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Original Article

Novel Antibacterial Activity and Time Kill Kinetics of Ethanolic Extract of *Ipomea marginata* against Select Oral Micro-organisms Causing Gingivitis-An *in vitro* Study

Vandana Sekizhar[1,*], Saravanakumar Ravindran[1], Saravanan Devarajan[2], Vijayan Venugopal[3], Raja Arun[4]

[1]Department of Oral Medicine and Radiology, Indira Gandhi Institute of Dental Sciences, Sri Balaji Vidyapeeth (Deemed to be University), Pillayarkuppam, Pondicherry, INDIA.
[2]Department of Pharmaceutical Chemistry, Tagore College of Pharmacy, Chennai, Tamil Nadu, INDIA.
[3]Department of Pharmaceutics, School of Pharmacy, Sri Balaji Vidyapeeth (Deemed to be University), Pillayarkuppam, Pondicherry, INDIA.
[4]Department of Microbiology, Helikem Biotek Industrial Research, Tiruchirappalli, Tamil Nadu, INDIA.

ABSTRACT

Background: Leaves of the plant *Ipomea marginata* is commonly used in Indian folklore medicine to treat skin infections however the antibacterial property of *Ipomea marginata* against oral microorganisms causing gingivitis and periodontitis has not been explored till date. Hence, the present study is planned to compare and evaluate the antibacterial efficacy of ethanolic extract of *Ipomea marginata* with 0.2% chlorhexidine mouth wash on select oral microorganisms. **Materials and Methods:** Phytochemical screening of ethanolic extract of leaves of *Ipomea marginata* showed significant levels of secondary metabolites accountable for the antibacterial property of the leaves. Identification of the volatile constituents of the leaves of *Ipomea margianta* was carried out using GC-MS and nine volatile compounds were identified. Antibacterial activity of ethanolic extract of *Ipomea marginata* was evaluated against *S. mutans, T. denticola, T. forsythia* and *P. gingivalis* which are the chief organisms indicated in the etiology of plaque induced gingivitis. The zone of inhibition and minimum inhibitory concentration was determined by disc diffusion and broth dilution method respectively and compared with gold standard 0.2% chlorhexidine. Time kills kinetics of test pathogens at 6, 12, 18 and 24 hr was also determined. The herbal extract exhibited statistically significant antibacterial property compared to standard chlorhexidine by agar disc diffusion method. **Results:** The Minimum Inhibitory Concentration (MIC) for the herbal extract for *S. mutans, P. gingivalis, T. denticola* and *T. forsythia* was 6.25 µg/mL, 100 µg/mL, 100 µg/mL and 50 µg/mL, respectively and for 0.2% chlorhexidine it was 25 µg/mL, 12.5 µg/mL, 200 µg/mL and 100 µg/mL respectively. Time kills kinetics revealed bactericidal activity of ethanolic extract of *Ipomea marginata* against oral microorganisms causing gingivitis in a time dependent manner. **Conclusion:** Ethanolic extract of leaves of *Ipomea marginata* showed promising antibacterial activity compared to standard 0.2% chlorhexidine mouth wash.

Keywords: Antibacterial agent, Cytotoxic, Mouth wash.

Correspondence:
Prof. Vandana Sekizhar
Department of Oral Medicine and Radiology, Indira Gandhi Institute of Dental Sciences, Sri Balaji Vidyapeeth (Deemed to be University), Pillayarkuppam-607402, Pondicherry, INDIA.
Email: drvandanapavit@gmail.com

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INTRODUCTION

Oral health is a significant determinant of general health, well-being and overall quality of life. Gingival disorders, sometimes referred to as gum disease, represent a prevalent chronic inflammatory illness affecting the oral cavity and are a frequent underlying factor contributing to oral malodor and poor oral health. Dental plaque is a structurally and functionally organized biofilm forms on the surface of teeth within few minutes after tooth brushing and is considered as the primary etiologic factor for gingivitis.[1] Gingivitis or gum inflammation frequently advances to periodontitis, which involves inflammation of the soft tissue and bone. Over time, persistent periodontitis has the potential to result in the loss of teeth. Oral biofilm serves as a protective barrier, impeding the effectiveness of antimicrobial agents and host immune responses however targeting biofilms for therapeutic purposes presents significant challenge as biofilms exhibit resistance to standard antimicrobials.

There exists a hypothesis suggesting that Low Grade Inflammation (LGI) may serve as a causal risk factor for chronic ailments, including diabetes mellitus, obesity and cardiovascular diseases.[2] Therefore, it may be argued that biofilm bacteria serve as a covert contributing component in the emergence and advancement of chronic systemic disorders. The prevention of periodontal problems is a public health concern because it has been shown

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that these conditions contribute to the development of various systemic diseases. Chlorhexidine-based antimicrobial agents have demonstrated moderate efficacy in eradicating dental plaque however achieving consistent and targeted removal of these pathogens while preserving the natural microbial balance in the oral cavity remains a formidable task.[3] One approach to address bacterial resistance is the utilization of herbal or natural products that include intricate chemical compositions thereby impeding the development of resistance in bacteria.[4] The growing desire for novel antimicrobials has prompted numerous studies to focus on exploring the antimicrobial properties of phytochemicals derived from various plant species.[5,6]

Though Chlorhexidine (CHX) has demonstrated antimicrobial action against *Porphyromonas gingivalis, Treponema denticola* and *Tannerella forsythia* commonly referred to as 'red complex' pathogens causing gingivitis and periodontitis, it is associated with side effects such as staining of teeth, unpleasant taste and most importantly change in oral microbiome. It was observed there was a major shift in oral microbiome, significant decrease in saliva pH and buffering capacity, increase in saliva lactate and glucose levels followed by increase in systolic blood pressure.[7] As the popularity of herbal products continue to rise mouthwashes made of natural, organic and herbal ingredients offer special therapeutic qualities which are free of alcohol, artificial preservatives, colors and flavors and are more preferred by todays comparatively conscious consumers. The practice of Indian folklore medicine has a long-standing history, dating back to ancient times, as evidenced by its mention in revered texts like the Vedas. Certain herbs utilized in traditional medicine have origins in domestic cultivation and are commonly referred to by local names. One example of a plant is the genus Ipomea, which is widely referred to as 'Morning glory' and is classified under the family *Convulvulacea*.[8] *Ipomea batatas* is a frequently documented and well-studied botanical specimen of the Ipomea genus. The plant popularly referred to as sweet potato is recognized for its notable antiulcer, antimutagenic, anti-diabetic and antioxidant qualities.[9] The main bioactive substances obtained from plants in the genus *Ipomoea* are alkaloids containing ergoline, alkaloids containing indolizidine, alkaloids containing nortropane, phenolic compounds, coumarins, norisoprenoids, diterpenes, isocoumarin and benzenoids, flavonoids and anthocyanins, glycolipids, lignan and triterpene compounds. Literature suggests these secondary metabolites have significant antibacterial properties.[10] Till date there is no research exploring the antibacterial potential of *Ipomea marginata* (*Ipomoea marginata* (Desr.) Verdcourt) on oral microorganisms which are the mainstay in causing multiple oral disorders ranging from dental caries to oral squamous cell carcinoma.[11,12] Hence, the present study is planned to compare and evaluate the antibacterial efficacy of ethanolic extract of *Ipomea marginata* with 0.2% chlorhexidine mouth wash on select oral microorganisms.

MATERIALS AND METHODS

Sample Collection

The utilization of *Ipomea marginata* leaves in traditional medicine for the treatment of abscesses and other inflammatory lesions is prevalent in the South India. The plants were identified and the leaves were harvested from the Auroville forest in Pondicherry, South India, under the assistance of local traditional healers. The authenticity of the specimens was additionally confirmed by a botanist affiliated with the French Institute of Pondicherry.

Extraction

The plant's leaves underwent a comprehensive washing process, followed by drying in a shaded environment. Subsequently, the leaves were transformed into a powdered form utilizing a high-speed blender manufactured by Warring. The Soxhlet extraction device (Biocoction Manufacturing Pvt. Ltd., India) was employed for the purpose of extraction. A quantity of 50 g of powdered leaf material was carefully inserted into a porous thimble within the apparatus located in the upper chamber. A volume of 200 mL of extracting solvent was introduced into the lower boiling flask. The flask was subjected to heat by the utilization of a heating mantle that was regulated by a thermostat. The extraction of oil and fats was conducted using petroleum ether, followed by the extraction of phytochemicals from the defatted sample using ethanol. The solvent was subjected to reflux and thereafter underwent extraction. The substance contained within the thimble was sequentially extracted using various solvents until a colorless extract was obtained and collected at the surface of the extractor. The solvent extract that was obtained subsequently underwent separate concentration under lower pressure. Following the process of complete evaporation, the remaining substance was carefully observed and subsequently stored in a sealed brown container at a temperature of 5°C for future utilization.

Phytochemical Analysis

The ethanol extract was tested for alkaloids, anthroquinones, flavonoids, phenols, steroils, tannins, terpenoids,[13] cardiac glycosides, saponins,[14] phlobatannin,[15] reducing sugars,[16] carbohydrates and protein/amino acids.[17]

Gas Chromatography-Mass Spectrometry (GC/MS) was used to analyze the chemical components using both quantitative and qualitative methods. By comparing the retention indices and mass spectra of the volatile compounds with the database of known components in the GC-MS NIST library, the volatile compounds were identified.

Antibacterial Effect of Extract by Disc Diffusion Method

The microbial strain used in the study was *S. mutans* (700610-ATCC), *P. gingivalis* (ATCC 33277), *T. denticola* (ATCC

35405) and *T. forsythia* (ATCC 43037) were selected as test organisms. 24 hr old test pathogens were inoculated on MH agar with blood at 0.5 McFarland optical density concentrations using sterile swab and allowed to dry for 15 min. Extract was prepared at 1 mg/mL concentration and from this 25, 50, 75 and 100 µL was loaded on sterile discs. All the discs were placed over the agar surface inoculated with the test pathogen and incubated at 37±2°C for 24-48 hr under anaerobic condition. Ethanol served as the negative control and the standard chlorohexidine was loaded on sterile disc to a concentration of 100 µg and served as the positive control. The zone of inhibition obtained after incubation was measured and recorded. The experiment was performed thrice in triplicates.

Minimum Inhibitory Concentration by Alamar Blue Oxidation Method

The Minimum Inhibitory Concentration (MIC) of the extracts was evaluated using the broth dilution technique, with concentrations ranging from 100 to 0.78 µg/mL. MH broth was mixed with extract to make a final concentration of 200 µg/mL. Serial two-fold dilutions were prepared to make final concentrations ranging from 200-0.78 µg/mL. In a similar manner, chlorhexidine was also prepared to a concentration of. In each dilution, a volume of 10 µL of bacterial solution containing 1×106 Colony-Forming Units per millilitre (CFU/mL) was introduced. The dilutions were then subjected to incubation for duration of 24 hr at a temperature of 37°C, while maintaining anaerobic conditions. A viability test was used to evaluate the growth of the bacterial isolates in the test tubes after incubation. 50 microliters of resazurin were added to the tubes, which were then incubated for duration of 15 min. The process of dye reduction was documented. The maximum dilution at which the dye does not undergo reduction was determined as MIC.

Time-Kill Kinetics Assay

100 mL of mouth wash made at the MIC level was combined with 1 mL of an inoculum size of less than 300 CFU/mL and it was then incubated at 37°C. At 0, 6, 12, 18 and 24 hr, 1.0 mL aliquots of the medium were obtained, aseptically plated onto 20 mL of nutritional agar and then incubated for 24 hr at 37°C. The Colony Forming Unit (CFU) was recorded. Three separate experiments were conducted in triplicate to complete the protocol and the log CFU/mL was plotted against time on a graph.

Statistical Analysis

Statistical analysis was done using SPSS software version 21 (IBM Corp, Chicago IL, USA). Mean±SD (Standard Deviation) was calculated for all variables. Independent '*t*' test was done to compare between the groups. The confidence interval was kept at 95% and *p* value≤0.05 was considered statistically significant.

RESULTS

Phytochemical Screening

After hot soxhlet extraction of the leaves of *Ipomea marginata* the preliminary phytochemical study revealed that ethanolic extract contained alkaloids, flavonoids, phenols, sterols, tannins, terpenoids, cardiac glycosides, phlobatannin, reducing sugars, carbohydrates and protein/amino acids. [Table 1].

GC-MS Analysis

The steam distillation of the dried precipitate of leaves of *Ipomea marginata* with an extraction yield of 13.28% was subjected to GC MS analysis and the results are summarized in Table 2 and Figure 1.

Zone of Inhibition

With regard to *S. mutans* the mean Zone of Inhibition (ZOI) was found to be more effective for herbal extract (18±0) compared to chlorhexidine mouth wash (12±1) (Figure 2a). This difference was also found to be statistically significant (p=0.00024). With respect to *P. gingivalis* also the mean Zone of Inhibition (ZOI) was found to be more effective for herbal extract (18.3±0.57) compared to chlorhexidine mouth wash (28.3±0.577) (Figure 2b) and (p=0.00001) with respect to *T. denticola* the mean Zone of Inhibition (ZOI) was found to be more effective for herbal extract (10.3±0.57) compared to chlorhexidine mouth wash (0) (Figure 2c) with (p=0.00001) and with respect to *T. forsythia* the mean Zone of Inhibition (ZOI) was found to be statistically significant for herbal extract (14±1.73) compared to chlorhexidine mouth wash (18±1) (Figure 2d) with (p=0.0128) (Table 3).

Minimum Inhibitory Concentration (MIC)

The Minimum Inhibitory Concentration (MIC) for the herbal extract for *S. mutans, P. gingivalis, T. denticola* and *T. forsythia* was 6.25 µg/mL, 100 µg/mL, 100 µg/mL and 50 µg/mL respectively

Table 1: Phytochemical constituents of Ethanolic extract of leaves of *Ipomea marginata*.

Carbohydrate	Positive
Glycoside cardiac	Positive
Glycoside	Positive
Alkaloid	Positive
Flavonoid	Positive
Terpenoid	Positive
Phenols and Tannins	Positive
Saponins	Negative
Terpenoids	Positive
Sterols	Positive
Phlobotanins	Negative
Anthraquinones	Negative

Table 2: **Volatile components identified in the ethanolic extract of *Ipomea marginata* by gas chromatography coupled to mass spectroscopy.**

Sl. No.	Compound name	Abundance (%)	Retention index
1.	2,3-Butanediol, 1,4-dimethoxy-	38.54	1094
2.	Ethyl 4-oxo-2-phenylpentanoate	42.72	1629
3.	Neophytadiene	22.31	1840.6
4.	n-Hexadecanoic acid	75.94	1968.2
5.	Hexadecanoic acid, ethylester	66.43	1993.5
6.	Phytol	49.35	2113.9
7.	3-Tridecen-1-yne, (Z)-	12.2	1319
8.	9,12,15-Octadecatrienoic acid, (Z,Z,Z)-	9.7	2154
9.	Squalene	26.28	2835.8

Table 3: **Zone of Inhibition (in mm) of Herbal Extract and Chlorhexidine at Different Concentrations.**

Pathogens	Herbal Extract				Chlorohexidine (100 µg)	*p* value
	10 µg	25 µg	50 µg	100 µg		
S. mutans	12.33±0.57	13.3±0.57	16±1	18±0	12±1	0.00024
P. gingivalis	0	0	0	18.3±0.57	28.3±0.577	0.00001
T. denticola	5±1	5±1	5±1	10.3±0.57	0±0	0.00001
T. forsythia	5±1	5±1	5±1	14±1.73	18±1	0.0128

The values are mean±SD (n=4).

Table 4: **MIC of Herbal Extract and Chlorhexidine.**

Pathogen	MIC of Herbal Extract (µg/mL)	MIC of CHX (µg/mL)
S. mutans	6.25	25
P. gingivalis	100	12.5
T. denticola	100	200
T. forsythia	50	100

Table 5: **Reduction in CFU of test pathogens (%) at different time intervals.**

Test pathogens	Percentage reduction (%)			
	6 hr	12 hr	18 hr	24 hr
S. mutans	36	64	79	97.5
P. gingivalis	31	47	77.8	99.65
T. denticola	13	40	71	99.67
T. forsythia	51	68.8	97.2	99.8

and that standard chlorhexidine was 25 µg/mL, 12.5 µg/mL, 200 µg/mL and 100 µg/mL respectively (Table 4). The MIC values determine the levels of susceptibility or resistance of a specific bacterial strain to a particular drug and drugs with lower MIC scores were more effective antibacterial agents. In the present study the herbal extract was found to be more effective against *S. mutans, T. denticola* and *T. forsythia* compared to chlorhexidine mouth wash. However, it was found chlorhexidine mouth wash was more effective against *P. gingivalis* compared to the herbal extract (Table 4).

Time kill curve

The results obtained for the time-kill study of test pathogens tested at MIC concentration is displayed in the growth curve in the form of log of colony forming unit (Figure 3). The reduction in Colony Forming Units (CFU) over a period of 24 hr showed

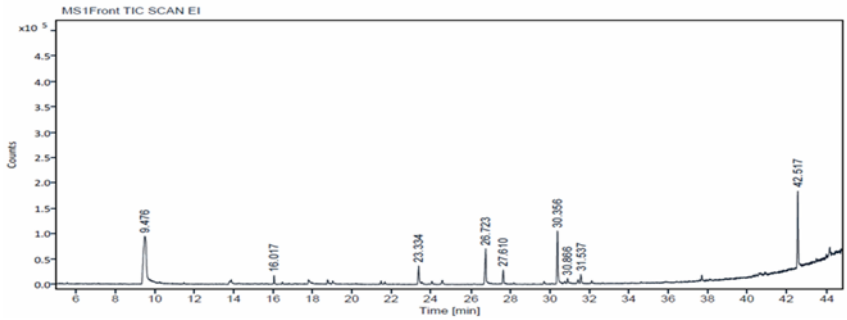

Figure 1: Gas chromatography/mass spectrometry chromatogram of ethanolic extract of *Ipomea marginata*.

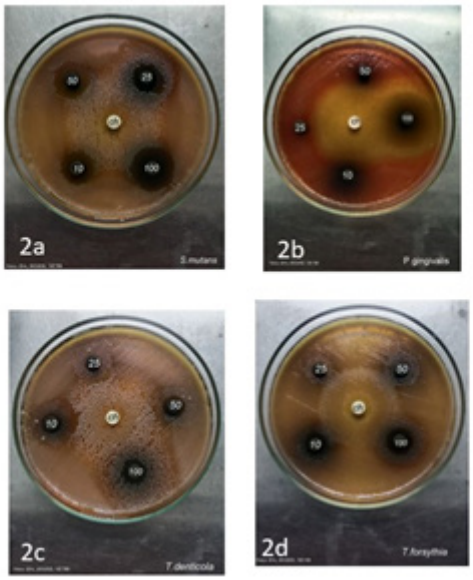

Figure 2: Zone of Inhibition of herbal extract versus 0.2% Chlorhexidine. Figure 2a: Zone of inhibition of herbal extract and 0.2% CHX mouth wash for *S. mutans*; Figure 2b: Zone of inhibition of herbal extract and 0.2% CHX mouth wash for *P. gingivalis*; Figure 2c: Zone of inhibition of herbal extract and 0.2% CHX mouth wash for *T. denticola*; Figure 2d: Zone of inhibition of herbal extract and 0.2% CHX mouth wash for *T. forsythia*.

97.5% reduction of *S. mutans*, 99.65% reduction of *P. gingivalis*, 99.67% reduction of *T. denticola* and 99.8% reduction of *T. forsythia* (Table 5).

DISCUSSION

Despite advances in dentistry dental plaque is a pertinent problem known to cause gingivitis progressing to periodontitis and tooth loss.[18] Though there are many methods of plaque removal, a combination of mechanical and chemical plaque control is found to be most effective.[19] Chlorhexidine gluconate is a commonly used antimicrobial mouth wash used for chemical plaque control.[20] Chlorhexidine is considered 'gold standard' antiplaque agent due to its bacteriostatic and bactericidal properties.[21] However the use of CHX for long-term therapy has been restricted due to its side effects, leading to the promotion of plant-based biologicals as mouthwashes.[22]

Preliminary phytochemical screening of leaves of *Ipomea marginata* showed presence of alkaloids, saponins, phenolic compounds and tanins which are known to have proven antimicrobial properties.[23] With this background the comparative evaluation of ethanolic extract of leaves of *Ipomea marginata* and chlorhexidine was carried out on the bacterial isolates commonly implicated in causing gingivitis.[24] Literature search revealed the present study is a pioneer study by itself as antibacterial effect of *Ipomea* species has not been tested on oral pathogens.

The observations of the present study indicate the Zone of Inhibition (ZOI) which is the area around the antibacterial agent in which the bacteria do not exhibit growth was statistically

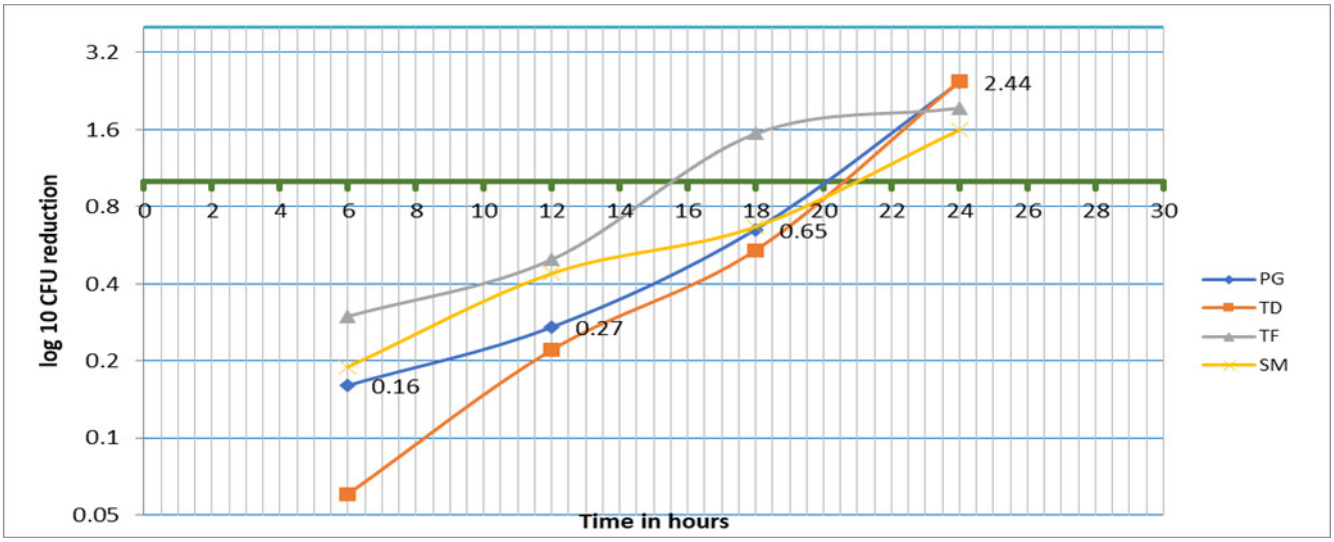

Figure 3: Time Kill Curve of Test pathogens. PG: *P. gingivalis*; TD: *T. denticola*; TF: *T. forsythia*; SM: *S. mutans*.

significant for the ethanolic extract of leaves of *Ipomea marginata* compared to 0.2% chlorhexidine mouth wash ($p<0.05$) for *S. mutans, T. denticola, P. gingivalis* and *T. forsythia* and MIC or the Minimum Inhibitory Concentration of the herbal extract was superior to chlorhexidine for *S. mutans, T. denticola* and *T. forsythia* however *P. gingivalis* exhibited a better MIC for chlorhexidine than herbal extract.

In one study crude acetone extract of leaves of *Ipomea carnea* showed antibacterial activity against *Ptroteus vulgaris* and *Salmonella typhimurium* and ethanolic extract of leaves of *Ipomea carnea* exhibited antibacterial activity against *Pseudomonous auroginosa*.[25] In another study to evaluate the antibacterial activity of different extracts of *Ipomea aquatica* leaves against *Escherichia coli* and *Salmonella typhi* leaves of *Ipomea aquatica* were extracted with 95% ethanol and 95% methanol. MIC was determined for both the extracts and the results revealed methanolic extracts of *Ipomea aquatica* showed significant antibacterial effect against *Salmonella typhi* compared to positive control ciprofloxacin (5 mg/disc).[26]

In another study chloroform soluble extracts of flowers of *Ipomea mucoides* exerted a potentiation effect on clinically useful antibiotics against Methicillin Resistant *Staphylococcus aureus* (MRSA) by increasing their antibiotic susceptibility up to fourfold at concentration 25 µg.[27]

There is growing literature evidence to support the role of organic and herbal based mouth rinses and their superiority to commercially available mouth rinses. In a study conducted to evaluate the efficacy of herbal mouth rinse formulated from extract of red ginseng extract against commercially available mouth rinses such as listerine, rexidine and colgate plax it was found that herbal mouth rinse significantly showed reduction in Colony Forming Units (CFU) of oral bacteria compared to commercially available mouth rinses.[28]

According to a systematic review that assessed the overall effects of these mouthwashes as supplements to oral hygiene compared with placebos and chlorhexidine mouthwash in the treatment of gingivitis it was observed herbal mouth wash had significant benefits in control of plaque and gingival inflammation.[29] Herbal mouthwash (Freshol) comprising of *staphysagria, chamomilla, echinacea, plantago, ocimum* and *cistus* was found to be superior to chlorhexidine in lowering the salivary *Streptococcus mutans* count and equally effective to chlorhexidine in changing plaque and gingival scores in a second double-blind study involving 55 healthy children. The study also assessed the efficacy of the herbal formulation on plaque accumulation, gingival inflammation and salivary *Streptococcus mutans* growth.[30] Time kill kinetics assay in the present study showed the herbal extract demonstrated bactericidal effect in a time dependent manner and the viability of pathogens was abolished within 24 hr in the time-killing test for the 5 log cycle reductions were detected. The findings of our study align with previous research which also observed a significant bactericidal impact when the concentration of the antimicrobial agent was doubled (2×MIC) and the duration of contact was extended. Different bacteria and plant extracts exhibited varying degrees of time-dependent microbial inhibition, according to the results of the time killing experiment. Response to microbial infection by plant secondary metabolites can be viewed as one of the contributing factors for antimicrobial properties exhibited by plants.[31,32]

CONCLUSION

Based on the findings of this investigation, it can be deduced that the ethanolic extract of *Ipomea marginata* exhibited demonstrable antibacterial activity against specific oral microorganisms that are known to be critical in the development of plaque, the primary etiologic factor for periodontitis and gingivitis. Mouthwashes made of natural ingredients, such as herbs and organic oils, are free of alcohol and chemical preservatives and offer special

medicinal benefits. As a result, they are growing in acceptance among the comparatively better-informed customers of today. Therapeutic formulation of mouthwash, oral gels and tooth paste can be carried out in the future after animal and human clinical trials.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

GC-MS: Gas chromatography mass spectrometry; **NIST:** National institute of standards and technology; **ATCC:** American type culture collection; **MIC:** Minimum inhibitory concentration; **ZOI:** Zone of inhibition; **CFU:** Colony forming units; **CHX:** Chlorhexidine.

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