

Exploring the Antioxidant Properties and Bioactive Compounds of Methanolic Extract of *Diplocyclos palmatus* Seeds

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ABSTRACT

Background: Concerns regarding the detrimental consequences of oxidative stress on human health have grown recently on a global scale and herbal treatment approaches to treat oxidative stress-related illnesses in humans are now a hot topic in traditional medicine. *Diplocyclos palmatus* is one such herbal remedy, it is utilised to treat a wide range of illnesses because of its antioxidant qualities. To provide scientific evidence for this claim the study was planned to evaluate the antioxidant capabilities of *D. palmatus* Seed Methanolic Extract (SME) using both *in vitro* and *in vivo* investigations. **Materials and Methods:** SME prepared with cold percolation method was first evaluated for phytochemical constituents. *In vitro* assays were carried out to get a preliminary indication of the antioxidant capacity of extract in a regulated environment whereas, *in vivo* analysis was done using an induced Wistar rat model to justify its biological relevance. The main bioactive components in SME were identified using the RP-HPLC technique. **Results:** SME has proven to be an effective antioxidant in both *in vitro* and *in vivo* experiments. The RP-HPLC analysis has revealed the presence of quercetin and vanillic acid as the main antioxidants in SME. All the results were obtained as Mean±Standard Deviation. Tests including Student's T-test (unpaired) and ANOVA were applied to compare the Statistical difference. A 'p' value of less than 0.05 was considered significant. **Conclusion:** The research findings indicate that *D. palmatus* SME demonstrates noteworthy antioxidant properties. Thus, the findings could contribute to developing a safe and effective natural health supplement.

Keywords: *Diplocyclos Palmatus*, *In vitro* and *in vivo* Antioxidant Assay, RP-HPLC.

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INTRODUCTION

Medicinal plants are widely used in both developed and developing countries for their healing properties (Bajpayee *et al.*, 2024). Before the 18th century, the therapeutic qualities of many plants were recognized, though their active ingredients were not yet understood (Salmerón-Manzano *et al.*, 2020). Modern techniques now allow for the extraction and identification of these compounds. (Balkrishna *et al.*, 2024). Phytochemicals can help combat oxidative stress caused by reactive oxygen species, which poses a significant global health concern (Syman *et al.*, 2024; Jomova *et al.*, 2024). Plant antioxidants can neutralize free radicals, reducing the risk of serious diseases and serving as potential therapeutic agents (Tiwana *et al.*, 2024; Chaachouay

et al., 2024). India's diverse climate makes it a prominent source of medicinal plants (Atanasov *et al.*, 2021; Varah *et al.*, 2023). *D. palmatus* (Shivlingi), from the Cucurbitaceae family, is traditionally used by tribal communities for various chronic ailments (Patel *et al.*, 2020; Kaurav *et al.*, 2021). This study aimed to investigate the antioxidant potential of *D. palmatus* extract through *in vitro* and *in vivo* assays and to evaluate its bioactive compounds using RP-HPLC.

MATERIALS AND METHODS

Plant collection and identification

Plants were collected in October and November 2022 from Badheri, Uttarakhand, India (29°53'54.3"N 77°59'08.0"E) and matched with a specimen (Accession No. 14405, Patanjali Research Institute, Panchayanpur) and identified as *D. palmatus*.

Chemicals used

Analytical grades of standards and other chemicals were procured from Sigma Aldrich Chemical Pvt. Ltd., India.



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Extract preparation

Air-dried seeds from healthy fruits were used to prepare SME by cold percolation method.

In vitro antioxidant assays

Three experimental replicates were used to calculate the mean value for the given below *in vitro* antioxidant assays.

DPPH (2,2-diphenyl-1-picrylhydrazyl) assay

Different concentrations (20-100 µg/mL) of extract and Ascorbic Acid (AA) were prepared from a stock solution (1 mg/mL) by dilution with methanol. A mixture of 1 mL of extract or AA and 3 mL of 0.2 mM DPPH was incubated for 30 min in the dark. Absorbance was measured at 517 nm against a methanol blank, with DPPH alone serving as the control. The percentage radical scavenging activity (% RSA) was calculated using the formula: % RSA = (abs. of control - abs. of sample) / abs. of control × 100. The IC₅₀ value was determined for both standard and extract using their calibration curve equation "y=mx+c." (Delarosa *et al.*, 2023).

ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) assay

The assay involved mixing 7 mM ABTS solution with 2.45 mM potassium persulfate and letting it sit for 12-16 hr at room temperature. After dilution with ethanol (1:89 v/v) and equilibrating at 30°C, the absorbance was set to 0.700±0.02 at 734 nm. Then, 1 mL of the extract and antioxidant (from 1 mg/mL stock) were combined with 3.5 mL of ABTS, and absorbance was read at 734 nm (Hussen *et al.*, 2023). % RSA and IC₅₀ values were calculated as given in DPPH assay.

NO (Nitric Oxide) assay

To perform the assay, a 5 mM sodium nitroprusside solution was prepared, which generates nitric oxide in the presence of oxygen, forming nitrite ions that react with Griess Reagent to produce a colored compound. Antioxidants in the extract compete with oxygen, decreasing nitrite ion formation and colored compound levels. A reaction mixture was created using 5 mM nitroprusside in phosphate-buffered saline (0.2 mM, pH 7.4) along with varying concentrations of the extract and Gallic Acid (GA) as a standard, and incubated at 25°C for 150 min. After adding 0.5 mL of Griess reagent, absorbance was measured at 546 nm (Das *et al.*, 2023) and % RSA and IC₅₀ values were calculated as given in the DPPH assay.

Total Phenolic and Flavonoid Content Assay

The TPC and TFC of the extract were measured at 100 µg/mL. For TPC, 5 mL of 10% Folin-Ciocalteu's reagent was added to the extract and Gallic Acid (GA) standard, incubated in the dark for 5-10 min, followed by 4 mL of 7% Na₂CO₃ and a 90-min incubation. Absorbance was recorded at 750 nm, with a blank

sample for comparison. The GA calibration curve "y=mx+c" determined mg GA Equivalent (GAE) per gram dry weight of extract.

For TFC, 0.3 mL of 5% NaNO₂ was mixed with the extract and quercetin standard, allowed to sit for 5-6 min, then 0.3 mL of 10% AlCl₃, 4 mL of 1M NaOH, and 10 mL of water was added and shaken. Absorbance was measured at 510 nm, again using a blank for comparison. The quercetin calibration curve "y=mx+c" calculated mg Quercetin Equivalent (QE) per gram dry weight of the extract (Islam *et al.*, 2023).

In vivo antioxidant assay

The study was approved by the Institutional Animal Ethical Committee (IAEC) of M.D. University, Rohtak (ref: IAEC/2023/46-60, dated 02/03/23). 15 rats were divided into Negative control, Positive control, Metformin and SME groups as shown in Figure 1. 12 rats received Letrozole (1 mg/kg/day) for 21 days to induce PCOS (Kakadia *et al.*, 2021), which causes oxidative stress (Sengupta *et al.*, 2024). After sacrificing one rat and confirming induction, each group contained 3 experimental replicates. Induced groups were treated with metformin (500 mg/kg) (Desai *et al.*, 2012) and SME (100 mg/kg) (Reddy *et al.*, 2010; Chauhan *et al.*, 2010; Sivakumar *et al.*, 2004) for 21 days. Post-dissection, liver and kidney homogenates were prepared to evaluate the impact of treatments on oxidative stress, compared with the untreated PCOS group. Following assays were conducted to assess *in vivo* antioxidant activity. In all the assays three experimental replicates were used to calculate the mean.

Superoxide Dismutase (SOD) assay

The main reagent in the assay was Pyrogallol, which auto-oxidizes to form free radicals and a brown Pyrogallol-quinone complex that absorbs at 420 nm. This auto-oxidation is inhibited by SOD, with one unit defined as the amount of enzyme that prevents 50% of the reaction. A reaction mixture (3 mL) was prepared with 1 mM EDTA, 50 mM Tris-HCl buffer (pH 8.2), 100 µL tissue homogenate, and 0.4 mM pyrogallol, topped with distilled water. Blanks excluded pyrogallol, while controls included it. Absorbance was measured at 420 nm for 2 min at 30±2°C. Following steps were used to calculate SOD activity (Siswoyo *et al.*, 2021):

The rate of absorbance/min was calculated for the control and extract by using the equation:

$$(A_{420 \text{ nm}} \text{cont. at } 2:30 \text{ min.} - A_{420 \text{ nm}} \text{cont. at } 00:30 \text{ min.}) / 2 = \Delta A_{420 \text{ nm}} \text{cont. / min.}$$

$$(A_{420 \text{ nm}} \text{ Test at } 2:30 \text{ min.} - A_{420 \text{ nm}} \text{ Test at } 00:30 \text{ min.}) / 2 = \Delta A_{420 \text{ nm}} \text{ Test / min.}$$

$$\text{Percentage inhibition by extract (\% inhibition)} = \frac{(\Delta A_{420} \text{ nmcont.} / \text{min.} - \Delta A_{420} \text{ nm Test/min.}) / \Delta A_{420} \text{ nmcont.} / \text{min.} \times 100}{}$$

SOD activity was calculated by using the equation.

$$\text{SOD activity (U/min/mg protein)} = \% \text{ inhibition} / 0.5.$$

Catalase Assay

Hydrogen peroxide is converted into oxygen and water by the enzyme catalase, found in the peroxisomes of eukaryotic cells. The reduction in absorbance at 240 nm correlates with the decomposition rate of hydrogen peroxide. For the assay, a mixture was prepared with 50 μL of tissue homogenate, 1 mL of 30 mM H_2O_2 , and 2 mL of phosphate buffer (pH 7.0). Phosphate buffer served as Blank, while the controls excluded the sample. Absorbance was measured at 240 nm for 1 min. Catalase activity (U/mg) was calculated by dividing the absorbance by the molar extinction coefficient ($43.6 \text{ M}^{-1}\text{cm}^{-1}$), indicating the degradation of 1 mM H_2O_2 per min (Tekin *et al.*, 2022).

GSH (reduced glutathione) Assay

GSH is an intracellular reductant that alleviates oxidative stress. When oxidized by DTNB (5,5'-dithio-bis-2-nitrobenzoic acid), it forms the yellow compound TNB, which has an absorbance peak at 412 nm. For the assay, 50 μL of tissue homogenate in 0.1 M phosphate buffer (pH 7.4) was mixed with an equal volume of 20% TCA and 1 mM EDTA to precipitate proteins. After standing for 5 min, the mixture was centrifuged at 2000 rpm for 10 min, and

the supernatant was collected. Then, 1.8 mL of 0.1 mM Ellman's reagent was added, and the volume was adjusted to 2 mL before measuring absorbance at 412 nm against a blank. GSH enzyme activity (U/mg protein) was calculated using the standard curve for GSH as shown in Figure 2 (Alahmadi *et al.*, 2023).

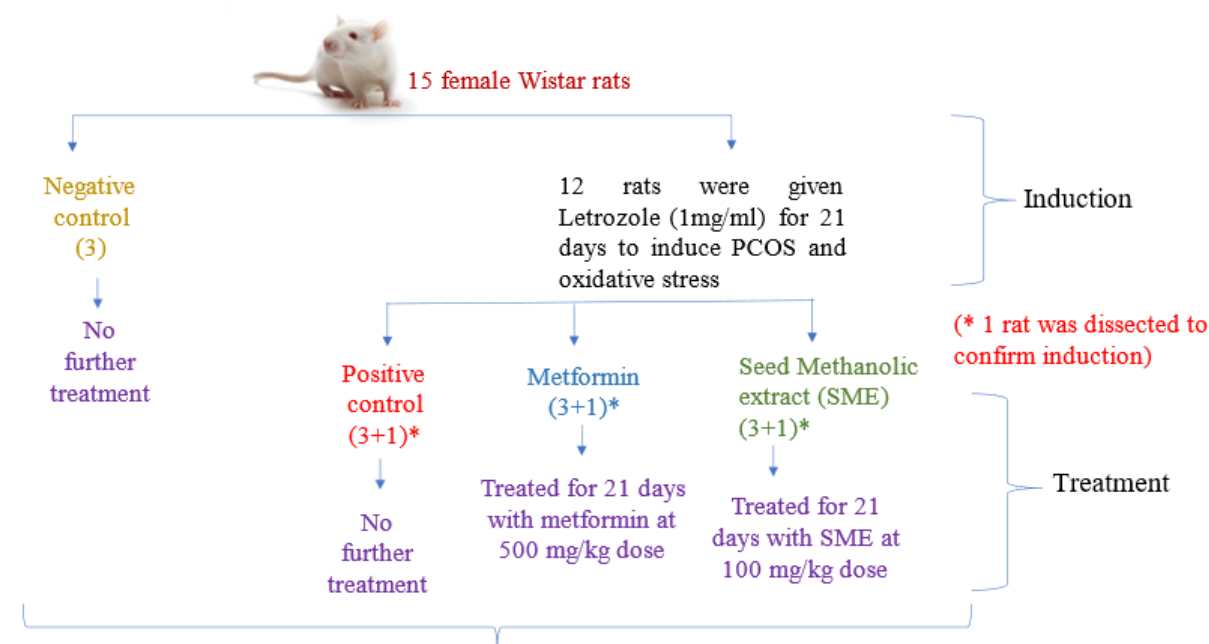
Chromatographic identification and quantification of bioactive Compounds

RP-HPLC instrumentation

HPLC (Agilent 1260 Infinity model, Agilent, USA), which was outfitted with a DAD G4212B detector, a G1311B quaternary pump, and an automatic injector (G1329B). The HPLC was operated by ChemStation software (Agilent, USA).

Preparation of solutions A standard stock solution of Quercetin and vanillic acid was prepared in methanol at 1 mg/mL. A calibration curve was created using a concentration range of 0.10-25 $\mu\text{g/mL}$. For each experiment, three experimental replicates were used. Extract solutions were made at 10 mg/mL in methanol. Both standard and sample solutions were homogenized for thirty seconds, and then filtered through a 0.22 μm membrane. An aliquot was placed in an amber vial and sealed before being injected into the HPLC unit.

Preparation of mobile phase Quercetin was separated from SME using an isocratic mobile phase of HPLC-grade acetonitrile and 0.3% Trichloroacetic Acid (TCA) in water (50:50, v/v) at a flow rate of 0.9 mL/min for 13 min, detected at 254 nm. Vanillic acid was separated using a mixture of distilled water, methanol, and



To assess the impact of treatment on oxidative stress, all rats were dissected, and the liver and kidney were removed. These tissues were then subjected to SOD, GSH, and Catalase assays.

Figure 1: Experimental setup for *in vivo* oxidative stress evaluation.

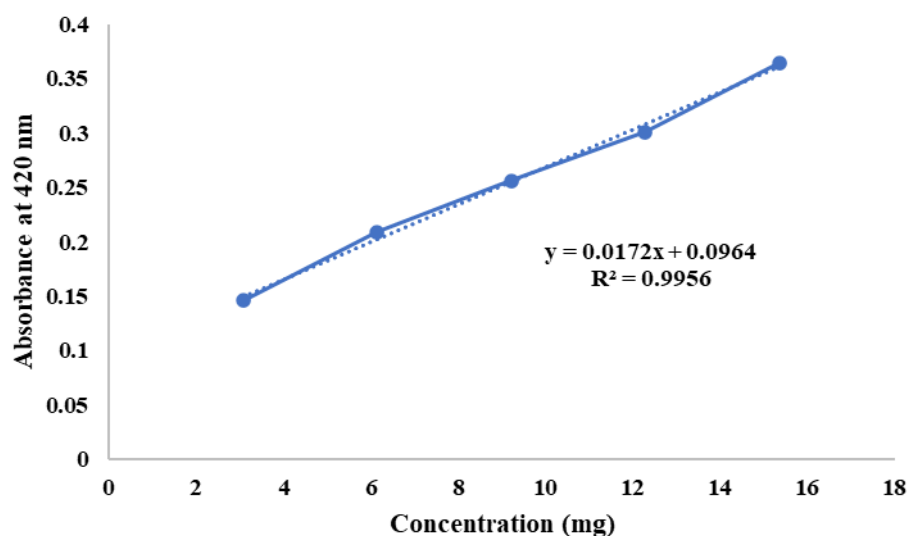


Figure 2: Standard curve for GSH.

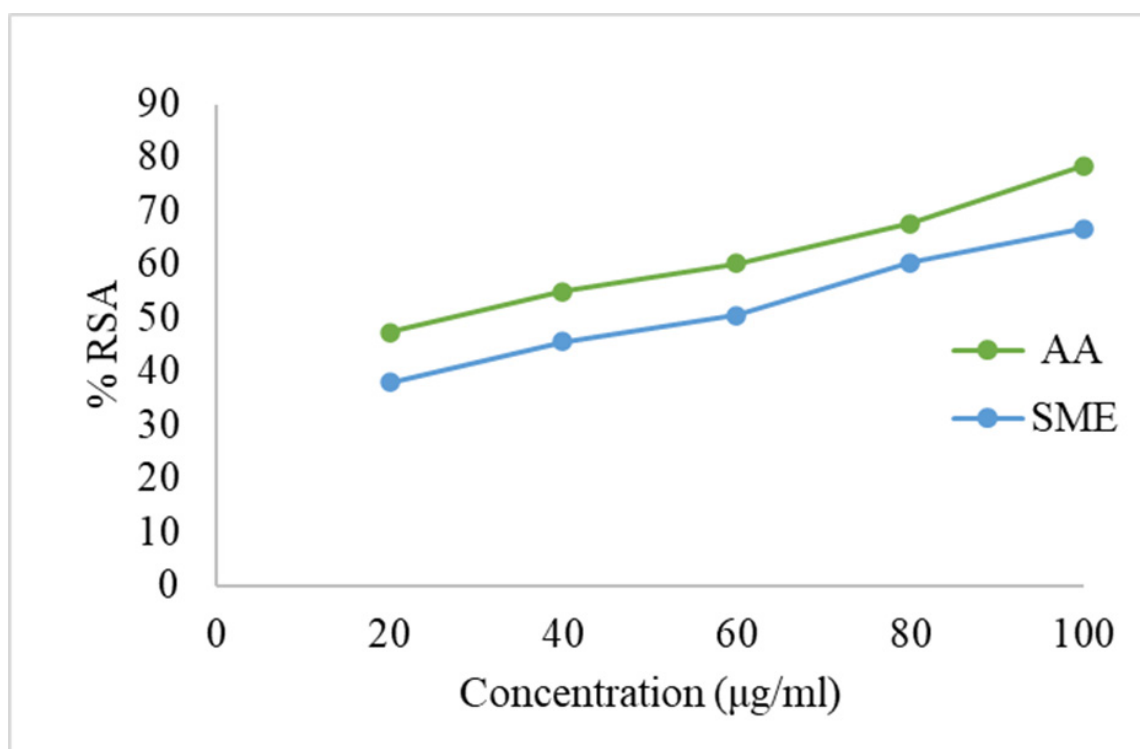


Figure 3: Calibration curve for AA and SME by DPPH assay.

Table 1: Effect of treatment on oxidative stress markers in kidney tissue homogenate.

Group	Oxidative stress marker levels (Kidney Tissue Homogenate) (After treatment)		
	Mean SOD (U/mg-protein)	Mean GSH (U/mg-protein)	Mean Catalase (U/mg-protein)
NC	12.23 ± 0.6 ^a	124.46 ± 0.002 ^{a1}	3.4 ± 0.04 ^{a2}
PC	5.47 ± 0.7 ^b	35.502 ± 0.001 ^{b1}	1.7 ± 0.03 ^{b2}
Met	10.96 ± 0.3 ^c	109.70 ± 0.004 ^{c1}	3.15 ± 0.03 ^{c2}
SME	10.319 ± 0.3 ^d	88.543 ± 0.004 ^{d1}	2.61 ± 0.004 ^{d2}

^a Data shown as Mean ± SD (n=3 experimental replicate). ^b differed significantly ($p < 0.05$) from ^a, ^{b1} and ^{b2} differed significantly ($p < 0.01$) from ^{a1} and ^{a2} respectively. ^c and ^d differed significantly ($p < 0.05$) from ^b, ^{c1}, and ^{d1} differed significantly ($p < 0.001$) from ^{b1}, ^{c2} and ^{d2} differed significantly ($p < 0.001$) from ^{b2}.

acetic acid (700:300:10 mL) at a flow rate of 1.0 mL/min for 14 min, detected at 260 nm.

Method validation Following ICH requirements, the method's accuracy, linearity, specificity, Limit of Detection (LOD), precision and Limit of Quantification (LOQ) were all validated.

Statistical analysis

Results were recorded as Mean \pm SD. Graph Pad (Version 3.0) was used to analyse the statistical data. Student's t-test determined the significant differences between the outcomes. $p < 0.05$ was considered as significant.

RESULTS

In vitro antioxidant assay

The DPPH, ABTS, and NO assays showed an increase in % RSA with increasing concentrations of the standard and extract

(Figures 3-5). The estimated IC₅₀ values were 28.69 μ g/mL for AA and 53.82 μ g/mL for SME in the DPPH assay, 21.2 μ g/mL for AA and 37.43 μ g/mL for SME in the ABTS assay, and 13.37 μ g/mL for GA and 26.16 μ g/mL for SME in the NO assay.

In TPC analysis, the calibration curve for Gallic Acid as in Figure 6(a) yielded the equation " $y=0.001x+0.0492$ " ($R^2=0.9901$) for calculating mg GAE/g of extract. For TFC analysis, the Quercetin calibration curve as in Figure 6(b) provided " $y=0.0873x+0.0579$ " ($R^2=0.996$) for calculating mg QE/g of extract. The results indicated that SME contains 28.13 \pm 0.2 mg GAE/g and 10.51 \pm 0.01 mg QE/g of dry extract

In vivo antioxidant assay

The PCOS induction in female Wistar rats has reduced the levels of oxidative stress markers SOD, GSH, and catalase in all groups except the Negative control. kidney and Liver tissue homogenate of different groups when subjected to SOD, GSH and Catalase

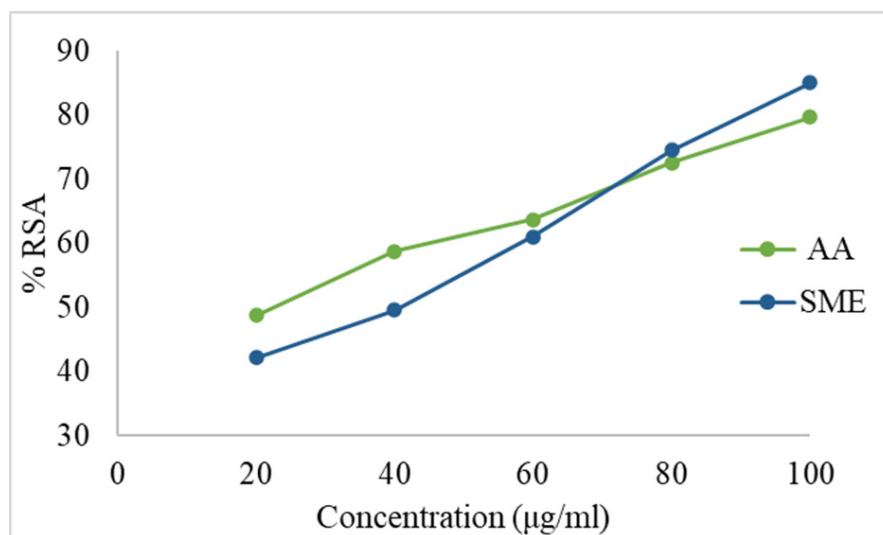


Figure 4: Calibration curve for AA and SME by ABTS assay.

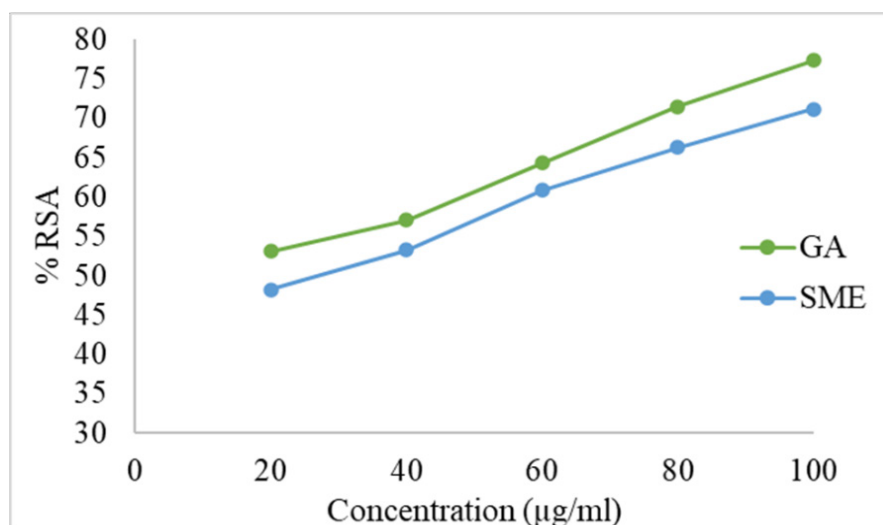


Figure 5: Calibration curve for GA and SME by NO assay.

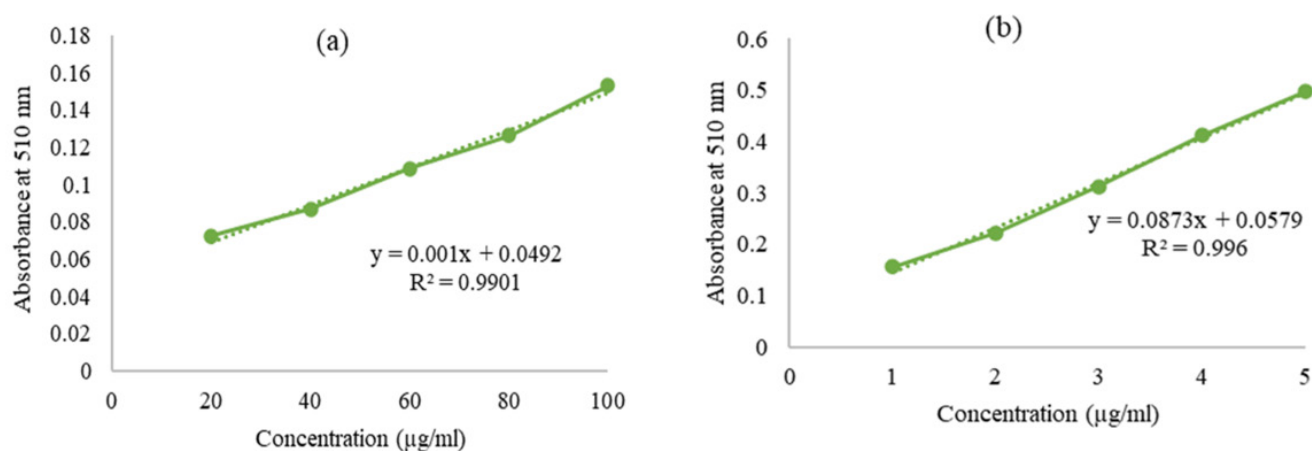


Figure 6: Calibration curve for (a) Gallic Acid (TPC assay) and (b) Quercetin (TFC assay).

Table 2: Effect of treatment on oxidative stress markers in liver tissue homogenate.

Group	Oxidative stress marker levels (liver Tissue Homogenate) (After treatment)		
	Mean SOD (U/mg-protein)	Mean GSH (U/mg-protein)	Mean Catalase (U/mg-protein)
NC	10.27 ± 0.8 ^a	110.50 ± 0.003 ^{a1}	3.1 ± 0.08 ^{a2}
PC	3.80 ± 0.5 ^b	27.26 ± 0.007 ^{b1}	1.6 ± 0.01 ^{b2}
Met	9.20 ± 0.7 ^c	96.15 ± 0.004 ^{c1}	2.96 ± 0.01 ^{c2}
SME	7.00 ± 0.6 ^d	90.24 ± 0.005 ^{d1}	2.90 ± 0.004 ^{d2}

Data shown as Mean ± SEM ($n=3$ experimental replicate). ^b differed significantly ($p<0.05$) from ^a, ^{b1} differed significantly ($p<0.01$) from ^{a1}, ^{b2} differed significantly ($p<0.001$) from ^{a2}, ^c and ^d differed significantly ($p<0.05$) from ^b, ^{c1} and ^{d1} differed significantly ($p<0.01$) from ^{b1}, ^{c2} and ^{d2} differed significantly ($p<0.001$) from ^{b2}.

assay the results indicated that the 21 days extract treatment had significantly raised the levels of these markers in all the treated groups compared to PC as shown in Tables 1 and 2 respectively.

RP- HPLC

Linearity A range of 0.10-25 µg/mL was taken as the calibration range. Vanillic acid and quercetin were determined to have 0.997 and 0.994 as coefficients of determination (R^2), respectively.

Specificity According to the specificity test, the well-shaped peak showed that the main peak of bioactive chemicals is not impacted by other components found in the extract.

LOD and LOQ Quercetin's LOD and LOQ were determined to be 0.184 ± 0.6 and 0.723 ± 0.9 µg/mL, respectively, while that for vanillic acid were 0.171 ± 0.2 and 0.732 ± 0.5 µg/mL, respectively.

Accuracy: The high recovery values demonstrated the method's accuracy for vanillic acid and quercetin, which ranged from 99.40 to 99.91% and 99.53 to 99.90%, respectively.

Precision: The remarkable repeatability of the procedure is confirmed by the finding that the percentage RSD of both intra-day and inter-day precision was less than 0.20%.

Robustness: The robustness was assessed by taking the standard solution ($n=3$) of quercetin and vanillic acid with slight variations of ± 2 in flow rate, column temperature, pH, and wavelength. However, no appreciable variations in the recovery, peak area, or retention time were noted.

The RP-HPLC analysis of SME showed Quercetin peak at a retention time of 2.643 min with a peak area of 24424 and the calculated value of the bioactive compound was 3.342%. Figure 7 represents the peaks for (a) Quercetin standard and (b) SME. Figure 8 represents the peaks for (a) Vanillic acid standard and (b) SME in similar chromatographic conditions. SME showed a vanillic acid peak at a retention time of 6.258 with a peak area of 3283 and the calculated value of the bioactive compound was 2.312%.

DISCUSSION

Phyto-antioxidants protect cells from oxidative stress by scavenging free radicals, with various phytochemicals playing a role in health promotion and disease prevention (Diba *et al.*, 2021; Mishra *et al.*, 2020; Pandey *et al.*, 2009; Bhattacharjee *et al.*, 2022). Research indicates essential phytochemicals are present in the methanolic extract of *D. palmatus* (Attar *et al.*,

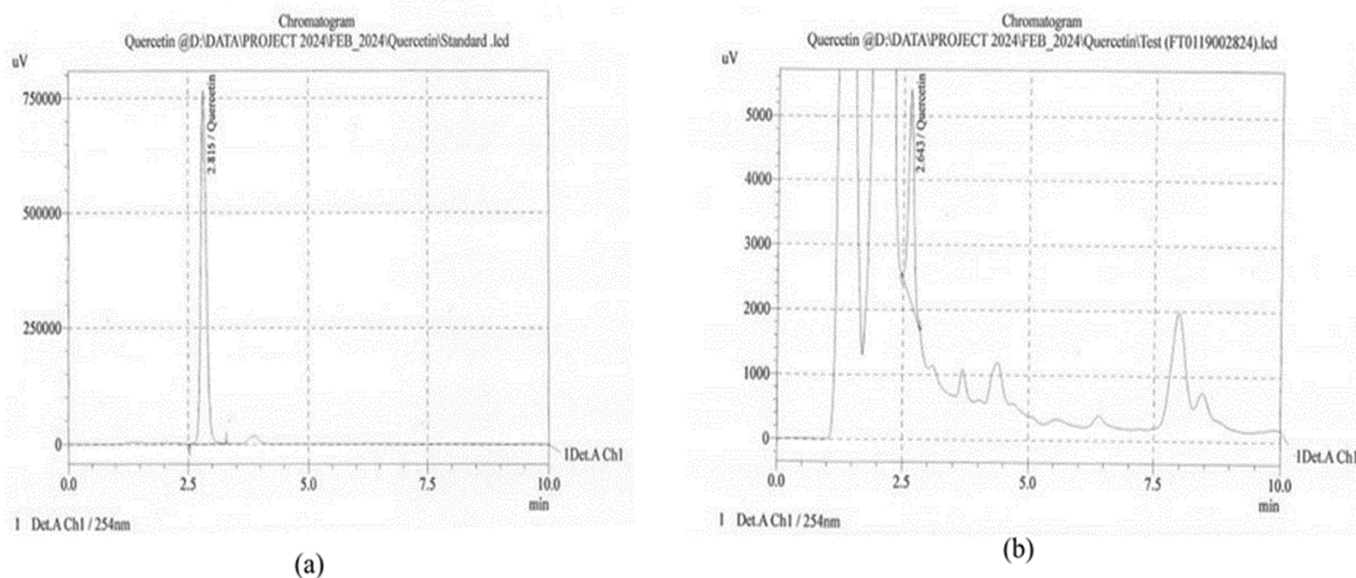


Figure 7: HPLC Peak for (a) Quercetin standard (b) SME.

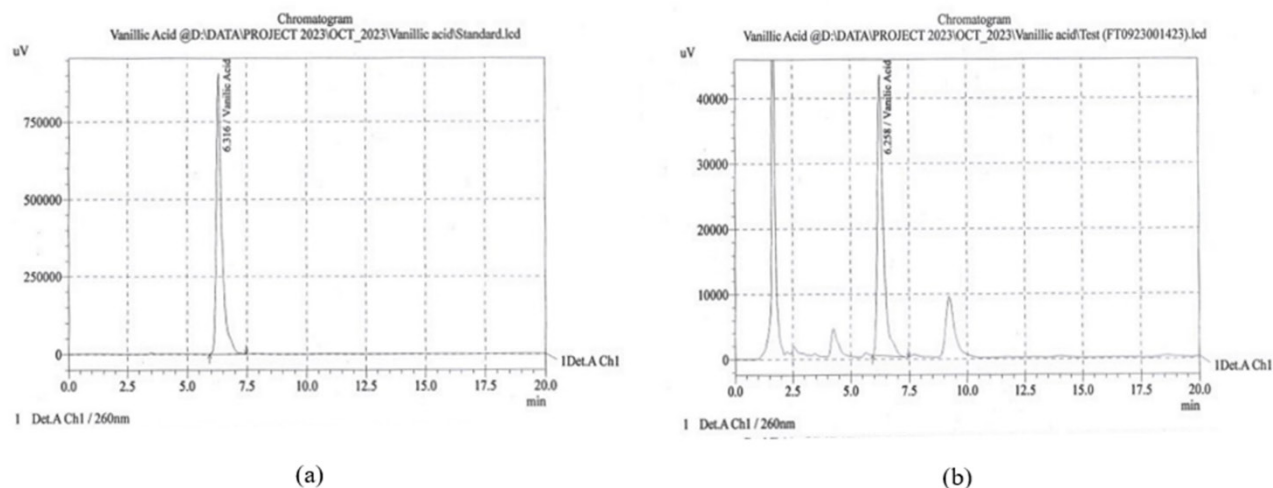


Figure 8: HPLC peak for (a) Vanillic acid standard (b) SME.

2017). *In vitro* assays (DPPH, ABTS, Nitric oxide) evaluated the antioxidant potential of SME, revealing IC_{50} values comparable to standards. TPC and TFC analysis showed SME has 28.13 ± 0.2 mg GAE/g and 10.51 ± 0.01 mg QE/g dry weight. The results were consistent with the earlier research demonstrating the presence of polyphenols, a class of antioxidants in the methanolic extract of *D. palmatus* (Upadhyay *et al.*, 2023). *In vivo* assays demonstrated reduced oxidative stress markers in PCOS female Wistar rats treated with SME, along with increased levels in liver and kidney tissue homogenate. RP-HPLC analysis confirmed vanillic acid and quercetin as the primary antioxidants in SME, supporting their high antioxidant capacity.

CONCLUSION

The study concluded that the SME of *D. palmatus* exhibits significant antioxidant activity with vanillic acid and quercetin identified as the main antioxidants by RP-HPLC. Therefore, the research promises to create a safe, all-natural health supplement.

ACKNOWLEDGEMENT

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

SME: Seeds Methanolic Extract; **DPPH:** 2,2-Diphenyl-1-picrylhydrazyl; **NO:** Nitric Oxide; **FRAP:** Ferric Reducing Antioxidant Power; **ABTS:** 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid); **TFC:** Total Flavonoid Content; **TPC:** Total Phenolic Content; **SOD:** Superoxide Dismutase; **RP-HPLC:** Reversed-phase high-performance liquid chromatography; **TLC:** Thin Layer Chromatography; **ANOVA:** Analysis of Variance; **ROS:** Reactive Oxygen Species; **TPTZ:** 2,4,6-Tripyridyl-S-triazine; **SNP:** Sodium Nitroprusside; **AA:** Ascorbic acid; **GA:** Gallic acid; **EDTA:** Ethylenediamine Tetra acetic Acid; **DTNB:** 5,5'-dithio-bis-2-nitrobenzoic acid; **TCA:** Trichloroacetic Acid; **IC₅₀:** Half-maximal inhibitory concentration; **PCOS:** Polycystic Ovary Syndrome; **Cont.:** Control; **LOD:** limit of detection, **LOQ:** limit of quantification; **ICH:** International Conference on Harmonisation; **LQC:** Low-quality control, **MQC:** Medium-quality control; **HQC:** High-quality control; **RSD:** Relative Standard Deviation; **SD:** Standard Deviation.

AUTHOR CONTRIBUTIONS

Richa Yadav: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Visualization along with Writing original draft. Jaya Parkash Yadav: Data curation, Methodology, Supervision, Validation, Writing-review and editing. All authors read and approved the final manuscript.

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