Bangladesh Pharmaceutical Journal ***29****(2): 228-234, 2026 (July)*

Phytochemical Profiling and Biological Activity Screening of *Diplazium splendens*: Insights into Antioxidant, Anti-inflammatory and Antidiarrheal Effects

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(Received: December 27, 2025; Accepted: March 25, 2026; Published (web): July 20, 2026)

Abstract

Ethnic communities of the Chittagong Hill Tracts (CHTs) in southern Bangladesh traditionally use *Diplazium splendens* (*D. splendens*) to treat inflammation, diarrhea, and cough; however, there is no scientific evidence regarding the bioactivity of these plants. This study aimed to investigate the phytochemical composition and evaluate the antioxidant, anti-inflammatory, and antidiarrheal activities of the ethanol extract of *D. splendens*. The extract was prepared using a Soxhlet apparatus with ethanol as the solvent. Phytochemical screening and bioactivity assessments were conducted using qualitative chemical tests, the DPPH free radical scavenging assay, inhibition of heat-induced protein denaturation, and the castor oil–induced diarrhea model in mice. Phytochemical analysis revealed the presence of alkaloids, glycosides, flavonoids, terpenoids, saponins, tannins, and vitamin C. The ethanol extract exhibited strong antioxidant activity with 51.46% DPPH inhibition compared to 65.77% for ascorbic acid. Significant ($p<0.05$) anti-inflammatory activity was observed, with 57.10% inhibition of protein denaturation of ethanol extract relative to aspirin (67.49%) at 1 mg/ml. The extract demonstrated dose-dependent antioxidant and anti-inflammatory effects. *In vivo* studies showed a significant reduction ($p<0.01$) of fecal percentage (42.02%), prolonged mean latency period, and delayed onset of diarrhea at a dose of 500 mg/kg body weight, although the effect was moderate compared to loperamide (78.26%) at 3 mg/kg. These findings provide scientific evidence supporting the traditional use of *D. splendens* as a potential natural source for antioxidant, anti-inflammatory and antidiarrheal agents.

Key words: *Diplazium splendens*, antioxidant, anti-inflammatory and antidiarrheal activity.

Introduction

Diplazium is a genus fern in the family Athyriaceae, approximately 400 species distributed globally, primarily in tropical and sub topical regions of Asia, Australia, and America (Praptosuwiryo, 2008). The genus name derives from the Greek word *Diplazium*, meaning "double," referring to the indusia that occur on both sides of the vein. Species of this genus are indigenous to Brazil, Newzeland, India, China, Bangladesh, Sri Lanka, Philippines, and Taiwan (Mildawati *et al.*, 2023). Although extensive research has documented various *Diplazium* species of its nutritional and therapeutic values—including protein (3.84%), fat (0.25%), fiber (5.05%), ash (1.33%), moisture (89.34%), and vitamin C (21.38 mg/100 g) ethnobiologists have largely overlooked its diverse culinary uses among various ethnic communities (Kumarihami *et al.*, 2022). *Diplazium splendens* (*D. splendens*), native to hilly regions, is found in Chattogram, the Chittagong Hill Tracts, Sylhet, and Cox's Bazar (Rudra *et al.*, 2021).

Several species of *Diplazium*, have been recognized for their medicinal and ethnobotanical significance, with documented applications in

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DOI: https://doi.org/10.3329/bpj.v29i2.91860

traditional medical systems such as Ayurveda and Materia Medica (Raina *et al.*, 2023). Ethanol, methanol, and aqueous extracts of whole plants of *D. esculentum* have shown strong antioxidant, anthelmintic, and cytotoxic activities, along with the isolation of bioactive compounds such as pentadecanoic acid, 1-heneicosanol, and phytol (Roy *et al.*, 2020). The root powder of *D. longifolium* is traditionally used as a hair tonic and an anthelmintic (Ullah *et al.*, 2022). Roots of *D. maximum* are used to treat gastrointestinal disorders (Rana *et al.*, 2015), while *D. polypodioides* is employed as a demulcent, hypotensive agent, and general tonic (Gul *et al.*, 2016). *D. stoliczkae* has been traditionally used for the treatment of dysentery and diarrhea (Rokaya *et al.*, 2010).

Ethnic communities of the Chittagong Hill Tracts used *D. splendens* for managing inflammation, diarrhea, and headaches (Yusuf *et al.*, 2006). Despite these traditional uses, no scientific studies have been reported on *D. splendens* to date. Therefore, the plant was selected for phytochemical profiling, antioxidant, anti-inflammatory and antidiarrheal activity in the present study.

Materials and Methods

Plant Identification and collection: D. splendens was selected for the present study based on its documented traditional medicinal use. The plant materials were collected from the hilly regions of Chittagong. Taxonomic identification was performed by botanists at the Bangladesh Forest Research Institute (BFRI) Herbarium, Chittagong. A standardized herbarium voucher specimen was prepared following institutional guidelines and deposited for future reference.

Collection and garbling: Fresh leaves of *D. splendens* were collected in October from the Sitakunda area near Chittagong. The collected materials were thoroughly cleaned to remove soil, debris, and extraneous plant parts. Garbling was performed to eliminate any unwanted, impure, or non-essential components, ensuring the purity of the plant sample and improving the reliability of subsequent analyses (Sasidharan *et al.*, 2015).

Drying and grindin:. The cleaned leaves were shade-dried at room temperature to prevent thermal degradation of heat-sensitive phytochemicals. After complete drying, the leaves were ground into a fine powder using an electric grinder. The powdered plant material was stored in airtight containers and kept in a cool, dry, and dark environment to preserve phytochemical integrity until further experimentation.

Hot Extraction with soxhlet extractor: For hot extraction, approximately 280 g of leaves powder of the *Diplazium splendens* was subjected with 1000 ml of ethanol (97.99%) and in a Soxhlet apparatus (SE-250~2000, China, Pyrex, 3.3 Glass). After collecting the material, filtering it with filter paper (Whatman), and evaporating it with a rotary evaporator (Wincom, RE 52A, China) at temperatures below 50°C (Kupchan *et al.*, 1973). The solvent was evaporated, resulting in a gummy concentrate that was labelled as hot ethanol crude extracts with a final extractive value of 22.25%.

Chemicals: All chemicals and solvents used in this study were of analytical grade and purchased from Merck, Germany. Standard drugs such as ascorbic acid, acetyl salicylic acid, loperamide were obtained from Square Pharmaceuticals Ltd as gift samples.

Experimental animals: 20-25 g of Swiss albino mice, aged 6-7 weeks old, was collected from the Bangladesh Council of Scientific and Industrial Research (BCSIR), Chattogram and Rajshahi. The mice were acclimatized to a controlled environment (temperature: 25±1°C, humidity: 55-65%, 12 hrs light/dark cycle) and provided a BCSIR-formulated meal and water for two weeks before the trials. Actions were taken to alleviate their discomfort. The institutional animal ethics committee approved all animal experimental methods that meet international standards, providing a strong ethical foundation for our study. The Institute of Biological Sciences' Ethical Research Committee at the University of Rajshahi in Bangladesh approved the study [Ref. No. 226/320/(69) IAMEBBC/IBSc].

Preliminary phytochemical analysis: For the preliminary phytochemical analysis, the crude ethanol extract was tested to a series of standard qualitative tests to identify its major phyto-chemical constituents (Ali, 1993).

*Test for antioxidant activity***:** The stable 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical was used to evaluate the ethanol extracts of *D. splendens* (Huiqing et al., 2013). 0.1 ml of ethanol extracts at different concentrations ranging from 20 µg/ml to 100 µg/ml was mixed with up to 3 mL of a 0.004% ethanol solution of DPPH. Ascorbic acid used as a positive control at similar concentration. Additionally, a control group received no extract treatment. After a 30 mins break, the absorbance at 517 nm was measured. The percent of inhibition was calculated (average absorbance [control-sample]/average absorbance control × 100).

*Test for anti-inflammatory activity***:** The reaction mixture contained 3 ml of 5% egg albumin solution and 3 ml of ethanol crude extracts of *D. splendens* to prepare final concentrations of 250, 500, and 1000 µg/ml. A similar volume was used standard (acetylsalicylic acid) respectively. Distilled water used as a control. To maintain consistency, all reaction mixtures' pH (6.2±6.5) was carefully adjusted with 0.1N HCl. The mixtures were incubated at 37±2ºC in a BOD incubator (Labline Technologies) for 15 mins before heating at 70ºC for 5 mins. After cooling, their absorbance was measured at 660 nm (*Shinde et al., 1999*)**.** The percent of inhibition was calculated (average absorbance [control-sample]/average absorbance control × 100).

In vivo anti-diarrheal activity: The castor oil-induced diarrhea technique developed by Shoba and Thomas was used to evaluate antidiarrheal properties (Shoba and Thomas, 2001). For the control, standard and test samples, swiss albino mice were selected randomely various groups of six mice each group. The doses for the extracts of *D. splendens* (500 mg/kg), control (10 ml/kg, 1% Tween 80), and standard (3 mg/kg) were determined based on body weight. Standard doses, control, and test samples were administered orally using a feeding needle. After 30 mins all mice were injected 0.4 mL castor oil orally. Blotting paper was placed on the bottom of each mouse cage. The lining of the blotting paper was replaced every hour. The inhibition of feces percentage was calculated (mean faces group [control-test]/mean faces control group × 100).

Statistical analysis: All results are expressed as mean ± standard error of the mean (SEM). Statistical analyses for the animal experiments were performed using one-way analysis of variance (ANOVA), followed by Tukey post hoc test to compare each treatment group with the vehicle control group. Differences were considered statistically significant at $p<0.05$ and $p<0.01$.

Results and Discussion

Preliminary phytochemical screening: The ethanol crude extract, when subjected to standard qualitative phytochemical tests, revealed the presence of alkaloids, glycosides, steroids, tannins, flavonoids, saponins, terpenoids, amides and vitamin C, as summarized in Table 1.

Table 1. Chemical analysis for phytoconstituents in the crude extract of *D. splendens*.

Experiment	Test performed	Results
Alkaloids	Dragendroff's test	+
	Mayer test	+
	Hager test	+
	Wagner test	+
Glycosides	NaOH test	+
Steroids	Libermann-burchard's test	+
Tannins	Ferric chloride test	+
Flavonoids	Conc. HCl and alcoholic test	+
Saponins	Froth test	+
Terpenoids	Salkowski's test	+
Amides	Sodium hydroxide test	+
Vitamin C	Dinitrophenyl hydrazine test	+

(+) = present; (-) absent.

In vitro antioxidant activity: In the antioxidant assay, the ethanol extract of *D. splendens* demonstrated notable free radical scavenging activity

in the *in vitro* DPPH test. The scavenging effect increased progressively with concentration, indicating a clear dose-dependent response. The standard antioxidant, ascorbic acid, produced DPPH inhibition values of 23.17, 34.23, 44.36, 53.05 and 65.77% at concentrations of 20, 40, 60, 80 and 100 µg/ml, respectively. In comparison, the ethanol extract of *D. splendens* produced inhibition values of 12.16, 23.56, 31.15, 42.05 and 51.46% at the same concentrations. Although the extract exhibited lower activity than the standard, it still showed substantial free radical scavenging potential, particularly at higher concentrations.

Table 2. Quantitative antioxidant activities of ethanol extracts of *D. splendens*.

Test Group	Crude extracts	% DPPH inhibition in different concentration (µg/ml)				
		20	40	60	80	100
Standard	Ascorbic acid	23.17	34.23	44.36	53.05	65.77
D. splendens	Ethanol	12.16	23.56	31.15	42.05	51.46

DPPH = 1, 1-diphenyl-2-picrylhydrazyl.

*In vitro anti-inflammatory activity***.** In the *in vitro* protein denaturation assay, both aspirin (ASA) and the ethanol extract of *D. splendens* exhibited concentration-dependent anti-inflammatory activity. The ethanol extract produced significant ($p<0.05$) percent inhibition protein denaturation values of 25.07, 46.52, and 57.10% at concentrations of 250, 500, and 1000 µg/ml, respectively.

Table 3. *In vitro* anti-inflammatory activity of ethanol extract of *D. splendens*.

Test Group	Crude extracts	Inhibition of Protein Denaturation (% IPD ± SEM)		
		250 µg/ml	500 µg/ml	1000 µg/ml
Standard	ASA	27.92 ± 0.02	52.01 ± 0.008	67.49 ± 0.026
D. splendens	Ethanol	25.07 ± 0.024	46.52 ± 0.007	57.10 ± 0.007

Values are expressed as percentage ± SEM (n = 3) each group; ASA = Acetyl salicylic acid, % IPD = % inhibition of protein denaturation, P-value $p<0.05$ considered significant.

In vivo anti-diarrheal activity: In the castor oil–induced diarrhea model, the ethanol extract of *D. splendens* demonstrated moderate antidiarrheal activity. The extract produced a 42.02% reduction in the number of fecal outputs, which was lower than the inhibition achieved by the standard antidiarrheal drug loperamide (78.26%). These findings suggest that *D. splendens* possesses measurable but comparatively weaker antidiarrheal efficacy (Table 4). Consistent with this, the extract produced a mean latency period of 24.33 ± 0.82 mins, whereas loperamide significantly delayed the onset of diarrhea to 48.00 ± 1.41 mins. Despite being lower than the standard, the prolonged latency associated with *D. splendens* was statistically significant ($p<0.01$), indicating a moderate capacity to delay diarrheal onset.

The present study demonstrated that the ethanol extract of *D. splendens* contains a wide range of bioactive phytochemicals, including alkaloids, glycosides, flavonoids, terpenoids, saponins, tannins, and vitamin C. The presence of these secondary metabolites is closely associated with the observed biological activities of the plant. Alkaloids are well known for their analgesic, anti-inflammatory, and antidiarrheal properties, whereas terpenoids possess broad pharmacological activities, including antibacterial, anti-inflammatory, analgesic, and antidiarrheal effects (Nassar *et al.*, 2010). Flavonoids, which are rich in polyphenolic compounds, play a crucial role in preventing cardiovascular disorders and inflammatory diseases due to their strong antioxidant potential (Genskowsky *et al.*, 2016).

Similarly, saponins have been reported to exhibit hypocholesterolemic and antihyperglycemic effects and contribute to antioxidant, antibacterial, antifungal, and weight-reducing activities (Iloki-Assanga *et al.*, 2016; Xiao *et al.*, 2017). Collectively, these phytochemicals may synergistically contribute to the antioxidant, anti-inflammatory, and antidiarrheal properties of *D. splendens*. Nevertheless, further detailed investigations are necessary to fully elucidate the underlying molecular mechanisms.

Table 4. *In vivo* antidiarrheal activity of *D. splendens*.

Medicinal Plants	Crude extract	Mean latency period (min)	No. of feces (4 hrs)	% defecation	% inhibition of defecation
Control	Tween 80	17.00 ± 1.87	23.00 ± 1.22	100	0
Standard	Loperamide	48.00 ± 1.41	5.00 ± 0.71	21.74	78.26
D. splendens	Ethanol	24.33 ± 0.82	13.33 ± 1.08	57.98	42.02

Values are expressed as percentage ± SEM (n = 6) each group; Standard (3 mg/kg) for standard; control (10 ml/kg) and ethanol extracts (500 mg/kg) respectively. Analysis using one-way ANOVA (graph pad prism software) followed by Tukey post hoc test to control. P-value $p<0.01$ considered significant.

In the DPPH free radical scavenging assay, the ethanol extract of *D. splendens* exhibited a DPPH inhibition of 51.46% at 100 µg/ml, which was a slight lower than standard (ascorbic acid). The DPPH assay is based on the reduction of the stable nitrogen-centered free radical DPPH, which changes color from violet to yellow upon accepting a hydrogen atom or electron from antioxidant compounds (Amri and Hossain, 2018). This reaction results in the formation of a colorless or bleached product, 1,1-diphenyl-2-picrylhydrazyl or a substituted hydrazine, leading to a decrease in absorbance at 517 nm. A rapid reduction in absorbance indicates strong hydrogen-donating and radical-scavenging activity. The observed activity suggests that *D. splendens* contains potent antioxidant constituents capable of neutralizing free radicals, thereby supporting its traditional medicinal use and highlighting its potential as a natural antioxidant source.

The ethanol extract of *D. splendens* also exhibited notable anti-inflammatory activity, as evidenced by its ability to inhibit heat-induced protein denaturation by 57.10%. This activity slightly lowers that of acetylsalicylic acid, indicating a strong anti-inflammatory potential. The inhibition of protein denaturation and stabilization of cell membranes against lysis are key mechanisms underlying anti-inflammatory activity, as previously reported for other medicinal plants (Leelaprakash and Das, 2011; Ngoua-Meye-Misso *et al.*, 2018). Protein denaturation is a common pathological event during prolonged inflammatory responses and can lead to impaired tissue function (Sangeetha *et al.*, 2016; Ikwegbue *et al.*, 2018). These results indicate that the extract possesses substantial anti-inflammatory potential, approaching the inhibitory activity of standard acetyl salicylic acid, a well-known cyclooxygenase (COX) inhibitor. Therefore, the ethanol extracts of *D. splendens* has potential in inhibiting protein denaturation and stabilizing cell membranes against lysis suggests its promise as a potential source for anti-inflammatory drug development (Ngoua-Meye-Misso *et al.*, 2018).

Castor oil-induced diarrhea is primarily mediated by ricinoleic acid, which irritates the intestinal mucosa, increases prostaglandin release, enhances intestinal permeability, and disrupts electrolyte transport (Khair *et al.*, 2014). Ricinoleic acid interacts with EP3 prostanoid receptors on intestinal smooth muscle cells, resulting in reduced absorption and increased secretion of fluids and electrolytes into the intestinal lumen (Tunaru *et al.*, 2012). In contrast, loperamide exerts its antidiarrheal effect by enhancing water and electrolyte reabsorption, reducing intestinal motility, prolonging the onset of defecation, and inhibiting prostaglandin-mediated

secretion (Tadesse *et al.*, 2017). Phytochemical analysis revealed that the ethanol extract of *D. splendens* contains alkaloids, glycosides, saponins, flavonoids, terpenoids, phenols, steroids, and tannins, all of which have been associated with antidiarrheal activity (Birru *et al.*, 2016). The extract demonstrated statistically significant ($p<0.01$) antidiarrheal activity, showing effects comparable to loperamide. However, the overall antidiarrheal efficacy of *D. splendens* was moderate, indicating the need for cautious use and further toxicological and pharmacodynamic evaluations

Conclusion

The present study revealed that the ethanol leaf extract of *D. splendens* exhibits significant antioxidant and anti-inflammatory activities, along with moderate antidiarrheal effects. These findings provide scientific support for the traditional medicinal use of this plant. The observed pharmacological activities may be contributed to the presence of bioactive constituents such as alkaloids, glycosides, flavonoids, terpenoids, saponins, and tannins. The results were validated through both *in vitro* and *in vivo* experimental models. Future research will focus on the isolation, characterization, and identification of the specific bioactive compounds responsible for these effects, using advanced phytochemical and analytical techniques.

Acknowledgements

The authors wish to thank the authority of the Institute of Biological Sciences, University of Rajshahi and Department of Pharmacy, Southern University, Bangladesh, Chattogram, Bangladesh for perform these investigations.

Funding

No funding sources.

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