UNVEILING THE BIOACTIVE POTENTIAL OF THE PTERIDOPHYTE *THELYPTERIS NUDATA* (ROXB.) C.V. MORTON: PHYTOCHEMISTRY AND PHARMACOLOGICAL EVALUATION

Bioresearch Communications
Volume 12, Issue 1, January 2026

DOI:
doi.org/10.3329/brc.v12i1.86764

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ABSTRACT

Pteridophytes, ancient vascular plants dating back to the paleozoic era, are recognized for their adaptability and production of diverse secondary metabolites with nutritional, agricultural, and medicinal significance. So, the present study was designed to investigate the phytochemical composition of *Thelypteris nudata* (Roxb.) C.V.Morton and evaluate its antimicrobial, antioxidant, and thrombolytic activities. The plant leaves were subjected to successive extraction using methanol, n-hexane, dichloromethane, ethyl acetate, and aqueous. Qualitative phytochemical analysis of methanol extracts of *T. nudata* exhibited the presence of flavonoids, alkaloids, phenolics, reducing sugar, diterpenes, coumarins, saponins, phlobatannins, and tannins. The Agar well diffusion method was performed for the antibacterial activity test. Antibacterial activity of methanolic extract showed greater zone of inhibition against all five bacterial strains (*Salmonella enterica*- 47 mm*, Shigella flexneri*- 37 mm*, Staphylococcus saprophyticus*- 43mm, *Enterococcus faecalis*- 39mm, *Vibrio parahaemolyticus*- 43mm). However, ethyl acetate and aqueous extracts also displayed substantial antibacterial activity, while n-hexane and dichloromethane fractions exhibited comparatively lower inhibition zones. The DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical scavenging method was used for antioxidant activity analysis. The antioxidant potential of different solvent extracts was evaluated based on their IC_{50} values. The methanol, ethyl acetate, and aqueous extract showed IC_{50} values 95.92 µg/mL, 21.4 µg/mL, and 22.92 µg/mL, respectively. However, the free radical scavenging activity of dichloromethane, and n-hexane extracts exhibited weak antioxidant activity, as evidenced by their higher IC_{50} values (319.89 µg/mL, and 236.42 µg/mL respectively) compared to standard butyl-1-hydroxytoluene (BHT) used. The thrombolytic activity of different solvent extracts was assessed using an *in vitro* clot lysis assay. The ethyl acetate extract showed the highest thrombolytic activity (11.44 ± 1.05 %) among the tested fractions.

KEYWORDS: Phytoprofiling, *Thelypteris nudata,* Antibacterial and Antioxidant activity, IC_{50}, Thrombolytic activity.

RECEIVED: 31 October 2025, ACCEPTED: 26 December 2025

TYPE: Original Article

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Introduction

The plant kingdom is a rich source of various therapeutic agents, and plants have been employed for their diverse healing properties for many centuries (Ghutke *et al*., 2023). Natural products and their derivatives have negligible side effects but higher effectiveness than other manmade chemical compounds (Batiha *et al*., 2020). The record shows that around 25-50% of today's clinically applied medicines are obtained from plants. According to WHO, about 11% of important medicines are purely plant-derived. Moreover, more than half of all anticancer and anti-infections drugs are derived from natural plant products. This emphasizes the important role of plants as a medicinal component in modern medicine (Górka *et al*., 2025). Although other plant categories have been thoroughly studied in the ethnobotanical use, pteridophytes are underrepresented in pharmacological surveys.

Ferns and their allies, with over 12,000 identified species in 250 genera are significant for their medicinal benefits (Sridhar *et al*., 2023). Pteridophytes hold considerable importance for their food, medicinal, and ornamental values (Jone *et al*., 2022). They have been utilized in traditional ethnomedicinal practices for managing numerous health conditions including infectious, respiratory, gastrointestinal, inflammatory, and ulcerative disorders, as well as snake bites. (Abraham and Thomas, 2022; Cao *et al*., 2017). In addition to this, potentiality of pteridophytes in therapeutic field is mainly attributed to flavonoids and terpenoids which exhibit strong antioxidant activity and play significant roles in anti-inflammatory, anticancer, antigenotoxic, and neuroprotective effects (Górka *et al*., 2025; Bandyopadhyay *et al*., 2022).

Bangladesh is a home to diverse biodiversity due to its varied climate, as well as its unique geographic location, situated

between the Indian subcontinent and south part of Asia (Mukul *et al*., 2008). Bangladesh is a home to 196 documented pteridophyte species, most of which thrive in diverse habitats ranging from water bodies and moist environments to tree surfaces and rocky areas (Jone *et al*., 2022) but most people are unaware of the possible use for ferns.

Thelypteris nudata (Roxb.) C.V.Morton belongs to the family Thelypteridaceae, under the genus *Thelypteris*. This fern is widely distributed in different multinational regions of Asia, such as India, Bangladesh, Nepal, and China (Fraser-Jenkins *et al*., 2015). The numerous species of the genus *Thelypteris* are well-known as traditional herbs which has been used to treat human diseases. For example, the paste of *T. arida* leaves and rhizomes is smeared on wounds and cuts (Giri et al., 2021), *T. parasitica* is used in the treatment of gout (Abraham and Thomas, 2022), *T. prolifera* has been used as a remedy for constipation, and *T. interrupta* possesses significant antimicrobial value (Marimuthu *et al.*, 2022). Another species, *T. nudata*, the focus of this work, has been described in traditional medicine. People use it in the treatment of headaches and pyorrhea (Kholia and Balkrishna, 2022). However, only a very few studies have been investigated to characterize its medicinal values. Our present study aimed to evaluate the phytochemical profile, antibacterial efficacy, antioxidant properties and thrombolytic potential of the fern *Thelypteris nudata.* Due to the genus's ethnomedicinal value, *T. nudata* in particular seems very promising to carry out detailed phytochemical and pharmacological studies.

Materials and Methods

Plant Collection and Identification

Specimens of *Thelypteris nudata* (Roxb.) were collected during October to November 2024 from the slope area adjacent to the faculty of Biological Sciences, University of Chittagong, Bangladesh. Healthy, mature plants were selected for leaf collection. The taxonomic identification was ensured by Professor Dr. Shaikh Bokhtear Uddin, Department of Botany, University of Chittagong. Besides, a voucher specimen (Accession No. SJR211223-627) was put in the Herbarium of the University of Chittagong.

Sample Preparation

Fresh leaves were separated from stems, washed thoroughly with fresh and distilled water, and cut into small fragments. The fragments were shade-dried for 3-4 weeks at ambient temperature. Dried material was prepared as a fine powder through high-capacity grinder. The prepared powder sample was preserved in an airtight amber glass container under cool, dry, and dark conditions until further extraction.

Preparation of methanolic extracts and fraction

At first, 300g of powdered leaves were saturated in 90% methanol at a ratio of 1:10. It was then stored in a covered glass bottle and left for 7 days with intermittent shaking and stirring for intensive absorption. Thereafter, using Whatman No. 1 filter paper, the solution was filtered and the residue was collected carefully. The residue was then reextracted two times, making a total of three extraction cycles. A rotary evaporator with a water bath at 40°C to obtain a dark green crude extract, which was then subjected to liquid-liquid partitioning. This is how, the combined filtrates were concentrated.

The liquid-liquid (solvent-solvent) partitioning technique was carrying out following the protocol developed by Kupchan and later modified by Van Wagnen *et al*. (1993) to fractionate the crude methanolic extract according to polarity. Firstly, the methanolic crude extract was diluted in distilled water (DW) and successively partitioned with solvents by increasing polarity: n-hexane, dichloromethane (DCM), and lastly ethyl acetate. For each solvent, an equal volume was added to the aqueous phase in a separating funnel, shaken thoroughly and allowed to separate into layers. After collecting the organic layer, the process was performed for three times for each solvent. Lastly, all fractions were concentrated using a rotary evaporator and then preserved at 4°C for biological evaluations later.

Microorganism

In this study, five pathogenic bacterial strains were used to examine the antibacterial potential. Bacteria used in this study were the pathogenic (gram-negative bacteria) *Shigella flexneri* (ATCC 12022), *Salmonella enterica* (ATCC 35664) and *Vibrio parahaemolyticus* (ATCC 17802), the gram-positive bacteria *Staphylococcus saprophyticus* (ATCC™ BAA-750™) and *Enterococcus faecalis* (ATCC 29212). The strains were obtained from the Bangladesh Council of Scientific and Industrial Research (BCSIR), Chattogram, Bangladesh. All the bacteria were cultured on nutrient agar slants at 4 °C followed by subcultured in nutrient broth for 24 hours. The final cellular concentration was standardized to 10^7 - 10^8 cfu/mL. Each suspension exhibited a concentration of 1 McFarland (OD600).

Qualitative phytochemical screening

The existence of major secondary metabolites or phytochemicals in the methanolic extract of *Thelypteris nudata* leaf was evaluated using standard procedures described by Shaikh *et al*. (2020).

***Detection of Alkaloids*:** The methanolic extract, dissolved in diluted HCl was filtered and filtrate extracts were subsequently studied separately using Wagner's and Hager's reagents. The existence of alkaloid was confirmed by the development red and yellow color respectively

***Detection of Reducing Sugar*:** About 5 mL of distilled water was added to methanolic extracts to dissolve it. The filtrates were then treated with Fehling's Reagent and kept in a water bath to provide heat. The development of a brick-red precipitate proved the existence of reducing sugar.

***Detection of Saponins*:** Saponin was detected by foam test where distilled water (5 mL) and aqueous extract (10 mL) were added and shaken strenuously. The foam development indicated the existence of saponins.

***Detection of Flavonoids*:** The flavonoids were confirmed by lead acetate test where a yellow precipitation was observed when a few drops of 10% lead acetate was mixed to the 1 mL of extract.

***Detection of Phenolic Compound*:** The phenolic compound was detected by two different tests: the lead acetate test and the ferric chloride test. In lead acetate test, aqueous extract (crude + 5mL distilled water) was treated with 3mL of 10% of lead acetate solution and in ferric chloride test, 1mL plant extract mixed with 5% ferric chloride. Phenolic compounds were detected by formation of white precipitate and a bluish-black color, respectively.

***Detection of Tannin*:** Braymer's test was done to detect tannin. An aqueous extract (5mL crude + 5mL distilled water) was boiled and filtrated. Tannins were confirmed when the mixer of 1mL of filtrate sample and 3 mL of distilled water extract produced a dark green color upon addition of ferric chloride.

***Detection of Phlobatannins*:** Phlobatannin was confirmed by the formation of red precipitate when mixture of 2 mL of aqueous extract of crude and 2 mL of 1% HCl was boiled properly.

***Detection of Diterpene*:** Emerald green color indicated the presence of diterpenes when 3-4 drops of copper acetate solution were mixed with the aqueous extract of the crude.

***Detection of Coumarin*:** 1 mL crude extract and 1 mL 10% sodium hydroxide (NaOH) solution was mixed and allowed to stand for a few minutes. Yellow coloration confirmed presence of coumarins.

Determination of antibacterial potential

The antibacterial potential of each solvent fraction of *Thelypteris nudata* leaf extract was assessed using the agar well diffusion method as used by Valgas *et al*. (2007) and Balouiri *et al*. (2016). Mueller-Hinton Agar (MHA) medium, composed of starch (1.5 g/L), casein hydrolysate (17.5 g/L), beef infusion solids (2.0 g/L), and agar (17.0 g/L) was prepared following standard procedures, and approximately 20 mL was dispensed into sterile Petridishes under aseptic conditions. Each plate was inoculated with the test bacterial suspension using a sterile swab after solidification.

Reservoir wells (6 mm) were made in the agar using sterilized corkborers, and each well was loaded with of extract (80 µL) solution (100 mg/mL in 10% DMSO). Ciprofloxacin (0.05 mg/well) functioned as the positive control, while 10% DMSO was considered as the negative control. The plates were kept at room temperature for 30 minutes to permit diffusion of the samples and then incubated at 37 °C for 24 hours. Antibacterial activity was determined by measuring the diameter of the inhibition zones (mm).

In vitro antioxidant activity (DPPH Radical Scavenging Assay)

The antioxidant activities of the methanolic crude extracts of *T. nudata* and Tert-butyl-1-hydroxytoluene (used as the standard antioxidant) was assessed based on their hydrogen donating or free radical scavenging (ROS) capacity by using the stable DPPH radical by method described by Brand Williams *et al*. (1995) with a few minor modifications. 2 mL methanolic plant extract at various concentrations (0.977µg/mL to 250µg/mL) was added with 3 mL of 0.004% methanolic DPPH solution. Incubation of the mixture was maintained in the dark place at room temperature for 30 minutes to allow the reaction to occur. The absorbance was then measured at 517 nm using a spectrophotometer, with methanol serving as the blank. In this bioassay, methanol mixed with methanolic DPPH solution was considered as a negative control. The percentage (%) inhibition activity was calculated using the mentioned equation:

$$\% \, I = \frac{A - B}{A} \times 100$$

Where, A = absorbance of the control, and B = absorbance of the extract/standard.

Lastly, Thee % inhibitions were plotted against the concentration and IC_{50} was calculated using a calibration curve from the graph.

Determination of thrombolytic activity

The evaluation of the thrombolytic activity of plant extract was done using *in vitro* clot lysis assay as described by Prasad *et al*. (2006) with a little modification. The fresh venous blood (6mL) was collected from healthy adult volunteers and transferred into six pre-weighted sterile 1.5 mL microcentrifuge tubes (1 mL/tube). To allow clot formation, the tubes were then incubated for 45 minutes at 37 °C followed by complete serum removal without disturbing the clot. The tubes were reweighed to determine clot weight. Therefore,

Clot weight = weight of clot containing tube − weight of empty tube

Next, 100 µL of the sample extracts (methanolic, n-hexane, dichloromethane, ethyl acetate and aqueous fractions) were added to tubes containing the preformed clot. 100 µl of streptokinase (Sk, 30,000 IU) was used as a positive control whereas 100 µl of distilled water was added as a negative control. After incubating the tubes for 90 minutes at 37 °C, released fluid was carefully removed and tubes were weighed again. The difference in weight at pre and after treatment represented the amount of clot lysis.

So, the percentage of clot lysis was calculated using the following formula:

$$\% \text{ Clot lysis} = \left(\frac{\text{Weight of the lysis clot}}{\text{Weight of clot before lysis}}\right) \times 100$$

Statistical analysis

Most of the experiments were carried out in triplicates. Experimental results were expressed as the mean ± standard error of the mean (SEM) of the three replicates.

Results and discussion

Qualitative Analysis

The qualitative phytochemical profiling of the *Thelypteris nudata* extract indicated the various secondary metabolites present in the sample (Table 1). Especially reducing sugar, flavonoids, saponins, tannins, phenolic compounds, diterpene, alkaloid, phlobatannins, and coumarins has wide range of potential activities. The strong color development and formation of precipitates confirmed the presence of flavonoids, phenolic compounds, and tannins are noteworthy. In the medicinal spectrum, all these compounds have been previously well studied. For instance, flavonoids have shown anticancer, antioxidant and antimicrobial activities. Tannins are known for their astringent and antimicrobial properties. Again, phenolic compounds are also considered as an antioxidant component. The obtained results show that *T. nudata* has a rich chemical composition, justifying the use of the plant by the traditional medicine.

Table 1. Phytochemical screening of *T. nudata* leaf extract

S. N.	Phytochemical test	Reagents used	Observed result	Inference
1	Alkaloid	Wagner's Reagent	Reddish brown precipitate	Present
		Hager's Reagent	No precipitate	-
2.	Reducing sugar	Fehling's Reagent	Red precipitate	Present
3.	Saponin	Foam Test	Stable foam (>1hour)	Present
4.	Flavonoids	Lead Acetate Test	Yellow precipitate	Present
5	Phenolic compound	Ferric Chloride Test	Dark Green/Bluish Black coloration	Present
		Lead Acetate Test	White precipitate	Present
6.	Tannin	Braymer's Test	Blue-black precipitate	Present
7.	Phlobatannins	HCL Test	Red precipitate	Present
8.	Diterpenes	Copper acetate test	Emerald Green coloration	Present
9.	Coumarin	NaOH test	Yellow coloration	Present

Antibacterial potential Test

The antimicrobial evaluation of *T. nudata* leaf extracts was confirmed a broad inhibitory effect on both pathogenic (Gram-positive and Gram-negative bacterial strains) (Table 2 and Figure 1). The methanolic extract showed the highest antibacterial activity among all the solvent fractions analyzed, with inhibition zones ranging from 37 ± 0.60 mm to 47 ± 0.78 mm. The ethyl acetate extracts showed inhibition zones between 32 ± 1.09 mm and 40 ± 0.67 mm comparatively. The higher level of activity suggested that the methanol effectively extracted a wide range of bioactive plant metabolites, including flavonoids, alkaloids, and terpenoids, which are commonly known for their robust antimicrobial potential.

Notable activity has been found from the aqueous extract though slightly reduced (27 ± 0.86 mm to 36 ± 0.72 mm) are likely due to the extraction of polar constituents like tannins and saponins. The smaller inhibition zones, ranging from 11 ± 0.16 mm to 20 ± 0.57 mm (using extracts of dichloromethane and n-hexane), indicating that non-polar constituents contributed negligible to antibacterial activity. No activity against *Vibrio parahaemolyticus*, suggesting a restricted antimicrobial spectrum in case of extract using n-hexane.

The Gram-positive bacteria, including *Staphylococcus saprophyticus* and *Enterococcus faecalis*, exhibited significant susceptibility to the methanolic and ethyl acetate extracts in regard to microbial susceptibility. Likewise, Gram-negative strains (*Salmonella enterica* and *Vibrio parahaemolyticus)* showed noteworthy sensitivity. Compared to the positive control, Ciprofloxacin (22–26 mm), the methanolic and ethyl acetate extracts exhibited comparable or greater inhibition zones which indicates significant antibacterial activity. The observed inhibition zones were exclusively due to the bioactive compounds present in the extracts where the absence of activity in the negative control authenticates that. These findings highlight *T. nudata* leaves as a possible source of antimicrobial agents, particularly in the methanolic and ethyl acetate fraction.

Table 2. Antimicrobial activity of test samples of leaves *T. nudata*.

		Zone of inhibition (mm) for 24-hour incubation period						
S. N	**Test organisms**	**Methanolic extract**	**N-Hexane extract**	**Dichloromethane extract**	**Ethyl Acetate extract**	**Aqueous extract**	**Positive Control**	**Negative Control**
Gram positive bacteria								
1	*Staphylococcus saprophyticus*	43 ± 1.16	14 ± 0.16	15 ± 0.37	40 ± 0.67	32 ± 1.16	22	0

2	*Enterococcus faecalis*	39 ± 0.72	11 ± 0.28	17 ± 0.35	35 ± 0.33	34 ± 1.45	22	0
Gram negative bacteria								
3	*Shigella flexneri*	37 ± 0.60	12 ± 0.16	16 ± 0.44	32 ± 1.09	36 ± 0.72	26	0
4	*Salmonella enterica*	47 ± 0.78	11 ± 0.44	12 ± 0.16	37 ± 1.25	27 ± 0.86	23	0
5	*Vibrio parahaemolyticus*	43 ± 0.57	Nill	20 ± 0.57	39 ± 0.77	30 ± 0.93	25	0
N.B: All zones of inhibitions were expressed without subtracting 6 mm (the diameter of well)								

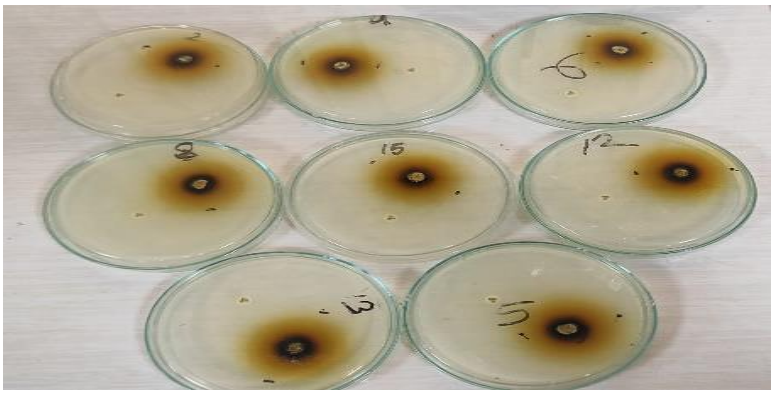

Figure 1. Agar well diffusion method of different extracts of *T. nudata* against pathogenic bacteria

Antioxidant activity

The scavenging activity of all samples is concentration dependent in case of this experiment (Figure 2-7). The IC_{50} findings of *antioxidant activity* from the *T. nudata* leaf extracts are illustrated in Figure 8. Particularly, the extract of ethyl acetate and aqueous solution showed the highest antioxidant potential with IC_{50} values of 21.4 ± 0.24µg/mL and 22.92 ± 0.18µg/mL respectively. These values are relatively close to the IC_{50} value of the standard (BHT) which is 18.26 ± 0.12µg/mL. Conversely, methanol, dichloromethane, and n-hexane extract exhibited moderate to weak antioxidant activity with higher IC_{50} values 95.92 ± 0.82µg/mL, 319.89 ± 1.49µg/mL, and 236.42 ± 1.25µg/mL respectively. Although these extracts still demonstrated some degree of antioxidant potential, their efficacy fell short in contrast to the ethyl acetate and aqueous extracts, underscoring the importance of extraction solvents in capturing bioactive antioxidant compounds effectively.

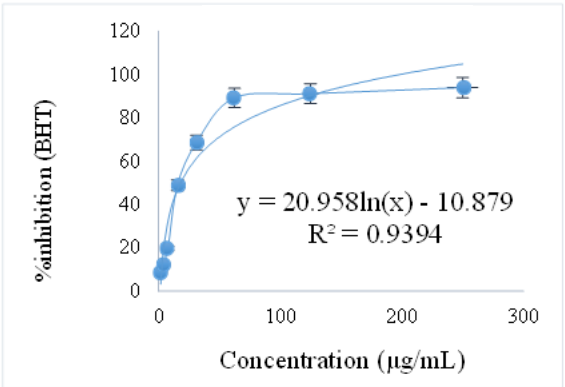

Figure 2. Scavenging activity of *tert*-butyl-1-hydroxytoluene (BHT).

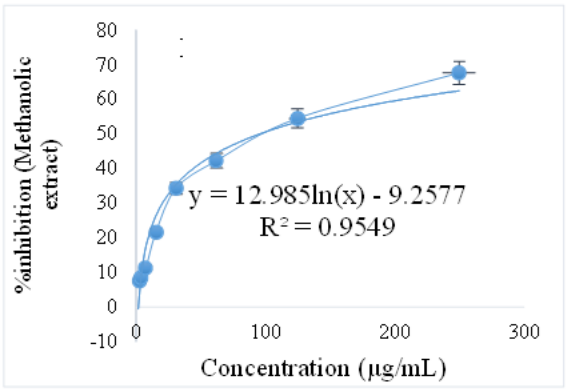

Figure 3. Scavenging activity of methanolic extract of leaves *T. nudata*

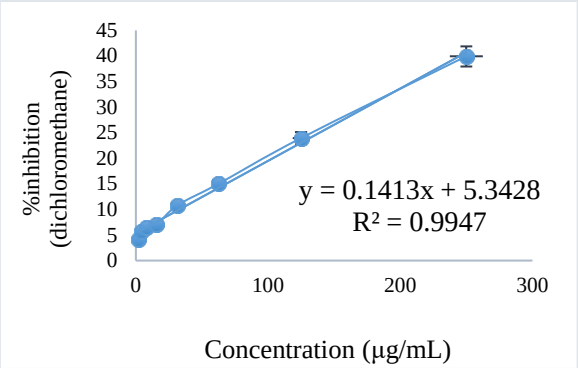

Figure 4. Scavenging activity of dichloromethane extract of leaves of *T. nudata*

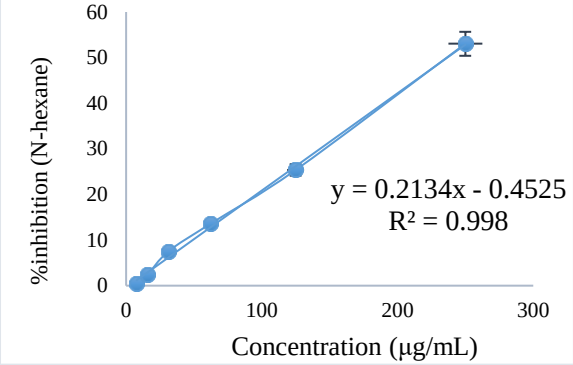

Figure 5. Scavenging activity of n-hexane extract of leaves *T. nudata*

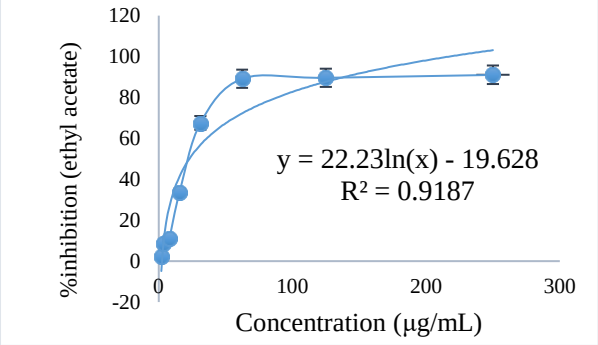

Figure 6. Scavenging activity of ethyl acetate of leaves *T. nudata*

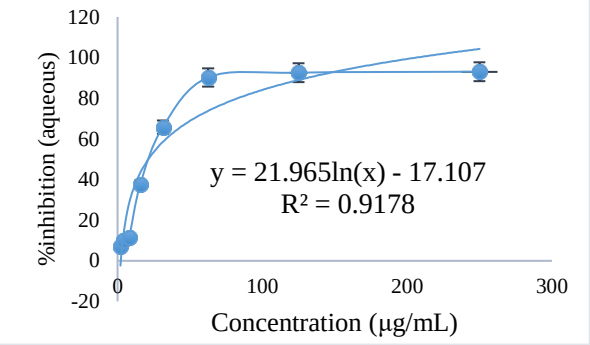

Figure 7. Scavenging activity of aqueous extract of leaves *T. nudata.*

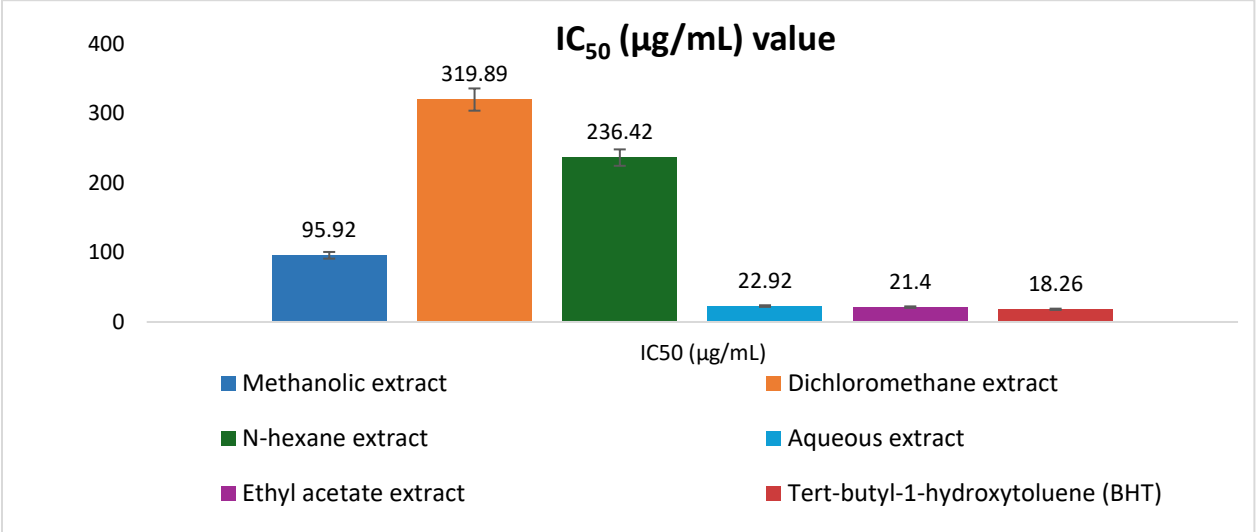

Figure 8. IC_{50} values of the standard and extracts of leaves of *T. nudata*

Thrombolytic activity test

Thrombolytic activity of crude methanolic extract and various solvent fractions of *Thelypteris nudata* was evaluated to explore its potential as a source of cardioprotective agent, and results are shown in Figure 9. Positive control, Streptokinase (30,000 I.U.) exhibited 65.86 ± 1.64% clot lysis after 90 minutes incubation at 37 °C, while negative control, distilled water showed negligible activity (1.15 ± 0.23 % clot lysis). Among the tested fractions, ethyl acetate fraction demonstrated the highest thrombolytic activity (11.44 ± 1.05 %), which was notably higher than the negative control. Methanolic, dichloromethane, n-hexane, and aqueous fractions displayed mild clot lysis activity compared to the standard ranging from around 4 to 8%.

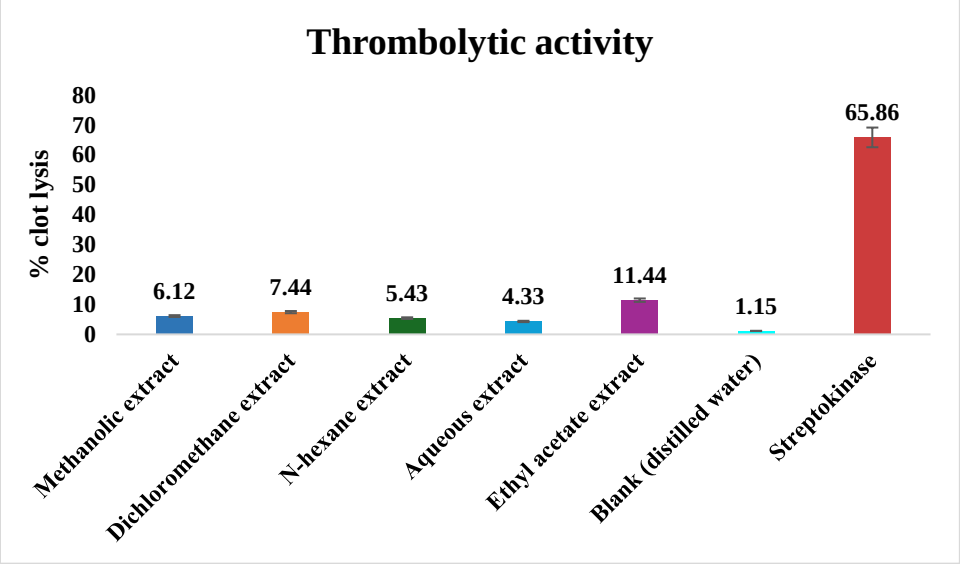

Figure 9. Thrombolytic activity of *T. nudata*

Conclusion

Medicinal plants always represent the potential source of therapeutic agents. The present study validates the novel status of *Thelypteris nudata* in the field of pharmacognosy using in vitro approaches. The results showed significant antimicrobial and antioxidant properties, which can be explained by the presence of various phytochemical compounds that were detected in the plant extract. However, the thrombolytic activity was however relatively weak and was not as effective as was expected. The present study serves as only a preliminary step toward the understanding of the medicinal aspect of this sample. Hence, further research is mandatory involving cytotoxicity analyses, in vivo studies, and active compounds isolation to fully determine the pharmacological properties and safety of T. nudata. In a nutshell, the *Thelypteris nudata* plant extract exhibits a great possibility as a potential candidate for the discovery and development of novel plant derived pharmaceuticals that could contribute to harmless, and more sustainable healthcare solutions.

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