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RESEARCH ARTICLE

Phytochemical Screening, Antioxidant, and Antibacterial Activity of *Dioon spinulosum* Dyer ex Eichl.

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ABSTRACT

The present study was aimed to investigate the phytochemical, antioxidant, and antibacterial studies of leaf and rachis of *Dioon spinulosum* Dyer ex Eichl. The phytochemical screening of the plant extracts revealed the presence of alkaloids, flavonoids, tannins, terpenoids, carbohydrate, and phenols, whereas saponin was absent. The phenolic content expressed as mg/g gallic acid equivalent was determined and was more in methanolic extract of leaf (29.40 mg) than rachis (8.76 mg). Flavonoid contents were also greater in leaves than in rachis and methanol extract contained higher content (2.812 mg/g) than water (1.923 mg/g). Terpenoids were more in the aqueous extracts of both leaf and rachis when compared to methanol extracts. Antioxidant activity of both leaf and rachis extracts was conducted using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing antioxidant power assay. Leaf extract showed more DPPH radical scavenging activity with IC_{50} value of 130 µg/ml when compared to the rachis of *D. spinulosum* Dyer ex Eichl. The reducing capability of the leaf extract was found to be more when compared to rachis. The antibacterial potential was evaluated with *Staphylococcus aureus* and *Streptococcus mutans* by agar well diffusion method. Antibacterial activity was observed only at higher concentration (1000 µg/ml) with inhibition zones of 12 mm and 13 mm.

Keywords: Antibacterial, antioxidant, *Dioon spinulosum*, phytochemical

INTRODUCTION

The present research in drug development involves a multifaceted approach which includes different branches of science. Several drugs have been isolated from plants and are still in use. Many more plants can be evaluated for a wide range of biological activities due to the development of screening tests now used in industry. Searching for new drug candidates from natural products is often made difficult by the complexity of the molecular mixtures. The therapeutic activity of plant extracts is usually due to the synergistic and simultaneous action of several chemicals.[1] The advent of novel technologies including quantum computing, profiling techniques, computational biology techniques, and artificial intelligence, enables scientists to use a combinatorial approach to harness the therapeutic properties of plant-based natural products.[2] As evident from the available literature, the medicinal properties of gymnosperms have not been studied properly when compared with angiosperms. Gymnosperms are a group of vascular plants with naked seeds and some secondary metabolites have been reported from them.[3] This type of research needs a multidisciplinary approach and since not much work on *Zamiaceae* has been done; an attempt was made to study the phytochemical constituents, their antioxidant nature, and antibacterial property of *Dioon spinulosum* belonging to the family *Zamiaceae* [Figure 1]. The giant *Dioon*, or gum palm, is one of the tallest cycads in the world, growing to 12 m in height. The tree is found at low elevations to 300 m above

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Figure 1: Photograph of *Dioon spinulosum* Dyer ex Eichl.

sea level. *D. spinulosum* prefers well-drained soil with regular water. Since there are certain reports regarding the ethnobotanical significance of these plants, there is an urgent need to study the bioactive potential of this plant.

MATERIALS AND METHODS

Preparation of extracts

After collection, the plant materials were thoroughly washed, shade dried, ground to get a coarse powder, and then subjected to Soxhlet extraction using methanol and distilled water as solvents. Each of the solvent was taken separately for extraction. The whole apparatus was kept over a heating mantle and was heated continuously for 8 h at boiling point of each solvent. The extract was concentrated to dryness and the residue was transferred to a sample bottle and stored in refrigerator for further studies.

Preliminary qualitative phytochemical analysis

Qualitative analysis of the phytochemicals was carried out to identify the secondary metabolites present in the extracts of leaf and rachis of *D. spinulosum*.[4-6]

Test for alkaloids

Approximately 50 mg of extract was dissolved in 5 ml of distilled water followed by 2 ml of hydrochloric acid to initiate an acid reaction and filtered. The filtrate was subjected to qualitative analysis to determine the presence of alkaloids as detailed below.

- Dragendorff's test: Dragendorff's reagent (1 ml) was added to 2 ml of the filtrate. A red precipitate formation indicates the presence of alkaloid
- Wagner's test: Two drops of Wagner's reagent were added to 1 ml of the test solution. The formation of brown precipitate confirmed the test as positive for alkaloids.

Test for flavonoids

About 100 mg of each extract was heated with 10 ml of ethyl acetate in boiling water bath for 3 min. The mixture was filtered and the filtrate was used for the following tests:

- Ammonium test: The filtrate was shaken with 1 ml of dilute ammonia solution (1%, v/v). The layers were allowed to separate. A yellow color observed in the ammonia layer suggested the positive result
- Alkaline reagent test: Few drops of 20% (w/v) NaOH solution were mixed with 2 ml of extract. Formation of intense yellow color, which became colorless on addition of dilute HCl, indicated the presence of flavonoids.

Test for terpenoids

Liebermann–Burchard's test

About 2 mg of the extract was dissolved in 2 ml of acetic anhydride, heated to boiling, cooled and then, 1 ml of concentrated sulfuric acid was added along the side of the test tube. A brown ring formation at the junction confirmed the presence of terpenoid.

Test for tannins

Ferric chloride test

A few drops of 5% (w/v) ferric chloride solution were added to 2 ml of the test solution. Formation of bluish-black color indicated the presence of hydrolyzable tannin.

Test for saponin

Foam test

About 5 ml of the test solution was taken in a test tube and shaken well for 5 min. Formation of stable foam confirmed the test.

Test for carbohydrates

Molisch's test

To 1 ml of the test solution was added, a few drops of 1% alpha naphthol followed by 2 ml concentrated sulfuric acid along the sides of the test tube. A reddish-violet ring at the junction of two liquids confirmed the test.

Test for phenol

Extracts were treated with 3–4 drops of 10% (w/v) ferric chloride solution. Formation of greenish-black color indicated the presence of phenol.

Quantitative estimation of chemical constituents

Determination of alkaloids

To 5 g of powdered sample, 200 ml of 20% acetic acid was added and kept for 40 h. It was filtered and the volume was reduced to 50 ml using water bath. To this sample, concentrated ammonium hydroxide was added dropwise until precipitation was complete. The precipitate was allowed to settle, collected by filtration, and weighed.[7] The percentage of total alkaloid content was calculated as follows:

$$\text{Percentage of total alkaloids}(\%) = \frac{\text{Weight of residue}}{\text{Weight of sample taken}} \times 100$$

Total flavonoids

The total flavonoid content was estimated using the procedure described by Okwu.[8] To 1 ml of the plant extract, 150 µl of sodium nitrite (5%) solution was added and incubated for 5 min and then, 150 µl of aluminum chloride (10%) solution was added and allowed to stand for 6 min. Then, 2 ml of sodium hydroxide (4%) solution was added and made up to 5 ml with distilled water. The mixture was shaken well and left for 15 min at room temperature. The absorbance was measured at 510 nm.

Total terpenoid

Estimation of total terpenoid content was carried out following the protocol of Ghorai *et al.*[9] About 1 g of each extract was dissolved in 10 ml methanol and 15 ml water. Mixture was shaken well and centrifuged at 10,000 rpm for 10 min. Filtrate was taken. To 1 ml of extract, 2 ml of chloroform and 3 ml concentrated sulfuric acid were added. A reddish-brown color developed indicated the presence of terpenoids and was estimated by reading the absorbance at 538 nm against blank of 95% methanol.

Total phenol

The total phenol content was estimated using Folin–Ciocalteu reagent.[10] To 1 ml of the plant extract, 0.5 ml of Folin–Ciocalteu reagent was added followed by 2 ml of 20% sodium carbonate and mixed thoroughly. The reaction of phenol with phosphomolybdic acid in alkaline medium produced a blue colored complex. The tubes were kept in boiling water bath for 1 min, cooled and centrifuged, supernatant was taken and the absorbance was measured at 650 nm. Gallic acid was used as the standard to express the total phenol content.

Total tannins

About 0.1 ml of the sample extract was added to a volumetric flask (10 ml) containing 7.5 ml of distilled water and 0.5 ml of Folin–Ciocalteu reagent. To this, 1 ml of 35% sodium carbonate solution was added and diluted to 10 ml with distilled water. The mixture was shaken well and kept at room temperature for 30 min. Absorbance were measured against the blank at 700 nm. The tannin content was expressed in terms of mg of tannic acid equivalents/g of dried sample.[11]

Assesment of antioxidant activity

2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

This test was measured as described by Blois.[12] Different concentrations of the sample (100, 200, 300, 400, and 500 µg/ml in methanol) were taken and made up to 1 ml using methanol. To this, 2 ml of DPPH solution (0.1 mM in methanol) was added and after 30 min of reaction at room temperature, the absorbance of the solution was measured at 517 nm. The free radical scavenging activity of each fraction was determined by comparing its absorbance with that of a blank solution. The ability to scavenge the DPPH radical was calculated using the following equation:

DPPH scavenging activity (%) = (A0−A1)/A0 × 100

Where, A0 is the absorbance of the control and A1 is the absorbance of the sample.

Reducing power assay

Reducing power was determined by the method prescribed by Oyaizu.[13] The sample in 1 ml of methanol at various concentrations was mixed with phosphate buffer (5 ml, 0.2 M, pH 6.6) and potassium ferricyanide (5 ml, 1%), and the mixture was incubated at 50°C for 20 min. About 5 ml of trichloroacetic acid (10%) was added to the reaction mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of solution (5 ml) was mixed with distilled water (5 ml) and ferric chloride (1 ml, 1%), and the absorbance was measured at 700 nm. A stronger absorbance indicates increased reducing power.

Antibacterial analysis

Agar well diffusion method was used for the antibacterial analysis.[14] Petri plates containing 20 ml Mueller-Hinton agar medium were seeded with bacterial culture of *Staphylococcus aureus* and *Streptococcus mutans* (growth of culture adjusted according to McFards Standard, 0.5%). Wells of approximately 5 mm were bored using a well cutter and samples of 250 µg/ml, 500 µg/ml, and 1000 µg/ml concentrations were added. The plates were then incubated at 37°C for 24 h. The antibacterial activity was assayed by measuring the diameter of the inhibition zone formed around the well.[15] Streptomycin was used as a positive control.

RESULTS AND DISCUSSION

The phytochemical screening of leaf and rachis of *D. spinulosum* was carried out. The methanolic and aqueous extract of rachis and leaves showed the presence of alkaloids, terpenoids, flavonoids, tannins, carbohydrates, and phenols. Saponins were absent in both leaf and rachis extracts [Table 1].

The major phytochemicals which were found in the samples were quantitatively determined by standard procedure. The total alkaloid content was found to be more in leaf (21.5 mg/g) when compared to the rachis (2.2 mg/g) [Figure 2]. The total phenolic content ranged from 29.243 mg in methanol extract of leaf to 7.583 mg in water

Table 1: Qualitative analysis of phytochemicals present in the leaf and rachis of *D. spinulosum* Dyer ex Eichl.

Phytochemicals	Leaf		Rachis	
	Methanol	Water	Methanol	Water
Alkaloids	+	+	+	+
Flavonoids	+	+	+	+
Terpenoids	+	+	+	+
Tannins	+	+	−	−
Saponins	−	−	−	−
Carbohydrate	+	+	+	+
Phenols	+	+	+	+

D. spinulosum: *Dioon spinulosum*

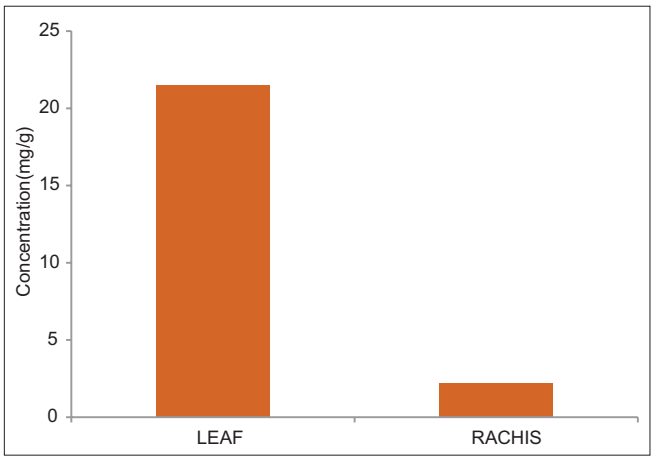

Figure 2: Total alkaloid context in leaf and rachis of *Dioon spinulosum* Dyer ex Eichl.

Table 2: Quantitative data of phytochemicals present in different extracts of *D. spinulosum* Dyer ex Eichl.

Phytochemical	Leaves (mg/g)		Rachis (mg/g)	
	Methanol extract	Water extract	Methanol extract	Water extract
Tanning	0.113±0.002	0.136±0.136	Absent	Absent
Flavonoid	2.77±0.053	1.911±0.017	0.789±0.007	0.314±0.002
Terpenoid	0.182±0.003	0.474±0.002	0.093±0.003	0.218±0.002
Phenol	29.243±0.221	13.522±0.006	8.786±0.025	7.583±0.020

Values are mean±standard deviation of three replicates. *D. spinulosum*: *Dioon spinulosum*

extract of rachis [Table 2]. The result indicated the influence of the extraction solvent on the total content of phenolic compounds. High phenolic content has been reported from three taxa of gymnosperms which could be considered as a valuable source of antioxidants.[16] Water and methanol extract of leaf showed positive result against tannin while both extracts of rachis showed negative result. It was reported that tannins possess physiological astringent properties which hasten wound healing and ameliorate inflamed mucous membranes.[17] Flavonoid concentration was found to be higher in methanol extract of leaf when compared to the rachis. The observations from quantitative analysis suggest that methanol can effectively extract flavonoids and phenolics than water. Flavonoids and other phenolic compounds are potent antioxidants and free radical scavengers, which prevent oxidative cell damage and have strong anticancer activity.[18]

Antioxidant activity

DPPH radical scavenging activity

The principle of DPPH method is based on the reduction of DPPH in the presence of a hydrogen-donating antioxidant. The DPPH radical has been widely used to test the free radical scavenging activity or which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom.[19] IC_{50} value is defined as the concentration of substrate that causes 50% loss of the DPPH activity. In the present study, leaf extract exhibited more DPPH radical scavenging activity with the determined IC_{50} value of 130 µg/ml when compared with rachis extract showing IC_{50} value of 300 µg/ml [Figure 3]. Flavonoids have been reported to exhibit multiple biological effects such as anti-inflammatory, antiallergic, antiviral, antioxidant, and anticancer activities.[20] Thus, the presence of flavonoids and its effect correlates with the present study.

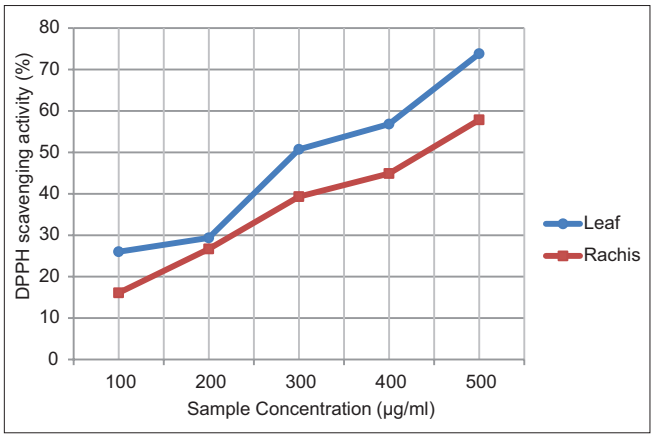

Figure 3: Scavenging effect on 2,2-diphenyl-1-picrylhydrazyl radical of extracts from leaf and rachis of *Dioon spinulosum* Dyer ex Eichl.

Reducing power assay

The antioxidant activity was also measured by the ferric reducing antioxidant power (FRAP) method. Reductive ability was measured by the reduction of ferricyanide complex/Fe^{3+} to the ferrous form (Fe^{2+}) in the presence of antioxidant (reductant). Basically, reducing power is associated with the presence of reductones that break the free radical chain by donating a hydrogen atom. Reducing power assay was carried out in methanol and water extract of leaf and rachis of *D. spinulosum* Dyer ex Eichl. The higher the FRAP value greater the antioxidant activity of sample extracts. The leaf extract displayed higher antioxidant capacity in both methanol and water extract when compared with rachis extract [Figures 4 and 5]. The absorbance of leaf extract clearly increased, due to the formation of the Fe^{2+}-TPTZ complex with increasing concentration. There was a strong

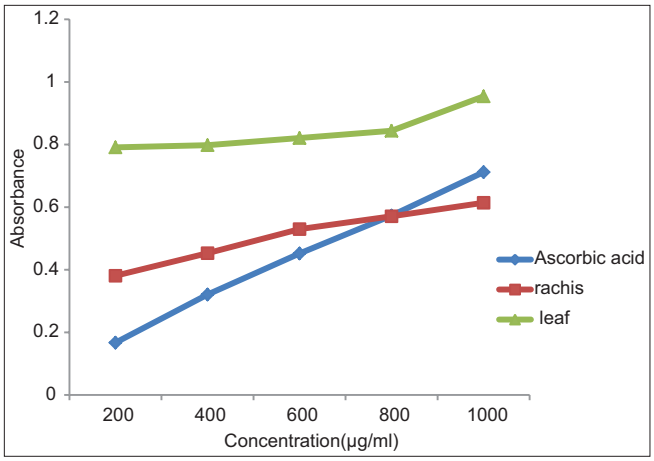

Figure 4: Reducing power assay of methanol extracts of *Dioon spinulosum* Dyer ex Eichl.

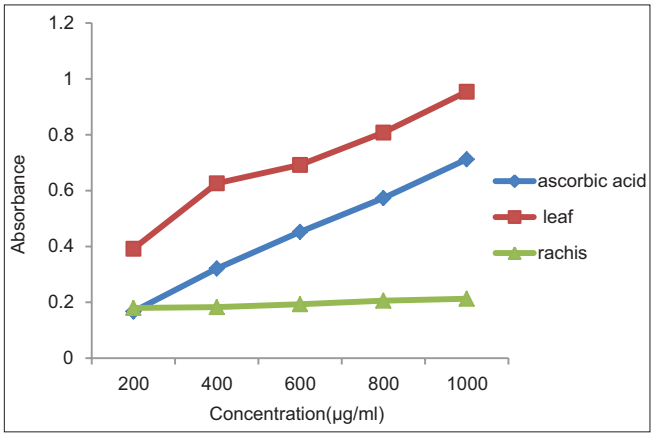

Figure 5: Reducing power assay of aqueous extracts of *Dioon spinulosum* Dyer ex Eichl.

correlation between phenolic content, flavonoid content, and FRAP values supporting the fact that phenolics and flavonoids are potent antioxidants.

Antibacterial assay

Antibacterial activity of methanol and water extract of leaf and rachis was studied against *S. aureus* and *S. mutans*. The methanolic extracts of leaf and rachis of *D. spinulosum* were investigated for its antibacterial activity by measuring the zone of inhibition [Table 3]. The zone of inhibition produced by streptomycin was 29.0 and 30 mm against *S. aureus* and *S. mutans,* respectively, and was larger than those produced by all the plant extracts which was between 11 mm and 13 mm. The zone of inhibition >5 mm diameter was reported to be having significant activity against particular bacteria.[21] Based on the results, rachis extract did not show any zone of inhibition against *S. mutans* and also, inhibition zone against *S. aureus* was less (11 mm) when compared with leaf extract (13 mm), which can be correlated with the lesser amount of phenol and flavonoid content in rachis extract.

Table 3: Antibacterial activity of leaf and rachis extract of *D. spinulosum* Dyer ex Eichl.

Phytochemical	Concentration (µg/ml)	Zone of inhibition (mm)	
		S. aureus	*S. mutans*
Control	Streptomycin (100)	29	30
Leaf	250	Nil	Nil
	500	10	10
	1000	13	11
Rachis	250	Nil	Nil
	500	10	Nil
	1000	11	Nil

D. spinulosum: *Dioon spinulosum*, *S. aureus*: *Staphylococcus aureus*, *S. mutans*: *Streptococcus mutans*

The study indicates that *D. spinulosum* Dyer ex Eichl. is an interesting source of secondary metabolites with potential for use as a medicinal plant. The results indicated that the methanol extract of leaf of *D. spinulosum* has richer source of flavonoids and phenolics than rachis which could be attributed to the better solubility level of these constituents in methanol than in aqueous medium. The extract possesses antioxidant activity that is less potent when compared with the standard. Therefore, an increase in concentration is required for an increase in the radical scavenging effect. The *in vitro* antibacterial evaluation confirms that higher concentration of plant extract exhibits antibacterial activity and *S. mutans* was found to be resistant against rachis extract even at higher concentration.

CONCLUSION

The reducing capability of the leaf extract was found to be more when compared to rachis. The antibacterial potential was evaluated with Staphylococcus aureus and Streptococcus mutans by agar well diffusion method. Antibacterial activity was observed only at higher concentration (1000 µg/ml) with inhibition zones of 12 mm and 13 mm. The antioxidant activity was also measured

by the ferric reducing antioxidant power (FRAP) method. The extract possesses antioxidant activity that is less potent when compared with the standard. Therefore, an increase in concentration is required for an increase in the radical scavenging effect. The in vitro antibacterial evaluation confirms that higher concentration of plant extract exhibits antibacterial activity and S. mutans was found to be resistant against rachis extract even at higher concentration.

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