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In vitro and in vivo evaluation of *Ficus religiosa* stem bark extracts, including antioxidant, anti-inflammatory, antimicrobial, antidiarrheal, and antipyretic activities

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ABSTRACT

Introduction: *Ficus religiosa* has traditionally been used for skin ailments, menstrual disorders, infections, and inflammatory conditions. This study aimed to explore its antioxidant capacity, anti-microbial activity, thrombolytic potential, anti-inflammatory effects, and antipyretic activity.

Methods: Antioxidant potential of methanolic and n-hexane extracts was assessed through total phenol, flavonoid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), and nitric oxide (NO) scavenging assays. Anti-microbial activity was tested on selected microbes, cytotoxicity on brine shrimp, and anti-inflammatory properties through membrane-stabilizing potential. Analgesic, antidiarrheal and antipyretic effects were evaluated in mice.

Results: The methanol extract had higher total flavonoid and antioxidant capacity content than the n-hexane extract. Both extracts showed substantial total phenolic, flavonoid, and antioxidant content. Methanol and n-hexane extracts showed free radical scavenging capacity against DPPH (IC_{50}: 24.524 µg/mL and 319.669 µg/mL) and NO (IC_{50}: 463.45 µg/mL and 69.45 µg/mL). n-Hexane extract exhibited antimicrobial efficacy against all tested pathogens. At 250 µg/disc, there was a 7 mm zone of inhibition against *Pseudomonas* spp. and *Candida albicans* and an 8 mm zone of inhibition against *Bacillus subtilis*, *Escherichia coli*, and *Streptococcus aureus*. Methanol extract had greater anti-inflammatory and thrombolytic activity than n-hexane extract. Maximum antidiarrheal activity was shown by methanol extract (83.48% defecation inhibition, $P<0.001$), whereas n-hexane extract demonstrated notable peripheral analgesic activity (72.55% writhing inhibition, $P<0.01$).

Conclusion: The extract of *F. religiosa* stem bark has significant therapeutic potential, which might be explored to develop natural remedies for inflammation, microbial infections, and oxidative stress-related disorders. Further study is needed to evaluate its isolated bioactive components.

Implication for health policy/practice/research/medical education:
Medicinal properties exhibited by the stem bark of *Ficus religiosa* in both in-vitro and in-vivo methods were noteworthy, providing a good option for bioassay-guided isolation and characterization of its active substances. Moreover, this study might also guide the establishment of this plant as a medicinal one.

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Introduction

Over three-quarters of people worldwide use plant extracts for healthcare, and historically, over 30% of plant species have been used for their therapeutic purposes (1). The bioactive phytochemicals found in plants have been extensively studied to understand their potential to prevent illness and promote overall well-being (2). Among these plants, *Ficus religiosa,* commonly known as the 'peepal tree', is particularly notable. This sacred plant, belonging to the Moraceae family, has been recognized for its rich bio-nutrient composition and therapeutic potential. Native primarily to India, *F. religiosa* is a large

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perennial tree found across the plains and up to 170 m in the Himalayas (3). Traditionally, different parts of the plant, including its leaves, fruits, and bark, have been used in herbal medicine, particularly for their anti-inflammatory, antidiarrheal, antimicrobial, and analgesic properties. The presence of bioactive compounds like flavonoids, saponins, tannins, and alkaloids has been attributed to these therapeutic effects and significant antioxidant properties due to phenolic compounds (4,5).

Despite its longstanding use in traditional medicine, the pharmacological potential of *F. religiosa*'s stem bark has been relatively underexplored, especially concerning its antioxidant, anti-inflammatory, thrombolytic, and antipyretic activities (6,7). The current study aims to bridge this gap by comprehensively evaluating the pharmacological properties of the methanol and n-hexane extracts of *F. religiosa's* stem bark. Through a series of in vitro and in vivo assays, we explored the extracts' antioxidant capacity, antimicrobial activity, thrombolytic potential, anti-inflammatory effects, and antipyretic activity. The findings from this study contribute new insights into the bioactivity of the stem bark, highlighting its significant therapeutic potential.

The results of this study can validate the traditional medicinal uses of *F. religiosa* and provide a scientific foundation for its potential application in modern medicine. This study paves the way for future research to identify and describe the particular bioactive substances that cause its pharmacological effects, potentially developing novel treatments for a wide range of medical conditions.

Methods

Sample collection and extraction

Stem bark of *F. religiosa* was collected from Ramna Park, Dhaka. After identification by a taxonomist (Accession number: UAP Herb/1054_24), the collected and dried stem barks were milled into coarse powder. 600 grams of crushed stem bark powder was then soaked in 2.5 L of methanol and n-hexane, in separate containers for 14 days with random shaking. After 14 days, the supernatant was collected following cotton filtration. The filtrate was left at room temperature to evaporate the solvents, leaving behind the concentrated extracts and then preserved accordingly (8).

Phytochemical screening

The phytochemical screening methods for various compounds in plant extracts are as follows: For carbohydrates, Molisch's test was done by adding 2 drops of Molisch's reagent and 1 mL of concentrated H_2SO_4 to the extract. A red ring and dark purple solution upon shaking indicated the presence of carbohydrates. Glycosides were tested by adding NaOH to the extract; yellow colour formation signified their presence. To test for glucosides, the extract was boiled with dilute H_2SO_4, followed by NaOH and Fehling solution.

A brick red precipitation confirmed the presence of glucosides. Saponins were detected by vigorously shaking of the extract with water; stable bubbles indicated the presence of saponins. The steroid test involved adding chloroform and concentrated H_2SO_4 to the extract, with a red colour in the chloroform layer confirming steroids. For tannins, adding distilled water and 5% $FeCl_3$ to the extract produced a blue/black/green colour or precipitation, indicating the presence of tannins. To identify flavonoids, a few drops of HCl were added to the extract, and the immediate development of a red colour signalled their presence. For alkaloids, the extract was neutralized with dilute H_2SO_4, followed by the addition of specific reagents: Hager's reagent produced a yellow crystalline precipitation, Wagner's reagent gave a brownish-black precipitation, and Dragendorff's reagent formed orange or orange-red precipitation, indicating the presence of alkaloids (9,10).

Total phenolic content

The plant extracts were examined to assess their total phenolic content by adding "Folin-Ciocalteu reagent (FCR)" as the testing reagent (11). Standard gallic acid and both extracted samples were diluted serially within the concentration range from 50 µg/mL to 250 µg/mL. After that, 1 mL of the diluted sample solutions of both extracts was treated with 5 mL of FCR (diluted 1:10 with water) and 4 mL of sodium carbonate preparation. Then, sample test tubes were placed for 1 hour at 20 °C, whereas the standard solutions were incubated for half an hour. Later, the tested samples' absorbance was recorded at 765 nm against the blank. The total phenolic content in extracts was expressed as gallic acid equivalent (GAE) and identified using the standard curve equation.

Total flavonoid content

The test samples were prepared by taking 1 mL of plant extracts in different test tubes at various concentrations. All test tubes were then filled with 3 mL of methanol solution individually, followed by 0.2 mL of 10% $AlCl_3$, 0.2 mL (1M) potassium acetate; distilled water up to 10 mL was added to adjust the final volume. The samples were rested at room temperature for half an hour. Afterward, the absorbance was recorded at 415 nm using a spectrophotometer. The content in plant extracts was quantified as milligrams per gram, stated as quercetin equivalent (QE) (12).

Total antioxidant capacity

Test sample, ascorbic acid standard (various concentrations ranging from 25 to 400 µg/mL), and methanol (0.3 mL) as blank solution were added to 3 mL of reagent solution (composed of ammonium molybdate, sodium phosphate,

and sulphuric acid) separately (13). The test samples were then incubated for 90 minutes at 95 °C. After the samples had reached room temperature, the spectrophotometer measured the absorbances against a blank at 695 nm. The efficiency in serving as a potential antioxidant was quantified in milligrams per gram of ascorbic acid equivalents using a formula based on the ascorbic acid reference standard.

DPPH scavenging capacity

Test tubes with 1 mL of plant samples or standard at varied diluted concentrations were prepared. Each test tube received a freshly formulated 0.004% DPPH solution of about 2 mL, resulting in 3 mL of solution. Then, the solution was rested for half an hour at room temperature, and the absorbance was recorded against the blank at 517 nm (14). The capacity of scavenging free radicals of both extracts was calculated as a percentage of inhibition and/or IC_{50} as per the equation stated below:

$$\% \text{ Inhibition} = (1 - A_1/A_0) \times 100$$

in which, A_1= Absorbance of extract or standard; A_0= Control sample's absorbance.

Nitric oxide scavenging capacity

In this method, test tubes with 4 mL of each sample or standard solution at different concentrations were combined with 1 mL (5 mM) sodium nitroprusside solution. The solutions underwent incubation for two hours at 30 °C. Subsequently, 2 mL of solution was mixed with 1.2 mL of reagent solution. With the help of a spectrophotometer, the absorbance of the concern solution was recorded at 550 nm against a blank. The % inhibition of nitric oxide (NO) radicals was determined accordingly (15).

Thrombolytic activity

One mL of freshly collected blood from healthy volunteers (n = 3) was dispensed into 8 pre-weighed vials, which were then incubated at 37 °C for about 45 minutes. After incubation, the clots settled at the base of all vials, and serum was collected from the top. Subsequently, the weight of the clots was recorded. In four vials (numbered from 1 to 4), 100 µL of sample extract solutions were added, while vials 5 and 6 received 100 µL of distilled water, and the last two vials (vials 7 and 8) were supplemented with 100 µL of streptokinase. All vials underwent a 90-minute incubation at 37 °C, after which the serum was again retrieved from the upper portion, leaving blood clots at the bottom of the vials. The vial weight was then re-measured, and the extent of clot lysis was determined (16).

Membrane stabilizing capacity

To induce hemolysis, 30 µL of stock erythrocyte suspension was mixed with 1 mL of 10 mM sodium phosphate buffer saline (pH 7.4) and 5 mL of hypotonic solution (50 mM NaCl). Different mixtures were prepared for plant samples (1 mg/mL) and the reference standard of acetylsalicylic acid (0.10 mg/mL). After 10 minutes at ambient temperature, the samples were centrifuged at 1500 rpm for 10 minutes. At 540 nm, the supernatant absorbance was measured. The membrane stabilization was then identified using the following equation (17).

$$\% \text{ Inhibition of haemolysis} = 100 \times \{(OD_1 - OD_2)/ OD_1\}.$$

In this equation, OD_1= Optical density of the hypotonic saline solution with buffer (control); OD_2= Optical density of the test sample in hypotonic solution.

To assess heat-induced hemolysis, 2 identical Falcon tubes were prepared, each with 5 mL of an isotonic buffer solution and 0.5 mL of plant extracts (dissolved in distilled water). 30 µL suspension of erythrocytes was then transferred to the tubes individually and gradually mixed by the inversion method. One pair of tubes was in a 54 °C water bath for 20 minutes, while the other received a 0-5 °C ice bath. Following incubation, the samples were placed in a centrifuge machine at 3000 rpm for 10 minutes, the supernatant was collected accordingly, and the absorbance was recorded at 540 nm. The % inhibition of hemolysis was determined accordingly.

Brine shrimp lethality bioassay

Dimethyl sulfoxide (DMSO) was used to prepare various concentrations of both sample extracts. Ten previously hatched nauplii were taken in different test tubes, filling the final volume with saline water. For positive control, vincristine sulphate was taken and evaluated at various low concentrations (0.06, 0.125, 0.25, 0.5, 1, 5, 12.5, 25 µg/mL). Negative control groups were set up by adding 50 µL of dimethyl sulfoxide to three tubes with 4.9 mL of simulated seawater and 10 shrimp nauplii. The live and dead nauplii were counted after allowing all test tubes to incubate for 24 hours. The percentage of lethality for each concentration was then computed, and the LC_{50} value was estimated using a linear regression equation (18).

Anti-microbial activity

In this approach, the samples were soaked onto filter paper discs with a 6 mm diameter, which were then positioned on "Mueller-Hinton agar media". The sample slowly diffused into the surrounding media from the disc, influenced by its solubility and size. Clear zones emerged where the concentration of test samples adequately inhibited microbial growth. The extent of these clear zones, measured in diameter, allowed for assessing the test substance's anti-microbial activity. Ciprofloxacin (5 µg/disc), Nystatin (50 µg/disc), and Ketoconazole (50 µg/disc) were employed as positive controls against bacteria (*Bacillus subtilis, Staphylococcus aureus, Escherichia*

coli, Pseudomonas spp) and fungi (*Candida albicans*), respectively; blank disc and negative control were prepared using the solvent disc (19).

Experimental animal

This study utilized 25 to 30 g male Swiss albino mice, collected from the "Animal Resources Facility at the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR'B)" in Mohakhali, Dhaka. The mice were kept in controlled conditions with 55-65% relative humidity, a temperature of 23.0 ± 2.0 °C, and a twelve-hour light-dark cycle, with unlimited access to feed and water.

Antidiarrheal activity induced by castor oil

The antidiarrheal effects of methanol and n-hexane extracts were evaluated by the "castor oil-induced diarrhea model" in Swiss albino male mice (20). The mice were divided into control, positive control, and test groups, with 4 mice in each group. The control group were given 10 mL/kg distilled water orally, while the positive control group received 3 mg/kg loperamide perorally. Plant sample as well as methanol and n-hexane extracts were given to the test group at 200 and 400 mg/kg body weight. The test animals were housed individually in jars lined with absorbent tissue paper, which was replaced hourly, and their stool production was monitored. Sixty minutes after consuming the test samples, all the subjected animals were treated orally with castor oil. Transparent containers were used to place the mice, where their fecal consistency and frequency were recorded over 4 hours.

Peripheral analgesic activity

Mice were assigned to six groups randomly, each comprising 4 mice, and the groups were given a specific treatment individually. The control group (group 1) received oral 1% Tween 80 at 10 mL/kg. Group 2 received 50 mg/kg diclofenac sodium as the standard. Methanol extract was given to groups 3 and 4 at 200 and 400 mg/kg body weight. Groups 5 and 6 received n-hexane extract at 200 and 400 mg/kg body weight, followed by a 10-mL/kg intraperitoneal injection of 0.7% acetic acid after 30 minutes of rest. Since mice did not always complete full-writhing responses, two half-writhing responses were counted as one. Fifteen minutes after administering the acetic acid injection, the writhing response was counted for a 5-minute period. For each group of mice, the average abdominal writhing response was calculated and documented (21).

Antipyretic activity study

A 15.0% w/v Brewer's yeast solution in distilled water was subcutaneously injected at 10 mL/kg BW to produce pyrexia. After 18 hours of Brewer's yeast injection, the mice's temperature climbed 0.6 °C. Paracetamol 50 mg/kg BW and Tween-80 (1.0%) were employed as controls. *F. religiosa* stem bark methanol preparations were taken orally. Mice in groups III to VI received oral dosages of 200 and 400 mg/kg BW *F. religiosa* methanol and petroleum ether stem bark extracts, respectively, and were compared before and after treatment (22).

Acute toxicity

The acute toxicity of the stem bark extracts of *Ficus religiosa* was evaluated using the procedure illustrated by Walum (22). Mice were randomly assigned to eleven groups, each containing four mice, and each group was subjected to a specific treatment. Ten mL/kg of standard saline was given to group I, which was the control. Mice of the groups II to VI were administered methanol extract of the stem bark at the doses of 500, 1000, 1500, 2000 and 4000 mg/kg, respectively. Similarly, groups VII to XI were administered n-hexane extract at the same doses. The animals were closely monitored for behavioural changes and mortality.

Statistical analysis

All the experiments are done twice to express mean ± SEM (standard error of mean). The results were statistically evaluated using Microsoft Excel 2010, and statistical comparisons were made between the treatment group and the control group. The LC_{50} and IC_{50} values of cytotoxicity and free radical scavenging properties were estimated from the dose-response curve.

Results

Phytochemical screening

The initial screening of *F. religiosa* verified the existence of specific secondary metabolites, including carbohydrates, glucosides, saponins, flavonoids, and alkaloids, which have been tabulated in Table 1.

Total phenolic content

The total phenolic content of the methanol fraction of stem bark was 46.48 ± 1.20 mg GAE/g ± SEM ($y = 0.0069x + 0.0366$, $R^2 = 0.9956$), and the n-hexane fraction of stem bark was 18.75 ± 0.23 mg GAE/g ± SEM. The methanol extract exhibited a higher total phenol content than the n-hexane extract (Table 2).

Flavonoid content

Based on the regression equation ($y = 0.0084x-0.1081$, $R^2 = 0.9878$) of quercetin, n-hexane extract had a greater mean flavonoid content (20.24 ± 0.12 mg QE/g ± SEM) than methanol extract (5.54 ± 0.04 mg QE/g ± SEM) (Table 2).

Total antioxidant capacity

The ascorbic acid calibration curve ($y = 0.0037x - 0.0054$, $R^2 = 0.9902$) was used to assess the overall antioxidant potential of the extracts. According to this study, n-hexane

Table 1. Phytochemical screening of *Ficus religiosa* extracts

Tests	Methanol	n-Hexane
Carbohydrate	+	+
Glycoside	+	-
Glucoside	+	+
Saponin	-	+
Steroid	+	+
Tannin	+	-
Flavonoid	+	+
Alkaloids		
Wagner's reagent	+	+
Hager's reagent	+	+
Dragendroff's reagent	+	+

extract exhibited higher total antioxidant capacity content than the methanol extract (Table 2).

DPPH scavenging capacity

The dose-dependent DPPH radical scavenging effect is shown in Figure 1. The calculated IC_{50} values (Table 2) for methanol extract and n-hexane extract were respectively 24.524 µg/mL (y = 35.905x+1.0689, R^2 = 0.9747) and 319.669 µg/mL (y = 18.442x + 3.8077, R^2 = 0.9396) compared to the reference value of ascorbic acid (15.595 µg/mL; y = 41.068x + 1.0066, R^2 = 0.9415). The current study revealed that the methanol extract had more potent DPPH free radical scavenging potential than the n-hexane extract.

Nitric oxide scavenging capacity

The data obtained from this study found that n-hexane had higher IC_{50} value (69.45 µg/mL, y = 32.206x - 9.3131, R^2 = 0.8776) than methanol extract (463.45 µg/mL, y = 20.61x - 4.9384, R^2 = 0.9141) against ascorbic acid standard (16.03 µg/mL, y = 35.297x + 7.471, R^2 = 0.9334) which are presented in Table 2 and Figure 2, determined by nitric oxide method.

Thrombolytic activity

Higher thrombolytic activity was exhibited by the methanolic extract than the n-hexane extract. The

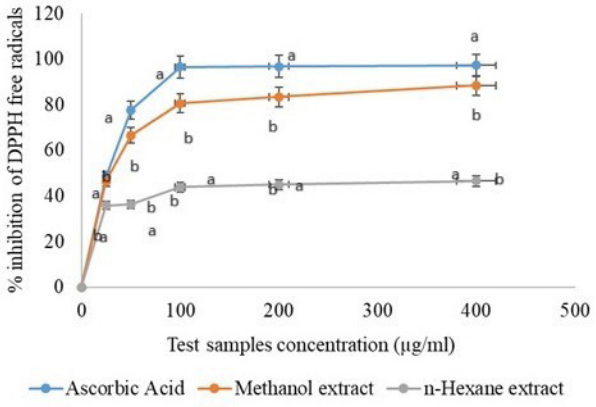

Figure 1. Percent inhibition of DPPH free radicals by the stem bark of *Ficus religiosa* at different concentrations. Values with similar superscripts in different lines indicate significantly different from others ($P < 0.05$; t-test).

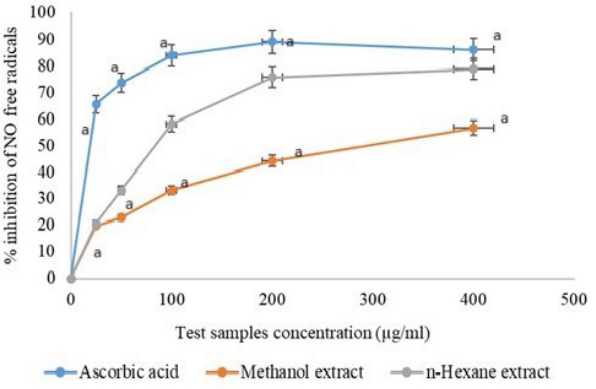

Figure 2. Percent inhibition of nitric oxide (NO) free radicals by the stem bark of *Ficus religiosa* at different concentrations. Values with similar superscripts in different lines indicate significantly different from others ($P < 0.05$; t-test).

methanol extract of the stem bark showed a mean % clot lysis of 43.649 ± 3.807% and the n-hexane extract 25.825 ± 1.408% against the standard streptokinase 66.381 ± 0.896% (Table 3).

Membrane stabilizing potential

Reference standard acetylsalicylic acid demonstrated

Table 2. Total phenolic and total flavonoid contents, antioxidant capacity, and free radical scavenging potential of methanol and n-hexane stem bark extracts of *Ficus religiosa*

Extracts/standards	Total phenol content (mg GAE/g)	Total flavonoid content (mg QE/g)	Total antioxidant capacity (mg AAE/g)	DPPH free radical scavenging assay (IC_{50} µg/mL)	Nitric oxide free radical scavenging assay (IC_{50} µg/mL)	Brine shrimp lethality bioassay (LC_{50} µg/mL)
Methanol extract	46.48±1.20	5.54 ±0.04	79.18 ±0.62	24.524	463.45	231.21
n-Hexane extract	18.75±0.23	20.24 ±0.12	125.51±0.57	319.669	69.45	109.40
Ascorbic acid	-	-	-	15.595	16.03	-
Vincristine	-	-	-	-	-	0.394

GAE: Gallic acid equivalent; QE: Quercetin equivalent; AAE: Ascorbic acid equivalent; IC_{50}: Inhibitory concentration 50%; LC_{50}: Lethal concentration 50%.

Table 3. Thrombolytic and membrane-stabilizing potential properties of various extracts and standards

Extracts	Thrombolytic activity	% Inhibition of hemolysis ± SEM	
	% Of clot lysis	Hypotonic solution	Heat induced
Methanol extract	43.649 ± 3.807	50.68%	38.968
n-Hexane extract	25.825 ± 1.408	34.77%	48.643
Streptokinase	66.381 ± 0.896	-	-
Distilled water	1.070 ± 0.211	-	-
Acetyl salicylic acid	-	55.708%	69.591

Mean ± SEM (Standard error of mean) value was considered.

55.708% and 69.591% hemolysis inhibition of RBC in hypotonic solution and heat-induced hemolysis; methanol extract showed 50.68% and 38.968% hemolysis inhibition; and n-hexane extract demonstrated 34.77% and 48.643% hemolysis inhibition (Table 3). This indicated the moderated anti-inflammatory potential of the plant extract.

Brine shrimp lethality bioassay

In this study, both extracts showed minimal cytotoxic activity as evident from the LC_{50} and LC_{90} values against the reference standard vincristine sulphate (Table 2).

Anti-microbial activity

The anti-microbial activities of methanol and n-hexane extracts at various doses (50, 100, 150, 200, and 250 µg/disc) were studied against two gram-positive bacteria, two Gram-negative bacteria, and one fungus. The n-hexane extract demonstrated relative antifungal activity against *Candida albicans*. The results of the anti-microbial activity of the methanol and n-hexane extracts are summarized in Tables 4 and 5, respectively.

Antidiarrheal activity

Both methanol and n-hexane extracts of the stem bark were dose-dependent, as the number of defecated pellets reduced compared to the Loperamide standard. However, higher anti-diarrheal activity was observed for the methanol extract at both doses. The methanol extract decreased defecated pellets by 64.35% and 83.48% at doses of 200 mg/kg and 400 mg/kg BW, respectively, while the n-hexane extract demonstrated 49.57% and 62.61% inhibition of defecation (Figure 3).

Peripheral analgesic effect

At 200 and 400 mg/kg body weight, the methanol extract achieved 45.10% and 64.71% writhing inhibition, respectively. Following the same dose, the n-hexane extract inhibited the writhing by 54.90% and 72.55%, respectively (Figure 3), which reflects the potent peripheral analgesic activity of the n-hexane extract.

Antipyretic activity

After 3 hours, the decrease in body temperature by methanol extract at the dose of 200 and 400 mg/kg was 3.79% and 5.69%, respectively. At the same dose, the

Table 4. Anti-microbial activities of the methanol extract of the stem bark of *Ficus religiosa*

Test microorganism	Zone of inhibition in mm							
	50 µg/disc	100 µg/disc	150 µg/disc	200 µg/disc	250 µg/disc	Ciprofloxacin 5 µg/disc	Nystatin 50 µg/disc	Ketoconazole 50 µg/disc
Bacillus subtilis	7	8	8	8	9	38	_	_
Staphylococcus aureus	Nil	Nil	Nil	Nil	Nil	39	_	_
Escherichia coli	7	7	8	9	10	41	_	_
Pseudomonas spp.	7	8	8	8	10	40	_	_
Candida albicans	7	8	9	9	10	_	12	12

Table 5. Anti-microbial activities of n-hexane extract of the stem bark of *Ficus religiosa*

Test microorganism	Zone of inhibition in mm							
	50 µg/disc	100 µg/disc	150 µg/disc	200 µg/disc	250 µg/disc	Ciprofloxacin 5 µg/disc	Nystatin 50 µg/disc	Ketoconazole 50 µg/disc
Bacillus subtilis	7	7	7	7	8	33	_	_
Staphylococcus aureus	7	8	8	8	8	36	_	_
Escherichia coli	6	7	7	7	8	35	_	_
Pseudomonas spp.	6	7	7	7	7	35	_	_
Candida albicans	6	6	7	7	7	_	12	12

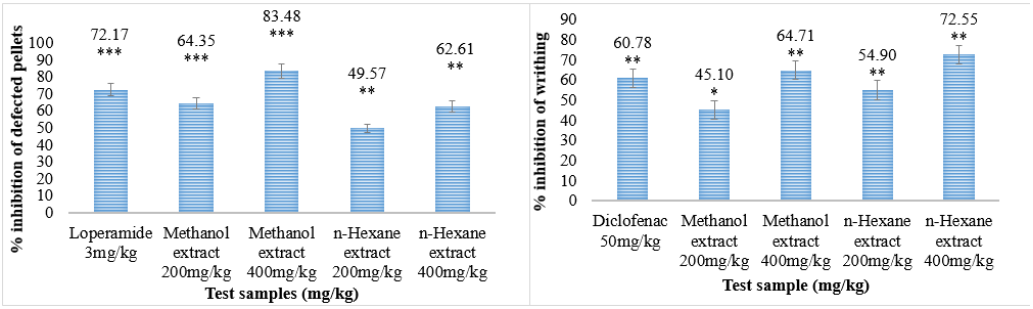

Figure 3. Percentage inhibition of defecated pellets by extracts (left) and % inhibition of writhing by extracts (right) against respective standards. Statistical comparisons are made between the treatment groups and the control group by a t-test of two equal variances. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ indicating statistical significance.

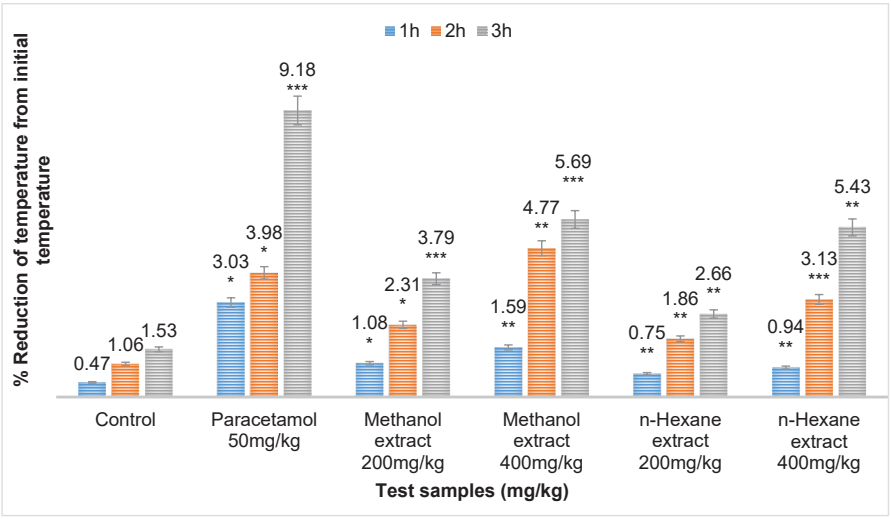

Figure 4. Percentage reduction of temperature by plant extract and standard. Statistical comparisons are made between the treatment groups and the control group by t-test of two equal variances. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ indicating statistical significance.

n-hexane extract lowered the temperature by 2.66% and 5.43% (Figure 4).

Acute toxicity study

Eleven groups were subjected to the study, with no record of mice death or toxic events during the test period. This showed that *F. religiosa* extracts were safe up to 4000 mg/kg body weight.

Discussion

The findings in this study are consistent with earlier studies, which also reported that leaves of *Ficus religiosa* from different extracts had several phytochemicals, namely, alkaloids, glycosides, flavonoids, phenols, tannins, etc., in moderate to high amounts (23). The in-vitro thrombolytic activities of the methanol and n-hexane extracts were 43.649% and 25.825%, respectively, and the positive control showed 66.381% clot lysis. A previous study showed that the methanol fraction of stem buds of *F. religiosa* has more significant thrombolytic activity than the chloroform fraction (24).

The leaf extract of *F. religiosa* was rich in tannins, phenols, triterpenoids, glucosides, and sterols. Previous studies have highlighted the analgesic and anti-inflammatory properties of flavonoids, steroids, and tannins (25). Both extracts exhibited high levels of phenolic content, moderate levels of flavonoids, and notable antioxidant capacity. The antioxidant activity of the extracts increased in a dose-dependent manner. However, the n-hexane extract of the stem bark showed higher antioxidant capacity due to the higher portion of flavonoid content found in the extract than methanol extract. A recent study revealed how phenolic compounds exert anti-inflammatory effects by inhibiting TNF-α production through TNF-α convertase suppression, affirming their role as effective anti-inflammatory agents (26).

The n-hexane extract of the plant sample exhibited a substantial peripheral analgesic effect at the higher dose than the methanol extract. Both extracts showed significant writhing inhibition compared to diclofenac at the dose of 50 mg/kg, which was 60.78% in this study. A similar result was observed for the different leaf extracts of

the concerned plant (27).

The antipyretic activity of the water and chloroform extract of leaves of *Ficus bengalensis* showed a significant decrease in elevated body temperature in mice induced by Brewer's yeast suspension (28). Similarly, in this study, the methanol extract of the stem bark showed a significant role in reducing elevated body temperature compared to the n-hexane extract at a dose of 400 mg/kg against paracetamol. However, the tendency to reduce the temperature of both extracts was lower than the standard.

In the case of the antidiarrheal effect, the methanol extract at the dose of 400 mg/kg showed a higher tendency to reduce the number of defecated pellets than the Loperamide standard itself at the dose of 3 mg/kg. Other species of this plant, namely, the extracts of *F. bengalensis* (bark), *F. racemosa* (leaves) and *F. carica* (leaves) showed significant inhibitory activities against castor oil-induced diarrhea in rats (27). Prostaglandins play a role in increasing gastrointestinal motility and fluid secretion, both of which contribute to diarrhea. By inhibiting prostaglandin production, *F. religiosa* may help reduce motility and fluid loss, alleviating the symptoms of diarrhea. The effect could be due to the presence of tannin and flavonoid content found during the phytochemical screening of both stem bark extracts (27). The extracts also showed minimal cytotoxicity in the brine shrimp lethality test against the vincristine standard; however, according to a previous study, the leaves of *F. religiosa* showed significant cytotoxicity in a similar experimental setup (29).

The study showed minimal antimicrobial activities against selected fungi and Gram-negative and Gram-positive bacteria. The anti-fungal effect of n-hexane extract showed a significant zone of inhibition of *C. albicans*. In previous studies, the leaves' aqueous extract has shown high antimicrobial activity against selected pathogens, including *E. coli* and *S. aureus* (30,31).

This study had certain limitations that should be considered when interpreting the results. First, the stem bark of *F. religiosa* was collected from only a single geographic location in Bangladesh, which may not reflect the phytochemical diversity and bioactivity that could occur in samples from different regions. Second, while our study confirmed the presence of several classes of bioactive compounds, we did not isolate or identify the specific individual constituents responsible for the observed pharmacological effects. Consequently, the precise mechanisms underlying these activities remain to be fully elucidated.

Conclusion

This study provides comprehensive evidence for the pharmacological potential of *F. religiosa* stem bark extracts, demonstrating significant antioxidant, anti-inflammatory, antidiarrheal, antipyretic, and antimicrobial activities in both in vitro and in vivo models. The methanol extract, in particular, exhibited notable antioxidant and anti-inflammatory properties, as well as pronounced antidiarrheal and antipyretic effects, while the n-hexane extract showed comparatively higher antimicrobial activity. Importantly, our results validate traditional medicinal uses of *F. religiosa* and suggest its potential as a source of natural therapeutic agents for oxidative stress, inflammation, infections, and gastrointestinal disorders. However, the study's limitations, such as the collection of plant material from a single region and the lack of specific compound identification, indicate the need for further research. Future studies should focus on isolating and characterizing the active constituents and investigating their mechanisms of action to better understand and harness the medicinal benefits of this species.

Authors' contribution

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Data availability statement

All data are securely retained by the authors and can be shared upon request.

Conflict of interests

The authors confirmed that they did not have any conflict of interest.

Ethical considerations

Ethical approval was given by the Research Ethics Committee, Department of Pharmacy, University of Asia Pacific (Ref: UAP/REC/2023/210) following the evaluation of the research protocol. Standard protocol was followed in handling of laboratory animals (ARRIVE Guideline). All the procedures were strictly followed as per the ethical guidelines.

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