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CLOT LYSIS AND MEMBRANE PROTECTION POTENTIALS OF *CHEILANTHES TENUIFOLIA* METHANOLIC LEAF EXTRACT

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Abstract

Cheilanthes tenuifolia is a little perennial fern that falls within the taxonomic classification of the Pteridaceae botanical family. The plant has a diverse array of phytochemical substances, including alkaloids, phenolic compounds, flavonoids, saponins, steroids, and triterpenoids, which have demonstrated promising medicinal properties. This study aimed to evaluate the *in-vitro* membrane-stabilizing and clot lysis activities of the methanol leaf extract of *C. tenuifolia* (MCT). For this, we performed hypotonic solution-induced erythrocyte lysing and human blood clot lysis methods to check the membrane stabilizing and clot lysis capacities of MCT using acetylsalicylic acid and streptokinase as standards, respectively. Additionally, we also checked its phytochemical groups. The results of a preliminary phytochemical screening indicate the presence of alkaloids, glycosides, tannins, flavonoids, and saponins in the MCT. MCT inhibited hemolysis in a concentration-dependent manner and inhibited 78.93 ± 0.01% hemolysis (IC_{50} = 46 ± 2.11 μg/ml) at the higher concentration (160 μg/ml), whereas the standard drug, acetylsalicylic acid (IC_{50} = 64.10 ± 2.08 μg/ml) inhibited 97.71 ± 0.01% at the same concentration. It also exhibited clot lysis in a concentration-dependent manner, where the maximum percentage of clot lysis was observed at 160 μg/100 ml where the IC_{50} value was 198.41 ± 1.87 μg/ml. The standard drug streptokinase showed 77.51 ± 0.01% clot lysis. *C. tenuifolia* possesses various important secondary metabolites and shows membrane stabilizing and clot lysis capacity. Further studies are required to elucidate its active principles and their biological effects.

Keywords: *Cheilanthes tenuifolia*, Clot lysis capacity, Membrane stabilizing activity

Introduction

In contemporary times, the utilization of plants, plant extracts, or isolated chemical compounds derived from natural sources has become prevalent in the treatment of many ailments, although conventional drugs are the most commonly used therapeutic system (Jamshidi et al., 2017). Plants include a diverse array of phytochemical compounds, such as vitamins, minerals, terpenoids, phenolic acids, lignins, stilbenes, tannins, flavonoids, quinones, coumarins, alkaloids, amines, betalains, and various other metabolites (Ahirwar and Tembhre, 2021). These phytoconstituents have been reported for diverse pharmacological activities such as anti-inflammatory, anti-atherosclerotic, anti-tumor, anti-mutagenic, anti-carcinogenic, antibacterial, and antiviral activities. These properties contribute to the reduction of cancer, heart disease, and diabetes risks (Tariq et al., 2021). As per the World Health Organization (WHO), it is currently observed that a significant proportion, namely three-quarters, of the global population relies on traditional remedies for the provision of healthcare (Shakeela and Sugumar, 2019). During the mid-nineteenth century, a significant proportion, around 80%, of medicinal substances were obtained from herbal sources. Consequently, chemicals originating from plants have consistently served as a crucial reservoir for pharmaceutical compounds (Gunjan et al., 2015). Numerous pharmacological categories of medications, such as morphine, digoxin, quinine, atropine, reserpine, physostigmine, pilocarpine, vincristine, vinblastine, artimesinin, and

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taxol, exemplify the contributions made by medicinal plants throughout history (Halder and Jha, 2023). The contemporary field of medicine heavily relies on the pharmaceutical industry for the production of medicinal drugs, a significant portion of which are derived from the bioactive compounds found in plants. Moreover, the escalating expenses associated with pharmaceuticals and the growing prevalence of treatment resistance in prevalent ailments such as malaria, bacterial infections, and sexually transmitted diseases have prompted a collective endeavor to explore novel chemical compounds (Thomford et al., 2015) Consequently, these plant-derived substances serve as essential raw materials in several instances (Atanasov et al., 2015).

Membrane stabilizing effects involve the inhibition or total abolishing of action potentials from being propagated across the membrane (Aronson, 2008). A membrane stabilizing drug can lessen aberrant or excessive electrical activity that protects heart from arrhythmia by attaching to the sodium channels and blocking their function. (Fouda et al., 2022). This helps to restore normal electrical signaling and prevent the uncontrolled firing of neurons or muscle cells (Nag et al., 2020). Membrane stabilizing activity is particularly relevant in the context of certain medical treatments, such as the management of analgesics, pain, and inflammation (Pogatzki et al., 2017). Certain medications, such as certain local anesthetics and non-steroidal anti-inflammatory drugs (NSAIDs), demonstrate membrane stabilizing capabilities in addition to their primary therapeutic effects (Patel and Day, 2018). Common side effects may include gastric ulcers, kidney damage, allergic reactions, dizziness, nausea, drowsiness, and an increased risk of heart attack and stroke (Bozimowski, 2015).

Atherothrombosis is a pathological state characterized by the formation of a thrombus on an unstable atherosclerotic plaque. The process in question is a widespread, diffused, and gradual phenomenon that impacts several vascular systems (Da et al., 2018). According to Eisen et al. (2016), the prognostication of the temporal progression of acute coronary syndromes (including unstable angina, acute myocardial infarction, and sudden cardiac death), ischemic stroke, and peripheral artery disease can pose challenges, given their potential life-threatening clinical manifestations. In the treatment of atherothrombosis, various drugs are utilized, but they can come with potential side effects. Antiplatelet drugs like aspirin and clopidogrel and anticoagulants such as warfarin may increase the risk of bleeding (Flora and Nayak, 2019). Statins can occasionally lead to muscle pain, liver enzyme abnormalities, or digestive issues (Sivashanmugarajah et al., 2019). ACE inhibitors and ARBs used to manage blood pressure can cause low blood pressure, coughing, or kidney problems. Beta-blockers may cause fatigue, slow the heart rate, or worsen asthma symptoms (Komajda et al., 2016).

Cheilanthes tenuifolia (Burm.f.) Sw., also known as the Sword Fern or Narrow-leaved Cloak Fern, is a small evergreen fern belonging to the Pteridaceae family (Jarial et al. 2018). It grows in the coastal areas of the Northern Territory and Queensland. Additionally, within the Asian region, there are several other geographic-entities, such Malesia, Melanesia, Polynesia, and Australia. Its fronds are pinnate, with lance-shaped, leathery leaflets displaying serrated edges. The fronds exhibit a length of up to 63 cm and a width of 17 cm. The stipe and rachis have a dark red-brown coloration, with a smooth surface or the occasional presence of sparse hairs consisting of 2-13 cells. Additionally, the fronds may possess very little thin scales. The lamina of the plant can take the form of a pentagon, triangle, or oval shape. It is 3-4-pinnate at the base and remains 3-pinnate for the majority of its length. The larger pinnae are triangular-ovate in shape, while the pinnules are lanceolate or ovate. The ultimate pinnules may occasionally exhibit a minor caudate characteristic. Typically growing to 15–30 centimeters in length, this fern is known for its ability to thrive in dry and rocky environments, often found in exposed locations like cliffs and rocky outcrops (Field et al., 2022). The primary constituents found in the methanolic extract of *C. tenuifolia* are phytochemical substances, including alkaloids, phenolic compounds, flavonoids, saponins, steroids, and triterpenoids (Ghorpade et al., 2015). The flavonoids derived from ferns have exhibited considerable promise in terms of their anti-cancer, anti-microbial, and anti-inflammatory properties, as well as their prospective therapeutic applications in the management of diabetes (Jarial et al., 2018). During ancient times, the liquid extracted from the rhizomes of ferns was employed for the treatment of gastrointestinal ailments, including stomach diseases and peptic ulcers. Furthermore, the utilization of rhizome paste derived from ferns has commonly been employed for the purpose of treating lacerations and injuries (Debbarma et al., 2017). Injuries and gastrointestinal ailments, including peptic ulcer are often characterized by inflammation (Serafim et al., 2020). The presence of diverse phytochemicals, such as flavonols, phenols, and alkaloids, in ferns is clearly apparent. These phytochemicals have been found to possess significant biological activity. As demonstrated by Jarial et al. (2018), the pure flavonoids derived from *C. tenuifolia* have notable properties as natural antioxidants and antibacterial agents, in addition to displaying potential efficacy in combating cancer. The objective of this work was to assess the membrane-stabilizing to verify its anti-inflammatory potentials and clot-lysis activities of methanol extracts from *C. tenuifolia* leaves using

the different *in-vitro* models. Furthermore, a preliminary phytochemical screening of the aforementioned extract was conducted. Therefore, this study was conducted on the traditional uses and scientific basement of the plant.

Materials and Methods

Collection and identification of plant materials

Fresh foliage was collected from the Bayazid hill tracts in Chittagong during July and August in 2021, which is the period when the plant grows the most. The plant was identified by a taxonomist at the Bangladesh Forest Research Institute Herbarium (BFRIH), Chittagong, Bangladesh (Voucher No. BFRIH-SA 577). The decayed leaves, stems, dust, and other parts of the plant were removed with great care. Subsequently, the plant constituents were subjected to a continuous stream of tap water for rinsing purposes, followed by a drying process conducted in a shaded environment at a temperature below 40 °C. Following the drying process, the materials underwent pulverization, resulting in a coarse powder. Subsequently, the pulverized components were carefully transferred into a hermetically sealed container possessing an amber hue. Subsequently, the container was stored in a cold and dry location until the commencement of the extraction procedure.

Extraction of plant materials

A quantity of 140 grams of leaf powder was immersed in 900 milliliters of 100% methanol within a glass bottle of amber hue, with a capacity of 2.5 liters. This soaking process lasted for duration of 14 days. The mixture was kept in a dark and cool place, and it was shaken once a day. On day 15, the mixture was shaken vigorously and collected in a large beaker. Subsequently, the content underwent filtration with Whattman Filter Paper No. 1. Finally, the filtrate was collected in a clean and dried beaker covered with aluminum foil paper to protect it from light. Its upper open part was covered with aluminum foil, making some small holes to allow the evaporation of methanol at room temperature. The determination of the % yield value was conducted in the following manner:

$$\%\text{Yield} = [\text{Weight of crude extract (gm)} / \text{Weight of powder taken (gm)}] \times 100$$

Reagents and chemicals

Streptokinase (Altepase®) was purchased from Beacon Pharmaceuticals Ltd., Bangladesh, while methanol, tween 80, acetyl salicylic acid, and other required reagents and chemicals were purchased from Merck (India).

Funding

The Bangabandhu Sheikh Mujibur Rahman Science and Technology University Research Center (BSMRSTU-RC) (Approval No. 2023-33) has approved and funded this study.

Phytochemical screening

The phytochemical screening was conducted following the methodology outlined by Islam (2021).

Clot lysis assay

This *in vitro* study was done according to the model developed by Prasad et al. (2007). In this case, we distributed 0.5 ml of fresh blood in pre-weighed microcentrifuge tubes from the non-contraceptive or anti-coagulant-treated humans. Following incubation of the blood sample at a temperature of 37 °C for duration of 45 minutes, the serum was carefully removed without disturbing the clot, and the tubes were subsequently weighed. Each tube was supplemented with 100 µl of extract at concentrations of 10, 20, 40, 80, or 160 µg/ml. A volume of 100 µl of streptokinase, equivalent to 30,000 International Units (IU), was added to the tube labeled as the positive control. In contrast, a volume of 100 µl of distilled water (DW) was added to the tube labeled as the control. Following a period of incubation at a temperature of 37 °C for duration of 90 minutes, the fluid that had been discharged from each tube was meticulously extracted, and subsequently, the tubes were subjected to reweighing. The calculation of clot lysis percentage was performed in the following manner:

$$\%\text{Clot lysis} = (\text{Weight}_{\text{clot after treatment}} \div \text{Weight}_{\text{clot before treatment}}) \times 100$$

The half-minimal inhibitory concentration (IC_{50}) was obtained using Graph Pad Prism software and a non-linear regression analysis.

Membrane protection assay

This study was conducted using the model developed by Shinde et al. (1999), with some minor adjustments. In the first step, a volume of 5 milliliters of freshly obtained blood was collected from a donor who was in good health. This blood sample was then combined with the dipotassium salt of ethylenediaminetetraacetic acid (EDTA) at a concentration of 2.2 mg/ml. The blood cells were subsequently collected using centrifugation at a force of 3000 times the acceleration due to gravity for duration of 10 minutes. Following this, the cells were subjected to three rounds of washing using an isotonic solution containing 154 mM concentration of sodium chloride, within a 10 mM sodium phosphate buffer at a pH value of 7.4. The cell suspension obtained was subjected to centrifugation at a force of 3000 times the acceleration due to gravity for 10 minutes. Subsequently, the resultant pellet was re-suspended in an equal amount of an isotonic buffer solution. Subsequently, a volume of 0.5 ml of the cellular suspension was introduced into a composite of 5 ml of a hypotonic solution containing 50 mM of sodium chloride, along with 0.5 ml of either a test or reference solution at concentrations of 10, 20, 40, 80, and 160 μg/ml, all within a 10 mM sodium phosphate buffered saline environment at a pH of 7.4, as explicitly indicated. The control tube was composed of a cell suspension volume of 0.5 ml and a hypotonic solution volume of 5 ml, both in the previously specified buffer. The reaction mixture was subjected to an incubation period of 10 minutes at ambient temperature, followed by centrifugation at a force of 3000 times the acceleration due to gravity for 10 minutes. The optical density (OD) of the supernatant was measured at a wavelength of 540 nm using a colorimeter (AE-11M, Japan). The calculation of the % membrane protection was performed using the following equation:

$$\%\text{Membrane protection} = 100 - \{(OD_{control} - OD_{test\ samples})/OD_{control}\} \times 100$$

The IC_{50} was determined as mentioned above.

Statistical analysis

Values were represented as the mean plus or minus the standard deviation (SD). The data analysis involved conducting a one-way analysis of variance (ANOVA) followed by a Newman-Keuls *post-hoc t*-test. The statistical software used for this study was Graph Pad Prism (version 6.5). The significance level was set at $p < 0.05$, with a confidence interval of 95%.

Results

Extraction and phytochemical screening

The yield of MCT was 4.73%. It was a brownish, gummy mass. Table 1 suggests that MCT possesses alkaloids, tannins, glycosides, flavonoids, and saponins (Table 1).

Table 1. Phytochemical groups in *Cheilanthes tenuifolia* leaf methanol extract

Examination	Name of the test	Consequences for MCT
Alkaloids	Mayer's test	-
	Dragendorff's test	+
	Wagner's test	+
	Hager's test	+
	Tannic acid test	-
Glycosides	Salkowski test	+
	Libermann-burchared test	+
Steroids	Salkowski test	-
	Libermann-burchared test	-
Tannins	Ferric chloride test	+
	Potassium dichromate test	+
	Keller-Killiani test	-
Flavonoids	Conc. HCl and alcoholic test	+
Saponins	Shake test (aq. solution)	+
Reducing sugars	Fehling's test	-
	Benedict's test	-
Gums	Molisch's test	-
Amides	NaOH test	-

'+'= positive response for respective component. '-'= negative response for respective component; MCT: Methanolic extract of Cheilanthes tenuifolia

Clot lysis assay

The control (vehicle) exhibited negligible clot lysing capacity (2.58 ± 0.01%). The MCT group demonstrated a statistically significant ($p < 0.05$) ability to lyse clots when compared to the control group. It showed a concentration-dependent clot lysis potential. At 160 µg MCT exhibited the highest clot lysis (31.56 ± 4.78%). However, the standard STK (equiv. 30,000 IU)/ 100 µl exhibited more clot lysis capacity (77.51 ± 0.01) than the test samples. The IC_{50} value of MCT was 198.41 ± 1.87 µg (Table 2).

Table 2. Clot lysis capacity of *Cheilanthes tenuifolia*

Sample/controls	Concentration	%Clot lysis	IC_{50} [CI, R^2]
Control (Vehicle)	100 µl	2.58 ± 0.01	-
STK (15,00,000 U/Vial/10 ml)	100 µl	77.51 ± 0.01*	-
MCT	10 µg	6.39 ± 1.40*	198.41 ± 1.87 µg [87.13 – 211.67 µg; 0.83]
	20 µg	8.93 ± 3.71*	
	40 µg	14.07 ± 1.13*	
	80 µg	16.71 ± 2.87*	
	160 µg	31.56 ± 4.78*	

*Values are mean ± SD (n = 5); One-way ANOVA followed by t-student post hoc test; *p <0.05 when compared to the control (vehicle) group; MCT: Methanolic extract of Cheilanthes tenuifolia; STK: Streptokinase; IC_{50}: Half-minimum inhibitory concentration; CI: Confidence of interval; R^2: Co-efficient of determination*

Membrane protection assay

The control (vehicle) showed a negligible membrane lysing inhibitory effect (1.42 ± 0.02%) on HRBCs. The MCT showed concentration-dependent membrane lysis inhibitory effects compared to the control group. The highest inhibition (78.93 ± 0.01%) was seen by MCT at 160 µg/ml. However, the standard drug ASA exhibited significant ($p < 0.05$), strong, and better inhibition at 160 µg/ml (97.71 ± 0.01%) than the MCT at the same concentration. The IC_{50} values calculated for the MCT and ASA were 64.10 ± 2.08 and 78.46 ± 2.11 µg/ml, respectively (Table 3).

Table 3. Membrane protection capacity of *Cheilanthes tenuifolia* and controls

Sample/controls	Concentration	%Membrane protection	IC_{50} [CI, R^2]
Control (Vehicle)	0.5 ml	1.42 ± 0.02	
ASA	10 µg/ml	17.71 ± 0.03*	64.10 ± 2.08 µg/ml [45.97 – 67.13 µg/ml; 0.94]
	20 µg/ml	28.91 ± 0.02*	
	40 µg/ml	35.21 ± 0.01*	
	80 µg/ml	68.69 ± 0.02*	
	160 µg/ml	97.71 ± 0.01*	
MCT	10 µg/ml	3.95 ± 0.01*	78.46 ± 2.11 µg/ml [49.91 – 88.19 µg/ml; 0.85]
	20 µg/ml	19.73 ± 0.01*	
	40 µg/ml	47.36 ± 0.01*	
	80 µg/ml	53.93 ± 0.01*	
	160 µg/ml	78.93 ± 0.01*	

*Values are mean ± SD (n = 5); One-way ANOVA followed by t-student post hoc test; *p <0.05 when compared to the control (vehicle) group; MCT: Methanolic extract of Cheilanthes tenuifolia; ASA: Acetyl salicylic acid; IC_{50}: Half-minimum inhibitory concentration; CI: Confidence of interval; R^2: Co-efficient of determination*

Discussion

The fern *C. tenuifolia* has bioactive compounds like flavonoids (quercetin), phenolic acids, tannins, alkaloids, and triterpenoids.

Alkaloids have several pharmacological activities, including anti-inflammatory, membrane-stabilizing, and clot-lysis activities (Vijayalakshmi et al., 2011). Certain alkaloids, such as quinidine and lidocaine, have been shown to possess membrane-stabilizing activity in cardiac tissues (Class, 2015). Alkaloids like tetrodotoxin and saxitoxin are used in the field of neurobiology to stabilize membranes by blocking voltage-gated sodium channels. This blockade inhibits the propagation of action potentials, leading to local anesthetic effects (Foadi, 2018). Certain alkaloids have been investigated for their potential clot lysis activity, including ephedrine, which enhances the activity of plasmin and promotes clot dissolution (Chiloane, 2019), and berberine, which exhibits antithrombotic effects and inhibits platelet aggregation (Kim and Park, 2019).

Flavonoids, natural compounds found in plants, possess membrane-stabilizing effects (Derouich et al., 2020). They inhibit enzymes like phospholipase A2, reducing the production of inflammatory mediators and

maintaining membrane integrity (Maleki et al., 2019). Flavonoids also scavenge free radicals, reducing oxidative stress and preventing membrane damage (Engwa, 2018). They may modulate ion channels and receptors, contributing to membrane stabilization (Diniz et al., 2015). Some studies suggest that certain flavonoids may have potential for promoting clot lysis or preventing excessive clot formation (Sharifi-Rad et al., 2022). Flavonoids such as quercetin and rutin have been investigated for their ability to enhance the activity of the body's natural clot-dissolving system (Kolodziejczyk et al., 2023; Patel et al., 2023).

Certain plant components have promising anti-inflammatory and immunosuppressive properties, as these can indirectly exhibit membrane-stabilizing effects (Saleem et al., 2020). While their main mode of action involves binding to glucocorticoid receptors and modulating gene expression, steroids can also suppress the production of inflammatory mediators like phospholipase A2, which indirectly helps stabilize cell membranes (Remesalet et al., 2016). Additionally, they can influence the activity of ion channels, potentially affecting nerve cell excitability and contributing to membrane stabilization (Friedrich et al., 2015). Cardiac glycosides like digoxin and digitoxin, derived from plants such as *Digitalis purpurea*, have been shown to have membrane-stabilizing activity in cardiac tissues (Cole and Roberts, 2017). The actions of these glycosides are exerted through the inhibition of the sodium-potassium ATPase pump. This inhibition leads to elevated levels of intracellular sodium, which subsequently impacts the electrical excitability of cardiac cells. Consequently, these effects can lead to heightened contractility and the manifestation of anti-arrhythmic properties (Dumotier, 2015). Urokinase, a glycoside enzyme that promotes clot dissolution by converting plasminogen into plasmin, and certain glycosides derived from medicinal plants like *Ginseng* and *Ginkgo biloba* can exhibit clot lysis or thrombolytic activity (Merlyn et al., 2018; Abd et al., 2023).

On the other hand, saponins, known for their foaming properties, have been studied for their potential clot lysis or thrombolytic activity (Ripa et al., 2022). Some saponins, such as those found in red ginseng, have shown promising effects in promoting clot dissolution by activating plasminogen and enhancing fibrinolysis (Kim et al., 2021). Tannins have the ability to interact with and bind to proteins, including those present in cell membranes. This protein-binding property of tannins can lead to the formation of complexes that can reinforce and stabilize the membrane structure (Adrar et al., 2019). Furthermore, tannins have antioxidant characteristics and have the ability to eliminate free radicals, hence diminishing oxidative stress and inhibiting membrane impairment (Akbari et al., 2022; Anosike et al., 2019).

In these studies, MCT possesses high intensity for alkaloids, glycosides, and falvonoids. MCT showed a concentration-dependent clot lysis and hemolysis inhibition capacity. The IC_{50} values of MCT were determined for clot lysis and membrane-stabilizing capacities of 198.41 ± 1.87 and 78.46 ± 2.11 µg/ml, suggesting its potentiality of clot-dissolving and membrane-protecting power. Clot lysis and membrane stabilizing activity may be possible due to the presence of phytochemical alkaloids, glycosides, flavonoids, and saponins in the MCT. Additional investigation is required in order to ascertain the precise bioactive chemicals that are accountable for the observed phenomena of clot lysis and membrane protection. This research endeavor aims to delve into the prospective therapeutic applications of these compounds in the context of inflammatory damage and atherothrombosis.

Conclusion

MCT exhibited significant clot-lysing capacity concentration-dependently. Furthermore, it demonstrated a significant ($p < 0.05$) concentration-dependent inhibition of hemolysis generated by hypotonic solution. MCT exhibits potential as a viable therapeutic option for the management of inflammatory disorders and atherothrombosis. Further studies are necessary to isolate its bioactive principles and elucidate molecular mechanisms for each biological activity.

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Conflict of Interest

The authors declare no conflict of interest.

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