

Evaluation of In-vitro Anti-arthritic, Anti-inflammatory, Anti-fungal, Thrombolytic, Membrane Stabilizing and Cytotoxic properties of *Camellia chrysantha* (Hu) Tuyama

ABSTRACT

The current study was set out to look into the anti-arthritic, 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-arthritic and anti-inflammatory activity tests were done using bovine serum albumin and egg albumin denaturation assays. In the result of MECCL's antiarthritic test the percent of inhibition was 84.68% compared to the standard Diclofenac sodium (DS)'s value 94.59%. Where's the anti-inflammatory result of MECCL 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 fungicidal 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 LC₅₀ value of 0.843 µg/mL when compared with the standard Vincristine sulphate (LC₅₀ 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 and pesticidal 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

Keywords: *Camellia chrysantha* (Hu) Tuyama, antiarthritic, anti-inflammatory, antifungal, thrombolytic

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1. INTRODUCTION

Plants continue to provide a rich supply of natural antioxidants that may pave the way for the development of new medicines, and traditional medicine is widely used. 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 their enormous number. 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%. The plant can alleviate the symptoms of atherosclerosis resulting from blood lipids, regulate blood pressure, and manage cardiovascular ailments and diabetes [4].

2. MATERIAL 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.

2.2 Preparation of plant extract

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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 let to air dry in a shaded area for twelve to fifteen days. 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 three fingers 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.

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2.3 Phytochemical screening

Following the procedures indicated in the preceding study, the phytochemical analysis was carried out [12]. 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-arthritis activity

Bovine serum albumin (0.45 ml, 5% aqueous solution) and *Camellia chrysantha* extract (0.05 ml, varying concentrations) made up the 0.5 ml reaction mixture. We left the samples to incubate at 37°C for half an hour. A 2.5 ml volume of phosphate buffer saline (pH 6.3) was added to each tube once the samples had cooled. For the control test, we used 0.05 ml of pure water instead of extracts and assessed turbidity spectrophotometrically at 660nm. On the other hand, for the ~~product~~ control test, we didn't utilize bovine serum albumin [5]. Here is the formula for determining the percentage inhibition of protein denaturation:

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$$\% \text{ of Inhibition} = [100 - (\text{OD of test solution} - \text{OD of product control}) \times 100]$$

In which optical density (OD) is the descriptor.

2.5 In-vitro anti-inflammatory activity

The previous study [15] reported using this approach with minimal modifications. 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:

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

Here, Abs= Absorbance

2.6 Antifungal activity test

2.6.1 Test microorganisms

4 fungi named *Penicillium chrysogenum*, *Aspergillus niger*, *Yeast budding* and *Mucor hiemalis* was collected from laboratory of microbiology, Stamford University Bangladesh and Bangladesh.

2.6.2 Disc Diffusion Method:

The disc diffusion experiment was used to test the antifungal activity of MECCL (Klančnik et al., 2010). 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.

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2.7 Cytotoxic assay

2.7.1 Brine shrimp lethality assay

Using brine shrimp lethality experiments, the cytotoxic activity of the methanolic crude extract at various doses was predicted. 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 LC₅₀.

2.8 Thrombolytic assay

2.8.1 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 μ L of fresh blood.

2.8.2 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.

2.8.3 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. A fresh weight measurement was made to determine the clot weight after the generated serum was emptied from the vials. Next, a 100 μ L 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.

2.9 Membrane stabilizing activity

2.9.1 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.

2.9.2 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% hemolysis control group. The incubation and centrifugation processes were carried out at 37°C for 30 minutes and 560 nm, respectively, to measure the hemoglobin content in the suspension. The formula used for estimating the percentage of hemolysis of HRBC membrane as follows:

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

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

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

2.10 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.

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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.

Table 1. Results of Different Chemical Group Test of MECCL

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

Gum	+
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Research has shown that compounds containing flavonoids provide various pharmacological effects. Some flavonoids have been shown to have analgesic and bactericidal effects [6]. It inhibits phospholipase A2, which blocks arachidonic acid metabolism, which may explain this action [7]. In addition to their potential anti-inflammatory effects, some alkaloids inhibit the metabolic pathway of arachidonic acid.

3.2. Anti-arthritis activity

Table 2 shows that MECCL and diclofenac sodium when tested for their antiarthritic effectiveness, reduced protein denature by a significant amount over a range of dosages.

Table 2. Percentage inhibition in protein denaturation of MECCL

Samples	Concentrations	% of inhibition
Diclofenac Sodium	62.5	84.64
	125	87.38
	250	89.18
	500	93.69
	1000	94.59
MECCL	62.5	74.77
	125	81.08
	250	81.98
	500	82.88
	1000	84.68

Prior studies have shown that protein denaturation is the first step in the chain reaction that starts with inflammation and arthritis and culminates in the development of auto-antigens and rheumatic diseases [8]. Hydrophobic, disulfide, hydrogen, and electrostatic bond changes may all play a role in denaturation processes [9]. The rate at which arthritic drugs prevent heat-induced protein denaturation is dose-dependent [10]. Therefore, compounds that inhibit protein denaturation might be useful in the development of drugs to alleviate arthritis.

3.3 Anti-inflammatory activity

Both MECCL had a more noticeable impact than standard, acetylsalicylic acid, according to the results shown in Table 3.

Table 3. Protein denaturation (egg albumin) assay results

Samples	Concentrations	% of inhibition $\mu\text{g/mL}$
Acetyl salicylic acid	62.5	93.52
	125	94.96
	250	95.68
	500	97.84

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	1000	98.56
	62.5	62.23
	125	74.44
MECCL	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 [11]. These antigens are linked to type-III hypersensitivity responses, which in turn are linked to certain illnesses including serum sickness and glomerulonephritis. Not only that but it has previously been shown that traditional 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 denatured [12].

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.

3.4 Antifungal activity

Using mm as a unit of measurement, the antibacterial and 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 4). Fungi had an inhibitory zone of 7–14 mm.

Table 4 Inhibition zone of MECCL against microorganisms.

Test organisms	Diameter of Zone of Inhibition (mm)			
	MECCL (300 µg/disc)	MECCL (500 µg/disc)	MCCL (700 µg/disc)	Griseofulvin (50 µg/disk)
Fungi				
<i>Penicillium chrysogenum</i>	07	09	14	19

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<i>Aspergillus niger</i>	07	08	11	20
<i>Yeast budding</i>	07	09	14	21
<i>Mucor hiemalis</i>	07	08	09	21

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 [13].

In a nutshell, the effectiveness of antimicrobial medications must be evaluated using antibacterial and antifungal assays. It is usual practice to assess the level of inhibition or kill rate of bacteria and 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 [14].

3.5 Cytotoxic Activity

In Table 5. The cytotoxic activity of MECCL against brine shrimp nauplii is summarised in Figure 2, while the impact of both standard and MECCL on brine shrimp nauplii is shown in Figure 1, respectively.

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Table-5. 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	

	0.98	20	
	1.95	20	
	3.91	40	
	7.81	50	
MCCL	15.625	70	0.843
	31.25	80	
	62.5	90	
	125	100	
	250	100	
	500	100	

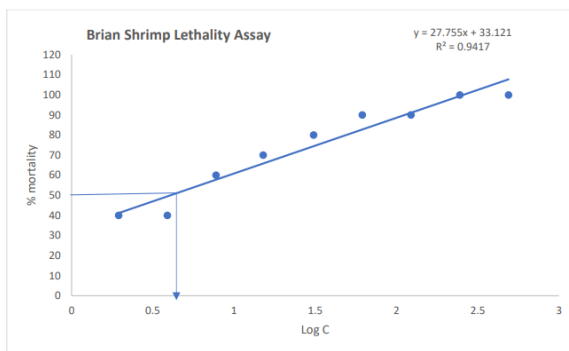


Figure 1: Effect of Vincristine sulphate on brine shrimp nauplii.

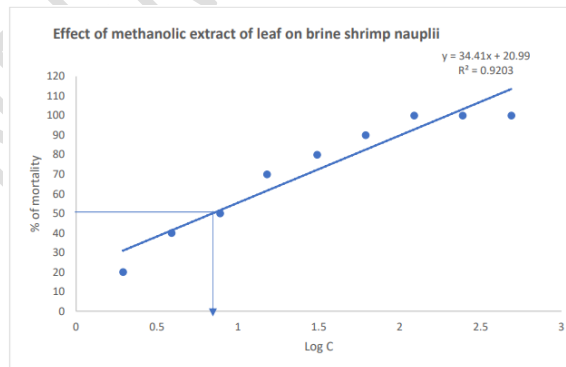


Figure 2: Effect of MECCL on brine shrimp nauplii.

A cytotoxic effect against brine shrimp nauplii was observed in the methanolic extract of *Camellia chrysantha* (Hu) Tuyama leaves, with an LC₅₀ 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 MECCL exhibits considerable cytotoxicity [15], [16].

3.6 Thrombolytic activity

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

Table 6. 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.

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 [17]. 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 [18].

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3.7 Membrane-stabilizing activity

Table 7 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 [19]. As a result, *Camellia chrysantha (Hu) tuyama* can be used as anti-inflammatory drug [20].

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-arthritis, 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.

7. 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. 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.

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