Asian Journal of Medicine and Health

Volume 22, Issue 7, Page 36-44, 2024; Article no.AJMAH.117292
ISSN: 2456-8414

Evaluation of *In-vitro* Anti-Inflammatory, Anti-Fungal, Thrombolytic, Membrane Stabilizing and Cytotoxic Properties of (*Camellia chrysantha Hu Tuyama*)

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Article Information

DOI: https://doi.org/10.9734/ajmah/2024/v22i71043

Open Peer Review History:
This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: https://www.sdiarticle5.com/review-history/117292

Original Research Article

Received: 23/03/2024
Accepted: 24/05/2024
Published: 03/06/2024

ABSTRACT

The current study was set out to look into the anti-inflammatory, anti-fungal, cytotoxic, thrombolytic and membrane stabilizing activities of the methanolic extract from the leaf of *Camellia chrysantha (Hu) Tuyama* (MECCL). Primary evaluation of MECCL was performed via phytochemical screening. Phytochemical analysis of the leaf extract revealed the presence of reducing sugar, flavonoids, alkaloids, and steroids. Anti-inflammatory activity test was done using egg albumin

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Cite as: *Takey, I. I., Shomudro, H. K., & Chowdhury, S. A. (2024). Evaluation of In-vitro Anti-Inflammatory, Anti-Fungal, Thrombolytic, Membrane Stabilizing and Cytotoxic Properties of (Camellia chrysantha Hu Tuyama). Asian Journal of Medicine and Health, 22(7), 36–44. https://doi.org/10.9734/ajmah/2024/v22i71043*

denaturation assay. In the result of MECCL's anti-inflammatory test showed 88.88% compared to the standard acetyl salicylic acid 98.56%. The anti-fungal activity test was performed by Disc Diffusion method using zone of inhibition against four fungi. MECCL showed moderate antifungal activity compared to standard Griseofulvin against *Aspergillus niger, Saccharomyces cerevisiae* (yeast), *Penicillium notatum*. Zones of inhibition of the fungi *Aspergillus niger*, *Saccharomyces cerevisiae* (yeast), *Penicillium notatum* is 11mm, 14mm and 14mm at the concentration of 700 µg/disc, which is close to the standard Griseofulvin 19mm, 20mm, and 21mm respectively at 50 µg/disc concentration. *Mucor hiemalis* showed mild zone of inhibition in 300, 500, and 700 µg/disc (7mm). Brine Shrimp lethality bioassay method was used to test the lethality of the methanolic leaf extract. The extract showed significant cytotoxic activity against brine shrimp nauplii with LC_{50} value of 0.843 µg/mL when compared with the standard Vincristine sulphate (LC_{50} value: 0.608 µg/mL). Comparison with positive control Vincristine sulphate signifies that cytotoxicity exhibited by MECCL and further bioactivity guided investigation can be done to find potent antitumor compounds. In thrombolytic activity test, MECCL demonstrated 95.69% thrombolysis which was significant compared to standard Streptokinase (SK) which demonstrated 91.304 % thrombolysis. While doing membrane stabilizing activity test, Heat induced hemolysis assay was performed and the percent of protection was 85.35 compared to the standard DS 73.63 which was very significant. The results highlight the potential of *Camellia chrysantha (Hu) Tuyama* as a valuable source of phytochemicals that possess notable anti-inflammatory, thrombolytic, and membrane-stabilizing properties. The methanolic leaf extract also shows moderate antifungal and cytotoxic effects, suggesting potential for future therapeutic advancements.

Keywords: Camellia chrysantha (Hu) Tuyama; anti-inflammatory; antifungal; hemolysis; thrombolytic; MECCL.

1. INTRODUCTION

Plants continue to provide a rich supply of natural antioxidants that may pave the way for the development of new medicines. A particular perspective that indicates that inflammation causes harm is that activated macrophages and neutrophils produce reactive oxygen species. Damage to tissues occurs as a result of lipid peroxidation of membranes and macromolecule damage caused by this overproduction. Furthermore, reactive oxygen species (ROS) promote inflammation by activating the secretion of cytokines, which in turn attract more neutrophils and macrophages. These cytokines include interleukin-1, tumor necrosis factor-, and interferon-. Therefore, antioxidants and radical scavengers may reduce inflammation by neutralizing free radicals, which are major mediators of inflammatory processes [1]. The treatment of rheumatoid arthritis often involves the use of non-steroidal anti-inflammatory medications (NSAIDs). Their therapeutic efficiency seems to be hindered, however, by a variety of undesirable and sometimes severe adverse effects, despite presence of their enormous amount. As a result, it's crucial to identify less harmful alternatives; certain medicinal plants may hold the key. Para immunity, the non-specific regulation of primary granulocytes, macrophages, natural killer cells, and complement activities, is supposedly induced by compounds found in abundance in medicinal herbs [2].

As a member of the Theaceae family*, Camellia chrysantha (Hu) Tuyama*, commonly referred to as "golden camellia," is a species of evergreen shrub or small tree that is endemic to moist forest regions beneath 500 meters in height. It is indigenous to Vietnam and China. This species was discovered in Ba Che (Quang Ninh) in Vietnam [3]. Numerous trace elements, including germanium, selenium, manganese, molybdenum, vanadium, and zinc, have been identified in *Camellia chrysantha*. The bioactive compounds present in this species exhibit the capacity to impede tumor growth by as much as 33.8% and aid in the reduction of blood cholesterol by as much as 35% [4]. The plant can alleviate the symptoms of atherosclerosis resulting from blood lipids, regulate blood pressure, and manage cardiovascular ailments and diabetes [5].

2. MATERIALS AND METHODS

2.1 Plant Material

Located at 1217 Moulana Bhasani Road, Romna Park, Dhaka, Bangladesh, the *Camellia chrysantha* sample was collected in October 2022. A team of specialists from Dhaka's

Bangladesh National Herbarium in Mirpur positively identified the plant (DACB 88045). The plant was let to dry in the shade for eleven days after accession, after which it was ground into a fine powder for use in experiments. After removing the dirt, the *Camellia chrysantha* was harvested as a full, fresh plant. After that, the whole plant was rinsed in room-temperature water to remove any remaining dust. Once the *Camellia chrysantha* was cleaned, it was set to air dry in a shaded area for twelve to fifteen days.

2.2 Preparation of Plant Extract

The plant was air-dried before being machine-pulverized into small pieces and mixed. Over three days, 58 grams of *Camellia chrysantha* powder was immersed in 750 mL of methanol. At regular intervals, stirring assisted the maceration process. The extract was filtered via filter paper after three days. The solvent was air-dried to produce 5.49 g of extract. Rested and protected from light, the unprocessed extract was preserved in a beaker [6].

2.3 Phytochemical Screening

Following the procedures indicated in the preceding study, the phytochemical analysis was carried out [7]. The presence of several phyto components such as alkaloids, steroids, saponins, reducing sugar, flavonoids, carbohydrates, tannins, gums, glycosides, and phenols was ascertained in samples of the methanolic extract of *Camellia chrysantha*.

2.4 *In-vitro* Anti-Inflammatory Activity

The previous study reported using this approach with minimal modifications [8]. To create the reaction mixture (5 mL), 0.2 mL of egg albumin (derived from a fresh hen's egg), 2.8 mL of phosphate-buffered saline (PBS, pH 6.4), and 2 mL of MECC were combined at concentrations of 31, 62.5, 125, 250, 500, and 1000 µg/mL. Comparative controls included the same amount of water that had been double-distilled. Afterwards, a BOD incubator (Labline Technologies) was used to incubate the solutions at 37°C for 15 minutes after heating them to 70°C for 5 minutes. After they had cooled, we measured their viscosity using an Ostwald viscometer and their absorbance at 660 nm (SHIMADZU, UV 1800) with a vehicle blank. Diclofenac sodium was used as a reference medicine at final concentrations of 31, 62.5, 125, 250, 500, and 1000 µg/mL while the viscosity and absorbance were determined. The following formula was used to ascertain the percentage of protein denaturation that was averted:

$$\% \text{ of Inhibition} = \frac{Abs\ of\ control - Abs\ of\ test\ sample}{Abs\ of\ control} \times 100$$

Here, Abs= Absorbance

2.5 Antifungal Activity Test

4 fungi named *Penicillium chrysogenum, Aspergillus niger, Yeast budding* and *Mucor hiemalis* was collected from laboratory of microbiology, Stamford University Bangladesh and Bangladesh. The disc diffusion experiment was used to test the antifungal activity of MECCL [9]. A Petri dish was used to create a solid agar medium using this approach. Then, 1mL of each fungus's culture was distributed evenly throughout the medium. A 6 mm diameter sterile filter paper disc was utilized, which was then saturated with 10 µL of diluted MECCL and placed on top of each agar plate. The MECCL was used in this experiment at concentrations of 300, 500, and 700 µg/mL. After that, the plates were placed in the incubator until the next day. The positive control group utilized an antifungal medication containing griseofulvin, whereas the negative control group employed methanol-containing discs. The antifungal activity was assessed after 24 hours by measuring the size of the inhibitory zone around the disc in millimeters.

2.6 Cytotoxic Assay

Using brine shrimp lethality experiments, the cytotoxic activity of the methanolic crude extract at various doses was predicted [10]. Various solutions were made using the serial dilution method with simulated seawater at various concentrations (400, 200, 100, 50, 12,5, 6,25, 3,13, 1.56, 0.78 µg/mL) to experiment. 4 mg of each extract was dissolved in dimethyl sulfoxide (DMSO). After that, the solutions were added to the corresponding vials containing 10 brine shrimp nauplii in 5 milliliters of artificial seawater. To determine how many nauplii survived, the test tubes were inspected under a microscope the next day. The percentage of brine shrimp nauplii that died at each concentration and in the control, group was calculated using these data. Vincristine sulfate was used as the reference standard. The concentration-death rate logarithmic plot was used to determine the LC_{50}.

2.7 Thrombolytic Assay

2.7.1 Blood sample

15 healthy human volunteers who had never used blood-thinning medication, nicotine, or oral contraceptives had their venous blood collected (with the help of a medical expert). Stamford University Bangladesh's Institutional Ethics Council gave institutional ethical approval to the whole procedure. 15 microcentrifuge tubes were then filled with 500 µL of fresh blood.

2.7.2 Affirmation of donors consent

Every single donor was supplied with a consent form that narrated the purpose of this research, title of this project, and the volume of blood that will be drawn. The illustration of this research includes whether or not volunteers will consume any therapy, any kind of irritation to the piercing area and the time period for blood collection.

2.7.3 Clot lysis method

Using pre-weighed sterile vials holding 1 mL each, blood was taken from healthy participants and then divided into vials of 5 mL. At 37°C, blood samples needed 45 minutes to coagulate [11]. A fresh weight measurement was made to determine the clot weight after the generated serum was emptied from the vials. Next, a 100 µL water solution containing the plant components (2 mg/mL) was added to the vials. One hundred microliters of a non-thrombolytic control and thirty thousand units of streptokinase were used as standards. The next step was to transfer the mixture to an incubator set at 37°C after 90 minutes. We measured the changed weights of the vials after incubation and drained the fluid that the clot had produced. To measure the thrombolytic activity, the following equation was employed to calculate the percentage of clot lysis.

$$\% \text{ of Clot lysis} = \frac{A}{B} \times 100$$

where A and B reflect, respectively, the weight of the released clot before treatment and after treatment.

2.8 Membrane Stabilizing Activity

2.8.1 Preparation of Human Red Blood Cells (HRBC) suspension

An equal volume of freshly extracted human blood was mixed with a sterile Alsever solution, which consists of 2% dextrose, 0.8% sodium citrate, 0.05% citric acid, and 0.42% sodium chloride in water. To prepare the packed cells for centrifugation, they were washed three times with iso-saline (0.85%, pH 7.2). A 10% volume-to-volume (v/v) suspension of iso-saline was prepared from the observed blood volume.

2.8.2 Heat induced haemolysis

The primary concept behind this method is that the stability of the human red blood cell membrane can be achieved through hypotonicity-induced membrane lysis. The reaction mixture consisted of the following: 1 mL of 0.15M phosphate buffer (pH 7.4), 2 mL of 0.36% hyposaline, 10% v/v HRBC suspension (0.5 mL) with plant extracts (0.5 mL), and distilled water (instead of hypo saline) to create a 100% haemolysis control group. The incubation and centrifugation processes were carried out at 37°C for 30 minutes and 560 nm, respectively, to measure the haemoglobin content in the suspension [12]. The formula used for estimating the percentage of haemolysis of HRBC membrane as follows:

$$\% \text{ Haemolysis} = \frac{Optical\ density\ of\ Test\ sample}{Optical\ density\ of\ Control} \times 100$$

The percentage of HRBC membrane stabilisation can be calculated as follows:

$$\% \text{ Protection} = 100 - \left[\frac{Optical\ density\ of\ Test\ sample}{Optical\ density\ of\ Control} \times 100\right]$$

2.9 Statistical Analysis

All experimental data were handled in triplicate, and mean, standard deviation was used to express tubular data. Excel also used for statistical analyses.

3. RESULTS AND DISCUSSION

3.1 Phytochemical Screening

The investigation of MECCL's phytochemical content revealed a wide array of substances, including flavonoids, reducing sugars, alkaloids, gums, and steroids. However, tannin, glycoside, saponin and carbohydrates were absent.

Research's result has shown in the Table 1 that compounds containing flavonoids, alkaloids &

steroids provide various pharmacological effects. Some flavonoids have been shown to have analgesic and antimicrobial effects [3], [13]. It inhibits phospholipase A2, which blocks arachidonic acid metabolism, which may explain this action. In addition to their potential anti-inflammatory effects, some alkaloids inhibit the metabolic pathway of arachidonic acid [14].

Table 1. Results of Different Chemical Group Test of MECCL

Phytochemical constituent	MECCL
Flavonoid	+
Tannin	-
Alkaloid	+++
Steroid	+++
Saponin	-
Carbohydrate	-
Glycoside	-
Reducing Sugar	++
Gum	+

3.2 Anti-Inflammatory Activity

Both MECCL had a more noticeable impact than standard, acetylsalicylic acid, according to the results shown in Table 2.

Table 2. Protein denaturation (egg albumin) assay results

Samples	Concentrations	% of inhibition µg/mL
Acetyl salicylic acid	62.5	93.52
	125	94.96
	250	95.68
	500	97.84
	1000	98.56
MECCL	62.5	62.23
	125	74.44
	250	83.33
	500	84.44
	1000	88.88

The anti-denaturation technique of egg albumin was selected for the assessment of MECCL's anti-inflammatory function. The anti-denaturation approach involves denatured egg albumin in the experiment. The denaturation process is brought about by applying heat. Proteins that have been denatured express certain antigens. These antigens are linked to type-III hypersensitivity responses, which in turn are linked to certain illnesses including serum sickness and glomerulonephritis [15]. Not only that but it has previously been shown that common NSAIDs such as indomethacin and phenylbutazone do more than only block the COX enzyme, which is responsible for the endogenous prostaglandin's synthesis. Plus, they prevent proteins from becoming denaturized [8], [16].

That is why the anti-denaturation test is the most practical way to screen for anti-inflammatory efficacy. The results of this investigation demonstrate that the extract has potent anti-inflammatory properties. Autoantigen synthesis may be regulated by *Camellia chrysantha (Hu) Tuyama*. Protein denaturation may therefore be prevented. A standard medication was used to compare this effect. The comparison was based on aspirin, the typical medicine. Preliminary phytochemical screening revealed the presence of secondary metabolites such as alkaloids and flavonoids [3].

3.3 Antifungal Activity

Using mm as a unit of measurement, the antifungal activity of varying concentrations of plant extract was tested against four distinct types of fungus. The findings demonstrated that the inhibitory zone expanded with increasing concentrations of plant extracts (Table 3). Fungi had an inhibitory zone of 7–14 mm.

Similar to the Kirby-Bauer technique used for bacterial testing, the agar diffusion method is also used to conduct antifungal tests. By placing discs containing the antifungal drug on top of an agar surface, the agar diffusion technique involves growing a fungal strain on an agar medium. To ascertain the substance's antifungal activity, the zone of inhibition around each disc is assessed [17].

In a nutshell, the effectiveness of antimicrobial medications must be evaluated using antifungal assay. It is usual practice to assess the level of inhibition or kill rate of fungi by using the agar diffusion and broth dilution procedures. These kinds of experiments are crucial for discovering and assessing potential novel antimicrobials and for choosing the right ones to treat microbial and fungal diseases [18].

3.4 Cytotoxic Activity

In Table 4. The cytotoxic activity of MECCL against brine shrimp nauplii is summarised in Fig. 2, while the impact of both standard and MECCL on brine shrimp nauplii is shown in Fig. 1, respectively.

Table 3. Inhibition zone of MECCL against microorganisms

	Diameter of Zone of Inhibition (mm)			
Test organisms	**MECCL (300 µg/disc)**	**MECCL (500 µg/disc)**	**MCCL (700 µg/disc)**	**Griseofulvin (50 µg/disk)**
Fungi				
Penicillium chrysogenum	07	09	14	19
Aspergillus niger	07	08	11	20
Yeast budding	07	09	14	21
Mucor hiemalis	07	08	09	21

Table 4. Effects of Vincristine Sulfate and methanolic extract on brine shrimp nauplii

Sample name	Concentrations	Mortality %	LC_{50} value
Vincristine Sulphate	7.81	40	0.608
	15.625	40	
	31.25	60	
	62.5	70	
	125	80	
	250	100	
	500	100	
MCCL	0.98	20	0.843
	1.95	20	
	3.91	40	
	7.81	50	
	15.625	70	
	31.25	80	
	62.5	90	
	125	100	
	250	100	
	500	100	

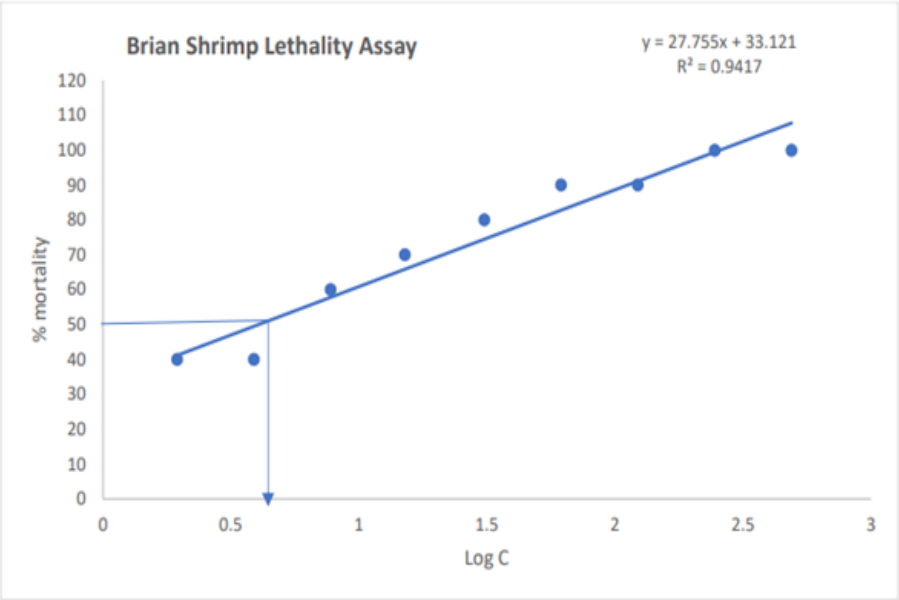

Fig. 1. Effect of Vincristine sulphate on brine shrimp nauplii.

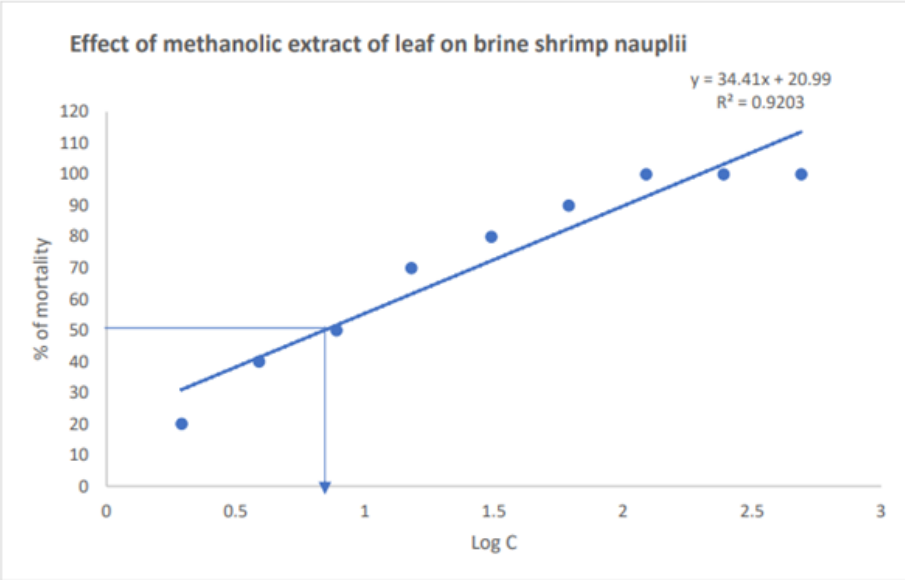

Fig. 2. Effect of MECCL on brine shrimp nauplii.

A cytotoxic effect against brine shrimp nauplii was observed in the methanolic extract of *Camellia chrysantha (Hu) Tuyama* leaves, with an LC_{50} value of 0.843 µg/mL, in comparison to the standard vincristine sulfate (0.608 µg/mL). Further bioactivity-guided research may be conducted to identify powerful anticancer and pesticidal chemicals; comparison with vincristine sulfate, a positive control, indicates that MECC exhibits considerable cytotoxicity [19].

3.5 Thrombolytic activity

Comparing MECCL to the standard streptokinase, the following data shows that MECCL has a much higher percentage of clot lysis.

Table 5. Thrombolytic activity (in terms of % of clot lysis) of the extractives of MECCL

Sample	% of clot lysis
Negative control	7.296
Streptokinase	91.304
MECCL	95.69

The methanol extract of *Camellia chrysantha (Hu) Tuyama* (leaf) showed thrombolysis of 95.69% respectively, in this study. Since this is just a preliminary examination, the chemical and pharmacological properties of the extract should be further investigated to maximise their medicinal and pharmaceutical potential.

An *In vitro* thrombolysis test is a controlled laboratory procedure that assesses the ability of plant extracts to dissolve blood clots. To prevent more bleeding after an injury, proteins and blood cells clump together to form a clot. On the other hand, conditions like heart attacks and strokes may be deadly if clotting occurs too often [20]. A wide range of research investigations have sought to identify natural food sources, supplements, and botanicals with thrombolytic action for the treatment of coronary events and strokes. The study found that MECCL can reduce blood clots. Table 6 shows that MECCL's thrombolytic potential was 95.69%, which is much higher than the usual value of 91.304%. This result was achieved because MECCL reduces blood clotting in laboratory studies, leading to the notion that it has cardioprotective properties [11]. The MECCL has great potential for improving cardiovascular health and might pave the way for the development of new thrombolytic medicines derived from the leaf of the *Camellia chrysantha (Hu)* plant.

3.6 Membrane-Stabilizing Activity

Table 6. Shows that compared to the reference medication, MECCL had a membrane-stabilizing activity of 63.14 %

Sample	% of hemolysis	% of protection
Diclofenac-sodium	26.36	73.64
MECCL	14.65	85.35

The method used to determine the percentage of membrane stabilization for MECCL and Diclofenac sodium was the suppression of HRBC membrane lysis, specifically the stabilization of the HRBC membrane caused by hypotonicity. As can be shown in Table 5, MECCL effectively reduces heat-induced hemolysis of HRBC. This demonstrated that MECCL had a range of protection of 58.87% compared to Diclofenac sodium's range of 73.63%, proving that the leaves of the *Camellia chrysantha (Hu) tuyama* plant had a significant stabilizing effect on cell membranes. It is possible to attribute this kind of action to flavonoids [21]. As a result, *Camellia chrysantha (Hu) tuyama* can be used as anti-inflammatory drug.

4. CONCLUSION

A member of the family Theaceae, the *Camellia chrysantha (Hu) Tuyama* plant has shown promise in in vitro research for potential health benefits. This plant has promising properties that might lead to novel therapeutic molecules. It is thrombolytic, anti-inflammatory, anti-arthritic, membrane stabilizing, and cytotoxic, with mild anti-fungal activity. Additional research into this plant could be useful. Research into the potential health benefits of traditional medicinal plants is vital, according to the research, as these plants might provide important foundations for the development of new medicines. More *in vivo* and clinical studies are needed to determine this plant's efficacy and safety for human use. In sum, our findings contribute to the growing amount of data suggesting natural products may have positive effects on human health and call attention to the need for more research into the medical plant industry's treatment potential.

CONSENT

Stamford University Bangladesh's Faculty of Science examined and accepted the research procedure and written consent form (reference number: SUB/SF/EC-2403/04). Everyone who took part in the study had to submit a documented consent form, and they had the right to withdraw at any moment.

ETHICAL APPROVAL

This research followed all rules set forth by the US Food and Drug Administration, the Declaration of Helsinki, and the International Conference on Harmonization.

ACKNOWLEDGEMENT

The authors would like to express their gratitude to the Laboratory of Microbiology and the Laboratory of Physiology and Pharmacology the Faculty of Science, Stamford University Bangladesh for their assistance, resources and infrastructural facilities necessary to perform this research.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
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