasing amount of evidence supporting therapeutic advantages of natural products for reducing chemotherapy adverse effects, potentially paving the way for developing novel strategies to protect kidney function in cancer patients receiving cisplatin treatment. Specifically, this study will examine the effects of *V. opulus* extract on key markers of renal function, oxidative stress and inflammation in rats that treats with cisplatin. The results of this investigation will shed important light on *V.*

opulus's potential function as a nephroprotective agent and might influence the creation of innovative treatment strategies to stop or cure cisplatin-induced nephrotoxicity.

MATERIALS AND METHODS

Collection of Plant Material

Bark of *Viburnum opulus* was procured from Vital herbs, New Delhi, ensuring proper identification and authentication by a botanist. To get rid of any dust or debris that could have adhered, the gathered plant material was carefully cleaned with distilled water. In order to preserve its phytochemical components and avoid deterioration, the bark was shade-dried for two weeks at room temperature ($25\pm 2^\circ\text{C}$). The dried material was then coarsely powdered using a mechanical grinder and stored in an airtight container for further extraction.¹¹

Preparation of Hydroalcoholic Extract

The powdered bark of *Viburnum opulus* was subjected to Soxhlet extraction using a hydroalcoholic solvent system (70:30 v/v ethanol:water) to ensure efficient extraction of both polar and non-polar phytoconstituents.¹² A Soxhlet apparatus was filled with around 100 g of the powdered material, and extraction was done for 8 hr at $60\pm 5^\circ\text{C}$ until a colorless solvent was seen in the siphon tube, signifying full extraction.

The extracted material was concentrated in a rotating evaporator at $40\pm 2^\circ\text{C}$ with lowered pressure and subsequently dried in a desiccator to remove residual moisture. The dried extract was weighed to determine the percentage yield and stored in an amber-colored glass container at 4°C for further phytochemical and pharmacological investigations.¹³

Methods for Qualitative Phytochemical Analysis

Alkaloids

Dragendorff's Test: 2 mL of Dragendorff's reagent are added in 1 mL of extract. When a reddish-brown or orange precipitate appears, alkaloids are present.¹¹

Mayer's Test: 2 mL of Mayer's reagent are added in 1 mL of extract. Alkaloids are confirmed by the development of a cream-colored precipitate.¹³

Wagner's Test: 2 mL of Wagner's reagent are added in 1 mL of extract. Alkaloids are confirmed by a reddish-brown precipitate.¹⁴

Carbohydrates

Molisch's Test: Two drops of Molisch's reagent and 2 mL of sulfuric acid concentration are added in 1 mL of extract. Carbohydrates are indicated by a purple or reddish-violet ring at the contact.¹¹

Fehling's Test: Add equal amounts of Fehling's solutions A and B to 1 mL of extract, and then heat. Reducing sugars are confirmed when a crimson or brick-red precipitate forms.¹³

Benedict's Test: Combine Benedict's reagent with 1 mL of extract and bring to a boil. When reducing sugars are present, a reddish-orange precipitate forms.¹⁵

Flavonoids

Alkaline Reagent Test: Use a sodium hydroxide solution to treat 1 mL of extract. Flavonoids are confirmed by formation of a bright yellow hue that converts into colorless when diluted acid is added.¹⁴

Lead Acetate Test: Combine 2 mL of lead acetate solution with 1 mL of extract. Flavonoids are confirmed by the development of a yellow precipitate.¹²

Phenols and Tannins

Ferric Chloride Test: To 1 mL of extract, add 3-5 drops of a 5% FeCl_3 solution. Phenolic substances are showed by a dark green or blue-black coloring.¹¹

Lead Acetate Test: 3 mL solution of lead acetate are added in 1 mL of extract. Tannins are present when a white or yellow precipitate forms.¹³

Saponins

Foam Test: 5 mL of distilled water should be shaken with 1 mL of extract. Saponins are indicated by persistent foaming that lasts for at least 10 min.¹⁴

Glycosides

Borntrager's Test: Shake together 2 mL of extract and 2 mL of chloroform. Pour in same amount of diluted ammonia. Anthraquinone glycosides are confirmed by a pink or crimson coloring in the ammoniacal layer.¹⁵

Steroids and Terpenoids

Salkowski's Test: 2 mL of chloroform and 2 mL of sulfuric acid are added in 1 mL of extract. Steroids are indicated by a reddish-brown interface, while terpenoids are indicated by a golden-yellow hue.¹³

Proteins and Amino Acids

Biuret Test: Add two drops of 1% solution of copper sulfate and 1 mL of 40% sodium hydroxide to 1 mL of extract. Proteins are indicated by a violet or purple hue.¹¹

Ninhydrin Test: 2 drops of a 0.2% ninhydrin solution are added in 1 mL of extract and heat it. Free amino acids are indicated by the presence of a purple tint.¹⁵

Determination of Total Phenolic Content

Principle: The Folin-Ciocalteu colorimetric technique is utilized to analyze the hydroalcoholic extract's TPC. In an alkaline media, extract's phenolic components combine with Folin-Ciocalteu

reagent to generate a blue complex with a measurement range of 765 nm.¹⁶

Procedure: Standard Curve Preparation: Methanol is utilized to make a stock solution of gallic acid (1 mg/mL). From the stock solution, various aliquots (20, 40, 60, 80, and 100 $\mu\text{g/mL}$) are transferred into test tubes. Each tube receives 0.5 mL of Folin-Ciocalteu reagent (1:10 dilution), which is then let to stand for 5 min. 2.5 mL of 7% solution of sodium carbonate is added and mixture is then allowed to sit at room temperature for 30 min. UV-visible spectrophotometer is utilized to calculate the absorbance at 765 nm. Plotting absorbance against gallic acid concentration results in a calibration curve.

Sample Preparation

Methanol is used to make the hydroalcoholic extract of *Viburnum opulus* (1 mg/mL). After combining 1 mL of extract with 0.5 mL of Folin-Ciocalteu reagent, mixture is incubated for 5 min. 2.5 mL of 7% solution of sodium carbonate is added and mixture is then let to stand at room temperature for 30 min. TPC is given as mg of GAE/g extract and absorbance is measured at 765 nm.

Determination of Total Flavonoid Content

Principle: TFC is ascertained by using a colorimetric method using aluminum chloride. When flavonoids and AlCl_3 combine, a yellow complex is created that can be seen at 415 nm.¹⁷ **Method:** Getting the Standard Curve Ready: Methanol is used to make a stock solution of quercetin (1 mg/mL). From the stock solution, various aliquots (20, 40, 60, 80, and 100 $\mu\text{g/mL}$) are transferred into test tubes. Each tube receives 0.5 mL of a 2% AlCl_3 solution, which is then allowed to sit at room temperature for 10 min. UV-visible spectrophotometer is utilized to calculate the absorbance at 415 nm. Plotting absorbance against quercetin concentration results in a calibration curve. **Preparing the Sample:** Methanol is used to make the hydroalcoholic extract of *Viburnum opulus* (1 mg/mL). 0.5 mL of 2% AlCl_3 solution is combined with 1 mL extract. 10 min are spent incubating the mixture at room temperature. The TFC is given as mg of QE/g extract, with absorbance being measured at 415 nm.

DPPH Radical Scavenging Assay

The antioxidant activity of hydroalcoholic extract of *Viburnum opulus* (VOE) was evaluated using the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay following the method described by Blois (1958) with slight modifications. Methanol was used to make a 0.1 mM DPPH solution, which was then kept in the dark. 1 mL of DPPH solution and one mL of VOE at several concentrations (10, 20, 40, 80 and 100 $\mu\text{g/mL}$) were combined in a test tube. The common antioxidant was ascorbic acid. The mixture was vortexed and then let to stand at room temperature for 30min. in the dark. Following incubation, UV-visible spectrophotometer was utilized

to calculate the absorbance at 517 nm. The percentage inhibition of DPPH radicals was measured using formula:

$$\% \text{ Inhibition} = (\text{Control's Absorbance} - \text{Sample's Absorbance}) / \text{Control's Absorbance} \times 100$$

Where A_{sample} is absorbance of reaction mixture containing VOE and A_{control} is absorbance of control sample (DPPH solution without extract). IC₅₀ values or concentration needed to block 50% of the radicals, were calculated after the experiment was run in triplicate.

ABTS Radical Scavenging Assay

The ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) radical scavenging activity of VOE was determined using the method by Re *et al.*, (1999). A 7 mM ABTS solution was mixed with 2.45 mM potassium persulfate and the mixture was incubated for 16hrs at room temperature in dark to produce ABTS radical cation. After then, ethanol was added to the ABTS solution until its absorbance at 734 nm was 0.70±0.02. 1 mL of VOE at several concentrations (10, 20, 40, 80 and 100 µg/mL) was combined with 1 mL of ABTS solution for the test. UV-visible spectrophotometer was utilized to calculate the absorbance at 734 nm after the mixture had incubated for 10 min at room temperature. The % inhibition was calculated using the same formula as in the DPPH experiment. IC₅₀ values were calculated using ascorbic acid as the standard. Every measurement was carried out three times.

Reducing Power Assay

The reducing power of VOE was assessed according to the method of Oyaizu (1986). Different concentrations of VOE (10, 20, 40, 80, and 100 µg/mL) were Together with 2.5 mL of potassium ferricyanide (1% w/v) and 2.5 mL of a phosphate buffer (0.2 M, 6.6 pH). The mixture was incubated at 50°C for 20 min. Following incubation, 2.5 mL of 10% w/v trichloroacetic acid was added and resulting mixture went through a centrifuge for 10 min at 3000 rpm. 0.5 mL of newly made ferric chloride (0.1% w/v) and 2.5 mL of distilled water were combined with the supernatant (2.5 mL). At 700 nm, the absorbance was identified with a UV-visible spectrophotometer. Stronger reducing power was indicated by a greater absorption. Every test was run in triplicate, with ascorbic acid serving as the standard.

Animals and Experimental Design

For this investigation, male adult Wistar rats of weight 180-220 g were employed. The animals were housed in standard housing with 12 hr Light/Dark Cycle, fed by regular pellet diet and given unrestricted water. The temperature was kept at 22±2°C. Animals were acclimated for seven days before the trial began. The Seven Hills College of Pharmacy (Autonomous) Institutional Animal Ethics Committee (IAEC) accepted the study protocol, assigning it the number SHCP/CCSEA/IAEC-2024-03.

Five groups (*n*=6 each group) were randomly selected from among the animals. For seven days, Group 1 (normal control) was given a single oral saline dosage (1 mL/kg body weight). On the fifth day, cisplatin (6 mg/kg via i.p.) was administered as a single dose to Group 2 (cisplatin-induced nephrotoxicity group), causing nephrotoxicity. Group 3 (cisplatin+standard silymarin group) was given silymarin (50 mg/kg, orally) from day 1 to day 7 and cisplatin on day 5. Cisplatin was administered on 5th day to Groups 4 and 5 (cisplatin+VOE 200 and 400 mg/kg), whereas VOE (200 and 400 mg/kg, orally) was administered from Day 1 to Day 7. Based on early research that demonstrated notable nephroprotective and antioxidant benefits at these concentrations, the VOE dosages were selected.

Treatment Protocol

On day 1, animals in the experimental groups were administered VOE or silymarin, as per the assigned group, orally using a gavage tube. On the 5th day, all groups, with the exception of normal control, received a single i.p dosage of cisplatin (6 mg/kg) to cause nephrotoxicity. Animals were slaughtered for biochemical and histological examinations at the conclusion of the trial after receiving therapy till day 7.

Assessment of Body and Kidney Weight

Body weight was measured on days 1, 3, 5 and 7 using a digital weighing scale. Following the animals' sacrifice at the conclusion of the trial, kidney weight was measured. The kidney-to-body weight ratio was calculated to assess any alterations in the kidney weight due to Nephrotoxicity caused by cisplatin and the impact of VOE therapy.

Serum Biochemical Markers

Animals were put to death under anesthesia at the conclusion of the treatment period, and heart punctures were used to obtain blood samples. Centrifugation was used to separate the serum for 10 min at 3,000 rpm. Standard laboratory kits were utilized to calculate the amounts of BUN, creatinine, uric acid and serum urea. Renal dysfunction brought on by cisplatin-induced nephrotoxicity indicated via raised levels of creatinine, blood urea, uric acid and BUN. A colorimetric test was utilized to ascertain the concentration of serum urea. A microplate reader was utilized to evaluate absorbance at 570 nm after 50 µL of serum samples had been incubated with reagents buffer in a 96-well microplate for 10 min. A colorimetric procedure involving phosphotungstic acid reduction was used to measure the blood uric acid level and absorbance was calculated at 520 nm. The modified Jaffe technique, in which creatinine and picric acid react in an alkaline media to generate a colored complex, was used to detect the levels of serum creatinine. Absorbance was determined at 490 nm. A urease-based test was used to quantify Blood Urea Nitrogen (BUN), in which ammonia released from urea interacted with a reagent to generate a blue-colored complex. The concentrations

of these factors were determined using standard curves created with known values and absorbance was measured at 650 nm. The nephroprotective effects of VOE were assessed by contrasting the control group with the treatment groups.

Kidney Tissue Biochemical Assays

After blood collection, the kidneys were excised, cleaned with ice-cold saline and then homogenised with a homogeniser in 0.1M phosphate buffer (7.4 pH). The supernatant, which was utilized for biochemical analysis, was then obtained by centrifuging the homogenates for 15 min at 4°C at 12,000 rpm.

Superoxide Dismutase (SOD) Activity

SOD activity in kidney tissue was measured based on inhibition of decrease of NBT by superoxide anion. A 50 µL kidney homogenate was mixed with a reaction buffer containing NBT and xanthine oxidase, which generates superoxide radicals. After 30 min of incubation, the absorbance at 560 nm was measured. SOD activity was measured in units per mg of protein, with one unit denoting the amount of enzyme needed to prevent 50% of NBT degradation.

Catalase (CAT) Activity

CAT activity was calculated by the rate of decomposition of H₂O₂ in kidney tissue. A 100 µL kidney homogenate was added to 3 mL of phosphate buffer containing H₂O₂. Over the course of 3 min, the absorbance at 240 nm decreased as a result of H₂O₂ breakdown. The molar extinction value for H₂O₂ (43.6 M⁻¹cm⁻¹) was utilized to compute the catalase activity, which was then reported as micromoles of H₂O₂ degraded per min per mg of protein.

Malondialdehyde (MDA) Level

To quantify MDA, a lipid peroxidation marker, the TBARS test was used. Kidney tissue homogenate (200 µL) was mixed with TCA and TBA solution, then heated at 95°C for 60 min. Butanol was added after cooling and mixture was centrifuged to extract the organic phase. After measuring the absorbance of top organic layer at 532 nm, the MDA levels were quantified using a standard curve established with known MDA values.

Reduced Glutathione (GSH) Level

Reduced Glutathione (GSH) levels were quantified using the Ellman's reagent method. Mixing 200 µL of DTNB reagent with 1 mL of phosphate buffer and 100 µL of kidney tissue homogenate produced a yellow result when it interacts with GSH. After 10 min of incubation, the absorbance at 412 nm was measured. GSH concentrations were identified by linking the absorbance values to a standard curve of known GSH concentrations and results were stated as µmol of GSH/gram of tissue.

Histopathological Analysis

Rats were put to sleep, and the kidneys were quickly separated, cleaned in cold saline and stored in 10% neutral buffered formalin for a full day in preparation for histological investigations. Following a 70% ethanol wash, the tissues were embedded in paraffin and dehydrated using a succession of alcohol grades ranging from 70% to 100%. Using a rotary microtome, sections remained sliced and mounted onto glass slides. Hematoxylin and eosin staining was useful to slides, and histopathological alterations were evaluated by light microscopy.

Statistical Analysis

The Mean±SEM was utilized to represent data. ANOVA and Tukey's *post hoc* test were utilized to calculate whether there was statistical consequence among groups. *p*-values <0.05 were regarded as statistically significant.

RESULTS

The phytochemical data represents that the VOE is rich in alkaloids, carbohydrates, tannins, saponins, terpenoids, and phenols. It does not contain steroids, amino acids, or fats. "+" indicates presence, and the number of plus signs may represent a relative abundance. "-" indicates the absence of the compound (Table 1a). These values represent the average amount of phenolics and flavonoids found in the sample, expressed in milligrams per gram. "GAE" refers to gallic acid equivalents, a common standard used for quantifying phenols. "QE" refers to quercetin equivalents, a standard for flavonoids (Table 1b).

In vitro antioxidant activity of ethanolic extract of *Viburnum opulus*

Figure 1a represents DPPH radical scavenging activity of *Viburnum opulus* Extract (VOE), indicating its antioxidant

Table 1a: Qualitative Phytochemical analysis of VOE.

Test for phytochemical	Result of VOE
Alkaloids	+++
Steroids	---
Carbohydrates	+++
Tannins	+++
Saponins	+++
Terpenoids	+++
Phenols	+++
Aminoacids	-
Fats	-

Table 1b: Quantitative phytochemical analysis of VOE.

Phytochemical	Quantity
Total phenolic content	169.7±4.66 mgGAE/g
Total flavonoid content	139.9±1.58 mgQE/g

potential. The x-axis shows concentration of VOE ($\mu\text{g/mL}$), while y-axis signifies percentage of DPPH radical scavenging. As concentration increases, the scavenging activity also rises, demonstrating a dose-dependent effect. With a R^2 value of 0.9904 and a linear regression equation of $y=0.9311x-1.8073$, concentration and activity are strongly correlated. The concentration required to neutralise 50% of DPPH radicals represented via IC_{50} value, which is 50.25 $\mu\text{g/mL}$ and illustrates the antioxidant efficacy of VOE.

The Figure 1b represents ABTS radical scavenging activity of *Viburnum opulus* Extract (VOE), indicating its antioxidant potential. The x-axis shows concentration of VOE ($\mu\text{g/mL}$), while the y-axis signifies percentage of ABTS radical scavenging. As concentration increases, scavenging activity also rises in a dose dependent manner. There appears to be a substantial association between concentration and activity, as indicated by linear regression equation $y=0.6306x+1.0508$ with a R^2 value of 0.9939. The concentration needed to neutralize 50% of ABTS radicals is represented by the IC_{50} value, which is 83.16 $\mu\text{g/mL}$ and illustrates the antioxidant efficacy of VOE.

The Figure 1c represents the ferric reducing power of *Viburnum opulus* Extract (VOE), demonstrating its electron-donating ability and antioxidant potential. The x-axis shows concentration of VOE ($\mu\text{g/mL}$), while the y-axis represents percentage of ferric reducing power. As the concentration increases, the reducing power also rises in a dose dependent manner. With r^2 value of 0.9834 and

a linear regression equation of $y=1.1355x+1.129$, concentration and activity are strongly correlated. The IC_{50} value, calculated as 41.72 $\mu\text{g/mL}$, represents the concentration required to achieve 50% ferric reduction, reflecting the antioxidant potency of VOE.

Effect of VOE on body weight and kidney weight

Cisplatin administration (7 mg/kg) resulted in a significant reduction of body weight from 170 g to 100 g and a substantial enhance in kidney weight to 2.96 gm compared with normal control (1.61 g) ($p<0.001$), indicating severe nephrotoxicity, likely due to kidney hypertrophy or fluid retention. In contrast, the Cisplatin+Silymarin (50 mg/kg) group showed only a slight reduction in body weight (172 g to 165 g), with kidney weight (1.50 g) remaining comparable to normal control, confirming the nephroprotective effect of standard treatment ($p<0.001$ vs. cisplatin group). Notably, the Cisplatin+VOE (200 mg/kg and 400 mg/kg) groups maintained or slightly increased their body weights over 30 days, and their kidney weights (1.64 g and 1.58 g, respectively) were knowingly lesser than diseased control ($p<0.001$) and closer to normal and standard control groups, suggesting a protective effect of VOE in contradiction of cisplatin-induced renal toxicity (Table 2).

Effect of VOE on renal functional parameters:

The creatinine plasma levels, serum urea, BUN and uric acid in each group of control and experimental rats were displayed

Table 2: Effect of VOE on body weight and kidney weight.

Groups	Body weight (g)		Kidney weight (g)
	0 th day	30 th day	
Normal control	168	171	1.61
Cisplatin 7 mg/kg	170	100 ^{***}	2.96 ^{***}
Cisplatin+Silymarin 50 mg/kg	172	165 ⁺⁺⁺	1.5 ⁺⁺⁺
Cisplatin+VOE 200 mg/kg	176	179 ⁺⁺⁺	1.7 ⁺⁺⁺
Cisplatin+VOE 400 mg/kg	180	183 ⁺⁺⁺	1.6 ⁺⁺⁺

A sample size of 6 is used, and values are displayed as mean $\pm$ SEM. Following a one-way ANOVA, Tukey's multiple comparison test was used to analyse the data. ^{**} $p<0.01$ compared to the normal control group, ^{***} $p<0.001$, and ^{^^^} $p<0.001$ compared to the cisplatin 7 mg/kg group are all statistically significant.

Table 3: Effect of *Viburnum opulus* on renal functional parameters.

Groups	Serum urea (mg/dL)	Uric acid (mg/dL)	Creatinine (mg/dL)	BUN (mg/dL)
Normal control	38 $\pm$ 1.2	1.2 $\pm$ 0.053	0.44 $\pm$ 0.044	18 $\pm$ 0.59
Cisplatin 7 mg/kg	145 $\pm$ 0.29 ^{***}	5.0 $\pm$ 0.045 ^{***}	2.2 $\pm$ 0.13 ^{***}	90 $\pm$ 3.1 ^{***}
Cisplatin+Silymarin 50 mg/kg	44 $\pm$ 1.1 ^{^^^}	1.7 $\pm$ 0.0058 ^{^^^}	0.62 $\pm$ 0.020 ^{^^^}	20 $\pm$ 0.35 ^{NS^^^}
Cisplatin+VOE 200 mg/kg	95 $\pm$ 0.24 ^{***^^}	3.0 $\pm$ 0.15 ^{***^^}	1.3 $\pm$ 0.020 ^{***^^}	55 $\pm$ 3.7 ^{***^^}
Cisplatin+VOE 400 mg/kg	45 $\pm$ 1.5 ^{***^}	2.0 $\pm$ 0.072 [^]	0.73 $\pm$ 0.019 [^]	27 $\pm$ 1.3 ^{NS^}

A sample size of 6 is used and values are displayed as mean $\pm$ SEM. After conducting a one-way ANOVA, Tukey's multiple comparison test was employed to analyze the data. ^{**} $p<0.01$ compared to the normal control group, ^{***} $p<0.001$ and ^{^^^} $p<0.001$ compared to the cisplatin 7 mg/kg group are all statistically significant.

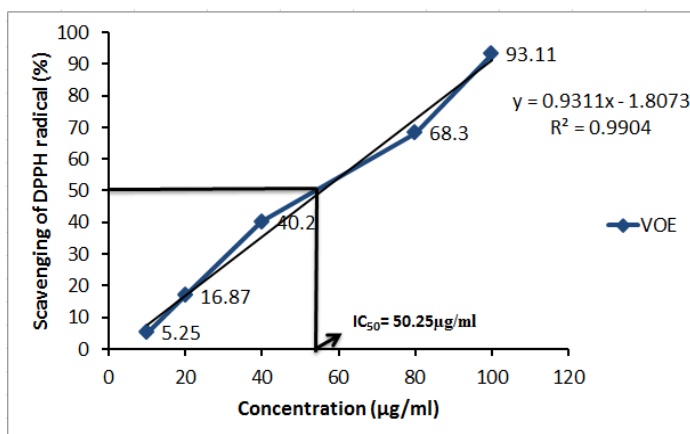


Figure 1a: DPPH radical scavenging activity of VOE.

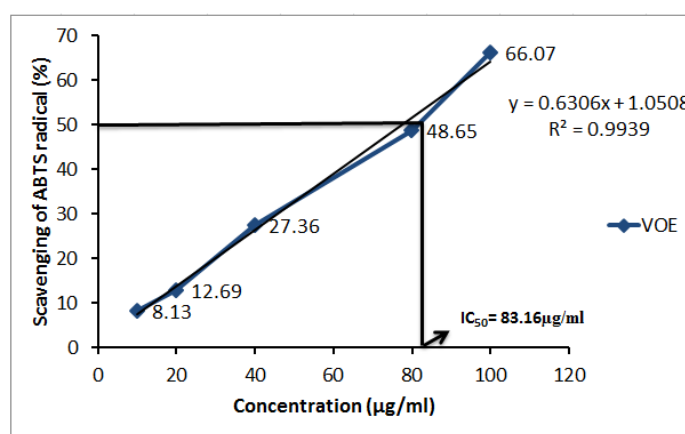


Figure 1b: ABTS radical scavenging activity of VOE.

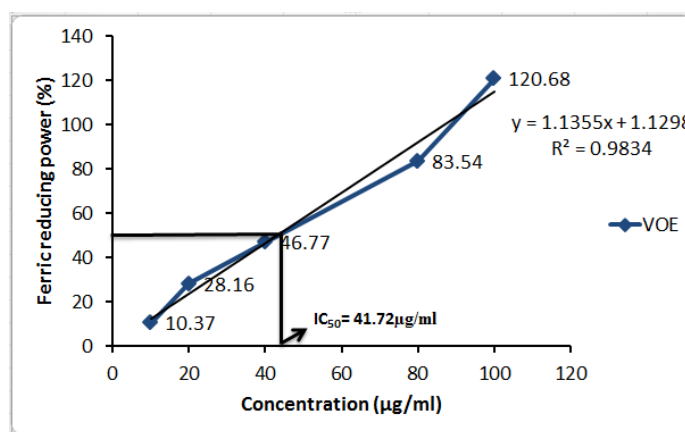


Figure 1c: Reducing power assay of VOE.

in Table 3. Following cisplatin induction, the cisplatin control group's creatinine serum levels, urea BUN and uric acid remained significantly greater ($p < 0.001$) than those of normal control group. These elevated levels of renal function parameters confirmed that nephrotoxicity had been induced. However, renal indicators such blood creatinine, urea, BUN and uric acid were significantly ($p < 0.001$) reduced after pretreatment with varying dosages of VOE (200+400 mg/kg).

Effect of *Viburnum opulus* on antioxidant status

Figure 2 shows the Cisplatin administration significantly altered enzymatic and non-enzymatic antioxidant levels in rat kidneys. Specifically, malondialdehyde levels were significantly elevated ($p < 0.001$, $p < 0.01$), while superoxide dismutase activity was meaningfully reduced ($p < 0.001$) compared with control group. Both tissue and serum glutathione levels were also significantly lower ($p < 0.001$) subsequent cisplatin treatment. Pretreatment via VOE at dose of 200 and 400mg/kg significantly modulated these effects, restoring SOD (Figure 2a), catalase (Figure 2b), MDA (Figure 2c), and GSH (Figure 2d) levels toward control values ($p < 0.05$, $p < 0.01$ and $p < 0.001$). This suggests a protective role of VOE counter to cisplatin-induced oxidative stress and resultant nephrotoxicity.

Histopathological Analysis of Renal Tissue

Hematoxylin and eosin discolored kidney sections from normal rats exhibited normal renal architecture of glomeruli and tubules with no structural abnormalities. In contrast, the Cisplatin treated control group displayed significant pathological changes, including severe Vacuolation (V), Degenerated Glomeruli (DG), Necrotic Tissue (N). Renal sections of silymarin and VOE treated animals showed Regenerative Glomeruli (RG), normal Interstitial Spaces (IS), normal Tubular Architecture (T) (Figure 3).

DISCUSSION

Cisplatin-induced nephrotoxicity is a well-documented model for studying Acute Kidney Injury (AKI), primarily due to oxidative stress-mediated damage. The present study evaluated the nephroprotective potential of *Viburnum opulus* Extract (VOE) against cisplatin-induced oxidative damage by assessing renal function markers, kidney weight, and oxidative stress parameters.

Phytochemical screening of VOE exposed the existence of polyphenols, flavonoids, tannins, alkaloids and saponins which is known for their potent antioxidant and nephroprotective activities. Quantitative analysis demonstrated high levels of TPC and TFC which contribute to its bioactivity.

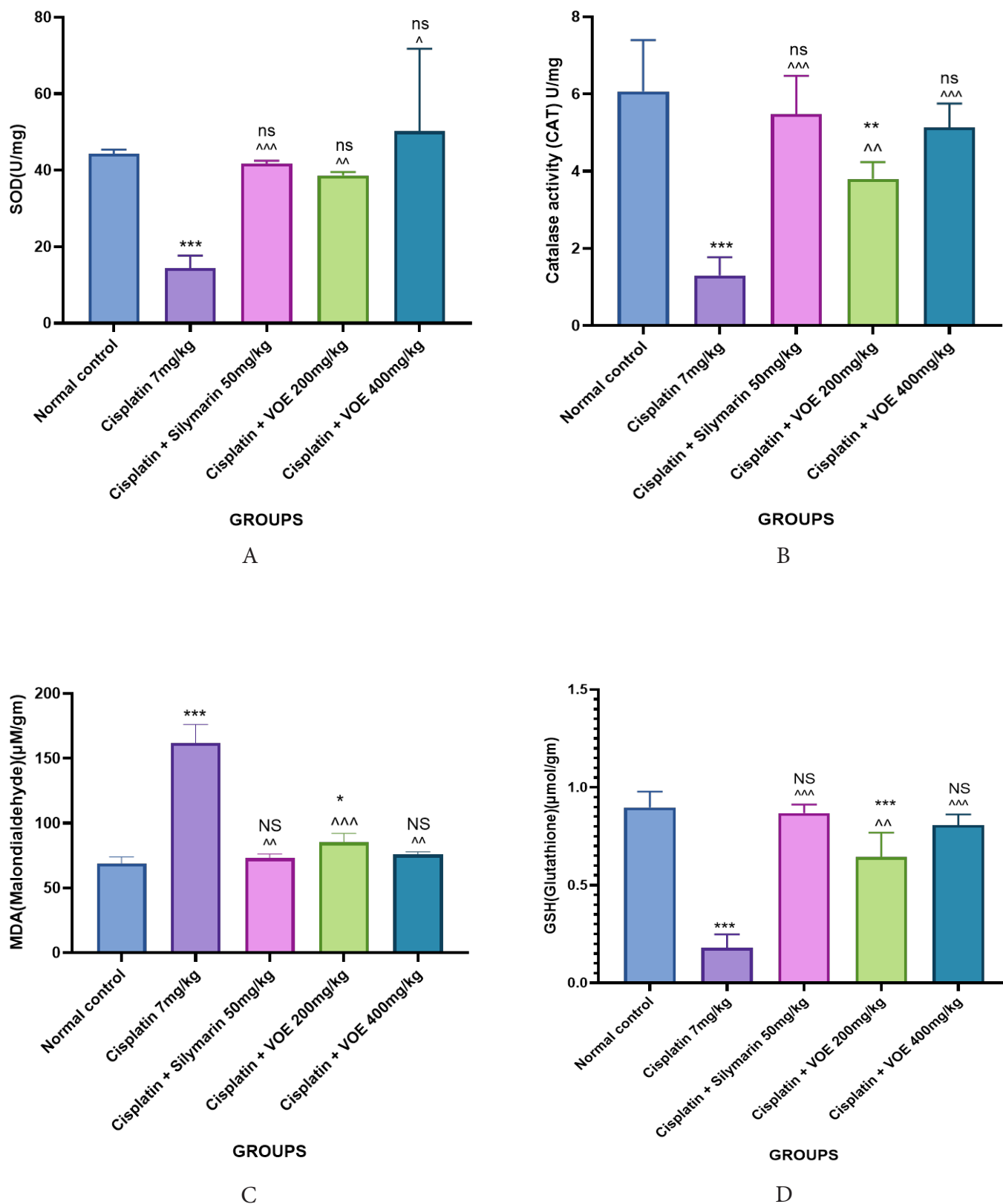


Figure 2: Effect of VOE on oxidative stress markers. 2a. SOD; 2b CAT 2c MDA 2d GSH levels. A sample size of 6 is used and values are displayed as mean $\pm$ SEM. After conducting a one-way ANOVA, Tukey's multiple comparison test was employed to analyze the data. ** $p < 0.01$ compared with normal control group, *** $p < 0.001$ and ^^ $p < 0.001$ compared to the cisplatin 7 mg/kg group are all statistically significant.

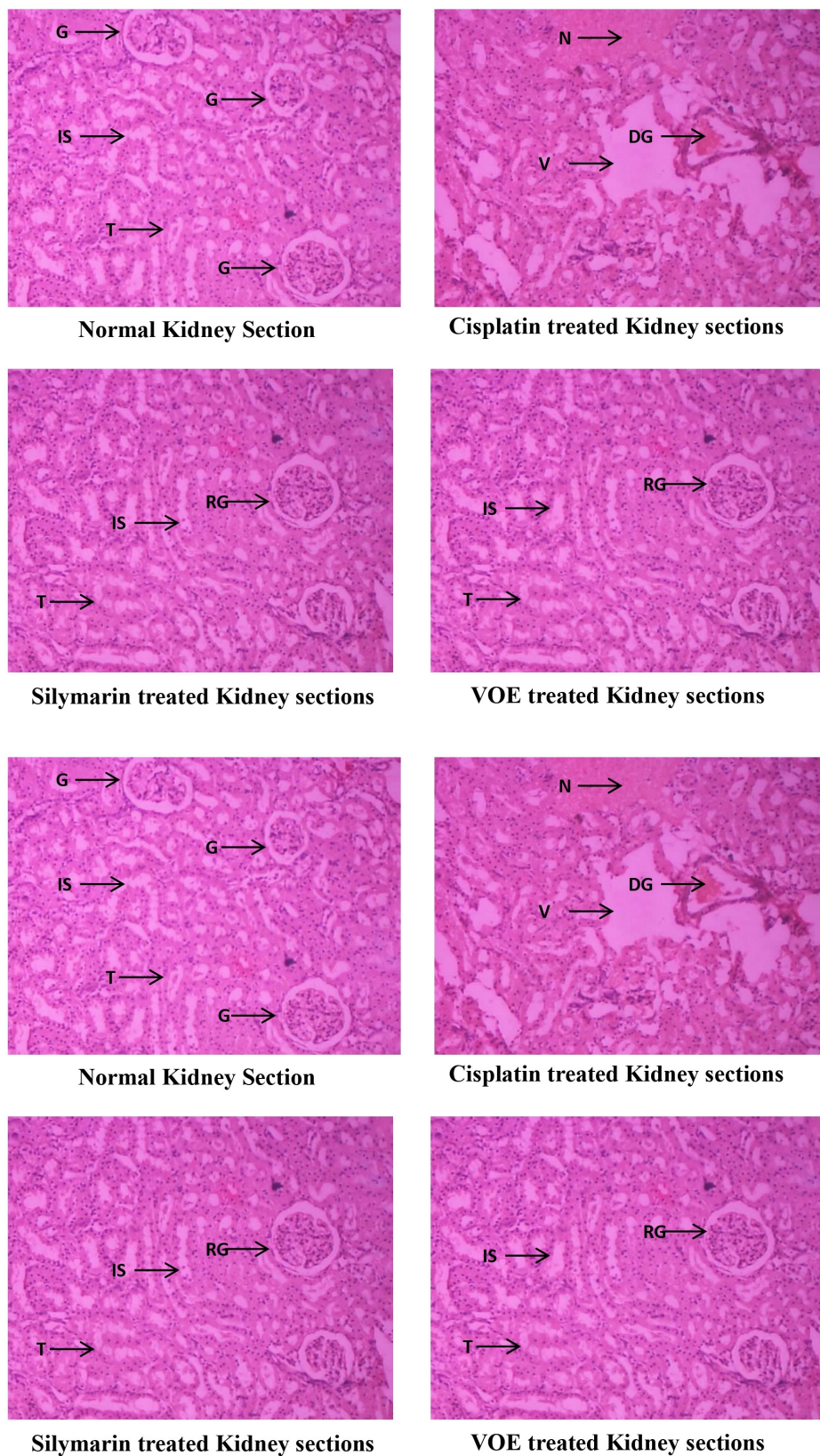


Figure 3: Hematoxylin and eosin discolored kidney sections from normal rats exhibited normal renal architecture of glomeruli and tubules with no structural abnormalities. In contrast, the Cisplatin treated control group displayed significant pathological changes, including severe Vacuolation (V), Degenerated Glomeruli (DG), Necrotic Tissue (N). Renal sections of silymarin and VOE treated animals showed Regenerative Glomeruli (RG), normal Interstitial Spaces (IS), normal Tubular Architecture (T).

To further confirm its antioxidant potential, *in vitro* assays such as DPPH, ABTS, and reducing power assays were conducted. The results disclosed significant free radical scavenging activity, indicating the capability of VOE to neutralize ROS and prevent oxidative damage. These results validate the theory that nephroprotective effects of VOE are due to its potent antioxidative constituents. The strong *in vitro* antioxidant activity of VOE suggests that it can effectively counteract oxidative stress, which is a key factor in cisplatin-induced nephrotoxicity.¹⁸

One of the important causes of cisplatin induced kidney damage is oxidative stress, primarily through excessive generation of ROS. These ROS cause lipid peroxidation, mitochondrial dysfunction, and apoptosis in renal tubular cells.¹⁹ Our study demonstrated a significant increase in Malondialdehyde (MDA) levels in cisplatin-treated rats, indicating enhanced lipid peroxidation and oxidative stress. This aligns with previous reports showing that cisplatin depletes antioxidant defense mechanisms, leading to renal dysfunction.²⁰

Treatment with VOE significantly reduced MDA levels while increasing antioxidant markers such as SOD, GSH and GPx. This suggests that VOE exerts protective effects by scavenging ROS and enhancing endogenous antioxidant defenses, which are crucial in mitigating cisplatin-induced oxidative damage.²¹ The potent *in vitro* antioxidant activity of VOE, as demonstrated by DPPH, ABTS, and reducing power assays, further validates its ability to counteract oxidative stress *in vivo*.²²

Serum creatinine and urea are key biomarkers of renal function, and their elevated levels indicate impaired kidney function.²³ In our research study, cisplatin administration led to a marked enhancement in serum creatinine and urea levels, suggesting nephrotoxicity. This is consistent with studies highlighting the detrimental effects of cisplatin on renal tubular epithelial cells, primarily due to oxidative stress.⁴

VOE supplementation significantly reduced serum creatinine and urea levels, indicating an improvement in renal function. Nephroprotective effects of VOE may be attributed to its antioxidative potential, which mitigates ROS-mediated renal injury and preserves kidney function. These findings are in agreement with studies demonstrating that natural antioxidants such as curcumin and resveratrol improve renal function by modulating oxidative stress and apoptosis pathways.^{24,25}

Renal hypertrophy is a key feature of nephrotoxicity, often resulting from oxidative damage and compensatory cellular proliferation.²⁶ In this research, cisplatin-treated rats showed a significant increase in kidney weight, indicative of renal inflammation and structural alterations. Similar results have been reported in previous studies, where oxidative stress-driven cellular damage led to kidney enlargement.²⁷

VOE administration significantly reduced kidney weight, suggesting a defensive effect against cisplatin-induced hypertrophy. This may be done due to its antioxidative properties, which mitigate cellular damage and glomerular expansion. Other plant extracts with nephroprotective effects, such as *Moringa oleifera*, have also been reported to attenuate kidney hypertrophy by reducing oxidative stress responses.²⁸

A defining feature of oxidative stress, lipid peroxidation is essential for cisplatin-induced nephrotoxicity. The increase in MDA levels observed in cisplatin-treated rats indicates significant lipid peroxidation, which can disrupt cellular membrane integrity and induce apoptosis.²⁹ The reduction in MDA levels following VOE treatment suggests a protective role in preserving membrane stability and preventing lipid oxidation.

Several phytoconstituents have been isolated from various medicinal plants and have shown promising nephroprotective effects, particularly against cisplatin-induced Acute Kidney Injury (AKI).³⁰ *Viburnum opulus* (VOE), a plant known for its rich composition of polyphenols, flavonoids, tannins, and other bioactive compounds,³¹ has garnered attention for its potential to prevent or mitigate nephrotoxicity, including that induced by cisplatin.

The key bioactive constituents of VOE, including flavonoids, phenolic compounds, and tannins, play a significant role in combating oxidative stress, which is a key mechanism underlying cisplatin-induced nephrotoxicity. Cisplatin triggers the generation of Reactive Oxygen Species (ROS), leading to oxidative stress, inflammation, and subsequent renal tubular cell damage. Recent studies have demonstrated that flavonoids such as quercetin, rutin, and kaempferol possess potent antioxidant properties that scavenge ROS, thereby reducing oxidative damage in kidney cells.^{32,33} For example, quercetin, a flavonoid found in VOE, has been shown to significantly mitigate cisplatin-induced renal dysfunction. It exerts nephroprotective effects by modulating key antioxidant enzymes, including Superoxide Dismutase (SOD) and catalase, and by reducing Malondialdehyde (MDA) levels, a marker of lipid peroxidation.³⁴ Moreover, flavonoids also inhibit NF- κ B signaling pathways, which are crucial in the inflammatory response during cisplatin-induced nephrotoxicity.³⁵ Phenolic compounds, another major group of bioactive compounds in VOE, further contribute to the nephroprotective effects by enhancing the activity of endogenous antioxidants and reducing oxidative damage. For instance, gallic acid, a phenolic compound, has been shown to reduce oxidative stress in cisplatin-treated rats by increasing Glutathione (GSH) levels and protecting renal tissue from apoptosis.³⁶ These effects are significant because oxidative stress is one of the key players in cisplatin-induced nephrotoxicity. Tannins, which are also present in VOE, possess anti-inflammatory and antioxidant properties that help protect against kidney damage.^{37,38} These effects further contribute to mitigating the kidney injury induced by cisplatin, which is

characterized by a marked inflammatory response and immune cell infiltration into renal tissue.

Histopathological examination of cisplatin treated group revealed severe kidney damage, including glomerular hypertrophy, tubular dilation, and inflammatory cell infiltration. VOE treatment significantly reduced proteinuria and maintained renal histology close to normal, reinforcing its potential renoprotective effects, likely through its antioxidant mechanisms that counteract oxidative stress-related renal damage.²⁹

CONCLUSION

The findings of this research study suggest that *Viburnum opulus* Extract (VOE) exhibits significant nephroprotective effects against cisplatin-induced oxidative stress and renal dysfunction. By enhancing antioxidant defense mechanisms, reducing lipid peroxidation, and improving renal function parameters, VOE demonstrates promising therapeutic potential in mitigating cisplatin-induced nephrotoxicity. The potent *in vitro* antioxidant activity of VOE, as evidenced by DPPH, ABTS, and reducing power assays, supports its role in combating oxidative stress. However, while these results are encouraging, further studies are warranted to explore the molecular mechanisms underlying VOE's nephroprotective effects. Additionally, toxicity profiling, bioavailability studies, and long-term studies are essential to confirm the clinical applicability and safety of VOE as a nephroprotective agent. These investigations will provide valuable insights into the therapeutic potential of VOE and its suitability for long-term use in clinical settings. These studies will be a significant part of the future scope of our work.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

AKI: Acute Kidney Injury; **BUN:** Blood Urea Nitrogen; **CAT:** Catalase; **DPPH:** 2,2-Diphenyl-1-Picrylhydrazyl; **GAE:** Gallic Acid Equivalent; **GPx:** Glutathione Peroxidase; **GSH:** Reduced Glutathione; **IC₅₀:** Half Maximal Inhibitory Concentration; **IAEC:** Institutional Animal Ethics Committee; **i.p.:** Intraperitoneal; **MDA:** Malondialdehyde; **NBT:** Nitroblue Tetrazolium; **p.o.:** Per Os (Oral Administration); **QE:** Quercetin Equivalent; **ROS:** Reactive Oxygen Species; **SEM:** Standard Error of Mean; **SOD:** Superoxide Dismutase; **TBA:** Thiobarbituric Acid; **TBARS:** Thiobarbituric Acid Reactive Substances; **TFC:** Total Flavonoid Content; **TPC:** Total Phenolic Content; **UV:** Ultraviolet; **VOE:**

Viburnum opulus Extract; **DTNB:** 5,5'-Dithiobis-2-Nitrobenzoic Acid.

AUTHOR CONTRIBUTIONS

Indhu DG was responsible for conducting the experiments, data collection, and initial compilation of results. G. Mallikarjuna oversaw the research, provided critical feedback on the report, and helped to conceptualize and design the study. Lavanya Yaidikar provided expertise in statistical analysis and data interpretation, contributing to the finalization of results. The final manuscript was reviewed and approved by all writers.

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Evaluation of Anti-Urolithiasis and Hepatoprotective Effects of *Mentha piperita* and Quercetin in Ethylene Glycol-Induced Urolithiasis in Wistar Rats

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ABSTRACT

Aim: Urolithiasis, a prevalent global health concern, is characterized by the formation of kidney stones. This study delves into the potential anti-urolithiasis properties of *Quercetin* and *Mentha piperita* (peppermint) using an ethylene glycol-induced model. The research aims to explore the combined effects of these natural compounds, comparing their efficacy with the conventional drug allopurinol. **Materials and Methods:** Male Wistar rats, exposed to ethylene glycol-induced urolithiasis, were administered extracts from *Mentha piperita*, *quercetin*, or a combination of the two. The investigation assessed indicators of oxidative stress, renal function, urinary parameters, and crystal deposition. **Results:** indicate a synergistic action of *quercetin* and *Mentha piperita*, effectively reducing crystal formation and preventing kidney damage. The treatment improved renal function and significantly decreased urine abnormalities induced by ethylene glycol. Furthermore, a reduction in oxidative stress markers suggests the combination possesses antioxidant properties. The co-administration of *Mentha piperita* and *quercetin* emerges as a promising therapeutic option for urolithiasis, offering a holistic approach that addresses both oxidative stress and crystal formation. While the study sheds light on the potential benefits of this natural combination, further investigation is needed to fully unravel the underlying mechanisms and therapeutic potential. **Conclusion:** In conclusion, this study underscores the potent anti-urolithiatic, antioxidant, and anti-hepatotoxic effects of *Mentha piperita* and *quercetin*. The reduction in levels of calcium, phosphate, oxalate, uric acid, alkaline phosphate, bilirubin, aspartate aminotransferase, alanine transaminase, superoxide dismutase, catalase, and glutathione in urine, serum, and kidney homogenate signifies the promising therapeutic impact of this natural combination.

Keywords: Urolithiasis, Ethylene glycol, *Mentha piperita*, *Quercetin*, Liver damage, Albino wistar rats.

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INTRODUCTION

A genitourinary system pathology called urolithiasis is defined by the development of calculi, or stones, in the urinary tract due to the accumulation of minerals and salts. This involves the development of kidney and ureteral stones, which obstruct urine flow and result in discomfort and other symptoms. In some cases, urethra or bladder urolithiasis can occur.¹

Urolithiasis is more common in males than in females. Patients with kidney stones tend to be between the ages of 30 and 50.²

In the United States, more than a million people visit doctors every year, and more than 300,000 people seek treatment for

kidney stones at emergency departments³. Urolithiasis affects approximately 2 million people in India, and the number of individuals suffering from kidney stones has significantly risen over the years. Additionally, kidney stones are often a recurring condition, with a 50% likelihood of recurrence within the next decade. Certain states in India, known as the “stones belt”, experience a higher prevalence of kidney stone cases, including Maharashtra, Gujarat, Rajasthan and northeastern states.³

However, bigger stones that are larger than 20mm may require more invasive treatments, such as surgery or scopes. There are several surgeries and surgical procedures that might increase the difficulties both during and following surgery.³

Allopathic medicine offers various treatments for kidney stones, employing a range of medications to address this condition. These include potassium citrate, sodium cellulose phosphate, oral calcium supplements, allopurinol, thiazides, thiol sodium bicarbonate, penicillamine, aceto-hydroxamic acid, and ketorolac,



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among others. Each of these medications plays a specific role in mitigating the formation or growth of kidney stones. The diverse nature of these pharmaceutical interventions highlights the complexity of managing and treating this health concern.⁴

These drugs do, however, have a variety of side effects. Other adverse effects of thiazide include weakness, blurred vision, and dizziness. With sodium cellulose phosphate, adverse reactions include convulsions, loss of appetite, weakness, and other issues. Other adverse effects of allopurinol include rashes, fever, joint pain, irregular bleeding, and others. Other symptoms that potassium citrate may cause include nausea, vomiting, and diarrhoea.

In addition to other adverse effects, penicillamine can cause fever, rashes, hives, joint pain, and ulcers. Shortness of breath, skin rashes, sudden weight gain, and other adverse consequences are possible side effects of ketorolac.⁴

During surgery, anesthesia is necessary, which may not be appropriate for older people. Shock wave lithotripsy can induce kidney damage after surgery, which can lead to diabetes and excessive blood pressure in some circumstances.⁴

As a result, it is necessary to research readily available alternatives so that their therapeutic benefits may be achieved and improved. The use of medicinal plants in pharmacological urolithiasis therapy is increasing for the reasons mentioned above.⁴

Herbal remedies are affordable and don't require hospitalization or surgery. They are known to have fewer side effects when compared to allopathic medications. The general population has easy access to and widespread availability of herbal treatments.⁴

Mentha piperita, commonly known as peppermint, is a widely utilized herb with a rich history of safe medicinal applications. It encompasses the dried leaves and flowering tops of *Mentha piperita* L. (peppermint) and *Mentha spicata* L. (spearmint), both belonging to the *Lamiaceae* family. Notably, *Mentha piperita* L., a sterile hybrid of *M. aquatica* L. and *M. spicata*, stands out as a paramount aromatic herb globally, particularly in terms of the extensive cultivation area dedicated to oil distillation. The peppermint leaves exude a distinctive sweetish, robust fragrance and possess an aromatic, warm, pungent taste, followed by a refreshing aftertaste. Beyond its sensory allure, peppermint leaves have been traditionally employed as a remedy for various ailments, including the common cold, inflammation of the mouth and pharynx, liver disorders, and gastrointestinal disturbances such as nausea, vomiting, diarrhoea, cramps, flatulence, and dyspepsia. Rich in polyphenols, peppermint serves as a source of highly effective antioxidants, exhibiting lower toxicity compared to synthetic counterparts. Its pharmacological properties encompass a spectrum of beneficial activities, including anesthetic, antimicrobial, antifungal, anthelmintic, and antioxidant attributes. Moreover, peppermint

demonstrates antinociceptive, anti-inflammatory, antimicrobial, anticarcinogenic, antiviral, anti-allergic, and antitumorigenic activities, underscoring its potential utility in both the prevention and treatment of various diseases.⁵

Quercetin, a vital bioflavonoid found in over twenty plant materials, is renowned for its diverse health benefits. Its anti-inflammatory, antihypertensive, vasodilator, anti-obesity, anti-hypercholesterolemic, and anti-atherosclerotic properties contribute to its significance in promoting well-being. The role of free radicals in the development of diseases such as hypertension, vascular disorders, and metabolic syndrome underscores the importance of antioxidants. *Quercetin*, as a potent antioxidant, combats free radicals, mitigating the risk of various ailments.⁶

Quercetin, a compound known for its diverse range of benefits, encompasses cardiovascular protection, antitumor and anticancer properties, anti-allergy and anti-ulcer characteristics, anti-inflammatory activity, antiviral capabilities, anti-diabetic attributes, gastroprotective effects, antihypertensive benefits, immunomodulatory features, and anti-infective potential. The extensive array of positive impacts underscores the versatility and significance of *quercetin* in contributing to various dimensions of health and well-being. Surprisingly, there is currently a lack of scientific studies or published work on the anti-urolithiatic potential of *Mentha piperita* and *quercetin*.⁷

Therefore, the primary objective of the present study is to assess the anti-urolithiatic effect of *Mentha piperita* and *quercetin*, along with their potential to ameliorate allopurinol-induced liver damage. This research endeavours to comprehensively explore the biological and pharmacological significance of *quercetin*, shedding light on its multifaceted potential for maintaining overall health. By investigating the anti-urolithiatic properties of *Mentha piperita* and *Quercetin*, this study aims to contribute valuable insights to the existing body of knowledge and further elucidate the holistic health benefits associated with these natural compounds.⁸

MATERIALS AND METHODS

Collection of plant material

***Mentha piperita*:** Collection of the marketed product from manufactured and distributed by Hindustan herbs.

***Quercetin*:** Collection of the marketed product from shrisha herbal (an ISO and GMP certified company).

Experimental Animal

Male Wistar rats, with an initial weight range of 150-200 g, were obtained from Krupanidhi College of Pharmacy in Bangalore, India. These rats underwent a period of acclimatization in a well-ventilated animal house, where laboratory conditions were carefully controlled for 10 days. The environment maintained a controlled temperature of 25±4°C, relative humidity between

50-60%, and a 12-hr light-dark cycle. The rats had access to food and water ad libitum during this acclimatization period. The study protocol received ethical approval from the Institutional Animal Ethics Committee, aligning with the guidelines provided by the Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA) under Ref. No. 378/PO/ReBi/S/01/CPCSEA. Throughout the experiment, the animals were housed and cared for in accordance with these approved standards within an animal facility.⁹

Grouping and dosing method

Wistar male rats were systematically divided into five groups, each comprising six animals. In Group I, considered the control, rats were provided with regular rat food and had access to drinking water ad libitum. To induce renal calculi over a 28-day period, Groups II–V were fed with ethylene glycol (0.75%). Group III received fixed doses of *Mentha piperita* (50 mg/kg) and *quercetin* (20 mg/kg) individually. Group IV was administered allopurinol alone at a fixed dose of 27 mg/kg. In Group V, a combination of *Mentha piperita*, *quercetin*, and allopurinol was given from the 15th day through the 28th day. This experimental design aimed to investigate the effects of these substances on ethylene glycol-induced renal calculi, providing valuable insights into potential preventive or therapeutic measures.¹⁰

Study Plan

General Parameters

Measurement of Body weight

The weight of the animals was recorded in grams on both the initial and final days of the treatment period, enabling the calculation of the percentage change in body weight. This approach serves as a valuable metric to assess and quantify any fluctuations or alterations in the animals' weight over the course of the study. By comparing the weights at the beginning and end of the treatment, researchers can gauge the impact of the experimental interventions on the overall body weight of the Wistar male rats. Such observations contribute to a comprehensive understanding of the potential physiological effects of the administered substances and their influence on the animals' well-being throughout the study duration.¹¹

Measurement of food intake

A measured quantity of food was provided to the rats daily, and the initial and final weights of the food were recorded over a 24-hr period. This systematic approach allowed for the calculation of food intake values, expressed in grams per 24 hr, representing the daily consumption of food by the Wistar male rats. By observing the changes in food weights within this specified timeframe, researchers could assess and quantify the rats' dietary habits and variations in food consumption. This data contributes to a comprehensive analysis of the animals' nutritional patterns, providing insights into potential correlations between the

administered treatments and their impact on food intake over the course of the study.¹²

Measurement of water intake

The study included the observation of both the initial and final amounts of water consumed by the rats over a 24-hr period. This systematic recording allowed for the calculation of water intake values, expressed in grams per 24 hr or grams per day. This approach serves as a crucial component in assessing the anti-urolithiatic activity of the substances under investigation. By quantifying the rats' water intake throughout the study, researchers gain valuable insights into potential correlations between the administered treatments and their influence on fluid consumption. This data contributes to a comprehensive evaluation of the impact of the experimental interventions on the rats' urolithiatic conditions, enhancing our understanding of the potential therapeutic effects of the substances involved in the study.¹³

Collection of urine

Urine samples were systematically collected over a 24-hr period on the 28th day, with each rat housed individually in a metabolic cage. Throughout the urine collection period, the animals had unrestricted access to drinking water. To preserve the samples, a drop of concentrated hydrochloric acid was added before storing them at 4°C. Subsequent analysis involved the estimation of various parameters from the collected urine, including urinary output, calcium, phosphate, oxalate, and uric acid. This meticulous approach to urine sample collection and analysis provides crucial data for evaluating the impact of the experimental interventions on these urinary parameters. The findings contribute to a comprehensive understanding of the potential anti-urolithiatic effects of the administered substances in the study.¹⁴

Collection of serum

Blood samples were obtained from the retro-orbital plexus of rats under light ether anesthesia, utilizing 1 mm glass capillaries. Collection was done in 2 mL Eppendorf tubes containing heparin to prevent clotting. Following a 15-min open clotting period, the blood samples were centrifuged at 5000 rpm for 20 min, facilitating the separation of serum. The obtained serum was then stored at -20°C until further analysis. Various biochemical parameters, including calcium, phosphate, oxalate, uric acid, bilirubin, AST, ALT, and ALP, were subsequently estimated from the stored serum. This meticulous collection and analysis of blood samples provide essential data for assessing the impact of the experimental interventions on these biochemical parameters, contributing to a comprehensive understanding of the physiological effects of the administered substances in the study.¹⁵

Procedure for estimation of biomarkers Estimation of Urine and Serum Calcium:

Calcium level in urine and serum of rats of different groups were estimated using the diagnostic kits (Erba diagnostic kit).

Estimation of urine and serum phosphate

Phosphate level in urine and serum of rats of different groups were estimated using the diagnostic kits (Erba diagnostic kit)
Estimation of Uric acid:

Uric acid level in urine and serum of rats of different groups were estimated using the diagnostic kits (Erba diagnostic kit).

Biochemical Estimation on Tissue parameters

Preparation of mitochondrial supernatant

At the conclusion of the study, the rats were humanely euthanized through an overdose of anesthetic ether. The kidneys were then carefully extracted, and subsequent to weighing, 10% kidney tissue homogenate was meticulously prepared in 0.1 M Tris HCl buffer with a pH of 7.8. Following the homogenization process, the resulting mixture underwent centrifugation at 10,000 rpm for 10 min at 4°C. The supernatant obtained from this centrifugation was utilized for assessing the activity levels of key enzymes, namely superoxide dismutase, catalase, and glutathione. This systematic approach to tissue preparation and enzyme analysis provides crucial data for evaluating the antioxidant status and overall health of the kidneys in the context of the experimental study

Estimation of SOD

In the SOD estimation procedure, the goal was to assess SOD activity by examining its ability to inhibit the reduction of a tetrazolium dye by superoxide radicals. The procedure involved the preparation of a reaction mixture comprising phosphate buffer, EDTA solution, NBT solution, riboflavin solution, and methionine solution. A tissue sample or cell extract was homogenized in the phosphate buffer, and the clear supernatant was obtained after centrifugation.¹⁶

Cuvettes or test tubes were labelled for the blank, sample, and positive control. The blank consisted of phosphate buffer only; the sample involved adding tissue homogenate or cell extract to the reaction mixture; and the positive control included a known SOD standard in the reaction mixture.

The reaction mixtures and the blank were incubated in a chamber with a light source for a specified period (e.g., 10 min). The light source initiated the reaction by generating superoxide radicals. The reaction was then stopped by placing the cuvettes or test tubes in the dark.

The absorbance of the reaction mixtures and the blank was measured at a specific wavelength (e.g., 560 nm) using a spectrophotometer. SOD activity was calculated by comparing the reduction in absorbance in the sample and positive control

with the blank, utilizing a formula or a standard curve generated from known SOD concentrations.

Throughout the experiment, duplicates or triplicates were used for accuracy, and efforts were made to maintain consistent incubation conditions. Fresh reagents were employed, and the reaction mixture was prepared just before the experiment. The procedure was adapted based on the specific requirements of the study, and reference to scientific literature or laboratory protocols was made for detailed and validated procedures.¹⁶

Estimation of Catalase

In the catalase estimation procedure, the primary objective was to assess catalase activity by observing its capability to decompose hydrogen peroxide, employing a spectrophotometer or colorimeter for precise quantification.¹⁷ The process commenced with the preparation of the sample, which could be fresh tissue, such as liver tissue, or an extract. In the case of tissue, it was homogenized in a buffer solution, while for extracts, a dilution was created in the buffer solution. Cuvettes or test tubes were labelled accordingly, designating a blank with only buffer solution, a sample with buffer solution and the tissue homogenate or extract, and a positive control with buffer solution and a known catalase solution for calibration. A known volume of hydrogen peroxide was added to each cuvette or tube, with mixing occurring promptly. The reaction was initiated by starting a stopwatch or timer upon hydrogen peroxide addition, allowing the reaction to proceed for a specific duration, typically around 1 min. The reaction was then stopped by introducing a catalase activity-inhibiting reagent, such as sulfuric acid, after the designated time. A spectrophotometer or colorimeter was utilized to measure absorbance at a specific wavelength, commonly around 240 nm. Catalase activity, proportional to the rate of hydrogen peroxide decomposition, could be calculated using an appropriate formula or a calibration curve. This method provided a streamlined and effective approach to estimating catalase activity, offering valuable insights into its potential therapeutic

implications. For detailed specifics and variations, reference to scientific literature or laboratory protocols was recommended.¹⁷

Estimation of GSH

In the GSH estimation procedure, the objective was to determine Glutathione (GSH) levels through a straightforward reaction with 5,5'-Dithiobis (2-Nitrobenzoic acid) (DTNB), forming a yellow-coloured complex. The process involved the preparation of a sample by homogenizing tissue or creating a cell extract in phosphate buffer, followed by centrifugation to obtain a clear supernatant.¹⁸

Cuvettes or test tubes were labelled for the blank, sample, and standard. The blank consisted of phosphate buffer, the sample involved adding the clear supernatant to DTNB solution, and the standard utilized a known GSH concentration.

Ellman's reagent was added to each cuvette or tube to initiate the reaction, and the mixtures were then incubated at room temperature for a specified duration (e.g., 10 min). The reaction was halted by adding a sodium bicarbonate solution to each cuvette or tube.

The absorbance of the reaction mixtures and the blank was measured at a specific wavelength (e.g., 412 nm) using a spectrophotometer. GSH levels were calculated by comparing the absorbance of the sample with the standard and blank, employing a formula or a standard curve generated from known GSH concentrations.

Throughout the experiment, duplicates or triplicates were used for accuracy, and efforts were made to maintain consistent incubation conditions. Fresh reagents were employed, and the reaction mixture was prepared just before the experiment. The procedure was adapted based on the specific requirements of the study, with reference to scientific literature or laboratory protocols for detailed and validated procedures.¹⁸

Estimation of Serum SGPT (UV- Kinetic method)

ALT level in urine and serum of rats of different groups were estimated using the diagnostic kits (Erba diagnostic kit).

Estimation of Serum SGOT (UV- kinetic method)

AST level in urine and serum of rats of different groups were estimated using the diagnostic kits (Erba diagnostic kit).

Estimation of Serum Alkaline Phosphatase (ALP)

ALP level in urine and serum of rats of different groups were estimated using the diagnostic kits (Erba diagnostic kit).

Statistical Analysis

The statistical analysis in this study involved the comparison of values using the mean±Standard Error of the Mean (SEM). This approach allowed for a comprehensive examination of the data, enabling a nuanced understanding of the central tendency while considering the precision of the measurements. Utilising mean±SEM values enhances the clarity of the results, providing a more detailed insight into the distribution of data points and contributing to the overall robustness of the statistical analysis using GraphPad version 10.2.

RESULTS AND DISCUSSION

Ethylene glycol induced urolithiasis

Rats with kidney stones were treated with various interventions as previously described. Electrolytes, including calcium, phosphate, oxalate, and uric acid, were assessed in urine, serum, and kidney homogenates using an auto analyser.

General Parameters

The following tables shows the effect of allopurinol, *Mentha piperita* and *quercetin* on biochemical variables.

Effect of *Mentha Piperita*, Quercetin and Allopurinolon body weight [on 14th day 28th day]

In this study, the observation of weight gain in all groups, including the one treated with *Mentha piperita* and *quercetin*, prompts a closer examination of each cohort. The normal group exhibited expected weight gain, while the positive group showed slightly lower weight gain, potentially due to experimental variations. Interestingly, the group treated with *Mentha piperita* and *quercetin* demonstrated weight gain similar to the normal and positive groups, suggesting no negative impact on overall weight gain. The unexpected finding was the allopurinol-treated group, which displayed weight gain comparable to the other groups, contrary to conventional expectations.

Moreover, the group receiving combined treatment with *Mentha piperita*, *quercetin*, and allopurinol showed weight gain similar to or slightly higher than other groups, indicating that this combined treatment did not significantly alter the general trend of weight gain. This leads to questions about the specific effects of *Mentha piperita*, *quercetin*, and allopurinol on body weight, emphasizing the need for a thorough review of existing literature on the subject.

Moving on to diuretic effects, the combination of *Mentha piperita*, *quercetin*, and allopurinol resulted in a significant increase in urine volume compared to the positive control group. While *Mentha piperita* and *quercetin* are known for their potential diuretic properties, allopurinol, not conventionally considered a diuretic, may indirectly impact renal function.

The observed synergy among these compounds suggests potential interactions enhancing diuretic effects, prompting exploration into the collective contribution of *Mentha piperita*, *quercetin*, and allopurinol to increased urine volume. This necessitates a thorough comparison with existing literature, considering previous studies on the diuretic effects of *Mentha piperita* and *quercetin*, either individually or in combination. Insights from reports on allopurinol influencing urine volume would further enrich our understanding.

The working hypothesis could pivot on synergistic interactions among *Mentha piperita*, *quercetin*, and allopurinol, potentially involving mechanisms such as increased renal blood flow, altered ion transport in the kidneys, or modulation of hormonal pathways regulating fluid balance. Acknowledging study limitations, including sample size and potential confounding factors, is imperative. This significant outcome calls for further exploration, paving the way for future research, including clinical trials or more in-depth mechanistic studies in diverse populations. Table 1 is mentioned below.

Effect of *Mentha Piperita*, Quercetin and Allopurinol on Urine (Calcium, Phosphate, Oxalate, Urine uric acid) on Ethylene Glycol Induced Urolithiasis

In this study, the evaluation of urine calcium, phosphate, oxalate, and uric acid levels among various treatment groups compared to a positive control and normal group sheds light on the potential therapeutic effects of *Mentha piperita*, quercetin, and allopurinol. The presentation of results in mean±Standard Error of the Mean (SEM) in mg/dl facilitates a comprehensive understanding of the observed patterns in Table 2.

Examining urine calcium levels, a significant increase in the positive control group, compared to the normal group, was noted. However, the combined treatment regimen remarkably and significantly decreased urine calcium levels compared to the positive control. This unexpected outcome necessitates further exploration, aligning with or deviating from existing literature and working hypotheses.

Similarly, urine phosphate levels exhibited a significant increase in the positive control group, with treatments, particularly the combination of *Mentha piperita* and quercetin, leading to an extremely significant decrease. This implies a potential therapeutic effect in mitigating elevated urine phosphate levels, inviting deeper exploration into underlying mechanisms and comparisons with prior reports.

The assessment of urine oxalate levels revealed an extremely significant increase in the positive control group, contrasting with an extremely significant decrease following treatment with *Mentha piperita*, quercetin, and allopurinol. This intriguing finding raises questions about the specific mechanisms at play, requiring alignment with or reconsideration of existing literature and working hypotheses.

Furthermore, urine uric acid levels showed an extremely significant increase in the positive control group, while the combined treatment regimen led to an extremely significant decrease. This result, consistent with or deviating from expectations, demands a thorough review of existing literature and working hypotheses to uncover the potential therapeutic impact of the treatment on uric acid metabolism.

In summary, the presented findings underscore the complexity of interactions between *Mentha piperita*, quercetin, and allopurinol in influencing urinary biomarkers. Comparative analyses with previous reports and critical reassessment of working hypotheses are essential for a comprehensive understanding of the observed outcomes and the potential therapeutic implications of the treatment regimen. Further exploration and validation through additional studies will contribute to refining our understanding of the intricate effects of these compounds on urinary parameters. Table 2 is mentioned below.

Table 1: Effect of *Mentha Piperita*, Quercetin and Allopurinol on Body Weight [on 14th day 28th day].

Treatment (P.O)	M+Q+A on body weight of rats on 14 th day	M+Q+A on body weight of rats on 28 th day	M+Q+A on urine volume
Normal group	185±0.846	200.66±0.49	6.9±0.25
Positive (EG induced)	175±0.447	182.6±0.66	4.8±0.38
<i>Mentha piperita</i> + quercetin (50+20mg/kg)	180.6±0.33	192.3±1.145	7.4±0.71
Allopurinol(27mg/kg)	182.5±1.02	195.6±1.085	9.2±0.81
<i>Mentha piperita</i> + quercetin + allopurinol(50+20+27mg/kg)	183.5±2.51	198±0.96	11.3±0.74

Table 2: Effect of *Mentha Piperita*, Quercetin and Allopurinol on Urine (Calcium, Phosphate, Oxalate, Urine uric acid) on Ethylene Glycol Induced Urolithiasis.

Treatment (p.o)	Urine calcium (mg/dL)	Urine phosphate(mg/dL)	Urine oxalate(mg/dL)	Urine Uric acid (mg/dL)
Normal group	6.2±0.31	3.6±0.29	2.0±0.11	0.7±0.13
Positive (EG induced)	14.4±0.72	9.5±0.71	4.5±0.12	5.2±0.31
<i>Mentha piperita</i> , quercetin (50+20mg/kg)	9.7±0.56	5.51±0.57	2.23±0.27	2.6±0.31
Allopurinol (27mg/kg)	8.9±0.51	4.6±0.43	2.15±0.20	2.3±0.19
<i>Mentha piperita</i> , Quercetin, allopurinol, (50+20+27mg/kg)	7.6±0.42	4.3±0.36	2.12±0.76	1.5±0.14

Effect of *Mentha piperita*, Quercetin and Allopurinol on Serum (Calcium, Phosphate, Oxalate and Uric acid) on Ethylene Glycol Induced Urolithiasis

In this study, the analysis of serum calcium, phosphate, oxalate, and uric acid levels among different treatment groups revealed unexpected yet highly significant outcomes, as depicted in Table 3. In the positive control group, a notable increase in all these parameters was observed compared to the normal group. However, treatments involving the combination of *Mentha piperita* and Quercetin, allopurinol alone, and the combination of *Mentha piperita*, Quercetin, and allopurinol with fixed doses exhibited an equally remarkable and extremely significant decrease in these serum levels when compared to the positive control group.

The unexpected reduction in serum calcium, phosphate, oxalate, and uric acid levels prompts a critical review of the working hypotheses. If the initial expectations aligned with a reduction in these parameters due to the treatment, the results validate the working hypotheses. Conversely, if the hypotheses predicted an increase or no change, the unexpected outcome requires reassessment to comprehend the specific mechanisms at play.

In summary, the substantial and consistent decrease in serum mineral levels following treatment with *Mentha piperita*, quercetin, and allopurinol raises intriguing questions. Comparative analyses

with previous reports will contribute to understanding the consistency of these findings. Revisiting and refining working hypotheses in light of these results will guide future research directions and enhance the comprehension of the therapeutic impact of this treatment regimen on mineral homeostasis. The observed effects underscore the potential of this combination in modulating mineral levels and highlight its significance in the context of urolithiasis management. Further investigations into the underlying mechanisms will be crucial for validating and expanding upon these promising findings. Table 3 is mentioned below.

Effect of *Mentha piperita*, Quercetin and Allopurinol on Kidney's (Homogenate Calcium, Homogenate phosphate, Homogenate oxalate and homogenate uric acid) on Ethylene Glycol Induced Urolithiasis

In this study, the examination of calcium, phosphate, oxalate, and uric acid levels in kidney homogenate across treatment groups, as presented in Table 4, reveals compelling results. In the positive control group, there was an extremely significant increase in these parameters compared to the normal group, indicating disrupted renal mineral homeostasis. However, treatments involving the combination of *Mentha piperita* and Quercetin, allopurinol alone, and the combination of *Mentha piperita*, Quercetin, and allopurinol with fixed doses demonstrated varying degrees of

Table 3: Effect of *Mentha Piperita*, Quercetin and Allopurinol on Serum (Calcium, Phosphate, Oxalate and Uric acid) on Ethylene Glycol Induced Urolithiasis.

Treatment (p.o)	Serum calcium(mg/dL)	Serum phosphate(mg/dL)	Serum oxalate(mg/dL)	Serum uric acid(mg/dL)
Normal group	3.81±0.34	5.61±0.37	2.71±0.29	2.82±0.07
Positive (EG induced)	13.1±0.71	14.92±0.82	5.21±0.63	5.72±0.30
<i>Mentha piperita</i> , quercetin (50+20mg/kg)	5.5±0.53	8.73±0.70	4.12±0.54	4.6±0.23
Allopurinol (27mg/kg)	4.9±0.47	7.5±0.55	4.1±0.45	4.21±0.19
<i>Mentha piperita</i> , Quercetin, allopurinol, (50+20+27mg/kg)	4.6±0.43	5.61±0.37	2.9±0.37	3.42±0.16

Table 4: Effect of *Mentha Piperita*, Quercetin and Allopurinol on Kidney's (Homogenate Calcium, Homogenate phosphate, Homogenate oxalate and homogenate uric acid) on Ethylene Glycol Induced Urolithiasis.

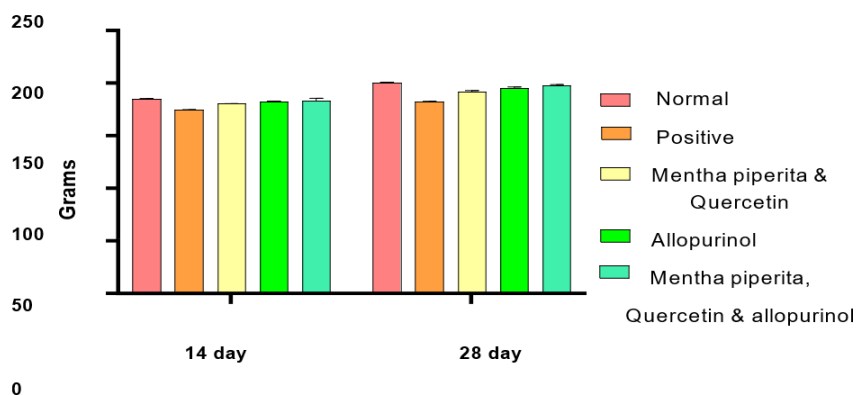
Treatment (p.o)	Kidney homogenate calcium(mg/dL)	Kidney homogenate phosphate(mg/dL)	Kidney homogenate oxalate(mg/dL)	Kidney homogenate uric acid(mg/dL)
Normal group	4.12±0.26	3.54±0.17	2.93±0.22	2.40±0.13
Positive (EG induced)	7.32±0.3	7.2±0.25	6.28±0.29	5.4±0.28
<i>Mentha piperita</i> , quercetin (50+20mg/kg)	6.51±0.6	5.2±0.23	4.41±0.25	3.55±0.23
Allopurinol (27mg/kg)	6.42±0.1	4.62±0.19	4.3±0.21	3.1±0.19
<i>Mentha piperita</i> , Quercetin, allopurinol, (50+20+27mg/kg)	4.40±0.3	4.42±0.18	3.52±0.17	2.66±0.17

Table 5: Effect on Superoxide Dismutase, Catalase and Glutathione.

Treatment (p.o)	SOD (unit/mg protein)	Catalase (unit/mg protein)	Glutathione (unit/mg protein)
Normal group	70.01±1.01	0.62±0.02	80.12±1.61
Positive (EG induced)	11.14±0.98	0.13±0.01	25.31±1.51
<i>Mentha piperita</i> , quercetin (50+20mg/kg)	50.12±1.02	0.51±0.01	53.4±1.02
Allopurinol (27mg/kg)	33.10±0.13	0.42±0.01	44.61±1.12
<i>Mentha piperita</i> , Quercetin, allopurinol, (50+20+27mg/kg)	64.6±0.91	0.53±0.01	55.41±3.01

Table 6: Effect of *Mentha Piperita* + Quercetin on Bilirubin, AST, ALP and ALT in Allopurinol Induced Liver Toxicity.

Treatment (p.o)	Bilirubin (U/L)	AST (U/L)	ALP (U/L)	ALT (U/L)
Normal group	1.35±0.017	67.46±0.59	96±1.06	32.5±0.76
Allopurinol (27mg/kg)	2.03±0.005	130.66±1.45	175.3±1.70	94.66±1.45
<i>Mentha piperita</i> + quercetin (50+20mg/kg)	1.42±0.0070	82.5±0.76	128.5±0.99	55.16±1.47

Body weight**Figure 1:** Effect of *Mentha piperita*, Quercetin and Allopurinol on body weight of rats on 14th and 28th day.

decrease in these renal levels compared to the positive control group.

This unexpected outcome prompts intriguing questions about the potential influence of these treatments on renal mineral regulation. To interpret these findings effectively, it is crucial to refer to existing literature and revisit the working hypotheses. If prior reports have indicated the efficacy of *Mentha piperita*, quercetin, and allopurinol in modulating renal mineral levels, the current results may align with established knowledge. Conversely, if the findings deviate from existing reports, a deeper exploration of the underlying mechanisms is warranted.

A critical review of the working hypotheses in light of the observed decrease in renal mineral levels is essential. If the initial

expectations were aligned with a reduction in these parameters due to the treatment, the results may support the working hypotheses to some extent. On the other hand, if the hypotheses predicted an increase or no change, a reassessment is necessary to understand the specific mechanisms at play.

In summary, the varying degrees of decreased renal mineral levels following treatment with *Mentha piperita*, quercetin, and allopurinol raise intriguing questions. Comparative analyses with previous reports will contribute to understanding the consistency of these findings. Revisiting and refining working hypotheses in light of these results will guide future research directions and enhance the comprehension of the therapeutic impact of this treatment regimen on renal mineral homeostasis. The unexpected outcomes highlight the complexity of interactions and emphasize

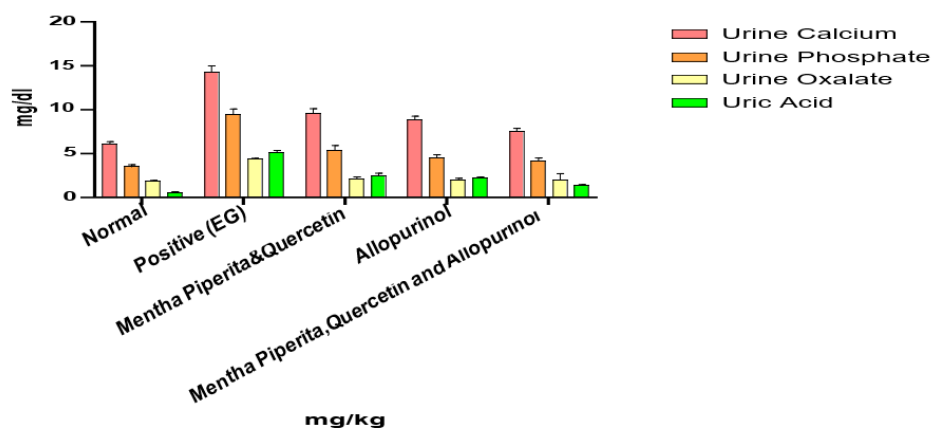


Figure 2: Effect of *Mentha piperita*, Quercetin and Allopurinol on urine calcium, phosphate, oxalate and uric acid.

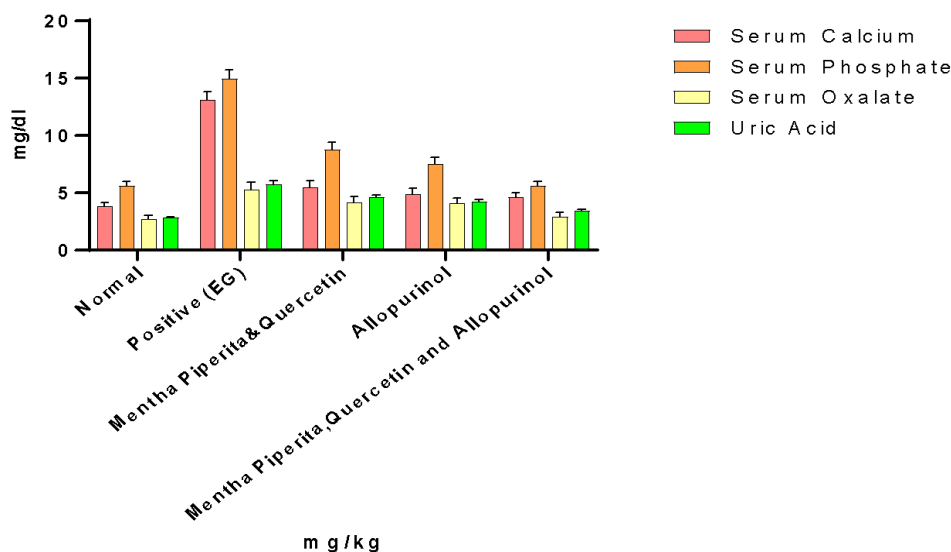


Figure 3: Effect of *Mentha piperita*, Quercetin and Allopurinol on serum calcium, phosphate, oxalate and uric acid.

the need for a nuanced interpretation of the data in the context of existing literature and hypotheses. Table 4 is mentioned below.

Effect on Superoxide Dismutase (SOD), catalase and Glutathione (GSH)

In this study, the thorough assessment of antioxidant enzymes-Superoxide Dismutase (SOD), catalase, and Glutathione (GSH)-in kidney homogenate, as presented in Table 5, provides valuable insights into the potential therapeutic impact of the combined treatment with *Mentha piperita*, *quercetin*, and allopurinol on renal antioxidant defense mechanisms.

Superoxide Dismutase (SOD) levels demonstrated an extremely significant decrease in the positive control group, indicating a compromised antioxidant defense system in renal tissues. However, the treatment with the combination of *Mentha*

piperita, *quercetin*, and allopurinol unexpectedly led to an extremely significant increase in SOD levels. This suggests a potential restoration or enhancement of the antioxidant defense mechanism in the kidneys, challenging existing hypotheses and prompting a need for their re-evaluation and refinement based on observed effects.

Similarly, catalase levels exhibited an extremely significant decrease in the positive control group, indicative of impaired antioxidant defense in renal tissues. The treatment with the combination of *Mentha piperita*, *quercetin*, and allopurinol resulted in an unexpected but extremely significant increase in catalase levels, emphasizing a potential therapeutic effect on catalase activity. This finding calls for a critical reassessment of working hypotheses to align them with the observed increase in antioxidant activity.

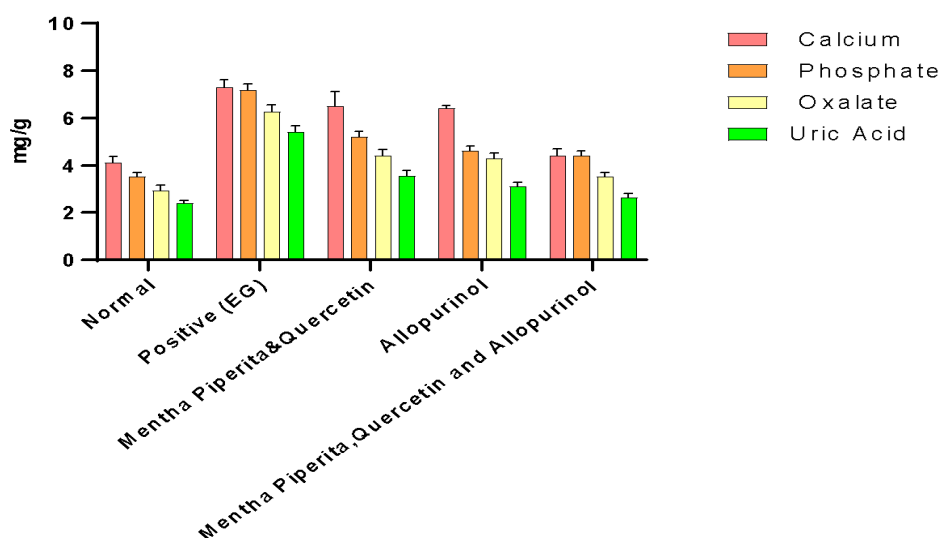


Figure 4: Effect of *Mentha piperita*, Quercetin and Allopurinol on kidney homogenate calcium, phosphate, oxalate and uric acid.

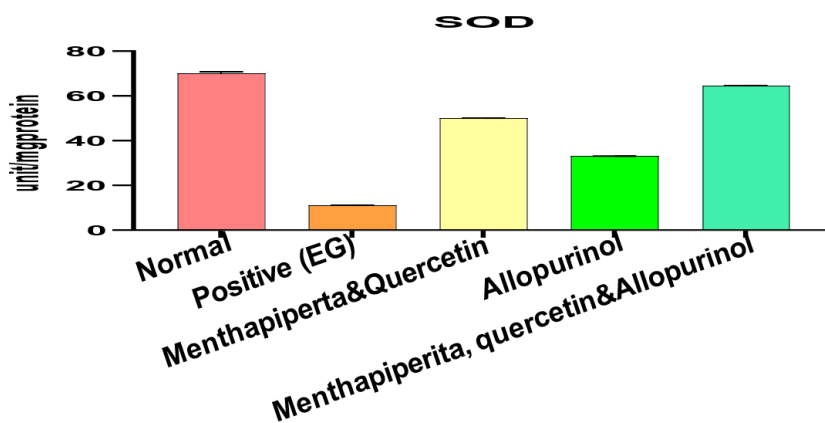


Figure 5: Effect of *Mentha piperita*, Quercetin and Allopurinol on superoxide dismutase (SOD).

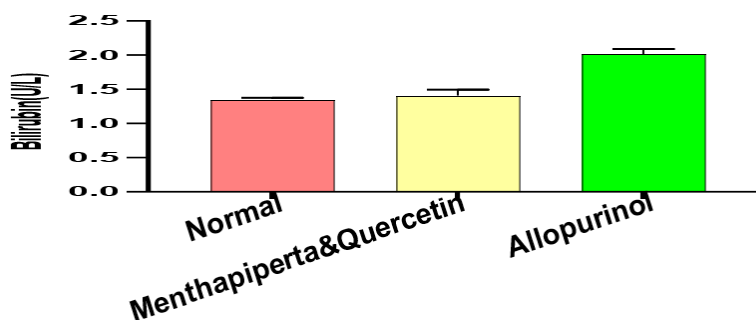


Figure 6: Effect of *Mentha piperita*, Quercetin and Allopurinol on bilirubin.

Glutathione (GSH) levels showed a significant decrease in the positive control group, suggesting a depletion of this crucial antioxidant in renal tissues. However, the treatment with *Mentha piperita* and *quercetin* exhibited an extremely significant increase in GSH levels, pointing towards a potential therapeutic effect in restoring or enhancing GSH levels and contributing to the antioxidant defense mechanism in the kidneys. As with SOD and catalase, these results necessitate a thorough revision of working hypotheses to capture the nuanced impact of the treatment.

To interpret these findings effectively, a comprehensive review of the existing literature is imperative. If prior reports support the efficacy of *Mentha piperita*, *quercetin*, and allopurinol in enhancing antioxidant levels, the current results align with established knowledge. However, if deviations are noted, further exploration of novel mechanisms is warranted to elucidate the specific pathways influenced by the treatment regimen.

In summary, the unexpected yet consistent increase in SOD, catalase, and GSH levels following treatment with *Mentha piperita*, *quercetin*, and allopurinol challenges existing hypotheses. Comparative analyses with previous reports will contribute to understanding the consistency of these findings. Revisiting and refining working hypotheses in light of these results will guide future research directions and enhance the comprehension of the therapeutic impact of this treatment regimen on renal antioxidant defense mechanisms. The unexpected outcomes highlight the complexity of interactions and emphasize the need for a nuanced interpretation of the data in the context of existing literature and hypotheses. Table 5 is mentioned below.

Effect of *Mentha piperita* + *Quercetin* on Bilirubin, AST, ALP and ALT in Allopurinol Induced Liver Toxicity

In this study, the assessment of the combined effects of *Mentha piperita* and *quercetin* on liver parameters-bilirubin, AST, ALP, and ALT-in allopurinol-induced liver toxicity, as presented in Table 6, reveals promising outcomes with potential therapeutic implications.

In the toxic control group, there was an extremely significant increase in bilirubin, AST, ALP, and ALT levels compared to the normal control group, indicating severe liver damage induced by allopurinol and substantiating its hepatotoxic effects under experimental conditions.

However, the treatment with *Mentha piperita* and *quercetin* at doses of 50 mg/kg and 20 mg/kg, respectively, demonstrated an extremely significant decrease in bilirubin, AST, ALP, and ALT levels compared to the toxic control group. This suggests a remarkable hepatoprotective effect of the combined treatment, mitigating the adverse impact of allopurinol-induced liver toxicity.

To interpret these findings effectively, it is crucial to refer to existing literature and revisit the working hypotheses. If previous reports have indicated the efficacy of *Mentha piperita* and *quercetin* in reducing liver enzyme levels and bilirubin, the current results align with established knowledge. Conversely, if the findings deviate from existing reports, a deeper exploration of the underlying mechanisms is warranted.

The working hypotheses should be critically reviewed in light of the observed decrease in liver parameters. If the initial expectations were aligned with a reduction in bilirubin, AST, ALP, and ALT levels due to the treatment, the results support the working hypotheses. On the other

hand, if the hypotheses predicted an increase or no change, the unexpected outcome necessitates a reassessment to understand the specific mechanisms at play. Table 6 is mentioned below.

CONCLUSION

The present study's findings lead to the conclusion that *Mentha piperita* and *quercetin* exhibit significant anti-urolithiatic and antioxidant effects against ethylene glycol-induced urolithiasis in male Wistar rats. Furthermore, the study highlights the hepatoprotective action of *Mentha piperita* and *quercetin* against allopurinol-induced liver damage. Notably, the combination of *Mentha piperita*, *quercetin*, and allopurinol effectively lowers high concentration of calcium, phosphate, oxalate, and uric acid in urine, serum, and kidney homogenate. Additionally, there is an increase in serum levels of antioxidant enzymes such as Superoxide Dismutase (SOD), catalase, and reduced Glutathione (GSH).

Importantly, the combination of *Mentha piperita* and *quercetin* demonstrates a notable reduction in the levels of AST, ALP, and ALT, whereas the allopurinol-induced group of animals exhibits increased levels of these liver enzymes (AST, ALP, and ALT). These results suggest that the administration of *Mentha piperita*, *quercetin*, and allopurinol has both protective and curative effects. The comprehensive effects observed in this study underscore the potential therapeutic benefits of the combined treatment approach in addressing urolithiasis and associated liver damage.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

ANOVA: Analysis of variance; **ALT:** Alanine aminotransferase; **ALP:** Alkaline phosphatase; **AST:** Aspartate aminotransferase; **CPCSEA:** Committee for the Purpose of Control and Supervision on Experiments on Animals; **DNA:** Deoxyribonucleic acid; **ESWL:** Extracorporeal shock wave lithotripsy; **gm/kg:** Grams per kilogram; **GFR:** Glomerular filtration rate; **GSH:** Glutathione; **HED:** Human equivalent dose; **HD:** High dose; **HPT:** Human

proximal tubule; **i.p**: Intraperitoneal; **IAEC**: Institutional Animal Ethics Committee; **IU/L**: International unit per litre; **KTH**: Kidney tissue homogenate; **LD**: Low dose; **mg/dl**: Milligram per deciliter; **mg/ml**: Milligram per milliliter; **µg**: Microgram; **Min**: Minutes; **µl**: Microliters; **p.o**: Per oral; **PCNL**: Percutaneous nephrolithotomy; **PTH**: Parathyroid hormone; **s.c**: Subcutaneous; **SEM**: Standard error of mean; **SGOT**: Serum glutamic oxaloacetic transaminase; **SGPT**: Serum glutamic pyruvic transaminase; **SOD**: Superoxide dismutase; **STD**: Standard dose; **SWL**: Shock wave lithotripsy; **UPJ**: Uretero pelvic junction; **UTI**: Urinary tract infection; **UTS**: Ureteroscopy; **UVJ**: Uretero vesical junction; **M+Q+A**: *Mentha piperita* + Quercetin + Allopurinol.

SUMMARY

In summary, the extremely significant decrease in liver parameters following treatment with *Mentha piperita* and *quercetin* in allopurinol-induced liver toxicity is a promising indication of hepatoprotective effects. Comparative analyses with previous reports will contribute to understanding the consistency of these findings. Revisiting and refining working hypotheses in light of these results will guide future research directions and enhance the comprehension of the therapeutic impact of this treatment regimen on liver function in the context of allopurinol-induced liver toxicity. The unexpected outcomes highlight the potential of *Mentha piperita* and *quercetin* as hepatoprotective agents, warranting further exploration and validation in broader contexts and populations.

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A New Spin on Gout: Exploring the Therapeutic Potential of a Novel Kaempferol-Molybdenum (VI) Complex

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ABSTRACT

Aim/Background: This study embarked on a journey exploring the therapeutic potential, developing and defining a novel kaempferol and molybdenum to scope out its uses in therapy in a rat model of gout brought on by potassium oxonate. It compared its impact on whether it changes certain conditions and learned about the promise it holds in preventing arthritic disease and gout. **Materials and Methods:** A kaempferol and molybdenum (VI) complex was thoughtfully defined and characterized using FTIR and UV-vis spectroscopy. A rat model of gout was created by inducing hyperuricemia, exposing the rats to Potassium Oxonate (PO), and was split up into several treatment groups, including those that received allopurinol and kaempferol, and molybdenum (VI) for 28 days. Parameters assessed included body weight, motor coordination, hematological parameters (RBC, WBC, MPV, ESR), serum uric acid, radiographic changes, and histopathological analysis of tibiotarsal joints. **Results:** A favorable therapeutic profile was revealed. The complex demonstrated a remarkable ability to lower uric acid levels, a key player in gout pathogenesis. Moreover, evidence of potent anti-inflammatory effects emerged, suggesting a great impact on the inflammatory cascade linked to gout. Intriguingly, radiographic along histopathological analyses revealed promising signs of joint protection, hinting at a potential for not only managing symptoms but also mitigating the long-term consequences of this debilitating disease. **Conclusion:** These preliminary findings ignite a spark of excitement, suggesting that this novel kaempferol and Molybdenum (VI) complex may offer a groundbreaking approach to gout treatment. Further investigations, such as pharmacokinetic studies, are crucial to unravel the intricate mechanisms underlying these promising effects and pave the way for their potential translation into clinical practice.

Keywords: Hyperuricemia, Gout, Potassium oxonate, Kaempferol, Molybdenum (VI) Complex.

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INTRODUCTION

A class of substances found in nature called flavonoids has garnered significant scientific and therapeutic attention in recent years and is widely distributed in plants.¹ As secondary metabolites of plants, flavonoids make up an extensive group of natural substances. A variety of fruits, vegetables, and even some drinks include them, and they have a polyphenolic structure.² They are predominantly located in the vacuoles of roots, flowers, leaves, or stems. Notably, flavonoids are responsible for the blue, brilliant red, and purple hues observed in diverse plant parts, such as seeds, leaves, flowers, and fruits.³

Flavonoids have long been extensively studied and found to exhibit diverse pharmacological properties. These include antioxidant properties,⁴ antiviral activity,⁵ anti-inflammatory properties,⁶

anticancer effects,⁷ antidiabetic effects⁸ antimutagenic activity, antithrombotic effects,⁹ antiatherosclerotic activity,¹⁰ vasodilatory effects¹¹ and neuroprotective properties.¹²

Kaempferol, a member of the flavonol subclass, is one particular flavonoid of interest.¹³ Kaempferol, which bears the name of German botanist and naturalist Engelbert Kaempferol, is well known for its various pharmacological characteristics and potential health advantages.¹⁴ Kaempferol (KMF) is a lipophilic compound with a yellowish crystal-like appearance and with 286.239 g/mol molecular weight. Its chemical structure is characterized by a diphenylpropane framework (C₁₅H₁₀O₆) that contributes to its unique properties. According to the IUPAC nomenclature, it is identified as 3,5,7-trihydroxy-2-(4-hydroxyphenyl) chromen-4-one.¹⁵ This structural arrangement shown in Figure 1¹⁶ is responsible for its distinct lipophilicity, which influences its behavior in biological systems.^{17,18}

Kaempferol can be found in many different plant sources, including tea,¹⁹ fruits,²⁰ vegetables,²¹ and medicinal herbs.²²⁻²⁴ It has sparked substantial curiosity due to its potential health benefits and therapeutic properties, and studies suggest this chemical may



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typically be consumed by humans at a level of 10 mg daily through diet. Based on epidemiological and preclinical studies, kaempferol exhibits an extensive array of health benefits and demonstrates diverse biological activities.¹⁷ Extensive research has revealed the pharmacological potential of kaempferol, highlighting its antioxidative,²⁵ anti-inflammatory,^{26,27} antimicrobial,²⁸ anticancer,^{29,30} hepatoprotective³¹ and neuroprotective properties.³² Its capacity to neutralize free radicals and regulate cellular signaling pathways has garnered considerable interest in the field of drug discovery using natural products.

Furthermore, research has looked into kaempferol's potential for treating and preventing several illnesses, including cardiovascular conditions,³³ cancer,³⁴ neurodegenerative disorders,³⁵ and diabetes.^{36,37}

The majority of bioflavonoids are notable for their intrinsic antioxidant qualities and their effective scavenging of free radicals, along with displaying strong metal chelating capabilities.³⁸ Flavonoids' role in metal chelation is widely acknowledged as a fundamental element of their antioxidant mechanism. The interaction between flavonoids and metal ions is acknowledged to exert influence over both the antioxidant attributes and the biological responses elicited by these compounds.³⁹ It is postulated that an organic ligand's biological functionality can be heightened by its coordination or interaction with a compatible metal ion, which can be attributed to its function as a receptor for free radicals.⁴⁰⁻⁴²

Despite the promising properties of kaempferol, its low bioavailability and chemical instability limit its practical applications in pharmaceutical formulations.⁴³ To get beyond these restrictions, researchers have investigated the formation of metal complexes with kaempferol.⁴⁴ In metal complexation, metal ions coordinate with organic ligands to form complex structures, resulting in the formation of stable complexes. This strategy offers several advantages, including improved solubility, enhanced stability, altered pharmacokinetics, and potential synergistic effects between the metal and the ligand.⁴⁵ So far it is reported that many metal ions are complexed with kaempferol which includes, Zn (II), Sb (V), La (III), Ru (II), Cu (II), Eu³⁺, Al (III), Ga (III), Fe (III) and many other metal ions.⁴⁶⁻⁵⁶

Classified as an essential trace element with intrinsic toxicity, Molybdenum (Mo) is a component of numerous enzymes in both plant and human systems, including oxidases. Its participation is integral to numerous enzymatic reactions, thereby underscoring its significance.⁵⁷

Earlier research has confirmed the substantial *in vitro* inhibitory effect of kaempferol on Xanthine Oxidase (XO). The catalytic conversion of xanthine into uric acid is catalysed by xanthine oxidase, an essential enzyme engaged in the breakdown of purines that is extensively dispersed throughout the human tissues and contributes to the onset of illness, including gout as

well as hyperuricemia. The inhibition kinetics assay revealed that kaempferol exerts reversible competitive inhibition on XO activity.⁵⁸ Xanthine oxidase inhibition activity is beneficial in gout as it reduces the production of uric acid, a key contributor to the development and exacerbation of gout symptoms. By lowering uric acid levels, restricting XO helps to reduce gout's associated pain and inflammation.

The objective of our investigation was to synthesize Kaempferol-Molybdenum complex, characterize, and evaluate the anti-gout activity of Kaempferol-Molybdenum Complex using a potassium oxonate-induced gout rat model.

MATERIALS AND METHODS

Synthesis and Characterization of Kaempferol-Mo (VI) Complex

A methanolic solution of kaempferol was prepared by dissolving 286 mg (equivalent to 1.00 mmol) of kaempferol in 25 mL of methanol, with 139 μ L of triethylamine, which equates to 1.00 mmol. Simultaneously, Molybdenum (VI) oxide, weighing 143.94 mg and equal to 1.00 mmol, was also dissolved in a separate container containing 25 mL of methanol. The Dropwise, the Molybdenum (VI) oxide solution was introduced into the prepared kaempferol solution. Stirring was employed with the reaction mixture for a duration of 10 hr at room temperature, with aluminum foil employed to shield it from light. As the reaction unfolded, the mixture's color shifted from pale yellow to a reddish-brown shade. Using rotary evaporation, the solvent was removed, ultimately yielding the crude product. The UV-vis spectra were recorded using a UV-visible spectrophotometer, UV 1800 Shimadzu, ranging from 200-400 nm. To capture the infrared spectra of the synthesized compound, the Bruker Alpha II FT-IR spectrometer was utilised, which covered the 400-4000 cm^{-1} range.

In vivo Studies

All experiments were conducted with approval by the Institutional Animal Ethic Committee bearing KCP/IAEC/PCOL/108/2022, Krupanidhi College of Pharmacy, Bengaluru. Sprague Dawley Rats (male) weighing 180-250 g, were housed in polypropylene cages lined with husk, replaced every 24 hr under a 12 hr cycle of light and dark at about 24°C with 50% humidity. In addition to receiving a standard pellet meal, the rats had unfettered access to tap water.

Preparation of Drugs

The experiment aimed to induce gout by Intraperitoneally (IP) administering 250 mg/kg body weight potassium oxonate, while allopurinol, serving as a positive control, was orally (PO) given at a dose of body weight-based dose of 5 mg/kg. Both substances were dispersed in a 0.9% saline solution for their delivery.^{59,60} The test drug, kaempferol and Molybdenum (VI) complex,

was administered orally (PO) of 5 mg/kg of body weight as the dosage. The selection of the 5 mg/kg body weight dose for the Kaempferol-Mo (VI) complex was guided by previous research that has consistently shown the high efficacy of kaempferol as an osteogenic agent at this particular dosage.⁶¹

Potassium oxonate induced Gout

By using potassium oxonate to block the uricase enzyme, gout was brought on in rats.⁵⁹

This model was employed to evaluate the Kaempferol-Mo (VI) Complex's efficacy against gout. Potassium oxonate, produced in a 0.9% saline solution, was administered intraperitoneally to all rats for the 28-day study period, with the exception of those in the vehicle control group, at a dosage of 250 mg/kg body weight. 1 hr later, the test compound Kaempferol-Mo (VI) Complex was administered to these rats, which was dissolved in 10% DMSO.

Experimental Design

Four groups of experimental rats were randomly selected. (a) Normal control group (b) Potassium oxonate induced gout group, (c) Kaempferol and Mo (VI) 5 mg/kg⁶² body weight- test drug treated group and (d) Allopurinol 5 mg/kg body weight treated group. Prior to initiating the efficacy study, the acute toxicity of the kaempferol-Mo (VI) complex was evaluated in accordance with OECD guidelines to ensure safety at the selected dose. From day 1 to day 28 all rats from (b)-(d) received once daily with potassium oxonate 250 mg/kg body weight I.P.⁵⁹ And after 1 hr of this the group (c) & (d) were treated with kaempferol-Mo (VI) complex 5 mg/kg body weight and Allopurinol 5 mg/kg body weight through oral gavage. On Day 1, 7, 14, 21, and 28th days the body weights were noted as well and motor coordination tests were performed on all animals.

Motor Co-ordination Test

Motor coordination assessments, such as the rotarod test, are significantly relevant in gout models as they provide a functional measure of joint inflammation-induced impairments.⁶³

A rod that revolves at 4 rpm and speeds to 40 rpm in 300 sec was used to move animals from the same cage into different lanes.

The animals' acceleration marked the start of the experiment, and their fall off the rod marked its conclusion. The animal gripped the rod and spun completely passively, causing the timekeeper to pause. The animal was returned to its original cage after passive rotation was recorded. Different trial cut-offs and re-run trial configurations were used, such as allowing the animal to turn passively once or three times in a row or fall off less than 5 sec into the trial. In total, three trials were traversed by the same process, with 15-min inter-tribal breaks. The trial's end was where the weight was taken.^{64,63}

Estimation of hematological Parameters

The trial came to a conclusion on the 29th day when all animals were anesthetized and blood samples were collected through retro-orbital plexuses.

The Mean Platelet Volume (MPV), Erythrocyte Sedimentation Rate (ESR), Red Blood Cells (RBC), and White Blood Cells (WBC) were calculated after blood was drawn.

Serum biochemical parameter, like uric acid was estimated using the ERBA semi-autoanalyser.

Radiographic and histopathological study

After the collection of blood samples, all rats were put down and photographed from each group. the knee joints were chosen for the examination, and representative rats from each group were photographed using the X-ray technology, after being cleaned in ice-cold saline, the tibiotarsal joints were removed from each group. Promptly kept in 10% buffered neutral formaldehyde solution, the joint samples were then prepared for histopathological analysis.

Statistical analysis

The mean $\pm$ SEM (Standard Error of Mean) was used to display the values. One-way Analysis of Variance (ANOVA) was used to examine the results, followed by Dunnett's multiple comparison and a *p*-value of 0.05 was considered significant.

RESULTS

Synthesis and characterization

The Kaempferol-Molybdenum (VI) complex was successfully synthesized, and the resultant reddish-brown colored end product was confirmed following the removal of residual solvents through the employment of a rotary evaporator.

Similar to Kaempferol, the Kaempferol Molybdenum (VI) Complex displayed characteristic absorption peaks. For the Kaempferol Molybdenum (VI) Complex, six absorption peaks were observed at wavelengths of 364 nm, 340 nm, 266 nm, 221 nm, 209 nm, and 204 nm. This observation was in contrast to the absorption pattern of Kaempferol, which exhibited peaks at 363 nm, 266 nm, 215 nm, and 204 nm.

In the presented FTIR spectrum of the kaempferol-Mo (VI) complex, the broad O-H stretching band typically observed between 3200-3600 cm⁻¹ appears significantly diminished or shifted, with only a weak absorption near 3301 cm⁻¹ shown in Figure 3.

The reduction in intensity is likely due to the coordination of the hydroxyl groups of kaempferol with the molybdenum ion, which reduces free-OH vibrational freedom. Such shifts or weakening of O-H bands upon metal complexation have been reported

in previous studies and are considered indicative of successful complexation.⁶⁵

At 1654 cm⁻¹, the carbonyl stretching peak is prominent in pure kaempferol, shown in Figure 2, but the kaempferol Molybdenum (VI) Complex in Figure 3a shows that the carbonyl stretching peak is shifted to 1647.5 cm⁻¹. This observation in Table 1 clearly points to the direct coordination of the carbonyl functional group in kaempferol with the Mo center.

In vivo studies

Change in Body weights

Table 2 shows the effect of kaempferol Mo (VI) complex and Allopurinol on PO-induced changes in body weight on 1st, 7th, 14th, 21st, 24th and 28th day. Rats treated with potassium oxonate had a significantly ($p < 0.05$) higher body weight than rats in the vehicle control group.

Treatment with kaempferol and Molybdenum (VI) complex and allopurinol revealed a significant change in body weight to levels close to normal ($p < 0.01$). When comparing the positive control group to the vehicle control group, 'a' represents $p < 0.05$, 'b' represents $p < 0.01$, 'c' represents $p < 0.001$, and 'ns' indicates no significant difference. When comparing the test group or the standard group to the positive control group, " represents $p < 0.05$, " represents $p < 0.01$, and " represents $p < 0.001$.

Fall time change

Table 3 shows the effect of kaempferol Mo (VI) complex and Allopurinol on PO-induced changes in fall of time on 1st, 7th, 14th, 21st, 24th and 28th day.

Rats given potassium oxonate had significantly ($p < 0.05$) less fall time than rats given a vehicle control. Allopurinol and Kaempferol Mo (VI) complex treatment markedly ($p < 0.01$) raised fall time

levels. Comparing the positive control to the vehicle control, 'a' signifies $p < 0.05$, 'b' signifies $p < 0.01$, 'c' signifies $p < 0.001$, and 'ns' indicates no significant difference. When comparing the test or standard groups to the positive control, " signifies $p < 0.05$, " signifies $p < 0.01$, and " signifies $p < 0.001$.

Effect of kaempferol Mo (VI) complex and Allopurinol on PO induced changes in RBC, WBC, MPV, ESR and Serum Uric Acid levels.

Table 4 shows the effect of kaempferol Mo (VI) complex and Allopurinol on PO-induced changes in RBC, WBC, MPV, ESR & Serum Uric Acid levels.

The PO induced group's RBC levels showed a significant drop ($p < 0.05$) in comparison to the vehicle control rats. The levels of RBC were restored to normal in diseased rats following the administration of the kaempferol and Mo (VI) complex and allopurinol. The elevation in WBC count, MPV, ESR, and Serum Uric Acid as effectively alleviated in both the test and standard groups, with significance at $p < 0.05$. For comparisons between the positive and vehicle control groups, 'a' denotes $p < 0.05$, 'b' denotes $p < 0.01$, 'c' denotes $p < 0.001$, and 'ns' indicates no significant difference. For comparisons between the test or standard groups

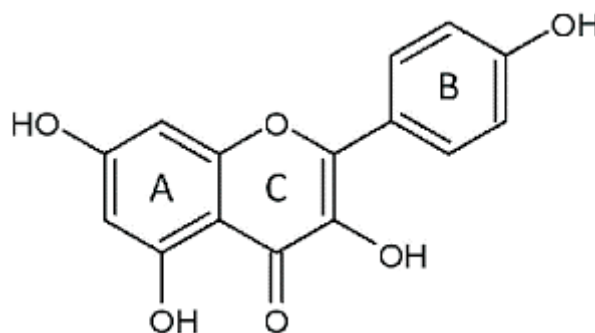


Figure 1: Structure of Kaempferol.

Table 1: Frequencies of characteristic absorption bands in IR Spectra of kaempferol and kaempferol-Mo (VI) complex.

Composition	ν (C=O)	ν (C=C)	ν (C-O-C)
Kaempferol	1654 cm ⁻¹	1619 cm ⁻¹	1219 cm ⁻¹
Kaempferol-Mo (VI) complex	1647.5 cm ⁻¹	1599.58 cm ⁻¹	122.73 cm ⁻¹

Table 2: Effect of kaempferol Mo (VI) complex and Allopurinol on PO induced changes in body weight on 1st, 7th, 14th, 21st, 24th and 28th day.

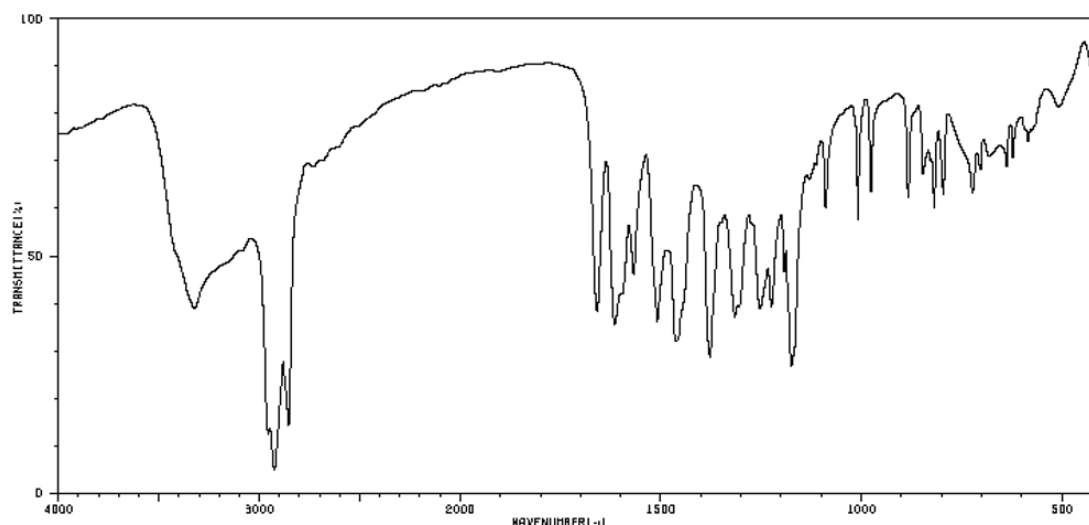
Body Weight (g)	1 st day	7 th day	14 th day	21 st day	24 th day	28 th day
Normal Control	200±2.63	202.5±2.82	207±2.35	211.66±2.54	212.8±3.22	212.66±3.04
Positive Control (Potassium oxonate induced)	205± 3.33	208.33±4.02 ^c	213.5 ±3.23 ^b	225.66±3.6 ^a	235.16±3.40 ^a	246.33±3.40 ^a
Kaempferol Mo (VI) complex (5 mg/kg)	198.33±3.04 ^{ns}	201.83±3.26*	204.8±3.16**	207.66±3.38*	209.5±3.17**	210.5±3.38***
Standard (Allopurinol -5 mg/kg)	202.5±3.48 ^{ns}	208.33±3.09*	210.16±2.68*	211.66±2.45*	213.66±2.84**	216.33±2.785***

Table 3: Effect of kaempferol Mo (VI) complex and Allopurinol on PO induced changes in fall of time on 1st, 7th, 14th, 21st, 24th and 28th day.

Fall Time	1 st day	7 th day	14 th day	21 st day	24 th day	28 th day
Vehicle Control	81.50±4.326	74.66±6.075	69.66±4.417	74.00±3.119	74.3±2.88	74.83±3.13
Positive Control (Potassium oxonate induced)	48.00±4.187	34.33±3.955 ^a	26.33±2.485 ^b	32.50±5.578 ^b	19.33±3.96 ^c	15.83±2.63 ^b
Kaempferol Mo (VI) complex (5 mg/kg)	52.83±2.072	54.83±2.613*	60.33±2.973*	64.00±2.00**	76.5±1.04***	75.5±3.51**
Standard (Allopurinol -5 mg/kg)	62.00±1.897	63.66±4.715*	67.83±3.070**	72.00±2.875**	81.5±1.98***	77±2.260**

Table 4: Effect of kaempferol Mo (VI) complex and Allopurinol on PO induced changes in RBC, WBC, MPV, ESR & SERUM URIC ACID levels.

Blood Parameters	RBC (106×μ)	WBC (103×μL)	MPV (μ3)	ESR (mm/hr)	Serum Uric acid (mg/dL)
Vehicle Control	7.275±0.347	9.418±0.876	6.16±0.370	0.83±0.51	1.21±0.72
Positive Control (Potassium oxonate induced)	3.94±0.462 ^a	19.27±2.074 ^a	9.84±0.858 ^a	2.55±2.857 ^a	3.48±3.95 ^a
Kaempferol Mo (VI) complex (5 mg/kg)	6.66±2.769**	12.15±0.800**	7.21±0.882*	1.59±2.305**	2.05±2.98**
Standard (Allopurinol -5 mg/kg)	6.91±0.498**	11.36±0.303**	7.05±0.693*	1.34±0.276**	1.71±3.2***

**Figure 2: FTIR graph of kaempferol.**

and the positive control group, " denotes $p < 0.05$, " denotes $p < 0.01$, and " denotes $p < 0.001$."

Radiographic and Histopathological analysis

Based on X-ray observations shown in Figure 3b, animals in the normal group exhibited no signs of soft tissue swelling or bony destruction. The PO induced group animals were presented with soft tissue swelling in addition to a reduction in joint space. The Kaempferol-Mo (VI) Complex treated rats shown prevention

against the narrowing of joint spaces was observed with less soft tissue swelling.

Histopathology

Group 1: Vehicle Control

Figure 4: A - Rat ankle joint synovium: Normal architecture of Bone; The normal (areolar) Synovium normal histology is observed-NAD+ (50X).

Figure 4: B - Rat ankle joint synovium: normal (areolar) synovial lining cells (cartilage)-NAD+ (100X) articular cartilage with regular arrangement of uniform chondrocytes.

Group 2: Positive Control (PO Induced)

Figure 5: A - Rat ankle joint synovium: synovium membrane is oedematous, Thickened, and hyperplastic with congestion-moderate (3+) (50X).

Figure 5: B - Rat ankle joint synovium: showing synovial cartilage cells hyperplasia with collection of neutrophils infiltration-inflammatory response-moderate (3+) (100X).

Group 3: Test drug- Kaempferol-Mo (VI) Complex 5 mg/kg

Figure 6: A - Rat ankle joint synovium: epiphysis and bone marrow spaces-normal; (areolar) synovial chondrocytes cells-NAD+ (50X) with normal in joint space-NAD+

Figure 6: B - Rat ankle joint synovium: synovial lining cells (cartilage) chondrocytes-under active proliferation(100X).

Group 4: Standard-Allopurinol 5 mg/kg

Figure 7: A - Rat ankle joint synovium: epiphysis and bone marrow spaces-normal-NAD+ (50X)

Figure : B - Rat ankle joint synovium: Normal the normal (areolar) synovial lining Cells (cartilage) chondrocytes-normal-NAD+ (100X).

DISCUSSION

In our investigation, we synthesised and examined the possible therapeutic effects of the Kaempferol Molybdenum Complex (VI) in a rat model of gout caused by Potassium Oxonate (PO). Our findings reveal several noteworthy observations and implications.

The initial confirmation of complex formation between kaempferol and molybdenum (VI) was substantiated by visible color changes and in the case of the UV-vis spectra of kaempferol

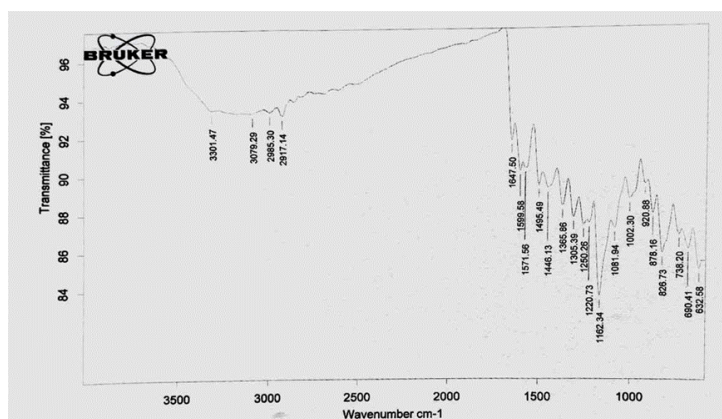


Figure 3a: FTIR graph of kaempferol Molybdenum (VI) Complex.

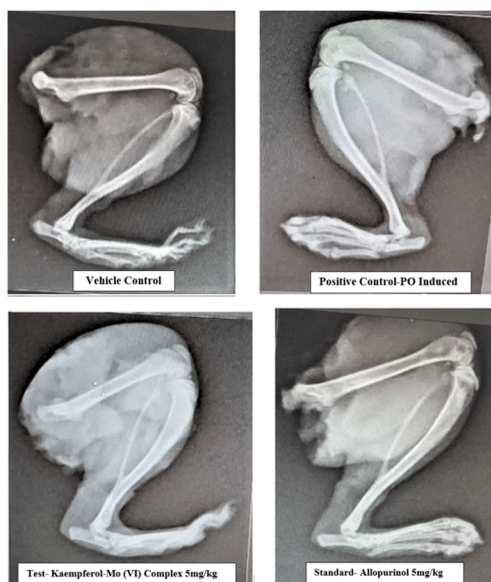


Figure 3b: Radiographic Images.

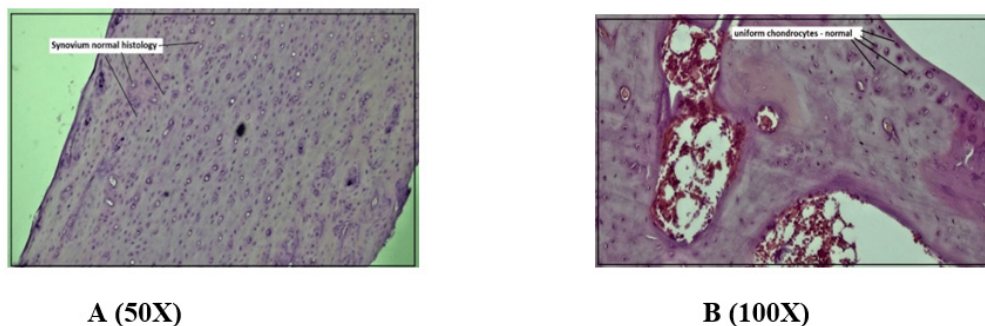


Figure 4: A, B: Histopathology (Group 1: Vehicle Control).

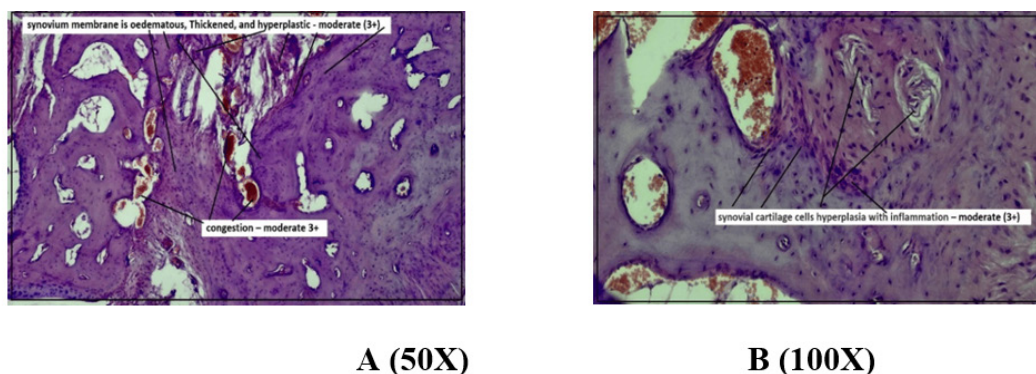


Figure 5: A, B: (Group 2: Positive Control PO Induced).

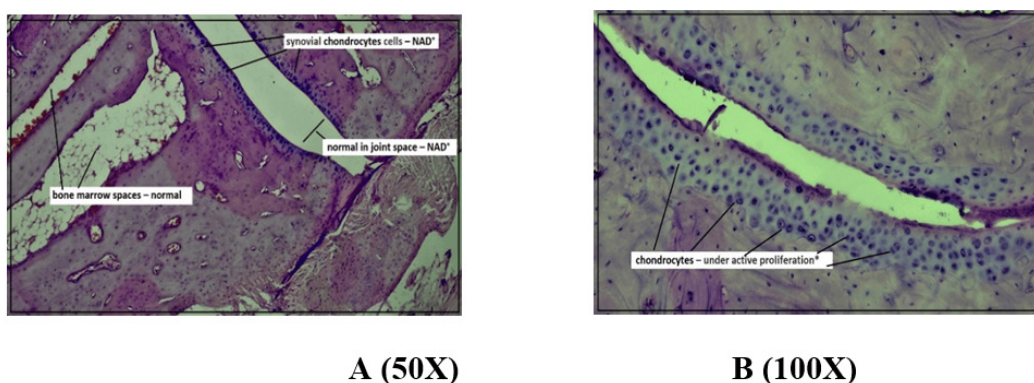


Figure 6: A, B: (Group 3: Test drug- Kaempferol-Mo (VI) Complex 5 mg/kg).

and molybdenum (VI) Complex, a connection between the band II absorption peak at 266 nm and the benzoyl system (ring A) was established, similar to what was observed in the kaempferol. However, it was noted that the absorption peak at 364 nm (band I) in the kaempferol and molybdenum (VI) Complex was indicative of the cinnamoyl system (ring B), which was distinct from the pattern seen in Kaempferol.

When Kaempferol formed a complex with Molybdenum (VI), it underwent a notable change in its absorption peaks. Due to a red shift, band II, which was initially at 266 nm, migrated slightly to 271 nm (band IV), suggesting that the benzoyl system (ring A) stayed mostly unaltered during the chelation process. The interaction between Kaempferol and Molybdenum (VI), however, led to a significant shift of the absorption peak at 364 nm (band I).

The 3-hydroxy-4-oxo portion of kaempferol may also be the main site involved in the coordination process with metal, according to the structural properties of the compound. The 3-OH group's acidic nature and the 4-CO's favourable location are the reasons behind this.

The significant electron delocalization of the oxygen electron clouds of the 3-OH group likely prompted π -electron delocalization. After the 3-OH group bound with Molybdenum (VI), it led to the development of an extended π -bond system via electronic redistribution. This, in turn, resulted in an enhanced conjugative effect involving 3-Hydroxy and 4-Carbonyl in ring C. Consequently, a novel ring emerged in the Molybdenum (VI) complex with Kaempferol, providing additional molecular stabilization at a lower energy state.

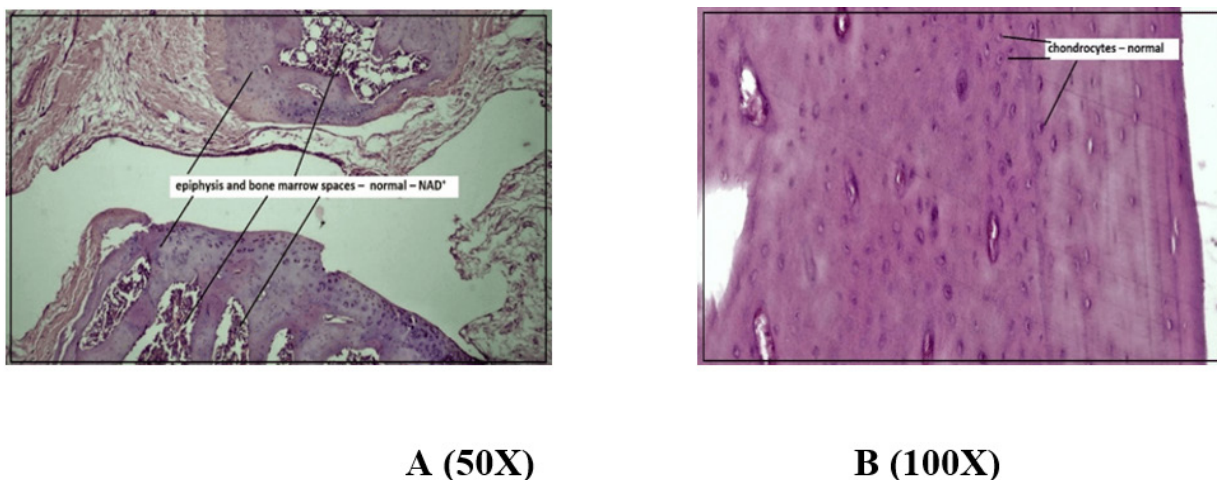


Figure 7: A, B: (Group 4: Standard-Allopurinol 5 mg/kg).

The FTIR spectral information provided strongly supports the conclusion that the carbonyl group in Kaempferol is in direct association with the Molybdenum (VI) center in the Kaempferol Molybdenum (VI) Complex. The coordination in the complex is indicated by the shift in the carbonyl stretching peak in pure Kaempferol at 1654 cm^{-1} and it shifted to 1647.5 cm^{-1} for the Kaempferol Molybdenum (VI) Complex, suggesting coordination between the carbonyl group and the metal ion.

A coordination link between the carbonyl oxygen and a metal ion is known to cause the carbonyl stretching peak to shift to a lower frequency (1647.5 cm^{-1}). This shift occurs because the formation of this bond causes electrons to be drawn away from the center of the bond and toward the oxygen atom. This redistribution of electrons results in a reduction in the electron density within the C=O bond, leading to a shift in the absorption band to a lower frequency.

In this case, the observed shift in the carbonyl stretching vibration frequency offers strong proof that the carbonyl functional group and the Molybdenum (VI) centre in the Kaempferol Molybdenum (VI) complex are coordinated.

In our study, we observed that rats treated with the Kaempferol Molybdenum (VI) complex exhibited comparatively lower body weight gain than the untreated gout brought on by potassium oxonate. This effect may be indirectly related to the complex's uric acid-lowering activity, which addresses a key pathological factor in gout. The association between hyperuricemia, body weight, and muscle mass is well-established,⁶⁶ and our results suggest that the complex may mitigate these interconnected aspects.

The Kaempferol Molybdenum (VI) Complex demonstrated anti-inflammatory properties, as evidenced by its effect on Erythrocyte Sedimentation Rate (ESR) and leukocyte count. A lower ESR in treated groups indicates a potential to reduce

inflammation, which is a hallmark of gout. Additionally, the decrease in leukocyte count, particularly in groups treated with the complex and Allopurinol, suggests an immunomodulatory effect, warranting further exploration. Although the kaempferol-molybdenum (VI) complex demonstrated significant biological activity in this study, the pharmacokinetic behavior and bioavailability of the complex were not evaluated. Future investigations are warranted to determine whether complexation improves the solubility, absorption, or metabolic stability of kaempferol, thereby contributing to its enhanced therapeutic efficacy.

Radiographic assessments in the context of gouty arthritis serve as valuable diagnostic tools that provide insights into the disease's severity the specific context of the rat joints subjected to the Potassium Oxonate (PO)-induced gout model, pronounced radiographic manifestations were evident. These included soft tissue swelling and the narrowing of joint spaces, suggestive of significant bony damage in the gout-afflicted joints. However, the administration of kaempferol and molybdenum (VI) Complex at a dosage of 5 mg/kg and allopurinol at 5 mg/kg exhibited a marked protective effect against bony destruction. This protection was evident in the reduced extent of soft tissue swelling and joint space narrowing when compared to the PO-induced arthritic rats. These radiographic findings underscore the potential of these compounds in mitigating the structural damage associated with Gouty arthritis, further emphasizing their therapeutic promise in managing the disease.

Histopathological analysis revealed promising insights into the impact of treatment on joint pathology. The complex demonstrated the preservation of joint architecture, synovium, and even chondrocyte proliferation. This not only suggests potential anti-inflammatory effects but also hints at the promotion of joint repair processes, an aspect that requires further investigation.

CONCLUSION

In conclusion, the Kaempferol Molybdenum (VI) Complex displayed significant promise as a therapeutic agent for gout, effectively mitigating key disease characteristics from biochemical markers to structural joint integrity. The findings underscore its potential and open valuable avenues for future pharmacological research in gout treatment.

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The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC) bearing the approval number KCP/IAEC/PCOL/108/2022.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

PO: Potassium oxonate; **RBC:** Red blood cells; **WBC:** White blood cells; **ESR:** Erythrocyte sedimentation rate; **MPV:** Mean platelet volume; **OECD:** Organisation for Economic Co-operation and Development; **ERBA:** External ratings-based approach; **SEM:** Standard error of the mean; **ANOVA:** Analysis of Variance; **FTIR:** Fourier Transform Infrared Spectroscopy; **UV-vis:** Ultraviolet-visible.

SUMMARY

This study rigorously investigated the therapeutic potential of the Kaempferol Molybdenum (VI) Complex in a rat model of gout, induced by Potassium Oxonate. Initial spectroscopic analyses, including UV-visible absorption and FTIR, definitively confirmed the successful formation of the complex. These analyses revealed specific binding interactions, particularly involving the 3-hydroxy-4-oxo section and the carbonyl group of kaempferol with molybdenum (VI), contributing to the complex's stability.

The complex demonstrated potent multi-faceted anti-gout activity. It significantly reduced elevated serum uric acid levels, a hallmark of gout. Furthermore, its anti-inflammatory effects were evidenced by decreased Erythrocyte Sedimentation Rates (ESR) and reduced leukocyte counts, indicating modulation of the immune response. Radiographic assessments highlighted its protective role, showing reduced joint damage, including less soft tissue swelling and narrowed joint spaces, in treated rats compared to untreated gout models. Histopathological evaluations corroborated these findings, revealing preserved

joint structures and even suggesting a potential for chondrocyte growth, pointing towards regenerative properties.

The study proposed that the complex's anti-gout activity, particularly its uric acid-lowering effect, is likely linked to the inhibition of xanthine oxidase, an enzyme central to uric acid production. This mechanism, potentially enhanced by the complexation of kaempferol with molybdenum (VI), suggests a novel approach to gout management. While the study established strong efficacy, it noted a research gap: the pharmacokinetics and bioavailability of the complex were not evaluated, necessitating further investigation to fully understand its *in vivo* behavior.

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Quantification of Carcinogenic Organic Impurities in Fruit Puree Nutraceutical Pouches Using Modified QuEChERS Method and GC-MS/MS

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ABSTRACT

Aim/Background: The present work focuses on a complex analytical method for quantifying Polycyclic Aromatic Hydrocarbons (PAHs) in fruit puree nutraceuticals, as significant dangers to human health are posed by PAHs, which the International Agency for Research on Cancer (IARC) has designated as carcinogens. Long-term exposure is a concern since these environmental contaminants can inadvertently find their way into food products through a variety of pathways. Therefore, it is essential to accurately quantify PAHs in food matrices in order to protect public health. **Materials and Methods:** A modified extraction technique called QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) was developed to handle the challenges of testing PAHs in fruit purees. Because fruit purees contain lipids and polyphenols, which might interfere with the detection of PAHs, they pose analytical problems. To improve the elimination of these matrix interferences, a novel sorbent material was added to the process for dispersive solid-phase extraction. By reducing matrix effects, this change increases the method's accuracy and selectivity. **Results:** At trace quantities, important target PAHs such as benz[a]pyrene, chrysene, and benz[a]anthracene were detected with good sensitivity and specificity. After extensive validation, the method's performance showed outstanding linearity, recovery, and precision and verified through the measurement of method uncertainty analysis. **Conclusion:** More precise risk assessment and of PAH exposure from consuming fruit puree nutraceuticals is made possible by the improved analytical capacity, which also provides useful information for evidence-based regulatory actions. This research is to increase consumer safety by providing a trustworthy instrument for tracking PAH levels in frequently consumed food items.

Keywords: PAHs (Polycyclic Aromatic Hydrocarbons), QuEChERS method, Food safety assessment, IARC, Dispersive solid-phase extraction.

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INTRODUCTION

Fruit puree nutraceuticals, becoming increasingly popular because of their reputed health benefits, can be unhealthy due to the impression of carcinogenic Polycyclic Aromatic Hydrocarbons (PAHs). In this study, the quantitation of these dangerous compounds, viz., Acenaphthylene [ACN], Acenaphthene [ACNT], Anthracene[ANTC], Benzo[b]fluoranthene [B[b]FT], Benzo[k]fluoranthene [B[k]FT], Benzo[a]pyrene [B[a]P], Fluorene [FRE], Phenanthrene [PHNT], Fluoranthene [FLU], Naphthalene [NAPL], Pyrene [PYRE], Benz[a]anthracene [B[a]AC], Chrysene [CHRS] is aimed.¹ PAHs can reach fruit purees either through contamination in the environment or through processes. Through

precise determination of PAH contents, the purpose of this work is to bring useful insights for consumers and government agencies, aiding in fruit puree nutraceutical safety assurance and Risk Assessment.² This research introduces a solid way to formulate an effective method of determining the concentrations of such oncogenic organic impurities, mainly Poly-cyclic Aromatic Hydrocarbons, in fruit puree nutraceuticals along with their Risk Assessment measurement as per Regulation (EU) No 835/2011.

According to The European Union and the literature the maximum limits for Benzo[a]pyrene (B[a]P) ranging from 1 to 10 µg/kg, with a 20 µg/kg limit for B[a]P and Benzo[a]anthracene (B[a]A) in liquid smoke flavoring. The European Union places a high priority on monitoring Polycyclic Aromatic Hydrocarbons (PAHs) in a wide range of foods.²

Different member states have different recommendations for daily B[a]P intakes. For example, as accordance with Spain recommends 8.6 µg/day for total PAHs in common baked goods



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and 0.14 µg/day for B[a]P, while Italy suggests 0.04 to 0.42 µg/day. PAH contents in toasted bread ranged from 0.323 to 9.40 µg/kg, according to analytical methods including fluorimetric detection and supercritical fluid extraction combined with HPLC. This emphasizes the significance of continuous monitoring and regulatory actions to guarantee food safety.³⁻⁵

Accurate PAH analysis requires careful sample preparation, with an emphasis on eliminating matrix complexity to allow for trustworthy qualitative and quantitative measurements. By using acetonitrile as an effective extraction solvent following the addition of salt, QuEChERS procedures have become a more straightforward substitute for conventional extraction methods, simplifying sample handling. These enhanced extraction and analytical techniques, when combined with sophisticated analytical tools such as gas chromatography-tandem mass spectrometry using a triple quadrupole mass analyzer, rigorous method validation, and uncertainty analysis, improve the accuracy and dependability of PAH determination in food matrices.⁶⁻¹⁴ This help consumers and regulatory agencies make informed decisions about the safety of such products. Through the measurement of PAH levels, this study contributes to the safety of fruit puree nutraceutical's and is useful to consumers and regulatory agencies.

MATERIALS AND METHODS

Materials

PAH standards

Acenaphthylene (ACE), Acenaphthene (ACNT), Anthracene (ANT), Fluorene (FLU), Fluoranthene (FLO), Naphthalene (NAP), Pyrene (PYR), Phenanthrene (PHE), Benz[a]anthracene (B[a]A), Chrysene (CHRS), Benzo[b]fluoranthene (B[b]FT), Benzo[k]fluoranthene (B[k]FT), Benz[a]pyrene (B[a]P), Indeno[1,2,3-cd]pyrene (IndcPy), Dibenz[a,h]anthracene (DiahAc), and Benzo[g,h,i]perylene (BghiPL).

Solvents: Acetonitrile (ACN), acetone, methanol, ethyl acetate, and distilled water were used for extraction and sample preparation.

QuEChERS kits: Sigma-Aldrich QuEChERS kits for extraction and cleanup.

Sorbents: Graphitized Carbon Black (GCB) and DSC-6S were used for dispersive solid-phase extraction to remove interfering compounds.

Sample Selected: Fruit puree nutraceutical pouch of different brand each labeled as FP¹, FP², FP³, FP⁴, FP⁵, FP⁶, FP⁷, FP⁸, FP⁹, FP¹⁰.

These samples were selected based on their higher processing levels, including the presence of added colorants, smoked

flavorings, and a higher number of preservatives, antioxidants, and acidifiers.

Methods

Standard solution preparation

Individual 16 Polycyclic Aromatic Hydrocarbon (PAH) stock solutions were meticulously prepared by liquefaction of 10 mg of each PAH in 10 mL of acetone, making the concentration resultant to 1 mg/mL, and obtained solutions were stored at -20°C to minimize degradation. Subsequently, a standard mixture was created by combining 50 µL of each individual PAH stock solution and diluting it with 900 µL of acetone, yielding a concentration of 0.05 mg/mL (50 µg/mL) for each PAH. This standard mixture served as the base for creating working standard solutions, which were obtained through serial dilutions with acetone, aiming for concentrations ranging from 0.05 mg/mL up to 1mg/mL to achieved serial dilutions of the stock solutions to the target range. These working standard solutions are crucial for establishing a calibration curve, necessary for the quantitative chemical analysis of PAHs in various samples. Throughout the preparation process, accuracy in pipetting and the use of high-purity materials are paramount, along with careful storage to ensure the stability of the PAH solutions.¹⁵⁻¹⁹

Sample Extraction procedure-QuEChERS Method

This multi-step procedure is meticulously designed to isolate and concentrate PAHs from fruit samples, ensuring accurate and reliable quantification.²⁰⁻²²

Fortified Fruit Sample extract with Spiked PAHs

1 g of fruit pulp pouch of different brand each labeled as FP¹, FP², FP³, FP⁴, FP⁵, FP⁶, FP⁷, FP⁸, FP⁹, FP¹⁰ is fortified with a precise concentration of PAH standards (2 µg/g). This spiking procedure is vital for determining the extraction recovery efficiency, serving as internal standard for the accurate quantification of native PAHs.

Mix and Incubate 30 min

The fortified sample is thoroughly mixed to guarantee uniform distribution of the spiked PAHs. A 30-min incubation at room temperature allows the PAHs to equilibrate within the fruit matrix, simulating the binding of inherent PAHs.

Homogenization

The initial step involves adding 5 mL of distilled water to facilitate sample homogenization by increasing volume.¹⁶ Homogenization, using a blender or homogenizer, disrupts fruit tissue, releasing PAHs for extraction. Subsequently, 5 mL of Acetonitrile (ACN) and acetone of 5 mL volume was added, and vigorously mixed for 1 min. The combination of these solvents, with differing polarities, ensures efficient extraction of a broad range of PAHs.

Salting out

Transfer the supernatant into 15 mL tube considering the volume of 5 mL into a QuEChERS salts. Then add accurate amount of 0.5 g of NaCl and 3 g of MgSO₄ and shake for a minute. The ionic strength of the aqueous phase increases with addition of NaCl promoting the "salting-out" effect, which drives organic compounds into the solvent. MgSO₄ acts as a dehydrating agent, removing water from the organic phase and enhancing phase separation.

Centrifugation

Centrifugation at 3400 rpm for 10 min separates the organic phase, containing the PAHs, from the aqueous phase and solid debris. Maintaining a temperature of 20°C helps preserve the stability of the PAHs.

Combine Extract

The organic extract (supernatant) is carefully collected, minimizing contamination. 150 mg of MgSO₄ and 120 mg of Na₂SO₄ (desiccants) are added to further remove residual water, ensuring a dry extract. The dried organic supernatant is then carefully isolated.

Add NaOAc and Agitate

7.721 g of sodium acetate (NaOAc) is added as a buffering agent, maintaining a stable pH critical for PAH stability. The mixture is agitated for thorough mixing. Subsequent centrifugation removes the sodium acetate, retaining the supernatant.

Dispersive solid-phase extraction

30 mg of GCB and 100 mg of DSC-6S (sorbents) are added for cleanup and the mixture is agitated. GCB removes planar interfering compounds like pigments, and DSC-6S removes other interferences like fatty acids and sugars as they may co-elute with PAHs or can clog columns and compete during extraction, leading to overlapping signals and reduced accuracy.

The Agitation ensures optimal contact between the sorbents and the extract. Then the sorbents are then allowed to settle, facilitating the separation of the cleaned extract. The cleaned supernatant is collected as removing these compounds during sample cleanup improves the clarity, precision, and reliability of PAH measurements.

Vacuum-Assisted Solvent Removal (Evaporation)

The solvent is evaporated under vacuum, concentrating the PAHs and enhancing the sensitivity of the analysis. The PAHs are left as a dry residue. This residue is then redissolved in 2 mL of diethyl ether, with diazomethane added if derivatization is necessary to enhance the volatility and GC-MS analysis of certain PAHs. The solution is then subjected to a nitrogen gas purge and concentrated to 200 µL, maximizing PAH concentration and analytical sensitivity.

The final, purified, and concentrated extract is ready for analysis using a techniques of GC-MS/MS.

Table 1: Gas Chromatographic Retention Times and Ionization Parameters for Polycyclic Aromatic Hydrocarbons (PAHs).

PAH	RT (min)	QI (m/z)	Qn Ion (m/z)	CE (eV)
Naphthalene (NAP)				
Acenaphthylene (ACE)	3.5	126	106	15
Acenaphthene (ACN)	8.6	153	154	20
Fluorene (FLU)	9.2	152	125	20
Phenanthrene (PHE)	11.2	166	155	20
Anthracene (ANT)	14.3	173	166	25
Fluoranthene (FLO)	18.3	173	170	25
Pyrene (PYR)	15.22	202	202	25
Benz[a]anthracene (BaA)	17.23	202	119	25
Chrysene (CHR)	21.2	221	202	30
Benzo[b]fluoranthene (BbF)	9.26	226	221	30
Benzo[k]fluoranthene (BkF)	23.2	256	251	35
Benzo[a]pyrene (BaP)	23.2	254	250	35
Indeno[1,2,3-cd] pyrene (IndPy)	22.3	256	231	35
Dibenz[a,h]anthracene (DiahAc)	24.5	265	265	40
Benzo[g,h,i]perylene (BghiPL)	24.2	256	251	40

RT: Retention time, QI: Qualifier ion, QnI: Quantifier ion, CE: Collision Energy.

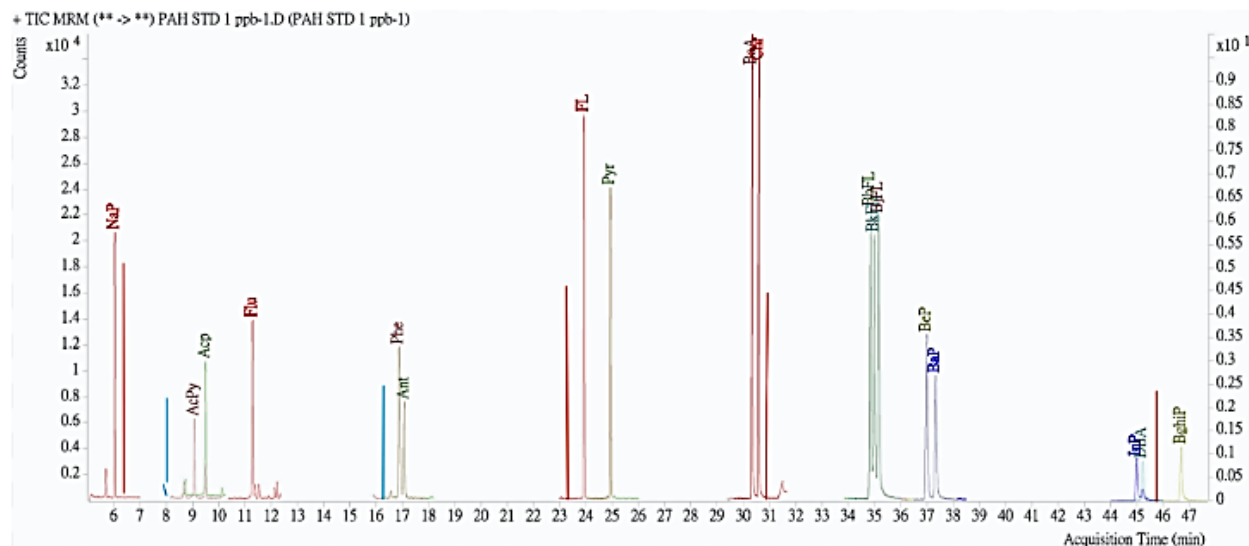


Figure 1: Total ion chromatogram of 16 PAHs.

GC-MS analysis

The GC system - Agilent 6890 is used in combination with a single quadrupole Agilent 5793N mass spectrometer. The operational conditions of the GC-MS analysis for desired analytical outcome include Ionization mode with Electron Ionization (EI) following split-less injection mode of injection volume of 1 μ L are used. Helium is utilized as the carrier gas at a flow rate of 1.2 mL/min. The monitoring mode is Selected Reaction Monitoring (SRM) with a dwell time of 5 min. Separation is performed using an HP-5MS column with precise construct: 30 meters long, 0.25 mm in diam, and a film thickness of 0.25 mm. 5 min of solvent delay and a detector gain of 10 are applied.

Polycyclic Aromatic Hydrocarbons (PAHs) were investigated using Selected-Ion Monitoring (SIM) acquisition at preselected mass-to-charge ratios (m/z) for the target PAHs as well as an internal standard, with each PAH's base peak documented in Table 1. Quantifier ions, which were chosen to be the primary target ions to be quantitated, were chosen for each PAH and are presented in Figure 1, Confirming the qualifier ion. Peak recognition was attained when the retention time coincided with the calibration standard within a tolerance of ± 0.05 min shown in the Figure 2. Total ion chromatogram of 16 PAHs and quantification only took place the quantifier to qualifier ion ratio was within $\pm 20\%$ of the standard and Signal-to-Noise ratio (S/N) was ≥ 3 and Samples with poor responses or concentrations less than the quantification limit were classified as non-detected, and only verifiable and valid data were included in the analysis.

GC-MS Procedure Performance

GC-MS procedure was executed for analyzing 16 Polycyclic Aromatic Hydrocarbons (PAHs) in fruit puree nutraceutical

pouches. The sample total ion chromatogram and mass peak is represented in Figure 3. The consistency in chromatographic behavior between PAH standards and the samples (FP1-FP10) across a wide volatility range indicates a reliable and stable analytical method shown in Figure 4. The inclusion of a blank sample for background correction is crucial for accurate PAH quantification, and the use of an internal standard for determination of concentration given in Table 2. According to EU Regulation 835/2011, the maximum allowed levels of Benzo[a]Pyrene (BaP) in general food products is 2.0 μ g/kg, and the combined total of PAH4 (BaP, BaA, CHR, BbF*) should not exceed 10.0 μ g/kg. Based on the detected values, all fruit puree samples in this study had BaP levels below the general EU limit, and in samples where PAH4 components were detected, the total was also below the 10.0 μ g/kg threshold. Notably, FP2 (11.8 μ g/kg) and FP7 (15.2 μ g/kg) exceed the PAH4 limit. These findings indicate non-compliance in some samples and highlight the need for tighter quality control.

Method Validation

The developed analytical procedure for the quantitation of 16 Polycyclic Aromatic Hydrocarbons (PAHs) in fruit puree nutraceutical samples was validated for linearity, Limit of Quantification and recovery.

Uncertainty of the method

Evaluating repeatability by generating ten replicate data points at two distinct levels, measured on two different days. Standard deviations were determined for each group of ten replicates. The uncertainty calculation will be based on this formula:

$$U(c) = \sqrt{[U(p)^2 + U(rep)^2 + U(STD)^2 + U(INSTD)^2]}$$

This uses several sources of uncertainty related to the root-sum-of-squares method. This is a common way to combine independent uncertainties.

RESULTS

Linearity and Correlation Coefficient

The method is of higher linearity with evidence of correlation coefficients of more than 0.996 in specified ranges of concentrations, demonstrating a uniform correlation between analyzed signals and PAH levels. Moreover, the method has good precision as testified by low interday and intraday coefficients of variation confirming its ability to provide consistent and reproducible results.

Limit of Quantification and Recovery

The LOQs range from 0.05 to 0.2 mg/kg. This means that the lowest amount of each PAH that can be reliably measured using this method is between 0.05 and 0.2 mg/kg. To obtain accurate recoveries further increasing the validity of the data. Average recoveries of the PAHs ranging from 92.11% to 96.56% are found within acceptable range, indicating effective extraction. The Relative Standard Deviation (RSD) of 7.2% to 7.9% shows good precision in recovery process. Recovery studies of Day 1 and Day 2

indicate the percentage of analyte recovered during the analytical process shown in Table 3. Values are generally high, ranging from 85% to 110%, suggesting good accuracy of the method.

Estimation of Daily Dose, Daily Intake and Toxicity Equivalency Factor (TEF)

These combined aspects of good linearity and precision render the method a precise and reliable tool for PAH quantitation in nutraceutical, crucial in ensuring product quality and safety by way of reliable analytical outcomes, together with aiding in estimation of Daily Dose, Daily intake and Toxicity Equivalency Factor (TEF) as presented in Table 4. In totality, this method seems robust and viable in determining PAHs in fruit puree nutraceutical samples, offering credible data for quality control and safety determination.

Limits of Method Uncertainty According to Validation Parameters

The combined Uncertainty (U(c)) for the analytical method used in this study was calculated to be 7.3490%. This value was derived by evaluating the contributions of various sources of uncertainty, including Repeatability (U(rep)), Standard Uncertainty (U(STD)), and Internal Standard Uncertainty (U(INSTD)).

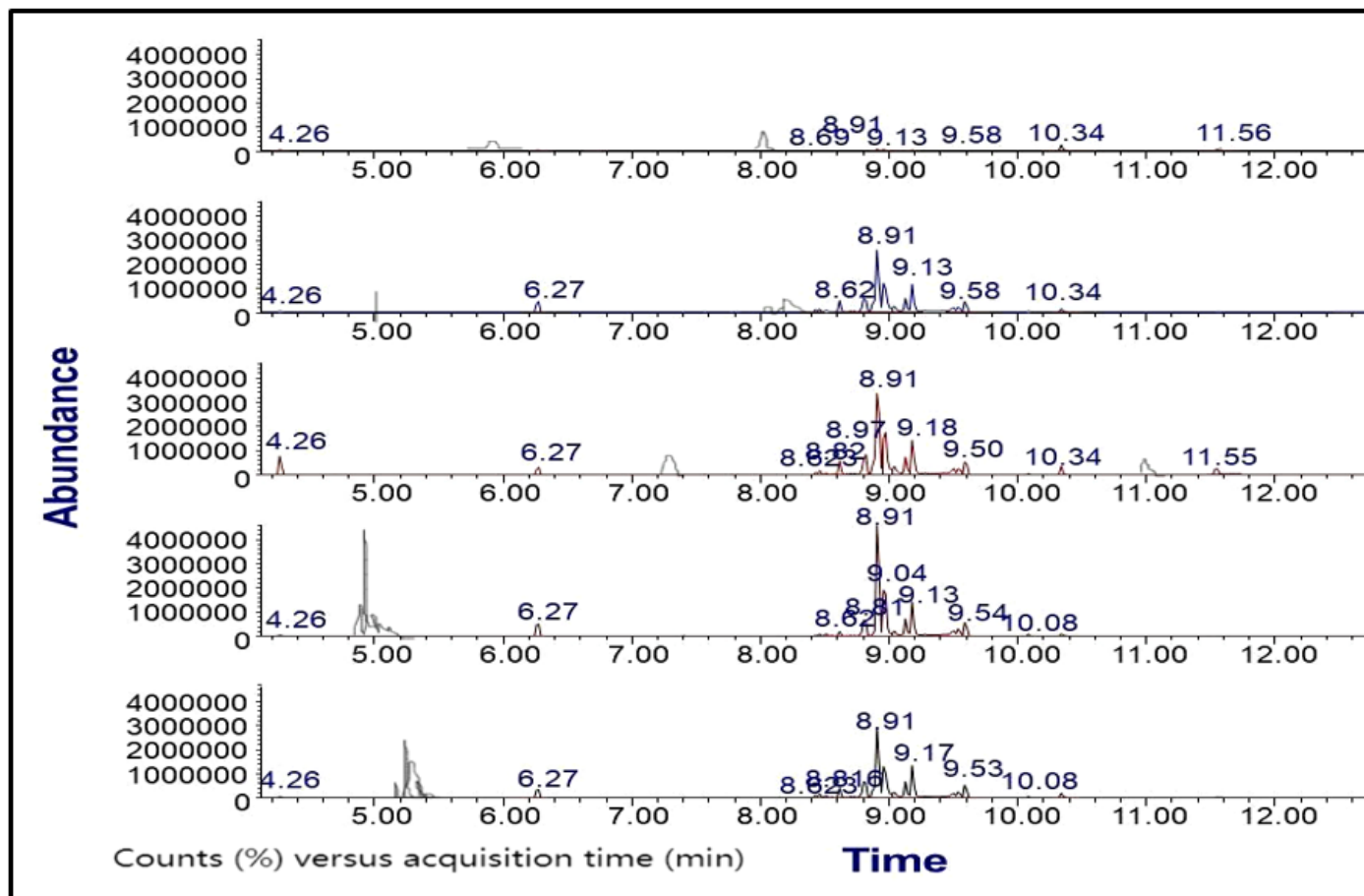
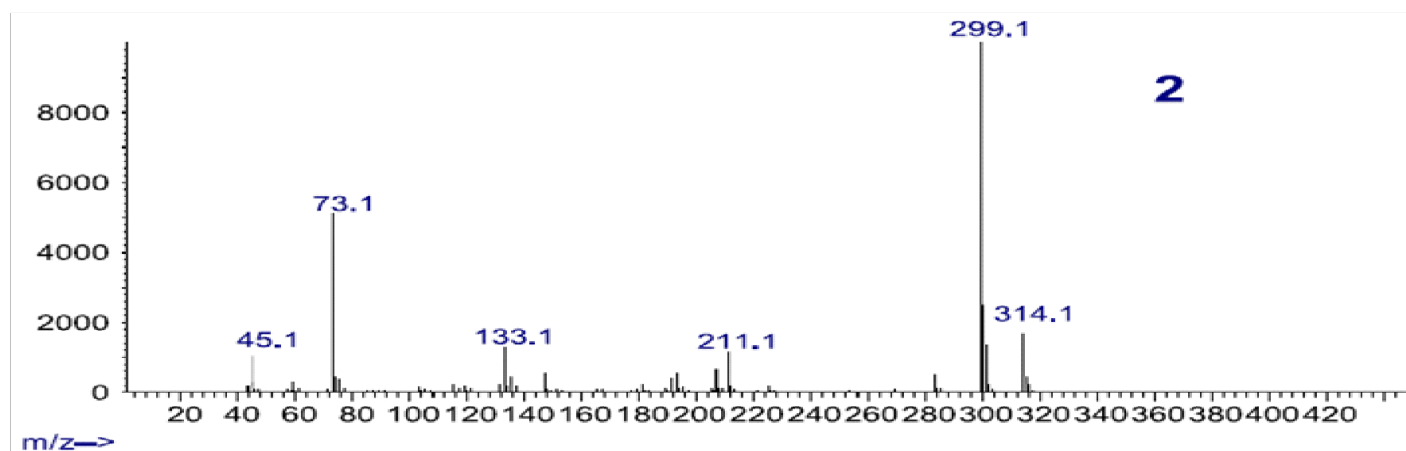


Figure 2: Sample ion peak with Counts (%) Vs Acquisition time.

Table 2: Individual PAH'S Levels detected in different sample of selection.

PAHs												
FP ¹	ACE	ACN	BaA	BaP	CHR	DahA	FLU	FLO	NAP	PYR	PHE	ΣPAH
FP ²	NI	1	1.88	0.39	NI	NI	1.58	NI	NI	0.89	1.02	6.76
FP ³	4.23	NI	NI	0.36	0.03	0.63	1.73	0.77	1.56	1.05	1.45	11.8
FP ⁴	1.32	NI	NI	NI	NI	NI	1.47	NI	NI	0.51	NI	3.3
FP ⁵	1.25	0.28	2.22	NI	0.2	NI	NI	0.77	2.86	1.13	NI	8.7
FP ⁶	NI	NI	NI	0.35	NI	0.55	NI	0.22	NI	NI	1.01	2.68
FP ⁷	NI	NI	NI	NI	NI	NI	1.22	NI	NI	NI	NI	1.22
FP ⁸	4.0	NI	2.6	0.7	NI	NI	2.2	NI	5.7	NI	NI	15.2
FP ⁹	NI	NI	NI	NI	NI	0.12	NI	0.6	NI	NI	NI	0.84
FP ¹⁰	NI	NI	NI	NI	NI	NI	3.34	NI	NI	NI	NI	3.34
ΣPAHs	NI	NI	NI	NI	NI	NI	NI	NI	4.2	NI	1.56	5.76
Averg. concentration	51.74	1.28	6.7	1.85	0.23	1.3	11.6	2.36	14.35	3.58	5.93	102.22
SD	17.25	0.64	2.2	0.46	0.115	0.43	1.9	0.59	3.6	0.90	1.1	29.7
Frequently detected (%)	7	0.51	0.4	0.2	0.12	0.3	0.8	0.3	1.8	0.3	0.30	12
FP: Fruit puree, NI: Not identified, SD: Standard Deviation	30	10	40	40	20	30	60	40	40	40	50	430

**Figure 3:** Gas chromatography-mass spectrometry total ion chromatogram of PAHs and samples FP1-FP10.

Specifically, the repeatability uncertainty was 0.9%, based on an RSD of 7.909%, while the standard uncertainty and internal standard uncertainty were 0.423% and 0.473%, respectively. These individual uncertainties were combined using the standard formula, resulting in a combined Uncertainty (U(c)) of 7.3490%.

To estimate the expanded uncertainty, a coverage factor of 2 was applied, corresponding to a 95% confidence interval. This adjustment resulted in an expanded Uncertainty (Uexp) of 14.69%. The expanded uncertainty provides a broader estimate that accounts for potential variability in the measurement process and reflects the uncertainty in the results with a higher

level of confidence. This expanded uncertainty is important for assessing the method's reliability and reproducibility, 95% of the results would fall within the reported range (Result $\pm$ 14.69%), particularly when the method is employed in regulatory or industrial contexts, where such uncertainties must be accounted for in decision-making.

DISCUSSION

This study developed and validated a method for quantifying Polycyclic Aromatic Hydrocarbons (PAHs) in fruit puree nutraceuticals using a modified QuEChERS extraction technique

Table 3: Validation data for the analysis of various PAHs found in various fruit puree nutraceutical pouches representing Relative Standard Deviation (RSD %) and Pooled RSD % and Recovery percentage.

Comp												
ACE	Recovery 1 st Day (1 ng/g)	RSD 1 st Day (1 ng/g)	Recovery 2 nd Day (1 ng/g)	RSD 2 nd Day (1 ng/g)	Recovery 1 st Day (10 ng/g)	RSD 1 st Day (10 ng/g)	Recovery 2 nd Day (10 ng/g)	RSD 2 nd Day (10 ng/g)	Pooled RSD (%)	Recovery Range (%)	RSD Range (%)	Range
ACN	95	5	98	7	102	3	105	4	5.2	95-105	3-7	5
BaA	102	8	99	6	108	4	103	5	6.5	99-108	4-8	6
BaP	88	12	92	10	95	7	98	9	10.5	88-98	7-12	9.5
CHR	91	9	95	11	98	6	101	8	9.2	91-101	6-11	8.5
DahA	105	4	102	5	110	3	108	4	4.5	102-110	3-5	4
FLU	93	10	96	8	100	7	103	9	9.5	93-103	7-10	8.5
FLO	97	6	94	7	101	5	98	6	6.8	94-101	5-7	6
NAP	85	15	89	12	92	10	95	11	13.2	85-95	10-15	12.5
PYR	100	7	103	6	106	4	109	5	6.1	100-109	4-7	5.5
PHE	92	11	95	9	99	8	102	10	10	92-102	8-11	9.5
AVG	98	5	101	6	104	3	107	4	5.5	98-107	3-6	4.5
RSD: Relative Standard Deviation, AVG: Average, Comp: Compound	96.72727			AVG		7.90909			AVG			7.22727

Table 4: Estimating the Daily Dose, Daily Intake and Toxicity Equivalency Factor (TEF).

PAH										
ACE	Con (mg/ kg)	LOQ (mg/ kg)	PW (g)	A (kg)	C (kg)	DD (A)	DD (C)	DI (A)	TEF	
ACN	51.74	0.05	100	70	15	73.9	344.9	0.012	0.001	
BaA	1.28	0.05	100	70	15	1.8	8.5	4.787	0.01	
BaP	6.7	0.05	100	70	15	9.6	44.7	9.146	0.1	
CHR	1.85	0.05	100	70	15	2.6	12.3	3.312	0.01	
DahA	0.23	0.05	100	70	15	0.3	1.5	26.64	0.01	
FLU	1.3	0.05	100	70	15	1.9	8.7	0.471	0.001	
FLO	1.3	0.05	100	70	15	1.9	8.7	0.471	0.001	
NAP	11.6	0.05	100	70	15	16.6	77.3	0.053	0.001	
PYR	2.36	0.05	100	70	15	3.4	15.7	0.260	0.001	
PHE	14.35	0.1	100	70	15	20.5	95.7	0.043	0.001	
TED: Toxicity Equivalence factor, PW: Product Weight, A: Adult, C: Children, D: Daily Dose, DI: Daily Intake, LOQ: Limit of Quantification [Estimation of daily dose (DD) = C X IR/BW R= weight of the product], C=Concentration [Estimation of daily Intake (DI) = (AVERAGE OF C X TEF) X IR/BW].	3.58	0.1	100	70	15	5.1	23.9	0.171	0.001	

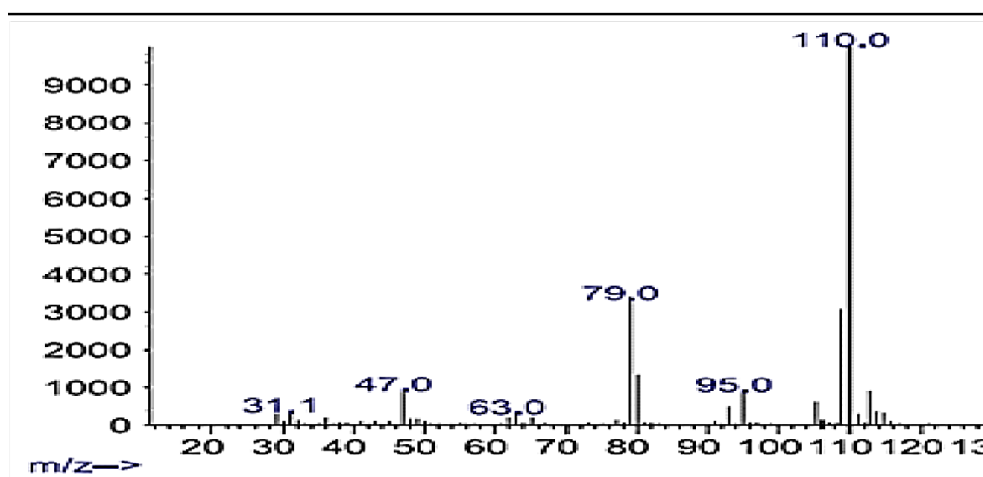


Figure 4: Gas chromatography-mass spectrometry total ion chromatogram of PAHs and samples FP1-FP10 in SIM mode.

coupled with GC-MS/MS. The results demonstrate that this method is effective, precise, and reliable, particularly for complex matrices like fruit purees, where matrix interferences such as lipids and polyphenols are common.

The inclusion of dispersive Solid-Phase Extraction (d-SPE) sorbents, like Graphitized Carbon Black (GCB), was crucial in enhancing extraction efficiency and removing interfering substances, ensuring accurate PAH quantification. GC-MS/MS, using Selected Reaction Monitoring (SRM) mode, provided high specificity and sensitivity, with detection limits ranging from 0.05 to 0.2 mg/kg, which meets regulatory sensitivity standards.

The method validation showed excellent linearity ($R^2 > 0.996$), recovery rates between 92.11% and 96.56%, and Relative Standard Deviations (RSDs) between 7.2% and 7.9%, confirming the method's robustness and precision. The expanded uncertainty of 14.69% falls within acceptable limits for food safety testing, enhancing the reliability of results.

Compared to traditional methods like Soxhlet extraction and SPME, the QuEChERS method is faster, simpler, and more cost-effective. It also outperforms liquid chromatography techniques in terms of sensitivity and throughput.

Given the carcinogenic nature of PAHs, this method is valuable for monitoring food safety and ensuring compliance with regulatory limits. The findings indicate that some fruit purees may exceed PAH safety limits, highlighting the need for stricter regulations. The validated QuEChERS-GC-MS/MS method provides a robust tool for routine PAH monitoring in fruit puree nutraceuticals, crucial for quality control. Its established performance metrics, including a 14.69% expanded uncertainty, enable standardized testing, realistic quality benchmarks, and consistent batch assessment. Manufacturers can utilize this method for supplier quality assurance, ensuring the safety of incoming materials. Future research should expand PAH analysis to diverse food matrices (oily, processed, cereal, dairy, beverages) and broaden the range of target PAHs. Optimizing the method for specific

food categories and developing higher-throughput or on-site techniques are also crucial. Integrating the method with studies on PAH formation and migration pathways, and comparing it with other analytical techniques, would further enhance its utility in ensuring food safety across the supply chain.

CONCLUSION

In conclusion, the developed GC-MS/MS method demonstrates high sensitivity and accuracy for the quantification of PAHs in fruit pulp nutraceuticals, providing a reliable tool for routine monitoring of food contaminants. The validation parameters, including low detection limits and satisfactory recovery rates, confirm the robustness of the method. Despite its effectiveness, the analysis revealed the presence of PAHs in fruit pulp samples, some of which exceeded the established safety levels, raising concerns about potential health risks. This underscores the importance of regular surveillance and stricter regulatory controls on food products to minimize consumer exposure to harmful substances. The findings call for further investigation into the sources of PAH contamination and the development of strategies to reduce exposure, ensuring the safety and health of consumers.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

ACE: Acenaphthylene; **ACN:** Acenaphthene; **FLU:** Fluorene; **PHE:** Phenanthrene; **ANT:** Anthracene; **FLO:** Fluoranthene; **PYR:** Pyrene; **BaA:** Benz[a]anthracene; **CHR:** Chrysene; **BbF:** Benzo[b]fluoranthene; **BkF:** Benzo[k]fluoranthene; **BaP:** Benzo[a]pyrene; **IncdPy:** Indeno[1,2,3-cd]pyrene; **DiaHAc:** Dibenz[a,h]anthracene; **BghiPL:** Benzo[g,h,i]perylene; **RT:** Retention time; **QI:** Qualifier ion; **QnI:** Quantifier ion; **CE:** Collision Energy; **FP:** Fruit puree; **NI:** Not identified; **SD:**

Standard Deviation; TEF: Toxicity Equivalency Factor; TED: Toxicity Equivalence factor; PW: Product Weight; A: Adult; C: Children; D: Daily Dose; DI: Daily Intake; LOQ: Limit of Quantification; DD: Estimation of daily dose; W: Weight of the product; C: Concentration; (U(c)): Uncertainty.

AUTHORS CONTRIBUTIONS

The authors shared equal responsibility for the conception, execution, and writing of this study.

SUMMARY

This study successfully developed and validated a modified QuEChERS-GC-MS/MS method for quantifying Polycyclic Aromatic Hydrocarbons (PAHs) in fruit puree nutraceuticals. This method proved highly effective, precise, and reliable, especially for complex fruit puree matrices, by utilizing d-SPE sorbents like GCB to mitigate interferences.

The GC-MS/MS in SRM mode offered high specificity and sensitivity, achieving detection limits from 0.05 to 0.2 mg/kg, which meets regulatory standards. Validation results showed excellent linearity ($R^2 > 0.996$), good recovery (92.11%-96.56%), and low RSDs (7.2%-7.9%). The expanded uncertainty of 14.69% was within acceptable limits for food safety.

This QuEChERS-based method provides a robust tool for routine PAH monitoring, quality control, and supplier quality assurance is superior to traditional and liquid chromatography techniques due to its speed, simplicity, cost-effectiveness, and higher sensitivity/throughput. Its value lies in monitoring food safety and ensuring compliance with regulatory limits, especially since some fruit purees were found to exceed safety limits, underscoring the need for stricter regulations.

Future research should extend this analysis to diverse food matrices and target a broader range of PAHs, optimize the method for specific food categories, and develop higher-throughput or on-site techniques.

In conclusion, this highly sensitive and accurate GC-MS/MS method is a reliable tool for routine PAH monitoring in fruit puree nutraceuticals. The detection of PAHs, some exceeding safety levels, highlights the critical need for continuous surveillance, stricter regulations, and strategies to reduce consumer exposure to these harmful contaminants.

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Isolation and Standardization of Phytocompounds from the Bark of *Stereospermum suaveolens*

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ABSTRACT

Background: The tree species *Stereospermum suaveolens* (Patala), which belongs to the Bignoniaceae family, is indigenous to tropical areas of Southeast Asia, India, and parts of Africa. Its bark is a vital part of Ayurvedic medicine and is widely known for its therapeutic qualities. **Objectives:** The purpose of this study is to identify, define, standardize, and confirm the structures of bioactive chemicals derived from *Stereospermum suaveolens* bark. **Materials and Methods:** A water-ethyl acetate-butanol system was used to partition the bark's methanolic extract. The ethyl acetate fraction was then separated by column chromatography. Using HPTLC and HPLC, the isolated compound was identified, and mass spectrometry, NMR, and FT-IR were used to confirm its structure. **Results:** A triterpenoid was isolated using the fractionation technique. Hexane-ethyl acetate (80:20, v/v) was used as the mobile phase in HPTLC, which produced discrete bands with an R_f of 0.39. Spectrophotometric studies validated the compound's structural integrity, while HPLC provided a retention time of 9.684 min. **Conclusion:** The novelty of this research revolves around isolation of 3 β -taraxerol from Patala bark and the comprehensive analyses confirming its identity and structural integrity, establishing a foundation for further therapeutic investigations.

Keywords: *Stereospermum Suaveolens*, 3- β -taraxerol, HPTLC, Column chromatography, HPLC, FT-IR.

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INTRODUCTION

The isolation and standardization of phytocompounds are crucial for ensuring the quality, safety, and effectiveness of herbal medicines and supplements. Isolation refers to the process of extracting and purifying specific bioactive compounds from plants for therapeutic and analytical applications. Techniques such as column chromatography, High-Performance Thin-Layer Chromatography (HPTLC) and High-Performance Liquid Chromatography (HPLC) are widely used in this process.¹

Column chromatography effectively isolates individual compounds based on their interaction with the stationary phase, while HPLC provides high sensitivity and reproducibility for quantifying active constituents. HPTLC enables accurate separation and quantification of closely related compounds, ensuring consistency in final products. Standardization ensures that phytocompounds meet established quality benchmarks, maintaining their potency and therapeutic efficacy. This process is vital for ensuring the safety and therapeutic efficacy of

plant-derived substances used in pharmaceuticals, nutraceuticals, and herbal medicines.²

Stereospermum suaveolens called as Patala is a large deciduous tree of the Bignoniaceae family, extensively distributed across the tropical and subtropical areas of India and Southeast Asia.^{3,4} The tree has a tall, robust trunk with a dense canopy, reaching heights of up to 20 meters. The species is characterized by large, compound leaves with opposite leaflets, and its flowers are large, showy, and typically yellow or white, forming in clusters. The fruit is a capsule, containing winged seeds that aid in the plant's propagation. The bark of *Stereospermum suaveolens* is thick, rough, and brownish-gray to reddish-brown in color. It has a fissured and scaly texture, with prominent longitudinal cracks and irregular peeling. The surface is often rough and corky, while the inner bark is lighter in color, fibrous, and softer.^{5,6}

Indigenous populations have used various parts of the plant various parts of the plant such as the bark, leaves, and roots, have been traditionally utilized by indigenous communities for treating a diverse range of ailments⁷ and traditionally, the bark of *Stereospermum suaveolens* has been traditionally utilized in folk medicine for treating a variety of ailments such as treating fever, respiratory conditions, digestive disorders, and inflammation.⁸

The pharmacological potential of *Stereospermum suaveolens* is attributed to its rich array of bioactive compounds such as



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alkaloids, flavonoids, saponins, tannins, and terpenoids,^{9,10} contributing to its medicinal properties including anti-obesity,¹¹ anti-inflammatory,^{12,13} anticancer,⁷ antimicrobial, antidiabetic, antioxidant⁷ and hepatoprotective effects. Although *Stereospermum suaveolens* has been extensively studied for its leaves and roots, limited research exists on the bark. This study aims to isolate and standardize a significant phyto compound from the bark, focusing on verifying its structural integrity and identity.

MATERIALS AND METHODS

Plant material

Dried barks of *Stereospermum suaveolens* were collected from Mysore, Karnataka, India. The plant material was identified and authenticated by a Plant Taxonomist at Natural Remedies Private Limited, Bengaluru, and the sample was then deposited in the herbarium of the Natural Product Botany Department.

Chemicals and Reagents

Commercial grade solvents like methanol, Butanol, petroleum ether, ethyl acetate, and Demineralized (DM) water were used. Laboratory Reagent (LR) grade chemicals included chloroform, Silica gel 60-120 mesh, Silica gel 120-200 mesh, Silica gel 230-400 mesh.

Instrumentation requirements

Distillator, Heidolph Rotary evaporator, Glass column, Beakers, Vacuum tray dryer, precoated silica gel 60F₂₅₄ Thin layer chromatography plates, Shimadzu LC 2010A for HPLC, HPTLC silica gel 60F₂₅₄ plates, CAMAG Scanner III for HPTLC, Shimadzu FTIR (KBr), Bruker AMX-400 (400 MHz) instrument for ¹H NMR and LC-MSD-Trip-XCT-plus for Mass Spectroscopy.

Extraction and Partitioning of plant material

The dried bark was coarsely powdered and subjected to pilot-scale methanolic extraction using a Heidolph rotary evaporator at 60°C.¹⁴ The concentrated extract was dried in a vacuum tray dryer at 65°C.¹⁵ Liquid-liquid partitioning was performed using ethyl acetate (1:3), butanol (1:3), and water (1:4). The fractions were dried at 65°C using a vacuum tray dryer.^{14,16}

Isolation of phyto compound using Column Chromatography

Column-1: Column chromatography of ethyl acetate partition fraction using silica gel column

The ethyl acetate partition dried powder was adsorbed onto silica gel 60-120 mesh at the ratio 1:3. It was then subjected to column chromatography to carry out broad fractionation.¹⁷ Elution was performed using petroleum ether, ethyl acetate, and methanol in increasing polarities. Thin layer chromatography and HPTLC

was performed for each fraction and compared with the standard compound using toluene: ethyl acetate: formic acid (7:3:1) solvent systems.^{18,19}

Column-2: Re-chromatography of fraction-2 (fraction 75% PE/ 25%) using silica gel column

The fraction-2 of column-1 was dissolved in methanol and adsorbed onto silica gel (120-200 mesh) in the ratio 1:3. The mixture was then loaded onto the column, and elution was performed using petroleum ether and increasing the polarity with chloroform. Thin layer chromatography was performed for each fraction and compared with the standard compound using n-hexane and ethyl acetate (80:20 v/v) solvent systems.^{18,19}

Column-3: Purification column of fraction-2 to 5 (fractions 10% to 40% CHCl₃/PE) of column-2 using flash grade silica gel

Fraction-2-5 of column-2 were combined and dissolved in methanol and adsorbed onto flash grade silica gel (230-400 mesh) in the ratio 1:3. The mixture was then loaded onto the column, and elution was performed using Petroleum ether and increasing the polarity with chloroform. Thin layer chromatography and HPTLC was performed for each fraction and compared with the standard compound using n-hexane and ethyl acetate (80:20 v/v) solvent systems.¹⁸

HPTLC Fingerprinting

Sample preparation: 10 mg of the sample was dissolved in 1 mL of methanol, heated, and sonicated. The resulting solution was then filtered through a syringe equipped with a 0.45-micron filter.

Standard preparation: 1 mg of the sample was dissolved in 1 mL of methanol and heated with sonication until the standard completely dissolved in the solvent.

Procedure: Silica gel (20×10 cm, 60F 254) pre-coated HPTLC plates were used for chromatographic separation and Vision CAT software, version 2.5.18072.1 (CAMAG, Switzerland), was used to analyze the data. A 100 µL Hamilton syringe attached to a Linomat 5 sample applicator was utilized to spot the samples within the TLC plates with an 8mm band, with solvent front distance-70 mm. The development tank (ADC 20×10) had been saturated for about 20 min prior to chromatography. Mobile phase for Column 1 was composed of toluene, ethyl acetate, and formic acid in a 7:3:1 ratio, while for Column 3, a mobile phase of n-hexane and ethyl acetate in an 80:20 (v/v) ratio was employed. The plates were subjected to drying for 5 min at room temperature after development and derivatized in a derivatization chamber using 3 mL of anisaldehyde sulfuric acid. These plates had been heated on a TLC heater to enhance spot visibility. Finally, they were transferred to the visualizer, and the R_f values along with fingerprint data were recorded using VisionCATS software.^{19,20}

HPLC

The samples were analyzed using an HPLC Shimadzu LC 2010A system, equipped with a UV and PDA detector, and operated with LC Solution software aided in detection of percentage purity of the compound at the detection wave length of 205 nm on kromasil C8 column with a particle size of 5 µm and dimensions of 250 mm×4.6 mm.

Mobile phase preparation: The isocratic mobile phase is composed of:

a) A total of 0.14 g of anhydrous KH_2PO_4 was mixed with of HPLC-grade water (900 mL) until completely dissolved. Subsequently, H_3PO_4 (0.5 mL) was incorporated into the solution. Water was added to bring the final volume up to 1000 mL. The prepared solution was passed through a 0.45 µm membrane filter and subjected to sonication for 3 min to remove any dissolved gases.

b) HPLC grade Methanol.

Sample preparation: 20 mg of sample dissolved in 4 mL of methanol & injected (20 µL) to HPLC Shimadzu system.²¹

Spectroscopic analysis

Spectroscopic methods, including IR, ^1H NMR, ^{13}C NMR and MASS Spectrometry, validated the compound's structure.⁹

RESULTS AND DISCUSSION

Extraction and Partition

The extraction of dried *Stereospermum suaveolens* bark with methanol resulted in 15.87% of powdered extract. Using ethyl acetate and water, this extract was subsequently put via liquid-liquid partitioning, and the yield percentages were noted as indicated in Table 1.

Isolation

Column chromatography was performed for the ethyl acetate partitioned fraction, the fraction-2 (fraction 75% PE/25% EA) obtained demonstrated a significant enrichment of 3-β-taraxerol, which was confirmed by Thin-Layer Chromatography (TLC). Further re-chromatography of fraction-2 (fraction 75% PE/25%EA) showed enrichment of 3-β-taraxerol in fractions 2 to 5 (fractions 10 to 40% CHCl_3 /PE) which upon purification by column chromatography resulted in the isolation of pure 3-β-taraxerol from fraction-5 (20% CHCl_3 /80% PE), yielding 1.525 g of pure compound.

HPTLC ANALYSIS

HPTLC analysis of the fractions obtained from column-3 was conducted using n-hexane and ethyl acetate as mobile phase in a ratio 80:20 (v/v). The fingerprint patterns were recorded at

Table 1: Yield data of the extract and partitioned samples.

Fraction	Colour and texture	%Yield
Methanolic extract	Dark brown powder	3.32%
Butanol fraction	Dark brownish powder	22.05
Ethyl acetate fraction	Dark brown powder	53.4
Water fraction	Blackish brown powder	8.02

Table 2: HPLC profile: 3-β-taraxerol.

Compound.	λ_{max}	Time (min)/Rt.	Area.	%purity
01	205 nm	09.684	7181.6620	97.6%

Table 3: Chemical shifts and Peaks in ^1H NMR.

Chemical shift (δ ppm)	Peaks
0.80	Dd
0.82	Dd
0.90	S
0.92	S
0.95	S
0.97	S
1.02	S
1.09	S
4.14	S
5.56	S

254 nm, 366 nm, and under white light. The isolated chemical was found to have an R_f value of 0.39, matching exactly with the R_f of the standard 3- β -taraxerol. The Figure 1 (a-e) displays the HPTLC analysis at 254 nm, 366 nm and white light.

HPLC Analysis

HPLC analysis indicated that the isolated compound SSL-01 is 97.6% pure as mentioned in Table 2. The isolated compound compared with standard for its purity. The peaks of HPLC are represented in the Figure 2 (b).

Characterization

Spectroscopic methods, including IR, ^1H NMR, ^{13}C NMR and MASS spectrometry, validated the compound's structure.

IR ν/cm^{-1} = 3600 cm^{-1} O-H stretching, 1540 cm^{-1} C = C stretching and 2900 cm^{-1} C-H stretching as shown in the Figure 2 (c).

The ^1H NMR values of isolated compound SSL-01 is described in Table 3, the ^1H NMR spectrum shown in the Figure 2 (d).

The ^{13}C NMR spectrum (CDCl_3 , 200 MHz) exhibited the following signals: δ 15.62, 17.68, 18.98, 21.49, 26.08, 27.34, 28.18, 28.98, 30.10, 33.28, 33.53, 33.88, 35.31, 35.97, 36.88, 37.80, 37.91, 38.04, 37.91, 38.82, 38.95, 41.51, 48.95, 49.47, 55.72, 79.25, 117.06, and 158.27 as shown in the Figure 2 (e).

MASS: The molecular formula of 3- β -taraxerol was calculated to be $\text{C}_{30}\text{H}_{50}\text{O}$ and their peaks are shown in the Figure 2 (f).

HRMS ESI calculated for $\text{C}_{30}\text{H}_{50}\text{O}$ $[\text{M}-\text{H}_2\text{O}]$ is 408m/z and the molecular ion peak was observed at 427m/z with melting point of 260-262°C.

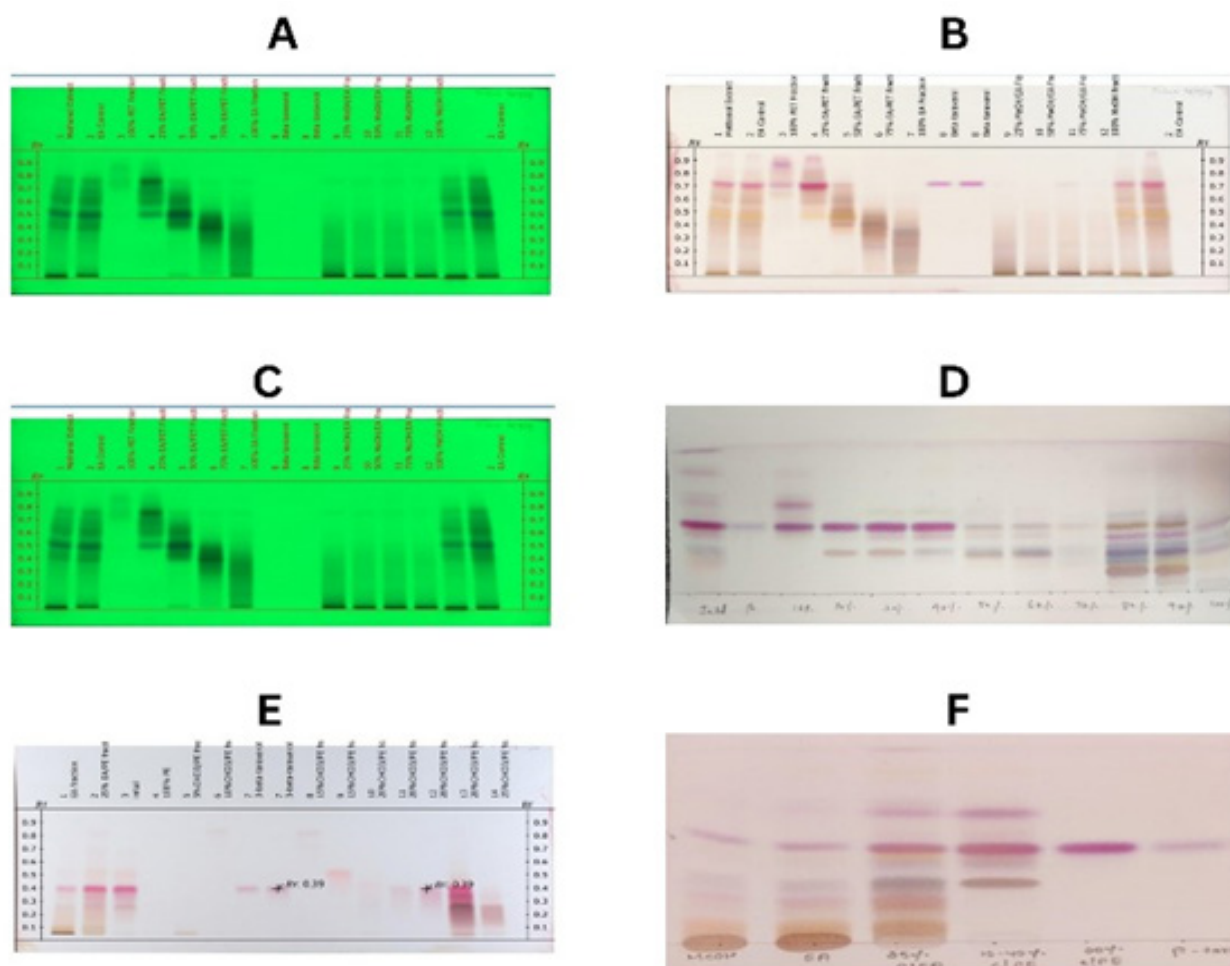


Figure 1: (A) HPTLC fingerprinting analysis of column-1 fractions at 254nm, (B) HPTLC fingerprinting analysis of column-1 fractions in white light, (C) HPTLC fingerprinting analysis of column-3 fractions at 254nm, (D) TLC of column -2 fractions at white light, (E) HPTLC fingerprinting analysis of column-3 fractions in white light, (F) Final TLC of isolated 3 β -taraxerol.

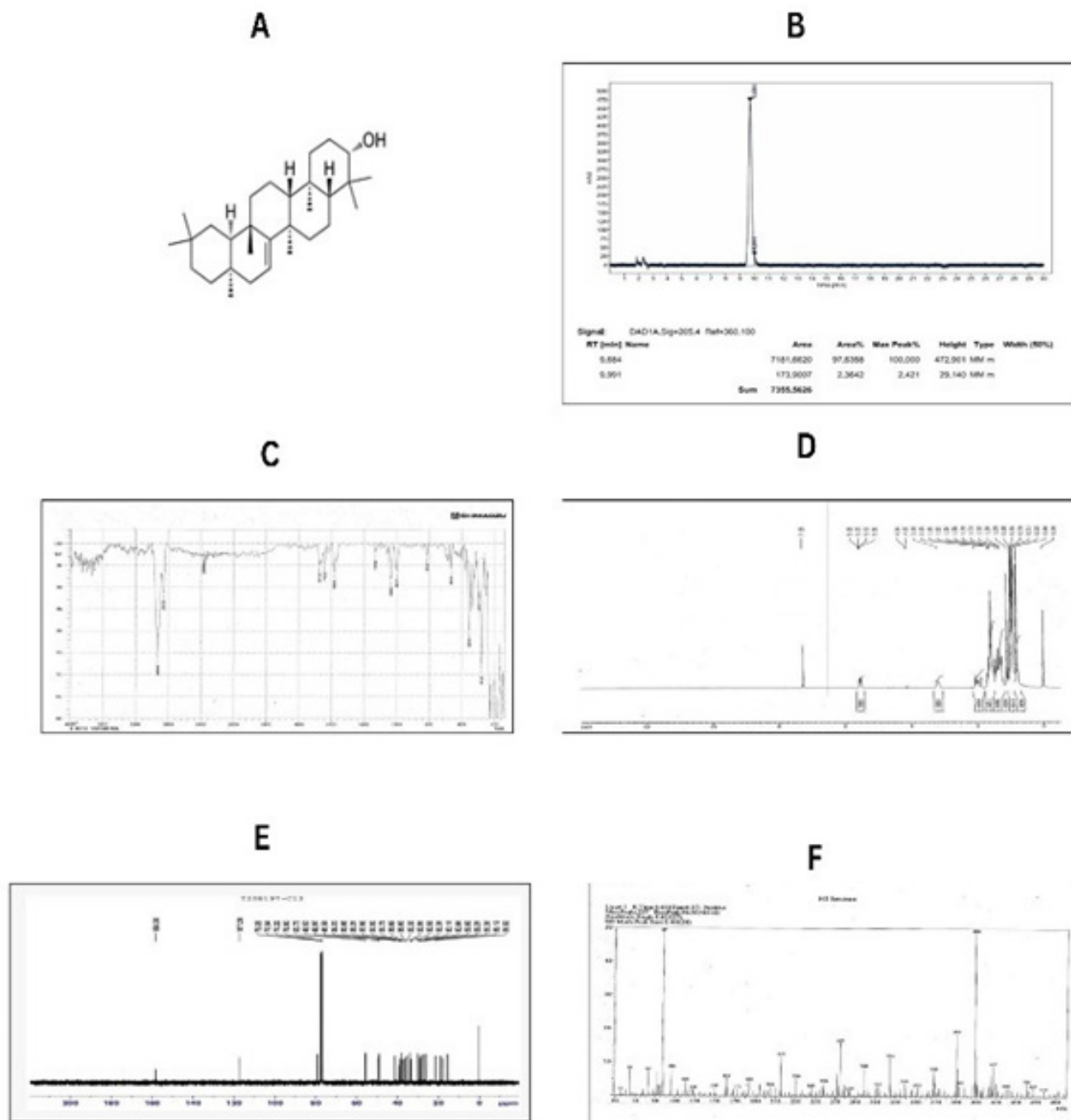


Figure 2: (A) Structure of isolated 3-β-taraxerol, (B) HPLC chromatogram of purified 3-β-taraxerol in comparison to standard, (C) FT-IR spectrum of 3-β-taraxerol, (D) ^1H NMR spectrum of 3-β-taraxerol, (E) ^{13}C NMR spectrum of 3-β-taraxerol, (F) Mass spectrum of isolated 3-β-taraxerol.

CONCLUSION

In this study, 3- β -taraxerol was isolated from the bark of *Stereospermum suaveolens* using a methanolic extract. TLC confirmed that the ethyl acetate phase from column chromatography, fraction 2 (75% PE/25% EA), considerably enhanced 3- β -taraxerol. Fractions 2-5 (10-40% CHCl₃/PE) were further concentrated by re-chromatography, and Fraction 5 (20% CHCl₃/80% PE) was purified to provide 1.525 g of pure 3- β -taraxerol (R_f 0.39), a high purity that was verified by HPLC. Using a variety of analytical methods, such as mass spectrometry, FT-IR, ¹H NMR, and HPTLC, we confirmed the compound's identity and structural integrity, providing a strong basis for more therapeutic research.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

%: Percentage; °C: Degree Celsius; HPTLC: High Performance Thin Layer Chromatography; HPLC: High Performance Liquid Chromatography; R_f : Retention factor; FT-IR: Fourier Transform Infrared Spectroscopy; DM: Demineralized; LR: Laboratory reagent; MHz: Mega hertz; ¹H NMR: Hydrogen Nuclear Magnetic Resonance; ¹³C NMR: Carbon Nuclear Magnetic Resonance; v/v: Volume/volume; mL: Milli liter; μ L: Micro liter; mg: Milli gram; g: Gram; mm: Milli meter; cm: Centimeter; nm: Nano meter; UV: Ultraviolet; PE: Pet ether; EA: Ethyl acetate; CH₃Cl: Chloroform.

SUMMARY

The present study was undertaken to isolate the phytoconstituents from *Stereospermum suaveolens* and to develop standardization profile for them. Percentage yield of methanolic extract obtained by refluxing of 45 kg was found to be 3.3% (1.496kg). Toluene: ethyl acetate: Formic acid (7:3:1) solvent system indicated the clear separation of bands. Partitioning of methanolic extract by using ethyl acetate, n-butanol and water, as there was no further separation, ethyl acetate partition fraction was subjected to column chromatography using silica gel as absorbent and eluted with 10 different combinations of solvents with increasing order of polarity using petroleum ether, ethyl acetate and methanol. Evaluation of above column eluent by TLC and HPTLC indicated that fraction fraction-2 [SPS/C1/Fr2 (25%EA/PE)] has shown prominent band of compound 01 upon visualization using ANS spray respectively, Hence the above fraction subjected to re-chromatography using silica gel as adsorbent and 10 different

combinations of solvent systems containing petroleum ether: chloroform increasing order of polarity as eluent. Evaluation by TLC and HPTLC indicated that fractions-2 to 5 [SPS/C2/Fr2 to SPS/C2/Fr5 (10 to 40%CHCl₃/PE)] was having prominent band with fewer impurities. Fraction-2 to 5 [SPS/C2/Fr2 to SPS/C2/Fr5 (10 to 40%CHCl₃/PE)] were further purified by column using flash grade silica as absorbent and solvent systems as petroleum ether: chloroform in increasing order of polarity. Evaluation by TLC indicated that fraction fraction-5 [SPS/C3/Fr5 (20% CHCl₃/PE)] was having single band. By HPLC analysis it was found to be 97.6% pure. A new HPTLC method was developed using hexane: ethyl acetate (8:2) as mobile phase, compound-01 was confirmed by distinct HPTLC bands with R_f of 0.39. SSL-01 is Soluble in Chloroform, methanol and has Melting point of 260- 262°C. Spectroscopic analysis confirmed the isolated compound SSL-01 as 3- β -taraxerol.

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Comparative Validation of Amlodipine and Valsartan by UV-spectrophotometry and RP-HPLC Methods Analysis by Statistical Method

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ABSTRACT

Aim: This study aims at method Validation and development of RP-HPLC and UV-spectrophotometry for the simultaneous detection of Valsartan (VAS) and Amlodipine (AMD) in tablet formulations. **Materials and Methods:** Two UV-spectrophotometric methods, first-order derivative spectroscopy and zero-order by simultaneous equation by Vierordt's approach, were created, and it was verified that they were linearity, precision, and accuracy were confirmed. AMD and VAS were successfully separated and simultaneously quantified using a reliable RP-HPLC method with a mobile phase. **Results:** For both medications, the absorbance ratio and First Order Derivative techniques showed low percentage R.S.D, excellent accuracy, and adequate sensitivity. Likewise, good resolution, accuracy, and robustness were attained by the RP-HPLC approach. Statistical analysis using ANOVA confirmed there is no noticeable distinction in the three approaches in precision and accuracy. The relevance of routine quality control was further confirmed when they were used to analyse commercial formulations. **Conclusion:** The validated methods offer sensitive, accurate, and exact procedures for estimating AMD and VAS simultaneously, which makes them appropriate for regulatory compliance and pharmaceutical quality control.

Keywords: Amlodipine, Valsartan, UV-spectrophotometry, RP-HPLC, Comparative Validation.

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INTRODUCTION

Amlodipine (AMD) prevents voltage-gated L-type calcium channels from opening; the oral dihydropyridine calcium channel blocker shows sustained efficacy. This function sets it apart within its pharmacological class by stopping the first calcium influx. Unlike shorter-acting competitors like nifedipine, Amlodipine has a half-life of 30 to 50 hr, making once-daily treatment convenient.¹

Valsartan (VAS) is a member of the Angiotensin II Receptor Blocker (ARB) class, which also includes [irbesartan], [Olmesartan], and [telmisartan]. ARBs prevent the hypertensive effects of the protein angiotensin II, which include vasoconstriction, cardiac stimulation, the generation of aldosterone and ADH, and renal reabsorption of sodium, by selectively binding to Angiotensin Receptor 1 (AT1).²

According to a thorough review of the literature, all spectroscopic techniques have been described for Amlodipine, either by itself or in combination with other medications. Developing and validating spectrophotometric techniques and the RP-HPLC techniques for the simultaneous determination of AMD and VAS by ICH guidelines is the objective of this work.^{3,4}

MATERIALS AND METHODS

Chemicals used: Amlodipine (AMD) and Valsartan (VAS) standards were obtained from a certified supplier. Analytical grade methanol, orthophosphoric acid, and acetonitrile were used. All chemicals and reagents were of pharmaceutical grade.

Instrumentation: A Shimadzu 1800 double-beam UV-visible spectrophotometer was used for spectrophotometric analysis. Chromatographic separation using a Waters HPLC system, and UV detector and a C18 column (150 mm x 4.6 mm, 5 μ m). An electronic analytical balance (Shimadzu AX-200) was used.

Analysis of Variance is used to compare all approaches statistically (ANOVA)

A statistical test called One way-ANOVA is used to look at how the means of three or more groups differ from one another. A $p < 0.05$ of significance level was considered statistically



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significant. It is a continuation of the t-test for two independent samples. One-way ANOVA was performed using Microsoft Excel 2016. The Hypothesis (H_1) and null Hypothesis (H_0) approaches suggest that at least one method differs significantly from the others. To analyse variance, the Sum of Squares (SS) is calculated as follows.⁶

Total Sum of Squares (SS_{total}): Measures the total variability in the data and is computed as:

$$SS_{\text{total}} = \sum X^2 - \frac{(\sum X)^2}{N}$$

Where $\sum X^2$ represents the sum of squared individual observations, and $(\sum X)^2/N$ represents the squared sum of all observations divided by the total observations (N).

Between-Groups Sum of Squares (SS_{between}): Measures the variability due to differences between the group means and is computed as:

$$SS_{\text{between}} = \sum X_{g1}^2 + \sum X_{g2}^2 + \dots + n - \frac{(\sum X)^2}{N}$$

Where X_{g1} , X_{g2} , etc., are the sums of Values within each method, and n represents the number of observations per method.⁶

Within-Groups Sum of Squares (SS_{within}): Captures the variability within each group.

Preparation of Standard and Sample Solutions: UV-Spectrophotometry

Two UV-spectrophotometric methods were validated: the absorbance ratio method and the first-order derivative method. Calibration curves were plotted and regression coefficients recorded. Precision, accuracy, and sensitivity Limit of detection (LOD), Limit of Quantification (LOQ) as per ICH Q2(R1).⁵

Calibration of curves for standard

The AMD calibration curve aliquots of the AMD working standard solution (0.2 to 1.0 mL) were taken into volumetric flasks. To get appropriate concentrations of 0.6, 1.2, 1.8, 2.4, and 3.0 µg/mL of AMD and for the VAS calibration curve. Aliquots of the VAS working standard solution (0.3 to 1.5 mL) were put into volumetric flasks. To get the appropriate concentrations of 9.6, 19.2, 28.2, 38.4, and 48.0 µg/mL of VAS, the volume was adjusted using methanol. Concentrations should be at a ratio of 1:16. Standard solutions' absorbances were determined using methanol as a blank at wavelengths 246 nm for AMD is an iso-absorptive point, where 238 nm is λ_{max} for VAS.⁵

Sample preparation

Tablets were opened and precisely weighed to determine the average weight. A 100 mL graduated flask containing 5 mg of AMD and 80 mg of VAS. After adding 50 mL of blank. To create a solution with 16 µg/mL of each medication, 1 mL of the filtered

solution and 15.5 mL of the standard stock solution of AMD (100 µg/mL) were combined in a 50 mL graduated flask.⁵

Sample calibration curve

In a separate volumetric flask of 10 mL volume, the standard AMD and VAS stock solutions were prepared to create calibration curves. To achieve the final concentrations of 10.2, 19.2, 28.2, 38.4, 48.0 µg/mL of AMD and 0.6, 1.2, 1.8, 2.4, and 3.0 µg/mL of VAS, these aliquots were then diluted with mobile phase. Chromatograms were produced using a 1.0 mL/min flow rate after standard solutions ($n=6$) were fed through a 20 µL loop device. At 234 nm, the effluent was observed.⁵

Validation Summary

All three analytical methods (absorbance ratio, first-order derivative, and RP-HPLC) were validated according to ICH Q2(R1) guidelines. All UV data were recorded using a double-beam spectrophotometer in the 200-400 nm wavelength range, with the slit size adjusted to 1 nm and a medium scanning speed. Methanol was taken as a blank. Each method exhibited excellent linearity across specified ranges with regression coefficients exceeding 0.998. Recovery studies confirmed accuracy with values between 98.2% and 102.6%. Sensitivity was supported by low LODs and LOQs: 0.26-0.53 µg/mL (LOD) and 0.80-1.97 µg/mL (LOQ) across the methods. All methods proved reliable for routine analysis of AMD and VAS.⁶

RESULTS AND DISCUSSION

Description of the API

FTIR data of Amlodipine and valsartan. The identity of Amlodipine and Valsartan was confirmed by FTIR spectroscopy through the presence of characteristic functional group vibrations. For Amlodipine, absorption bands corresponding to C=O (ketone), C-H stretching, and S=O (besylate group) were observed. Valsartan exhibited distinct peaks for O-H (carboxylic acid), C=N, and C-O stretches.⁷

Vierordt's method by simultaneous estimation of Amlodipine and valsartan

In two different 50.0 mL volumetric flasks, a precisely determined quantity of VAS and AMD in a 1:16 ratio was transferred and diluted with methanol. The iso-absorptive point can be found in Figure 1 compared to a blank. The levels of VAS and AMD were calculated by varying the parameters in the following formula.⁸

$$\text{Absorptivity} = \frac{\text{Absorbance}}{\text{Concentration}}$$

The following formulas can be used to determine the concentration of two medicines in the combination.

Diluted mixture's absorbances at 238 nm and 249 nm are denoted by A1 and A2, respectively. AMD and VAS. C_x and C_y

$$\text{For AMD, } C_x = \frac{A_{1ax2} - A_{2ax1}}{ax2ay1 - ax1ay2}$$

$$\text{For VAS } C_y = \frac{A_{1ax2} - A_{2ax1}}{ax2ay1 - ax1ay2}$$

concentrations (100 g/mL), respectively. The wavelengths were chosen based on the maximum absorbance ($\lambda_{\max}$) observed in the UV spectra for each drug with minimal spectral interference, ensuring optimal sensitivity and specificity. AMD and VAS have absorptivity of 238.0 and 249 nm, respectively, denoted by ax1, ax2, ay1, and ay2.

Method Validation

Linearity and range

It was discovered that the linearity range for AMD and VAS was between 0.2 and 3.0 $\mu\text{g/mL}$ for AMD and 9.6-48.0 $\mu\text{g/mL}$ for VAS (Figure 4 VAS and AMD linearity Values are provided). This implies that the analytical method accurately and consistently records concentration changes with absorbance variations, improving accuracy and dependability. The inclination for AMD and VAS is constant at various wavelengths, suggesting that the analytical method's sensitivity for both chemicals is comparable.⁸

Intra-day precision and inter-day precision

The RSD Values of intra-day for VAS vary from 1.60 to 0.45%. In contrast, AMD's intra-day percentage RSD Values range from 1.60 to 0.45% at 238 nm and from 1.44 to 0.93% at 238.01 nm. Intra-day and inter-day precision were evaluated at three concentration levels. %RSD values for both drugs were consistently 2% or less, confirming repeatability and intermediate precision.⁸

Accuracy

Recovery studies conducted at 80%, 100%, and 120% levels yielded recovery values ranging from 96.0% to 102.6% for AMD and 99.12% to 101.1% for VAS, indicating high method accuracy.⁸

Limit of detection and limit of quantification

Final uniform LOD/LOQ values of Amlodipine and valsartan are shown in Table 1. The LOD and LOQ Values are shown in Table 1. It is determined that the LOD and LOQ for AMD are 0.0265 $\mu\text{g/mL}$ and 0.803 $\mu\text{g/mL}$, respectively. For VAS, the LOD is 0.285 $\mu\text{g/mL}$ and the LOQ is $\mu\text{g/mL}$, whereas the LOD is lower at 238 nm at $\mu\text{g/mL}$ with a LOQ of 0.414 $\mu\text{g/mL}$.⁸

Analysis of tablet formulation

By examining the formulation of a commercially available tablet, the applicability of the suggested method was evaluated. These results show how accurate, precise, user-friendly, and quick the developed approach. It may be used for routine dosage form quality checking in industries.⁸

- First-order derivatives to estimate VAS and AMD simultaneously.
- Finding a wavelength to estimate VAS and AMD simultaneously.

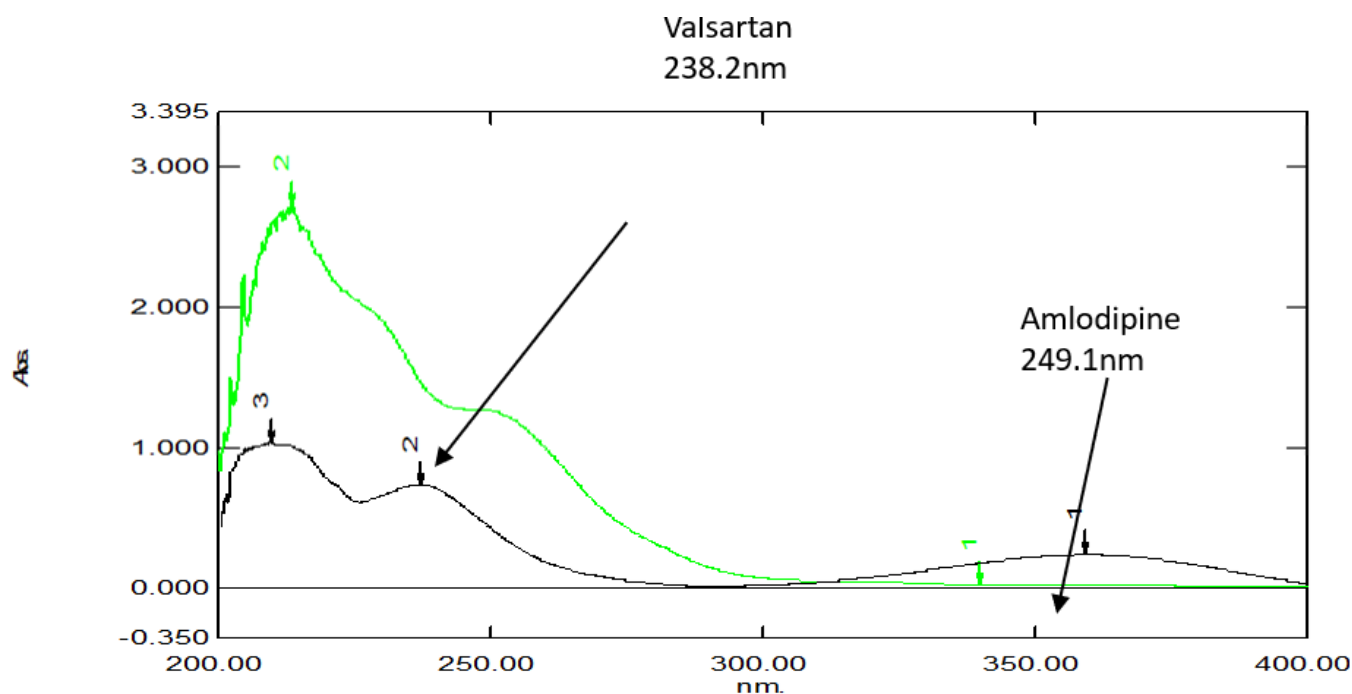


Figure 1: Overlay of AMD and VAS.

First-order overlay spectrum refers to the plotting of first-order derivative absorbance values across wavelengths, enhancing the resolution between overlapping spectra of Amlodipine and Valsartan, thus allowing for their simultaneous determination. The drug overlay spectra make it evident that the Zero-Crossing Point (ZCP) is present at 254.62 nm for VAS and 241.42 nm for AMD (Figure 2). The absorbances were measured at 254.62 nm (ZCP of VAS) and 241.42 nm (ZCP of AMD). The calibration curves from five working standards at the chosen wavelengths are used to compute the absorptivity coefficients. By using wavelengths that correspond to each compound ZCP, this method enhances specificity and sensitivity while ensuring accurate and trustworthy simultaneous measurements of AMD and VAS

concentrations in samples. This strengthens the quantitative analytical process resilience for analysis.⁹

Method Validation

Linearity and range

The linearity of AMD and VAS is across a range of concentrations to which the analytical technique reacts linearly to concentration changes. The linearity range for AMD and VAS in this investigation was found to be 3-30 µg/mL. It was discovered that the linearity range for AMD and VAS was between 3 and 30 µg/mL. The correlation coefficients (0.9884 for VAS and 0.9860 for AMD) demonstrate a linear relationship between absorbance and concentration, confirming the regression model's reliability. The method's sensitivity is further supported by the slope values

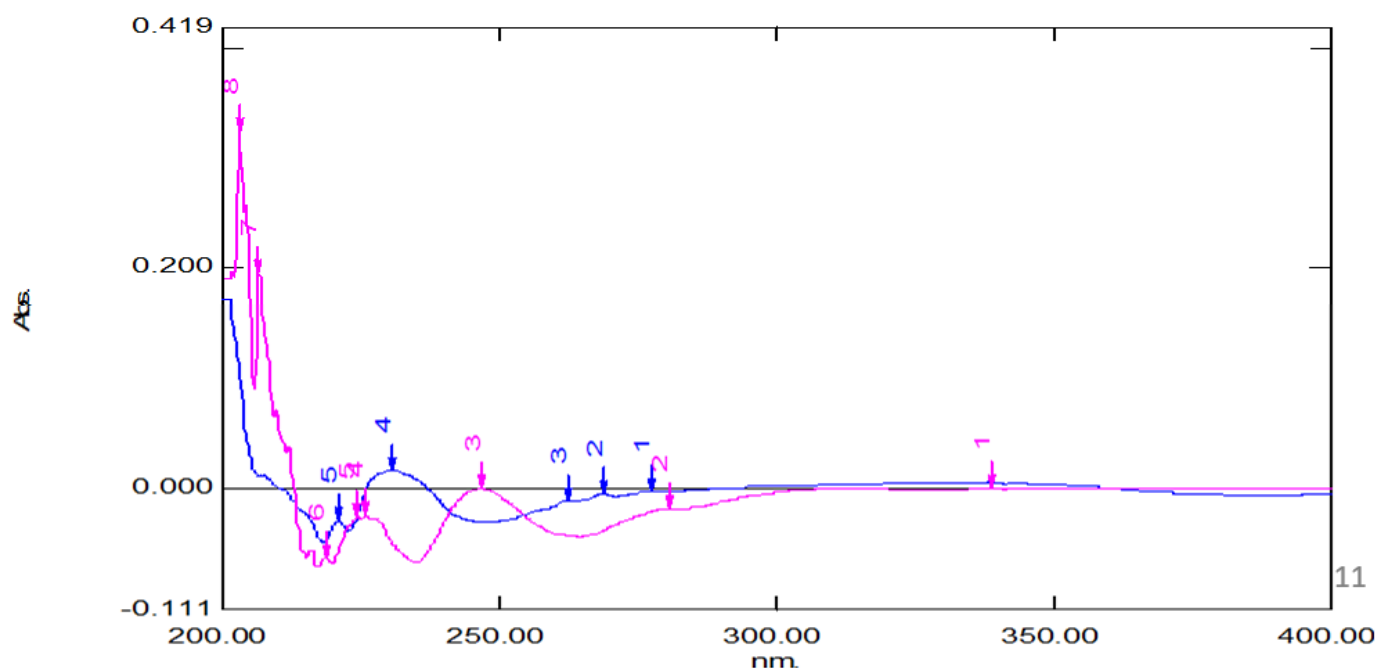


Figure 2: 1st Overlay for zero crossing point.

Table 1: Validation Parameters summary.

Sl. No.	Parameter		AMD (238 nm)		AMD (249 nm)		VAS (238 nm)		VAS (249 nm)	
1	Linearity Range (µg/mL)		5-25		5-25		5-25		5-25	
2	Precision (% RSD)		0.152-0.97		0.101-0.618		0.210-0.830		0.108-0.210	
3	LOD (µg/mL)		0.265		0.264		0.285		0.414	
4	LOQ (µg/mL)		0.803		0.800		0.864		0.124	
5	Accuracy (% Recovery, <i>n</i> =5)		96-102.6				99.12-101.1			
Evaluation of the tablet's composition										
6	Tablet	Label claim	Amount found	% label claim		Tablet	Label claim	Amount found		
	AMD + VAS	AMD	VAS	AMD		AMD + VAS	AMD	VAS		
		5 mg	80 mg	4.80			5 mg	80 mg		

(0.0010 for AMD and 0.0030 for VAS), (Figure 4) indicating consistent absorbance changes per unit concentration.⁹

Precision

Percent RSD for AMD ranged from 0.516-1.589% and for VAS from 0.33-0.546% at 237 and 246 nm. Inter-day Relative Standard Deviation (%RSD) ranged from 0.68-1.14% for AMD and 0.516-1.589% for VAS. VAS consistently showed lower %RSD values at 246 nm (0.51-1.58%), and AMD showed similarly low values at 247 nm (0.65-1.45%). These results indicate excellent precision with minimal variability in measurements across both intra- and inter-day analyses. All things considered, the findings show that the analytical approach has outstanding precision, which qualifies it for precise and trustworthy determination of VAS and AMD concentrations.⁹

Accuracy

A recovery study using a commercial tablet at three concentration levels confirmed the method's accuracy. Recovery rates exceeded 98.2%, with values of 98.2% (AMD) and 99% (VAS) at 80%, 100.4% (AMD) and 100.1% (VAS) at 100%, and 103.3% (AMD) and 102.75% (VAS) at 120%.⁹

Limit of quantification and limit of detection

The LOD values for AMD and VAS was 0.261 µg/mL, 0.371 µg/mL, indicating the lowest concentrations detectable with confidence. The LOQ is 0.791 µg/mL for AMD and 0.796 µg/mL for VAS, confirming the sensitivity and its ability to accurately and reliably quantify low analyte concentrations.⁹

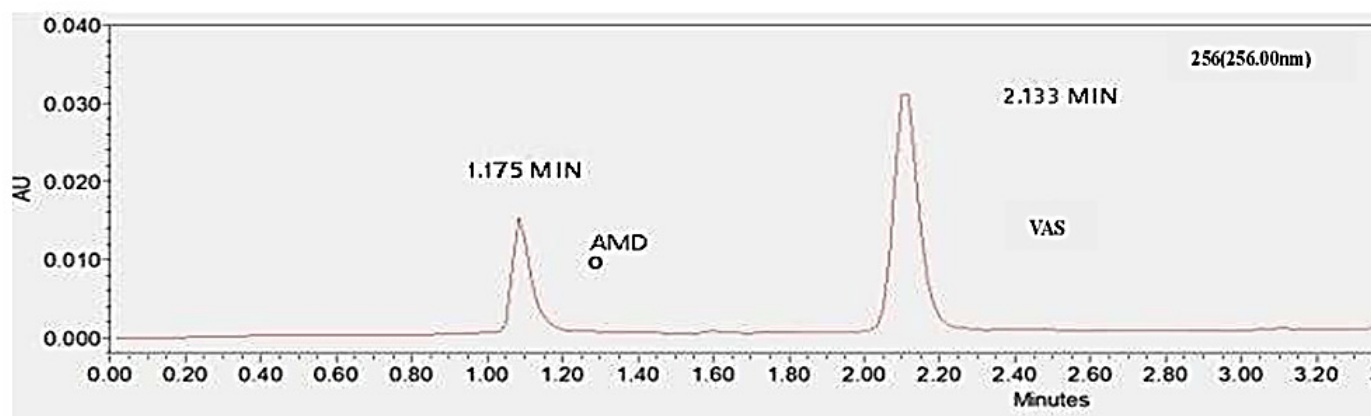


Figure 3: RP-HPLC overlay spectra for AMD and VAS.

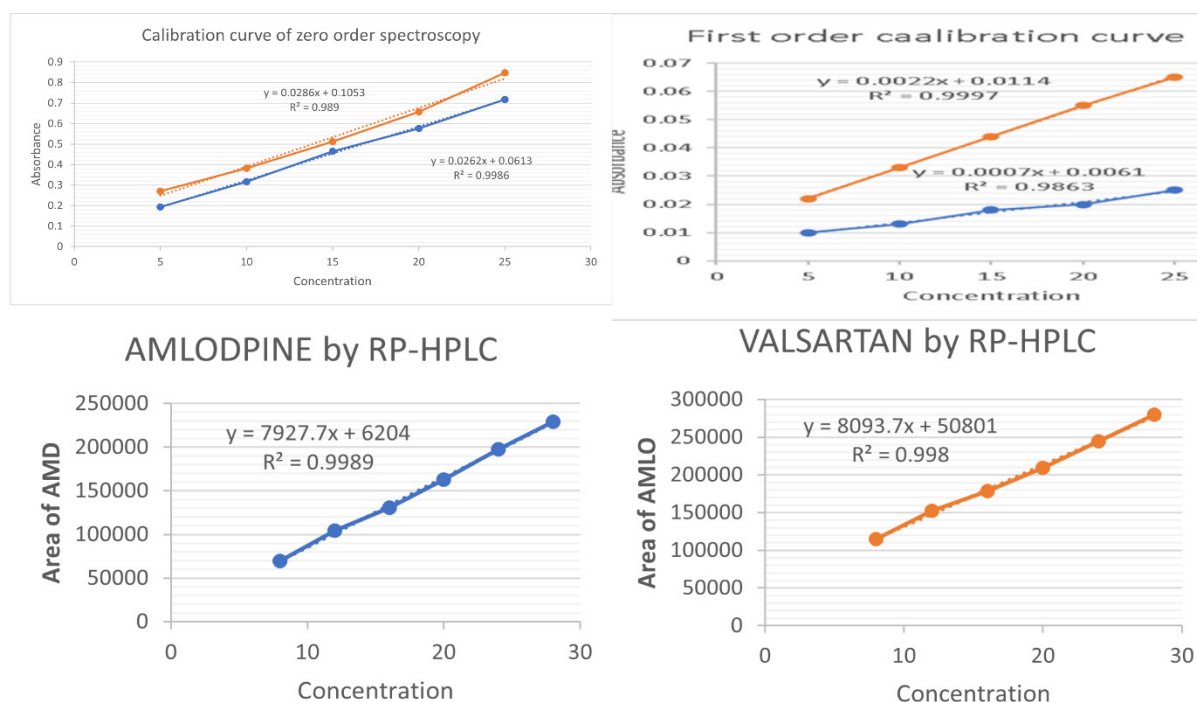


Figure 4: Overview linearity of AMD and VAS across all method.

Analysis of marketed formulation

The applicability of the method was evaluated using a commercially available tablet containing AMD and VAS. As shown in Table 2, the formulation contained 98.2% of AMD (5.02 ± 0.61 mg) and 99% of VAS (80.1 ± 2.28 mg), closely matching the label claims.⁹

Summary of Validation Parameters

Precision is measured for both intraday and interday measurements. It is expressed as the per R.S.D. The low values

of R.S.D. found for both compounds show excellent analytical technique precision. Lowest concentrations of the substances that can be accurately identified and measured are denoted by the LOD and LOQ, respectively. The high recovery percentages, which range from 98.2% to 103.6% for AMD and 98.89% to 102.75% for VAS. Taking everything into account, the validation criteria show that the analytical technique for assessing AMD and VAS is accurate, sensitive, and precise, making it suitable for implementation.

Table 2: Summary table.

Limit of detection and Limit of quantification					
Parameters	AMD			VAS	
Linearity Range	5-25 µg/mL			5-25 µg/mL	
Precision	0.516-1.589 (<i>n</i> =5) at 246 nm			0.821-1.189 (<i>n</i> =5) at 237 nm	
LOD (µg/mL)	0.261 (246 nm), 0.371 (295 nm)			0.262 (246 nm), 0.120 (249 nm)	
LOQ (µg/mL)	0.791 (both wavelengths)			0.796 (246 nm), 0.285 (249 nm)	
Accuracy (% Recovery)	98.2%-103.6%			99%-102.75%	
Analysis of a commercial tablet.					
Tablet	Label claim	Quantity found	% Label claim	Tablet	Label claim
AMD + VAS	AMD	VAS	AMD	AMD + VAS	AMD
	5 mg	80 mg	4.91 mg		5 mg

Table 3: Evaluation summary.

Parameter			AMD			VAS		
Retention Time (Flow Rate)								
1.0 mL/min			1.15 min (%RSD: 0.7)			2.51 min (%RSD: 0.7)		
0.8 mL/min			1.60 min (%RSD: 0.5)			2.69 min (%RSD: 0.6)		
System Suitability								
Injection 1-3 (Mean Area)			164,348±164.6 (%RSD: 0.10)			211,306±184.5 (%RSD: 0.08)		
Precision (Intra-day)								
16 µg/mL			179,845 (%RSD: 0.56)			178,895 (%RSD: 1.05)		
Linearity Parameters								
Standard Deviation (SD)			1283.6			1664.5		
Slope			7830.1			8922.3		
LOD (µg/mL)			0.525			0.654		
LOQ (µg/mL)			1.691			1.970		
Recovery data								
Level of recovery	Quantity of drug taken (µg/mL)		Quantity of drug added (µg/mL)		Quantity of drug added (µg/mL) (µg/mL) <i>n</i> =		% Recovery	
	AMD	VAS	AMD	VAS	AMD	VAS	AMD	VAS
50%	5	80	8	8	9.94	8.03	99.3	100.4
100%	5	80	16	16	15.76	15.88	98.2	99.3
150%	5	80	24	24	23.75	23.78	98.9	99.4

RP-HPLC

Validation of the RP-HPLC method

Chromatographic conditions and method development

Amlodipine (AMD) and Valsartan (VAS) were separated using a Sunfire C18 column (150 mm × 4.6 mm, 5 µm) in isocratic mode. The mobile phase comprised 0.02 M potassium dihydrogen phosphate buffer (pH 3.0) and acetonitrile (56:44 v/v) pH was adjusted using H₃PO₄. (Figure 3) The mobile phase was filtered (0.45 µm) and ultrasonicated before use. Flow rate at 1.0 mL / min, with UV detection at 234 nm. Column temperature was maintained at 25°C, and 20 µL was injected. Acetonitrile provided low viscosity and sharp, symmetrical peaks. The C₁₈ column was preferred for its efficiency with polar analytes.¹⁰

Optimisation of mobile phase

The mobile phase, composed of acetonitrile and H₃PO₄ buffer in mobile phase (56:44 v/v), for Amlodipine and Valsartan. The solvent was sonicated for ten to 20 min. Following ultrasonication degassed.¹⁰

Validation of the method

Range and Linearity

The linearity range for AMD was 8-28 µg/mL, while for VAS, it was 8-28 µg/mL. The calibration curves for both AMD and VAS were generated, with retention times of 1.16±0.02 min for AMD and 2.13±0.02 min for VAS (Figure 4). Regression analysis showed satisfactory correlation coefficients of 0.9897 for AMD and 0.9898 for VAS, indicating a strong linear relationship between chromatographic peak area and concentration. Precision data are within acceptable limits, with intra-day values from 0.30% to 1.15% for AMD at 10, 15, and 20 µg/mL.¹⁰

Limit of Detection and Limit of Quantification

The RP-HPLC method was validated using the determination of the LOD and LOQ Values for VAS and AMD. LOD and LOQ values of 1.3363 µg/mL, 4.0495 µg/mL. Similarly, LOD and LOQ values for AMD, which show how sensitive the approach is at identifying and measuring AMD, were measured to be 0.525167µg/mL and 4.1089 µg/mL. Linearity ($r^2 > 0.999$), Precision (%RSD < 1%), Accuracy (Recovery 98.2%-99.4%), and Sensitivity.¹⁰

Table 4: Calculation of ANOVA for Amlodipine and Valsartan.

Amlodipine						
Method	Values			Mean	ΣXg	ΣX ²
Vierordt's method	96	96.3	96.8	96.3	289.1	27763.38
First-order Derivative	98.2	98.6	98.4	98.4	295.2	29047.76
RP-HPLC	99.6	98.5	98.9	99.0	297.0	29403.62
Valsartan						
Method	Values			Mean	ΣXg	ΣX ²
Vierordt's method	97.8	97.2	96.6	97.2	291.6	28344.24
First-order Derivative	98.5	98.9	98.7	98.4	295.2	29166.02
RP-HPLC	99.3	99.1	98.6	99.3	298.0	29601.46
Calculation of ANOVA						
Source of Variation	Sum of Squares (SS)	d _f	Mean Square (MS)	F-Calculated	F-Critical (α=0.05)	p-Values
Amlodipine						
Between Groups	17.6511	5	3.5302	43.6	5.14	1.775 × 10 ⁻⁵
Within Groups	2.0867	12	0.1739	-	-	
Total	19.7378	17	-	-	-	
Valsartan						
Between Groups	10.8511	5	2.1702	8.60	3.1059	0.00116
Within Groups	3.0267	12	0.2522	-	-	
Total	13.8778	17	-	-	-	

Robustness

Robustness of the method's consistency and dependability by allowing it to withstand minor modifications in the experimental setup. Some adjustments to the composition of the Buffer: ACN: Methanol produced 98.2% and 99.3% in Table 3. Despite slight variations in the experimental conditions, the assay percentages generally stayed within a range of fluctuation, demonstrating the resilience in producing consistent and trustworthy results.¹⁰

Accuracy

The recovery statistics for AMD and VAS from tablets utilising the RP-HPLC are shown in Table 3. The total quantities found by analysis after known doses of drugs were added to pill formulations at different recovery levels. For AMD, the total quantities detected at corresponding recovery levels ranged from 98.3% to 99.3%, with respective Values of 9.94 ± 0.14 $\mu\text{g/mL}$, 15.76 ± 0.02 $\mu\text{g/mL}$, and 23.75 ± 0.06 $\mu\text{g/mL}$. With a percentage recovery near 100%. With recoveries ranging from 99.4% to 100.4%, the total quantities for VAS at 50%, 100%, and 150% recovered amount 8.03 ± 0.14 $\mu\text{g/mL}$, 15.88 ± 0.06 $\mu\text{g/mL}$, and 23.78 ± 0.12 $\mu\text{g/mL}$.¹⁰

Statistical comparison by ANOVA

As indicated in Table 4, the RP-HPLC method and UV spectrophotometry were created for the determination of AMD and VAS in combined forms. At a significance threshold of 0.05, the computed F-values for Valsartan and Amlodipine fell between 0.36 and 2.048, all of which were below the critical F-values of 4.25. This suggests that no appreciable variations were found between the approaches for AMD and VAS for the simulations. These results imply that the accuracy and precision of the three approaches are similar. To evaluate the differences in the effectiveness of various techniques in analysing the content of VAS and AMD, an ANOVA analysis was performed. With three degrees of freedom (d_f), the between-groups variance for VAS produced a Mean Square (MS) of 3.5302 and a Sum of Squares (SS) of 17.6511, reflecting the variations between the approaches. The calculated F-values of 8.60 were less than the crucial F-values of 43.6 at a significance level of 0.05, indicating that there was no appreciable difference between the techniques. Similarly, the AMD between groups variation yielded a lesser SS of 2.0867 with 2 d_f and an associated F-value, which was again below the necessary F-values.¹¹

Comparing test results using three distinct approaches using a one-way ANOVA

The One-way ANOVA test was used to evaluate the use of RP-HPLC and UV spectroscopic methods for determining the concentration of VAS, AMD. The mean Values for each strategy were 96.7, 98.4, and 99.2, based on three observations each. It may be concluded that there is no discernible difference between the means obtained using the three-method statistical comparison,

using one-way ANOVA, the three analytical techniques did not significantly differ from one another. Since the null hypothesis is true, it was determined that there are no appreciable differences between the three devised methods for determining Amlodipine and valsartan. *Post-hoc* tests were not conducted because the (F calculated < F critical) ANOVA results showed no notable differences between the methods.¹¹

CONCLUSION

The current study effectively established and validated UV spectrophotometric and RP-HPLC techniques for Amlodipine and Valsartan estimation. Validation studies were found to provide excellent accuracy with good recoveries of precision with low %RSD, and thus, these methods are appropriate for quick quality control in resource-poor environments. The RP-HPLC method offered superior specificity and resolution, while the UV methods provided cost-effective and rapid alternatives with comparable reliability. These validated methods are employed in standard quality control and stability analysis of combined dosage forms of AMD and VAS in pharmaceutical industries. All methods were qualified according to Q2(R1) guidelines of ICH and satisfied all regulatory requirements. Although UV spectroscopy offers a convenient and cost-effective method of routine analysis, HPLC is a more accurate alternative, particularly where greater sensitivity and resolution are needed.

ACKNOWLEDGEMENT

We thank the Department of Pharmaceutical Analysis and Principal, Krupanidhi College of Pharmacy, for constant support throughout the study. No particular funds from the public, private, or nonprofit sectors were awarded for this study.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

SUMMARY

The study focuses on the development and validation of UV-spectrophotometric and RP-HPLC methods for the simultaneous estimation of Amlodipine (AMD) and Valsartan (VAS) in bulk and tablet formulations. Employing approaches like first-order derivative spectroscopy and Vierordt's method, the research ensures precision, accuracy, and sensitivity as per ICH Q2(R1) guidelines. Key findings include high linearity, low Limits of Detection (LOD) and Quantification (LOQ), and excellent recovery percentages, demonstrating the method's reliability for quality control. RP-HPLC offered superior specificity and resolution, with chromatographic conditions optimised for AMD and VAS separation, while UV techniques provided cost-effective alternatives.

Statistical analysis via ANOVA confirmed no notable differences in accuracy and precision among the methods, underscoring

their equivalency in analytical performance. Validation outcomes like low %RSD and robust recoveries validate the suitability of these methods for routine pharmaceutical analysis. The study concludes that while UV spectroscopy methods are rapid and economical, RP-HPLC offers greater precision and resolution, making both valuable for industrial applications in assessing AMD and VAS in combination drug formulations.

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In silico and *In vitro* Screening of Selected Plant Leaves of Indian Origin: A Prospective in Malaria

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ABSTRACT

Background: Malaria is one of the leading parasitic infections caused by *Plasmodium* species, which is still a major threat, especially in wet tropical region. There is a need for better and more widely accessible anti-malarial treatments to combat drug-resistant strains of the parasite. Natural plants have various phytochemical constituents with different pharmacological properties. **Objectives:** The computational screening, selected plant leaf extraction and *in vitro* evaluation for anti-malarial activity. **Materials and Methods:** Molecular docking studies were performed for 10 chemical constituents against Enoyl-acyl carrier protein reductase using Molegro Virtual Docker 6.0, followed by Molecular Dynamic Simulation to evaluate binding stability, RMSD, RMSF and MM-PBSA binding free energy and the physicochemical properties were evaluated for ADME and toxicity studies using online server SWISS ADME and ProTox-II and *in vitro* antimalarial activity was assessed by β -haematin inhibition assay. **Results:** The molecular docking study the best binding score for quercetin (-147.48 Kcal/mol) from leaves of *P. guajava*. MD simulations further validated quercetin's stable binding conformation, ADMET results are within the Lipinski rule of 5 and *in vitro* studies showed good inhibition scores. **Conclusion:** The preliminary *in silico* and *in vitro* study from current research gave valuable insight about antimalarial chemical constituents from leaves of Guava which showed notifiable activity against malarial parasites. Further pre-clinical studies need to be performed for the new lead development for malarial activity.

Keywords: β -haematin, Docking, Malaria, MM-PBSA, Molecular dynamic simulation, Plasmodium, Phenols, RMSD, RMSF.

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INTRODUCTION

Malaria stands out as the most parasitic disease caused by Plasmodium species leading to huge number of deaths according to WHO most recent World Malaria Report, there were 247 million cases of malaria in 2021 as opposed to 245 million cases in 2020 with an estimated 619,000 deaths in 2021 compared to 6,27,000 deaths due to which showed an increase compared to the previous year.¹ However, malaria is treatable and preventable, therefore, for the growing resistance to current antimalarial medications, it is now necessary to find and develop novel alternative medications in order to potentially prevent drug resistance issues.² Malarial infection caused by *P. vivax* and *P. falciparum* are much more acute and severe than the protozoal infection causes by other Plasmodium species. They can cause serious and life-threatening

illness which can lead to large number of deaths directly related to severe complications.^{3,4}

In the intra-erythrocytic life cycle of the malarial parasite, during the trophozoite stage, the Plasmodium species digest haemoglobin within the acidic food vacuole leading to the formation of monomeric heme units known as ferri heme. These heme gets converted quickly into an inert non-toxic polymer called haemozoin as malarial parasites cannot tolerate the toxic effects of the free heme. In biological test determination, β -haematin, a synthetic form of this haemozoin, was utilized. Antimalarial medications thereby prevent the production of β -haematin, which eventually results in parasite destruction.^{5,6}

The different plant constituents having antimalarial properties were reported as antimalarial compounds. Nimbolide (*Azadirachta indica*),^{7,8} *M. oleifera* (Quercetin and Kaempferol),⁹⁻¹¹ Catechin (Green tea),¹²⁻¹⁴ Piperine (curcumin).¹⁰ Important antioxidants such as protocatechuic acid, quercetin, morin, ferulic acid, ascorbic acid, gallic acid, and caffeic acid are abundant in guava¹⁵ in Figure 1. Morin, a constituent of guava leaves shows significant antimalarial property.^{8,10} Hence based on the above information the extracts of *Psidium guajava* was selected for the *in vitro*



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β -haematin assay due to the evidence of previous studies on its potential as an antimalarial agent.¹⁶

The current study aims to investigate docking studies and the β -haematin inhibition activity of the guava leaf extract, using semi-quantitative and quantitative screening methods.

MATERIALS AND METHODS

Materials

Fresh guava leaves were collected from medicinal garden of Krupanidhi college of pharmacy and authenticated from IIHR, Bangalore. After submitting the request to the chemical-reagent procurement department, all chemicals of the LR grade were acquired from private vendors including Merck and Sigma-Aldrich. The normal agents approved by the institute provided these substances. Analytical TLC plate with silica gel 60 was used to determine the TLC profile. The spots on TLC plates were visualized using a UV lamp at 254 nm. spectrophotometric study was performed on double beam UV-vis spectrophotometer (Shimadzu UV-1800 spectrophotometer). Computational tools used for molecular docking and simulations are Molegro Virtual Docker 6.0, RCSB-Protein Data, ZINC 15 database and GROMACS v5.0.4. softwares. ADME and Toxicity studies were conducted using webserver SwissADME and ProToxII.

Methods

Screening of different plant constituents for their use in antimalarial activity

A survey of numerous research publications, journals, theses, and review papers using search engines like Google Scholar, PubMed, Science Direct, and others in order to find information on the usage of various plants to treat malaria was performed. Molecular docking studies were done for the various constituents present in the different plants.

Molecular docking

Target preparation

The target of the designed work plan was determined through a comprehensive literature review. The 3D structure of the selected protein, 1VRW, from the enzyme Enoyl-acyl carrier protein reductase, was obtained in .pdb format from the RCSB-Protein Data Repository.¹⁷

Preparation of Ligand molecule

The nine ligands were selected for antimalarial activity as a potent flavonoid based on literature studies. The 3D structures of various ligands were retrieved from ZINC 15 database.¹⁸

Molecular docking Study

Molecular docking study between the receptor and ligands using computational platform enables the precise determination of

ligand geometry and provides a robust docking environment. Prior to docking, water molecules and cofactors were excluded to enhance the accuracy of the interaction analysis. MVD calculates key docking parameters, including the MolDock and hydrogen bond score, while optimizing ligand binding geometry at the receptor's active site. Post-docking refinement was performed to minimize energy and enhance hydrogen bond interactions. The docking results, including hydrogen bond and MolDock scores, were recorded to evaluate the binding efficacy of plant-derived phytoconstituents. A lower MolDock and hydrogen bond score correspond to a higher binding affinity between the receptor and ligand. Furthermore, ligand geometry was analysed and compared with positive control targets based on the docking scores obtained from MVD.¹⁹

Evaluation of ADME and Toxicity studies of the phytoconstituents

The ADME and Toxicity studies was based on Lipinski's concept, the phytoconstituents were examined. The molecules were transformed into the format known as SMILE. Using SwissADME, the compounds *in silico* pharmacokinetics were estimated, including the amount of hydrogen acceptors and donors, Log P, Mol. Refractivity, GI absorption, TPSA and drug-likeness. The toxicity of the identified phytoconstituents was predicted using ProTox II.¹⁸

Molecular Dynamic Simulation

The dynamic interactions between the ligand and protein were explored through molecular dynamics simulations utilizing GROMACS v5.0.4. The AMBER03 force field was employed for the simulations, Acpye server was used to generate the topology parameters of the ligand. The protein-ligand complex exhibiting the highest binding affinity (MolDock score) was selected for a 10,000-picosecond simulation. The simulation was conducted within a cubic simulation box containing SPC water molecules, ensuring system neutrality by incorporating 7 chloride and 24 sodium ions at 300K and 1 bar pressure and the system was equilibrated for a duration of 100 ps to allow stabilization of temperature and pressure before proceeding to the production run. The LINCS algorithm, with a 2-femtosecond time step, was utilized to constrain bond lengths. The trajectory data was collected at 2-picosecond intervals during the production dynamics phase and analyzed using a van der Waals cutoff of 1.4 nm.²⁰

Extraction

The leaves were washed thoroughly with distilled water. Then the leaves were dried in the shade. After the leaves are dried, they were crushed into fine or coarse powder. The powder was sieved in a mesh size of 17 to get a coarse powder of guava leaves. This powder was weighed and added in the round bottomed flask with distilled water. Then the round bottomed flask was connected to

reflex condenser and boiled at 45°C for 12 hr. The solution was taken out and filtered with a muslin cloth to get the extract. This extract was concentrated by keeping it in the steam bath or also by using a lyophilizer.²¹

Phytoconstituent analysis

To indicate the presence of flavonoids, 1 mL of extract was taken and few drops of dilute sodium hydroxide was added, the colour change was observed.²²

Qualitative analysis

UV test

1g of sample dissolved in a 10 mL of water was introduced to a test tube with 0.1 mL of 10% aluminum nitrate, 0.1 mL of 1 mol/lit aqueous potassium, and 4.3 mL of 80% ethanol and placed in a dark at room temperature.²³

In vitro β -haematin inhibition assay

Freshly prepared 3 mM Haematin in 0.1M NaOH was dissolved in 10 mM oleic acid and 1M HCl. Given guava leaf extract with different concentrations was dissolved in 0.5% DMSO mixed with 3 mM Haematin solution in 1 mL Eppendorf tubes and the final volume was adjusted to 1 mL with Sodium acetate buffer (pH-5). Three assay controls were used for this study a blank control (only 0.5% DMSO), negative control (3 mM Haematin in 0.1M NaOH) and Positive control (3 mM Haematin in 0.1M NaOH and 0.5 mg/mL of Chloroquine phosphate). Chloroquine phosphate with 0.5 mg/mL was used as the standard control. The tubes were incubated at 37°C overnight in a gyratory shaker. After the incubation period, the tubes were centrifuged at 14,000 rpm for 10 min at 21°C. Resulted hemozoin pellet was washed with 2.5% SDS prepared in 1M PBS which was centrifuged again at 14,000 rpm for 10 min and the obtained pellet were washed with 0.1M Sodium bicarbonate until supernatant becomes clear (5-6 times).

The pellets were dissolved in 1 mL of 0.1M NaOH to determine the hemozoin content. The absorbance on a microplate reader 400nm and % beta haematin inhibition activity was calculated using the formula.²⁴⁻²⁶

$$\beta - \text{haematin inhibition} = \left[\frac{(\text{Abs of Control} - \text{Abs of Sample})}{\text{Abs of Control}} \right] \times 100$$

Concentrations employed for the study

One test sample (guava leaf extract) was evaluated to measure the β -haematin inhibitory activity. The concentrations used for the study are shown in Table 1.

RESULTS AND DISCUSSION

Molecular docking

Molecular docking performed with Molegro Virtual Docker 6.0 (MVD) demonstrated different binding affinities. The binding energy and the hydrogen bond interactions of the various constituents with the protein 1VRW are given in Table 2. Among the 9 compounds quercetin from Guava leaves exhibited better binding affinity to the target with a binding score of -147.48 kcal/mol (Figure 2) and the lowest score was exhibited by α -pinene from long pepper of about -58.91 kcal/mol. Hence the plant extract of guava leaves is considered as the potential candidate for *in vitro* study of antimalarial activity.

ADME and Toxicity profile

The maximum TPSA value (190.28) was noted for Quercitrin, followed by Quercetin (131.36). Additionally, Quercetin was identified to have a molecular mass of 302.04 Daltons, featuring five hydrogen bond donors and seven acceptors. It was classified as mildly toxic, falling under toxicity class 3. The ADMET and toxicity profile of the plant phytoconstituents are given in Tables 3 and 4, respectively.

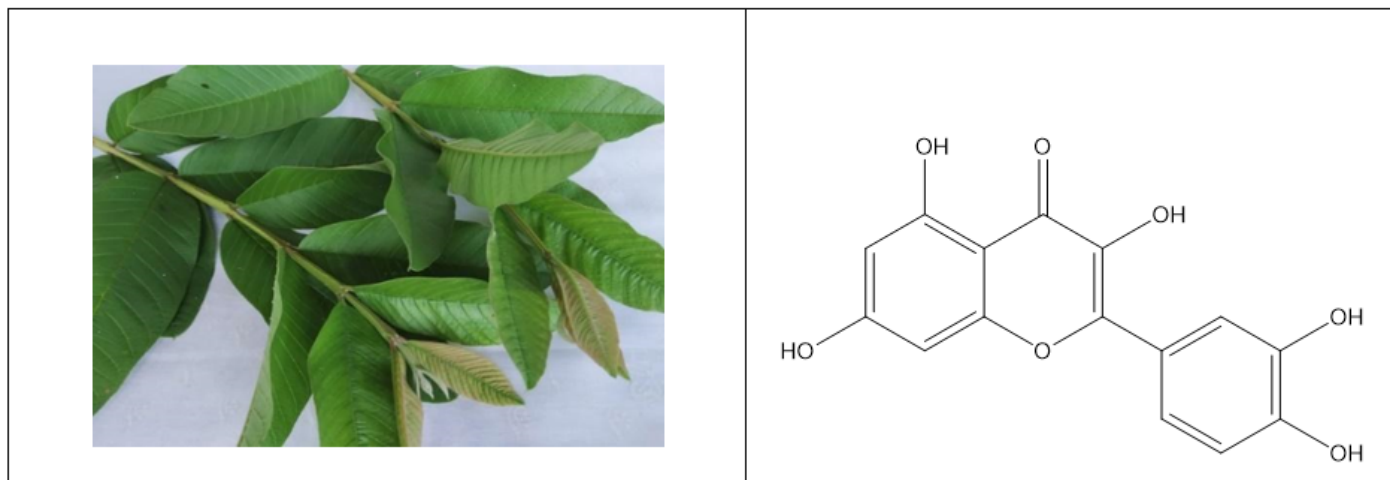


Figure 1: Quercetin obtained from leaves of *Psidium guajava* (Guava).

Molecular dynamic simulation

The Root-Mean-Square Deviation (RMSD) of every trajectory frame during the Molecular Dynamics Simulation (MDS) was used to evaluate the stability of the docked protein-ligand complex. RMSD serves as a critical metric to evaluate the conformational stability and interactions of biomolecules over time. The analysis was conducted over a 10,000 ps simulation to examine the dynamic behavior of both the protein and the ligand.

The RMSD profile of the protein as depicted in Figure 3, exhibits a steady rise within the initial 2000 ps, indicating structural adjustments as the system equilibrates. Beyond this period, the RMSD values stabilize, fluctuating around 0.35-0.45 nm, suggesting that the protein maintains a relatively stable conformation. Minor fluctuations observed in the later stages are attributed to inherent protein flexibility, yet no significant deviations indicative of unfolding or major structural disruptions were detected.

The ligand RMSD in Figure 4 remains consistently low throughout the simulation, fluctuating within a narrow range

of approximately 0.01-0.03 nm. This minimal deviation signifies that the ligand remains securely bound within the active site, undergoing only minor positional adjustments. The absence of substantial fluctuations confirms strong binding affinity and limited conformational alterations within the protein's binding pocket.

The RMSD analysis of both the protein and ligand highlights a stable complex formation. The protein undergoes initial conformational adaptations before stabilizing, while the ligand exhibits minimal fluctuations, reinforcing the robustness of the interaction between the two. These findings indicate that the ligand maintains its binding pose effectively, contributing to the overall stability of the system.

The Root-Mean-Square Fluctuation (RMSF) analysis was conducted to assess the flexibility of individual residues within the protein-ligand complex throughout the molecular dynamics simulation. RMSF provides insight into localized structural variations, highlighting regions of high mobility and potential functional significance.

The RMSF profile as shown in Figure 5, exhibits fluctuations across the protein structure, with most residues maintaining stability within a range of 0.1-0.3 nm. Several peaks exceeding 0.4 nm are observed, indicating regions of pronounced flexibility. These fluctuations are typically associated with loop regions, surface-exposed residues, or terminal regions, which are inherently more dynamic. In contrast, lower fluctuations are observed in core structural elements such as α -helices and β -sheets, suggesting their role in maintaining overall protein stability.

Table 1: Details of the various concentrations of samples used in the study.

Sl. No.	Test compounds	Concentration
1	β -haematin	3 Mm
2	Standard (Chloroquine phosphate)	0.5 mg/mL
3	Blank	0.5% DMSO
4	Sample (Guava leaf extract)	0.25, 0.5, 1 and 2 mg/mL

The study results were presented as mean $\pm$ SEM.

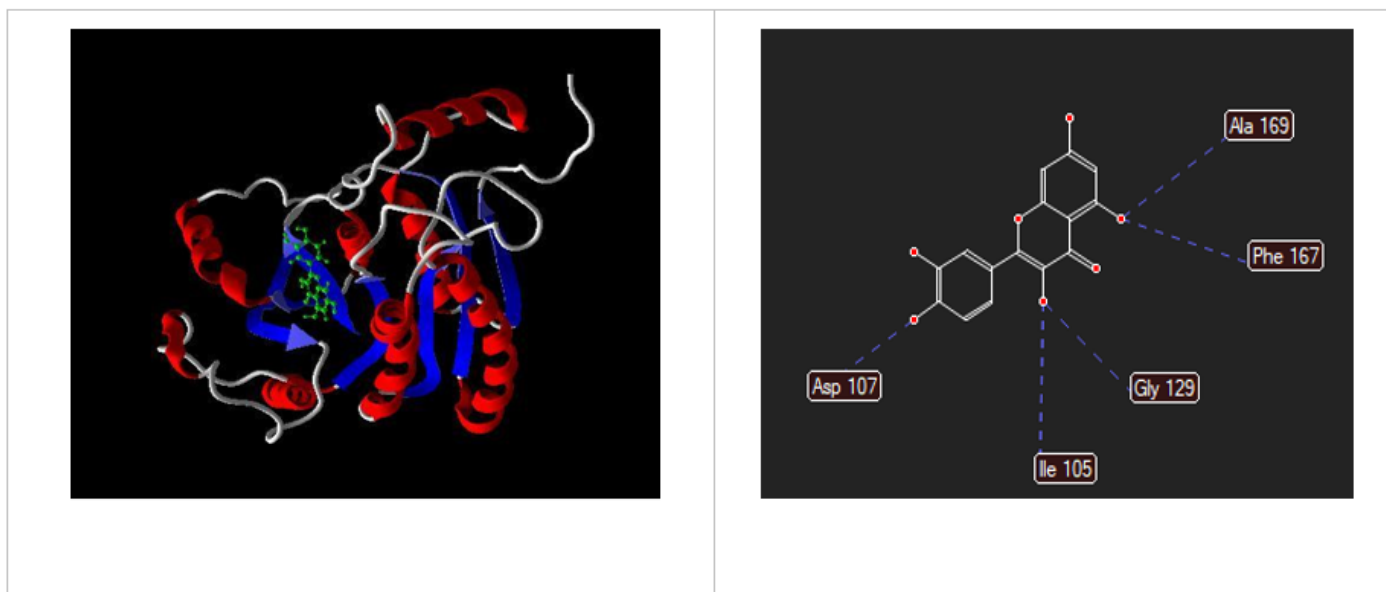
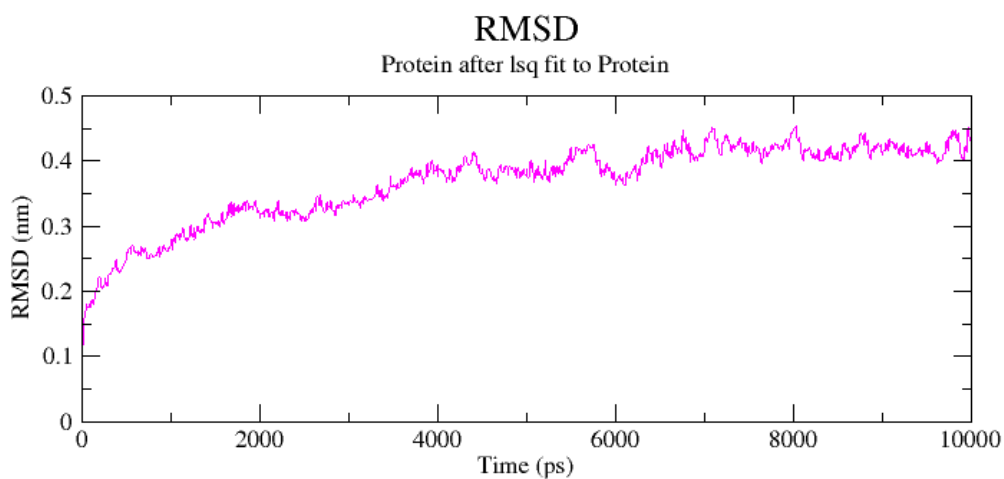
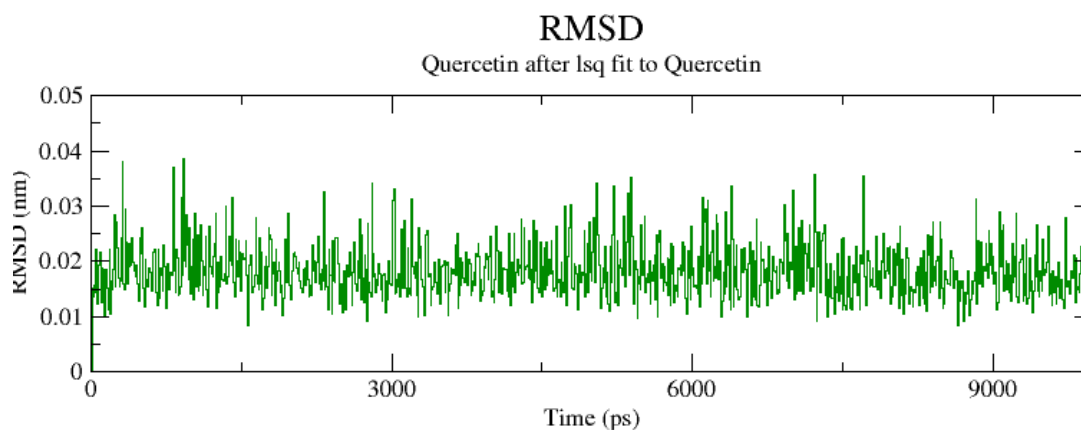


Figure 2: Binding energy and hydrogen bond interactions of the quercetin with protein 1VRW.

Table 2: Plant constituents along with their ligand interactions and docking scores.

Sl. No.	Constituent	Docking/binding score (Kcal/mol)	H-bond
1	Nimbolide	-194.24	-6.57
2	α -pinene	-58.91	0
3	Thymol	-85.12	-2.97
4	Kaempferol	-124.0	-8.94
5	Morin	-126.3	-8.90
6	Quercetin	-147.48	-5.39
7	Catechin	-122.22	-9.26
8	Quercitrin	-129.98	-9.38
9	Piperine	-137.61	-6.20

**Figure 3:** RMSD analysis of protein.**Figure 4:** RMSD analysis of ligand.

Stability of protein ligand complex

Stability was shown by the protein-ligand complex during a molecular dynamics simulation that lasted 2000 ps. The stable conformation was shown by the protein's RMSD stabilising at 0.35-0.45 nm, while the ligand's RMSD stayed low at 0.01-0.03 nm, indicating robust binding within the active site. A well-retained posture and strong affinity are indicated by minimal ligand position variations. The RMSF analysis confirmed the overall integrity of the complex by demonstrating modest flexibility near the binding site, which supports ligand accommodation, but core structural elements remained stable.

Qualitative test for flavonoids

The laboratory test procedure showed intense yellow colour which represents the presence of flavonoids in the extraction. The spectrophotometric study confirmed the presence of characteristic peak of quercetin at 256 nm and 373 nm (Figure 6).

In vitro antimalarial activity of crude extract of leaf of *Psidium guajava*

The β -haematin study was performed by Colorimetry. The anti-malarial activity of the given sample (guava extract) was compared with the standard potent drug chloroquine at 0.5 mg concentration which inhibited the β haematin at about 54.18%.

RMS fluctuation

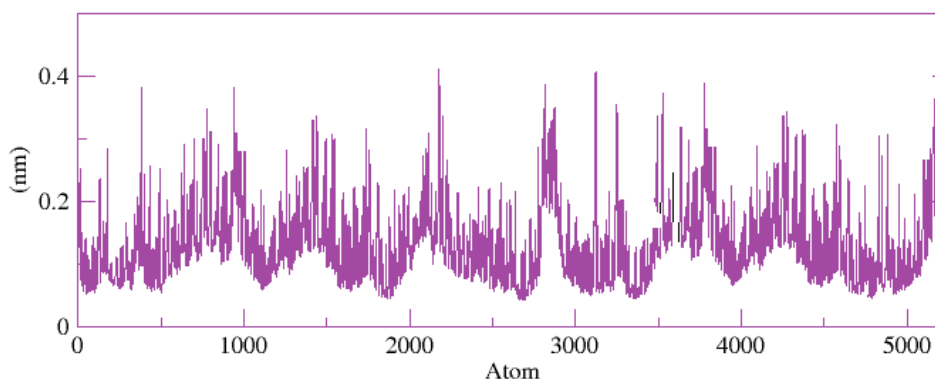


Figure 5: RMSF analysis of protein-ligand complex.

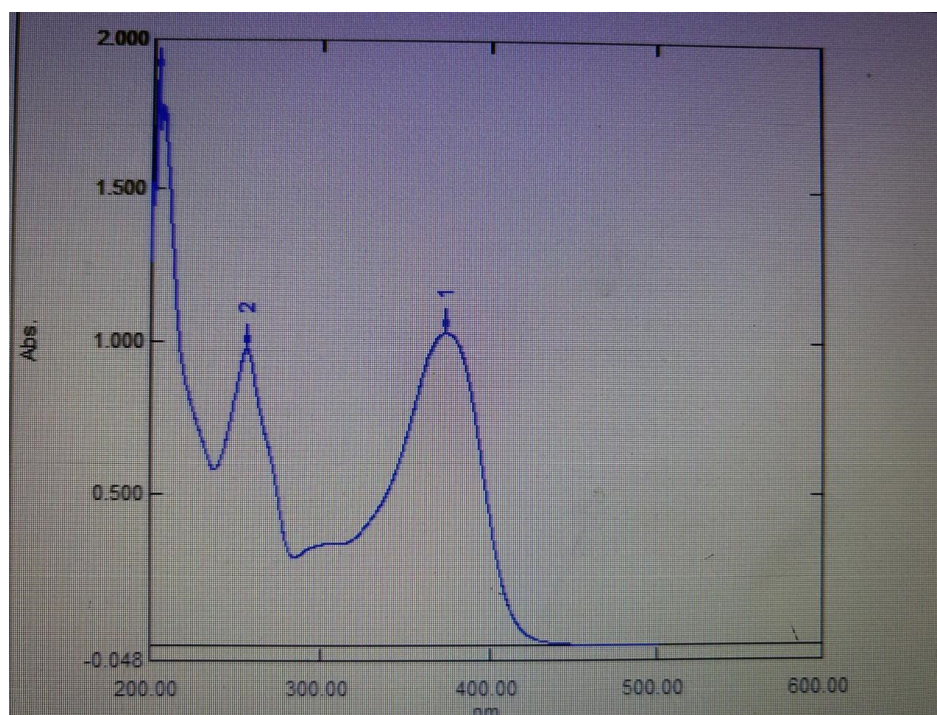


Figure 6: Graph showing absorbance range of Quercetin in the leaves of Guava.

The extract gave the antimalarial activity from the concentration of 0.25 mg and was gradually increased to concentrations of 0.5 mg, 1 mg and 2 mg. The increase in the concentration of the extract showed enhanced potency to inhibit β -haematin as mentioned in Table 5 and Figure 7. The incubation period was of

12 hr and was done for 3 independent individual experiments and the average of 3 experiments was considered. The observed values should at least give the half maximum inhibitory concentration in order to have positive inhibitory function. The extract showed antimalarial activity in a dose-dependent manner with the

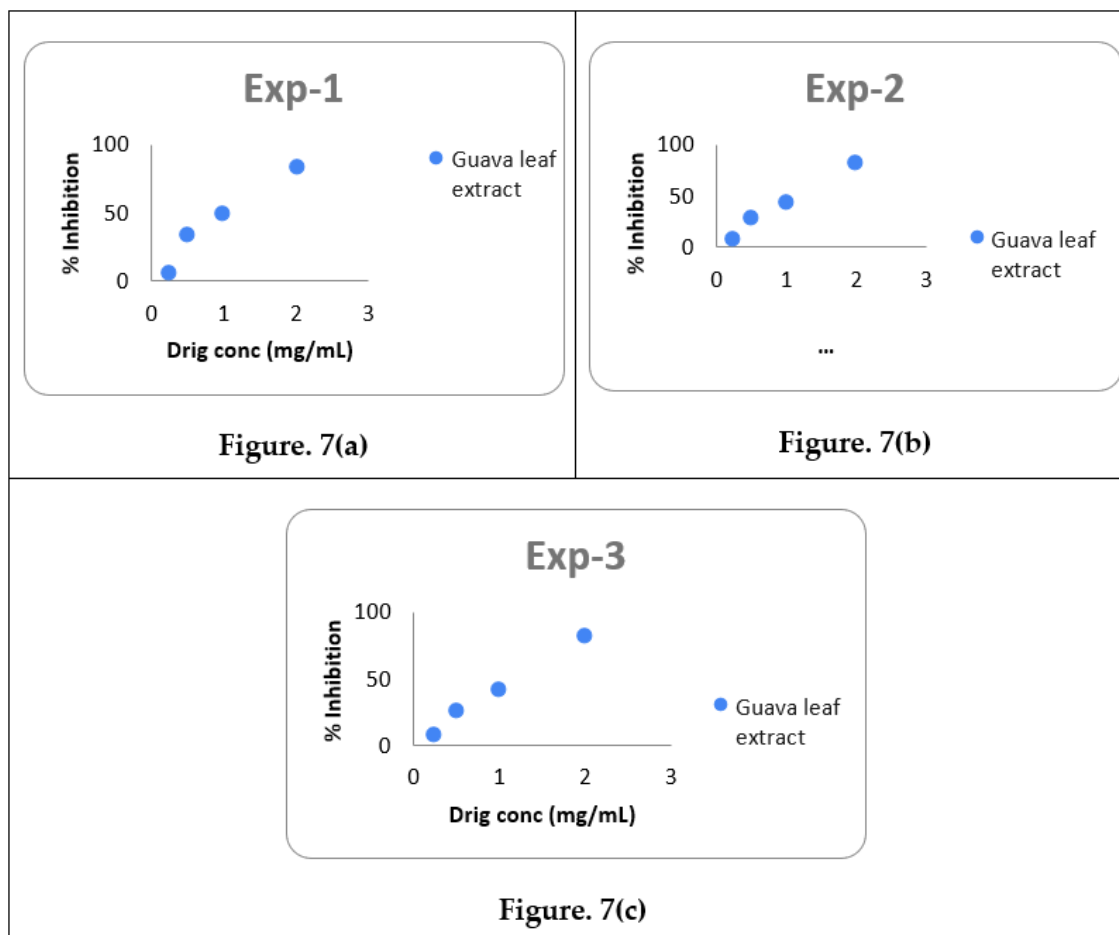


Figure 7: Graphical representation of triplicate values of the extract.

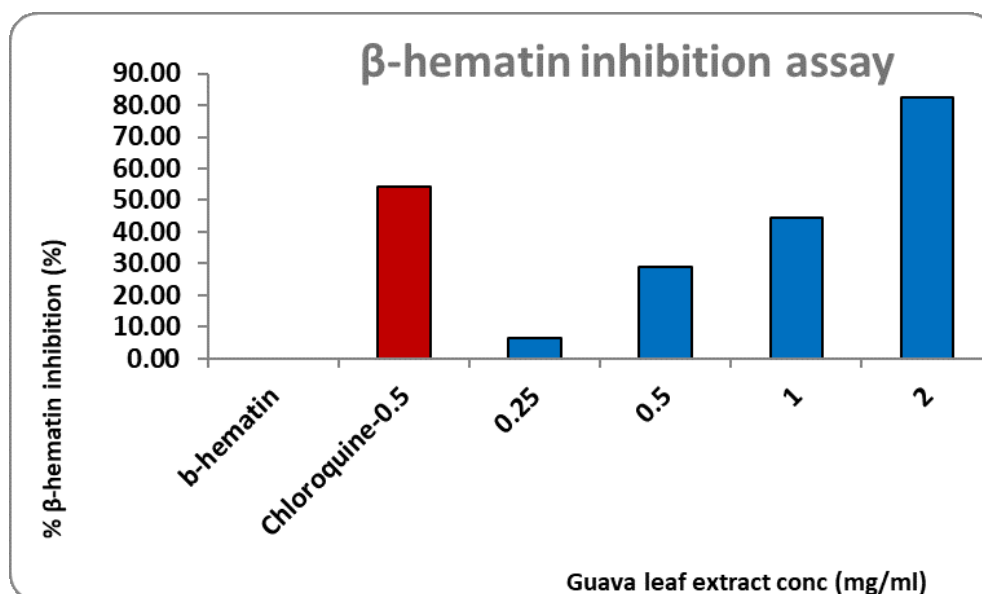


Figure 8: Bar graph representing the β -haematin inhibition by Guava leaf extract.

maximum dose administered demonstrating the highest activity as given in Table 6 and Figure 8.

From the provided data we concluded that chloroquine at 0.5 mg concentration gave 54.18% (± 1.71) inhibition score as a

standard concentration and the concentration of the extract will be increased till it reaches the optimum β -haematin inhibition score. The extract at concentrations of 0.25 mg, 0.5 mg, 1 mg, 2 mg gives 6.54 (± 1.31) %, 29.03 (± 4.22) %, 44.41 (± 4.06) % and 82.32 (± 0.61) % of beta hematin inhibition respectively. When

Table 3: ADME profile of phytoconstituents.

Ligand	Chemical formula	Swiss ADME Filters							
NA	NA	Molar mass (g/mol)	H bond acceptor	H bond donor	iLogP	Mol. Refractivity	GI absorption	TPSA ($\text{\AA}^2$)	Drug-like ness
Nimbolide	$\text{C}_{27}\text{H}_{30}\text{O}_7$	466.20	7	0	2.57	119.38	high	92.04	Yes
α -pinene	$\text{C}_{10}\text{H}_{16}$	136.13	0	0	4.49	43.75	high	0	Yes
Thymol	$\text{C}_{10}\text{H}_{14}\text{O}$	150.10	1	1	3.43	150.22	high	20.23	Yes
Kaempferol	$\text{C}_{15}\text{H}_{10}\text{O}_6$	286.05	6	4	1.61	74.57	high	111.13	Yes
Quercetin	$\text{C}_{15}\text{H}_{10}\text{O}_7$	302.04	7	5	1.19	76.24	high	131.36	Yes
Morin	$\text{C}_{15}\text{H}_{10}\text{O}_7$	302.04	7	5	1.64	76.24	high	131.36	Yes
Catechin	$\text{C}_{15}\text{H}_{14}\text{O}_6$	290.08	6	5	1.53	72.62	high	110.38	Yes
Quercitrin	$\text{C}_{21}\text{H}_{20}\text{O}_{11}$	448.10	11	7	0.32	107.56	high	190.28	Yes
Piperine	$\text{C}_{17}\text{H}_{19}\text{NO}_3$	285.14	3	0	3.47	81.17	high	38.77	Yes

Table 4: LD₅₀ and toxicity class predicted by Protox-II for the phytoconstituents.

Ligand name	LD ₅₀ mg/kg	Toxicity class
Nimbolide	1000	4
α -pinene	3700	5
Thymol	640	4
Kaempferol	3919	5
Quercetin	159	3
Morin	3919	5
Catechin	10000	6
Quercitrin	5000	5
Piperine	330	4

Table 5: The observed absorbance reading with the calculations are given below.

Average of 3 experiments							
Experiment	β -hematin	Chloroquine (mg/mL)	Guava leaf extract (mg/mL)				IC ₅₀ (mg/mL)
		0.5	0.25	0.5	1	2	
Exp-1	0.00	53.26	5.08	33.78	48.81	82.90	1.11
Exp-2	0.00	53.13	7.62	27.56	43.63	82.36	1.17
Exp-3	0.00	56.15	6.92	25.74	40.79	81.69	1.21
Average	0.00	54.18	6.54	29.03	44.41	82.32	1.16
SD	0	1.7083654	1.312883	4.218644	4.063772	0.60754	0.0503
Std error	0	0.9863252	0.757993	2.435635	2.346220	0.35076	0.0290
Average $\pm$ SD	0	54.18 $\pm$ 1.71	6.54 $\pm$ 1.31	29.03 $\pm$ 4.22	44.41 $\pm$ 4.06	82.32 $\pm$ 0.61	1.16 $\pm$ 0.05

Table 6: % β -Haematin inhibition values of the extract.

Condition	% β -haematin inhibition	IC ₅₀ Conc (mg/mL)*
β -haematin	0	1.16 $\pm$ 0.05
Chloroquine-0.5 mg	54.18 $\pm$ 1.71	
Extract-0.25 mg	6.54 $\pm$ 1.31	
Extract-0.5 mg	29.03 $\pm$ 4.22	
Extract-1 mg	44.41 $\pm$ 4.06	
Extract-2 mg	82.32 $\pm$ 0.61	

*The presented values were the average of 3 independent individual experiments.

at IC₅₀ concentration of 1.16 mg/mL of Guava leaf extract is considered as the expected concentration to act as a β -hematin inhibitor compared to chloroquine.

CONCLUSION

The present study demonstrated the *in silico* and *in vitro* studies of selected leaves as a prospective in malarial treatment. From the docking studies we gather that out of all the 9 compounds screened, Quercetin, the chief constituent of *Psidium guajava* showed better docking scores, with the stability of the protein-ligand complex confirmed by Molecular Dynamic Simulation and hence the plant extract of guava has been studied for its β -haematin inhibition activity. Results of the given test compound showed promising results of β -haematin inhibition activity on dose dependent manner with IC₅₀ concentration of 1.16 $\pm$ 0.05 mg/mL. Percentage of inhibition of the guava leaf extract ranged from 6.54 $\pm$ 1.31% to 82.32 $\pm$ 0.61% with chloroquine phosphate used as the positive standard control for the current study. In summary the obtained results are directly proportional to the anti-malarial efficacy of guava leaf extract and showed satisfactory antimalarial effect invitro. These findings further validate the traditional use of various medicinal plants in the treatment of malaria. The interaction-based structural models at the Enoyl-acyl carrier protein reductase active site (1VRW) can be used for future design of antimalarial drugs.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

WHO: World Health Organization; **3D structure:** 3-Dimensional structure; **RMSD:** Root Mean Square Deviation; **RMSF:** Root Mean Square Fluctuation; **IC₅₀:** Half-maximal inhibitory concentration.

SUMMARY

Malaria remains a serious parasitic disease, especially in tropical regions, with increasing resistance to current treatments. This study focused on identifying potential anti-malarial agents from natural plant sources, particularly guava (*Psidium guajava*) leaves. Computational screening, including molecular docking and dynamic simulations, was conducted on ten plant-derived compounds targeting the *Enoyl-acyl carrier protein reductase* enzyme. Quercetin showed the strongest binding affinity (-147.48 Kcal/mol), confirmed by stable molecular dynamics simulations and favorable ADMET profiles. *In vitro* β -haematin inhibition assays further supported its anti-malarial potential. The findings suggest that quercetin from guava leaves is a promising lead compound, warranting further pre-clinical studies.

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Computational and Experimental Evaluation of Phytoconstituents Isolated from *Cassia* Species Leaves: Pharmacokinetic Predictions, Molecular Docking, and *in vitro* Assessment

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ABSTRACT

Aim/Background: This study investigates the phytochemical and pharmacological properties of *Cassia* species (Fabaceae) to assess its antiasthmatic potential as reported in traditional medicinal literature. **Materials and Methods:** Extracts from *Cassia* leaves were subjected to preliminary phytochemical screening to identify bioactive compounds. High-Performance Thin Layer Chromatography (HPTLC) was performed to confirm the presence of key phytoconstituents. *In silico* pharmacokinetic prediction and molecular docking were conducted for selected compounds. The antihistaminic activity of petroleum ether and methanol extracts was evaluated using an *in vitro* goat tracheal chain model. **Results:** Petroleum ether extract primarily contained steroids, triterpenoids, and carbohydrates, while the methanol extract exhibited the presence of saponins, anthraquinones, alkaloids, flavonoids, tannins, and phenolic compounds. HPTLC analysis verified the existence of gallic acid, quercetin, and resveratrol. Computational studies indicated that selected phytoconstituents exhibit properties suitable for drug development. The methanol extract significantly reduced histamine-induced contraction in goat tracheal chain assays. **Conclusion:** This research provides substantial evidence supporting the antiasthmatic efficacy of *Cassia* species through phytochemical characterization, computational modeling, and *in vitro* pharmacological evaluation.

Keywords: *Cassia*, Antiasthma Activity, *In silico* Pharmacokinetic Prediction, Molecular Docking, *In vitro* study.

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INTRODUCTION

Medicinal plants play a vital role in healthcare systems worldwide, with the World Health Organization reporting that more than 80% of the global population depends on remedies derived from plants.¹ India, known for its vast herbal resources, has extensively used plant-based medicine for therapeutic purposes. Phytoconstituents derived from herbal sources have demonstrated potential in modern drug discovery.²

Various species of the *Cassia* genus, commonly found in tropical and subtropical regions, have a long history of traditional use in managing ailments such as fever and respiratory disorders.³

Literature reports indicate that *Cassia* species contain anthraquinones,⁴ mucilage,⁵ flavonoids, and polysaccharides. However, limited research has been conducted on some species, necessitating further exploration of their bioactive components and pharmacological effects. This study focuses on the phytochemical and pharmacological evaluation of *Cassia siamea*, particularly its potential for treating asthma and allergic conditions.

Cassia siamea is native to Asia, as illustrated by the aerial parts shown in Figure 1. The African and Asian populations used to make decoctions of the leaves of this plant along with lemon juice as home remedies to treat malaria and liver diseases.⁶ This decoction was used in a dose of 0.5 L to alleviate cough, stomach ache, and malarial fever (0.5 L in morning and night)⁷ in Côte d'Ivoire. In India, combination therapy of leaf decoction with honey is consumed (150 mL, in morning, afternoon and night



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to cure anemia.⁸ Other authors reported that *Cassia siamea* leaf decoction can act as a laxative and antihypertensive.^{9,10}

As per a literature survey, Ethnobotanical study states use of leaf decoction of *Cassia siamea* in treatment of fever, cough and cold,^{10,11} but this plant was not evaluated for Antihistaminic and Antiasthmatic activity. The *Cassia siamea* leaves were conventionally used as a home remedy for malaria, liver disorders, fever, and cold in African and Asian populations underscores the need for scientific validation of its therapeutic properties.

In this research work, *Cassia siamea* was evaluated using phytochemical, pharmacological, and computational methods of analysis. Phytochemical evaluation of pharmacologically active extracts is essential for identifying the bioactive phytoconstituents responsible for these effects. The separation, identification, and quantification of these compounds are crucial steps in establishing the plant's pharmacological profile. Additionally, computational studies can be employed to assess the binding affinity of these compounds with target receptors, supporting predictions of their potential efficacy as antihistaminic and antiasthmatic agents. The antihistaminic activity can also be assessed *in vitro* using an isolated goat tracheal chain preparation, which provides a basis for understanding its potential antiasthmatic effects and helps validate the ethnobotanical claims associated with this plant.

Our research findings will contribute to a better understanding of the pharmacological basis for the traditional use of this plant in fever and cough.

MATERIALS AND METHODS

Plant collection, preparation and extraction

Cassia siamea samples were collected from Nashik, Maharashtra, and authenticated at the Botanical Survey of India, Pune (Voucher specimen no. BSI/WRCIDEN.CER/2018/112/89). The leaves were cleaned, dried, and pulverized prior to undergoing sequential solvent extraction using petroleum ether, chloroform, and methanol via Soxhlet extraction.

Phytochemical analysis

Chemical tests were conducted to detect the presence of carbohydrates, proteins, steroids, alkaloids, flavonoids, and saponins.^{12,13} Fractions collected from *Cassia siamea* extracts were further purified by thin layer chromatography¹⁴ and subjected to HPTLC analysis for the identification of separated phytoconstituents. Following parameters were used for HPTLC analysis (Table 1).

Computational evaluation

Selected phytoconstituents, standard drugs like Dexamethasone and Chlorpheniramine were evaluated based on Lipinski's rule-of-five using SwissADME, Molinspiration, and Osiris DataWarrior tools.¹⁵ The docking interactions of these compounds

with histamine H1 receptors, glucocorticoid receptors, and Interleukin-13 (IL-13) receptors were analyzed using AutoDock Vina software.¹⁶ The natural ligand and crystalline water were withdrawn and then samples were subjected to docking experiments. The possible interactions, binding and stability were determined by applying Auto-Dock vina software.

The Three-Dimensional (3D) crystallographic structures of potential target i.e. H1 receptor (PDB code: 3rze),¹⁷ Glucocorticoid receptor¹⁸ (PDB code: 5ufs) & IL-13¹⁹⁻²¹ (PDB code: 5l6y) were taken from the Protein Database (PDB). For ligand generation the 3D structure of selected phytoconstituents were obtained from Pubchem in SDF format, and Biovia Discovery Studio visualizer (3.5) converted it to PDB format. Pymol software was used to transform the PDBQT files of selected proteins (by using previous PDB files as downloaded from the databank). With the help of a grid box, Autodock-vina was used to create nine configurations for each Protein-Ligand complex.

Pharmacological Evaluation

The antihistaminic activity was assessed using tracheal chains obtained from goats. Goat tracheal tissues were obtained from a slaughterhouse and suspended in a Krebs solution bath at about 37.5°C. Histamine dose-response curves (log M vs % Contraction) were recorded in the presence and absence of pet ether and methanol plant extracts.²²⁻²⁴

RESULTS AND DISCUSSION

Phytochemical evaluation

Preliminary tests confirmed the presence of bioactive compounds viz. Steroids and triterpenoids in trace amounts, while alkaloids, tannins, phenolic compounds, carbohydrates, and proteins were found in trace amounts in chloroform extract. Steroids, triterpenoids, saponins, anthraquinones, alkaloids, flavonoids, tannins, phenolic chemicals, carbohydrates, and proteins were found in modest amounts in a methanolic extract of *Cassia siamea* as per Table 2.

HPTLC analysis of extract revealed presence of rutin with $R_f = 0.2$, Gallic acid with $R_f = 0.6$, Resveratrol with $R_f = 0.8$ (Figure 2).

Computational Studies

To assess the drug-like properties of the identified phytoconstituents, their adherence to Lipinski's Rule of Five was evaluated. This rule posits that most orally active drugs exhibit: a MW below 500 daltons, a partition coefficient (log P) less than 5, NMT 5 hydrogen bond donors and NMT 10 hydrogen bond acceptors. Furthermore, TNRB and TPSA are also important factors affecting drug bioavailability.^{18,25}

For drug similarity experiments, all of the chosen phytoconstituents had log *p* values below 5, indicating that they adhere to rule mentioned above. A drug with outstanding oral bioavailability

Table 1: HPTLC Parameters for analysis of phytoconstituents.

Stationary Phase	Merck's silica gel 60 F 254 plate
Mobile Phase	Toluene: Ethyl acetate: Methanol: Formic acid (6:3:0.8:2.6 v/v)
Band width	6 mm
Development distance	85 mm
Saturation time	20 min
Scanning wavelength	305 nm

is produced by determining the molecule's flexibility based on TNRB. Rutin is the only chemical that has seven rotatable bonds. Because all of the chemicals studied have rotatable bonds less than 5, they are unlikely to have bioavailability issues. Other parameters like molecular weight, hydrogen donor sites and hydrogen bond acceptor sites were within the acceptance criteria of Lipinski's rule except for rutin.

The TPSA, which reflects the addition of the surface areas of atoms (typically O₂, N₂, S, and H), is related to the H-bonding capacity of a compound. TPSA is a valuable reflector of transport properties, including a drug's blood-brain barrier penetration, permeability in cells, intestinal absorption, as well as overall bioavailability. Molecules with a TPSA value exceeding 140 Å² are generally predicted to exhibit poor intestinal absorption.

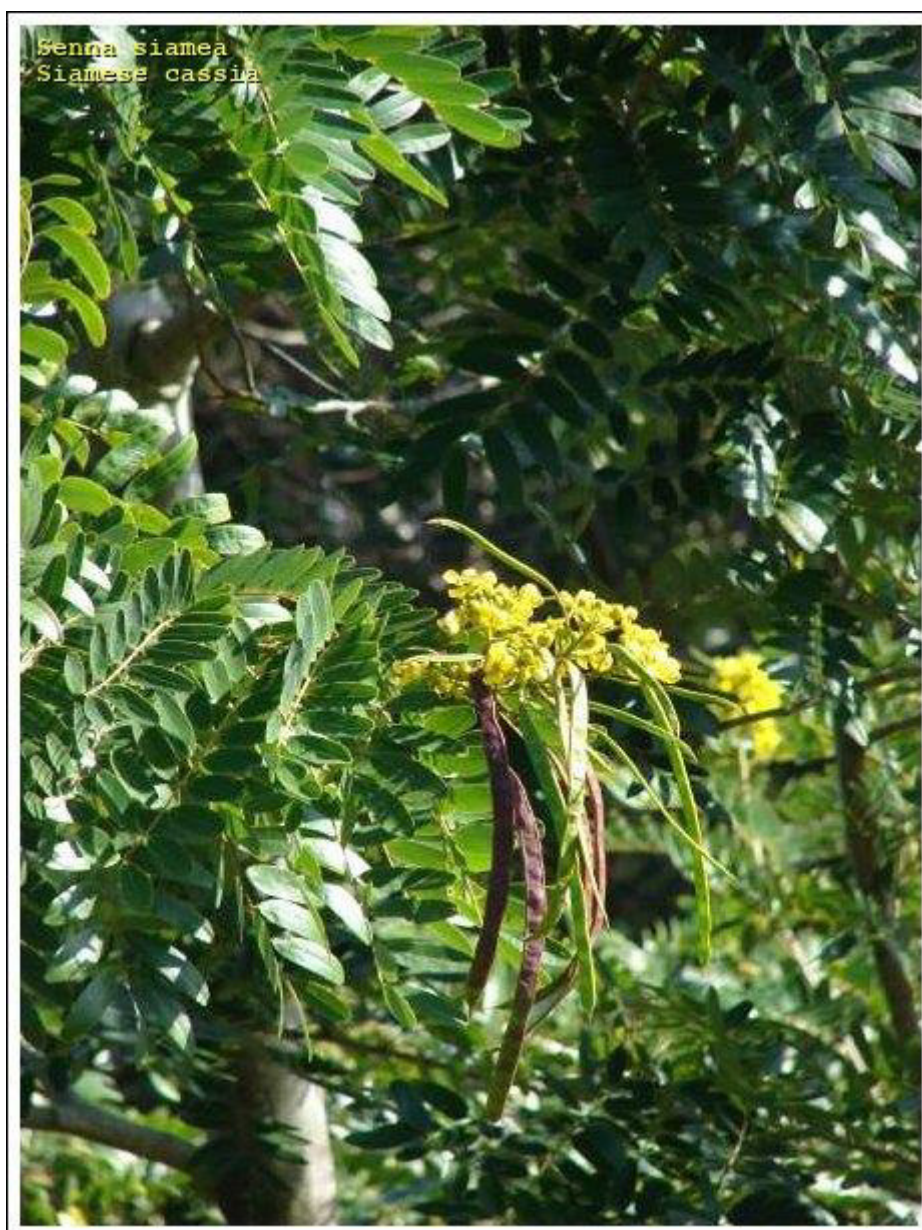
**Figure 1:** Aerial parts of *Cassia siamea*.

Table 2: Phytochemical evaluation of *Cassia siamea*.

Test for active constituents	Petroleum ether extract	Chloroform extract	Methanol extract
Steroids	+	-	+
Saponins	-	-	+
Alkaloids	-	+	++
Flavonoids	-	-	+
Glycosides	-	-	+
Phenolic compounds	-	+	++
Anthraquinones	+	+	+

Table 3: Physicochemical properties calculated based on the Lipinski's rule of 5.

Ligands	Mol. Wt. ≤ 500	Log P ≤ 5	No. of HBA ≤ 10	No. of HBD ≤ 5	TPSA
Resveratrol	228.08	2.048	3	3	60.69
Apigenin	270.05	1.138	5	3	90.9
Gallic acid	170.02	0.964	5	4	97.99
Quercetin	302.04	1.834	7	5	131.36
Rutin	610.15	0.735	16	10	269.43
Dexamethasone	392.2	1.138	5	3	94.83
Chlorpheniramine	274.12	1.366	2	0	16.13
Cetirizine	388.16	0.891	5	1	53.01

Table 4: Toxicity prediction of phytoconstituents.

Ligands	Mutagenicity	Tumorigenicity	Irritancy	Reproductive effects
Resveratrol	1	1	1	0.6
Quercetin	0.6	0.6	1	1
Rutin	1	1	1	1
Gallic acid	0.6	1	1	0.6
Apigenin	0.6	1	1	1
Dexamethasone	1	1	1	1
Cetirizine	1	1	1	1
Chlorpheniramine	1	1	1	1

Table 5: Bioactivity score of phytoconstituents.

Ligands	GPCR	Ion channel	Kinase inhibitor	Nuclear receptor	Protease inhibitor	Enzyme inhibitor
Resveratrol	-0.2	0.02	-0.2	0.01	-0.41	0.02
Quercetin	-0.06	-0.19	0.28	0.36	-0.25	0.28
Rutin	-0.05	-0.52	-0.14	-0.23	-0.07	0.12
Gallic acid	-0.77	-0.26	-0.88	-0.52	-0.94	-0.17
Apigenin	-0.07	-0.09	0.18	0.34	-0.25	0.26
Dexamethasone	0.03	-0.21	-0.81	1.59	0.76	0.78
Cetirizine	0.14	-0.03	-0.28	-0.13	-0.06	0.01
Chlorpheniramine	0.26	0.22	-0.01	-0.32	-0.09	0.15

According to these criteria, the results presented in Table 3 show that all tested phytoconstituents, with the exception of rutin, exhibit TPSA values below the threshold of 140 Å², suggesting efficient intestinal absorption.¹⁸

Drug distribution, largely governed by tissue diffusion and influenced by drug size and lipophilicity, is a critical determinant of drug action. Consequently, assessing permeability across the blood-brain barrier and gastrointestinal epithelial cells is essential for interpreting pharmacokinetic results and predicting how these compounds will behave in the body.^{18,26}

The toxicity risk predictor searches for fragments in a molecule that could signal toxicity.²⁷ Toxicity risk signals highlight that the isolated phytoconstituents may be hazardous to the risk category in question.¹⁸ As per Table 4 phytoconstituents were identified as non-irritant. Only resveratrol and gallic acid had shown toxicity to reproductive effects. Quercetin, gallic acid and

apigenin had shown mutagenic toxicity while quercetin had shown tumorigenicity.

Additionally, the BAS of the compounds (Table 5) for the therapeutic targets have been predicted. According to Paramashivam *et al.*, (2015), a molecule is considered to have high biological activity if its BAS is greater than 0.00, moderately active if BAS is between -0.50 and 0.00, and not active if BAS is less than -0.50.²⁸ All of the chosen phytoconstituents have demonstrated bioactivity scores more than -0.5 for enzyme inhibitors, ion channel modulators, and nuclear receptor ligands. Except for gallic acid, all GPCR ligands and protease inhibitors had scores greater than -0.5.

The ability of a medicine to permeate the intestinal barrier and reach systemic circulation is known as oral bioavailability (Table 6). P-gp (P-glycoprotein) belongs to the ABC transporters, which are among the numerous transporters found in the gut membrane. It can be found in the apical membrane of epithelial cells of the GI. It protects the body by preventing potentially harmful chemicals from entering the bloodstream from the intestine. Many medications (lopinavir, dexamethasone, etc.) are substrates for this transporter, limiting their absorption and reducing treatment efficacy.²⁹ Software demonstrated that all selected phytoconstituents were not shown as substrates of P-gp except rutin and dexamethasone (A conventional marketed drug evaluated computationally as a standard to compare with selected phytoconstituents), which may limit their intestinal permeation.

There are many CYP enzymes in humans, a limited number are primarily responsible for the metabolism of most drugs. Specifically, approximately 12 CYP enzymes (only a small number CYPs) (belonging to the CYP1, 2, and 3 families) are responsible for metabolizing a significant portion of commonly used drugs. Drug clearance and serum levels may change if these enzymes involved in drug metabolism are inhibited. A drug's or its active form's therapeutic efficacy may also vary. Therefore, concurrent use of a CYP3A4 inhibitor and a medication that is a substrate for the same isoenzyme may cause a significant rise in the drug's

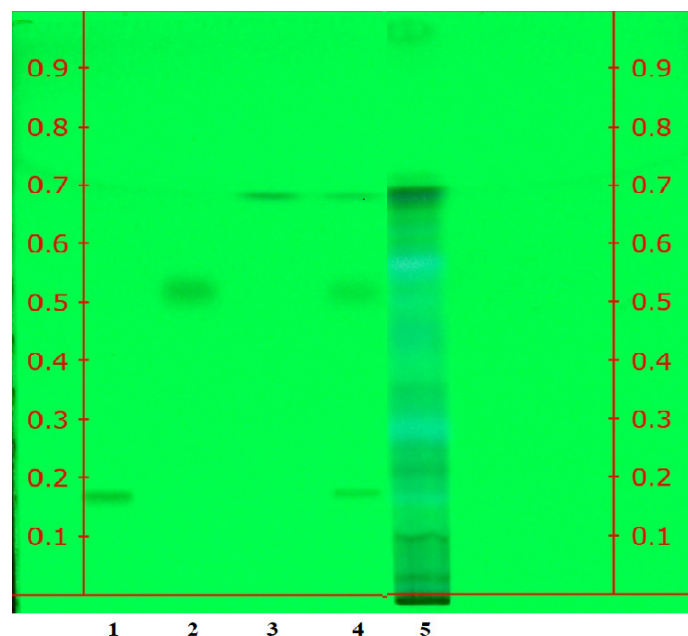


Figure 2: HPTLC analysis of *Cassia siamea* leaf extract (Band 1: Rutin, Band 2: Gallic acid, Band 3: Resveratrol, Band 4: Mixture of all 3 standards, Band 5: Extract).

Table 6: ADME profile.

Tin Nad Ligand	Silicos-IT class	GI absor-ption	BBB permea-tion	Bioavail -ability Score	Pgp	CYP1A2	CYP2C19	CYP2C9
Resveratrol	Soluble	High	Yes	0.55	No	Yes	No	Yes
Apigenin	Moderate	High	No	0.55	No	Yes	No	No
Gallic acid	Soluble	High	No	0.56	No	No	No	No
Quercetin	Soluble	High	No	0.55	No	Yes	No	No
Rutin	Soluble	Low	No	0.17	Yes	No	No	No
Dexametha-sone	Soluble	High	No	0.55	Yes	No	No	No
Chlorpheni-ramine	Poorly	High	Yes	0.55	No	No	No	No
Cetirizine	Moderate	High	Yes	0.55	Yes	No	No	No

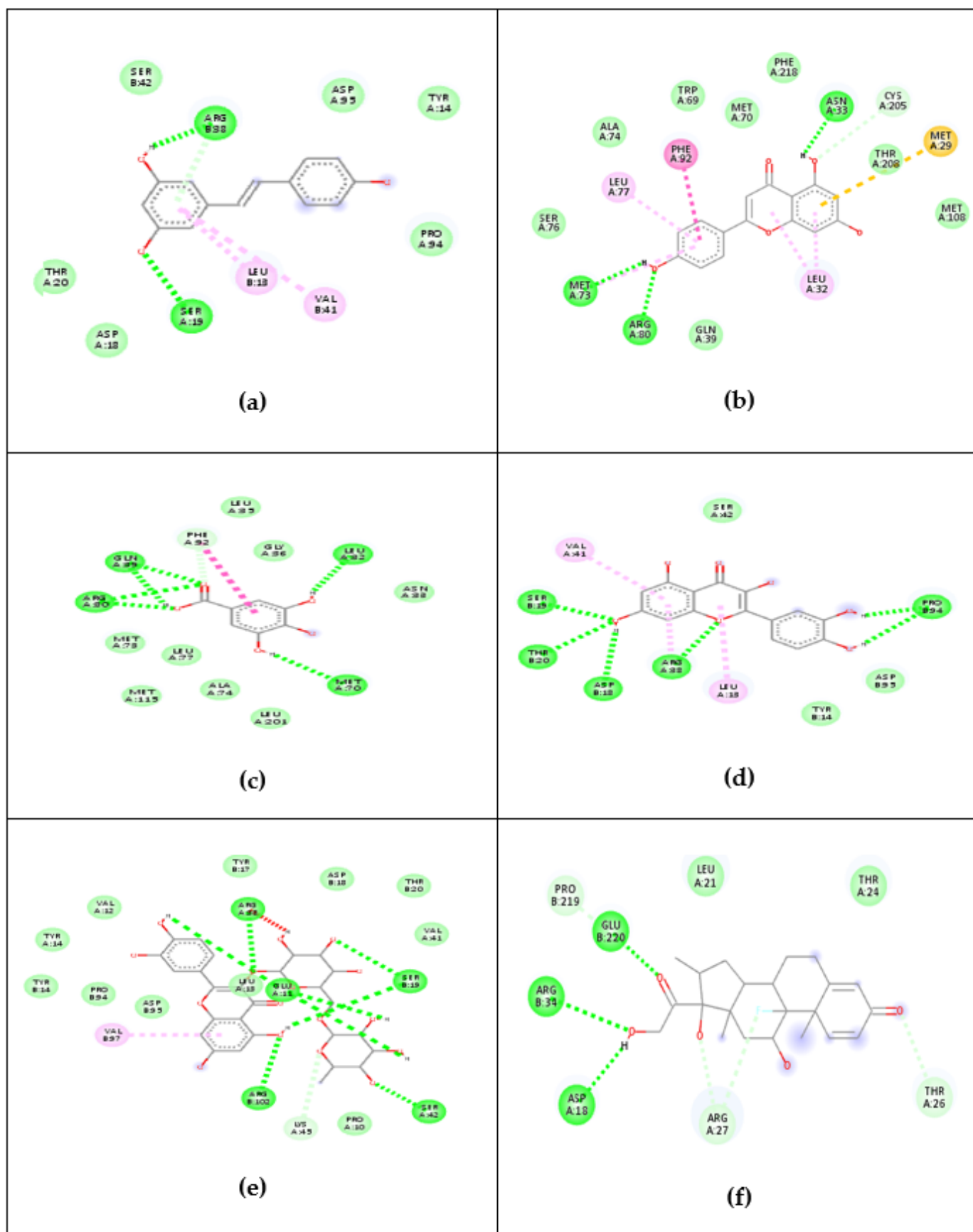


Figure 4: 2D Interaction of Phytoconstituents with Glucocorticoid Receptor with (a: Resveratrol, b: Apigenin, c: Gallic acid, d: Quercetin, e: Rutin, f: Dexamethasone).

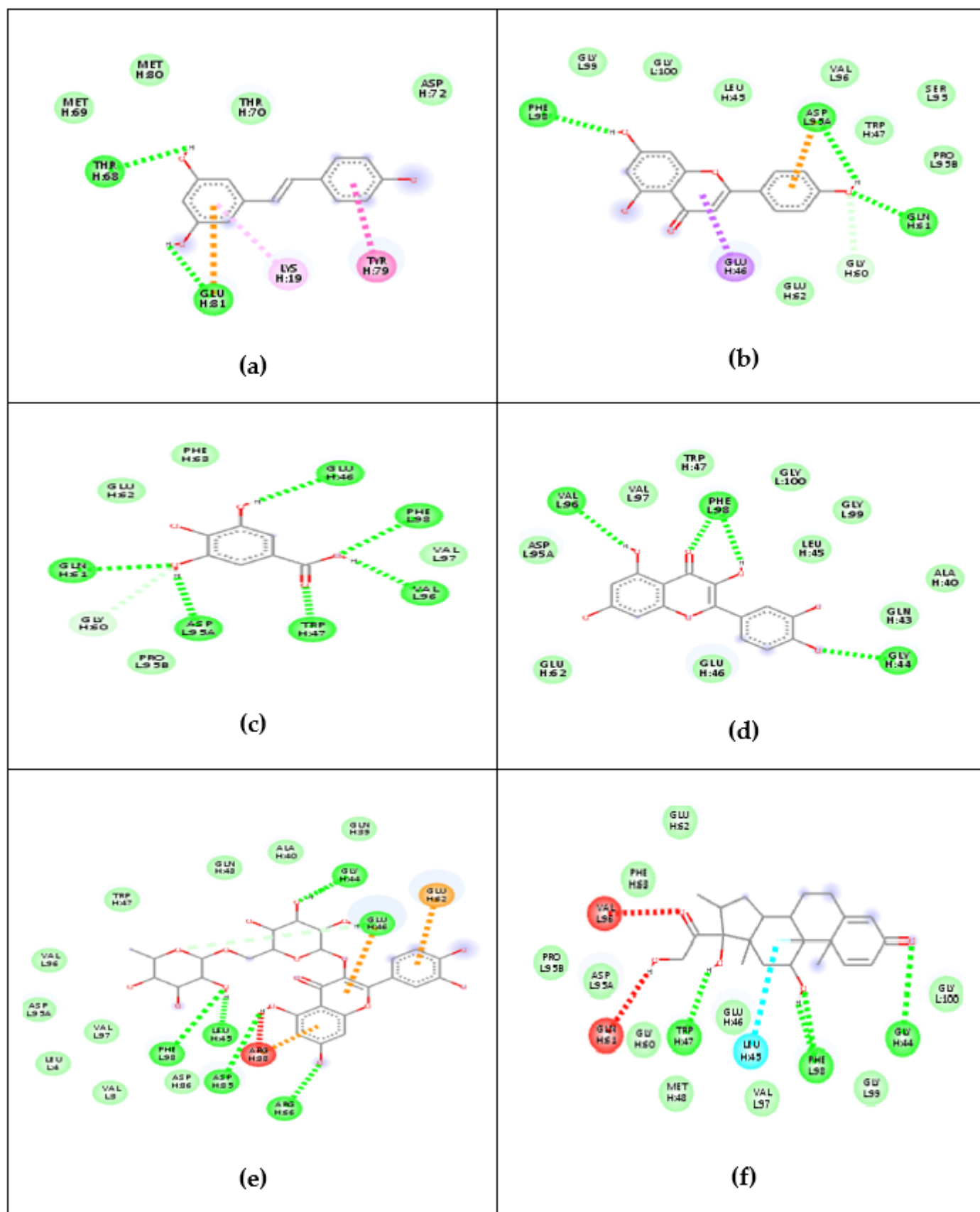


Figure 5: 2D Interaction of Phytoconstituents With IL-13 Protein Receptor with (a: Resveratrol, b: Apigenin, c: Gallic acid, d: Quercetin, e: Rutin, f: Dexamethasone).

Table 7: Analysis of molecular interaction of phytoconstituents with H-1 receptor.

Ligand	H-bond	Residues involved in H-bond interaction	Docking score
Resveratrol	7	A: LYS179, A: TYR108, A: PO41202, A: SER111, A: ASP107, A: TYR458, A: TYR431	-8.4
Apigenin	5	A: THR194, A: TYR431, A: TYR458, A: SER111, A: TYR431	-9.1
Gallic acid	5	A: TYR431, A: TYR431, A: THR112, A: ASP107, A: ASP107	-6.3
Quercetin	9	A: TYR431, A: TYR458, A: ILE454, A: ASP107, A: ASP107, A: LYS191, A: THR194, A: SER111, A: TYR431	-9.1
Rutin	2	A: ASN198, A: TRP158	-3.9
Dexamethasone	2	A: THR147, A: PRO202	-6.5
Cetrizine	4	A: THR194, A: LYS191, A: THR194, A: TYR108	-5.9

Table 8: Analysis of molecular interaction of phytoconstituents with Glucocorticoid receptor.

Ligand	H-bond	Residues involved in H-bond interaction	Docking score
Resveratrol	3	A: SER19, B: ARG38 B: ARG38	-7.6
Apigenin	4	A: ARG80, A: MET73, A: ASN33, A: CYS205	-8.2
Gallic acid	6	A: GLN39, A: ARG80, A: LEU32, A: GLN39, A: MET70, A: PHE92	-5.9
Quercetin	6	A: ARG38, B: SER19, B: THR20, B: PRO94, B: PRO94, B: ASP18	-7.1
Rutin	10	A: ARG38, A: SER42, B: SER19, B: ARG102, A: GLU11, A: GLU11, A: GLU11, B: SER19, A: LYS45, B: VAL97	-7.6
Dexamethasone	7	B: ARG34, B: GLU220, A: ASP18, A: THR26, A: ARG27, A: ARG27, B: PRO219	-6.7

Table 9: Analysis of molecular interaction of phytoconstituents with IL-13 protein.

Ligand	H-bond	Residues involved in H-bond interaction	Docking score
Resveratrol	2	H: GLU81, H: THR68	-6.4
Apigenin	4	H: GLN61, L: ASP95A, L: PHE98, H: GLY60	-7.0
Gallic acid	7	H: TRP47, H: GLN61, L: PHE98, L: ASP95A, L: VAL96, H: GLU46, H: GLY60	-6.1
Quercetin	4	H: GLY44, L: PHE98, L: PHE98, L: VAL96	-6.9
Rutin	8	H: GLY44, H: ARG66, L: PHE98, H: LEU45, H: ASP85, H: GLU46, H: GLY44, H: GLU46	-7.0
Dexamethasone	4	H: GLY44, L: PHE98, L: PHE98, H: TRP47	-8.3

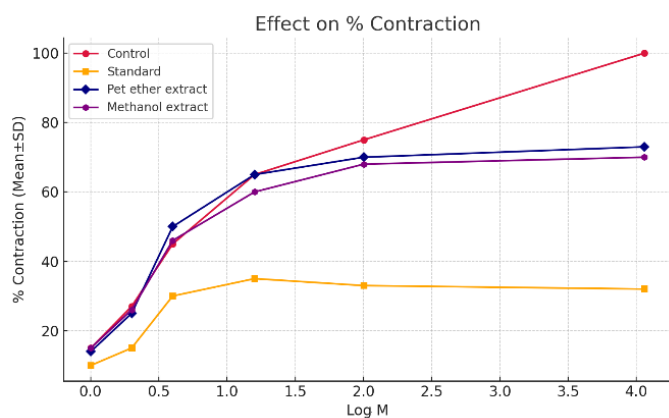


Figure 6: Pharmacological evaluation by using isolated goat tracheal chain preparations.

plasma concentration and, consequently, increased toxicity. As a result, phytoconstituents were tested to see if compounds with CYP inhibitors might block CYP in human hepatic microsomes. Phytoconstituents, including conventional medications, have been found to be CYP inhibitors.¹⁸

Molecular docking is a technique for projecting ligand binding mode within a target protein's binding site. For proper docking, protein enhancement and grid creation are critical processes. The Autodock MGL tool was used to refine the structure while water was removed and the H-bond was optimised. Water molecules were removed, other conformations were removed, and hydrogen was added to the protein.¹⁸

Autodock vina 4 was used to determine the docking scores of the examined phytoconstituents (Tables 7-9). It was observed that all compounds had positions in the receptor site, with two molecules scoring higher than the conventional medicines Dexamethasone and Cetrizine. Furthermore, analysis of the selected phytoconstituents and marketed compounds molecular docking-based interaction profiles indicated that they generated H-bond interactions with multiple amino acid residues of the selected target proteins (Figures 3-5). Most phytoconstituents and the target protein have similar amino acids, according to a three-dimensional docking model.¹⁸

Pharmacological investigation

Histamine induced a dose-dependent increase in the contractile response of the isolated goat tracheal chain. This is evidenced by the typical sigmoidal curve observed in the control group (Figure 6), where contraction increases with the log molar concentration (Log M) of histamine. This confirms the responsiveness of the tissue to histamine, likely mediated via H1 histamine receptors, which are known to stimulate smooth muscle contraction. Histamine is the primary mediator released during allergy and stimulates hyper-responsiveness. The effects of various spasmogens like Histamine, 5-HT, ACH etc. can be studied on this *in vitro* model.

When the tissue was pre-treated with the petroleum ether extract, the contractile response to histamine was slightly reduced, suggesting mild antihistaminic properties. However, this inhibition was not statistically significant. In contrast, pre-treatment with the methanol extract resulted in a significant inhibition of histamine-induced contraction ($p < 0.001$). This inhibition indicates a substantial antihistaminic effect, potentially through antagonism of histamine receptors. This effect is likely due to the presence of active phytoconstituents in the methanol extract, as suggested by our phytochemical analysis. methanol extract produces significant bronchodilation as compared to the control group (Figure 6).

CONCLUSION

Ethnobotanical studies have reported the use of *Cassia siamea* leaf decoctions in traditional medicine for the treatment of fever and cough.⁷ The present study offers phytochemical, computational, and pharmacological evidence supporting the biological activity of this plant. Phytochemical analysis using chromatographic techniques identified key bioactive constituents such as rutin, resveratrol, and gallic acid in *Cassia siamea*. Computational studies demonstrated that these phytoconstituents possess favorable pharmacokinetic properties and exhibit strong receptor-binding affinities in comparison to standard drugs like dexamethasone and chlorpheniramine. Furthermore, *in vitro* pharmacological evaluation showed significant inhibition of histamine-induced contractions in isolated goat tracheal chains. The findings of this study reinforce the therapeutic potential of traditional Indian medicinal practices and provide a foundation for future investigations into underlying molecular mechanisms, potentially contributing to the development of novel treatment strategies for asthma and allergic disorders.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

HPTLC: High-Performance Thin Layer Chromatography; **PDB:** Protein Data Bank; **3D:** Three-Dimensional; **MW:** Molecular weight; **NMT:** Not More Than; **TNRB:** Total Number of Rotatable Bonds; **TPSA:** Topological Polar Surface Area; **BAS:** Bio-Activity Score; **GPCR:** G-Protein-Coupled Receptor; **P-gp:** P-glycoprotein; **CYP:** Cytochrome P450; **GI:** Gastrointestinal; **SDF:** Structure Data File; **PDBQT:** Protein Data Bank, Partial Charges and Torsions; **ACH:** Acetylcholine; **5-HT:** 5-Hydroxytryptamine (Serotonin).

SUMMARY

This study investigates the antiasthmatic potential of *Cassia siamea*, guided by its traditional use for respiratory ailments. Researchers performed phytochemical screening, HPTLC analysis, *in silico* pharmacokinetic prediction, molecular docking, and *in vitro* goat tracheal chain assays. Phytochemical analysis using chromatographic techniques identified key bioactive constituents such as rutin, resveratrol, and gallic acid in *Cassia siamea*. Computational studies suggested that key phytoconstituents (rutin, quercetin, apigenin, resveratrol) have favorable drug-like properties and receptor-binding affinities. The *in vitro* results showed that methanolic extract significantly inhibited histamine-induced contraction, indicating antihistaminic activity. These findings support the traditional use of *Cassia siamea* for respiratory conditions and point to its potential as a source of antiasthmatic compounds.

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Meropenem Allergic Test for Cefoperazone Allergic Case: Understanding Cross-Reactivity among Beta-Lactam Antibiotics-A Case Report

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ABSTRACT

Objectives: This study aimed to assess cross-reactivity among beta-lactam antibiotics, with a focus on cephalosporins and carbapenems. This study also aimed to evaluate the safety of alternative antibiotics in individuals with cephalosporin allergies. **Case Study:** A 35-year-old male who had multiple right renal calculi and Autosomal Dominant Polycystic Kidney Disease (ADPKD) was scheduled for Percutaneous Nephrolithotomy (PCNL) surgery. The patient had no history of allergy to penicillin and was not on any concomitant medications before the procedure. Prior to the procedure, the patient received cefoperazone for surgical prophylaxis, which triggered an allergic response. Antibiotics were stopped, and the symptoms subsided after treatment with antihistamines and corticosteroids. Following surgery, a test dose of meropenem was administered to prevent postoperative infection. Within 2 hr, the patient experienced allergy, indicating an Immunoglobulin E (IgE)-mediated hypersensitivity reaction. Meropenem was discontinued and ciprofloxacin was introduced as an alternative prophylactic measure. **Analysis:** The patient demonstrated Type I hypersensitivity reactions to both cefoperazone and meropenem, suggesting cross-reactivity between cephalosporins and carbapenems. Ciprofloxacin was well tolerated for prophylaxis of post-operative infections without additional adverse effects. This case underscores the significance of allergy testing in the prevention of severe adverse drug reactions and emphasizes the necessity of personalized antibiotic selection for patients with a history of beta-lactam allergies. **Conclusion:** Despite its uncommon occurrence, this case emphasizes the clinical significance of cephalosporin-carbapenem cross-reactivity. To ensure safe antibiotic selection, patients with beta-lactam hypersensitivity should be evaluated for preoperative allergy testing.

Keywords: Renal calculi, Allergic Test, Cross-reactivity, Beta-lactam allergy, Prophylaxis.

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INTRODUCTION

Cephalosporins and carbapenems, which belong to the beta-lactam antibiotic family, are essential for preventing post-surgical infections due to their wide-ranging antibacterial effects.¹ These drugs are particularly useful in procedures where bacterial contamination is likely to be high.² Nevertheless, allergic reactions to beta-lactams present a significant medical issue, potentially restricting treatment options for patients who need infection prevention or treatment.³ Beta-lactam allergies are classified based on the immune system mechanisms involved. Type 1 hypersensitivity, or immediate allergic reactions, are triggered by an allergen that activates IgE antibodies on mast cells and basophils. The allergen then links with these IgE

molecules, causing histamine and other inflammatory substances to be released. This can lead to symptoms ranging from itching and swelling to bronchial constriction and anaphylaxis in severe cases. Type IV reactions, or delayed hypersensitivity responses, involve T-cell activation and can manifest as skin rashes, fever, or more serious conditions such as Stevens-Johnson syndrome.⁴ The potential for cross-reactivity between cephalosporins and carbapenems remains a concern, especially for healthcare providers treating patients with known cephalosporin allergies. While research indicates a low occurrence of cross-reactivity, real-life cases underscore the need for caution when switching between different types of beta-lactams.⁵ This research presents a case of cross-reactivity between cefoperazone and meropenem in a patient undergoing Percutaneous Nephrolithotomy (PCNL) and investigates alternative antibiotic choices to ensure safe and effective surgical infection prevention. The objective of this study was to assess the cross-reactivity among beta-lactam antibiotics, specifically cephalosporins and carbapenems, in patients who experienced allergic reactions.⁶ Comprehending



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this cross-reactivity is vital for patient safety and optimal antibiotic selection. Furthermore, the study evaluates the safety of alternative antibiotics for individuals allergic to cephalosporins, emphasizing the importance of identifying well-tolerated substitutes to avoid adverse drug reactions.⁷ This study highlights the importance of preoperative allergy testing and cautious antibiotic selection to reduce risks and enhance patient outcomes by providing clinical insights into antibiotic selection for surgical prophylaxis in patients with beta-lactam allergies.

CASE STUDY

A 35-year-old male patient with multiple right renal calculi and Autosomal Dominant Polycystic Kidney Disease (ADPKD) was prepared for PCNL surgery. The patient had no history of allergy to penicillin and was not on any concomitant medications before the procedure. The patient was administered an intravenous dose of cefoperazone as part of the standard preoperative protocol. Shortly thereafter, the patient experienced an allergic reaction, displaying periorbital edema, itching, and allergic rhinitis. Antibiotics were promptly stopped, and the symptoms subsided following treatment with antihistamines and corticosteroids. After surgery, a test dose of meropenem was administered to evaluate its effectiveness in preventing postoperative infection. Within 2 hr, the patient showed signs of mild hypotension and itching, indicating a possible IgE-mediated hypersensitivity reaction. This reaction was quickly resolved by discontinuing meropenem and administering an antihistamine. Subsequently, ciprofloxacin has been introduced as an alternative prophylactic antibiotic. The patient responded well to ciprofloxacin with no further allergic reactions or postoperative infections.

ANALYSIS

The phenomenon of cross-reactivity between cephalosporins and carbapenems is well-established, although uncommon. Despite the theoretical structural disparities between Cefoperazone and Meropenem, including differences in their fused rings and side chains, clinical observations have shown instances of cross-reactivity⁸ (Figures 1 and 2).

The shared beta-lactam core may still elicit an immune response in sensitized individuals, even though the likelihood is reduced because of differing stereochemistry and side chains (Tables 1 and 2).

The case report demonstrates that a patient experienced IgE-mediated hypersensitivity reaction to both antibiotics, indicating that structural differences do not entirely eliminate the risk.⁹ This emphasizes the intricacy of immune recognition, as even minor similarities can result in cross-reactivity in certain patients. Consequently, healthcare providers should be cautious and consider allergy testing before transitioning between cephalosporins and carbapenems in patients with allergies. Evaluating Adverse Drug Reactions (ADRs) and confirming the

probability of a specific drug causing hypersensitivity requires causality assessment. In this instance, the WHO-UMC criteria were employed to evaluate reactions to cefoperazone and meropenem. These assessments indicate a high likelihood that both antibiotics will trigger hypersensitivity reactions in patients.

Table 1: Core structure differences between Cefoperazone and Meropenem.

Feature	Cefoperazone (Cephalosporin)	Meropenem (Carbapenem)
Beta-Lactam Ring	Present	Present
Fused Ring System	Dihydrothiazine Ring (6-membered).	Pyrrolidine Ring (5-membered)
Sulphur Atom in Core	Present	Absent
Double Bond in Core	No	Yes (conjugated with the beta-lactam).

Table 2: Side chain differences between Cefoperazone and Meropenem.

Feature	Cefoperazone	Meropenem
R1 Side Chain (C-7 position)	Methoxy-imino group	Hydroxyethyl group
R2 Side Chain (C-3 position)	Piperazine group	Methyl group

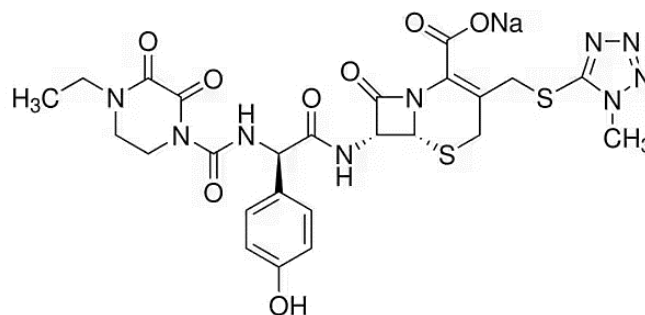


Figure 1: Chemical structure of Cefoperazone. (6R,7R)-7-[[[(2R)-2-(4-Ethyl-2,3-dioxo-1-piperazinyl)-2-(1H-tetrazol-1-yl)acetamido]-2-carboxy-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-3-carboxylic acid.

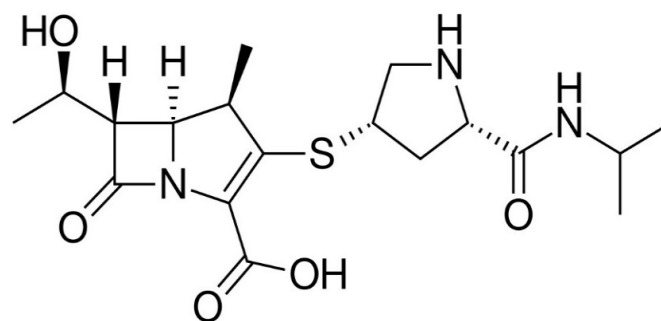


Figure 2: Chemical structure of Meropenem. (4R,5S,6S)-3-[[[(3S,5S)-5-(Dimethylcarbamoyl)pyrrolidin-3-yl]sulfanyl]-6-[(1R)-1-hydroxyethyl]-4-methyl-1-7-oxo-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid.

For patients exhibiting cross-reactivity between Cefoperazone and Meropenem, it is essential to choose an alternative antibiotic for surgical prophylaxis to prevent hypersensitivity reactions, while ensuring effective infection control. Fluoroquinolones (such as Ciprofloxacin and Levofloxacin) are appropriate alternatives due to their structural differences and immune recognition patterns, particularly for urinary tract or abdominal surgeries, given their broad-spectrum activity against gram-negative pathogens.¹⁰ Aminoglycosides (such as Amikacin and Gentamicin) can also be considered for their synergistic antibacterial effects, especially when combined with other agents.¹¹ In this case, ciprofloxacin was selected because of its broad-spectrum activity against gram-negative uropathogens and favorable safety profile in patients with beta-lactam allergy. When Gram-positive coverage is required, particularly for individuals susceptible to Methicillin-Resistant *Staphylococcus Aureus* (MRSA) infections, glycopeptides such as Vancomycin, Teicoplanin are preferred. Additionally, Clindamycin or Metronidazole may be included to address anaerobic bacteria when needed.¹² The selection of antibiotics should be guided by local resistance patterns, infection risk, and patient-specific factors, with allergy testing being recommended before considering any beta-lactam alternatives.

CONCLUSION

This report underscores the clinical relevance of cross-sensitivity between cephalosporins and carbapenems, although such occurrences are infrequent. The observed allergic reactions to both cefoperazone and meropenem underscores the necessity of conducting preoperative allergy assessments to ensure appropriate antibiotic selection. Furthermore, this case reinforces the importance of non-beta-lactam alternatives, such as fluoroquinolones in surgical prophylaxis for patients with known beta-lactam allergies. Subsequent studies should aim to enhance the cross-sensitivity risk classification and establish standardized protocols for antibiotic choice in allergic patients. Furthermore, implementing preoperative allergy screening protocols could reduce the incidence of adverse events while improving patient safety and surgical outcomes. Antibiotic allergies pose significant challenges in perioperative settings, necessitating a comprehensive approach for patient management and risk mitigation. Healthcare providers must carefully evaluate patients' allergy histories, considering both the reported allergies and potential cross-sensitivities between different antibiotic classes. This thorough assessment is crucial for developing individualized treatment plans that balance the need for effective antimicrobial prophylaxis with the risk of allergic reactions. When alternative antibiotics are required due to known allergies, it is critical to choose appropriate alternatives depending on the patient's individual allergy profile, the nature of the surgical treatment, and local antimicrobial resistance patterns. To guarantee the best possible patient outcomes, anesthesiologists, surgeons, and clinical pharmacists frequently collaborate during the

decision-making process. Even when administering alternative antibiotics, healthcare practitioners should continue to monitor patients for potential adverse reactions. This vigilance extends throughout the perioperative period from preoperative assessment to postoperative care. Prompt recognition and management of allergic reactions are critical to prevent severe complications and ensure patient safety. Continued research in the field of antibiotic cross-sensitivity is vital for expanding our understanding of allergic mechanisms and for improving risk stratification. This research should focus on identifying novel biomarkers for predicting cross-reactivity, developing more accurate diagnostic tools, and exploring new desensitization protocols for patients with multiple antibiotic allergies. Education plays a crucial role in enhancing healthcare providers' knowledge and skills in managing antibiotic allergies. Regular training sessions, workshops, and updates on current guidelines can help ensure that all members of the perioperative team are well-equipped to handle complex allergy cases. Additionally, patient education is essential for improving the accuracy of self-reported allergies and promoting adherence to alternative antibiotic regimens, when necessary. Implementing standardized protocols and decision support tools can further improve the perioperative management of antibiotic allergies. These tools can guide healthcare providers in the process of allergy assessment, alternative antibiotic selection, and monitoring for adverse reactions, thereby reducing the risk of errors and improving patient outcomes. Finally, fostering a culture of open communication and collaboration among different healthcare specialties is crucial for optimizing the care of patients with antibiotic allergies. This interdisciplinary approach ensures that all aspects of patient care are considered from preoperative planning to postoperative follow-up, leading to more comprehensive and effective management strategies. Additionally, even while administering alternative antibiotics, medical professionals should continue to closely monitor patients for any allergic reactions. Continued research and education on cross-sensitivity patterns and management strategies are essential for improving patient care and reducing the incidence of perioperative allergic reactions.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

FUNDING STATEMENT

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CASE STUDY

This work was carried out under research program of Department of Pharmacy Practice, MVM College of Pharmacy.

ABBREVIATIONS

ADPKD: Autosomal Dominant Polycystic Kidney Disease; **PCNL:** Per-cutaneous Nephrolithotomy; **IgE:** Immunoglobulin E; **ADR:** Adverse Drug Reaction; **WHO-UMC:** World Health Organisation -Uppsala Monitoring Centre; **MRSA:** Methicillin-resistant *Staphylococcus-aureus*.

ETHICAL COMPLIANCE

The 1964 Helsinki Declaration and its subsequent amendments, the institutional and/or national research committee's ethical requirements, and similar ethical criteria were all followed in this case study involving human participants. Informed consent was obtained from the patient.

AUTHOR CONTRIBUTIONS

Akash H Nataraj and Soumya Morabad contributed to the case data selection, observation, data collection and analysis. Rajeswari Ramasamy to the analysis of the case and to the writing of the manuscript.

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A Rare Case Report on Occupational and Life Style Habits Inveterate Induced Bethesda Category V, Papillary Thyroid Carcinoma in a 43-Year-Old Male

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ABSTRACT

Introduction: A male, 43 years old had a history of K/L/O chronic smoking, consuming one pack of cigarettes daily past 10 years this prolonged exposure to tobacco been the main reason for Papillary Thyroid Carcinoma (PTC) required a definitive thyroidectomy and lymph node dissection. **Objectives:** This case report aims to highlight the clinical, histopathological, and radiological aspects of Papillary Thyroid Carcinoma (PTC) with pulmonary metastases. It evaluates the role of FNAC and the Bethesda classification in diagnosing thyroid malignancies, particularly Category V lesions. The study assesses the effectiveness of total thyroid removal and RAI therapy while emphasizing the significance of immune histochemical markers in confirming metastasis. Additionally, it underscores the challenges of managing concurrent thyroid malignancies and the importance of a multidisciplinary approach for accurate diagnosis, treatment, and vigilant post-treatment monitoring to improve patient outcomes. **Methodology:** A retrospective study was conducted for 1 month using case report of inpatient. Histopathology revealed neoplastic cells positive for TTF-1, Napsin A, CK7, and thyroglobulin. Computed Tomography (CT) scans identified pulmonary nodules and a hypo dense left lobe nodule, indicative of PTC metastasis (Bethesda Category V). **Results:** Treating PTC, especially in concurrent conditions, is difficult because of the scarcity of guidelines. The cancer breached the thyroid capsule and spread to the left lung, necessitating radioactive-iodine treatment and recurrence monitoring. The potential co-occurrence of PTC with Bethesda Category V nodules warrants vigilant monitoring for additional diseases in cases with PTC. **Conclusion:** This case spots the need for a heightened clinician, radiologist, and pathologist awareness of simultaneous thyroid tumours and suspicion of malignancy to improve evaluations, diagnoses, and research contributions.

Keywords: Papillary thyroid carcinoma, Pulmonary metastases, Immunohistochemistry, Thyroid malignancy, Bethesda category V, Diagnostic approach.

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INTRODUCTION

The Thyroid cancer represents the incredibly prevalent endocrine malignancy, with DTC reason for over 90% of diagnosed cases. The escalating incidence of thyroid cancer, particular papillary carcinoma, has led to speculation regarding the potential influence of specific carcinogens in driving distinct molecular aberrations characteristic of PTC. This hypothesis is substantiated by the observed increase in BRAF (B-Raf proto-oncogene serine) mutations in PTC cases.¹ Papillary thyroid carcinoma is observed three times high over women than compared to men. In 851

patients diagnosed with PTC, 158 (19%) were male, 693 (81%) were female. the average age was 48 years, with a defined standard deviation.² TTF1 and Thyroglobulin immune histochemical staining are highly effective in confirming the diagnosis of PTC, especially in metastatic sites. PTC frequent type of thyroid cancer, involving more than 70% of all thyroid malicious.³ PTC predominantly high-flown in women when compared to men, in 2006 it was estimated to be among the top seven most frequently diagnosed cancers in women, comprising 3% of all female's cancers, while accounting for around 1% of cancers in men.⁴ The highest standardized mortality rate for males was seen in south East Asia (0.8%) west Asia (0.7%) per 100000 respectively.⁵ As per WHO classification in latest 5th edition of endocr and neuroendocrine tumors released at online beta version in march 2022.⁶ The retrospective study identified in 1104 case who undergone FNA later thyroidectomy by surgeon's part of surgical oncology and endocrine surgery from 2009 to 2016. The Bethesda



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System for Reporting Thyroid Cytology (TBSRTC) was initiated in 2007.⁷ As FNA procedure is an old for examining patients with thyroid nodules it was in use for more than 50 years. Total classes of Bethesda category v are suspicious v164 (3.7%), suspicious N(55(1.2%), and suspicious of PTC 109 (2.5%).⁸ Tumors duplicated outside neck in patients most repeatedly in lungs that ensures death of about half the patients rates were reduced when it was detected prior by radio iodine scan other than clinical hint. This ensures top action for most patient with diffetiated TC almost total thyroidectomy continued RAI attrition of the thyroid that looks reduction in the recurrence rate and the increase in survival rate by continuous follow-up.⁹ Men was notably correlated with perilous to suspicious of malignancy cases aged less than 47 odd ratios 1.38%.¹⁰ The histopathological diagnosis showed 823 patients 73(4.73%) suspicious of malignancy 607 (39.34%) benign.¹¹

MATERIALS AND METHODS

A retrospective case study was carried out in an inpatient setting of a tertiary hospital in Bangalore, India, after obtaining approval from the institutional ethical committee. The study was conducted for 1 month. The data sources required for the source of data were collected from the hospital medical records

(prescribing medications, diagnosis report, case sheets, laboratory investigations, medication charts).

CASE REPORT

A male, 43 years old had a history of K/L/O chronic smoking, consuming one pack of cigarettes (20 cigarette) daily for past 10 years. Prior to this cigarette habit first five years he had habit of bidi smoking, with daily consumption of one full pack. This prolonged exposure to tobacco have played a pivotal role in the onset of symptoms presented with unexplained cachexia, persistent refractory paroxysmal cough, and dyspnoea for one month. CT tomography revealed numerous nodular opacities in the lung, suggesting metastasis. HRCT confirmed multiple 5-15 mm pulmonary nodules without cavitation, calcification, pleural effusion, or significant mediastinal lymph nodes. A thyroid examination revealed a normal right lobe and a benign 10×11 mm hypo attenuating nodule in the left lobe. Oncologist consultations suggested thyroid carcinoma. Lung biopsy (Figure 1) using IHC indicated metastatic carcinoma from the thyroid, confirmed by positive TTF1, Napsin A, CK7, and thyroglobulin markers, and negative p40 and CK20. FNAC of the thyroid lesion revealed nuclear abnormalities (Bethesda category V), indicating a stage 4 papillary carcinoma treatment involving thyroidectomy and radioiodine therapy. Fifteen days later, thyroidectomy was



Figure 1: Digital X-ray of the chest in a Posterior Anterior (PA).

performed, followed by a week of radioiodine therapy. Postsurgical (Figure 2) Radioactive Iodine Uptake (RAIU), PET-CT showed no additional metastases. The subject was initiated with LT4 (100 µg/day) and planned in order to systematic follow-up, including open neck ultrasonography and serum FT3, TG, FT4, TSH, and calcitonin monitoring. Follow-up investigations showed no abnormalities on neck ultrasonography after nuclear therapy this Radioactive Iodine Uptake (RAIU) (Figure 3) post therapy scan report it has shown its effect and removed the dead cells

but the spread ratio was not decreased and calcitonin levels. The physician's thorough evaluation considered the slow progression of the ailment based on the symptomatology and assessments.

DISCUSSION

We reported a rare case of Papillary Thyroid Carcinoma (PTC, Bethesda Category V) coexisting with thyroid carcinoma, highlighting the necessity for heedfulness in identifying various cancers. Imaging suggested that PTC in left lobe, with immune

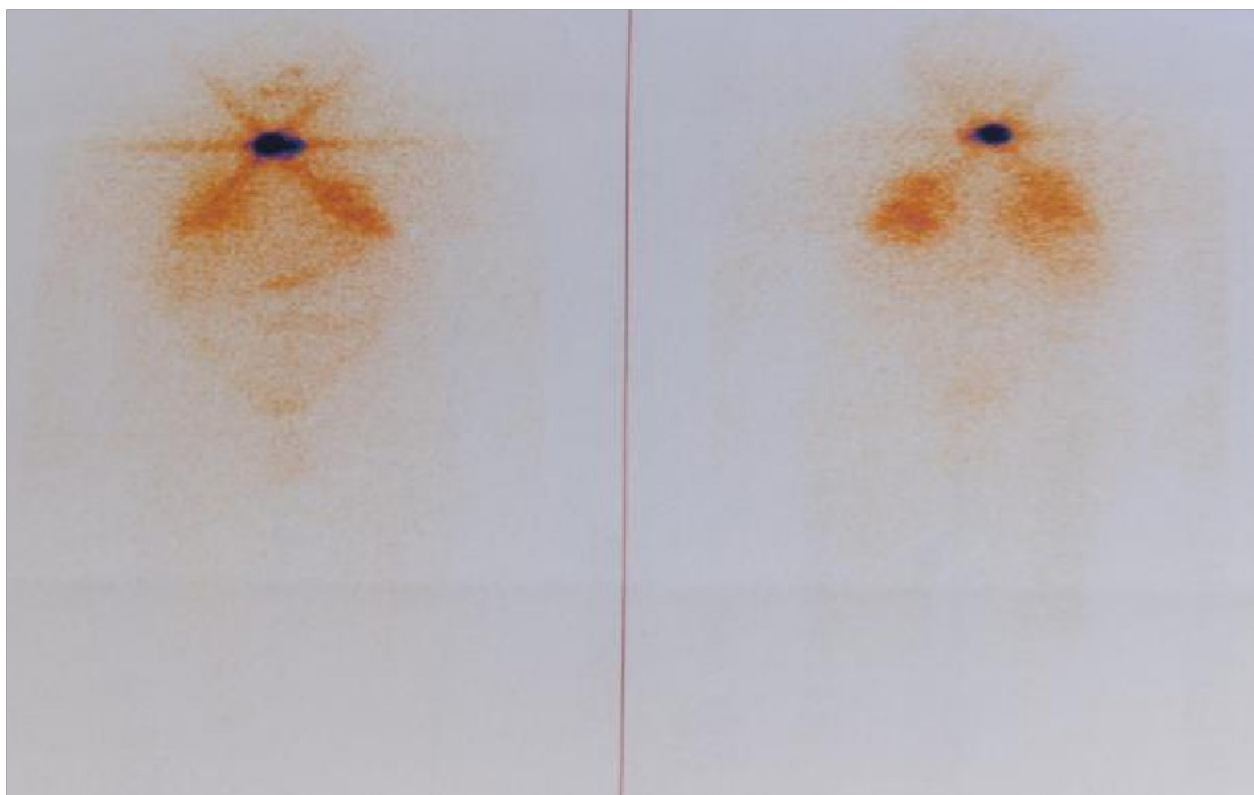


Figure 2: Radioactive Iodine Uptake (RAIU) Post Therapy (1) 18th May, 2024.

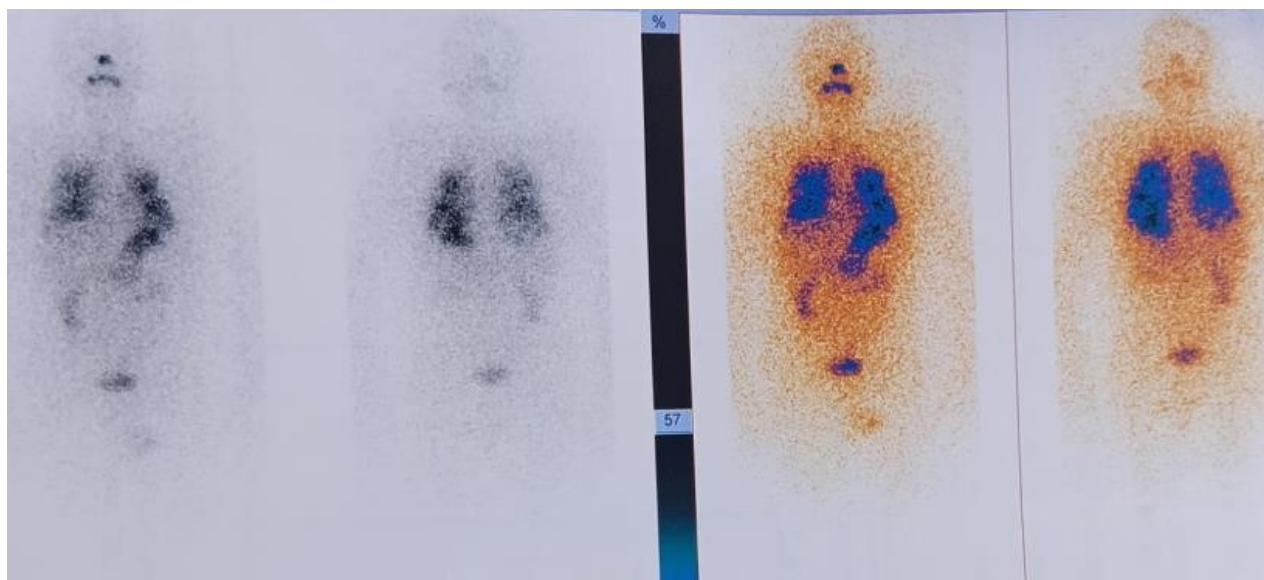


Figure 3: Radioactive Iodine Uptake (RAIU) Post Therapy (2) 27th December, 2024.

histochemical staining reveal tumour cells positive to TTF-I and CT negative for thyroglobulin. The left lobe nodule was identified as classical PTC, alongside simultaneous metastatic carcinoma and SLL in lymph nodes.

Parallel diagnoses of PTC and thyroid carcinoma are scarce, accounting for less than 3% of thyroid tumors. Our case presented a typical PTC pattern, with the right lobe's nodule diagnosed as low-grade classical PTC. Various hypotheses explain thyroid collision tumors, including tumor-induced tumor theory and common stem cell origin theories.

Treatment strategies for concurrent PTC and thyroid carcinoma are not standardized, with some recent studies revealing that separate treatments and others favoring targeting the most aggressive tumor.

Monitoring calcitonin and thyroid globulin levels for tumor recurrence is recommended; in our case, the PTC seized the capsule of the left lobe and disperse widely, leading to radioactive iodine therapy consideration, though the potential negative impacts were acknowledged.

The follow-up revealed no lymphoma progression after six months, and treatment strategies focusing on tumor stage and condition are necessary. However, an ideal approach would be to optimize treatment for both tumors. Boundary of this case include the lack of similar studies and a relatively short follow-up period with plans for long-term monitoring.

In this study of categories V which was 100% each after addition of papillary thyroid carcinoma. This study was able to accurately predict the range of motion for suspicious for malignancy.

CONCLUSION

Early detection and timely management are key to preventing the spread of PTC, especially with Bethesda Category V cases, which have a dangerous of malignancy. This case highlights importance of regular thyroid check-ups, especially for those with lifestyle or occupational risk factors, to catch abnormalities before they worsen. Simple precautions like routine ultrasound scans, FNAC testing for suspicious nodules, and monitoring thyroid-related biomarkers can help in early diagnosis and treatment. Promoting awareness, healthy lifestyle choices, and regular screenings can reduce the chances of late-stage thyroid cancer. A team-based approach among doctors, radiologists, and pathologists ensures better care, early treatment, and improved recovery.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

PTC: Papillary Thyroid Carcinoma; **CT:** Computed Tomography; **TTF-1:** Thyroid Transcription Factor-1; **IHC:** Immunohistochemistry; **CK7:** Cytokeratin 7; **FNAC:** Fine Needle Aspiration Cytology; **RAIU:** Radioactive Iodine Uptake; **HRCT:** High-Resolution Computed Tomography; **TBSRTC:** The Bethesda System for Reporting Thyroid Cytology; **FNA:** Fine Needle Aspiration; **SLL:** Small Lymphocytic Lymphoma; **TSH:** Thyroid Stimulating Hormone; **TG:** Thyroglobulin; **WHO:** World Health Organization; **FT3:** Free Triiodothyronine; **FT4:** Free Thyroxine; **LT4:** Levothyroxine.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The institutional ethics clearance was obtained for study the approval number IEC/TOCP/001/2024.

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Chronic Consumption of Alcohol Leading to Nutritional Deficiencies and Onset of Peripheral Polyneuropathy in a 28 Years' Man

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ABSTRACT

Introduction: Alcohol is injurious to health and its chronic consumption can cause harmful effects on body. It can directly or indirectly affect various organs mainly liver, gastrointestinal tract, brain and the nervous system. **Objectives:** in this case report we initially assess the condition of the patient based on the complaints and background information provided by the patient and the health care professionals to study the diagnosed disease by inter-connecting the conditions with the main cause and follow up the therapeutic intervention with the treatment provided. **Background:** A 28-year-old man presents with chief complaints of weakness, swelling and pitting edema. The patient was admitted to the general medicine ward in a multi-speciality hospital and research center. The patient is also exposed to nutrition deficiency due to alcohol consumption, which leads to nerve damage and neurodegeneration. The prescriber recommended a psychiatric review with strong evidence of alcohol dependence syndrome. **Discussion and Treatment Plan:** The general physician reviewed the complaints, and as mentioned above, the patient and further investigation were shifted to the male general ward. The appropriate treatment started with diuretics, supplements (thiamine and multivitamin), and antibiotics as a prophylaxis. The psychiatrist's opinion was taken in the view of alcohol abuse and advised to take benzodiazepine and tapered. The surgical opinion was taken, given left upper limb pain and swelling. The patient was closely monitored for vital signs of alcohol withdrawal syndrome. **Conclusion:** The patient was monitored symptomatically and hemodynamically stable throughout treatment in the multi-speciality hospital. The physician advised for 2 weeks of observation in hospital. The physician reviewed the OPD and recommended CBC, LFT, serum analysis, thyroid profile and a vitamin B12 test. Further, the patient was advised for neurological analysis- rhomberg's sign to identify the motor response.

Keywords: Alcohol, Peripheral polyneuropathy, Vitamin deficiency, Benzodiazepines, Alcohol dependence syndrome.

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INTRODUCTION

Alcohol consumption is one of the most widely abused substance globally and causes severe implications for individuals and society.¹ Metabolism of alcohol takes place by mainly the following pathways which are formation of acetaldehyde from alcohol, catalyzed by alcohol dehydrogenase I in the cytosol which converts to acetate by the influence of acetaldehyde dehydrogenase which is further converted to carbon di oxide and water.² Under chronic alcoholic consumption, microsomal ethanol oxidizing system is activated, which regulates Cytochrome P450

2E1 enzyme metabolizing alcohol to reactive oxygen species.³ Alcohol substantially aids to emergence of cancer in the oral cavity, pharynx, oesophagus, liver, colon.⁴ The toxic metabolites of ethanol also transforms the metabolism of components such as nucleus, cytoplasm, peroxisomes, glycosomes, endoplasmic reticulum.⁵ The other disorders associated are Liver diseases, kidney diseases, reprotoxicity, respiratory conditions, cardiovascular disorders, and haematological conditions.⁶

Polyneuropathy is a form of peripheral neuropathy, signatored by symptoms associated with sensory neurons, leading to numbness, pain and weakness, especially in the neurons residing in the distal part of the arms and legs.⁷ Alcoholic polyneuropathy is a disorder oriented with the Peripheral Nervous System (PNS), associated with the disruption of nerve transmission in peripheral nerves.⁸ Moreover, intake of alcohol also accounts for



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the toxin accumulation⁹ in the body, which may, which implicates to several risk factors such as thiamine deficiency,¹⁰ absorption impairment which is said to be profusely more common in chronic alcoholism.^{11,12}

Chronic alcohol consumption leads to Alcohol Dependence Syndrome (ADS), marked with a desire to ingest alcohol regardless of the outcomes. Sudden withdrawal of alcohol consumption leads to cognitive impairment and motor changes like tremors, sleep disturbances, and anxiety.¹³ The neuromuscular nerve group function related to alcohol-induced neuropathy is mainly linked to the deterioration of the axonal part, but can also be associated with demyelination or a combination of both.¹⁴ The degeneration of the lower extremity is an early sensory sign, later affecting the upper part,¹⁵ and gradual as time progresses with Total Life Dose of Ethanol (TLDE), sensory motor, and autonomic nervous distortion.¹⁶

METHODOLOGY

A Retrospective Observational study was been executed in a patient admitted to a multispeciality hospital of Bangalore, India, approved by the Institutional Ethical Committee, IEC/TOCP/23/2024-25.

CASE REPORT

A 28-year-old man was experiencing bilateral limb swelling and pitting edema for the past 15 days, along with growing bilateral upper and lower limb weakness that has been developing over the previous month. The patient claimed being in good health before these symptoms started, and the weakness gradually developed, first affecting the distal muscles and then moving to the proximal muscle groups. Significant functional damage has resulted from this trend, most notably the patient's inability to walk around freely. The patient now needs assistive aids for mobility and has trouble walking and getting out of a squat position.

The patient has no noteworthy medical history and denies any family history of similar neuromuscular or systemic problems with no substance abuse, however, persistent alcohol use (whiskey) throughout the previous five years is shown by the social history. Furthermore, the patient was admitted to a rehabilitation facility a year ago, most likely for alcohol-related reasons. The clinical picture of malnutrition is further supported by the patient's three-year history of marked decrease in appetite along with insomnia during the previous six months.

On physical examination, Both the lower and upper limbs have noticeable pitting edema. Vital signs were within normal ranges in spite of these observations, suggesting stable hemodynamic. The patient was attentive and cognizant of time, location, and people, according to a comprehensive systemic neurological examination. However, the patient displayed subtle tremors when moving, which may indicate cerebellar or extrapyramidal dysfunction. A positive Romberg sign, which indicates poor proprioception, was seen during additional neurological testing.

Additionally, the patient exhibited an ataxic gait, which is indicative of vestibular or cerebellar impairment and is characterized by wide-based, unstable steps and evident lateral swaying. A provisional diagnosis of alcohol-induced polyneuropathy secondary to alcohol dependence syndrome associated with liver injury is strongly suggested by the patient's history of chronic alcohol abuse, malnutrition, bilateral limb edema, progressive neuromuscular weakness, and positive neurological findings.

Laboratory Parameters

Hematological investigations: decreased levels of hematocrit and hemoglobin and elevated monocytes depicted in Table 1.

Liver function tests: elevated levels of total bilirubin (both direct and indirect), SGOT and reduced alkaline phosphatase depicted in Table 2.

Table 1: Hematological Investigations.

Blood Tests	Normal range	Day 1	Day 5
Hematocrit	38.3-48.6	34.2%	-
Hemoglobin	13.2-16.6	11.3g/dL	12.1g/dL
Total leucocyte count	4000-11000	5800%	6200%
Neutrophils	40-70	64%	70%
Lymphocytes	20-30	25%	30%
Eosinophils	1-4	1%	2%
Monocytes	1-2	10%	9%
Basophils	0-1	0%	0%
Platelet	1,00,000- 3,00,000	1,10,000	1,56,000

From Table 1, the hemoglobin levels are slightly reduced due to the malabsorption due to effect of alcohol on Brush Border membrane of small intestine following folate deficit disrupting RBC precursor cells in the bone marrow. Alcohol use also leads to the bacterial colonization in the gut followed by the release of toxin which increases the monocyte level which contributes to immunosystemic effect.

Table 2: Liver and Renal function tests.

Liver and Renal Function Tests	Normal range	Day 1	Day 2
Urea	15-4	15 mg%	21 mg%
Serum creatinine	0.6-1.4	1.02 mg%	1.1 mg%
Uric acid	2-7	4.5%	-
Total Bilirubin	0.1-1.2	1.62%	1.30%
Direct bilirubin	<0.3	0.91%	0.75%
Indirect bilirubin	0.2-0.8	0.71%	0.55%
A:G	1.1-2.5	1.86	1.81
SGOT	5-37	67 U/L	72 U/L
SGPT	5-40	19 U/L	9 U/L
Alkaline phosphatase	84-306	36 U/L	36 U/L

Chronic alcohol use also damages the hepatocytes leading to the impairment of detoxification process leading to elevated levels of SGPT and ALP along with the hindrance in the ability of liver to process and eliminate bilirubin, hence the levels are elevated as mentioned in Table 2.

Table 3: Thyroid profile and other serum components.

Components In Serum	Normal Range	Values Obtained
MCV	80-100	121 fl
MCH	27-33	40.1 pg
MCHC	32-36	33.1 g/dL
RBS	90-110	90 mg%
PT time	<14	13.3 sec
INR	0.8-1.1	0.89
Total triiodothyronine (T3)	80-220	0.88 ng/dL
Total thyroxine (T4)	5-12	7.74 mg/dL
TSH	0.5-5	5.08 mIU/l

Table 3 portrays that MCV and MCH is increased and it also depicts with a decrease in T3 and increase in TSH manifesting subclinical hypothyroidism, hence suggestion of monitoring over time is been suggested.

Serum components: elevated MCV and MCH levels with low levels of T3 are shown in Table 3.

TREATMENT PLAN AND COURSE

The plan was to restore medical and social well-being control, preventing further damage to peripheral nerves and to restore vitamin and mineral requirements and prevent the further complications. The treatment course commenced with a thorough assessment of the patient's chief complaint, followed by the initiation of therapy based on the established management plan shown in Table 4.

The patient was closely monitored for vitals, signs and symptoms over the hospitalization period throughout the final stages of treatment course. The patient was also monitored symptomatically and hemodynamically and discharged with medication to be followed.

Tab. Thiamine 200 mg 1-0-1(2weeks),

Tab. Pan 40 mg 1-0-0(5 days),

Tab. Neurobion forte 0-1-0(1 month).

DISCUSSION

This case report explains the manifestation of alcohol induced polyneuropathy which is a devastating health condition leading to the additive effects of nutritional deficiencies, neuronal damage, hepatic dysfunction, haematological abnormalities, subclinical hypothyroidism further aiding in nerve degeneration which effects the sensory motor and autonomic functions of the body.

Haematological abnormality due to Nutritional deficiency

Due to the toxic alcoholic on the small intestine, we observe nutritional deficiency along with alteration and suppression of erythropoiesis leading to reduced haemoglobin levels (12.1g/dL) with abnormal MCV (121fl) and MCH (40.1pg). levels. This condition was managed with ablation therapy with dietary support. Due to Malabsorption leading to iron and folate deficiency significantly reducing RBC synthesis, hereby concluding, heavy drinkers of alcohol have been prone to affecting the biochemical hematology.¹⁷

Immunoactivation

Release of endotoxins from liver and generation of ROS from mitochondria and Kupffer cells and cytokines leading to disrupt in microbiota of the gut contributing to the activation and release of monocytes (9%) into the systemic circulation. Alcohol causes gut barrier dysfunction and Kupffer cells activation which triggers monocytes thereby increasing its levels.^{18,19} upon intervention prophylactic treatment was given (ceftriaxone injection 1g IV).

Hepatic dysfunction

Upon observation from the LFTs we find mild elevation in SGOT (72U/L) and bilirubin levels (1.30%) followed by its decline over

Table 4: Treatment Plan.

Generic Name	Dose and Dosage	Category	Days of Treatment	Purpose
Pantoprazole	40 mg Inj	Proton pump inhibitor	1,2,3,4,5,6 (TID)	To relieve from gastric irritation
Furosemide	20 mg Inj	Loop diuretics	1,2,3,4 (OD)	Increases urine output and reduces fluid leakage into tissues and reduces edema.
Thiamine	200 mg Inj	Vitamin B1 Supplement	1,2,3,4,5,6 (OD)	Ablation therapy, supportive care And fulfilling nutritional requirement.
Multivitamin	500 mL in NS	Vitamin supplement	1,2,3,4,5,6 (OD)	
Thiamine+ Pyridoxine+ D-pantenol	100 mg+100mcg+50mg Inj	Vitamin, mineral supplement	1,2,3,4,5,6 (OD)	
Ceftriaxone	1gm Inj	3 rd generation cephalosporin antibiotic	1,2,3,4,5,6 (BID)	Prophylaxis
Trypsin+ Chymotrypsin	1 lakh AU Tab	NSAIDs	2,3 (TID)	Reduce inflammation providing Symptomatic relief.
Haloperidol	2.5 mg Inj	Anti-Psychotics	SOS	To manage the
Lorazepam	2 mg Inj	Benzodiazepines	SOS	Withdrawal Symptoms of Alcohol.
Pregablin+ Nortryptaline	75 mg+ 10 mg Tab	Tricyclic antidepressants	6,7,8,9,10,11 (OD)	Psychological support

the consecutive days. this is due to liver's regenerative capacity to combat the toxic metabolites of alcohol hereby reporting Chronic Alcohol consumption is associated with mild liver dysfunction.²⁰⁻²²

Subclinical hypothyroidism

Under observing the thyroid profile in the Table 3, the signs of mild elevation of TSH being asymptomatic. A study proved the evidence for the alterations of thyroid hormones due to the alcoholic toxic effect causing subclinical hypothyroidism on the thyroid gland due to heavy drinking is proved.²³

Neurological Weakening

Chronic exposure to alcohol damages the peripheral nerves, which lead to distal muscle weakening, progressing to proximal muscle is a characteristic of peripheral neuropathy. The positive Romberg sign and ataxia gait with lateral swaying indicates cerebellar and sensory ataxia, most likely caused by persistent alcohol use. Alcohol is known to have neurological effects contributing to peripheral nerve dysfunction and cerebellar degeneration.²⁴

Social and Psychological abnormalities

The patient's history of chronic alcohol consumption over five years, along with insomnia persisting for three years and decreased appetite for six months, highlights a pattern of declining physical and mental health. Insomnia may be attributed to alcohol induced sleep disruption, psychological stress, or withdrawal effects.

CONCLUSION

In this case study, we identified chronic alcohol consumption as the main cause for the disease by recognizing the patient history and interconnected the symptoms and conditions of the patient with the main cause. we identified the outcomes of alcohol consumption and assessed the nutritional deficiencies and noting the neurological symptoms to confirm alcohol dependence syndrome. The nutritional deficiencies were managed by providing nutritional supplementation along with addressing dietary changes to improve nutrition whereas, neurological symptoms were managed by providing, benzodiazepines, anticonvulsants, antidepressants and conventional antipsychotic medications along with providing symptomatic treatment, prophylaxis and supportive care with discharge plan after the recovery from his conditions

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

ALP: Alkaline phosphatase; **SGPT:** Serum Glutamate Pyruvate Transaminase; **SGOT:** Serum Glutamic Oxaloacetic Transaminase; **A: G-Albumin:** Globulin ratio; **ROS:** Reactive Oxygen Species; **MCV:** Mean Corpuscular Volume; **MCH:** Mean Corpuscular Hemoglobin; **MCHC:** Mean Corpuscular Hemoglobin Concentration; **RBS:** Random Blood Sugar; **PT:** Prothrombin Time; **INR:** International Normalized Ratio; **TSH:** Thyroid Stimulating Hormone.

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Hemorrhagic Cysts Accelerate Polymenorrhea and Development of Bilateral Polycystic Ovarian Disease

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ABSTRACT

Introduction: The young population is prone to stress, lifestyle changes and occupation, thus increasing risk of polymenorrhea associated Bilateral Polycystic Ovarian Disease (BPOD).
Objectives: To assess retrospective case report in young patients from level three hospital.
Methodology: The present epidemiological study investigated a case of 24-year-old corporate woman having a history of emesis, abdominal cramps and irregular menstruation which occurred over 6 weeks. The gynecologist suggested Pelvic Abdominal Ultrasound Scanning (PA U/S). **Results:** The scanned report revealed that presence of hemorrhagic cyst measuring 55×56×39 mm with renal calculi ranging from 3-4 mm in size associated with polymenorrhea. Based on diagnostic parameter the obstetrician prescribed desogestrel and ethinyl estradiol for 30 days and recommended for follow-up. During treatment the patient experienced with irregular menstrual cycle, and consulted with physician. The gynecologist suggested again with PA U/S for further deep investigation of uterus. Patient was diagnosed with bilateral polycystic ovarian disease for which she was prescribed with L-arginine, folic acid, Vit-B12, selenium with micronutrients. **Conclusion:** The patient was recommended with regular follow-up and symptoms-based treatment for the underlying conditions.

Keywords: Lifestyle patterns, Hemorrhagic cyst, Polymenorrhea, Renal calculi, Bilateral polycystic ovarian disease.

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INTRODUCTION

Menstruation is the shedding of endometrium followed by bleeding occurring in every month except during pregnancy. Menstruation begins at age of 11 and continues until age of 45-50 years (menopause). 90% of women experience menstrual cycle which lasts for 25-30 days, while only 10-15% have 28-day cycle. Some women experience irregular menstrual cycle due to variety of medical and psychological health conditions. Menstrual irregularity may occur due to underlying causes like hemorrhagic with polymenorrhea leading to development of bilateral polycystic disease. The development of hemorrhagic ovarian cyst and abnormalities in menstrual cycle along with polymenorrhea may be due to imbalance in hormonal factors, stress, BMI, and lifestyle factors.¹ Hemorrhagic ovarian cysts are functional cysts which occurs when a cyst bleed. These are generally benign and can be treated on their own but develop symptoms like abdominal pain and pelvic pain. Around 8% of women develop

large cyst before menopause. In around 16% women are found to develop cancerous ovarian cyst after menopause.² Surgical procedures should be delayed in regulation of HOCs because most of them can be cleared with regular follow-up, so patient should be exposed to sonographic and clinical diagnosis to avoid unnecessary surgery.³

All these conditions are symptoms of hormonal imbalances or gynecological conditions. Lifestyle factors such as stress and diet were identified as etiological factors. This case study explores the factors causing hemorrhagic ovarian cyst with polymenorrhea leading to development of bilateral polycystic ovarian disease.

METHODOLOGY

An observational, retrospective study was carried out in an inpatient setting in a tertiary hospital in Udipi, India. The study was conducted for one month.

CASE REPORT

A 24-year-old female patient presented with a history of unceasing vomiting and abdominal pain which had worsened over six weeks. Additionally, the patient reported experiencing menstruation twice monthly. After these symptoms, she sought



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consultation with a gynecologist who recommended a pelvic and abdominal ultrasound examination.

The ultrasound results indicated the presence of a hemorrhagic cyst in right ovary, characterized by a thick-walled cyst measuring approximately 55×56×39 mm and possessing a volume of approximately 64cc, located posterior to uterus were shown in Figure 1. The cyst was observed to contain turbid fluid. Small left renal calculi ranging from 3-4 mm was identified in conjunction. The patient was prescribed with Tab. Desogestrel and ethinyl estradiol and syrup menopaus, with directives to adhere to medication regimen for a duration of 21 days and to return for follow-up on 3rd or 4th day of her menstruation.

One month later, the patient continued to experienced irregular menstrual cycles, necessitating another abdominal ultrasound scanning. The result of this examination revealed a diagnosis of bilateral polycystic ovary disease, as indicated by enlarged ovaries: the right ovary measuring 11cc and left ovary measuring 14.2cc, each containing multiple cysts (exceeding 15) with sizes ranging from 4-7mm arranged in a peripheral pattern shown in Figure 2. Additionally, small right renal calculi measuring 3mm were detected. Consequently, the patient was prescribed with L-arginine, folic acid, Vit-B12, selenium with micronutrients and syrup menopaus to address her condition.

Ultrasound sonography of abdomen and pelvis

Liver: Normal in size, shape and echo pattern. No evidence of focal lesion. Biliary radicals on portal system are normal in their course and caliber.

Gall bladder: Normal in size and shape. No evidence of cholelithiasis. The CBD is normal.

Kidney: a small 3mm calculus is noted in right and left kidney.

Spleen: Normal in size, shape and echo pattern.

Urinary bladder: Normal in size, shape and outline. No evidence of fibroids or any focal lesions. The endometrium is normal in thickness, measures 4.2mm. No evidence of free fluid noted in pouch of Douglas.

Adnexa

Initially right ovary shows a thick-walled cyst measuring about 55×56×39 mm (volume about 64cc) in size posterior to uterus, contains turbid fluid. Left ovary is normal. This developed into bilateral polycystic disease of ovaries where both ovaries are enlarged. Right ovary measures 11cc and left ovary measures 14.2cc in volume. Both contains multiple cysts (>15) ranging from 4-7 mm in size arranged peripherally. No evidence of any adnexal mass.

DISCUSSION

There is no exact cause for manifestation of PCOD till date.⁴ The disease is becoming more prevalent worldwide due to environmental changes and lifestyle disease as a result of modernization. Although the disease occurs frequently in a community, etiology of few cases remain unknown.⁵ This case presented involves a complex medical scenario of hemorrhagic ovarian cyst, polymenorrhea with renal calculi leading to development of bilateral polycystic ovarian disease. HOCs arise as a result of hormonal changes. It is not surprising that these are

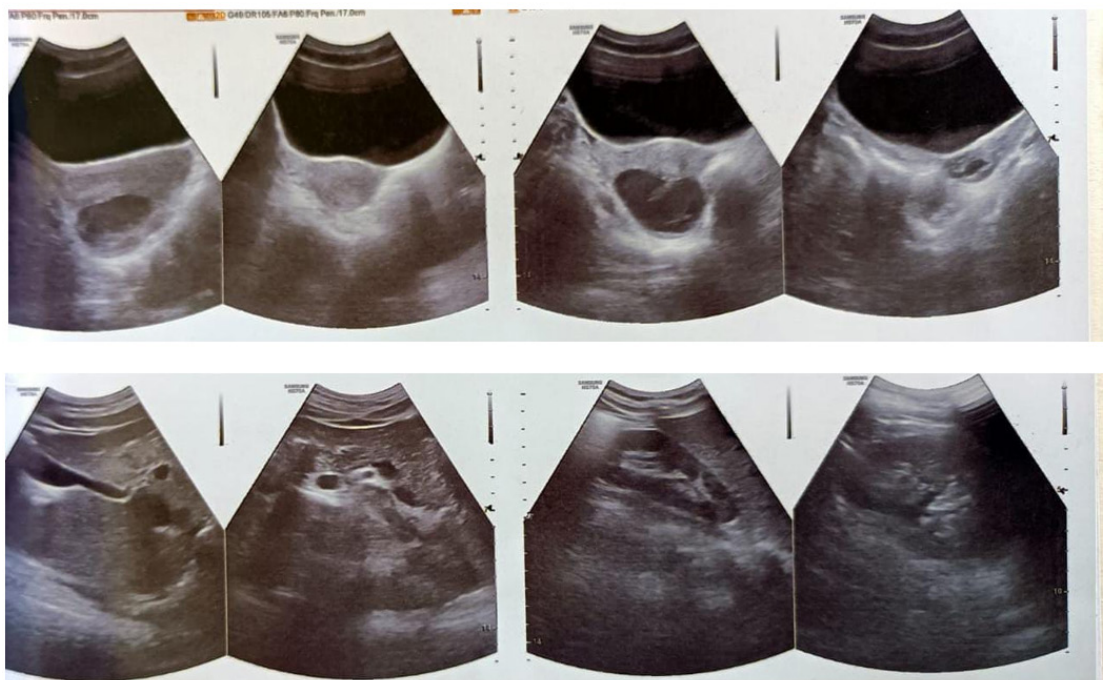


Figure 1: Ultrasound scanning of uterus (hemorrhagic ovarian cyst).

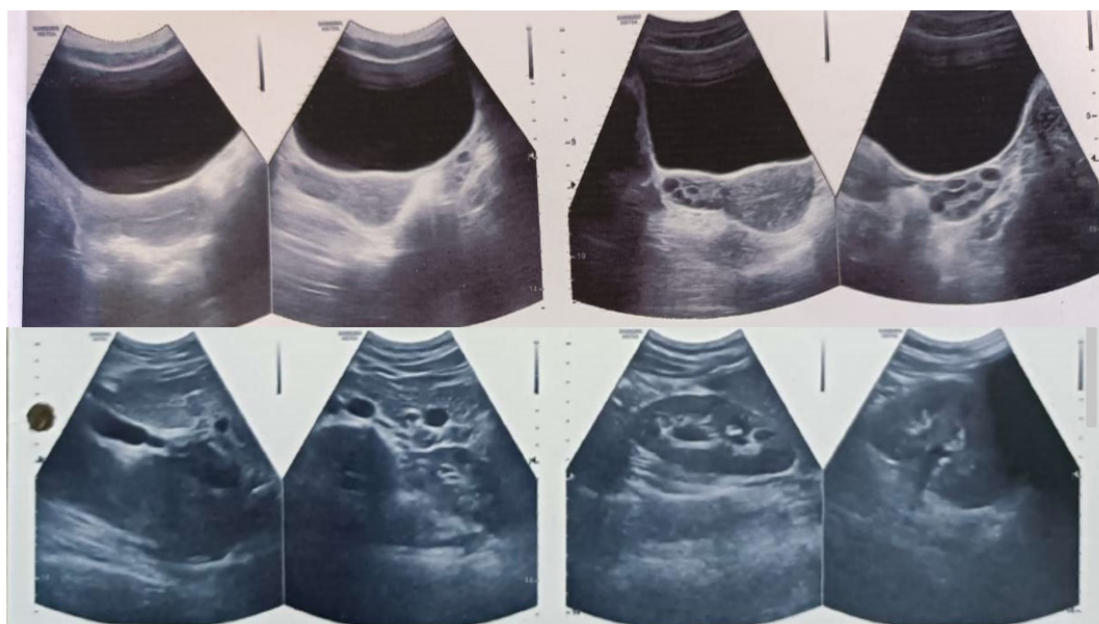


Figure 2: Ultrasound scanning of uterus (bilateral polycystic disease).

mainly discovered in adolescent females during their reproductive years.⁶ This is associated with acute pelvic pain, bloating, heavy, irregular or lighter than normal periods and a frequent need to urinate.² Most of HOCs were initially identified during luteal phase (68.8%) and a few during first weeks of pregnancy.⁷ In this case, polymenorrhea may be due to stress, chronic illness and uterine abnormalities causes ovulation problems and shorter luteal periods. These lead to development of bilateral polycystic ovarian disease that affect women of reproductive age. It is categorized by existence of cyst in both the ovaries.

The patient was also diagnosed with renal calculi, hard deposits that develop in the kidney leading to discomfort and problems in urine.⁸ Renal calculi and gynecological disorders may affect different body systems but share same risk factors such as underlying metabolic disorders, hydration and nutrition.⁹ Lifestyle factors and gynecological conditions plays an important role in development of bilateral polycystic ovarian disease.

Patient should be recommended with ongoing care plan which involve regular follow-ups and comprehensive treatment approach.¹⁰

CONCLUSION

This retrospective study concludes that prevalence and risk of hemorrhagic cysts accelerate polymenorrhea and development of BPOD. The gynecologist feedback was influenced by the suggestion provided. There is a need of awareness among young adults for the risk of BPOD and medication intervention. Health care providers should consider the complexity between lifestyle factors and gynecological conditions contributing to development of BPOD. Health care specialists and patients should work in collaboration for effective management.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

HOCs: Hemorrhagic ovarian cysts; **BPOD:** Bilateral polycystic ovarian disease; **PA U/S:** Pelvic abdominal ultrasound scanning.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The institutional ethical clearance was obtained with the approval number IEC/TOCP/23/2024-2025.

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Emerging Dynamics of the Medical Devices Sector in India

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ABSTRACT

With a current compounded annual growth rate of more than 16% and a projected market value of 12 billion US\$, the medical device industry in the Indian subcontinent alone is projected to reach a market size of over 50 billion US\$ by 2030. Despite being a vast market, emerging markets like India rely heavily on imports to meet most of their medical device needs. To bridge this gap, the government has envisioned developing innovative policies, indigenous facilities, practices, rules, and regulations to foster the production of medical devices. Keeping this in view, a series of initiatives have been rolled out, concealing the aspects related to classification, registration, manufacturing, pricing, sales, and market supplies. This review provides a comprehensive 360-degree understanding of the Dynamics of the medical device perspective, using a case study of an emerging market like India. The primary objective is to outline the ongoing regulatory amendments, new policy enforcement, adopted quality management tools, and quality standards required for medical device products. This review will also assist as a guide for stakeholders in subjects related to this sector, providing insight into the federal government's efforts to strengthen the country's medical device production capabilities, thereby improving access and affordability. It is anticipated that continued debate and effective action will benefit diverse sectors and ultimately enhance the worldwide development of medical device industries.

Keywords: Medical Devices, Medical Device Regulations, Government Initiatives, Federal Policies.

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INTRODUCTION

Medical device refers to an instrument, appliance, apparatus, material, implant, or other article, that is employed as such or in association with other modalities and proposed to be used in humans or animals for some specific applications.¹⁻³ These applications may serve various purposes, including diagnostics, preventive measures, monitoring, treatment, and the mitigation of specific diseases or ailments. It can be applied for diagnostic, monitoring, therapeutic, or mitigation purposes in the context of an injury or disability. It also denotes to an instrument, appliance, apparatus, material, implant, or other article for examination, modification, replacement, or support of anatomical/physiological processes. These also involve their application as life support or for life sustenance, disinfection, or as the controller of conception.⁴

Notably, it should not achieve the primary activity in humans or animals through pharmacological, immunological, or metabolic approaches; however, the activity of such instruments, apparatuses, appliances, implants, materials, or other articles may be assisted by such means in mediating their intended purpose. The term "medical device" often refers to instruments, appliances, apparatuses, materials, implants, or other articles used for diagnostic purposes, including reagents, calibrating machines, standards, assessment kits, or equipment. These can be employed alone or in combined form with each other for inspection and providing medical or diagnostic information by examining specimens derived from humans or animals.⁵

Recently, medical devices have emerged as a crucial component of the healthcare sector, addressing a wide range of healthcare needs. With a growing population and an ever-increasing demand for affordable and high-quality healthcare, these sectors have demonstrated significant potential. The medical device industry requires substantial capital investment and has a lengthy development process, often necessitating the introduction of new technologies.⁶ Over the past decade, the global medical devices sector has experienced substantial growth. The medical devices



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sector is projected to grow at a Compound Annual Growth Rate (CAGR) of 7% between 2025 and 2030, driven by robust technological advancements. Furthermore, the rapid growth in the global aging population, as well as the increasing prevalence of chronic diseases, further enhances the potential of the medical device industry. Notably, the medical device sector is primarily occupied by the USA, accounting for a 40% share, followed by the European market and Japan, with shares of 25% and 15%, respectively.⁷ With a current compounded annual growth rate of more than 16% and an estimated market value of US\$12 billion, the medical device industry in the Indian subcontinent alone is projected to reach a market size of over US\$50 billion by 2030.⁸

INDIA AS AN EMERGING MEDICAL DEVICE MARKET

The Indian medical device sector is one of the top twenty global medical device markets.^{9,10} The rising global market for medical devices opens opportunities for the Indian medical device sector to achieve new heights of growth.⁹ India is positioned as one of the rapidly rising medical device industry markets globally, with an anticipated CAGR of 15%. In India, the factors driving this growth include the increasing prevalence of chronic diseases, technological advancements, and improvements in healthcare infrastructure. Medical device manufacturers are increasingly attracted to establishing manufacturing facilities in India,

primarily due to its sizable population and favorable regulatory environment.¹¹

The medical device sector in India encompasses a diverse range of products, spanning from consumables to implantable devices. These medical device products fall under various segments that differ in terms of their share in the Indian market. The most significant segment is medical instruments and appliances, which account for approximately 35%. This is followed by investigative imaging and diagnostic devices (30%), implants and consumables (20%), patient aids (15%), and other categories. The country focuses its manufacturing endeavours on disposable items such as syringes, needles, catheters, extensions, cannula, feeding tubes, and perfusion sets. Moreover, India plays an active role in the production of implants, including orthopaedic implants, cardiac stents, drug-eluting stent devices, and ocular lenses.

Based on the projected application of the medical device, its underlying risks, and the prominent parameters listed in the Indian Medical Device Rules (IMDR), the Indian Central Licensing Authority categorizes Medical Devices and *in vitro* Diagnostic Medical Devices (IVDMDs) into four major risk classes. Namely, (i) low risk-Class A Low; (ii) moderate risk-Class B; (iii) Moderate to high risk-Class C, and (iv) High risk-Class D. Figure 1 demonstrates the principle on which the classification of Medical Devices or IVDMDs is based. It is worth noting that combination hybrid devices must be classified distinctly. The

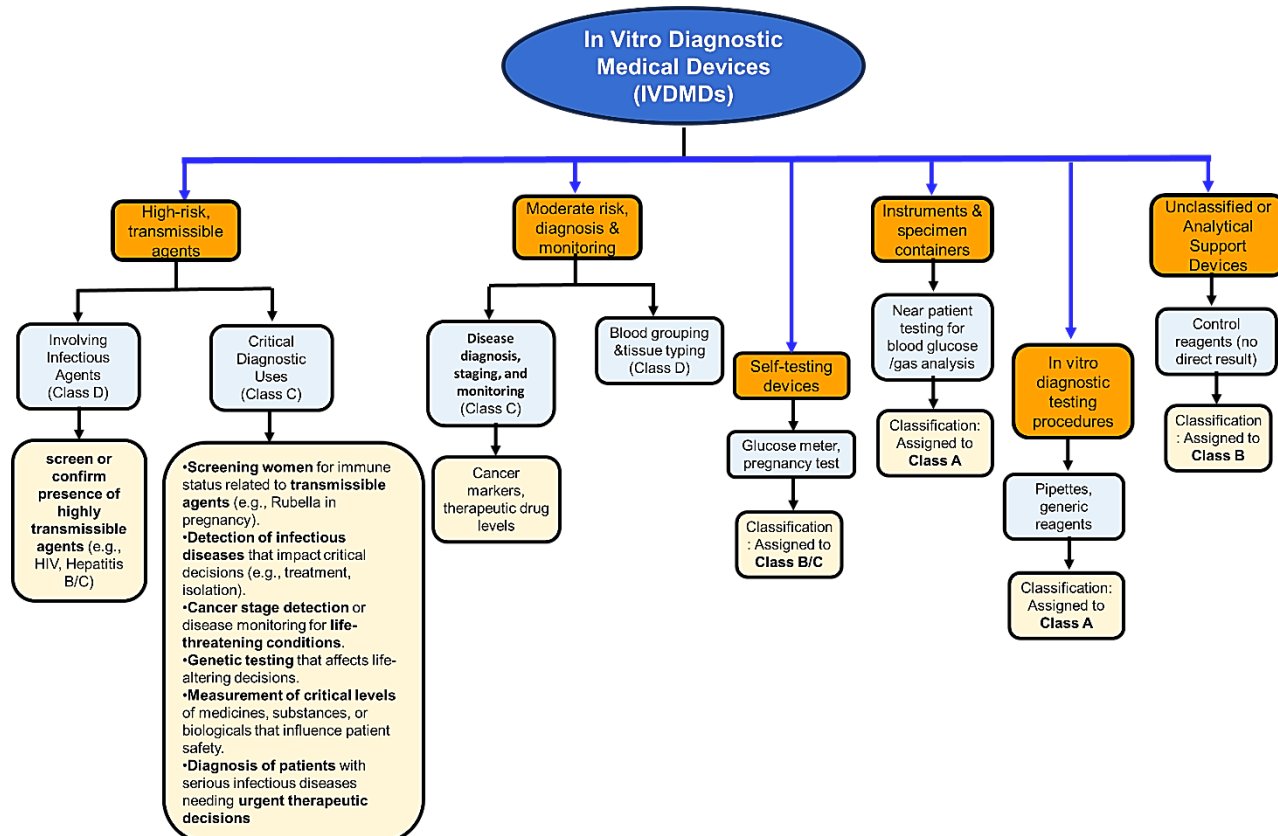


Figure 1: Distinct parameters involved in the classification of *in vitro* diagnostic medical devices.

software must be classified in the same class as its associated devices. Furthermore, the calibrators used in conjunction with the reagent must have the same class as the IVDMD reagents. The device with multiple specified applications must be classified according to its most critical application. If multiple guidelines apply to a specific class, the most stringent rule that results in an advanced classification of the medical devices must be adopted.

It is evident from the above discussion that today, India is one of the leading global medical device markets.^{3,10} However, despite being such a vast market, India relies heavily on imports to meet most of its medical device needs. The existing gap between medical device import and manufacturing presents a significant opportunity for medical device manufacturers to bridge it through indigenous manufacture and sales.^{12,13} To bridge this gap, the Indian government and manufacturers have long envisioned developing India-centric policies, indigenous facilities, practices, rules, and regulations to foster the production of medical devices in India. Keeping this in mind, a series of policies have been recently rolled out, concealing aspects related to classification, registration, manufacturing, pricing, sales, and market supplies, among others. It must be noted that the process of innovating and developing a new medical device is an intricate, as well as a lengthy, laborious, and time-consuming operation.

Notably, its approval is one of the most precise and exceedingly regulation-driven processes, governed by the IMDR and the Medical Devices Amendment Rules released in 2020. These rules encompass various aspects of medical device-related regulations, including the designated classification system, registration protocol, manufacturing leads, importation, labeling requirements, sales prerequisites, and post-market obligations.

Lately, the Indian government has taken giant initiatives to promote the domestic manufacturing of medical devices through campaigns like "Make in India." This increased investments in the medical device sector and created opportunities for domestic and international companies. Moreover, implementing policies such as the National Health Protection Scheme, which aims to provide health insurance coverage to millions of Indian citizens, is expected to further boost the prospects of medical devices in India. Although the statistical data on India's import and export of medical devices suggests a rising importance in the medical devices sector, achieving a balance between domestic and global demand and supply remains a concerning challenge. However, the timely government initiatives being undertaken to foster the growth of medical devices will undoubtedly be fruitful in their purpose. The enduring dynamics of demand and supply provide a significant opportunity and rationale for manufacturing medical devices in India.

This manuscript aims to provide a comprehensive 360° understanding of the dynamics from a medical device perspective, using a case study of an emerging market like India. The primary

objective is to expound on the ongoing regulatory amendments, new policy enforcements, adopted quality management systems, and quality standards required for medical device products. It is also anticipated that this manuscript will serve as a comprehensive guide for stakeholders in cases related to medical devices, providing insight into the federal government's efforts to enhance medical device production capabilities and improve access and affordability within the country. It is anticipated that continued debate and effective action will improve the medical device industry, benefiting the medical device sectors worldwide.

REGISTRATION OF MEDICAL DEVICES IN INDIA

In India, the Central Drugs Standard Control Organization (CDSCO) is the regulatory body responsible for regulating pharmaceuticals, medical devices, and cosmetics. Recently, the CDSCO has organized its norms for regulating the medical device sector in the nation, aiming at ensuring the identity, purity, safety, efficacy, and quality norms. Previously, manufacturers were allowed to sell medical devices in the country without any regulations.^{13,14} It was later made compulsory that the medical devices entering India must comply with the Medical Device India 2017 Regulations set forth by the CDSCO. There are two ways to register medical devices in India, which are as follows:

Notified Medical Device Registration

There are thirty-seven device categories listed as notified medical devices by the CDSCO department. In this category, medical devices are classified according to the Medical Devices Rules 2017. Medical Devices are classified in a process.^{15,16} as per the Global Harmonised Task Force (GHTF) risk-based assessment.

Non-Notified Medical Device Registration

In this legislation, the CDSCO has amended the new rules 2020 by introducing a new set of rules for the rest of the devices which is not included in the above 37 notified devices, which include apparatus, instruments, appliances, and implants for different applications like analysis, treatment, prevention, replacement, and investigation. This set of rules is known as the Medical Device (Amendment) Rules, 2020. The previous medical device Rule 2017 incorporated Chapter IIIA in Medical Device (Amendment) Rules, 2020, which was imposed on 1st April 2020.^{16,17}

As of October 2023, it is compulsory for all medical devices classified as Class A, B, C, and D to hold an MD-14/15 Import License for import purposes. The formal procedure for the MD-14 application mandates submitting a detailed Device Master File (DMF) for each device. It is worth noting that the regulatory body also requires the submission of a DMF, along with an explicitly detailed Plant Master File (PMF). The authority grants an MD-15 Import License after a rigorous approval process to a local license holder for the manufacturing site, with an inclusive list of all approved devices. The circular released by the Indian

Ministry of Health and Family Welfare in October 2023 [(G.S.R. 777(E))] exempts Class A non-measuring and non-sterile medical devices from the MD-15 import licensing.

On the contrary, Class A devices (non-measuring and non-sterile) do not require an importation license; however, they must obtain registration and provide self-certification to the applicable standards. Additionally, in the case of medical devices imported into India, the registration process primarily involves a re-examination of the existing documents filed in the country of origin. For the same, the CDSCO critically scrutinizes the established (Predicate) device section of the application to compare the product under consideration. Therefore, it is recommended that manufacturers carefully identify the predicate device for comparison and provide necessary proof to establish equivalency.

Appropriate certification (e.g., ISO 13485) is mandatory to confirm the quality management system of both the legal and actual manufacturing facilities.¹⁸ To demonstrate compliance, the applicant must provide a detailed Plant Master File (PMF) for the manufacturing site. After approval of the PMF and other formalities, the medical device is added to the license list, and an Importation License is allotted. It may be noted that the medical device product manufactured at two separate sites will need to have two separate PMFs as well as be separately listed on the Import License once approved.

In the context of medical devices that are new to the Indian medical device sector, the material of construction, mechanism of action, or application of these devices are regarded as 'investigational devices'. Such products must undergo additional clinical studies to demonstrate their safety and efficacy in India. The list of clinical investigation studies is usually determined by a Subject Expert Committee (SEC) of CDSCO.

MEDICAL DEVICE REGULATIONS IN INDIA AND THEIR CURRENT STATUS

Medical devices have a direct connection to the health and safety of the subject; therefore, their production must be carried out in a strictly controlled environment that meets regulatory requirements and established guidelines. Medical devices fall under Schedule M-III under the Drug and Cosmetic Act of 1940, but there are no specific rules and regulations for the manufacturing and monitoring of medical devices under the Drug and Cosmetic Act of 1940.¹⁹ In India, the guidelines and intriguing laws for drug manufacturing have been in place for decades, but a clear regulation for medical devices has been lacking for a prolonged period.

Nonetheless, the regulatory landscape of medical devices in India has recently transformed into a very dynamic and continually evolving.^{14,20,21} Therefore, to overcome this barrier, the CDSCO has framed the IMDR 2017 and Medical Devices (Amendment)

Rules 2020 for the effective regulation of Medical Devices.^{1,2} These rules cover various perspectives of medical devices, like registration, classification, import, manufacturing, sales, labelling, and post-market surveillance.^{22,23}

The CDSCO department under the Directorate General of Health Services in the Ministry of Health and Family Welfare is the National Regulatory Authority that accounts for the regulation of import, manufacturing, licensing, sales, clinical trials, and distribution of medical devices via the imposition of Medical Devices Rules, 2017.^{24,25}

The medical devices and diagnostics division of CDSCO has framed guidelines for medical devices, in the form of IMDR, which were released in 2017 and came into full enforcement from 2018. Later, IMDR was also amended in 2020 as "Medical Devices (Amendment) Rules, 2020" which came into enforcement in 2020. It is worth noting that the 2020 IMDR amendment was introduced by adding "registration of certain medical devices". Interestingly, many medical devices are still considered controlled substances and are therefore regulated under the D&C Act of 1940.¹⁴ The drafting of the IMDR and other auxiliary guiding principles has facilitated the Indian medical device market to take patient safety-centric steps concerning medical devices. In the years to come, amendments to the IMDR are anticipated to focus on aligning the regulations of the MDR and IVDR, the most recent European Union (EU) international regulations establishing the safety and intended performance of medical devices.²⁶ A schematic representation of the evolution of the regulatory landscape of medical devices in India is shown in Figure 2.

According to IMDR, guidelines are valid for both medical devices and IVDMDs, which can be licensed for production, distribution, import, sale, as well as for stock, exhibition, or offer for sale. This also applies to medical devices and IVDMDs, which can be manufactured for clinical investigations, assessment, training, or for specific demonstration purposes. The IMDR primarily discusses the regulatory features of the medical devices, including classification, classification parameters; their sub-grouping, underlying critical principles, applied standards; applicable governing subsidiaries; registration protocols; manufacturing standards; import framework; labelling requirements; clinical investigation; obligations of governing bodies; registration of laboratories; medical device sales; underlying fees; licensing; QMS-quality management system; PMS-post-approval changes. A depiction of these guiding principles is illustrated in Figure 3.

MEDICAL DEVICES AMENDMENT RULE 2020

The CDSCO enrolled Medical Devices Rules, 2017, have been amended as Medical Device (Amendment) Rules, 2020,²⁷ which consists of the chapters as presented in Table 1. Moreover, the Government of India established the National Medical Device Promotion Council in January 2020 to promote the manufacture

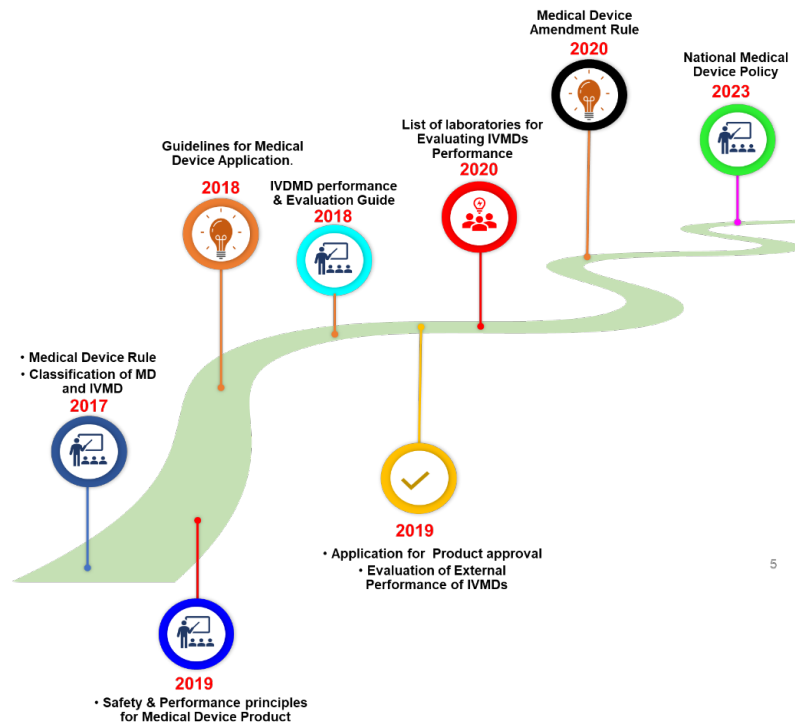


Figure 2: India's Roadmap for Medical Device Legislation. MD: Medical Device; IVMD: *In vitro* Diagnostic Medical Device; IVDs: *In vitro* Diagnostics.

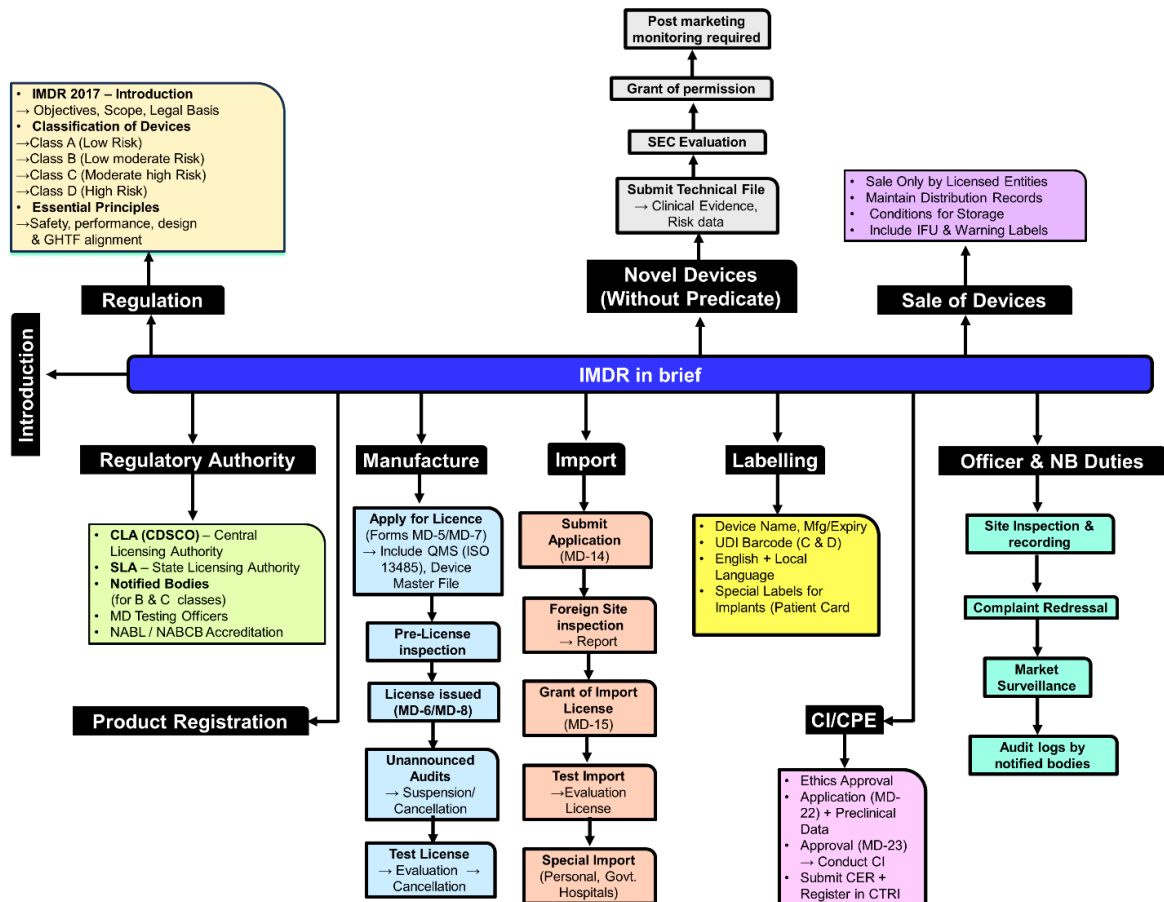


Figure 3: Summary of IMDF. CLA: Central Licensing Authority; MD: Medical Devices; NABL: National Accreditation Board for Testing and Calibration Laboratories; CER: Clinical Evaluation Report; NABCB: National Accreditation Board for Certification Bodies; SLA: State Licensing Authority.

Table 1: Medical Devices Amendment Rule 2020.

Chapter	Chapter Title	Role
I	Preliminary	Introduction
II	Medical Devices Regulation	Classes of Medical Devices and <i>in vitro</i> Diagnostic Medical Devices.
II	Authorities, Officers, and Regulatory Bodies	Central Licensing Authority Controlling Officer National Accreditation Body Notified Body Medical Device Testing Officers Central Medical Device Testing Labs.
III A	Registration of Certain Medical Devices	This chapter applies to all non-notified medical device registrations that are exempted by 37 categories of already existing medical devices.
IV	Import of Medical Devices	Application for the Import Licence grant .
V	Labelling of Medical Devices	All the essential information shall be printed with indelible ink on the packed cover of the medical device.
VI	Clinical Assessment and Performance Valuation of an <i>in vitro</i> Diagnostic Medical Device	Application to grant permission to conduct a Clinical Trial.
VII	Import/ Production of Medical Devices that do not have a Predicate device	Permission to import/ manufacture a medical device that do not have a predicate device.
VIII	Registration of a Laboratory to perform Testing	Application for Medical Device Testing Laboratory Registration with Central Licensing Authority (CLA).
IX	Sale of Medical Devices	Provisions for the license of sale of medical devices from the State Licensing Authority of Medical Devices.
X	Miscellaneous	Exemptions from provisions related to medical devices.

of indigenous medical devices. It has been regulated by the Department for Promotion of Industry and Internal Trade (DPIIT). The National Medical Device Promotion Council was reconstituted by the Department of Pharmaceuticals in August 2022.

As a result, the Union Cabinet approved the new National Medical Devices Policy, 2023, on April 26, 2023, to accelerate the growth of the medical devices sector from its current US\$11 billion to US\$50 billion by 2030. This policy aims to improve the public health sector by escalating the affordability, innovation, accessibility, and quality of medical devices.²⁸

REGULATORY AUTHORITIES FOR MEDICAL DEVICES

For the controlled regulation of medical devices, CDSCO has assigned two regulatory authorities, which are as follows:²⁷

Central Licensing Approval Authority

Central Licensing Approval Authority (CLAA) is a sub-division of CDSCO that serves as the main regulatory body for medical devices, as well as the competent authority which is responsible for the enforcement of medical device rules, like:

- Import all medical device classes,
- Manufacturing of Class C and Class D devices,
- Clinical testing and licensing of experimental medical devices,
- Assessing clinical performance and approving newly developed *in vitro* diagnostic medical devices,
- Collaborating with state licensing authorities.¹⁷

State Licensing Approval Authority

The State Licensing Authority is the governing body responsible for regulating medical devices within the state. The State Controller is the competent authority for the imposition of certain rules applied to (i) manufacturing for the sale of Class A and B Medical Devices, and (ii) stock, sale, or exhibit for sale of medical devices. Therefore, both authorities work simultaneously, i.e., the Central Licensing Authorities delegation of licensing powers has been given to the Drug Controller General of India (DCGI). Likewise, the State Licensing Authority has to get prior approvals from the State Government, and power has been given to Drug Controllers under its name and seal.^{17,29}

Over the past decade, India has made enormous strides in the fields of medical equipment and healthcare. Notably, India has set a new standard in medical equipment with the implementation of Medical Device Rules in 2017. Nowadays, governments are incentivizing manufacturers to prioritize the indigenous production of medical devices over imports. However, India has

ample opportunities to capitalize on unexplored sectors of federal, transparency, policy accessibility, and ease of doing business. Therefore, India has revised the Medical Device Rules 2020, as well as the National Medical Device Policy 2023, and introduced Unique Device Identification (UDI) to navigate the regulatory landscape more effectively, facilitating traceability, harmonizing practices, safeguarding safety, effectiveness, and approachability of medical devices.

INVESTMENT CLIMATE FOR MEDICAL DEVICES IN INDIA

Foreign Direct Investment (FDI) refers to the ownership stake in a foreign establishment or venture undertaken by a stakeholder, investor, company, or even the government of another country. It typically describes a business's aspiration to acquire a substantial stake in a foreign company or to expand its operations into a new market. In this regard, FDI has now been permitted in almost all sectors in India, without the need for prior regulatory approvals, except in exceptional cases such as defence, housing, and media. If the FDI is not in compliance with the stated guidelines, approval from the competent government authority must be obtained.

Recognizing the need to cut dependency on imports, the Indian Government has taken several decisive steps to make the investment environment more favorable, particularly for foreign investors. A detailed account of this can be traced back to the Press release note issued by the Department for Promotion of Industry and Internal Trade (DPIIT), which became operative from the first quarter of 2015. It may be noted that, as per this modification, the FDI in the manufacturing of medical devices is permitted to the extent of one hundred percent.

This signifies that foreign companies may invest in or establish a new manufacturing facility in India without obtaining formal prior approval from the government. This move was made to substantially create a business-friendly environment by attracting global players to the Indian market. Notably, the norms in the medical device division were explicitly defined from the pharma sector to enable funding and investment. Moreover, these FDI policies apply to both greenfield and brownfield projects, without any limitations specific to the pharmaceutical industry, such as the non-competitiveness section or former agreements for acquiring any existing company.

Notably, greenfield projects refer to initiatives undertaken from scratch on undeveloped property without any existing building structure. Such projects offer absolute autonomy in designing and construction efforts, such as a new factory, building, power plant, or housing society on designated agricultural or unoccupied land. On the other hand, the brownfield projects refer to the renovation or extension of a preexisting facility with a likelihood of contamination or outdated infrastructure. Such projects comprise refurbishing old plants, upgrading facilities

for API synthesis, and upgrading buildings for new purposes. The Production Linked Incentive Scheme (PLI) Scheme of the government of India under the medical device sector offers ground for financial incentives on the basis of incremental market and sale of medical devices internally manufactured in Indian settings to generate robust in-house manufacturing base for high-end medical devices (dialysis machine, diagnostic and imaging tool, etc., to name the few).

The Indian Government has also initiated the notion of dedicated Medical Device Parks across the country. Today, the concept of such parks has been implemented in states such as Tamil Nadu, Gujarat, Himachal Pradesh, Andhra Pradesh, Telangana, and Madhya Pradesh. Such parks are equipped with top-notch facilities, high-end testing equipment, and accreditation services. These facilities are designed to enable cost-effective manufacturing processes, support basic research, and reduce product development time to market, thereby contributing to India's development as an internationally competitive destination for the production of medical devices.

Ayushman Bharat is one of the world's most significant federally funded healthcare insurance initiatives, expanding access to healthcare nationwide. In line with this vision, the Indian government is continually seeking potential trade partners to ensure that Indian-originated medical devices gain wider global acceptance and deeper market access. To achieve this goal, the Indian federal agency, the Indian Brand Equity Foundation (IBEF), is making exceptional efforts to streamline the regulatory approval process and location valuation, as well as provide hand-holding support for easy market entry and operation thereafter. India's emphasis on self-sufficiency, as marked by initiatives such as "Make in India" and "Atmanirbhar Bharat", has further strengthened the country's investment climate.

The globally harmonized regulatory policies also play a prominent role in creating a transparent and progressive investment environment. In this regard, as discussed, the Medical Devices Rules of 2017 have helped establish a required regulatory framework for the previously under-regulated medical device market. It assigns governing responsibilities in terms of regulation to the CDSCO, describes the licensing protocol for domestic medical device manufacturers, and outlines the medical device import policies. These policies outline the practices, as per the International Medical Device Regulators Forum (IMDRF), for making Indian medical device products more acceptable in global markets.

Furthermore, the CDSCO has also launched platforms like SUGAM to assist with registration, licensing, and post-marketing observations, aiming to generate transparency and reduce delays due to administrative processes. This platform has also facilitated smooth and well-organized interaction between national and international partners. Due to these coordinated efforts, India

has today emerged as a dynamic ecosystem with ease of doing business and an investment-friendly sector in the medical devices industry.

DRIVERS OF THE MEDICAL DEVICES INDUSTRY IN INDIA

The amended FDI policy by the Government of India, backed by major regulatory reformations and lucrative incentive schemes, has played a vital role in transforming India into a key hub. Furthermore, the existence of a vast consumer base, skilled manpower, and technical knowledge is anticipated to further enhance medical device innovation and manufacturing in the country over the upcoming decade.^{3,23} This section explicitly expounds the key drivers of the medical devices industry in India.

Government initiatives for the promotion of medical devices

Over the years, the medical device industry has become a vital component of India's healthcare system. However, producing medical devices with international quality standards at an affordable cost has been an area of concern for the industry. Given this fact, the Government of India has designated the medical device industry as an essential focus area for its signature "Make in India" initiative to bolster manufacturing facilities.

Regarding market share, India ranks fourth in Asia in terms of medical equipment. Though exports have increased recently, the Indian market still depends heavily on imports. India is gaining momentum toward its goal of developing into a global centre for medical device manufacture, thanks to the Atma Nirbhar Bharat initiative. This is demonstrated by recent programs, such as the Encouragement of Medical Device Parks Scheme and the Production Linked Incentive Scheme (PLI). These programs have been thoughtfully designed to encourage massive production and provide the necessary facilities for establishing industrial hubs in India. Extending these actions requires a comprehensive policy framework to facilitate this expansion and realize the sector's full potential.

The present strategy aims to establish a comprehensive set of priorities for the industry to develop organizationally, despite multiple government agencies having implemented proactive measures to support the industry. Furthermore, because the field of medical devices is diverse and multifaceted, legislation, talent development programs, and promotional efforts are dispersed across multiple ministries at the state and federal levels. It is necessary to organize the variety of actions logically so that the corresponding agencies may provide targeted, practical assistance as well as encouragement for the industry.³⁰

The National Medical Devices Policy of 2023 is projected to support the logical expansion of the medical device industries, to foster the attainment of the public health goals, viz accessibility,

affordability, excellence, and research-centric innovations. With the tactics that follow, this industry is expected to reach its full potential: establishing a strong and efficient legal structure, supporting development and training programs, encouraging higher learning to develop skills and proficient assets tailored to the sector's needs, and establishing a structure that fosters innovation.

National Medical Device Policy 2023 and Its Key Elements

The vision of this policy is a patient-centred, expedited expansion strategy to become a world pioneer in medical device invention and manufacture, securing a 10-12% revenue share in the growing worldwide marketplace over the next twenty-five years. By 2030, the strategy is projected to assist the expansion of the medical device sector from its current eleven billion USD to fifty billion USD. To accomplish these goals, the strategy puts out a mission for the quick expansion of the medical device industry: accessibility and inclusivity; economy; excellence; patient-centred and high-quality care; proactive and promotional healthcare; safety; investigation and innovations; and qualified workers.^{3,23,31,32} The medical device sector is assisted and directed by various plans encompassing six primary categories of government measures. These measures can be visualized primarily under the following heads:

- **Facilitating infrastructure:** To improve progress and retrogression with the medical device industry, the State Governments, industry, and the National Logistics Policy 2021 under the horizon of Gati Shakti program will work together to build and fortify massive medical device parks, as well as medical device clusters outfitted with worldclass infrastructure/ amenities that are lying in a closer proximity to commercial areas with adequate transport access.
- **Encouraging Research and Development:** The strategy aims to support Research and Development (R&D) in India and supplement the Department's National Policy on R&D and Innovation in the pharmaceutical and medical technology (Pharma-MedTech) Sector of India. In addition, it seeks to create innovative centres, "plug and play" infrastructures, Centres of Excellence (CoE) established in association with institutions of higher learning and research, and business assistance.
- **Human Resources Development:** The policy envisions the following as a means to maintain a consistent supply of competent workers throughout the entire value chain, including scientists, regulators, health experts, managers, technicians, and others. Investors can utilize the assets offered by the Ministry of Skill Development and Entrepreneurship to provide expertise, retrain, and upgrade medical device industry professionals.

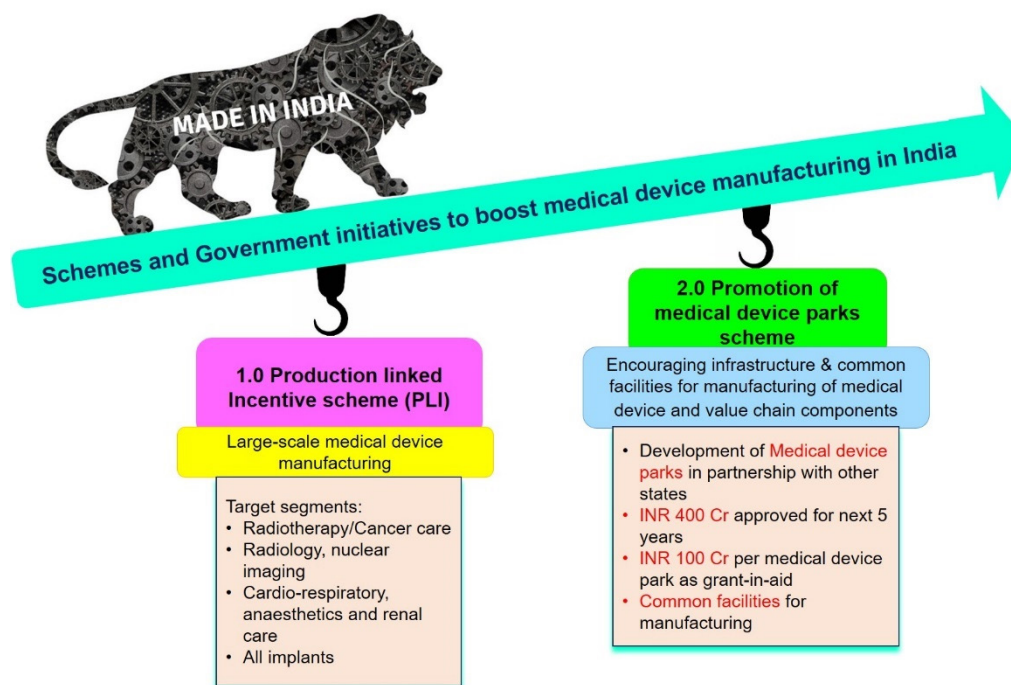


Figure 4: Government initiatives for the production and promotion of medical devices.

To guarantee a steady supply of qualified workers for contemporary medical and healthcare technologies, high-end equipment manufacturing, and cutting-edge research, and to create future-ready MedTech human resources, the policy will promote specialized, multifaceted training in medical devices within existing institutions. Forming alliances with overseas academic and business institutions to create medical technology and meet global demand jointly.

- **Bringing in Investments:** In addition to current programs and initiatives, the Indian government has implemented several measures to develop further initiatives, including Ayushman Bharat, Make in India, Startup India, and Heal-in-India. The policy also promotes venture capitalist funding, private investments, and Public-Private Partnerships (PPP).
- **Positioning the brand and raising awareness:** The strategy calls for the department to establish a special Export Promotion Council for the industry, which will facilitate the resolution of several accessibility concerns in markets.
- **Regulatory streamlining:** Strategies like "Single Window Clearance System" to license medical devices, which includes participation from every relevant agency and organization, including MeitY (Ministry of Electronics and Information Technology), AERB (Atomic Energy Regulatory Board), etc., taken to enhance the ease of conducting studies and business, regulate patient security with new product development, and strengthen

the function of Standards of India like Bureau of Indian Standards (BIS).

The PLI and the Promotion of Medical Devices Parks are the two crucial and skillfully designed schemes laid out by the Indian Government to encourage industrial production as well as provide the necessary infrastructure for the growth of manufacturing hubs, as shown in Figure 4.

Production-Linked Incentive (PLI) scheme to promote domestic manufacturing of medical devices

To encourage substantial investments in medical device segments, such as implants, radiation therapy, imaging, cancer treatment, analgesics, and others, the PLI Scheme for Medical device manufacturing facilities offers a monetary reward. This PLI scheme shall offer a 5% incentive on the incremental sales of goods manufactured in India. The program's assistance will be provided to authorized businesses that produce target categories in India and have a total asset value of at least Rs. 18 crores (net cost of the candidate business plus the net cost of member firms included).

Promotion of medical device parks

Marketing of health-related products parks aims to establish a robust infrastructure and foster a thriving local medical device manufacturing ecosystem. Creating outstanding standard testing and infrastructure assets through grants under the Scheme will accelerate local manufacturing and expand India's medical device value chain. These are also expected to drastically

lower production costs, thereby improving the availability and affordability of medical devices nationwide. To create Common Infrastructure Facilities (CIF), which would lower the production costs of medical devices made in the country, the Scheme for Promotion of Medical Device Parks has been notified. The budget of the entire scheme is approximately 400 crores of Indian rupees. The tenure for this scheme is the financial year 2020-2021 to 2024-2025. This scheme shall cover products from three categories: Category 1 includes orphan drugs, biopharmaceuticals, phytopharmaceuticals, and other similar products. Category 2 contains APIs and drug intermediates. Category 3 includes *in vitro* diagnostic devices, repurposed drugs, anti-cancer drugs, and other related products.³³

Foreign Direct Investment (FDI) for medical devices

India now holds the top spot in the global pharmaceutical industry. Nevertheless, the medical device industry could not replicate this success. The nation boasts a significant number of researchers and engineers who can propel the medical device industry to unprecedented heights. The local financial system fails to supply the industry with vital investment. By press note no. 2 of 2015, the Government of India has examined the FDI policy for pharmaceutical companies and determined that a particular breakout would be for medical devices. Up to 100% FDI is allowed to produce medical devices in the automated route. Both brownfield and greenfield developments are exempt from the following requirements:³⁴

- Non-compete provisions would only be permitted under certain conditions and with the Foreign Investment Promotion Board's permission.
- The document must be submitted with the FIPB petition by both the potential investee and the investor.
- The federal government can include suitable FDI requirements when approving a brownfield project.

Export opportunity for medical devices

India has become a hub for affordable and high-quality healthcare products, resulting in an increased export of medical devices that offer a diverse range of products, from diagnostic equipment to hospital furniture. However, exporting material from India requires proper arrangements and adherence to the rules. Notably, CDSCO is responsible for issuing the NOC for the exportation of medical devices from India.^{30,35}

Requirements for medical device export in India compliance by licensees

The Medical Devices Rules, 2017, govern the export conditions for medical devices in India. Some general conditions that apply include: (i) *Registration*: Medical device exporters must have a valid license for CDSCO-registered devices; (ii) *Quality Management System*: Ensuring compliance manufacturing and

export procedures requires an efficient CDSCO import quality control system, frequently in line with ISO 13485; (iii) *Product Classification*: Due to varying laws and regulations, an exporter is required to appropriately categorize the medical supplies according to its expected application and safety grade; (iv) *Standards Compliance*: Indian Pharmacopoeia, IEC standards, and device-specific standards are only a few examples of the pertinent national and international standards that the medical device must meet.

Furthermore, it may be noted that other conditions also play pivotal roles, including (v) *Labelling and Packaging Requirements*: The exporter must ensure proper labeling and packaging of the medical device, with essential information included; (vi) *Clinical Data*: For legal compliance, scientific information or security and efficacy proof may be needed, based on the risk category and purpose of the device; (vii) *Export Documentation*: For exporting reasons, the correct documentation, such as bills, packaging lists, details about shipping, as well as other required records, must be kept; and (viii) *Adverse Event Reporting*: It is crucial to set up a system for gathering, evaluating, and reporting adverse events or accidents involving the medical equipment.^{36,37}

Essential documents for exporting medical devices from India

In India, the documents required for exporting medical devices may differ depending on the specific device. A significant set of essential documents must include (i) *Free Sale Certificate*: This certificate is issued by the regulatory authority to authorize that the medical device is freely sold or marketed in India; (ii) *Manufacturing License*: A duplicate copy of the production license that was acquired from the concerned Indian authorities; and (iii) *Registration Certificate*: If the medical device is registered with the CDSCO, a copy of the registration certificate must be provided.

Furthermore, documentation on product specification and appraisal must include documents ranging from (iv) *Technical Specifications*: Comprehensive practical specifications of the medical device, including plan, parts, and industrial procedure; (v) *Product Information*: Product leaflets, labeling, and directions for use; (vi) *Quality Certifications*: Any quality certifications obtained

Table 2: Classification of medical devices in India.

Class	Risk	Examples
A	Low-risk medical devices	Bandages, syringes
B	Low-to-moderate risk medical devices	Non-invasive devices like stethoscopes
C	Moderate-to-high risk medical devices	Orthopaedic implants, X-ray machine
D	High-risk medical devices	Cardiac implants, implantable defibrillators

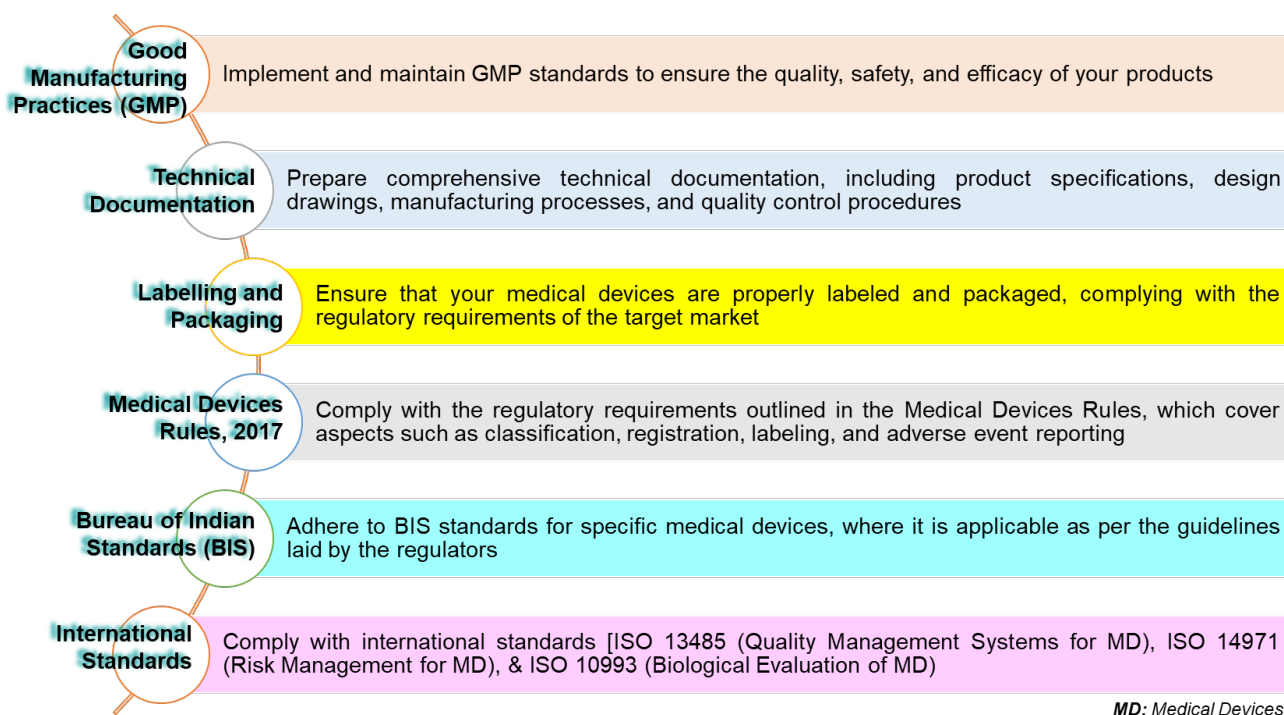


Figure 5: Various quality standards and regulations must be met by medical devices.

for the medical device, such as ISO 13485 (Quality Management System for Medical Devices); (vii) *Clinical Data*: Clinical trial information or other relevant clinical proofs giving safety confirmation and efficacy of the medical device; (viii) *Regulatory Approvals*: Documents of regulatory approvals obtained from international regulatory authorities, if applicable;^{3,11,23} and (ix) *Importer/Exporter License*: A proof of the exporter or importer license obtained from a suitable consultant.

Process for exporting medical devices

It is imperative to understand the process and protocols needed to be followed for exporting medical devices in India, which essentially can be subdivided into the following significant steps:

Step 1: It is necessary to understand India's regulatory framework for exporting medical devices to India. The CDSCO regulates medical devices in India and has established rules to ensure safety, quality, and efficacy. After this, market Research and product selection will be initiated. Research potential export markets for your medical devices, considering demand, competition, pricing, and regulatory requirements. Assess compliance with international standards and regulations for your specific devices.

Step 2: The medical devices in India are categorized into four levels based on the level of risk associated with their intended use. This classification determines the documentation and other regulatory requirements needed for exporting. The classification is shown in Table 2.

Step 3: Obtaining the licenses and registrations to export medical devices from India legally.

The following are some essential requirements:

- **Medical Device Manufacturing License:** The CDSCO license or the State licensing authority ensures compliance with quality standards and is mandatory for manufacturing or selling medical devices.
- **Import-Export Code (IEC):** To obtain an IEC, the applicant needs to register with the DGFT or the Director General of Foreign Trade. This code is essential for export transactions and can be obtained online by submitting the required documents.
- **Free Sale Certificate (FSC):** The application is made to the CDSCO to get an FSC, which certifies that the medical devices are freely sold in India. This certificate is often required by foreign authorities for the clearance of imports.
- **ISO Certification:** Obtain ISO 13485 certification, which demonstrates compliance with international quality standards for medical devices.
- **Goods and Services Tax (GST) Registration:** Register for GST with the appropriate tax authority if your turnover exceeds the prescribed threshold.

Step 4: Ensure your medical devices meet quality standards and regulations Figure 5.^{11,21,36}

Step 5: Conduct market research to identify potential export markets for your medical devices. Consider factors like demand, competition, regulations, and reimbursement policies. Identify



Figure 6: Documentation and logistics requirements for exporting medical devices.

partners who can assist with market entry and distribution in target countries. Documentation and logistics requirements for exporting medical devices are listed in Figure 6.

Step 6: Customs Clearance: Ensure compliance with customs rules and procedures. Submit the obligatory papers to the customs authorities, including the shipping documents, invoice, packing list, and other relevant certificates. Pay any applicable customs duties and taxes. If necessary, engage a licensed customs broker to facilitate a smooth customs clearance process. Then, one needs to evaluate the post-export responsibilities. The shipment is to be tracked until it reaches its destination. Maintain communication with the importer to address any concerns or inquiries.

Role of the Department of Pharmaceuticals (DoP) in Medical Device Policy

To improve and move forward with the implementation of the National Medical Devices Policy, 2023, the DoP has released an expanded approach document that focuses on privacy and security policies, the implementation of national designation for medical devices, a plan to manage e-waste of obsolete medical devices, and the enhancement of technology transfer capabilities within the medical device sector.

According to the strategy document, consistent with the approach paper on the NMDP, which was published for stakeholder

consultation, and the NMDP, 2023, which was notified on May 2, 2023, the government would consider aligning medical device regulations with national laws for personal health data security. It stated that appropriate data security and data annotation procedures will be attempted. It should be emphasized that, with the rapidly increasing impact and the use of digital technology platforms in the medical and healthcare sector, there is a rising need for sufficient security protocols to safeguard the confidential data of patients.

Furthermore, the DoP also stated that it will work closely with the CDSCO and the Department of Health and Family Welfare to develop a National Nomenclature for Medical Devices. As part of its regulatory restructuring efforts, the policy also encourages the implementation of Global Medical Devices Nomenclature (GMDN) or Universal Medical Device Nomenclature System (UMDNS) and establishes guiding rules under Standard Setting Organizations, such as the BIS, to guide innovators and entrepreneurs throughout the developmental stages of a medical device product. It also aspires to prepare researchers, innovators, and entrepreneurs during the testing phase.

These guidelines aim to encourage the research and innovation fraternity to consider regulatory compliance prerequisites from the outset of product development, employing programs such as course content, skill development, routine training, and

seminars. The NMDP-2023 encompasses six broad areas aimed at promoting the medical devices industry for general awareness, investment climate, infrastructure development, facilitating R&D, motivating need-oriented innovation, trained manpower generation, regulatory streamlining, and brand management.

The strategy paper specifies that, as part of infrastructure development, mechanisms for e-waste management of old medical devices would be created in close collaboration with the Ministry of Environment, Forest, and Climate Change. It emphasizes that fostering the tech-transfer competences would create additional chances to translate R&D set-up grown ideas and knowledge into goods and technologies to support innovation. Technology transfer agencies established by the Indian Council of Medical Research (ICMR), Patent-Mitra, DBT-PACE, DBT-BIRAC, and AGNI would be engaged to facilitate technology transfers. The program aims to build and foster an R&D-centric environment to mediate Innovation as part of the policy of these departments to catalyze research and innovation in the medical device segment of the country. It aspires to create a framework that can support inventors and IP holders in commercializing their inventions. It looks at incubation support for startups in R&D and innovation, mentorship for entrepreneurs along with skill development, such as business management, market access, and commercialization efforts for the innovation solutions development, technology transfer mechanism for innovative solutions, and relations for startups in compliance and regulatory regime as financial and non-financial measures to invite and motivate investments in this sector. As human resources in the field of design are scarce, the National Institute of Design (NID) and private design schools are motivated and encouraged to provide design-centric curricula and courses to fill the gap, according to the human resources development strategy.

One noteworthy intellectual property development plan is to collaborate with DPIIT on medical devices, stimulating innovation by incentivizing both institutions and inventors, and building a strong technology transfer ecosystem within the country to capitalize on the utilization of government-funded discoveries. Basically, the focus is towards developing creative products that create new IPR and enter the market. This will necessitate improved support for startups looking to expand globally.

ESTABLISHING THE CLINICAL PERFORMANCE OF MEDICAL DEVICES

A clinical trial of an investigational medical device must be conducted on human volunteers to establish its biosafety and effectiveness. This applies to novel IVDMD, where clinical trials must be performed on samples from volunteers to assess its performance. Such clinical evaluation is performed based on protocols as detailed in the clinical investigation plan, which governs their conduct. During this time, the constitutional rights

and safety of human volunteers must be protected as per the ethical protocols mentioned in the Declaration of Helsinki.³⁸

Medical device products considered under IMDR are required to undertake (i) a pilot investigation and (ii) a critical clinical study. For all completed clinical trials, the sponsor needs to provide the clinical trial report and clinical performance evaluation report to the human ethics committee, volunteers, and the central licensing authority of the country.³⁹

In India, clinical investigation data is mandatory for all class B, C, and D categories of medical devices that do not have a predicate product or if the medical device product falls under the new IVDMD category. On the other hand, if the medical device has been successfully available in the market of the countries such as the USA, Australia, Japan, Canada, or in Europe for more than two years, and the regulatory bodies of the respective Central Licensing Authorities in that country are contented with the available clinical investigation evidence, then a separate set of Clinical report is not warranted to issue the import license. However, in these cases, the Central Licensing Authority requires the post-marketing investigation report, review report of the concerned subject expert committee, and their recommendation.

To obtain an import license for medical device products from other countries, a detailed clinical investigation must be conducted in India for class C and D medical devices to establish their safety and effectiveness. For Class A and Class B medical devices, an import license can be approved if the safety and performance have been proven via a clinical investigation in the country of origin. Furthermore, the Free Sale Certificate is a mandatory export document required by law for exporting products to a specific country. This Free Sale Certificate must also be provided by the country of origin, along with documents that support the claim of a clinical investigation conducted in the country of origin for the issuance of an import license, in cases of Class A and Class B medical devices.

PROTOCOLS FOR REPORTING POST-MARKETING SURVEILLANCE OF MEDICAL DEVICES

A post-market surveillance report is required from the manufacturer for all medical devices introduced into the market. This report must comprise the details of the reporting protocol, complaints, and the corrective and preventive measures taken to address the complaints.⁴⁰ For medical devices that do not have a predicate, manufacturers must also conduct a post-marketing clinical investigation to ensure ion safety and performance, with special emphasis on drug-device interactions, safety studies, mortality and morbidity studies, among other aspects.

A periodic safety update report is a pharmacovigilance document that provides a comprehensive and critical assessment of the risk-benefit ratio of a product at a given time after its

authorization.⁴¹ The periodic safety update report considers all newly established safety norms to provide cumulative evidence on associated risks and benefits. For clinical safety monitoring, a Periodic Safety Update Report for each medical device product is mandatory to report relevant data collected by all stakeholders and correlate this information with patient exposure.

The periodic safety update report must also provide a summary of remarks on the existing market authorization status of the product in other countries, highlighting notable discrepancies related to safety. The report should also highlight whether any deviations will be applied to the product information to adjust its usage. Any substantial variations in the reference safety information relating to contraindications, adverse reactions, precautions, warnings, or any significant observations from ongoing/completed clinical investigations or substantial nonclinical findings observed within the reporting period must also be recorded in the report.

Continuous monitoring facilitates the timely detection and assessment of adverse effects of medical devices, malfunctions, and other issues.^{42,43} In this regard, to foster medical device monitoring, minimize risk, and ensure a tolerable risk-benefit ratio, the Indian Pharmacopoeia Commission has introduced the Materio-vigilance Program of India (MvPI). Since its launch, this program has been vital in screening as well as the recall of several non-compliant medical device products from the Indian market.⁴⁴

INDIAN PERSPECTIVES OF INDUSTRIAL-ACADEMIA COLLABORATIONS FOR DEVELOPING MEDICAL DEVICES

Recently, in India, the collaborative efforts between industry and academia for the development of medical device products have been increasing. Such collaborations play a crucial role in fostering innovations and bridging the gap between academic research and its industrial application to address healthcare concerns. Such collaborations are expected to leverage resources, facilities, and expertise from both industry and academia, thereby enhancing innovations and research capabilities in the development of medical devices. Industrial experts can thus work closely in collaboration with academia and contribute to curriculum development, ensuring that learners are equipped with the relevant skills and knowledge to make a better contribution to the progress of the medical devices sector within the country.

In one such instance, Boston Scientific teamed up with the Indian Institute of Technology (IIT)-Ropar and launched a customized course for engineers at Boston Scientific.⁴⁵ This initiative was first conceived in 2021 and has continued since then. Boston Scientific intends to scale up these efforts and develop customized, multidisciplinary courses that cover various domains of medical device design and development. Such efforts to upskill the workforce help industries achieve accelerated employee learning and promote academic institutions to produce industry-ready talent. Such collaborations are necessary to align research and development activities with international standards and trends, ultimately ensuring the global acceptance of Indian medical devices. Table 3 provides a few examples of industry-academia collaborations in the medical device sector.

Table 3: Some industry-academia collaborations in the domain of medical devices.

Collaborating Bodies	Product developed	Description of product	References
Boston Scientific, India, and IIT-Ropar	Customized course	A customized course for the engineers at Boston Scientific India covering various domains of medical device design and development.	45
Tata Medical Center, Kolkata, and IIT, Kharagpur	CHAVI	India's first imaging bank for oncological imaging data, with access to researchers all over the globe for research purposes.	46
ICMR, DHR, and IIT-Delhi	Pragati	medical device manufacturing and testing facility at IIT, Delhi, to provide support for the translation of technologies and products from the proof-of-concept stage to the clinical evaluation stage.	47
IIT Bombay and Effecmed Pvt. Ltd.,	Bone Screws	low-cost, bioresorbable bone screws for soft tissue and bone fixation applications especially in bone fracture surgeries.	48

Tata Medical Centre, Kolkata, and the Indian Institute of Technology (IIT), Kharagpur, have collaboratively developed India's first cancer imaging bank- CHAVI.⁴⁶ CHAVI was created as part of the National Digital Library of India (NDLI). CHAVI is a comprehensive archive database of imaging that enables researchers worldwide to freely access patient images and relevant medical data for investigation purposes. The image bank permits researchers to evaluate the effectiveness of existing treatment regimens and promote the evolution of novel protocols to improve therapeutic effects. Although CHAVI is a database specifically for oncological imaging data about India, such collaborations at the international level would contribute immensely to global cancer care.

Department of Health Research (DHR), ICMR, and IIT-Delhi jointly established the MedTech Product Development Acceleration Gateway of India (mPRAGATI), which is a medical device manufacturing and testing facility to provide support for the translation of technologies and products from the ideation, proof-of-concept, to the clinical evaluation stage.⁴⁷ The mPRAGATI offers services, including product conceptualization, design, prototyping, testing, and regulatory compliance, all under one roof. This enables the rapid conception, execution, and application of innovative ideas and medical device technologies. These initiatives created fertile ground to flourish the medical devices sector in terms of research and development, as well as the commercialization of products.

The researchers at IIT Bombay, in collaboration with Effecmed Pvt. Ltd., a startup registered at Society for Innovation and Entrepreneurship (SINE), IIT Bombay, have developed low-cost, bioresorbable bone screws for soft tissue and bone fixation applications.⁴⁸ This opens the gateway to immense opportunities in orthopedic applications owing to the product's superiority over the existing ones in terms of biodegradability and patient compliance. This technology has been patented, and collaborators are planning to file another patent for the improved design of bone screws, aiming to enhance function in human physiological settings.

The Memorandums of Understanding (MoUs) between industry and academia ensure close cooperation between the two institutions to enhance the mutual skills and knowledge of the domain of medical devices. Given this, IIT Bombay has signed an MOU with Max Healthcare to foster joint research endeavors, skill development and training, technology transfer, and commercialization.⁴⁹ Similarly, IIT Madras has signed an MoU with MGM Healthcare.⁵⁰

Considering the recent launch of the medical science and technology department at IIT Madras, this MoU holds immense importance. The MoU will facilitate a platform for student-employee exchange between the two parties. IIT Jodhpur

has signed a MoU with Johari MedTech to establish the Johari Incubation Centre at IIT Jodhpur.⁵¹ This incubation centre will serve as a bridge between these two bodies, facilitating the development of innovative products and technologies related to medical devices. It will also serve as a funding source for aspiring startups in the medical device sector. Similarly, IIT Goa has signed an MoU with Sciverse Solution for the design, development, and fabrication of lab-on-chip and microfluidics-based technologies for rapid, cost-effective, and sensitive detection of infectious diseases and pathogens such as Human Immunodeficiency Virus (HIV) and tuberculosis.⁵¹

The National Institute of Pharmaceutical Education and Research, Ahmedabad (NIPER-A) has signed multiple MoUs with Johnson and Johnson and Sahajanand Laser Technologies to develop industry-oriented skilled human resources in the medical device sector.⁵² Such MoUs, being signed between academic institutes and industries, facilitate personnel exchange programs and the development of a skilled workforce.

The Tekade lab at NIPER-A is actively developing nanotechnology-assisted platforms for the diagnosis and treatment of various ailments, including cancer, arthritis, diabetes, and topical conditions, among others.⁵³⁻⁵⁷ Recently, NIPER-A and Madhya Pradesh Industrial Development Corporation (MPIDC) have entered into an MoU under which NIPER-A will provide technical and academic support to the Ujjain Medical Device Park. NIPER-A has also offered testing and certification services to the Government of Madhya Pradesh for the MD Park. The MoU was signed during the Spiritual and Wellness Summit in Ujjain by Dr. Shailendra Saraf, Director of NIPER Ahmedabad, in the eminent presence of the Chief Minister of Madhya Pradesh, Dr. Mohan Yadav.

Although collaborations between academia and industry are crucial to achieving India's goal of a higher contribution to the global medical devices market and improved patient care, it is the responsibility of policymakers to frame and implement policies and regulations that are conducive to these goals. The union cabinet of the government of India, given the expanding medical devices sector, has come up with the National Medical Devices Policy (NMDP) 2023, with the idea to lead the medical devices sector on a path of fast-tracked growth with a patient-centric approach to serve the growing healthcare needs by building an innovative and globally competitive industry in India. NMDP 2023 serves as a stepping stone in establishing India as a leading player in the global medical devices market.

INNOVATION TRENDS IN MEDICAL DEVICES: "CASE STUDIES"

India has witnessed several innovative trends in the medical device industry, with a particular emphasis on addressing healthcare issues, enhancing accessibility, and improving patient

care. In India, a few innovative trends in medical equipment are as follows:

TTK Chitra heart valve

The TTK Chitra heart valve is among the most reasonably priced heart valves available worldwide and the first heart valve to be manufactured in India. The Sree Chitra Tirunal Institute for Medical Sciences and Technology (SCTIMST), a nationally significant institution housed within the Department of Science and Technology (DST) of the Government of India, is responsible for its design and development. Since 1990, it has been implanted in approximately 300 cardiac clinics throughout India. The production plant is situated in Trivandrum's KINFRA International Apparel Park. The three parts that make up the heart valve are the sewing ring, the frame, and the disc. Its design incorporates excellent hemodynamics, structural integrity, a low profile, and smooth operation. The special plano-conical disc is positioned to maximize flow through the small aperture. The disc has thromboresistance due to its highly polished, slick surface that resembles wax.⁵⁸

Altius™ (Cemented Hip Replacement System)

RCH Orthopaedics, one of India's leading manufacturers and international suppliers of hip replacement implants, is the developer of the Altius Cemented Hip Replacement System. Altius is a High-nitrogen Stainless Steel (HNS) double-tapered stem that has been cemented and fitted with a PMMA/UHMWPE distal centralizer to assist with stem placement and equal distribution of cement mantle. Hip replacement has been successfully using a collarless, highly polished, double-tapered design that served as the model for the cemented stem for over 45 years. Because ALTIUS is a collarless stem, it makes it easier for surgeons to deal with a wide range of patient anatomy and indications, including primary cases and fractures. In a surgeon's practice, it can be selected as a whole or partial hip replacement method.⁵⁹

SanketLife Pro⁺

A unique Electrocardiogram (ECG) gadget, the SanketLife Pro⁺ can be employed for wired and touch-based ECG and stress and HRV level monitoring. To use the gadget for wire-based 12-lead ECG, attach a cable and converter. Both on-site and remote ECG testing can be performed with this portable ECG gadget. SanketLife Pro⁺ is a simple device for immediate cardiac monitoring at home or in an ambulance. The highest quality requirements were taken into consideration when creating this ECG monitor. This gadget works with both iOS and Android smartphones. It also provides the opportunity to have your ECG professionally reviewed, and may also immediately communicate with a cardiologist to have your report evaluated.⁶⁰

SSi Mantra

SS Innovations, an Indian medical technology firm, is the creator of the "SSi Mantra," an affordable and adaptable surgical robotic device. SSi Mantra represents the first surgical device manufactured in India. It is among the few systems worldwide created for broad surgical usage and was notably cost-effective in design and construction. The CDSCO in India has granted regulatory approval to the technique, which has also undergone hundreds of clinical validations in the country through its use in over 40 distinct surgical operations, including cancer, spinal, head, and heart surgeries. The SSi Mantra Surgical Robotic device, the flagship innovation of the SSi Team, is designed with numerous advanced and intuitive features for the entire surgical team. The company thinks that many more hospitals, doctors, and patients will be able to take advantage of this cutting-edge non-invasive approach because it additionally offers multi-specialty usage, including cardiac surgery, but also costs less than half of the world's best-selling surgical robot.⁶¹

US 111

The US 111 Medical Ultrasound Therapy equipment is a well-known medical breakthrough that offers a natural, non-invasive solution for treating chronic muscle pain. With the help of crystals vibrating within the probe's head, this handheld ultrasound treatment device, featuring advanced technology, produces both pulsed and continuous sound waves at a frequency of 1 MHz. These waves subsequently penetrate through the skin to a depth of 1.6 inches (4 cm) into the body tissues and muscles, leading to deep heating that the patient typically does not feel. When used promptly and consistently, US 111 can help accelerate the recovery of muscle pain and reduce swelling and inflammation.

Using the settings on the gadget, one can adjust the duration, intensity, and mode of treatment according to the patient's pain level. US 111 has been scientifically shown to be a highly effective pain reliever. Pain management specialists, including doctors, chiropractors, and physical therapists, have tested, authorized, and even utilized US 111. Due to its fundamental functionality, the device's operation requires no specialized knowledge or assistance. A Naturecure ultrasonic gel, a power adapter, an ultracool pack, an instruction booklet, and a stylish carrying pouch are included with the US 111 set.⁶²

AyuLynk

The AyuLynk Wired Digital Stethoscope is a specially designed medical device for caregivers working in demanding situations, such as emergency rooms. In addition to supporting noise reduction, the denoise feature and the in-app heart and lung filters can detect minute sounds that confirm the diagnosis. With the help of an amplifier, this cardiac stethoscope can improve audio quality by up to 40 times, which can help prevent late diagnoses

by conducting more accurate auscultation examinations. The visually impaired digital stethoscope on the Ayu device may be used to record and share auscultated sounds when linked to a smartphone or tablet via an OTG connection with the AyuShare app. In addition, the app displays the phonocardiogram of the auscultated sounds, which aids in objectively assessing the sounds of the heart and lungs. AyuSynk is the most cost-effective diagnostic instrument for normal practice and telemedicine to improve diagnosis and prompt detection.⁶³

DRISTI

Diabetic Retinopathy Screening (DRISTi) is an artificial intelligence tool designed to instantly identify patients with Diabetic Retinopathy (DR) at an early stage during the eye examination procedure. They have brought DR screening to the most remote corners of the world by breaking the cord and developing the first AI on a chip. Their goal is to reach out to communities without access to DR specialists by offering early screening at no cost. This application is simple to use, and integration with retinal cameras on smartphones and skilled technicians for prompt and early assessment is also offered.⁶⁴

MARKET SIZE AND GROWTH RATE OF MEDICAL DEVICES

The Indian medical device market is projected to approach US\$50 billion by 2030 at a CAGR of 16.4% from its estimated value of ₹90,000 crores (approximately US\$11 billion) in 2022. India is projected to account for 1.65% of the global medical device market. India ranks among the foremost 20 medical device markets worldwide and the fourth-largest Asian market for medical devices, behind China, South Korea, and Japan. In general, India relies on imports to meet 75-80% of its medical device needs. India is a major importer of medical devices to the US, China, Germany, Iran, Brazil, and other nations.

The Indian states that produce the majority of medical devices are Gujarat, Andhra Pradesh, Maharashtra, Tamil Nadu, Haryana, Karnataka, and Telangana. During a medical device panel discussion at BioAsia 2021, major stakeholders projected that India would achieve self-sufficiency in medical device production by 2025-2026. The panel noted that to support the overall development of India's domestic medical device market, the government is implementing supportive measures, such as encouraging the manufacture of high-tech medical devices domestically, offering Production-Linked Incentive Schemes (PLIs) on medical equipment, and enhancing novel medical device parks.⁶⁵ Under the Medical Device and *in vitro* Diagnostic (IVD) laws, the Health Ministry of India has classified the Indian medical device market into four distinct groups.⁶⁶

While addressing the India MedTech Expo 2023 gathering in August 2023, Dr. Mansukh Mandaviya, Union Health Minister of India, declared that the country is set to emerge as an

international hub for medical device technologies. Medtronic announced in May 2023 that the company would invest over US\$350 million, or approximately ₹3,000 crore, to develop the Medtronic Engineering and Innovation Centre (MEIC) in Hyderabad. MEIC is Medtronic's biggest R&D facility outside of the United States. Omron Healthcare, a Japanese distributor and manufacturer of personal healthcare goods, announced in May 2023 that it will establish a medical device production facility in Tamil Nadu for US\$15.5 million (₹128 crores).

Siemens Healthineers, a medical technology company focused on digitalizing healthcare, developing care delivery, enhancing patient experience, and precision medicine, announced in March 2023 that it would invest ₹1,300 crore (US\$157.2 million) at Bommasandra, Bengaluru, to establish a campus. Wipro GE Healthcare announced in August 2022 that it has partnered with Boston Scientific, a leading manufacturer of medical devices, to deliver comprehensive, state-of-the-art cardiac interventional care solutions to India. Northern India's first medical instrument and system manufacturing park is anticipated to open in Gautam Budh Nagar, Noida, by 2022. A mission scheme of around ₹5,000 crore (US\$685.35 million) is expected to be introduced by the Yamuna Expressway Industrial Development Authority (YEIDA) in March 2021. The central government is expected to contribute approximately ₹100 crore (US\$13.71 million) of this amount.⁶⁷

SOME PLAYERS IN THE MEDICAL DEVICES MARKET IN INDIA

There are several National and International critical players in the Indian medical device market. The national vital players are the med-tech companies, usually startups or developing. In contrast, international vital players are the internationally based med-tech companies that have established their headquarters in India. Both key players are discussed in this section as follows:

Monitra Healthcare Private Limited

The Hyderabad-based medical technology company Monitra Healthcare is creating tools to record cardiac occurrences anywhere. These devices identify cardiovascular disease and cardiac arrhythmias in real-time to provide preventive care. The diagnosis of heart illness, the assessment of therapeutic medications' effects on cardiac activity, and the monitoring of cardiac activity before and during surgery, as well as other cardiac operations, are all common uses for cardiac monitoring. The company's goal is to significantly lessen the impact of cardiovascular disease by integrating preventive cardiac care and monitoring into daily living.⁶⁸

Robo Bionics

Robo Bionics is a medical technology firm established in 2016 in Mumbai, with the tagline "to give a hand to those who are

missing a hand. They developed Grippy, a Functional Upper Limb Prosthetic Hand. Grippy is India's first 3D-printed prosthetic hand with multi-grip control and a tactile sensation, approved by the NABL Lab for safety testing. It is created, developed, and manufactured with pride in India. Grippy is a lightweight, reasonably priced, battery-operated prosthesis currently accessible in the Indian market for patients with below-elbow amputations who are at least 15 years old.⁶⁹

Brainiac Healthcare

Brainiac Healthcare, a medical technology firm based in Ahmedabad, specializes in developing cutting-edge, innovative medical equipment for healthcare professionals. They have developed the world's first smart capnometer gadget. The Internet of Things powers this respiratory CO₂ gas monitoring equipment (capnometer) for anesthesiologists and can be operated via a mobile app. They have created a little hardware piece that is attached to the patient in the operating room during the operation. The patient's data will be wirelessly transmitted via the hardware to the mobile application, where it will be processed and shown in both numerical and graphical form.⁷⁰

Team Spectral Insights

Team Spectral Insights is a 2016-founded medical technology firm in Bangalore, specializing in digital pathology and analytics to serve the pharmaceutical, hospital, and laboratory industries. They provide the pathology area with a completely vertically linked digital solution. One standard hardware unit and multiple software modules are required to handle the range of tests carried out under a microscope in a pathological lab-biopsies, pap smears, blood smears, bone marrow smears, TB sputum smears, etc. They have developed an extremely inexpensive, completely automated digital tool that creates stitched panoramas, photos from glass slides, and tools for objectively classifying objects and producing results for users. Machine learning and Artificial Intelligence (AI) algorithms have been developed to assist with a rapid assessment of pap smear slides to identify questionable cells, count morphologies of Red blood cells and White blood cells on blood smears, and identify cancer spots on tissue biopsies.⁷¹

Designocare Innovative Devices Pvt. Ltd.,

Designocare Innovative Devices is a Gurgaon-based medical technology startup firm focused on the research and development of medical devices. The Unique Hospital Bed is the company's debut product, and creativity in the healthcare industry is its foundation. Moving a patient from a stretcher or bed is typically done manually or with the assistance of complex transfer technologies that have limited application and unique challenges. The bed, which incorporates the stretcher into its design, is their solution. Therefore, the patient will not be manually moved from their bed to a stretcher.⁷²

Transasia*

Transasia is India's leading diagnostic company, established in 1979, and a member of the Erba Mannheim GmbH Group, a leading IVD solution supplier in Germany. Transasia Bio-Medicals Ltd., announced in December 2022 that it would be collaborating with the Andhra Pradesh Medical Services and Infrastructure Development Corporation (APMSIDC) to enable the distribution of the Erba range of fully and semi-automated biochemistry analyzers, urine chemistry analyzers, and coagulation analyzers. In August 2022, Transasia introduced the first domestically manufactured Reverse Transcription-Polymerase Chain Reaction (RT-PCR) kit for monkeypox testing at the Andhra Pradesh Medtech Zone (AMTZ). The Mumbai-based IVD company Transasia Bio-Medical Ltd. announced in March 2021 that it would invest ₹150 crore (approximately US\$21 million) to establish its manufacturing facility at Telangana's Medical Devices Park in Sultanpur. The company intends to produce cutting-edge, high-tech analysis equipment in the facility for biochemistry, hematology, immunology, and molecular testing, as well as for COVID-19, dengue, HIV, and tuberculosis testing, serving both local and international markets.⁷³

Medtronic

In 1979, Medtronic India Pvt. Ltd. started conducting business in India, and it is an entirely operated subsidiary of the Irish-American medical device manufacturer Medtronic Plc. Across India, the organization is present in cities like Gurgaon, Vadodara, Hyderabad, Pune, Ahmedabad, New Delhi, Bangalore, Chennai, Kolkata, Cochin, Dhaka, and Mumbai. The Cardiac and Vascular Group (CVG), the Diabetes Group, the Restorative Therapies Group (RTG), and the Minimally Invasive Therapies Group (MITG) are the four business areas that make up this organization. Medtronic India and Qure.ai announced their partnership in April 2023 to incorporate AI for improved stroke treatment in India. The MiniMed 780G, a future-oriented closed-loop insulin pump structure, was introduced by Medtronic India in March 2022 to treat type 1 diabetes in patients aged 7 to 80. Medtronic India Pvt. Ltd. opened a robotic surgery experience hub in Gurugram in September 2021, to aid physicians with surgery performed with robotic technology.⁷⁴

Johnson and Johnson

Johnson and Johnson is one of the world's leading manufacturers in the healthcare industry, having been founded in 1886. The company is the largest provider of medical devices in India, offering products that target unmet demands in the areas of diabetes management, metabolic and bariatric surgery, hernia surgery, cancer surgery, general surgery, vision care, urologic surgery, cardiovascular disease, peripheral vascular and obstructive disease, coronary artery disease, neurovascular disease, orthopedics, arrhythmias, and infection prevention. According to information released in March 2022, Stayfree

and United Nations International Children's Emergency Fund (UNICEF) have taught over 1.7 million girls about Menstrual Hygiene Management (MHM) during the previous seven years. The firm stated in June 2021 that it is in discussions with the Indian government to introduce the COVID-19 vaccine as a single dose. India approved Johnson and Johnson's COVID-19 single-dose vaccine for emergencies in August 2021. They established a public-private collaboration in 2015 with the National Institute of Pharmaceutical Education and Research (NIPER) in Ahmedabad, India, for a postgraduate training curriculum for future regulators of the medical device industry.⁷⁵

Siemens Healthineers

Siemens Healthineers, a German enterprise, offers services and solutions for the healthcare industry. Their production plants are located in Bangalore and Vadodara, India, for the medical imaging and diagnostic products. Siemens Healthineers and the Manipal Academy of Higher Education (MAHE) signed a Master Research Collaboration (MRC) in August 2023 to further enhance their future collaboration and achieve shared objectives for stakeholders. Siemens Healthineers stated in September 2021 that its Vadodara, Gujarat, facility will produce molecular diagnostic kits. Siemens Healthineers and SyntheticMR expanded their partnership in September 2021 with the signing of a new license agreement for product distribution. In 2020, Siemens Healthineers, a multinational med-tech firm, committed an investment of ₹1,300 crore (US\$177 million) in Bangalore, Karnataka, over the following five years, aiming to establish India as one of its four primary global hubs for digital innovation.⁶⁷

MAJOR CHALLENGES FOR THE MEDICAL DEVICE INDUSTRY

Medical device manufacturing can be a highly intricate and complicated business. The medical devices sector in India is a vital and essential component of the Indian healthcare sector. As per the Government's medical devices draft policy, the share of India in the total market is approximately two percent, while the sector is still in its infancy phase in the country.^{76,77} The country is heavily dependent (approximately 80%) on imports from other countries supply of sophisticated equipment (viz: diagnostic devices, imaging modalities, etc.). Local companies are mostly restricted from manufacturing low-end products for domestic and international consumption.⁷⁸

Recently, the government launched a PLI scheme to motivate investments, boost indigenous manufacturing towards reducing dependency on medical device imports. Nevertheless, the "Make in India" and "Atmanirbhar Bharat" programs are admirable, domestic production of medical devices is still distant from achieving self-reliance in producing world-class medical devices.⁷⁹ While the government is attempting to simplify regulations and

paperwork, the landscape remains complex, characterized by the presence of numerous high-level government bodies at both the state and central levels. Also, India's per capita expenditure on health is among the lowest in the world. The government's focus on self-reliance is appreciated, but the changes need sustained financial and policy support from the government.

Notwithstanding the strong growth of the medical device industry, it is facing abundant challenges.⁸⁰ There is a continually rising demand for technology, which paints a positive outlook for future income, but many obstacles stand in the way. The following are the challenges of medical device invention in India.

Slow, Expensive R&D

More than any other industry, medical device manufacturing relies heavily on thorough research and development. Conducting the necessary clinical trials is often a slow and expensive process, which can delay time to market and hinder profitability. Our government spending on healthcare is only 3% of Gross Domestic Product (GDP), compared to the world average of 11%.⁸¹ This suggests that India has a significant shortage of hospitals and medical devices. One of the primary challenges encountered by the Indian medical device manufacturing industry is the deficiency of a robust research and development ecosystem. However, research and development efforts in the medical devices industry are limited, which hampers the development of advanced technologies and new products.⁷⁹ Many Indian medical device manufacturing companies give emphasis on producing cheaper, low-tech devices rather than investing in more advanced research and development.

Lack of supply chain visibility and product traceability

The recent COVID-19 pandemic faced by humankind emphasized the necessity for vigorous supply chain systems and has impacted medical equipment manufacturers. The existing size of the medical industry in India is approximately US\$12 billion, compared to a global market size of around US\$500 billion. Therefore, India accounts for just 2.5% of the global total available market.⁷⁶ Operating at this size makes the supply chain expensive and non-competitive. Indian manufacturers are often overlooked by global suppliers. As most components are imported into our country, we also suffer geopolitical risks. This resulted in delayed delivery and rising prices. Several firms were affected by the ambiguous supply chain, as well as the rising prices of raw/ starting materials, labor, and the end product, reaching sky-high levels. The end-user market, specifically hospitals, in India is highly fragmented, with numerous small hospitals spread across various geographies, resulting in an expensive downstream supply chain. Some companies ramped up operations to meet the requirements of demand and supply, to safeguard the timely Medicare to the patients.⁸²

Duplication, Imitation, and Counterfeit Devices

We live in an age of IP theft and iteration. Too often, new and innovative products are only seen by counterfeiters and imitators before they are released. This is a devastating blow to the medical device market, and these 'copies' are responsible for providing inferior medical care. These lookalike devices deceive patients and exacerbate the challenges of inadequate healthcare facilities, leading to further illness or injury. India has been criticized on global forums for its deficiencies in protecting intellectual property rights. This needs the immediate attention of the government to encourage global houses to bring innovation to India. Responsible manufacturers create the device with utmost care and compliance.^{79,83}

Regulatory challenges

Some life science segments are profoundly regulated, while others have very light regulatory oversight. One of the most pervasive challenges in medical device manufacturing is navigating the industry's challenging regulatory environment.¹¹ Manufacturers often face uneven regulations with inconsistent language and standards. The Indian government has recently introduced new regulations for medical devices; however, some confusion and uncertainty remain regarding the approval process and compliance requirements.

In India, the inconsistent structure and lack of global harmonization make it difficult for manufacturers to determine the correct regulatory pathway. This inconsistency also challenges patients and clinicians as they cannot determine what device is optimal for their healthcare. This gap further widens due to the costs associated with regulatory requirements. Exponential increases in time, cost, and effort are required to manage regulatory compliance, affecting the timely launch of medical devices.⁸²

Import-centric

Kits designed in other countries may fail to perform effectively due to India's unique climate and environment. Additionally, the kits are manufactured for well-equipped laboratories, but proper maintenance is often not performed in Indian laboratories. The regulator is challenged to tackle both the flood of substandard imports and the production of poor-quality medical devices in the country.^{81,82}

Proper training and education

A deficiency in training and education is another challenge for medical device invention. Developing and maintaining a skilled workforce is vital for the growth of a robust medical devices industry. However, India faces a shortage of specialized engineers, technicians, and healthcare professionals with expertise in medical device technology, which increases the risk of damage to

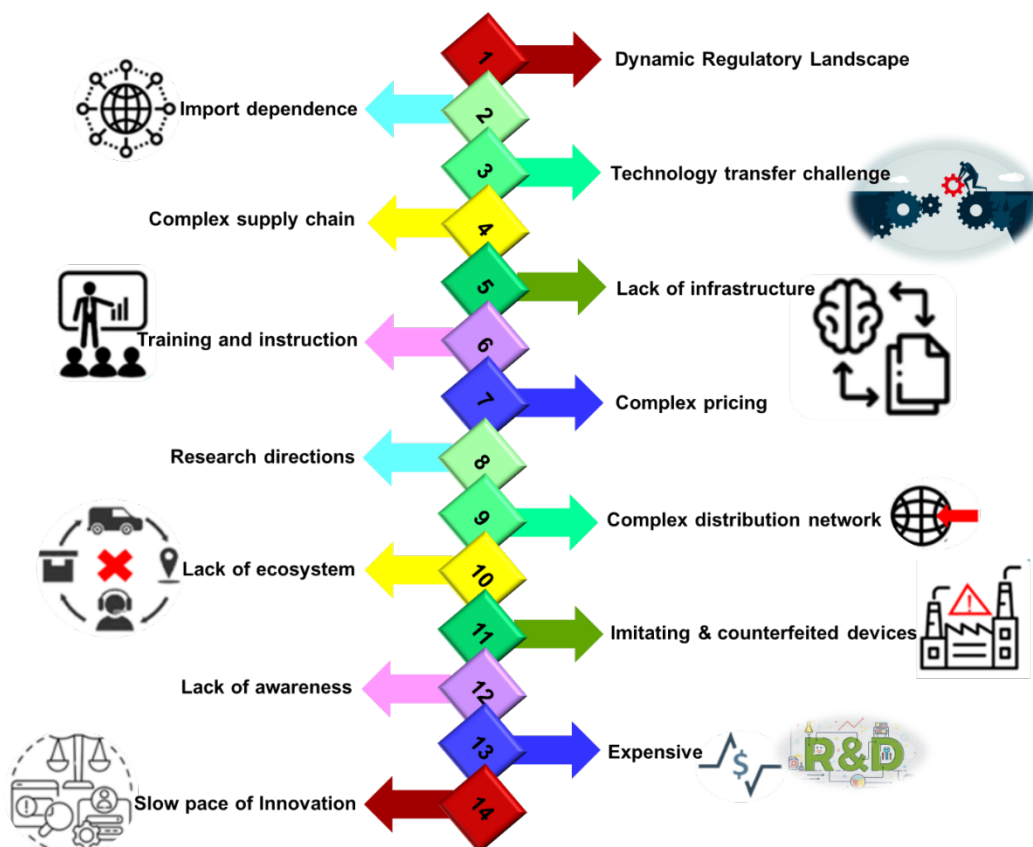


Figure 7: Challenges of the medical device industry.

the kits. This scarcity limits the industry's capacity to undertake and drive meaningful research projects. Bridging this skill gap through training and educational initiatives is essential.

Weak technology transfer process

Technology has spun out MedTech innovations. The integration of AI, ML, and other technological advancements in the medical industry is lucrative, but it also has an unexplored grey area. These advancements are designed to improve the patient experience; however, several factors should be considered. There is a huge gap between industry and academia. A World Intellectual Property Organization (WIPO) document stated that most Indian universities have not encouraged in-house projects.

Complex pricing and distribution

High costs are another significant challenge in the healthcare industry. Different states in India often have distinct pricing policies, resulting in a fragmented market. This non-uniformity complicates the pricing strategies for medical device companies nationwide. This fragmented distribution network can lead to higher mark-ups, inefficiencies, and complexities in reaching end-users. Medical device manufacturers face increasing pressure to lower product costs, but that can be a challenge with high production expenses.⁸¹

Lack of infrastructure and resources

India has a large population, and the demand for healthcare services is consistently increasing. However, the country's healthcare infrastructure is inadequate to meet the needs of its population. The Indian medical devices manufacturing industry lacks the necessary infrastructure to produce high-end medical devices. The country needs modernized testing facilities, proper storage facilities, and skilled workspace, which are currently insufficient.⁷⁹

Lack of awareness

Some medical devices are complex and may require specialized knowledge for their proper use. The lack of awareness about these devices can stem from their technical intricacies. The Indian population lacks awareness about the importance of medical devices, which results in a low demand for these products. This low demand makes it difficult for companies to invest in research and development, which ultimately affects the growth of the industry.⁷⁹

Lack of ecosystem

Healthcare costs are trending upwards because managing chronic diseases requires heavy deployment of medical devices. Many factors, from the planning phase to the use of medical devices, contribute to this upward trend in costs. However, the Indian medical devices industry struggles to attract investments due to the lack of a robust ecosystem for venture capital and angel

investors (Figure 7). Due to a lack of ecosystem, limited profit margins, decreased visibility across assets, and inability to optimize utilization and operational performance, medical device companies face some daunting challenges.⁸⁴ Manufacturers are vulnerable to the increasing costs of raw materials, innovation, and logistics, while patients are vulnerable to rising insurance, device, and healthcare costs. These costs must be adjusted to ensure economic stability in demand and supply.⁷⁹

GLOBAL HARMONIZATION INITIATIVES AND THEIR IMPACT

Establishing a reliable and consistent quality practice with minimum variation in safety assessment protocol significantly improves the patient safety record and diminishes the chances of associated risks.⁸⁵ Global harmonization initiatives play a crucial role in ensuring the availability of safe and efficacious products that meet the required quality standards, which are in line with global regulatory standards. The product manufactured with this thought process enhances patient acceptance and ensures the availability of a compliant product, while providing significant impetus for global trade.⁸⁶ The adoption of globally harmonized practices significantly nurtures innovation, owing to the simplification of the regulatory trail and the easy translation of newly developed products into the market. Eventually, this leads to development as well as compliance-enhanced access to novel and advanced technologies in the market.⁸⁷

It plays a vital role in reducing manufacturing cost, time-to-market cost, and promoting cost efficiency. A well-informed and integrated reporting system improved the post-market surveillance by rationalizing the follow-up procedure for reporting adverse events. This significantly safeguards the operation of the medical device monitoring throughout its lifespan. The following section provides an overview of various global harmonizing regulatory forums for medical devices.

Global Harmonization Task Force (GHTF)

Medical devices are an intricate and industrially advanced sector. Hence, the regulators and the governing bodies overseeing this sector must be the first to adapt to the review and regulation of these devices to confirm safety and efficacy. The quality management system is becoming increasingly complex and stringent, particularly in manufacturing, clinical investigation protocols, and post-marketing surveillance.¹⁴ This makes the review and harmonization of the regulatory progression process a mandatory formula to ensure a balance.

The Global Harmonization Task Force (GHTF) is a globally recognized forum established in 1993 by representatives from federal governments and industry nominees from the United States, Canada, Japan, Australia, and the European Union. The GHTF provides for both regulatory bodies and representatives from the medical device industry to address complex issues. The

primary objective of GHTF is to promote the harmonization of standards and regulatory practices concerning the performance, safety, and quality control of medical devices. The GHTF also works to foster high-tech innovation and facilitate international trade. To achieve this goal, it circulates harmonized documents for regulatory rules, regulations, and practices.

It promotes the alignment among affiliate countries, inducing regulatory frameworks and structuring the medical device regulation worldwide.⁸⁸ Initially, the forum comprises intriguing members from Canada, Japan, the USA, the European Union, and Australia. Over time, the membership expanded to include other governments from various countries, thereby enhancing its role and influence in global regulatory practices.⁸⁹ The GHTF played a vital role in laying the foundation for the IMDRF and continues to lead the international regulatory union, thereby enhancing the trade of medical devices (Chettri and Ravi 2024).

International Medical Device Regulators Forum (IMDRF)

The IMDRF was established in 2011 and has since played a key role as a global harmonizing regulatory body for medical devices. Its primary objective is to accelerate international regulatory convergence and promote consistent standards for medical devices that enhance user safety and efficacy.^{21,90}

In the evolving landscape of the medical device sector, IMDRF facilitates collective knowledge sharing for the development of effective global regulatory policies by fostering discussions among regulators from key markets. IMDRF clarifies the expectations of regulatory agencies, facilitating the easy alignment of regulatory requirements and promoting market access for medical device companies.⁹¹ It primarily focuses on aspects related to developing guidance documents that serve as internationally recognized references for regulatory agencies. IMDRF also emphasizes initiatives aimed at regulatory capacity-building and supporting emerging medical device markets in firming up their regulatory frameworks.^{13,92} Its collaborative approach helps address evolving challenges and discrepancies that arise during the innovation and commercialization cycle of a new medical device product, with due regard for public health.

The Medical Device Single Audit Program (MDSAP) is a notable initiative of the IMDRF that enables a single audit for medical device manufacturers to meet the regulatory requirements of multiple countries. This initiative significantly helped in avoiding redundant audits, thereby reducing the time and costs associated with launching a new medical device to market (Sethi *et al.*, 2017). The development of a universally harmonized UDI system is another key accomplishment of MDSAP. This led to an enhancement of the informed traceability of medical devices, ensuring reliable communication of critical information on medical device products across global markets.^{93,94}

The IMDRF guidelines have also aided global regulators in developing a classification system for medical devices based on associated risk concerns, enabling them to conduct thorough inspections to ensure patient safety through a regulated regulatory procedure. The ongoing efforts of IMDRF in harmonizing international medical device regulations are significantly enhancing patient safety and facilitating the regulated distribution of medical devices worldwide. Companies like Medtronic have effectively utilized MDSAP to streamline their regulatory procedures. It is anticipated that through unified initiatives like IMDRF, MDSAP, and UDI, the regulatory landscape will continue to evolve, ensuring it remains compliant to ongoing technological advancements and public health requirements.

AI IMPLEMENTATION AND FUTURE OUTLOOK IN MEDICAL DEVICES

The potential of a machine to replicate human intelligence and behavior, including decision-making and task execution, is known as Artificial Intelligence (AI). The fundamental algorithm serves as the basis for the strategies employed to integrate AI technologies into medical equipment. Neural network-based advanced machine learning methodologies are gaining popularity in the medical device industry. A collection of algorithms known as neural networks is created to recognize connections and is based on a loose approximation of the human cognitive system.

Machines' deep learning approaches can replicate how humans learn and adapt without programming when they receive access to information via neural networks. With deep learning processes and neural networks, AI is utilized in medical devices to augment or replace human tasks. These functions involve, but are not limited to, disease forecasting and identification, evidence grouping and interpretation for disease epidemics, and healthcare optimization. According to studies, these applications include the processing of natural language, customized medicine, pharmaceutical research and development, surgical support, diagnostic assistance, clinical trial optimization, and cybersecurity and compatibility.⁹⁵⁻⁹⁷

India stands to gain significantly from the application of AI in medical devices, which will enhance patient outcomes, improve diagnosis, and enhance the provision of healthcare. Numerous elements demonstrate AI's potential and possibilities in India's medical gadgets. AI can potentially enhance the speed and precision of medical imaging, enabling the prompt diagnosis of health problems, including cancer, cardiovascular issues, and neurological abnormalities. Radiological services provided by AI systems can help radiologists evaluate patients more quickly and accurately by assisting them in analyzing and interpreting medical images.^{98,99} AI-powered gadgets can continuously screen patients, enable immediate intervention, and reduce the need for hospital visits. By offering symptomatic investigation, therapeutic suggestions, and tools to support decision-making, AI can enhance telemedicine consultations.^{100,101}

AI machines can analyze vast datasets to create personalized treatment programs tailored to an individual's genetic composition, lifestyle, and medical history. AI can expedite the process of drug discovery and development by analyzing massive datasets to identify prospective potential treatments.^{102,103} Machine learning algorithms can save time and money compared to more labor-intensive approaches by predicting the safety and efficacy of novel medications. AI can enhance overall effectiveness, streamline hospital operations, and allocate resources more efficiently. AI has the potential to enhance inventory control, ensure an adequate supply of essential healthcare products, and reduce waste.^{104,97}

AI-powered healthcare information analyses can forecast and track illness outbreaks, enabling the implementation of preventive public health strategies. According to,¹⁰⁵ AI can help with epidemiological research and population health trend analysis. AI in medical devices has the potential to reduce healthcare costs by increasing productivity, decreasing the need for manual labor, and optimizing resource utilization. Utilizing telecommunication and AI-driven diagnostics, AI can play a crucial role in providing healthcare facilities to underprivileged and remote areas.^{106,107} Strong rules and regulations must be established before AI may be used in healthcare to guarantee patient safety, confidentiality of information, and address ethical issues.

Advancements in AI for medical devices can be promoted by fostering partnerships among the public, commercial, and academic sectors. To fully benefit from new technologies, healthcare workers must be educated and trained in the use of AI.¹⁰⁸ Conclusively, India and the rest of the world have great prospects for AI in medical equipment. This may lead to increased accessibility, improved health outcomes, and breakthroughs in medicine and therapy. Governmental authorities, healthcare facilities, technology developers, and regulatory organizations must collaborate to create an environment that supports the moral and practical application of AI in the medical field.

CONCLUSION

Recently, India has witnessed significant progress in its capabilities as both a producer and consumer of healthcare and medical devices. With thriving support from the development and implementation of medical device policies, India has made constructive progress in developing quality, affordable, and regulated medical devices. Government policies are exceedingly supportive, goal-oriented, and business-friendly, encouraging the shift towards indigenous manufacturing rather than importing. The objective is to reduce import dependency by fostering indigenous production capability. It is primarily advocated that there is a big room for countries like India to tap the untouched areas relating to government policies, business, and collaboration protocol, ways to foster ease of business, and increase reach and compliance with the regulatory guidelines. Still, grounds for

improvement exist in the areas of evaluating clinical performance, certification, safety, reporting, and planning; efforts in this area are indeed in progress at the national level to bring them into line with global policies.

Today, many of the devices in India are still defined as drugs as per the Drugs and Cosmetics Act 1940, while others are defined under the IMDR as devices. In subsequent revisions of the guidelines, the CDSCO may also realign these definitions and consolidate all medical devices under a single umbrella, making the rules and norms for all these devices uniform. This will enhance the ease of doing business in the medical device industry in India and attract more manufacturers to invest in this segment. Furthermore, India's new medical device regulatory guidelines certify the introduction of novel and innovative medical devices. It is anticipated that in the years to come, India will develop itself as a major player in manufacturing and emerge as one of the primary consumers of indigenous healthcare medical devices.

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ABBREVIATIONS

AERB: Atomic Energy Regulatory Board); **AI:** Artificial intelligence; **AMTZ:** Andhra Pradesh Medtech Zone; **APMSIDC:** Andhra Pradesh Medical Services and Infrastructure Development Corporation; **BIS:** Bureau of Indian Standards; **CAGR:** Compound annual growth rate; **CDSCO:** Central Drugs Standard Control Organization; **CER:** Clinical Evaluation Report; **CIF:** Common infrastructure facilities; **CLA:** Central Licensing Authority; **CLAA:** Central Licensing Approval Authority; **CVG:** Cardiac and Vascular Group; **DCGI:** Drug Controller General of India; **DHR:** Department of Health Research; **DMF:** Device Master File; **DoP:** Department of Pharmaceuticals; **DPIIT:** Department for Promotion of Industry and Internal Trade; **DPIIT:** Department for Promotion of Industry and Internal Trade; **DRISTi:** Diabetic Retinopathy Screening; **DST:** Department of Science and Technology; **ECG:** Electrocardiogram; **EU:** European Union; **FDI:** Foreign direct investment; **FSC:** Free Sale Certificate; **GHTF:** Global Harmonised Task Force; **GMDN:** Global Medical Devices Nomenclature; **GoI:** Government of India; **GST:** Goods and Services Tax; **HIV:** Human immunodeficiency virus; **HNS:** High-nitrogen Stainless Steel; **IBEF:** Indian Brand Equity Foundation; **ICMR:** Indian Council of Medical Research; **IEC:** Import-Export Code; **IIT:** Indian Institute of Technology; **IMDR:** Indian Medical Device Rules; **IMDRF:** International Medical Device Regulators Forum; **IVDMDs:** *In vitro* Diagnostic

Medical Device; **IVDs**: *In vitro* Diagnostics; **MAHE**: Manipal Academy of Higher Education; **MD**: Medical Device; **MPIDC**: Madhya Pradesh Industrial Development Corporation; **MDSAP**: Medical Device Single Audit Program; **MEIC**: Medtronic Engineering and Innovation Centre; **MeitY**: Ministry of Electronics and Information Technology; **MHM**: Menstrual hygiene management; **MITG**: Minimally Invasive Therapies Group; **MoHFW**: Ministry of Health and Family Welfare; **MoUs**: Memorandums of understanding; **mPRAGATI**: MedTech Product Development Acceleration Gateway of India; **MRC**: Master Research Collaboration; **GDP**: Gross Domestic Product; **NABCB**: National Accreditation Board for Certification Bodies; **SLA**: State Licensing Authority; **NABL**: National Accreditation Board for Testing and Calibration Laboratories; **NDLI**: National Digital Library of India; **NID**: National Institute of Design; **NIPER-A**: National Institute of Pharmaceutical Education and Research, Ahmedabad; **PLI**: Production Linked Incentive Scheme; **PMF**: Plant Master File; **PMS**: Post-approval changes; **PPP**: Public-private partnerships; **QMS**: Quality management system; **R&D**: Research and development; **RTG**: Restorative Therapies Group; **RT-PCR**: Reverse transcription-polymerase chain reaction; **SCTIMST**: Sree Chitra Tirunal Institute for Medical Sciences and Technology; **SEC**: Subject Expert Committee; **SINE**: Society for Innovation and Entrepreneurship; **UDI**: Unique Device Identification; **UMDNS**: Universal Medical Device Nomenclature System; **UNICEF**: United Nations International Children's Emergency Fund; **WIPO**: World Intellectual Property Organization; **YEID**: Yamuna Expressway Industrial Development Authority.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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DECLARATION OF CONFLICTING INTERESTS

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

SUMMARY

The Indian medical device industry is an emerging and rapidly growing healthcare sector. However, to meet the rising demands of this sector, India relies primarily on imports. Considering this situation, the Indian Government has initiated several measures and policies to boost the production of indigenous medical devices. This manuscript offers an all-inclusive crosstalk on the emerging dynamics of medical devices in India with the help of thoughtfully presented case studies. It is anticipated that this manuscript will also serve as a ready reference source for all shareholders of the medical devices sector in India.

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