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Original Article

Evaluation of phytochemical and pharmacological properties of seeds of *Nephelium lappaceum* L.

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Abstract: This study aimed to evaluate the antioxidant, analgesic, neuropharmacological, antihyperglycemic, anti-inflammatory, and antidiarrheal properties of the methanolic extract of *Nephelium lappaceum* L. seeds (MSNL). *In vitro* antioxidant was determined using the DPPH free radical scavenging test. Analgesic, antihyperglycemic, antidiarrheal, and anti-inflammatory activities were evaluated using the acetic acid-induced writhing test, oral glucose tolerance test, castor oil-induced diarrhea, and carrageenan-induced paw edema, respectively, using rodents. Neuropharmacological activity was investigated in mice using both open-field and hole-cross methods. MSNL demonstrated strong DPPH scavenging capacity (IC_{50} = 53.92 µg/mL) compared to standard ascorbic acid (IC_{50} = 41.10 µg/mL). In the acetic acid-induced writhing test, the highest dose (600 mg/kg) showed 59.89% inhibition of abdominal constrictions compared to indomethacin (81.97%). MSNL showed a significant ($P<0.05$, $P<0.01$) diminution in the locomotion of rodents in both open field, and hole cross methods at the highest dose compared to the control group. MSNL significantly reduced blood glucose levels in mice ($P<0.01$, $P<0.05$) in a dose-dependent manner. The highest dose 600 mg/kg of MSNL showed a significant 53.46% reduction in diarrhea. MSNL 600 mg/kg displayed significant inhibition of inflammation at the 3rd hour (65.22%). The findings demonstrated that the extract has potential bioactivities and can be considered as the benchmark for the isolation of pure bioactive compounds from this plant to discover new drugs.

Keywords: *Nephelium lappaceum L.;* Antioxidant; Analgesic; Neuropharmacological activity; Anti-inflammatory; Antihyperglycemic properly.

1. Introduction

Researchers are always looking for novel medications with enhanced or superior therapeutic effects. Medicinal plants are a great source of lead compounds for the development of novel, noble medications with comparatively few side effects or unfavorable effects [1, 2, 3]. Since ancient times, traditional medicines have been widely used for their reliability in treating various illnesses and sufferings in humans. Medicinal plants yield a variety of bioactive natural chemicals that are used as starting points for the development of novel drugs [4, 5, 6]. Different kinds of medicinal plants are used to synthesize phytochemicals, which are used in the treatment of various disorders. These phytochemicals include alkaloids, saponins, carbohydrates, glycosides, flavonoids, gums, steroids, tannins, phenolic compounds, volatile oils, etc. The potential health advantages of natural compounds with antioxidants, as well as their antibacterial, analgesic, neuropharmacological, anti-inflammatory, anticancer, and anti-diabetic properties, have received much attention in the past few years. As a result, there has

been a massive surge in studies on many medicinal plants to identify the key chemicals causing this pharmacological activity [3, 4, 5, 6].

The fruit known as rambutan, or *Nephelium lappaceum* L., is indigenous to tropical areas including Indonesia, China, India, Australia, Malaysia, Mexico, and Thailand. It belongs to the *Sapindaceae* family [7]. The fruit known as rambutan is also called hairy lychee since its name comes from the Malay-Indonesian word "rambut", which means hairy [8]. Its fruit is an ovoid berry with colors ranging from brilliant crimson, maroon, yellow, and orange-red [9]. Its leathery skin, about 3 mm thick, is completely covered in splinters ranging from 0.5 to 2.0 cm. The flesh has a sweet to slightly sour flavor and is juicy and translucent white in color. The almond line seed in the fruit core is rectangular, measuring 2.5–3.4 cm in length and 1.0-1.5 cm in breadth [10, 11]. There are several bioactive ingredients in the rambutan peel, seed, and pulp. Phenolic chemicals were the most significant phytochemical component examined. Among other phytoconstituents, the phytochemical investigations identified reducing sugar, monosaccharides, carbohydrates, phenols, proteins, tannins, alkaloids, flavonoids, steroids, saponins, and glycosides. Antioxidant, antibacterial, anticancer, antidiabetic, antiviral, anti-inflammatory, and antiproliferative actions are just a few of the medicinal and nutritional qualities of phytochemicals [12, 13, 14, 15].

Traditionally, not much thought has been given to how the seeds and rind, which are frequently the wasted portion of the fruits, may be recycled or utilized instead of being thrown away. Interestingly, some fruits' seeds and rinds contain more vitamins, fibers, minerals, and other vital components than the pulp parts [15, 16]. Because rambutan seeds don't contain any toxins and provide protein, fat, and carbohydrates that the body requires, they are safe to eat [16]. Rambutan seeds have a high polyphenol content. The chemicals known as polyphenols, which include anthocyanins, complex polyphenols, leucoanthocyanidin (3%), and catechin (3%), are highly astringent [16]. The primary components of polyphenols are flavonoids and tannins, which have been identified as ellagic acid, geraniin, and corilagin [14, 15, 16]. The various polyphenols, such as antioxidant, anti-inflammatory, anticarcinogenic, and other bioactivities, demonstrated suggest that they may have beneficial effects on human health and provide protection against such chronic diseases as cardiovascular diseases, neurodegenerative disorders, and cancers [16].

The literature review revealed that, despite the abundance of bioactive components, there had been relatively little research on *N. lappaceum* seeds. This motivated us to investigate the antioxidative, analgesic, neuropharmacological, antihyperglycemic, anti-inflammatory, and antidiarrheal properties of rambutan seeds and their methanolic extractive in a comprehensive manner, keeping in mind the need for natural mineral supplements and bioactive compounds from seeds on a global scale.

2. Materials and Methods

2.1 Collection, authentication, and extraction of plant sample

The Fresh ripe *N. lappaceum* fruits were collected from Dhaka district, Bangladesh, in April 2024, and were identified by a taxonomist from the Bangladesh National Herbarium in Mirpur, Dhaka (Accession no. DCAB 36575). After precise washing, seeds were separated from fruits. Following a week of drying in the shade, they were ground into a fine powder and kept in sealed containers in a dark, cold, and dry room until they were processed. Extraction was carried out based on the maceration method [17]. Five hundred grams of powdered sample was macerated in two liters (95%) of methanol for 14 days with random shaking and stirring. After two weeks, the entire mixtures were separately filtered using a clean cotton bed and Whatman filter paper number 1. The filtrate was concentrated to dryness in a rotary evaporator at 40°C under reduced pressure. The dried methanolic extract of *N. lappaceum* fruit (MSNL) was preserved in the laboratory to conduct *in vivo* and *in vitro* pharmacological experiments.

2.2 Chemicals

Diazepam, Indomethacin, Metformin, and Loperamide were generous gifts from Bangladesh's Square Pharmaceuticals Ltd. We bought DPPH, ascorbic acid, acetic acid, and methanol (95%) from Merc (Germany). Analytical grade materials were used for all other reagents in the investigations.

2.3 Experimental Animals

All *in vivo* pharmacological studies were conducted on young, healthy Swiss-albino mice aged 4-5 weeks (weight, 25-30 g) and rats aged 3-4 months (weight, 120–130 g) of both sexes. They were purchased from the Animal Resources Division of the International Center for Diarrheal Disease Research, Bangladesh (ICDDR, B). The rodents had free access to ICCDR, B-formulated rodent feed, and water while being housed in a conventional laboratory environment (room temperature of (25±1)°C, relative humidity of 56%–60%, and a 12-hr light/12-hr dark cycle). Prior to pharmacological investigations, all rodents were housed under the mentioned conditions for approximately a week to acclimate to the laboratory environment.The animal ethical committee of the Southeast University (Dhaka, Bangladesh) authorized each animal experiment protocol (SEU/Pharm/CERC/111/2023).

2.4 Phytochemical screening

Conventional techniques were employed to investigate whether the investigated extract included any distinct bioactive components [18, 19]. Color or foaming was visually examined to determine if a particular phytochemical group was present or missing [20].

2.5 Evaluation of antioxidant activity

The antioxidant activity of *N. lappaceum* methanol seed extract was assessed using a slightly modified version of the previously published quantitative DPPH-scavenging approach [21]. In summary, 3.0 mL of a methanolic solution (20 g/mL) containing DPPH was combined with plant materials at different concentrations (500.0 to 0.977 g/mL). After vortexing the reaction mixture, it was kept in the dark for half an hour. The absorbance of each mixture was then measured at 517 nm using a UV-Vis spectrophotometer. Using the following formula, the free radical quenching capacity was determined:

$$\textbf{\% of inhibition} = \frac{\mathbf{A_{blank} - A_{sample}}}{\mathbf{A_{blank}}} \times \mathbf{100\%}$$

In this equation, A is the absorbance for each group. The IC_{50} value (50 percent inhibition) for each plant sample was then determined using a graph of the percent inhibition of DPPH scavenging vs. concentration of the test materials.

2.6 Evaluation of analgesic activity

The acetic acid-induced writhing test was used to measure the peripheral analgesic activity in accordance with the Haque et al., 2020 approach [5]. The experimental mice were given 200, 400, and 600 mg/kg body weight of the plant sample in addition to standard indomethacin orally during this test. Each mouse was given an intraperitoneal injection of 1% acetic acid at a dose of 10 mL/kg body weight, forty minutes after starting the treatments in order to induce writhing (abdominal constrictions). Each animal's writhes were counted over the course of the following thirty minutes. The following equation was used to compute the proportion of the treated group's writhing inhibition:

$$\textbf{\% of inhibition of writhing} = \frac{\mathbf{N_{Control} - N_{Test}}}{\mathbf{N_{Control}}} \times \mathbf{100\%}$$

Where N represents the average number of writings for each group.

2.7. Evaluation of neuropharmacological activity

The neuropharmacological activity of the plant extract was evaluated using the open-field and hole-cross methods.

2.7.1 Open field method

The open field behavioral test is widely used to assess the emotional state and locomotor activity of rats [22]. With a few minor modifications, the test was carried out utilizing the methodology outlined in Moniruzzaman et al. [23]. A half-square-meter hardwood field with rows of squares painted in black and white alternately used as the open field device. It was kept in a dimly lit room with a wall 50 centimeters high. The mice were free to move about the center of the box throughout the duration of the three-minute pretreatment reading, and the number of squares they visited was recorded. After the reading was taken, the rats were given a vehicle, extracts (200, 400, and 600 mg/kg), or diazepam (1 mg/kg) therapy. After that, they were viewed on a regular basis at 30, 60, 90, and 120 minutes later.

2.7.2 Hole cross method

The most persistent behavioral shift occurs from an intense emotional response to a novel setting. The hole-cross test was carried out using the Shahed-Al-Mahmud and Lina [22] protocol. A cage measuring 30 cm × 20 cm × 14 cm, with a partition in the middle. The apparatus is constructed from hardwood planks. A 3 cm-diameter hole was drilled in the center of the cage at a height of 7.5 cm. After placing a mouse in the middle of the cage and administering oral treatments (vehicle, extracts, and standard), the number of mice that passed through the aperture connecting one chamber to the next was counted for three minutes at 0, 30, 60, 90, and 120 minutes.

2.8 Evaluation of Anti-inflammatory Activity

By slightly altering the technique of Mondal et al. [24], inflammation caused by carrageenan in rat paws was used to test the anti-inflammatory properties of MSNL. Five groups of five rats each were randomly assigned, while group I was kept as the control group and was given only distilled water. The conventional medicine, indomethacin (5 mg/kg), was administered to Group II. The test sample was administered to groups III, IV, and V at doses of 200 mg/kg, 400 mg/kg, and 600 mg/kg body weight, respectively. Each rat received an injection of 1% carrageenan in saline into its left hind paw thirty minutes after the test materials were given orally. After carrageenan was administered, the amount of paw edema was measured using a water plethysmometer at 1, 2, and 3 hours. For comparison, the right hind paw was used as the non-inflamed reference. We computed the average percent increase in paw volume over time and compared the results to the control group. Percent inhibition was calculated using the formula:

$$\textbf{\% Inhibition of paw edema} = [(Vc - Vt) / Vc] \times 100.$$

Where Vc and Vt represent the average paw volume of the control and treated animals, respectively.

2.9 Hypoglycemic Test (Oral Glucose Tolerance Test-OGTT)

The Tesfaye et al. technique [25] was followed by an overnight fast (18 hours) before healthy mice were given the oral glucose tolerance test (OGTT). Five groups of mice, one for each sex, were randomly assigned. 1% tween 80 in normal saline, metformin, and extracts/fractions were given to the test, positive, and negative control groups, respectively. Mice in all test groups were given a 10% glucose solution orally, 30 minutes after the plant extract/drug was administered orally. A glucometer was then used to measure each animal's blood glucose levels at zero, 30, 90, and 150 minutes after the glucose load. The equation below can be used to calculate the percent drop in blood sugar level caused by *N. lappaceum* extract:

$$\textbf{\%reduction in blood glucose} = \frac{\mathbf{BGL_{control} - BGL_{test}}}{\mathbf{BGL_{control}}} \times 100$$

Here, BG is the average blood glucose level for each group.

2.10 Evaluation of Antidiarrheal activity

The method of Haque et al. [5] was followed for conducting the castor oil-induced diarrheal test. To put it briefly, 30 minutes after the proper doses and treatments were administered, each mouse was given 1 mL of castor oil orally to induce diarrhea. The mice were then kept in cages covered in absorbent materials for four hours in order to check for diarrhea, which is defined as sloppy, unformed feces. The control group's results were considered to be 100%. The percentage inhibition (%) of diarrhea was used to assess each group's performance. The percentage of defecation inhibition was estimated as follows:

$$\% \text{ Inhibition of defecation} = [(A\text{-}B)/A] \times 100$$

Where A represented the average number of feces induced by castor oil, and B represented the average number of feces caused by the drug or extract.

2.11 Statistical analysis

The findings were shown as mean ± SEM. The statistical analysis was carried out using the SPSS 26 program, and one-way analysis of variance (ANOVA) and Dunnett's post hoc test were utilized. Differences between groups were deemed significant at a level of $p < 0.01$ and $p < 0.05$.

3. Results

3.1 Preliminary phytochemical screening

Table 1 summarizes the findings of the phytochemical screening of MSNL extract. The crude methanolic extract showed the presence of numerous beneficial secondary metabolites. All of the examined phytochemicals had been identified in the experimented sample. Carbohydrate, glycoside, resin, steroid, terpenoids were the least abundant secondary metabolites among all the bio constituents while fixed oil, protein, phenol were the most prevalent the studied sample.

Table 1. Phytochemical screening test of methanolic extract of *N. lappaceum* seed

Phytocompounds	MSNL
Carbohydrate	+
Glycoside	+
Tannin	++
Protein	+++
Alkaloid	++
Saponin	-
Resin	+
Phenol	+++
Flavonoid	++
Steroid	+
Terpenoids	+
Fixed oil	+++

Here, "+" specifies the existence, and "-" shows the absence of any phytochemical group. Bioavailability key: (+++) ve = strong intensity, (++) ve = Moderate intensity, (+) ve weak intensity, (−) ve = Absence

3.2 DPPH radical scavenging assay

In the antioxidant experiment, the studied extract demonstrated concentration-dependent quenching characteristics against the DPPH radical. The ability of plant extracts to quench DPPH radicals is shown by the IC_{50} values (50 percent inhibition), which are displayed in **Table 2**. At every concentration point, ascorbic acid, a well-known antioxidant, had a higher level of free radical-scavenging activity than the plant extract. The normal ascorbic acid had an IC_{50} value of 41.10 µg/mL, while the seed methanol extract had an IC_{50} value of 53.92 µg/mL.

Table 2. DPPH scavenging capacity of MSNL extract

Sample	IC_{50} (µg/mL)
Standard (Ascorbic acid)	41.10
MSNL	53.92

3.3 Acetic acid induced writhing test

The tested extract considerably ($p<0.01$; $p<0.05$) reduced the number of writhes in mice in the acetic acid-induced writhing technique when compared to the negative control (**Table 3**) at all doses. Additionally, the reference medication, indomethacin, demonstrated a significant ($p<0.01$) antinociceptive activity compared to the negative control group. The MSNL extract showed a maximum percent of inhibition of 69.59% at the higher dose of 600 mg/kg.

Table 3. Effect of MSNL Extract on acetic Acid-induced writhing in mice

Group	Treatment	Number of writhing	% of inhibition
Negative control (I)	Tween 80 solution	30.83 ± 0.98	----------
Positive control (Standard) (II)	Indomethacin 10 mg/kg	6.33 ± 0.83**	84.11
III	MSNL 200 mg/kg	30.00 ± 0.33*	24.68
IV	MSNL 400 mg/kg	21.17 ± 1.33*	46.85
V	MSNL 600 mg/kg	14.50 ± 1.05**	69.59

The values are revealed as mean±SD (n=5); One-Way Analysis of Variance (ANOVA) followed by Dunnet's test. *P<0.05, **P<0.01 significant compared to the negative control.

3.4 Neuropharmacological activity

3.4.1 Open field method

The outcomes of the open field test are given in **Table 4**. At dosages of 200, 400, and 600 mg/kg, MSNL significantly reduced the locomotor activity in mice ($p < 0.01$; $p< 0.05$), and this effect was noticed from the initial observation (0 min) period and persisted through the fifth observation period (120 min). From the second to the fifth observation, mice were given diazepam (1 mg/kg, i.p.) showed a noticeably reduced ability to move around.

Table 4. Neuropharmacological effect of *N. lappaceum* L. seed extract on mice in the Open Field method

Groups	Treatment	Number of movements				
		0 min	**30 min**	**60 min**	**90 min**	**120 min**
Negative control (I)	Tween 80 solution	152.4 ± 0.83	150.0 ± 0.63	152.2 ± 0.74	149.4 ± 0.66	150.6 ± 0.33
Positive control (Standard) (II)	Diazepam 1 mg/kg, i.p.	142.80 ± 0.84*	100.2 ± 0.53**	69.2 ± 1.3**	62.2 ± 0.83**	10.2 ± 0.66**
III	MSNL 200 mg/kg	136.33 ± 0.52	99.8 ± 0.54*	83.0 ± 1.64*	76.0 ± 0.89**	50.60 ± 1.58*
IV	MSNL 400 mg/kg	130.00 ± 1.52*	87.2 ± 1.48**	72.4 ± 1.14**	66.2 ± 1.648*	49.0 ± 1.87**
V	MSNL 600 mg/kg	129.6 ± 0.89*	76.8 ± 0.83	70.2 ± 1.30	62.2 ± 1.58**	40.0 ± 0.71*

The values are revealed as mean±SD (n=5); One-Way Analysis of Variance (ANOVA) followed by Dunnet's test. *P<0.05, **P<0.01 significant compared to the negative control.

3.4.2 Hole cross method

The findings demonstrated that practically MSNL extract considerably (p 0.01; p 0.05) reduced the number of holes the mice crossed over time from their initial value. From the second to the fifth observation hour (30 to 120 min), at a dose of 600 g/kg, the locomotor activity of the experimental mice was noticeably decreased in the examined sample. With time, the CNS-depressant effects were significantly (p<0.01; p<0.05) diminished. The MSNL extract effectively reduced locomotor activity, and the results were significant compared to those obtained using diazepam as the reference medication (**Table 5**).

Table 5. Neuropharmacological effect of *N. lappaceum* L. seed extract on mice in hole cross test

Groups	Treatment	Number of movements				
		0 min	**30 min**	**60 min**	**90 min**	**120 min**
Negative control (I)	Tween 80 solution	28.0 ± 0.64	26.6 ± 0.80	29.2 ± 0.75	26.20 ± 0.49	26.8 ± 0.52
Positive control (Standard) (II)	Diazepam 1 mg/kg, i.p.	29.8 ± 0.75*	20.0 ± 1.40*	10.8 ± 0.75**	3.2 ± 0.40**	1.6 ± 0.80**
III	MSNL 200 mg/kg	30.4 ± 0.49*	25.2 ± 0.74*	20.2 ± 0.89*	15.60 ± 0.89	12.60 ± 0.89*
IV	MSNL 400 mg/kg	27.41 ± 1.02*	17 ± 1.66*	14.0 ± 0.97*	11.40 ± 0.49	9.40 ± 0.80**
V	MSNL 600 mg/kg	26.0 ± 1.02*	14.63 ± 0.48*	10.80 ± 0.49*	9.60 ± 0.80**	4.0 ± 0.63*

The values are revealed as mean±SD (n=5); One-Way Analysis of Variance (ANOVA) followed by Dunnet's test. *P<0.05, **P<0.01 significant compared to the negative control

3.5 Anti-inflammatory activity evaluation

Table 6 displays the anti-inflammatory effect of the investigated plant extract in the rat paw edema technique caused by carrageenan. According to the current investigation, after three hours, MSNL (600 mg/kg dose) significantly reduced the amount of edema (56.52%). At three hours, the standard anti-inflammatory medication (Indomethacin 5 mg/kg dose) showed effective inhibition (71.01%).

Table 6. Anti-inflammatory effect of *N. lappaceum L*. seed extract in the rat paw edema technique

Groups	**Treatment**	**Paw volume (mm)**	
		Change in paw edema mean (mm)	**% Edema inhibition relative to control at 3rd hr**
Negative Control (I)	Normal saline 0.9% 0.3 ml	1.38 ± 0.05	---
Positive control (Standard) (II)	Indomethacin 5 mg/kg	0.4 ± 0.02**	71.01
III	MSNL 200 mg/kg	0.9 ± 0.05*	34.78
IV	MSNL 400 mg/kg	0.7 ± 0.02*	49.27
V	MSNL 600 mg/kg	0.6 ± 0.02*	56.52

The values are revealed as mean±SD (n=5); One-Way Analysis of Variance (ANOVA) followed by Dunnet's test. *P<0.05, **P<0.01 significant compared to the negative control

3.6 Hypoglycemic test

From 30 minutes on, MSNL extract in the OGTT significantly reduced the plasma glucose levels (**Table 7**). In comparison to Metformin, the tested sample showed a substantial (p<0.01, p<0.05) and concentration-dependent glucose-lowering impact that continued for up to 150 minutes after the loading dosage.

Table 7. Effect of *N. lappaceum* L. seed extract on OGTT in healthy rodents

Groups	**Treatment**	**Blood glucose level in different time**			
		0 min	**30 min**	**90 min**	**150 min**
Negative control (I)	Tween 80 solution	8.32 ± 0.49	26.78 ± 0.48	20.5 ± 0.71	18.33 ± 0.62
Positive control (Standard) (II)	Metformin 10 mg/kg, i.p.	8.78 ± 0.63**	15.53 ± 0.67**	11.78 ± 0.77**	5.22 ± 0.89**
III	MSNL 200 mg/kg	9.58 ± 0.58	22.67 ± 0.69*	17.28 ± 0.38*	14.55 ± 0.25*
IV	MSNL 400 mg/kg	9.67 ± 0.66	19.78 ± 0.69**	12.27 ± 0.29**	10.95 ± 1.16**
V	MSNL 600 mg/kg	9.3 ± 0.36	16.27 ± 0.53	10.10 ± 0.35	9.2 ± 1.38*

The values are revealed as mean±SD (n=5); One-Way Analysis of Variance (ANOVA) followed by Dunnet's test. *P<0.05, **P<0.01 significant compared to the negative control

3.7. Antidiarrheal activity evaluation

At all tested doses, MSNL was found to be efficacious in a dose-dependent manner in treating experimental mice's diarrhea caused by castor oil. The extract significantly reduced the amount of diarrhea in albino mice, measured by the defecation rate and feces' consistency, at all doses (200, 400, and 600 mg/kg bodyweight). Compared to the standard drug Loperamide, which showed an 80.32% suppression of diarrhea, the highest dose of the extract (600 mg/kg) demonstrated a 59.03% inhibition of diarrhea (**Table 8**).

Table 8. Effect of MSNL extract at various dose levels on castor oil-induced diarrhea in mice

Groups	Treatment	Total no. of feces in 4 hours	% of inhibition
Negative control (I)	1% Tween 80 solution	20.33 ± 1.21	----------
Positive control (Standard) (II)	Loperamide 2 mg/kg	4.0 ± 0.63**	80.32
III	MSNL 200 mg/kg	15.56 ± 0.75*	21.84
IV	MSNL 400 mg/kg	12.16 ± 1.05*	40.19
V	MSNL 600 mg/kg	8.33 ± 0.84*	59.03

The values are revealed as mean±SD (n=5); One-Way Analysis of Variance (ANOVA) followed by Dunnet's test. *P<0.05, **P<0.01 significant compared to the negative control

4. Discussion

The use of medicinal plants has emerged as a fascinating avenue for the development of traditional and modern medications, and research has demonstrated the actual medical benefits of herbal medicines [26, 27, 28]. Our current study's objective was to look into the general *in vitro* and *in vivo* bioactivities of *N. lappaceum* L. seeds. The biological effectiveness of medicinal plants is mainly dependent on their phytochemical content. A key factor in the discovery of novel, uncommon, and active chemicals is phytochemical analysis. The existence of secondary metabolites in plants is associated with their biological significance [27]. The crude seed extract of the experimental plant exhibited the presence of numerous valuable secondary metabolites such as alkaloids, glycosides, tannins, reducing sugars, steroids, fixed oil, terpenoids, flavonoids, and phenols (**Table 1**). *N. lappaceum* L. plant parts are already reported as an ailment for various diseases in the traditional system [12, 13, 14, 15]. The seed of the plant is reported to have a diverse nature of compounds, including anthocyanins, complex polyphenols, leucoanthocyanidin, catechin, ellagic acid, geraniin, corilagin, etc. [14, 15, 16].

The most used technique for assessing the antioxidant capacity of plant materials is the DPPH scavenging assay. Based on the ability of 1, 1-diphenyl-2-picrylhydrazyl (DPPH), a persistent free radical that decolorizes in the presence of antioxidants, the DPPH antioxidant experiment was carried out [24]. The results of the DPPH scavenging test suggest that the plant may contain active ingredients that, because of their redox characteristics, exhibit antioxidant activity and are essential for absorbing and neutralizing free radicals. Using DPPH, we found, as previously reported by Mohan et al. [29], a direct correlation between dose and radical quenching potential in MSNL extract. Our study was able to show that the extract has a considerable scavenging effect in a dose-dependent manner, even if it is less active than ascorbic acid (IC_{50} 41.10µg/mL) (IC_{50} 59.92 µg/mL; **Table 2**). Free radicals are recognized to have a significant impact on a wide range of clinical symptoms. This becomes well-known when an excess of it occurs in a living organism and causes oxidative damage. It also weakens the body's defenses against disease, leading to the manifestation of a range of ailments such as ageing, cancer, Alzheimer's, atherosclerosis, angina pectoris, metabolic disorders, Parkinson's, complications from diabetes, rheumatoid arthritis, etc. Free radical-squelching antioxidants are essential for treating this pathological condition. Because of this, there is a growing interest in creating natural antioxidants derived from plants that can shield the body from oxidative damage brought on by free radicals [5, 30]. Previous studies have demonstrated the importance of bioactive phytochemical components, especially phenolic compounds (flavonoids, phenolic acids, and tannins), for plants' ability to scavenge free radicals and act as antioxidants (these compounds were also found in our studied extracts; **Table 1**) [2, 30, 31].

Acetic acid is the primary inducer of pain in an animal model, and the acetic acid-induced writhing response technique is a commonly used approach to assess the peripheral analgesic activity of any plant portion [32]. The acetic acid-induced writhing test is a useful paradigm for evaluating the peripheral analgesic potentials of test compounds because of its sensitivity and capacity to identify antinociceptive effects of natural products and test compounds at dose levels that are inert for other techniques [33]. Acetic acid injected intraperitoneally stimulates and irritates the peritoneal cavity, which in turn causes the production and release of a number of endogenous inflammatory mediators, including histamine, serotonin, bradykinin, substance P, and PGs [34, 35]. These different endogenous inflammatory mediators produced chemically induced visceral discomfort, which is characterized by the body lengthening and the forelimbs extending together with the abdominal muscles contracting. The acetic acid-induced writhing test is regarded as a model of visceral pain because of this [35], and furthermore, linked to elevated PGE and PGF2α levels in this model. Raising PG levels in the peritoneal cavity increases the intensity of inflammatory pain by activating primary afferent nociceptors and widening capillary permeability [33, 35]. When compared to the negative control, MSNL extract at all three dosages (200, 400, and 600 mg/kg) significantly ($p < 0.05$ and $p < 0.01$) demonstrated peripheral analgesic effects by lowering the number of writhing (**Table 3**) with respective values of 24.68%, 46.85%, and 69.59%. These results demonstrated that the extract's dosage-dependent peripheral analgesic effectiveness rose from the lower dose (200 mg/kg) to the higher dose (600 mg/kg). A rise in concentration of phytoconstituents that exhibit analgesic activity at the highest dose may be the cause of the extract's increasing analgesic effect with higher doses. The extract may have inhibited the synthesis and release of different endogenous inflammatory mediators as well as the sensitivity of peripheral nociceptors in the peritoneal free nerve endings to chemically induced pain, which could be the mechanism by which it produced peripheral analgesia in this model. These suggested pathways are consistent with the guiding principles, which claim that any substance that reduces the amount of writhing will exhibit analgesia by preventing the production and release of PGs and by preventing the transmission of pain to the peripheral areas [32, 33, 34, 35].

Mice's naturalistic locomotor behavior was used to study the CNS depressed effect of MSNL extract using two neuropharmacological models: open field and hole cross. These paradigms represent established conventional approaches to investigating neuropharmacological activity [35, 36]. The investigated extracts significantly induced substantial locomotor inhibition at the tested doses, and in both tests, this effect persisted for 30 to 120 minutes during the research period (**Tables 4 and 5**). Reduced locomotor activity is thought to be an indicator of awareness and is a symptom of CNS-depressing activity [37]. The CNS-depressing impact of the plant extract may be responsible for this decrease in spontaneous motor activity [38]. In the brain and central nervous system, GABA is an essential inhibitory neurotransmitter that plays a role in physiological processes related to neurological and psychiatric disorders [39]. Many medications can modify the GABA system at the molecular level by increasing GABA-mediated postsynaptic inhibition through an allosteric modification of GABA receptors. It can either improve chloride conductivity or increase GABA-induced chloride conduction when the voltage-triggered Ca2+ channel is blocked. Therefore, the extracts are probably going to cause a decrease in the firing rate of important brain neurons by either directly activating GABA receptors or amplifying GABAergic suppression in the CNS through membrane hyperpolarization [36, 38]. Prior studies on phytochemicals indicate that flavonoids, neuroactive steroids, triterpenoids, and saponins have agonistic effects at the GABA-A receptor complex. The depressive activity of the extracts is caused by the secondary metabolites (flavonoids, terpenoids, saponins, etc.) of the plant, which may have synergistic effects at one or more target sites related to a physiological function [36, 37, 38, 39]. These phytoconstituents may play a role in the CNS depressive effects observed in mice. Further investigation is required to pinpoint the specific phytoconstituents responsible for the neuropharmacological activities and the corresponding mechanisms of action.

An *in vivo* experimental model of acute inflammation known as carrageenan-induced paw edema has been widely used to assess the anti-inflammatory properties of novel investigational medicines. The urge to concentrate on herbal drugs with fewer side effects arises from the rise in the use of synthetic drugs and their associated adverse effects. According to the study's findings, methanolic seed extract of *N. lappaceum* L. has a strong anti-inflammatory effect on rats' paw edema caused by carrageenan. It is thought that rats' paw edema caused by carrageenan is biphasic. Bradykinin, protease, prostaglandin, and lysosome release are the causes of the second

phase, whereas histamine or serotonin release is responsible for the first. Other chemical mediators, such as hydroxyl radicals (OH^-) and oxygen-derived free radicals like superoxide anion (O^{2-}), are also generated during the late phase of inflammation and are crucial to the onset and development of acute inflammation [32, 33]. Beginning one hour after carrageenan induction, the extract at all test dosages used (200, 400, and 600 mg/kg) significantly ($p < 0.05$ and $p < 0.01$) reduced the formation of edema, and the effects lasted until the 150 minutes of observation. The extract's effects persisted from the first phase of inflammation, which lasted for one hour, until the second phase, which lasted three hours. This observation implied that the extract's bioactive components might inhibit the release and/or activation of chemical mediators, hence suppressing both stages of acute inflammation. At the third time of observation (150 minutes), the most significant percentage of edema inhibition by all extract doses was noted, with the corresponding values being 34.78%, 49.27%, and 56.52% (**Table 6**). These results confirmed that the extract has a dose-dependent anti-inflammatory effect. At the 3rd period of observation, the edema inhibition potential exhibited by the larger dose of the extract (600 mg/kg) exhibited good inhibition in comparison to that of the standard drug (Indomethacin 5 mg/kg), with respective values of 56.52% and 71.01%. Since endogenous inflammatory mediators like serotonin and histamine are involved in the early phase of inflammation, the extract and standard medication, indomethacin 5 mg/kg, both demonstrated significant anti-inflammatory effects. Additionally, the extract's effects on edema inhibition peaked at the third time point, suggesting that both the extract and the standard medication have potent anti-inflammatory effects against a variety of endogenous inflammatory mediators that involve in the late phase of inflammation such as, COX, different PG analogues, BK and/or leukotriene or they could have, free radical scavenging activities [33, 34, 35]. It follows that the MSNL extract may have inhibited carrageenan-induced inflammation by inhibiting the enzyme cyclooxygenase, which in turn may have inhibited the manufacture of prostaglandins.

A drug that effectively cures diabetes will be able to control the rise in blood sugar through several different pathways, and a glucose-loaded hyperglycemic mode may be used to evaluate an extract's potential to prevent hyperglycemia. The OGTT measures the speed at which the body can remove exogenous glucose from the blood after it has been eaten and is a commonly used test to diagnose diabetes mellitus [40, 41, 42]. This method is called physiological induction of diabetes mellitus [41] because it momentarily raises the animal's blood glucose level without harming the pancreas. Tracking changes in blood glucose levels in response to an oral glucose challenge is a common use for it [40]. During the glucose tolerance test, the crude MSNL extract showed a considerable hypoglycemic action ($p < 0.05$, $p < 0.01$; **Table 7**) in mice compared to the reference metformin, which lasted up to three hours. It is believed that secondary metabolites (tannin, flavonoids, phenol, sterol, etc.) enhance regulatory systems by an action akin to that of insulin, most likely by raising peripheral glucose consumption or cell glucose sensitivity [5, 40, 41]. We also validated the presence of hypoglycemic terpenoids, flavonoids, and tannins in our qualitative phytochemical screening of MSNL extract (**Table 1**). These compounds may work alone or in combination to lower blood glucose levels.

One of the most widely used methods for *in vivo* studies of the antidiarrheal properties of medicinal herbs is castor oil, which is used to cause diarrhea in animals. By promoting intestinal peristalsis and obstructing the absorption of fluids and electrolytes, castor oil causes diarrhea [5, 43]. Thus, the most crucial aspect of managing diarrhea is preventing castor oil-induced diarrhea. The crude extract of *N. lappaceum* L. seeds was administered, and the number of wet feces decreased, and the beginning of diarrhea was significantly ($p < 0.05$, $p < 0.01$) delayed. These results suggest that the extract had an antidiarrheal effect at the test dosages used. The ability of MSNL extract to enhance the gastrointestinal tract's absorption of fluids and electrolytes is one possible explanation for its anti-diarrheal properties. The antidiarrheal properties of crude extract (ME) and solvent fractions may be attributed to phytochemicals such as alkaloids, tannins, saponins, phenols, terpenoids, and flavonoids, per results from many investigations [5, 43, 44]. The reduction of total feces, including the wet and watery components, implies that an antisecretory mechanism may be involved in the antidiarrheal effect of the MSNL extract. Moreover, the examined extract's antidiarrheal properties may be explained by its inhibition of nitric oxide and platelet-activating factor synthesis [45, 46]. In all of the models used in this investigation, the MSNL demonstrated antidiarrheal action by lowering castor oil-induced diarrhea. The quantity and weight of wet and watery fecal matter were significantly reduced ($p < 0.05$, $p < 0.01$), and the onset of diarrhea was delayed. The most plausible explanation could be the presence of different phytochemicals in MSNL extract [47, 48]. For

instance, the antidiarrheal effect of flavonoids and phenolic compounds is probably due to their antioxidant characteristics [5, 43, 44, 45, 46, 47, 48, 49]. These phytochemicals may reduce the amount of fluid produced by castor oil by blocking enzymes or slowing the metabolism of arachidonic acid [48, 49, 50]. There have been prior reports of the anti-diarrheal effects of tannins and saponins. Furthermore, the results of this investigation are in line with previous studies on a variety of crude plant extracts that demonstrated dose-dependent antidiarrheal properties [5, 43, 44, 45, 46, 47, 48, 49, 50]. These might be explained by the fact that the test plants included a wide variety of phytochemicals.

5. Conclusions

Throughout human history, customary herbal remedies have been utilized to both prevent and cure a diverse array of ailments. Researchers are reportedly considering the development of plant-based medications as a major and demanding area of attention by considering the therapeutic benefits of herbs. The secondary bioactive metabolites found in large quantities in *N. lappaceum* L. seeds, including glycosides, alkaloids, tannin, flavonoids, terpenoids, resin, and others, have been shown to have a range of health benefits, including analgesic, anti-inflammatory, CNS depressant, hypoglycemic, and antidiarrheal properties. There is an instantaneous, widespread, and statistically significant effect at every experimental dosage that is investigated. Our research leads us to conclude that pharmaceutical companies may succeed in developing new, safer, more effective, and less toxic candidate drugs from the seeds of *N. lappaceum* L., hence reducing the cost of treating sickness. We will conduct an additional study to identify the bioactive compound(s) and comprehend the exact molecular mechanisms to develop a safe and effective dosage and confirm the likelihood of its usage in the prevention and treatment of various diseases.

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Review Article

A Comprehensive Review on *Tradescantia pallida:* Phytochemistry and Pharmacology

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Abstract: *Tradescantia pallida*, also known as Purple Queen, is a remarkable ornamental plant from the *Commelinaceae* family that boasts significant medicinal and ecological value. This review decisively underscores its phytochemical, pharmacological, traditional, and environmental applications, making it clear that consolidating existing knowledge and identifying areas for future research is essential. Extensive phytochemical studies have confirmed the presence of bioactive compounds like phenolics, flavonoids, and anthocyanins that drive its powerful antioxidant, antimicrobial, antifungal, and antidiabetic effects. It demonstrated cytotoxicity against tumor cells and effective larvicidal activity against *Aedes aegypti*, highlighting its essential roles in medical applications and pest control. In traditional medicine, *T. pallida* is well-recognized for its hepatoprotective, nephroprotective, and anti-inflammatory properties, which are fully supported by modern scientific findings. Moreover, its ability to synthesize therapeutic nanoparticles and produce natural dyes firmly establishes its significance in the field of green technology. Environmental studies robustly affirm its contribution to phytoremediation, air pollution biomonitoring, and soil detoxification, reinforcing its status as an eco-friendly solution. This review meticulously synthesizes information from esteemed databases such as PubMed, SciFinder, and Google Scholar, focusing on the most recent and impactful findings. By delving into its vast pharmacological activities and environmental applications, *T. pallida* emerges as a versatile resource poised for sustainable development. The plant's diverse properties present confident avenues for future research in medicine, industry, and ecological conservation.

Keywords: *Tradescantia pallida*, Phytochemical, Pharmacological properties

1.Introduction

Tradescantia pallida, commonly referred to as the purple queen, is a striking ornamental plant celebrated for its beneficial medicinal properties. It is often utilized for its anti-inflammatory and antitoxic effects, as well as to enhance blood circulation. Phytochemical analyses indicate that *T. pallida* is abundant in essential compounds

such as phenolics, flavonoids, tannins, alkaloids, and saponins. Additionally, it serves as a source of vibrant natural pigments, particularly anthocyanins[1]. *Tradescantia pallida* is a member of the family *Commelinaceae* which is also known as purple queen [2]. *T. pallida* grows annually in subtropical and tropical regions of Asian countries, including Pakistan, India, and Bangladesh [3]. The silver nanoparticles synthesized from the extract of *T. pallida* have antioxidant, antimicrobial, and antifungal activity. The volatile constituents of hexane extract are determined from *T. pallida* (HE-TP) aerial parts by gas chromatography spectrometry and gas chromatography-mass spectrometry flame ionization detection, which also shows antifungal activity. *T. pallida* also shows cytotoxic activity against tumor cells [4]. Many plants or parts of plants contain several activities. Availability of secondary metabolites, including flavonoids, in plants with antioxidant activity has been related to hepatoprotective effects [5]. Isoflavones, flavonoids, chalcones, and anthocyanins are among the common polyphenolic chemicals known as flavonoids; these latter compounds are found in particular species of *T. pallida* [6]. *Tradescantia pallida* plants are a rich source of bioinsecticide that causes cytotoxicity in *Aedes aegypti* (dengue mosquito), reducing the mosquito population.[7]. The use of *Tradescantia pallida* extract in the green synthesis of silver nanoparticles is possible. Due to the biological potency of *T. pallida* silver nanoparticles (TPAgNs), they are essential in assessing *T. pallida's* therapeutic potential [8]. *Tradescantia pallida* (Rose) D.R. Hunt offers a promising dual role as a natural alternative in aquaculture and a bio-monitor for air quality. The plant's bioactive compounds, such as phenolics and flavonoids, provide antimicrobial, antioxidant, and immune-boosting properties beneficial for aquaculture species [9]. Additionally, its phytoremediation capabilities enable it to absorb volatile organic compounds, making it an effective tool for improving indoor air quality and assessing environmental pollution through bioassays [10]. Synthesized nanoparticles provide antibacterial activity against gram-positive and gram-negative strains such as *E. coli*, *Staphylococcus aureus*, *Salmonella typhimurium*, and *Pseudomonas aeruginosa*, and for two antifungal strains, *Candida albicans* and *Aspergillus niger*. In this review, a scientific article from the last 5 years, including medicinal and evaluation of biological properties of *Tradescantia pallida* species through *in vitro* and *in vivo* models, is prioritized for subjection [11].

2. Materials and Methods

This literature review on *T. pallida* was compiled from literature from seven databases: SciFinder, PubChem, ScienceDirect, Scopus, PubMed, Google Scholar, and Web of Science. Articles included in the review were published in English before September 2024. Other records were discovered through other sources. Then, the duplicated records were expelled.

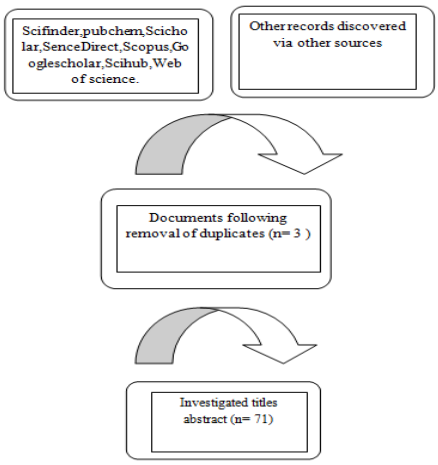


Figure 1: Flow chart of Data extraction

3. Botany [21]

Tradescantia pallida is a perennial herbaceous ornamental, a member of the *Commelinaceae* family. It is commonly distributed in tropical and subtropical regions, east of Mexico. The plant is known for having a strong pointed top, a deep royal purple color with a dusty green tinge, and bright purple leaves and pink to purple flowers in small dense cymose clusters [12]. Research on this species has traced the anatomical and physiological changes in the limbus under the action of light of different intensities. This species has a great capacity for adaptation, can colonize a wide range of environments, and grows very well in bright light and shaded places. The species is also known for the presence of calcium oxalate crystals in the parenchyma of all vegetative organs and flowers, in the form of raphides and tetragonal crystals [13].

3.1 Synonyms[13]

Preferred Scientific Name: *Tradescantia pallida* (Rose) D.R. Hunt, 1975; Preferred Common Name: purple queen; Other Scientific Names: *Setcreasea jaumavensis* Matuda, *Setcreasea lanceolata* Faruqi, Mehra & Celarier, *Setcreasea pallida* Rose, *Setcreasea purpurea* Boom, *Tradescantia purpurea* Boom

3.2 Scientific classification [13]

Domain: Eukaryota
Kingdom: Plantae
Phylum: Streptophyta
Subphylum: Angiospermae
Class: Monocotyledonae
Order: Commelinales
Family: Commelinaceae
Genus: Tradescantia
Species: *Tradescantia pallida*

Figure 2:(a) *Tradescantia pallida* (Purple Heart) (b) Flower

4. Traditional uses

Traditional medicine has been using medicinal herbs for ages because of their well-tolerated benefits, affordability, ease of use, and lack of severe adverse effects. There has been a surge in interest in medicinal plants worldwide in recent years. Because there aren't enough standards and authentication criteria to guarantee the quality and purity of herbal medications, questions have always been raised regarding their proper use. Furthermore, the absence of standards pertaining to the originality of drugs has made adulterations and substitutions in herbal remedies a major issue in recent years [12]. *Tradescantia pallida* is a perennial plant [15] widely distributed in the tropical and subtropical regions [12] of Pakistan, India, Bangladesh and Africa [16] Traditionally, *T. pallida* was thought to enhance blood circulation [12, 17, 18, 19], operate as an anti-inflammatory and anti-toxic supplement [20], and be an antioxidant [12, 16, 17, 18]. In Malaysia, the Ayta communities of Potrac, Pampanga, utilize this herb to heal injured eyes, mainly to prevent sore eyes [12, 16, 17, 20] and purified and cleaned airborne volatile organic compounds. *T. pallida* has been shown to be an effective option for *in situ* mutagenesis testing. Methanol extracts of the leaves show promising antioxidant, anticancer, and antibacterial [4, 12, 21] properties against both gram-positive and gram-negative bacteria [19, 22]. *T. pallida* mediated zinc oxide nanoparticles have been demonstrated to be efficacious against cervical cancer cell lines [17, 23, 12]. Malaysians recommend a decoction of the plant to improve kidney function [21], and it is thought to be beneficial in the treatment of venomous snakebites, leukorrhea, urinary tract infections [21], nephritis, and intestinal inflammation [3]. In various ethnic communities, *Tradescantia pallida* has been used as an antidiabetic ethnopharmacological fraction [16]. In terms of pharmacological benefits, the leaves of *T. pallida* have been used as a tincture and have acted as an anode against rheumatism and joint pain [4]. Anthocyanin from *T. pallida* is used as a natural food color, which may be potentially beneficial for heart patients and cancer prevention [19, 24, 25]. Chloroform extract of *T. pallida* has shown the most promising effect against *Labeo rohita* pathogens [19].

T. pallida is planted as a ground cover or as an ornamental plant [19, 26, 27] in hanging pots for its attractive foliage, traditionally at Maha Sarakham province in Thailand [28], and is highly adaptable, particularly to a shaded environment in Romania [23]. The people of this province believed that the therapeutic properties of *T. pallida* help to relieve dehydration and inflammatory syndrome. The recommended method of preparation is to boil the entire plant in water and then consume the resulting infusion to aid healing. This particular botanical specimen placed in a closed space, such as a room, and thus contributed to air purification and oxygen production at night [28] also renowned for effectively removing volatile organic pollutants from the air [18, 29] and this species is an excellent bioindicator of air pollution levels [4, 30]. *Tradescantia pallida* (Rose) D. R. Hunt var. purpurea Boom is a suitable alternative for genotoxicity testing of air pollutants [31] as it is well adapted and widely cultivated in tropical and subtropical regions [32].

5. Pharmacological activities

5.1 Antidiabetic Activity

The study indicates that niosomes loaded with the extract have a better antidiabetic effect than the crude extract and the standard drug. Chloroform extract of leaves of *T. pallida* helps to prepare phytoniosome, which is remarkable for its effect against alloxan-induced diabetes at a dose of 50 mg/kg. Study also indicates that *in vitro* models like alpha-amylase inhibition assay and non-enzymatic glycosylation of hemoglobin assay help to determine the antidiabetic activities of pure extract and niosomal formulation in the extract [3]. The antidiabetic compounds from *Tradescantia pallida* leaves were isolated using column chromatography. Syringic acid, p-coumaric acid, morin, and catechin isolated from the leaves of *T. pallida* have antidiabetic activity. Alpha-amylase and non-enzymatic glycosylation of hemoglobin protein assays were used to assess the *in vitro* antidiabetic potential of the phenolic compounds [34].

5.2 Antibacterial activity

This study determines the silver nanoparticles by using the aqueous extract of *T. pallida*, which is remarkable for its antibacterial activity. Silver nanoparticles are non-toxic, highly effective against bacteria, and are used as antibiotics [35]. Methanolic extract of *Tradescantia pallida* leaf appeared to have antibacterial activity against methicillin-resistant *Staphylococcus aureus, Bacillus subtilis, Enterococcus faecalis, Micrococcus luteus*, and *Staphylococcus epidermidis* [23]. *Tradescantia pallida* has antibacterial activities against gram-positive and *Staphylococcus* bacteria. *Pseudomonas aeruginosa* is a gram-negative bacterium that can form biofilm. The aqueous extract of *T. pallida* is used to prevent bacterial growth and the formation of biofilm [22]. The MIC for *Pseudomonas aeruginosa* is 64 microgram/ml [1]. Furthermore, the bacterial growth can be inhibited by using polyphenol-rich *T. pallida* extracts [38]. The 10 microgram/ml chloroform extract of *T. pallida* shows antibacterial activity against fish pathogens using well diffusion and disc diffusion assay [10].

5.3 Antioxidant activity

This study shows that the silver nanoparticles were biosynthesized from the $AgNO_3$ precursors using the *T. pallida* aqueous extract. AgNPs inhibited the DPPH free radical scavenging activity with an IC_{50}= 91.87 µg/ml. 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) free radical scavenging activity and reducing power assay are performed [1, 36]. *T. pallida* leaf extract shows the highest antioxidant activity for the ethyl acetate fraction (IC_{50}= 14.55±016µg/mL and Abs= 0.613 at 300µg). This Study also investigates that *Tradescantia pallida* methanol extract contains abundant antioxidants and can protect human dermal fibroblasts at the maximum dose of 50µg/ml [40].

5.4 Cytotoxic activity

Hexane extract from *T. pallida* shows cytotoxic activity against tumor cell lines [2]. This study used various types of human cells to determine the cytotoxicity of samples, including non-tumoral fibroblasts and cervical adenocarcinoma (HeLa). The evaluation of cytotoxicity was carried out by the colorimetric assay of the toxicology *in vitro* Kit XTT [1]. Anticancer activity of *T. pallida* leaf extract is also found. The synthetic nanoparticles based on *T. pallida* show cytotoxic activity in HeLa cervical cancer cells at a dose of 1000mg/mL. *T. pallida* extract exhibited good cytotoxicity with an IC_{50} value of 90.59 ± 1.6 µg/mL and a cell viability % of 27.4 ± 1.05 [23].

5.5 Antifungal activity

This study shows the antifungal activity of the Hexane extract from *T. pallida*. Hexane extract shows antifungal activity in *Penicillium digitatum* and *Sclerotinia sclerotiorum* at a dose of 400 µL. It also inhibits 92.6%. Growth in *Rhizopus stolonifer* at the dose of 400 µL [4]. The aqueous natural dye extracted from the leaves of *Tradescantia pallida*. Natural dye is extracted with a concentration range of 0.025-0.85g/ml. Natural dye, such as anthocyanin, is extracted from the leaves of *T. pallida*, which acts as a fungicide probe in *Fusarium solani, Sclerotinia sclerotiorum,* and *Colletotrichum gloeosporioides* fungi [38]. Nanoparticles are synthesized from the leaf of *T. pallida*, which are also used to check the antifungal activity against *Candida albicans* and *Aspergillus niger* [8].

5.6 Hepatoprotective activity

Tradescantia pallida has a high antioxidant content. Research has been performed on its dynamism, analyzing its histopathology, functional, and biochemical parameters to evaluate the hepatoprotective outcome of a *Tradescantia pallida* Ethanol Extract (TPEE). The researcher conducted the study assessing the efficacy of TPEE (50 mg/kg) in chronic induced hepatotoxicity in the Wistar rat model. TPEE administration decreased aspartate aminotransferase (AST), alanine transaminase (ALT), albumin, and alkaline phosphatase (ALP) levels.

The leading characteristic of CCl_4 propensity is fibrogenesis, but transaminase proven beneficial through functional and biochemical observations verified by histopathology analysis. TPEE increases genes involved in antifibrotic and antioxidant actions. These judgments note that TPEE has superior hepatoprotective characteristics equivalent to silymarin, a conventional hepatoprotective chemical [5].

5.7 Larvicidal activity

Recent research has revealed that *Tradescantia pallida* showed larvicidal activity against *Aedes aegypti* larvae [5]. Betim et al.(2019) provided the methodology that was modified for the quantitative degree of larvicidal activity. To assess extract activities, *A. aegypti* eggs were incubated in a BOD oven at 25 + 3 °C and 80 percent relative humidity. The larvicidal activities of Ethyl Acetate Extract (EAE) and Ethanolic Extract (EE) were evaluated at concentrations ranging from 10, 100, and 1000 micrograms per liter. *Tradescantia pallida* is considered as a bioinsecticide [7].

6. Other activities

Dye: This study isolates the anthocyanin from the aqueous extract of *Tradescantia pallida*. Anthocyanin is used as food colorants. Anthocyanin is also used to prevent coronary heart disease and cancer. The purity and identity of the isolated anthocyanin were determined by HPLC using diode array detection. *T. pallida* gives pH-dependent color. It is red at pH three and yellow at pH 8 [25, 38, 39]. *Tradescantia pallida* contains two major anthocyanins, one of which is cyanidin-3,7,3'-triglucoside with three molecules of ferulic acid and an extra terminal glucose unit. The other is similar but without the terminal glucose unit. For these two anthocyanins, *T. pallida* is used as a food colorant [24].

6.1 Multidrug resistance of T. pallida in the human body

According to a recent study, *T. pallida* has an antibiotic effect. Its root and stem extracts have antibacterial properties against vancomycin-resistant Enterococcus. It is isolated and identified that endophytic fungi from *T. pallida* leaves and identify their antagonistic effects on multidrug-resistant human pathogens [19]. It is also determined that *T. pallida* purpurea in chloroform extract inhibits the growth of fish pathogens [10]. Aqueous *T. pallida* extracts inhibit bacterial growth [19].

6.2 Pesticidal properties

In an investigation, researchers found that the *Tradescantia pallida* aqueous extract obtained through the infusion method (ETPI) yielded the most favorable outcomes, particularly in the context of overall insect development specially the mature period, dropping the number of individuals in the following generation, and so minimizing harm in the various *Brassicaceae* cultures grown organically [49]. The biological landmarks of insects, including mortality [40, 41, 42], deviations in food [43, 44] or oviposition [44, 45], favoritism, malformations, and morphological and physiological metamorphosis of *P. xylostella* [15, 41, 46, 47], are switched according to studies involving *T. pallida* plant extracts with insecticidal properties.

7. Environmental Effect

7.1 Effect on soil

The contaminants are deposited on the soil periphery when the soil becomes accumulated with organic materials, trace elements, and hazardous metals. Many metals, including iron, chromium, zinc, cadmium, arsenic, mercury, and copper, are known to degrade soil quality seriously and negatively impact human health and the health of other creatures that come into contact with them [48]. Heavy metals persist in the environment and may not decompose through chemical oxidation due to their non-biodegradability [49]. A collection of ecological

techniques known as phytoremediation uses plants to encourage the breakdown and immobilization of contaminants in order to remove them from the environment *in situ*. Through phytoextraction, which assembles pollutants like heavy metals from the environment into plant tissues, plants can directly influence the amounts of contaminants [50, 51]. With minimal impact on the surrounding environment, phytoremediation is an affordable remediation method for eliminating pollutants from polluted soils and streams at the site level, primarily heavy metals and organic compounds. Additionally, it lowers the price of disposing of hazardous waste in an off-site storage facility or landfill [52]. *T. pallida* is well known for its capacity to efficiently remove airborne volatile organic pollutants [53]. *T. pallida* can clean the air and inedible plants, develop quickly, and coexist in all seasons. The plants that can perform the phytoremediation process employ several processes, including: 1) tolerance mechanisms based on their capacity to withstand elevated concentrations of heavy metals that generally hinder or kill plants. 2) Detoxification mechanisms through respiration and photosynthesis contribute to preserving low levels of heavy metals in the cytoplasm, potentially serving as a detoxifying mechanism [54]. Current finding shows that different fertilizers have demonstrated varying capacities to remove distinct heavy metals from polluted soil when used to cultivate the violet plant *T. pallida*. The investigated soil's nickel (Ni) concentration was higher than the highest amount allowed by the WHO. When commercial fertilizer was applied, followed by cow dung and bird manure treatments, *T. pallida* demonstrated a greater capacity to extract nickel from the contaminated soil [55].

7.2 Effect on air

T. pallida is an effective bioindicator for air pollution biomonitoring in urban environments. As confirmed by neutron activation analysis, its leaves accumulated significant levels of trace elements like Ba, Cr, Fe, and Sb [56]. *T. pallida* has an effect on micronucleus frequency from vehicular traffic, which has an environmental effect and causes cardiovascular disease [57]. As it is a bioindicator Species, it is used in monitoring atmospheric pollution in forensic investigations [58]. *T. pallida* is used to detect and measure DNA damage by air pollution, specifically in vehicular traffic areas, to identify the genetic damage of the plant [59]. Pollution is detected by instrumental neutron activation analysis (INAA), which measures pollutants and is also seen as a cost-effective air quality monitoring in various regions [56]. There is a computer vision model for analyzing *T. clone* 4430 stamen hair cells sensitive to environmental stressors like air pollution [60]. *T. pallida* has an effect on micronucleus frequency from vehicular traffic, which has an environmental effect and causes cardiovascular disease [61]. Due to its properties, *T. pallida* can be an essential tool for forensic experiments to identify various environmental crimes [62].

7.3 Effect on water

T. pallida var. purpurea cuttings with flower buds are utilized in bioassays to diagnose genotoxic effects of water [63]. Biomimetic superhydrophobic materials are innovative solutions that mimic nature's ability to repel water, inspired by structures like those of lotus leaves that cause droplets to bead and roll off. In water harvesting, these materials enhance moisture capture from air and rain by preventing water from sticking to surfaces, allowing it to flow efficiently into storage systems. This approach maximizes water collection and minimizes waste [64]. *Tradescantia pallida* is a plant that grows well in dry areas. It captures moisture from the air using its special leaves, which have tiny hairs and grooves. This unique ability has inspired scientists to create surfaces that mimic these features for smart water-harvesting systems. Using these designs from nature, we can develop new technologies that help solve water scarcity and improve access to water in areas affected by drought [65]. Researchers found a crucial link between flooding intercellular spaces and stomatal closure in *Tradescantia pallida (T. pallida)*. Injecting water into these spaces caused rapid stomatal closure not only in *T. pallida* but also in other plant species. This response is primarily due to the dilution of potassium ions in guard cells, which are essential for stomatal regulation. A vapor-phase signal from the mesophyll may also play a role, highlighting the complexity of plant responses to excess water. This research underlines the need to understand plant physiology in managing water-related challenges [66]. Degradation of water quality at all sites, with exposure to effluents causing genotoxic effects in *Tradescantia pallida* [67]. These findings underscore the

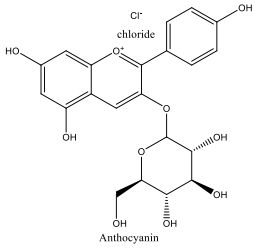
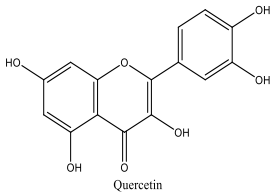
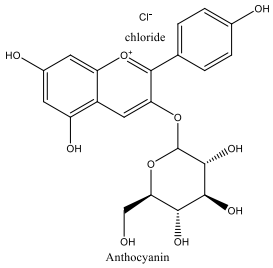
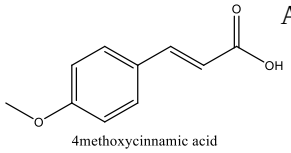
sensitivity of the Trad-MCN bioassay and its vital role in water quality monitoring, recommending its integration with standard physicochemical analyses for comprehensive evaluations [68].

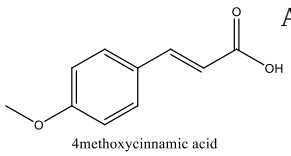
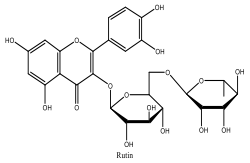
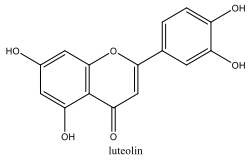
Table 1: Pharmacological activities *of Tradescantia pallida.*

SL. no.	Pharmacological Activity	Extract	Doses checked	Experimental Methods/ Models	Ref.
1	Antifungal	Hexane	400 µl	Disc-diffusion method in fungi of *P. digitatum, S. sclerotiorum, R. stolonifer*	[2]
2	Cytotoxic	Hexane	31.25-1000 µg/ml	Colorimetric assay in a human tumor cell line.	[2]
3	Antidiabetic	Chloroform	50 mg/kg	Alloxan induced diabetes in mice.	[3]
4	Antibacterial	AgNPs using aqueous extract	25-100 µg/ml	FTIr method, both gram-positive and gram-negative bacteria.	[4]
5	Antioxidant	AgNPs using aqueous extract	50 µg/ml	DPPH method in *P. aeruginosa.*	[1, 4]
6	Hepatoprotective activity	Ethanol	50 mg\kg	CCl_4 induced liver damage in Wistar rats	[5]
7	Larvicidal activity	Ethyl Acetate, Ethanol	10, 100, 1000 µg/L	Docking study in *Aedes aegypti* larvae	[5, 6]
8	Cytotoxic Activity	Ethyl acetate, ethanol	31.25-1000 (µg/L) concentration	*In vitro* cytotoxic activity to control the dengue mosquito	[6]
9	Antibacterial	Methanolic leaf extract	0.02-10 mg\ml	Antioxidant Content, Antioxidant Activity, and Antibacterial Activity of Five plants from the *Commelinaceae* family, 8 species of Gram-positive (Methicillin-Resistant *Staphylococcus aureus, Proteus vulgaris, Bacillus cereus, Aeromonas hydrophila, Bacillus subtilis, Enterococcus fascalis, Micrococcus luteus, Staphylococcus epidermis.*	[7]

10	Antifungal activity	*Tradescantia pallida* plant extract Biosynthesized *Tradescantia pallida* silver nanoparticles	40 µL, 5mg/ml	Agar well diffusion method in fungi	[1]

Table 2: Compounds isolated from *Tradescantia pallida*

SL. No.	Purified compounds	Parts of the plant	Model used	Doses checked	Pharmacologi--cal activity	Conclusion	Ref.
1	 Anthocyanin	Leave		0.025 - 0.85 g/ml	Fungicidal	Active against the tested strain	[8]
2	 Quercetin	Whole plant	*In vitro*	10, 100, 1000 µg/L	larvicidal	active against the tested strain	[5]
3	 Anthocyanin	Whole plant	*In vitro*	2.99 mg/ml	antioxidant	*In vitro* studies have shown that anthocyanin possess antioxidant activity.	[69]
4	 4methoxycinnamic acid	Aerial part	*In vitro*	400 µL	Antifungal activity	active against the tested strain	[10]

5	 4methoxycinnamic acid	Aerial part	*In vitro*	31.25-1000 µg/mL	Cytotoxic activity	Active against the tested strain	[42]
6	 Rutin	Whole plant	*In vivo*		Insecticidal	Active against *P. xylostella* larvae	[69, 71]
7	 luteolin	Whole plant	*In vivo*		Insecticidal	Active against *P. xylostella* larvae	[17]

8. Phytochemistry

Tradescantia pallida contains phenolic compounds. The chloroform extract of *T. pallida* leaves is rich in polyphenols, and the amounts of flavonoids and polysaccharides are lower. *T. Pallida* extract also contains tannins, alkaloids, and saponins [33]. These compounds of *T. pallida* extract reveal a great degree of effectiveness in antioxidant, antibacterial, antitumor, cytotoxic, and *in vivo* analgesic activities. The silver nanoparticles from the *T. pallida* have antibacterial and antioxidant activity [4]. Silver nanoparticles are obtained by using the aqueous extract of *T. pallida.* The colorimetric method can determine total flavonoid content, and quercetin can be used as a reference for determining the flavonoid [33]. The phenolic content of *T. pallida* extract can be determined by the Folin-Ciocalteu method. *T. pallida* extract is also a rich source of anthocyanins, which are naturally colored compounds. They are obtained by a pH-differential method [16].

9. Conclusion

Tradescantia pallida, or the Purple Queen, is remarkable for its aesthetic appeal and significant applications in medicine, ecology, and industry. This plant is rich in bioactive compounds that provide potent antioxidant, antimicrobial, and antidiabetic benefits, reinforcing its traditional medicinal use. Moreover, *Tradescantia pallida* plays a vital role in phytoremediation and enhances indoor air quality by filtering pollutants, highlighting its importance in sustainable practices. To fully realize the potential of *Tradescantia pallida*, focused research is essential. Bridging traditional knowledge with modern science will drive innovative applications in healthcare, environmental management, and industry, maximizing the benefits of this extraordinary plant.

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Review Article

Unused and Expired Medicines Disposal Practices in Asian Countries

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Abstract: Inappropriate disposal of medicines has an alarming impact on our environment. Unused and expired pharmaceuticals contaminate the soil and water that end up entering our food chain. Not only this, they pollute the air as well. The active ingredients diffuse in the human body and not only cause many diseases but also play a pivotal role in developing antibiotic resistance. These unused drugs are thrown into the trash, flushed down the toilet, poured into the sink, burned, or buried improperly. Proper disposal of these medications is a major challenge since many serious diseases continue to rise as medication consumption increases in Asian countries. This review writing integrates results from various research carried out between January 2010 and January 2025 across Asian countries, focusing on 23,825 samples. Household trash, followed by flushing, was the most common mode of disposal, with limited rates of returns to pharmacies. Some of the common trends include awareness, home storage, and poor disposal practices, which are primarily affected by the dosage forms of medication. Effects include pollution of the environment, increased cases of antibiotic resistance, and increased cases of diseases. Interventions mentioned as successful include learning, legal initiatives, environmentally friendly disposal solutions, and medication return campaigns. Stakeholders, including pharmacists, have a crucial role in the implementation of safe and effective disposal measures and overall proper disposal of pharmaceutical waste streams. This review study, therefore, calls for increased willingness and commitment in these regions to prevent further deterioration of health and the environment by taking adequate measures to dispose of medication properly.

Keywords: Medicines disposal, pharmaceutical waste, Asian countries, environment, pharmacist

1. Introduction

Unused medicines are those that are unwanted or discontinued by the patient but remain with them. Expired medicines are those that have lost their potency and stability**.** Non-adherence to the prescription, drug adjustment, and lack of sincerity, along with a sudden change of drug, result in the storage of expired and unwanted medicines. As the majority of the people do not know about the proper disposing system of these drugs, they simply dump them in the environment. This practice is very unsafe for both humans and the atmosphere. Some even donate them, which increases the risk of adverse or no therapeutic effects. The improper disposal of unused, unwanted, and expired medicines is a major environmental and public health threat. Contamination of water sources and the accidental swallowing of medicines by fish, children, and pets have long-term adverse effects. Again, contaminated soil and air have serious consequences. Considering the dangers associated with the above, awareness about the proper disposal measures and proper medication disposal programs is very important. Like many other South and South Asian countries, Bangladesh lacks explicit government policies and procedures for the disposal of unwanted and outdated pharmaceutical products [1]. As a result, many people choose hazardous methods to dispose of unwanted medications, such as dumping them into the trash, sink, or toilet without considering the consequences of doing so. This kind of disposal must be strictly prohibited because it endangers people's safety and the environment [2]. For example, the improper disposal of unwanted tetracyclines has been associated with renal tubular damage**,** as per a report originating from Pakistan, there has been a notable decline in the vulture population, primarily attributed to the consumption of cattle feed consisting of diclofenac. Another critical repercussion of improper medication disposal is that it increases the risk of unintentional poisoning and abuse, particularly among young children and adolescents. Additionally, a recent hypothesis suggests that the haphazard disposal of antimicrobials could potentially play a role in fostering antimicrobial resistance development effects [3]. Again, contaminated soil and air have serious consequences.

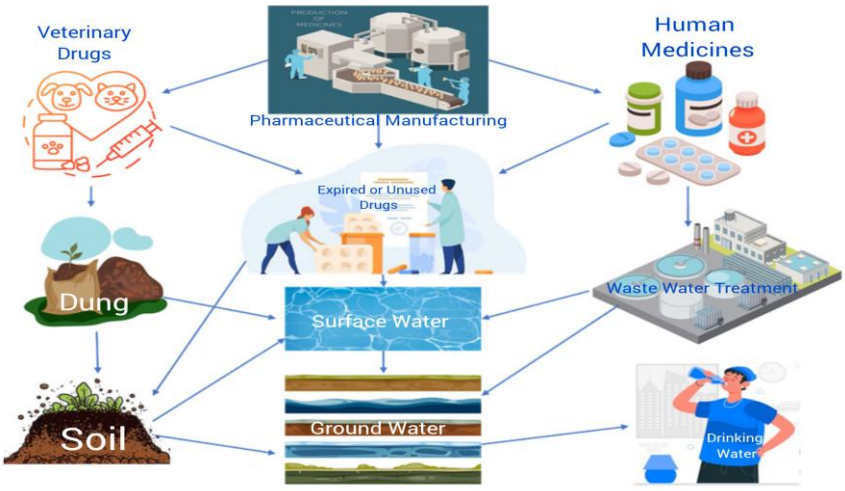

Figure 1: Consequences of Improper Medicines' Disposal Practices

In the South-Asian region, prompt actions are needed to be taken to mitigate the issues as early as possible. Addressing this problem will raise the level of living for the general population and aid in the global fight against drug desensitization, including antibiotic resistance. The pharmacists play a crucial role regarding this situation. Community pharmacists can successfully reduce the damaging consequences of medicine disposal by properly disposing of the medications they collect [4].

2. Method

A secondary data collection method was used. To meet the study's purpose, goal, and rationale, information was gathered from various search engines, including PubMed, Elsevier, Nature, and Springer Link, in addition to other suitable and relevant publications and guidelines. The relevant articles were found by searching "drug disposal", "unwanted medications", "expired medications", "disposal practice in South Asia", "waste disposal in Asia", "waste disposal", "pharmaceutical waste disposal", and many more. Various health-related websites were evaluated, research instruments such as structured interviews and questionnaires from research articles were analyzed, and articles were narrowed by focusing on specific topics. The collected data was carefully arranged and cited to provide a comprehensive picture of the Medicine disposal practices in South Asian nations as well as a few other nearby countries. The material was gathered from a majority of recent articles that contain current information. A search for appropriate references was also conducted using systematic reviews, and reference lists were examined for additional pertinent research.

2.1 Inclusion Criteria

The included studies (Jan 2010- Jan 2025) focused on questionnaire-based surveys that helped to explore how the general population of South Asia and Asia dispose of their pharmaceutical wastes. Also, it includes their attitude, knowledge, and awareness regarding improper disposal methods with percentages.

2.2 Exclusion Criteria

The articles that do not focus on discarding practices or disposal methods were excluded. Articles written in languages other than English were avoided. Writings that did not have percentages or enough data were excluded. Older data before 2010 were not included. Among 82 articles, 61 articles were found eligible to be included in this chapter. These 61 articles have answers and opinions from households, students, pharmacists, doctors, nurses, and the general public.

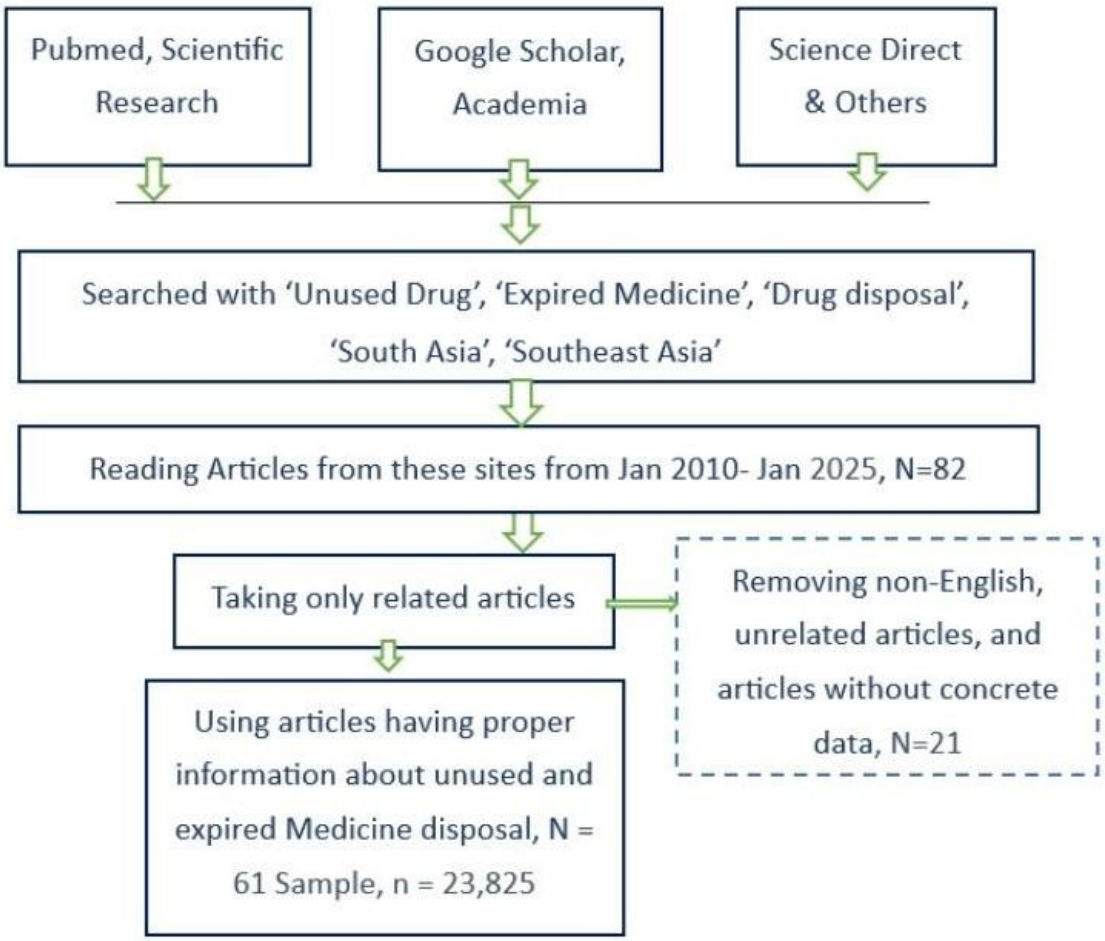

Figure 2: Flowchart of Method

3. Study outcomes

To know about the disposal practice among households and healthcare professionals, many studies have been done in several countries. In this section, the disposal practices, attitudes, methods, awareness, and knowledge of people of South Asia and Asia are discussed to decrease the detrimental effects on the environment and human health of inappropriately discarding unused and expired medications.

Table 1: Medicines' Disposal Practice in Different South Asian Countries

Region	Study Design & Sample Size (n)	Findings	References
Bangladesh	Cross-sectional study, structured questionnaires n=250	Here, the majority of students had very little knowledge and awareness about the disposal system of unused drugs.	[2]
Afghanistan	In-person interviews with pre-validated structured questionnaire n=301	Numerous respondents felt that government officials were responsible for educating people regarding how to properly dispose pharmaceuticals. 77.7% of those surveyed dispose of all their leftover medications in the household garbage bin.	[3]
India	Cross-sectional, questionnaires n=385	Some were aware, but mostly unaware. Dumping in the garbage was the most prevalent form of disposal. 82.1% of participants dispose of the unused medicines in trash cans, and only 2.6% return these medicines to pharmacies.	[5]
India	Cross-sectional, interviewer-administered questionnaire n=555	87% of the participants have unused medication in their houses and less than 46% of the respondents know about the denotation of expiry date.	[6]
India	Cross-sectional study, questionnaires n=118	A large proportion (63.5%) of the drugs stocked were topical formulations. 73% of the participants stated that they planned to get rid of excess medications in their home's dustbin.	[7]

India	Cross-sectional, interviewer-administered questionnaire n=200	39% of the respondents know proper disposal methods. 76% of the participants believe that there is a need for some facilities to collect waste medications.	[8]
India	Cross-sectional questionnaire-based study n=400	According to roughly 17% of participants, smash the pills before throwing them in the garbage. Only 5% return them to the pharmacy.	[9]
India	Questionnaires based on a cross-sectional study n=120	The two most popular approaches are flushing and throwing medications in the garbage. 66% of thought returning to the pharmacy was best.	[10]
India	Questionnaire-based cross-sectional study n=956	The maximum consumers kept their medications at home (87%). After keeping the expired medications for a few days, 92.6% of the customers dumped them elsewhere.	[11]
India (Kerala)	Cross-sectional survey, Interview n=30	66.66% were unaware of the proper elimination of out-of-date and unusable medicines. The participants who said they knew, actually know of the packaging disposal.	[12]
India	Cross-sectional Study n=372	The participants mostly throw their out-of-date and unusable medicines into the dustbin. They do not have adequate knowledge about proper disposal.	[13]
South-India	The questionnaires, in-person, semi-structured interview n=480	The majority of participants (45.97%) threw away unwanted medications in the garbage, and 83.3% were unaware of the harmful	[14]

		consequences drug abandonment has on the environment.	
North-India	The questions that are both open-ended and closed-ended n=236	94% of the population threw the unused drugs into trash cans, and most of the participants claimed that they were unaware of the environmental issues.	[15]
India	Cross-sectional study, interview n=56	The impact of unused drugs was unknown to 55% of individuals. 57.1% of the participants throw them into the garbage.	[16]
India	Cross-sectional study, interview n=150	Not-used medicines are thrown into the dustbin by 62% of those interviewed, and into the sink or toilet by 18%.	[17]
India (West Bengal)	Cross-sectional study n=143	67.1% of the responders had unused drugs in their homes. Discontinuation after feeling better and the potential for future use were the primary reasons for keeping leftover medicines in storage.	[18]
North India	Cross-sectional, structured questionnaires based n=84	Approximately 90% of the individuals surveyed kept some medicines at their homes, and the most typical way to get rid of them is by throwing them in the garbage.	[19]
India	Pre-cross-sectional survey n=150	84.62% of respondents store medicines in their rooms. 23.33% of students were ignorant about the expiry date of the medications.	[20]
South India	Face-to-face semi-structured interview n=127	63.9% were unaware of the environmental hazard	[21]
India (BG Nagar)	Descriptive cross-sectional questionnaire	91.41%, and 73.44% of respondents throw solid and liquid dosage forms	[22]

	n=128	in the garbage, respectively. Also, 1.56% of participants throw solid dosages in the sink and 3.91% in the toilet.	
India	Cross-sectional observational questionnaire-based study n=220	55.9% of those surveyed were unaware of the detrimental impacts of inappropriate medication elimination. The most popular way is to throw them in the trash.	[23]
Pakistan	Cross-sectional, questionnaire-based study n=676	Mostly unaware of safe disposal. 60.56% of female participants throw them in the dustbin, and 48.72% of male responders bury these medications on the ground.	[24]
Pakistan	Closed-end Question n=1022	Approximately 80% of those surveyed kept unneeded medicines where they live, whereas 20% utilized all the medicine they bought.	[25]
Pakistan	Cross-sectional survey n=830	27% of respondents kept medicine after expiration, and 88% of participants did not have knowledge about proper disposal methods. 58.1% throw these in the dustbin, and 14.5 % give them to relatives and friends.	[26]
Bangladesh	Cross-sectional study, questionnaires n=200	Mostly unaware of safe disposal. But more than 15% know and practice proper methods.	[27]
Bangladesh	Cross-sectional study, structured questionnaires, n=400	21% of those questioned returned expired prescribed drugs to the pharmacy requesting repayment, 19% put them through the window, and 47% threw them in the garbage without any additional action.	[28]

Bangladesh	Cross-sectional survey, questionnaires n=310	73% of the participants throw solid waste medications in the dustbin. 58% of the participants pour the liquid dosages into the sink or toilet.	[29]
Nepal	Cross-sectional, face-to-face interview n=210	65.2% of respondents did not know about proper unused medicine disposal. However, 59.5% of the participants check expiry dates of medicines.	[30]
Nepal	A web-based cross-sectional descriptive study n=294	Healthcare professionals tended to keep medicines at home. Their way of disposal was not appropriate.	[31]
Western Nepal (Pokhara city)	Cross-sectional, semi-structured questionnaires n=54	They lack awareness about the impact of improper disposal practices. 52.36% disposed of in municipal dump.	[32]
Myanmar	Cross-sectional questionnaire-based study n=183 healthcare institutions	They are knowledgeable, and most of the facilities maintain proper disposal. Only 0.55% of the participants throw these medicines into the dustbins. The use of incineration and burial pits is commonly practiced.	[33]
Sri Lanka	Face-to-face interview n=200	78% of participants had unused medicines in their homes. 57.5% said they had unused medicine at home because of self-discontinuation.	[34]
Sri Lanka	Cross-sectional study, n=40 pharmacies	25% of participants burn, and 22% throw the solid dosages in the landfill. For liquid dosages, 17% of participants burn them, and 28% throw the liquid dosages in the landfill.	[35]

Bangladesh	Questionnaire-based cross-sectional study n=150	Not more than 84 respondents threw expired medicines into the dustbin, and 13 of them returned them to the pharmacy.	[36]

Table 2: Medicines' Disposal Practice in Other Asian Countries

Region	Study Design Sample Size (n)	Findings	References
Malaysia	Cross-sectional study n=426	Over 80% of those who took the survey said they had knowledge about the mounting problems that affect both our surroundings and human well-being.	[37]
Malaysia	Cross-sectional study n=244	47.8% of the respondents throw their unused medication in the garbage. 26% user return their unused medicines to the pharmacies.	[38]
Malaysia (Rawang)	Cross-sectional, questionnaires (close ended questions) n=384	About 69.5% of the people surveyed had no idea what medication waste was, and most of them (74.3%) dumped them into trash cans.	[39]
Malaysia	Cross-sectional, structured questionnaires n=885	Merely 2% of the population utilized the drug take-back program, despite 87% of them being knowledgeable of drug waste. Furthermore, 83% of those interviewed fill landfills with unnecessary medications.	[40]
Malaysia	Cross-sectional study n=481	Among the 481 participants, 93.1% of participants store medication at home. Mostly educated female students practice this.	[41]
Malaysia	Cross-sectional study n=319	54% of the respondents throw the solid dosage forms in the dustbin. 46.4% of the respondent pour their unused liquid dosages into the sink.	[42]
Malaysia	Cross-sectional survey n=1184	84% of the respondents own unused medication. 21% of participants throw them in the household trash can.	[43]

Region	Study Design Sample Size (n)	Findings	References
Malaysia	Cross-sectional survey n=483	37% of participants throw the waste in the municipal hazardous waste collection.	[44]
Malaysia	Cross-sectional survey n=103	Of the people who took part, 25.2% returned the drugs to the pharmacies.	[45]
Malaysia	Cross-sectional survey n=1067	62% of those who were surveyed said unwanted liquid dosages are thrown in the sink and toilet. Only 6% of respondents return these medications to the pharmacy. 65% of participants' unused solid dosage forms end up in landfills.	[46]
Malaysia	Cross-sectional Survey n=200	For them, dumping drugs that are expired in the garbage is the most popular method of elimination. 60.6% of participants throw them in rubbish bins.	[47]
Indonesia	Cross-sectional study n=324	85% reported they store any unneeded medicines at home, and only 3% of participants return to pharmacies. 17.3% drain them into the toilet.	[48]
Indonesia	Cross-sectional study n=497	About 82% of the participating population discarded their unwanted drugs by throwing them in the trash cans. The percentage of respondents who returned the medicines to the drug store was just 0.2%.	[49]
Indonesia	Cross-sectional studies n=322	49.4% of the participants throw unused medicine in household garbage. 8.2% of the respondents drain them in the toilet, and 3.7% burn the medications.	[50]
Indonesia	Cross-sectional questionnaires-based survey n=497	82% of the respondents usually throw unused drugs in the trash. 5% of participants used the toilet, and 4% used other methods. Only 0.2% of the medications are returned to pharmacies.	[51]

Region	Study Design Sample Size (n)	Findings	References
Thailand	Cross-sectional, Structured interview n=331	A large number of them (89%) stated that they received no instruction on how to discard drugs properly. 73% were in favor of the "Take-back" initiative. Liquid doses were thrown into the drainage system by 7.4% of individuals.	[52]
Vietnam	Cross-sectional questionnaire-based Study n=525	83% of the respondents throw unused medicines in dustbins, and 24% use other methods of disposal. Only 5% of participants return these drugs to the pharmacy.	[53]
China	Cross-sectional Study n=613	71.6% of the respondents disposed of them in trash cans and sinks. 8.3% of the people return them to the collection point.	[54]
Saudi Arabia	Questionnaire-based Study n= 1105	79.5% of the respondents dispose of their unused medicine in the trash bin. Only 4% of the responders return them to the pharmacy.	[55]
Japan (Kobe)	Cross-sectional Study n=75	82.7% of participants throw the containers in the trash.	[56]
Lebanon	Cross-sectional Study n=450	78.9% of the participants discard the expired drugs into household garbage.	[57]
Iraq	Cross-sectional Study n=591	Approximately 70% of the respondents threw medicines in household garbage.	[58]
Qatar	Cross-sectional Study n=410	76% of the respondents threw medicines into their trash cans, and 79% of them kept medicines at home for future use.	[59]
Saudi Arab	Questionnaire-based cross-sectional study n=503	Almost half of the people (58%) kept expired medication in their homes. 86% of the respondents threw the expired drugs in the garbage.	[60]
UAE	Questionnaire-based cross-sectional study n=219	41% of people throw expired drugs in the trash, and very few flush them down the toilet. 101 people returned them to the pharmacy	[61]

Region	Study Design Sample Size (n)	Findings	References
Philippines	Questionnaire-based cross-sectional study n=709	11% of the respondents returned expired drugs to the Pharmacy, and 42.5% placed them in a resealable plastic bag.	[62]
Saudi Arab	Questionnaire-based cross-sectional study n=820	Seven hundred ninety-eight threw expired drugs in the garbage, and 595 threw unused but not expired medications into the trash.	[63]

In a cross-sectional questionnaire-based study conducted in India, where 385 participants took part. Among them, a vast number of respondents had leftover medicines at their houses. The leading cause is that they stop using medication after feeling better from the illness. Just 2.6% of the people surveyed brought their medicines back to pharmacy stores, while 82.1% discarded them in the trash [5]. A study was also conducted in India, where 956 respondents were included. 73% of the people surveyed threw the leftover medications in the trash can, and 20% of those individuals disposed of the drugs in sinks or toilets [11].

A questionnaire-based study was conducted in Pakistan, where 1022 respondents mentioned that they had leftover medications at home. They stock the medicines after they stop using medication after feeling better or the doctor changes the medicines. 83% of the answerers threw drugs in the dustbin, and only 2% returned them to pharmacies; 12% of them poured them down the toilet [25]. Another study in Pakistan included 676 participants. Here, 60.52% of the female participants threw the leftover medicines in the trash can, and 48.72% of the male participants disposed of the drugs by burying them [24].

A study was conducted in Bangladesh, where 200 participants took part. Among them, the majority had leftover drugs at home. 47% of the respondents threw drugs in the dustbin, 8% disposed of these drugs into a sink or toilet, 29% used other methods, and 16% returned them to pharmacies [26]. In another study in Bangladesh, including 400 samples, it was observed that 47% threw them in the garbage, 2% of the respondents burned them, and 21% of respondents returned their leftover and outdated prescription drugs to pharmacies [28].

In a cross-sectional study in Myanmar consisting of 183 samples, the study mentioned about throwing out-of-use and out-of-date medicines in dustbins, 49.18% of them practiced the burial of expired medication, and 55.9% used open pit burning [33].

An in-person interview-based study was conducted in Nepal, and 210 participants took part. Of these participants, 65.2 % did not know about proper medicine disposal. [30]. Another web-based cross-sectional descriptive survey was done in the same country, including 294 respondents. There 72% of the respondents threw

the medicines, 21% flushed them down the toilet, and just 2% brought these outdated and leftover drugs back to the pharmacy [31].

In Sri Lanka, a study was conducted including only 40 samples. Here, 25% of the participants burnt and 22% of the participants threw away the solid unused and expired medications. 17% of them burned, and 28% threw the liquid dosage forms in landfills [35].

In Bangladesh, a questionnaire-based cross-sectional study was performed among 150 people, of which 100 were doctors and 50 were nurses. Among the nurses, 56.82% threw medicines in dustbins, 38% flushed them in basins, and only 2.2% returned them to the pharmacy. On the other hand, 56.25%, 36.64%, and 20.83% of the doctors threw the medicines in the dustbins, flushed them down the basin, and returned to the pharmacy, respectively [36].

An in-person interview using a pre-validated structured questionnaire, including 301 participants, was conducted in Afghanistan. Here, 77.7% of people throw expired medicines in dustbins, 12% drain them in toilets, 3% use other methods, and 7.3% return them to pharmacies. 14.3% of the respondents threw unused medications in the trash, 1.3% drained them in sinks or toilets, and 63.1% used other methods. Only 21.3% of the medicines are returned to pharmacies [3].

In a structured questionnaire-based study in Malaysia containing 885 participants, just 2% of people used the drug take-back program, despite 87% of people being conscious of drug waste. Furthermore, 83% of the people surveyed discarded unneeded medicines in landfills [40]. Another study showed that among the 481 participants, 93.1% of participants store medication at home. Mostly, they were educated female students who performed self-medication [41]. Again, there was a study that had 483 participants among them 64% threw the medication in the dustbin, 10.6% flushed those in the toilet, 6% used other methods, and only 19.4% of them returned the expired and unused medicines to the pharmacy [44].

In a cross-sectional study in Indonesia consisting of 322 participants where 49.4% of the people who took part threw out-of-use and out-of-date medicines in dustbins, 8.2% poured these in a sink or toilet and 39.3% used other methods. Returning to the pharmacies was seen by only 3.1% of the respondents [50]. In another cross-sectional study in Indonesia consisting of 497 participants where 82% of the responding people threw left-out and outdated medicines in dustbins, 5% poured these in a sink or toilet and 4% used other methods. Returning to the pharmacies was seen by only 0.2% of the respondents [51].

In a study in Thailand, among 331 participants, 89.4% of the participants had leftover medicines at their residences. For them, throwing in the garbage was the most popular form of disposal. 7.4% of the unused liquid dosages were disposed of into the drain. 89% of the participants said they received no instruction on appropriate discarding methods [52].

In another study in Vietnam involving 525 participants, 84% of those questioned discarded their unwanted and outdated medicines in dustbins, and 23% used other methods. Only 5% of the respondents reported returning

to the pharmacies. Among the individuals, 70% had abandoned prescription drugs at their houses, 20% disposed of their medicines, and 15% gave those to others [53].

In a cross-sectional study in the UAE, 11% of the participants, among 709 respondents, returned expired medicines to the Pharmacy, 42.5% kept them in a resealable plastic bag, and 12% of them didn't know what to do with their medicines [61]. In another study conducted in Saudi Arabia, among 820 participants, 565 respondents threw unused medication into the garbage, and 331 kept it for future use. On the other hand, in the case of disposing of the expired medications, 798 people threw them in the garbage, and 28 returned to the pharmacy. The rest threw their medication in the toilet, burned it, and buried it in the soil. [62]

4. Discussion

In these regions, to prevent infections, medication abuse or misuse, health issues for the public, and environmental damage, it can be challenging to get rid of outdated and out-of-use pharmaceutical products properly. This study will examine how many South Asian and Southeast Asian countries, including India, Nepal, Bangladesh, Malaysia, Afghanistan, Indonesia, Pakistan, Malaysia, Indonesia, Vietnam, Myanmar, and Sri Lanka, discard abundant and outdated medicinal products. Data was collected through cross-sectional studies and questionnaire-based surveys conducted between 2010 and 2024. The research instruments were predominantly comprised of questionnaires, structured interviews, and closed-end questions, and they were administered to medical students, healthcare professionals, pharmacy and general students, doctors, staff nurses, pharmacists, and the general population. According to a survey done in India, topical medicines made up 63.5% of the prescriptions that were kept in storage, and 73% of participants preferred to throw all of them in residential garbage cans. Just 5% of healthcare workers took their medications back to the pharmacy, while 17% smashed them before discarding them, which is frightening. Furthermore, many medical personnel stored medications inside their homes using incorrect handling techniques. In one study in India, 385 participants took part. Among them, a large proportion of individuals have leftover prescription drugs at their residences. The main reason is that they stop using medication after feeling better from the illness. 82.1% of those questioned threw pharmaceuticals in the waste bin, and only 2.6% returned them to pharmacies [5]. Again, in another study in India, 956 respondents were included. 73% of the participants threw the unused medications in the trash can, and 20% of those people disposed of the drugs in sinks or toilets [11]. On the other hand, in a Pakistani study, 1022 participants took part. Among them, most individuals had unused and outdated medicines at their homes. They stock the medicines after they stop using medication after feeling better or the doctor changes the medicines. 83% of the respondents threw drugs in the dustbin, and only 2% returned them to pharmacies, which is almost similar to India. 12% of them poured into the toilet [25]. Again, another study in Pakistan includes 676 participants. Here, 60.52% of the female responders threw the out-of-use medicines in the trashcan, and 48.72% of the male participants disposed of the drugs by burying them [24]. Students of pharmaceutical sciences and others in Bangladesh showed a lack of understanding and awareness regarding appropriate drug management procedures. Again, lots of participants kept different kinds of medicines in their homes, which had the potential to cause health and environmental damage.

A cross-sectional questionnaire-based study was conducted in Bangladesh, where 200 participants took part. Among them, a lot of people kept medicines inside their houses. 47% of the respondents threw drugs in the dustbin, 8% disposed of these drugs into a sink or toilet, 29% used other methods, and 16% returned them to pharmacies [27]. Again, in another study in Bangladesh, including 400 samples, 47% threw them in the garbage, 2% of the respondents burned them, and 21% of the people involved gave the pharmacy their abandoned and outdated prescription drugs back [28]. On the contrary, in Sri Lanka, 25% of the participants burned, which is less than in Bangladesh, and 22% of participants threw solid unused and expired medications, which is almost similar. 17% of them burn, and 28% throw the liquid dosage forms in landfills [35]. In another cross-sectional study conducted on doctors and nurses in Dhaka, Bangladesh, it was found that neither doctors nor nurses are aware of these safe medicine exposures. The most shocking news is that being healthcare professionals, only 2.2% of doctors return their medicines to the pharmacy, and the rest of them throw them away as garbage in dustbins and basins [36]. More than 80% of people in Malaysia were conscious of the increasing problem of medicine waste, its consequences for consumers, and the present scenario. The fact is unpleasant that drugs are gotten rid of in India by pouring them into the sink as well as the toilets or by putting them in the dustbin. However, 66% preferred returning unused medicines to pharmacists. In Thailand, sadly, 89% of respondents stated that individuals received no instructions on how to discard unused medications properly. [52] According to a different survey, 92.6% of those surveyed in India and 77.7% within Afghanistan disposed of unwanted and out-of-date prescriptions in their residence's garbage cans, while 87% of buyers preserved pharmaceuticals at their houses [3]. Surprisingly, all the individuals agreed that inappropriate disposal methods could have negative effects on the ecosystem and human health. Then there are 82.1% of the participants from Indonesia who were students admitted that they dump undesired medication in the garbage, and about 80% of Pakistanis kept outdated and unwanted medications in their residences. In a cross-sectional study in Indonesia consisting of 497 participants, where 82% of the answerers threw outdated and out-of-use drugs in dustbins, 5% poured these in a sink or toilet, and 4% used other methods. Only 0.2% of the respondents reported returning to the pharmacies [51]. In Indonesia, 85% reported they saved any unneeded medications at their house, and only 3% of participants returned to pharmacies, 17.3% drain them into the toilet [48]. In another cross-sectional study in Indonesia consisting of 322 participants, where 49.4% of the participating personnel threw their unneeded medications in dustbins, 8.2% poured these in a sink or toilet, and 39.3% used other methods. Only 3.1% of the respondents reported returning to the pharmacies [50]. This throwing of medication into the garbage turned into a massive number in Saudi Arabia where among 820 respondents, 798 respondents threw their expired medications to the garbage and only 28, i.e. 3.4% return them to the pharmacy. It is quite shocking that 72.6% even threw their unused medicines into the garbage, where they could have easily kept them in their home for future use or given them back to the pharmacy [62]. To sum up, the most common disposal method among all the countries is throwing on the dustbin. Sadly, 83% of the respondents threw unused medicines in dustbins and 24% used other

methods of disposal in Vietnam whereas only 0.55% of participants in Myanmar threw the medication in dustbins [33,53].

4.1 Effects of improper medicine disposal on human health

Human health is seriously threatened by inappropriate medicine disposal, since recent studies have shown the intricate biological effects of pharmaceutical contamination. Active pharmaceutical ingredients (APIs) are introduced into water supplies by practices like flushing pharmaceuticals down the toilet or throwing them in the trash, which may interfere with endocrine function and cause developmental problems [64]. Pharmaceutical pollution also contributes to antibiotic resistance, which reduces the efficacy of necessary therapies and raises global health issues [65].

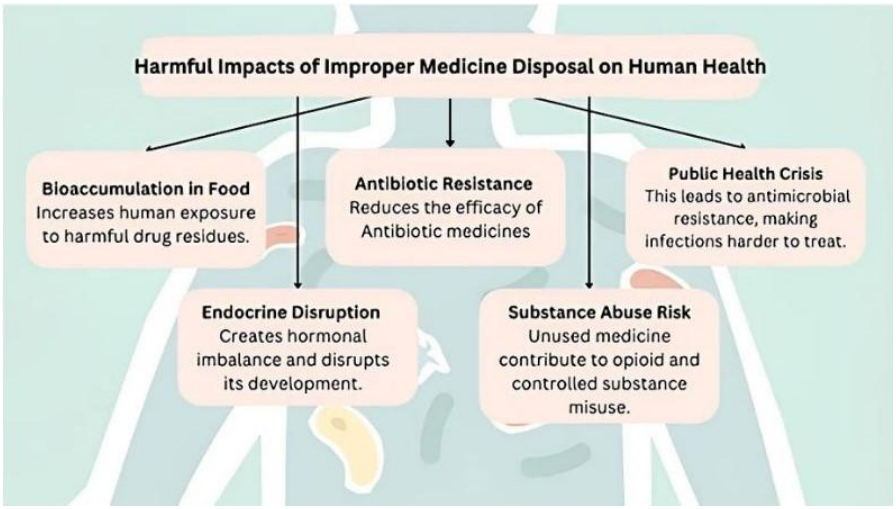

Figure 3: The harmful impact of medicine disposal on human health

In addition to contaminating water, inappropriate disposal can cause pharmaceutical residues to bioaccumulate in the food chain, raising human exposure and health hazards [66]. Concerns over long-term impacts are raised by persistent drug chemicals in the environment, highlighting the need for immediate public awareness campaigns and regulatory actions.

Moreover, inappropriate medicine disposal has an effect on society that goes beyond substance abuse. Prescription medications that are unused or expired in homes encourage the use of illegal drugs, especially opioids and other controlled substances [67]. This public health risk is linked to more general issues like antimicrobial resistance, reproductive abnormalities, and hormone disruption [64,65]. To address these issues and lessen the extensive health and societal repercussions, focused initiatives, education, and stricter disposal laws are needed.

4.2 Hazardous impact of improper medicine disposal on the environment

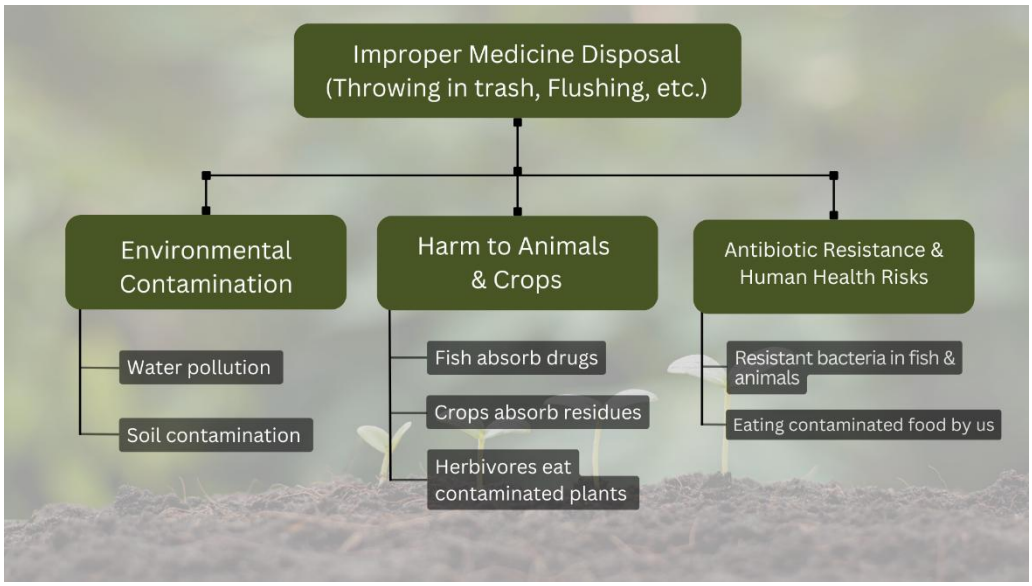

Figure 4: The impact of improper medicines disposal on the environment

Due to improper medicine disposal, our environment is facing an extreme risk, and day by day, it is increasing. The API (Active pharmaceutical ingredients) or the excipients that have been used by drugs will significantly contaminate water, soil, rivers, and our ecosystem as well [68]. It exerts its harmful effects mostly in the aquatic world and in agriculture [69]. The presence of drug residues in the ponds or river water can contaminate the entire ecosystem living within it. Thus, the fish living there will face its harmful impacts. Similarly, the contaminated soil will grow crops and grasses, which will be eaten by the herbivorous animals. So, they will face the side effects of those disposed of drugs. Most importantly, we can feel the danger when it comes to drugs having high potency and a narrow therapeutic index [70]. Its harmful side can also be understood very clearly when the animals or fish develop antibiotic resistance, and that will be eaten by us, so that we will develop the same harmful effects. Similarly, the irrigated crops will raise the harmful effects inside them, and we will face the same effects when we eat them as our food [64].

5. Recommendations

The following recommendations are made to address the significant issue of inappropriate disposal of unused and expired pharmaceuticals:

a. Educate and increase awareness regarding the drawbacks of hazardous disposal techniques. Seminars, workshops, and campaigns should be organized to raise people's social awareness. They must be informed of the physical well-being and the risks of the atmosphere linked to inappropriate pharmaceutical elimination.

b. Governments and healthcare institutions should establish easily accessible drug take-back programs [71]. It is vital to educate the public on the places of operation and benefits of these programs. Offering monetary incentives for returning unused or expired medications can encourage wider use.

c. Punitive measures can be used to discourage and promote responsible behavior by imposing fines on those who flush or dispose of medications in garbage cans.

d. Initiatives at the Pharmacy Level:

i. Inventory management: To minimize medication expiration, pharmacists must adhere to the First-In-First-Out principle [71].

ii. Prescription Practices: OTC drug distribution should be limited, and the sale of medications without a legitimate prescription should be prohibited.

iii. Buyback Programs: To encourage consumers to return leftover medications, the pharmacist may accept them in exchange for the price they paid.

e. Technological Solutions: One of the most important technical solutions for regulating medication disposal is the creation of online platforms that track disposal sites and enable returns. These services can guarantee that unused or expired prescriptions are disposed of appropriately by giving users up-to-date information on the closest drug take-back sites. They can also provide advice on how to effectively and safely return drugs. By making this information easily accessible, these platforms can significantly lower the risks to human health and the environment associated with improper medication disposal. They can also assist in tracking and analysing disposal patterns, which can improve waste management strategies [71].

By implementing these actions, we may reduce the adverse impacts of inappropriate medicine discarding and encourage ecologically beneficial and public health-protecting sustainable practices.

6. Limitations

Most of the studies used a limited number of populations from each country. These small groups are not proper representatives of a country. The articles used in this review article didn't have enough information regarding other types of disposal practices other than throwing in trash cans. In some Asian countries, not a single survey study has been conducted about inappropriate medicine disposal practices.

7. Conclusion

The issue of medication misuse that results from the improper disposal of unused and expired medicines is a serious threat to public health and the environment. It results in prescription drug abuse, poisonings, antibiotic-resistant bacteria, and contamination of the environment. Medical personnel, policymakers, pharmaceutical companies, and the general public should cooperate to popularize proper ways of medicine disposal and educate people on the importance of safe medication disposal practices. Responsible disposal practices need to be applied best until the risks have been completely resolved, thus protecting human health, as

well as the environment. In South and Southeast Asian nations, this problem is a major obstacle. There are no strong guidelines for many health hazards issues like medicine disposal practice, proper labelling of ingredients used in widely used energy drinks in a country like Bangladesh [72]. These unhealthy practices cause environmental pollution and public health issues. Importance should be given to addressing these issues and encouraging pharmacists working in policy-making sectors to come forward to implementing a medicine take-back program and other pragmatic initiatives. The suggested appropriate pharmaceutical waste elimination guidelines and preventative actions highlight how important it is for stakeholders to work together. This study highlights the ecological and public health hazards that concern the decision-making personnel. This would help to make prompt decisions regarding this and convey the implementation. It would open room for pharmacists and other healthcare practitioners to contribute to the environment's well-being and the people.

Statement and Declarations

Author Contribution: ST wrote the primary manuscript. RAS, TBS, AAMB, and NT revised the manuscript. MBU conceptualized, supervised the work, and edited the final draft.

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Original Article

Unveiling the therapeutic profile of *Oxyspora paniculata*: Insights into neuropharmacological, antidiarrheal, and thrombolytic activities *In Silico*

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Abstract: The application of supplementary herbal medicines has lately expanded in an effort to discover viable alternative therapies that lessen the side effects of chemical pharmaceuticals. *Oxyspora paniculata* is a rich source of phytochemicals with pharmacological action; therefore, it may have health advantages. This current research was performed to analyze the *in silico* evaluation of phytoconstituents present in *Oxyspora paniculata* for neuropharmacological, antidiarrheal, and thrombolytic activity. *In silico* activity of the isolated constituents for antioxidant activity was carried out by PyRx AutoDock Vina, and the protein-ligand interactions were examined using BIOVIA Discovery Studio Visualizer. ADME analysis was performed utilizing the SwissADME free online web server, and a toxicology study was done using the admetSAR online server. In the computational approach, among all the proteins, a docking score was found ranging from −4.6 to −10.3 kcal/mol. Besides, all the compounds were found safe in the ADME/T study. The absorption, distribution, metabolism, excretion/toxicity evaluation of phytoconstituents assures that they have obeyed Lipinski's guideline of five, suggesting their safe consumption. The results of our scientific research validate the suitability of this plant as an alternative source of novel therapeutics. It was determined that *Oxyspora paniculata* contained active phytochemicals, which might provide a variety of pharmacological uses as a hidden source of substances with significant medicinal value. This needs more investigation in order to identify secondary metabolites that can be used to treat various illnesses.

Keywords: Antidiarrheal, antidepressant, thrombolytic, SwissADME, molecular docking, PyRx Autodock Vina.

1. Introduction

Medicinal plants offer promising chemicals for medicinal and pharmacological applications [1]. According to estimates from the World Health Organization (WHO), up to 80% of people in underdeveloped

nations still get their primary medical treatment from locally grown medicinal herbs [2]. Plants are the source of around 25% of prescription medications and 11% of drugs classified as essential by the World Health Organization. Additionally, a significant portion of synthetic pharmaceuticals are generated from plant precursor molecules. The primary arguments in favor of traditional medicine over contemporary therapy are its accessibility, effectiveness, and low cost [3].

There are two distinct words for two different mental illnesses: anxiety and depression. That being said, they may both happen simultaneously. There is a complex relationship between these two conditions [4]. People with anxiety disorders often develop depression [5]. The sensation of being threatened never goes away for those who suffer from anxiety [6]. Furthermore, the World Health Organization lists depressive disorders as a major contributor to nonfatal illnesses globally [7]. Regular experience of anxiety may lead to its classification as a psychiatric condition. Anxiety is a natural emotional state. The presence of anxiety in conjunction with depression leads to a variety of symptoms, such as a decrease in response to treatment or medicine, a drop in the actual prognosis, and an elevated risk of suicide [8].

One of the most common infectious disorders among children in third-world nations is diarrhea, which is brought on by abnormalities in the intestine's secretion and absorption, increasing the volume of excrement produced [9]. According to the World Health Organization (WHO), Bangladesh has a high prevalence of diarrhea in children, with 17% of children under the age of five hospitalized in pediatric wards [10]. Diarrhea can be acute or chronic, with the former caused by epidemiological factors, including travel. Chronic diarrhea lasts for more than four weeks [11, 12]. Infections with bacteria, viruses, and parasites are the causes of diarrhea. Noroviruses and rotaviruses are two examples of viruses that can cause viral illnesses. The primary bacterial causes of diarrhea include species like *Salmonella typhi*, *Helicobacter pylori*, *Clostridium difficile*, and *Escherichia coli*; parasitic diseases are caused by *Entamoeba histolytica* and *Giardia intestinalis* [13]. Diarrhea is a significant issue in underdeveloped nations, despite progress in health services and economic prosperity.

Generally speaking, thrombosis is the term for localized blood clotting that can happen in the venous or arterial circulation and has serious medical consequences. Acute arterial clotting is the primary risk factor for myocardial infarction (heart attack), and 80 percent of strokes occur in the majority of affluent nations [14]. If treatment for this condition is delayed, it will finally result in death. Thrombosis is often brought on by a platelet abnormality or blood coagulation protein, which obstructs the circulatory vessel and prevents the body's normal blood flow. Nevertheless, in addition to heart attacks and strokes, thrombosis can result in cardiac impairment, bleeding ulcers, blindness, and a few other symptoms [15]. Antithrombotic medicines are used to treat thrombosis by targeting particular proteins in the human body's coagulation cascade. The administration of these medications causes the drug molecule to connect with a target protein, thus promoting clot breakup. There are several antithrombotic medications on the market that are effective in treating thrombosis in people with cardiovascular problems. However, several of the existing medications have been accused of causing severe bleeding after administration [14, 16, 17].

Molecular docking facilitates the identification, screening, designing, prediction, and synthesis of chemical compounds, among other processes that lead to the discovery of medicinal medications. Drugs with significant therapeutic potential can be designed, synthesized, and discovered more effectively with the help of molecular docking [18]. It is being used in several biological and medical domains, including bioremediation, protein engineering, medicinal chemistry, and cheminformatics. Using the molecular docking approach, potent therapeutic molecules, particularly those derived from naturally occurring chemicals, have been predicted to combat various diseases. Using molecular docking to assess the intricacy of protein-ligand interactions saves money and time. As a result, the neuropharmacological, antidiarrheal, and thrombolytic effectiveness of *Oxyspora paniculata* has been sought through an *in silico* docking model to determine how they are implicated in such biological activity.

2 Materials and Methods

2.1 Molecular docking: protein preparation

Human serotonin transporter (PDB ID: 5I6X) [19], potassium channel enzyme (PDB ID: 4UUJ) [20], μ-opioid receptor (μOR) (PDB ID: 5C1M) [21], and tissue plasminogen activator (PDB ID: 1A5H) [22] have been derived from RCSB Protein Data Bank (https://www.rcsb.org/structure) in PDB format for the antidepressant, anxiolytic, antidiarrheal, and thrombolytic studies, respectively. All of the water and heteroatoms have been removed from proteins using BIOVIA Discovery Studio 2020. Additionally, using SWISS PDB Viewer, all proteins were reduced to the lowest possible energy level in preparation for additional analysis [23].

2.2 Molecular docking: ligand preparation

Eight chemical components of *Oxyspora paniculata* were simultaneously determined in a previous study using the HPLC technique [24]. The structures of eight compounds of *Oxyspora paniculata*, namely gallic acid (PubChem CID: 370), dihydromyricetin (PubChem CID: 161557), protocatechuic acid (PubChem CID: 72), rutin (PubChem CID: 5280805), myricitrin (PubChem CID: 5281673), oleanolic acid (PubChem CID: 10494), quercetin (PubChem CID: 5280343), and kaempferol (PubChem CID: 5280863), were retrieved from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/). Besides, diazepam (PubChem CID: 3016), loperamide (PubChem CID: 3955), and streptokinase (PubChem CID: 9815560) have been studied to compare and contrast the docking of the compounds of *Oxyspora paniculata*. The ligands have been downloaded in 3D SDF format and minimized by the PyRx tool to find the best possible hit for these targets. The virtual screening software PyRx from MGLTools (https://sourceforge.net/projects/pyrx/) has been kept in default format [25].

2.3 Molecular docking: docking analysis

PyRx AutoDock Vina was used to dock the chosen protein-ligand complexes [25]. The docking study was carried out using a semi-flexible docking system. PDB files containing phytochemicals and proteins were

reduced and then converted to PDBQT format using the PyRx AutoDock Vina program. This analysis kept the protein stiff and the ligand flexible. The ligand molecules have been granted ten degrees of freedom. AutoDock describes measures for automatically transforming molecules into PDBQT-format molecules, such as box type and grid box construction. The grid box was designed with an active location in its middle. In addition, BIOVIA Discovery Studio Visualizer 2020 [26] was expedited to find the optimal docking places.

2.4 Pharmacokinetics and toxicity measurement

The pharmacokinetic properties of the substances were investigated using the SwissADME online method (ADME). Compounds having desirable drug-like properties are evaluated using Lipinski's five criteria (molecular weight < 500 daltons, H-bond donors ≤ 5, H-bond acceptors ≤ 10, molar refractivity between 40 and 130, and lipophilicity<5) [27]. Furthermore, the online application admetSAR (http://lmmd.ecust.edu.cn/admetsar2) was utilized to compute the toxicological properties of every material.

3. Results

3.1 Molecular docking analysis for antidepressant and anxiolytic study

The docking analysis results for antidepressant and anxiolytic activity are presented in **Table 3** and **Figure 1**. In this investigation, two receptors namely human serotonin transporter (PDB ID: 5I6X) and potassium channel (PDB ID: 4UUJ) have been used to screen antidepressant and anxiolytic docking analysis, respectively. In the case of the human serotonin transporter (PDB ID: 5I6X), the ranking of the docking score is as follows: rutin > diazepam> quercetin > dihydromyricetin > oleanolic acid > myricitrin > kaempferol > gallic acid > protocatechuic acid. On the contrary, the docking of the selected compounds against the potassium channel (PDB ID: 4UUJ) is as follows: oleanolic acid > diazepam >myricitrin > quercetin > rutin > dihydromyricetin > kaempferol > protocatechuic acid > gallic acid. Amino acid residues namely gly338, tyr95, phe341, val501 and ile172 established the interaction between rutin and 5I6X. Furthermore, oleanolic acid binds to the enzymatic pocket of 4UUJ receptor by means of phe103 and val106 residues with a docking score of (-7.2) kcal/mol.

3.2 Molecular docking analysis for the antidiarrheal study

The docking analysis of antidiarrheal activity has been illustrated in **Table 3** and **Figure 1**. In this investigation, μ-opioid receptor (μOR) (PDB ID: 5C1M) was used to quest of binding interaction with the selected compounds of *Oxyspora paniculata*. Oleanolic acid explores best binding affinity with the μ-opioid receptor (μOR) (5C1M) proteins which interact with the amino acid residues namely asn109, leu116, tyr149, ile146, met203, trp152 and leu112. The ranking of the docking score is as follows: loperamide > oleanolic acid > dihydromyricetin > kaempferol > quercetin > rutin > myricitrin > gallic acid > protocatechuic acid.

3.3 Molecular docking analysis for thrombolytic study

The docking analysis results for the thrombolytic activity have been presented in **Table 3** and **Figure 2**. In case of tissue plasminogen activator (PDB ID: 1A5H), the highest score has been obtained −8.9 kcal/mol for myricitrin. The ranking of the docking score is as follows: myricitrin > oleanolic acid > quercetin > rutin > dihydromyricetin > kaempferol > streptokinase > gallic acid > protocatechuic acid. Myricitrin interacts with the protein (1A5H) through a series of amino acid residues (tyr99, tyr151, leu41, gly219, gly216) of conventional hydrogen and van der Waals as well as cys191, trp215, and gln192 of amide-Pi stacked and carbon hydrogen bond.

3.4 Pharmacokinetics and toxicity measurement

The study shows that all the compounds agree with Lipinski's rules and claim that these compounds are orally bioavailable. In order to predict the toxicological properties of the eight compounds, the online admetSAR (http://lmmd.ecust.edu.cn/admetsar1) server is also used. The analysis showed that the selected compounds are non-Ames toxic and noncarcinogenic (**Table 1**). The investigation also revealed the GI absorption (GA) and blood-brain barrier (BBB) of eight phytoconstituents in *Oxyspora paniculata* (**Table 2**).

Table 1: Absorption, digestion, metabolism, excretion, and toxicological analysis of phytoconstituents in *Oxyspora Paniculata*

Ligands	Molecular formula	MW(g/mol)	HBD	HBA	LogP (o/w)	AMT	CAR	Lipinski violation
Gallic acid	$C_7H_6O_5$	170.12 g/mol	4	5	0.21	No	No	0
Dihydromyricetin	$C_{15}H_{12}O_8$	320.25 g/mol	6	8	0.9	No	No	1
Protocatechuic acid	$C_7H_6O_4$	154.12 g/mol	3	4	0.66	No	No	0
Rutin	$C_{27}H_{30}O_{16}$	610.52 g/mol	10	16	1.58	No	No	3
Myricitrin	$C_{21}H_{20}O_{12}$	464.38 g/mol	8	12	0.92	No	No	2
Oleanolic acid	$C_{30}H_{48}O_3$	456.70 g/mol	2	3	3.89	No	No	1
Quercetin	$C_{15}H_{10}O_7$	302.24 g/mol	5	7	1.63	No	No	0
Kaempferol	$C_{15}H_{10}O_6$	286.24 g/mol	4	6	1.7	No	No	0

Note: PID = PubChem ID, MW = molecular weight (acceptance range: <500), HBD = hydrogen- bond donor (acceptance range: ≤5), HBA = hydrogen-bond acceptor: (acceptance range: ≤10), LogP = high lipophilicity (acceptance range: <5), AMT, AMES toxicity; CAR = carcinogens

Table 2: GI absorption (GA) and Blood brain Barrier (BBB) of phytoconstituents in *Oxyspora Paniculata*.

Ligands	PubChem ID	Canonical SMILES	GA	BBB permeant
Gallic acid	370	C1=C(C=C(C(=C1O)O)O)C(=O)O	High	No
Dihydromyricetin	161557	C1=C(C=C(C(=C1O)O)O)C2C(C(=O)C3=C(C=C(C=C3O2)O)O)O	Low	No
Protocatechuic acid	72	C1=CC(=C(C=C1C(=O)O)O)O	High	No
Rutin	5280805	Oc1cc(O)c2c(c1)oc(c(c2=O)OC1OC(COC2OC(C)C(C(C2O)O)O)C(C(C1O)O)O)c1ccc(c(c1)O)O	Low	No
Myricitrin	5281673	Oc1cc(O)c2c(c1)oc(c(c2=O)OC1OC(C)C(C(C1O)O)O)c1cc(O)c(c(c1)O)O	Low	No
Oleanolic acid	10494	OC1CCC2(C(C1(C)C)CCC1(C2CC=C2C1(C)CCC1(C2CC(C)(C)CC1)C(=O)O)C)C	Low	No
Quercetin	5280343	Oc1cc(O)c2c(c1)oc(c(c2=O)O)c1ccc(c(c1)O)O	High	No
Kaempferol	5280863	Oc1ccc(cc1)c1oc2cc(O)cc(c2c(=O)c1O)O	High	No

Table 3: Docking scores of the selected ligands with the human serotonin transporter (PDB ID: 5I6X), potassium channel (PDB ID: 4UUJ), μ-opioid receptor (μOR) (PDB ID: 5C1M), and tissue plasminogen activator (PDB ID: 1A5H).

Ligands	**Antidepressant (5I6X)**	**Anxiolytic (4UUJ)**	**Antidiarrheal (5C1M)**	**Thrombolytic (1A5H)**
Gallic acid	-6	-4.6	-5.3	-6.4
Dihydromyricetin	-8.9	-5.7	-7.5	-8.1
Protocatechuic acid	-6	-4.7	-5.1	-6.2
Rutin	**-10.3**	-5.8	-6.6	-8.2
Myricitrin	-8.5	-6.3	-6.2	**-8.9**
Oleanolic acid	-8.7	**-7.2**	**-7.7**	-8.7
Quercetin	-9	-5.9	-6.9	-8.2
Kaempferol	-8	-5.6	-7	-7.4
Standard (Diazepam/Streptokinase/ Loperamide)	-9.1	-7	-8	-6.5

Note: The most significant values of docking scores have been marked in bold letter.

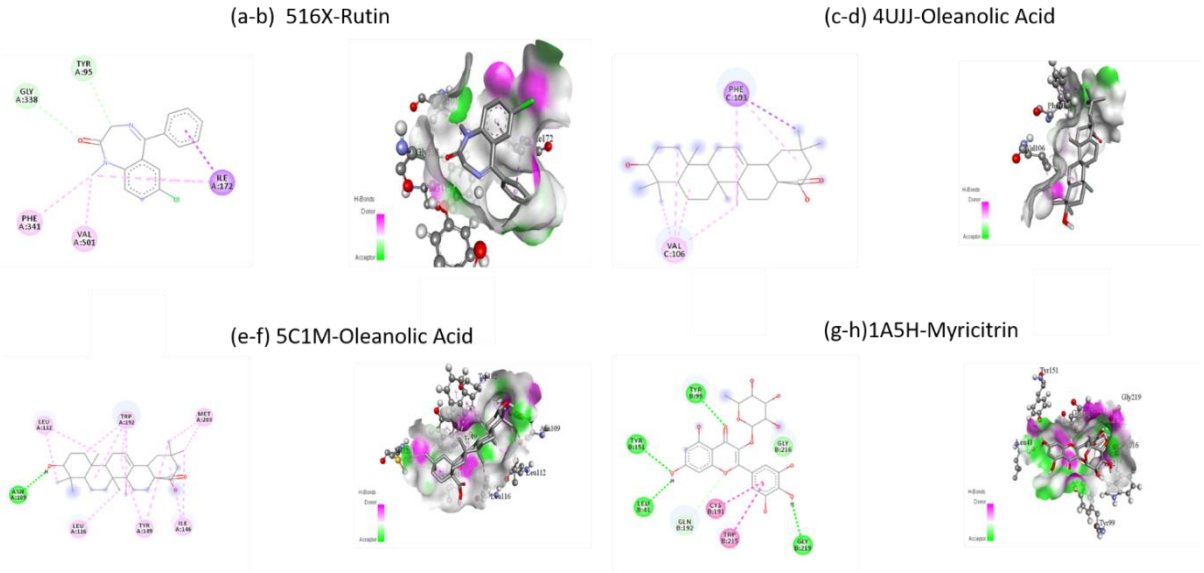

Figure 2: 3D and 2D presentations of the best ligand–receptor interactions (a, b, c, d, e, f, g, and h represent 5I6X-rutin, 4UUJ-oleanolic acid, 5C1M-oleanolic acid, and 1A5H-myricitrin interactions, respectively).

4. Discussion

Even if there are several therapy options for depression, anxiety, diarrhea, and coronary artery disease, it is still unclear how to completely relieve the symptoms of the condition without causing adverse consequences. As a result, these medications' poor pharmacokinetics and adverse effects limit their therapeutic use. Because of this, the need for new medications is growing, and concerns about the safety, effectiveness, duration of action, and side effects of existing medications have become crucial [28]. Herbal therapy has emerged as a potentially effective treatment for many ailments because of the variety of neurological targets it targets [29]. However, *Oxyspora paniculata*, a prominent ethnomedicinal plant with a wide range of therapeutic uses, was used in this investigation. The current research demonstrated that the neuropharmacological, antidiarrheal, and thrombolytic efficiency of *Oxyspora paniculata* was explored by an *in silico* docking model to establish whether they have been connected to such biological activity.

Molecular docking studies are commonly used to forecast ligand-target interactions and deepen our understanding of the bioactivities of natural products. It also provides more information on potential binding processes inside the protein binding pockets [30]. To understand the consequences of this result, biological investigations were described and validated using molecular docking research. Eight common *Oxyspora paniculata* compounds were chosen for the docking research in order to conduct a more in-depth analysis of their biological properties (antidepressant, anxiolytic, anti-diarrheal, and thrombolytic). The compounds were docked against four targets: human serotonin transporter (PDB ID: 5I6X), potassium channel (PDB ID: 4UUJ), μ-opioid receptor (PDB ID: 5C1M), and tissue plasminogen activator (PDB ID: 1A5H) for antidepressant, anxiolytic, antidiarrheal, and thrombolytic studies, respectively. Human serotonin transporters (PDB ID: 5I6X) interact with ligands through several linkages, resulting in docking values ranging from -6 to -10.3 kcal/mol. These findings suggest that these phytoconstituents play a significant role in antidepressant activity via their interactions with target proteins. To analyze the anxiolytic docking investigation, the chosen phytoconstituents were docked with the potassium channel (PDB ID: 4UUJ). The docking score ranged from -4.6 to -7.2 kcal/mol. The docking score for the antidiarrheal trial ranged from -5.1 to -7.7 kcal/mol. In the thrombolytics docking study, myricitrin, oleanolic acid, quercetin, and dihydromyricetin all had a significant docking score against plasminogen tissue activator (PDB ID: 1A5H), with myricitrin having the highest score. This study reveals that *Oxyspora paniculata's* thrombolytic effect may be attributed to its bioactive components (myricitrin, oleanolic acid, quercetin, dihydromyricetin, and rutin).

The pharmacokinetics and toxicological characteristics of the drugs have been verified in relation to the results of molecular docking investigations against the human serotonin transport, potassium channel, μ-opioid receptor, and tissue plasminogen activator. Every compound complied with Lipinski's guidelines for drug-likeliness. As a result, these analyses of the compounds that have been found are very helpful in the development of a novel pharmaceutical agent [27, 28, 29, 30, 31].

The results imply that the compounds are safe for oral use and satisfy Lipinski's criteria, which suggest that they may serve as useful medication candidates (**Table 1**). Additionally, as medication safety is a crucial component of what makes a successful medical product, we evaluated the toxicological parameters of the assigned plant components using the admetSAR online tool [32]. As a result, the thorough investigation produced important information on the chosen chemicals. The combined orientation of several phytochemicals, including established and unreported phytochemicals, may become a contributing factor in the outcomes.

5. Future implication and limitations of study

Our findings have interesting implications for future *tabble* and clinical studies, potentially leading to the development of new treatment drugs and combination therapies for neurological disorders, diarrhea, and thrombosis. However, the study's in-silico nature restricts its immediate usefulness, as the effectiveness and safety of the compounds must be verified in experimental and clinical trials. Further study might include *in vitro* and *in vivo* studies to establish the physiological consequences of these findings.

6. Conclusion

The biological results of this scientific study indicate that *Oxyspora paniculata* can be a significant source of antidepressant, anxiolytic, antidiarrheal, and clot-lytic medicines. Furthermore, the molecular docking analysis of the bioactive phytoconstituents revealed promising binding affinity to certain proteins, and the ADMET research demonstrated their drug-like properties. Thus, the computational investigation validated the experimental results of biological activities and gave a potential insight into contemplating *Oxyspora paniculata* as a significant therapeutic candidate. Additional research is also encouraged, as structural modifications to these compounds may result in a higher docking score and greater medicinal relevance.

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Original Article

HPLC-Analysis of Polyphenolic Compounds in *Crateva nurvala* and Evaluation of *In vitro* Antioxidant Activities of its Flash Column Chromatographic Fraction

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Abstract: *Crateva nurvala* (Barun), a medicinal plant extensively used in traditional medicine, is rich in phenolic compounds that exert antioxidant effects. The escalating demand for safer, natural antioxidants has renewed interest in medicinal plants. This study investigates the antioxidant potential of *Crateva nurvala* bark extract and its flash column chromatographic fractions, with emphasis on polyphenolic composition and *in vitro* radical scavenging activity. Ethanolic crude extract of *Crateva nurvala* bark was fractionated using gradient flash column chromatography into five major groups. Polyphenolic compounds were identified and quantified via HPLC-DAD. Antioxidant potential was evaluated using DPPH, nitric oxide (NO), hydroxyl radical (•OH) scavenging, ferric reducing antioxidant power (FRAP), and total antioxidant capacity (TAC) assays. IC_{50} values were calculated from dose-response curves. HPLC profiling revealed three major phenolics: p-coumaric acid (203.09 ± 0.67 mg/100g), (-)epicatechin (39.77 ± 0.46 mg/100g), and myricetin (37.35 ± 0.32 mg/100g). In the DPPH assay, IC_{50} values were lowest for the *Crateva nurvala* crude extract (3.276 µg/mL) and methanol fraction (3.436 µg/mL), compared to ascorbic acid (0.8579 µg/mL). NO scavenging results followed a similar trend: crude extract (IC_{50} = 7.865 µg/mL), methanol (8.371 µg/mL). For hydroxyl radicals, the crude extract showed IC_{50} = 156.5 µg/mL, approaching ascorbic acid (105.8 µg/mL). FRAP and TAC assays indicated the methanol fraction had the highest reducing potential and antioxidant capacity, respectively. The potent antioxidant activity of *Crateva nurvala*, supported by its rich polyphenolic content, underscores its value as a promising natural source of therapeutic antioxidants. Low IC_{50} values in multiple assays validate its potential for further development into nutraceutical or pharmaceutical applications.

Keywords: *Crateva nurvala*; Barun; *In vitro* assays; oxidative stress; antioxidant; polyphenols; total antioxidant capacity

1. Introduction

Oxidative stress is a pathological condition that arises due to an imbalance between the production of reactive oxygen species (ROS) and the body's ability to detoxify them through its antioxidant defenses [1]. ROS, including

free radicals like superoxide anion, hydroxyl radical, and hydrogen peroxide, are generated as natural byproducts of cellular metabolism. However, excessive ROS accumulation can lead to oxidative damage to lipids, proteins, and DNA, contributing to various chronic diseases such as cancer, neurodegenerative disorders, and cardiovascular diseases [2], and metabolic syndromes [3]. The detrimental effects of oxidative stress highlight the necessity of maintaining redox homeostasis to prevent cellular dysfunction and disease progression [4].

The body employs enzymatic and non-enzymatic antioxidant systems that neutralize ROS and repair oxidative damage to counteract oxidative stress. Antioxidants are classified into endogenous (produced within the body) and exogenous (obtained from diet or supplements). The primary enzymatic antioxidants include superoxide dismutase (SOD), catalase, and glutathione peroxidase, which catalyze the breakdown of harmful ROS [5]. Non-enzymatic antioxidants, such as vitamins C and E, polyphenols, and flavonoids, scavenge free radicals and mitigate oxidative damage. Plant-derived antioxidants are particularly significant due to their high bioavailability and minimal side effects [6]. Research has consistently demonstrated their ability to mitigate oxidative damage while exhibiting anti-inflammatory [7], anti-allergy, and anti-cancer [8] properties. These findings underscore the value of polyphenols as key contributors to human health and highlight their potential as therapeutic agents [9]. Various *in vitro* assays, such as the DPPH radical scavenging assay, ferric reducing antioxidant power (FRAP) assay, and nitric oxide scavenging assay, are commonly employed to assess antioxidant potential[10].

Among dietary antioxidants, polyphenols- abundant in fruits, vegetables, and plant-based foods- have gained prominence due to their potent antioxidant effects and additional health benefits [10]. Synthetic antioxidants, like butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), have been associated with toxicological concerns and carcinogenic risks [11]. Butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) have been extensively utilized for their free radical-scavenging abilities, however, multiple studies have raised concerns about their possible carcinogenicity and adverse metabolic effects [12, 13]. Toxicological studies have indicated that BHT and BHA, when administered at high doses (typically 0.1–0.5% in food), can induce liver toxicity, promote tumorigenesis, and disrupt endocrine signaling pathways [13]. These limitations highlight the urgent need for natural, biocompatible alternatives that not only combat oxidative stress effectively but also provide additional health benefits.

In contrast, plant-derived phytochemicals, including polyphenols, flavonoids, tannins, and terpenoids, have demonstrated significant antioxidant activity and health-promoting effects [10]. Other essential antioxidants, including vitamins C and E, beta-carotene, selenium, and zinc, play fundamental roles in sustaining oxidative balance and overall health. The increasing recognition of natural antioxidants highlights their potential in therapeutic applications, reinforcing their importance in maintaining cellular resilience against oxidative damage.

The Barun tree, or *Crateva nurvala*, is an important medicinal plant that has long been valued in traditional medical systems throughout many countries [14]. *Crateva nurvala* is frequently utilized in several Ayurvedic and Unani medicine formularies and has been characterized in numerous noteworthy qualitative studies as having a variety of therapeutic actions [15]. According to reports, *Crateva nurvala's* bark, leaves, and roots contain antioxidant compounds like phenolics (linoleic acid, oleic acid, stearic acid, lauric acid, rutin, quercetin, γ-taraxasterol, lupeol, β-sitosterol acetate, β-sitosterol, and β-epilupeol) and flavonoids (L-stachydrine, rutin, quercetin-3-O-α-D-glucoside, quercetin, isoquercetin, methyl pentacosanoate, kaempferol-O-α-D-glucoside, and dodecanoic anhydride) [16]. Alkaloids, flavonoid glycosides, glucosinolates, triterpenes, tannins, and phytosterols are all abundant in the plant [17]. This plant is also used by practitioners of traditional medicine to treat rheumatic fever, vomiting, gastrointestinal discomfort, kidney and bladder stones, and more [18]. In our recent study, in 2K1C rats, *Crateva nurvala* bark extract enhanced antioxidant capacity and decreased fibrosis, inflammation, cardiovascular problems, and renal impairment [19]. This study aims to bridge this knowledge gap by evaluating the antioxidant potential of Barun bark extracts and their fractions by column chromatography. By analyzing their ability to mitigate oxidative stress, this research seeks to provide a scientific basis for the potential therapeutic applications of Barun bark-derived antioxidants.

2. Materials and Methods

2.1 Plant Material Collection and Extraction

The bark of the *Crateva nurvala* plant was gathered from Mymensingh, Bangladesh's local marketplaces. The National Herbarium in Mirpur, Bangladesh, provided the initial identification and authentication of the plant. An accession number (DACB-78798) was deposited for future use. They sliced the bark into little pieces. All of the bark bits were then pulverised into a powder. After that, 200 gm of powder was immersed in 80% ethanol for seven days. After that, the solvent was filtered via filter paper and decanted. A rotary evaporator was used to condense the extract at 40°C. The result was a sticky, crude extract. The yield as a percentage was computed. The plant extract's final percentage yield from the soaked powder was 33.25% (w/w).

2.2 Different fraction preparation

2.2.1 Column Chromatography Preparation of reagent:

The crude extract was fractionated via column chromatography on silica gel, employing a gradient solvent system (hexane, ethyl acetate, and methanol). Initially, 1 gram of crude extract was processed through the column. Subsequently, a total of 8 grams of crude extract was fractionated in successive runs using the solvent gradient system. This process yielded 13 sub-fractions, which were later grouped into 5 major fractions based on their thin-layer chromatography (TLC) profiles and stored at 4°C for further analysis.

Solvent gradient system: The gradient solvent ratio to be applied in column chromatography is summarized in the following table.

Table 1: Gradient solvent system to be used in column chromatography

Solvent system	Ratio	Volume	Fraction No.
Hexane	100%	100 ml	1
Hexane: Ethyl acetate	90:10	100 ml	2
Hexane: Ethyl acetate	80:20	100 ml	3
Hexane: Ethyl acetate	70:30	100 ml	4
Hexane: Ethyl acetate	60:40	100 ml	5
Hexane: Ethyl acetate	50:50	100 ml	6
Hexane: Ethyl acetate	40:60	100 ml	7
Hexane: Ethyl acetate	30:70	100 ml	8
Hexane: Ethyl acetate	20:80	100 ml	9
Hexane: Ethyl acetate	10:90	100 ml	10
Ethyl acetate	100%	100 ml	11
Ethyl acetate: Methanol	90:10	100 ml	12
Ethyl acetate: Methanol	80:20	100 ml	13
Ethyl acetate: Methanol	70:30	100 ml	14
Ethyl acetate: Methanol	60:40	100 ml	15
Ethyl acetate: Methanol	50:50	100 ml	16
Ethyl acetate: Methanol	40:60	100 ml	17
Ethyl acetate: Methanol	30:70	100 ml	18
Ethyl acetate: Methanol	20:80	100 ml	19
Ethyl acetate: Methanol	10:90	100 ml	20
Methanol	100%	100 ml	21

2.3 Quantitative and Qualitative Analysis through HPLC

2.3.1 Chemicals

Gallic acid, 3,4-dihydroxybenzoic acid, catechin-hydrate, catechol, (-) epicatechin, caffeic acid, vanillic acid, syringic acid, rutin hydrate, p-coumaric acid, trans-ferulic acid, rosmarinic acid, myricetin, quercetin, trans-cinnamic acid, and kaempferol were purchased from Sigma–Aldrich (St. Louis, MO, USA). Acetonitrile (HPLC), methanol (HPLC), acetic acid (HPLC), and ethanol were obtained from Merck (Darmstadt, Germany). α-amylase, 1, 1-diphenyl-2-picrylhydrazyl (DPPH), and naphthyl ethylenediamine dihydrochloride were obtained from Sigma-Aldrich Chemical Co. (USA). Starch soluble (extra pure) was obtained from J.T. Baker Inc., Phillipsburg, USA. Other chemicals and reagents used were of analytical grade.

2.3.2 High-performance liquid chromatography (HPLC) Analysis

Detection and quantification of polyphenolic compounds in the *Crateva nurvala* bark extracts were determined by HPLC-DAD analysis as described by Ahmed et al. (2021) with some modifications [20]. HPLC analysis was performed on a LC-20A (Shimadzu, Kyoto, Japan) equipped with a binary solvent delivery pump (LC-20AT), an auto sampler (SIL-20A HT), column oven (CTO-20A) and a photodiode array detector (SPD-M20A) and controlled by the LC solution software (Lab Solution Separation was performed using Luna C18 (5µm) Phenomenex column (4.6 x 250 mm) at 33ºC. The mobile phase composed of A (1% acetic acid in acetonitrile) and B (1% acetic acid in water) with gradient elution: 0.01-20 min (5-25% A), 20-30 min (25-40% A), 30-35 min (40-60% A), 35-40 min (60-30% A), 40–45 min (30–5% A), and 45–50 min (5% A) was used in this study. The sample injection volume was 20µL, and the flow rate was set at 0.5 mL/min. The UV detector was set at 270 nm and applied for validation of the method and analysis. The mobile phase was filtered through a 0.45 µm nylon 6, 6 membrane filter (India) and degassed under vacuum. For the preparation of calibration curve, a standard stock solution was prepared in methanol containing Gallic acid (20 µg/ml); 3,4-dihydroxybenzoic acid (15 µg/ml); catechin hydrate (50 µg/ml); catechol, (-) epicatechin, rosmarinic acid (30 µg/ml each); caffeic acid, vanillic acid, syringic acid, rutin hydrate, p-coumaric acid, trans-ferulic acid, quercetin (10 µg/ml each); myricetin, kaempferol (8 µg/ml each); trans-cinnamic acid (4 µg/ml).

2.4 Total Antioxidant Capacity Assay

The phosphomolybdenum method, as described by Prieto and his colleagues, was used to assess the extract's antioxidant activity [34]. As the extract reduces Mo (VI) to Mo (V), a green phosphate/Mo(V) complex is formed at acidic pH, which serves as the basis for the assay. A reagent solution consisting of 0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate was mixed with 0.3 ml of extract. For ninety minutes, the reaction solution-containing tubes were incubated at 95°C. After the solution had cooled to room temperature, its absorbance at 695 nm was measured with a spectrophotometer against a blank. As a blank, 0.3 ml of methanol was used in place of the extract. The number of equivalents of ascorbic acid is used to express antioxidant activity.

2.5 In vitro Antioxidant Assays

2.5.1 DPPH (1, 1-Diphenyl-2- Picrylhydrazyl) Radical Scavenging Assay

In HPLC grade methanol, a DPPH solution(0.004% w/v) was prepared [21]. To make the stock solution (500 µg/mL), each of the five fractions (A, B, C, D & E) and the *Crateva nurvala* crude extract was combined individually with Milli-Q water 1mL extracts were taken to the test tubes of serially diluted different concentrations (12.5 µg/mL to 500 µg/mL), and then a freshly made 1mL DPPH solution (0.004% w/v) was added. So, the total volume was 2 mL, and after 10 minutes of incubation in a dark place, the absorbance was measured at 515 nm using a spectrophotometer (HACH 4000 DU UV-visible spectrophotometer). As a reference standard, ascorbic acid was dissolved in Milli-Q water to create a stock solution with the same strength (500 mg/mL). The percent scavenging of the DPPH free radical activity was measured by using the following equation:

% of inhibition = [(absorbance of the control – absorbance of the test sample) / absorbance of the control] X 100

Lower absorbance of the reaction mixture indicates higher free radical-scavenging activity. All the tests were performed in triplicate.

2.5.2 Nitric Oxide (NO) Radical Scavenging Assay

For the NO radical scavenging assay, a previously described method was used [22]. At a physiological pH, sodium nitroprusside solution produced nitric oxide (NO) radicals. In phosphate buffer, 1 ml of sodium nitroprusside (10 mM) was combined with 1 ml of ethanolic extract and fractions of various concentrations (12.5 - 150 g/ml) (pH 7.4). 150 minutes were spent incubating the mixture at 25°C. Griess' reagent (1% sulfanilamide, 2% o-phosphoric acid, and 0.1% naphthyl ethylene diamine dihydrochloride) was added to 1 ml of the incubated solution. The absorbance was measured at 546 nm, and the formula for % inhibition was applied are mentioned below:

% of inhibition = [(absorbance of the control – absorbance of the test sample) / absorbance of the control] X 100

2.5.3 Ferric Reducing Power Assay

The method outlined by Oyaizu was used to determine the reducing power of *Crateva nurvala* extract and fractions [23]. The reducing power of the fractions was evaluated by mixing 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of potassium ferricyanide (1%) with 1 mL of each fraction. The mixture was incubated at 50°C for 20 minutes, followed by the addition of 2.5 mL of trichloroacetic acid (10%). After centrifugation at 3000 rpm for 10 minutes, 2.5 mL of the supernatant was mixed with 2.5 mL of distilled water and 0.5 mL of ferric chloride (0.1%). The absorbance was measured at 700 nm. Ascorbic acid was used as the standard. A phosphate buffer (pH 6.6) was used as the blank solution.

3. Results and Discussion:

Reactive nitrogen and oxygen species (RNS/ROS) are double-edged biological entities that exert both beneficial and detrimental effects depending on their concentration. At physiological levels, they contribute to critical processes such as cell signaling, redox homeostasis, mitogenic responses, and immune modulation. However, under pathological conditions, excessive ROS and RNS production lead to oxidative and nitrosative stress, resulting in the oxidation of proteins, lipids, and nucleic acids, ultimately impairing cellular function and promoting disease progression [25, 26].

3.1 HPLC-Detected Polyphenolics

High-performance liquid chromatography (HPLC-DAD) analysis of *Crateva nurvala* bark extract revealed the presence of several potent phenolic compounds. The most abundant was p-coumaric acid (2)(203.09 ± 0.67 mg/100g) [27, 28], followed by (-) epicatechin (1) (39.77 ± 0.46 mg/100g)[29, 30] and myricetin (3) (37.35 ± 0.32 mg/100g) [31, 32]. These compounds are recognized for their strong antioxidant properties. p-Coumaric acid (2) has demonstrated the ability to directly scavenge reactive oxygen species (ROS) and upregulate endogenous antioxidant enzymes. It also exhibits anti-inflammatory and cardioprotective effects. Epicatechin (1) and myricetin (3), two well-known flavonoids (**Figure 1**), contribute to radical scavenging through their hydroxyl-rich structure and redox potential [10]. Their documented activities include inhibition of lipid peroxidation, ROS neutralization, and enhancement of cellular antioxidant defenses. The high concentrations of these compounds likely underpin the strong antioxidant performance seen across all assays in this study.

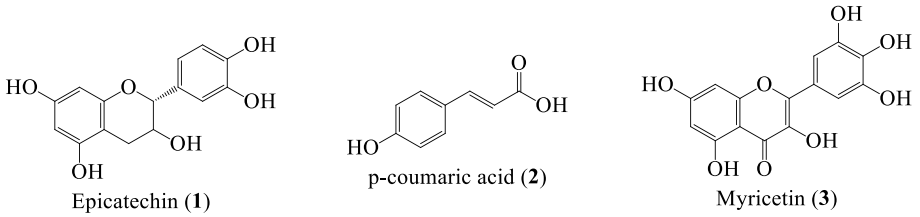
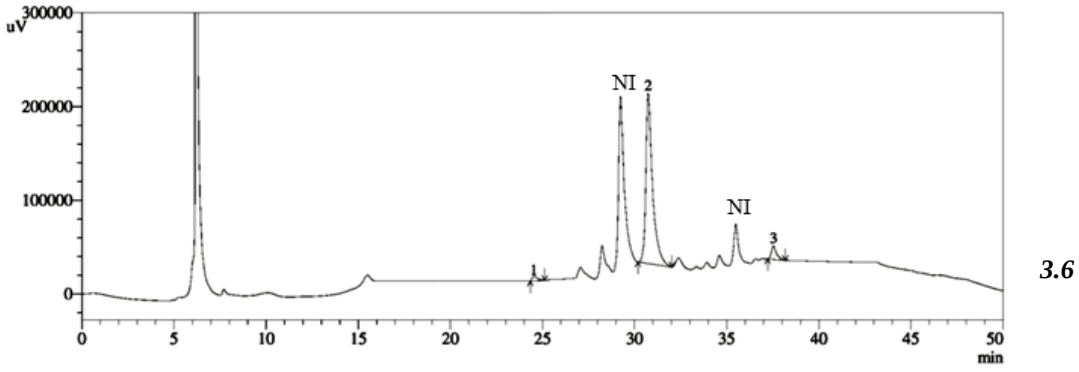


Figure 1: Identified phenolic acids and flavonoids in the ethanolic extract of *Crateva nurvala*

The HPLC - DAD chromatogram of *Crateva nurvala* extract is presented in **Figure 2**. Several bioactive antioxidants such as epicatechin, p-coumaric acid, and myricetin were detected in the sample. Among them, p-coumaric acid (203 mg/100 g dry extract) was found in high amount in *Crateva nurvala* extract.

Table 2: HPLC data of available standard compounds found in *Crateva nurvala* bark extract

Peak no.	Name of standard compounds	Retention time (min)	*Crateva nurvala* (mg/100g dry extract)
1	Gallic acid	ND	ND
2	3,4-Dihydroxybenzoic acid	ND	ND
3	Catechin hydrate	ND	ND
4	Catechol	ND	ND
5	(-) Epicatechin	24.56	39.77±0.46
6	Caffeic acid	ND	ND
7	Vanillic acid	ND	ND
8	Syringic acid	ND	ND
9	Rutin hydrate	ND	ND
10	p-Coumaric acid	30.75	203.09±0.67
11	trans-Ferulic acid	ND	ND
12	Rosmarinic acid	ND	ND
13	Myricetin	37.55	37.35±0.32
14	Quercetin	ND	ND
15	trans-Cinnamic acid	ND	ND
16	Kaempferol	ND	ND



3.6

Figure 2: HPLC chromatogram of *Crateva nurvala* bark extract showing detected phenolic compounds- p-coumaric acid, (-) epicatechin, and myricetin, ND = Not detected.

3.2 Total antioxidant capacity of Crateva nurvala bark extract and its fractions with ascorbic acid (standard)

The total antioxidant capacity of the crude ethanol extract of *Crateva nurvala* bark and its fractions with ascorbic acid is presented in **Figure 3**. The extract and sub-fractions showed increased total antioxidant capacity in a concentration-dependent manner compared to the ascorbic acid.

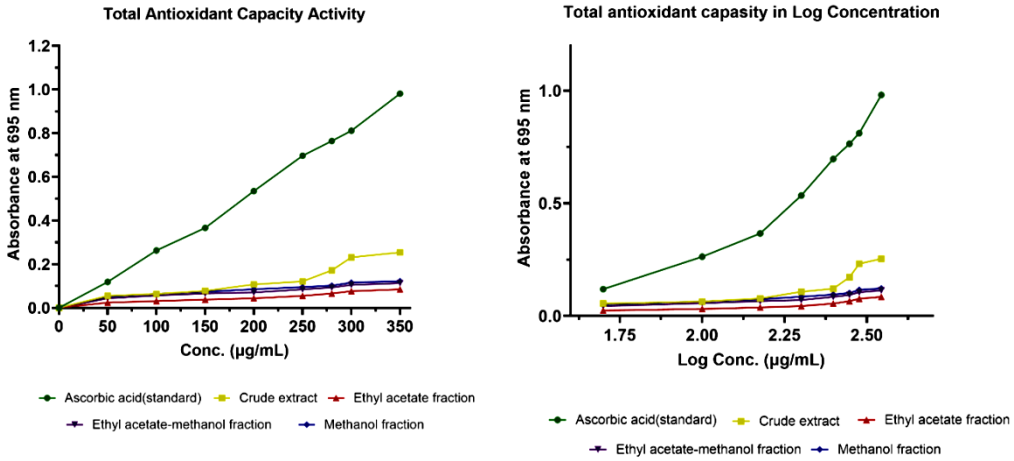


Figure 3: Total antioxidant capacity of *Crateva nurvala* bark crude ethanol extract and its fractions with ascorbic acid (standard)

The total antioxidant capacity, assessed using the phosphomolybdenum method, reflects the overall reducing capacity of all antioxidants in the sample. The assay revealed that the methanol fraction showed the highest TAC, followed by the crude extract and the ethyl acetate: methanol fraction. Results were expressed in µg/mL of ascorbic acid equivalent (AAE) per mg of extract, with values indicating strong reducing potential across all fractions. The TAC results complement the radical scavenging and FRAP data, suggesting that *Crateva nurvala* bark consists of several strong and complex phenolic compounds; for this, the antioxidant activity of the extract and its fractions is considerably high. This includes both high-molecular-weight polyphenols and low-molecular-weight secondary metabolites, many of which have been characterized in the HPLC chromatogram. The results are further supported by studies on other polyphenol-rich Bangladeshi medicinal plants, where TAC values showed similar or lower ranges [9, 20].

The antioxidant potential of *Crateva nurvala* bark extract and its chromatographic fractions was evaluated using multiple *in vitro* assays, and results indicated promising activity across all tested parameters. Each assay offers insight into different mechanisms of antioxidant defense, and when considered collectively alongside the HPLC-detected polyphenolics, these data support the extract's therapeutic potential against oxidative stress.

3.3 DPPH Radical Scavenging Activity of Crateva nurvala bark crude extract and its fractions with ascorbic acid (standard)

Currently, interest has vigorously increased in the naturally occurring antioxidants from natural sources due to their safety margin in the reduction of human oxidative stress and free radical scavenging DPPH method is an easy, rapid, and sensitive way to determine the antioxidant activity of a specific compound or plant extracts [24]. DPPH radical scavenging activity of the ethanolic extract of *Crateva nurvala* and its fraction was found to increase with increasing concentration, as shown in **Figure 4**. Crude extract showed the maximum DPPH scavenging activity of 85.46% at a higher concentration of 250 µg/mL **Figure 4** but the other fractions also showed a considerable quantity of DPPH scavenging activity. There was a respectable correlation revealed between the DPPH scavenging assay and the total antioxidant activity result **Figure 4**.

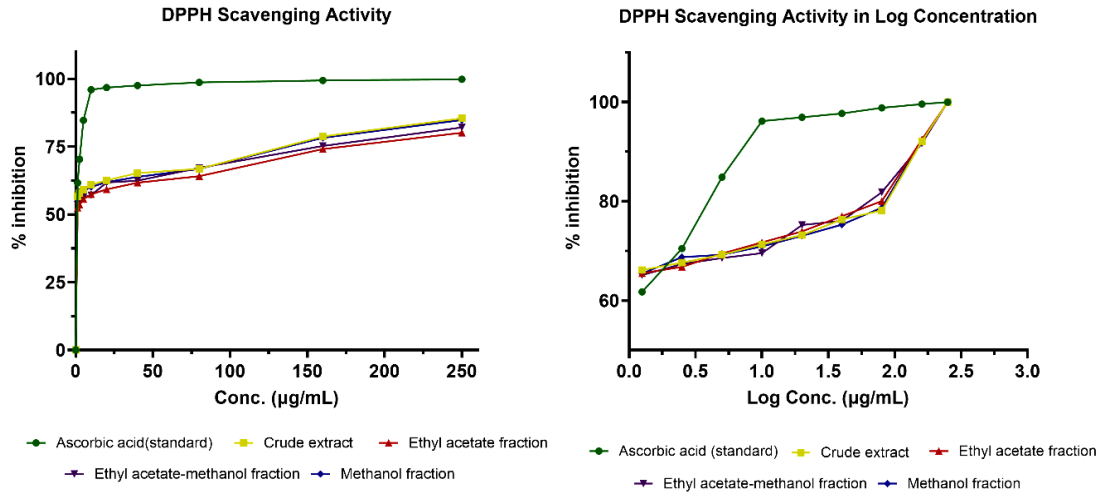


Figure 4: DPPH scavenging activity of *Crateva nurvala* bark crude ethanol extract and its fractions with ascorbic acid (standard).

The DPPH assay showed that all tested samples had significant radical scavenging capacity. The IC_{50} value for ascorbic acid was 0.8579 µg/mL, indicating high antioxidant potential. Among the test samples, the *Crateva nurvala* crude extract exhibited an IC_{50} of 3.276 µg/mL, followed closely by the methanol fraction (3.436 µg/mL). The ethyl acetate: methanol (4.291 µg/mL) and ethyl acetate (4.841 µg/mL) fractions also showed notable activity. Compared to previous reports, triterpenes from *Crateva nurvala* showed DPPH IC_{50} values near 95 µg/mL, the current results are remarkably potent. These low IC_{50} values suggest that the extract and its fractions possess excellent hydrogen-donating abilities, likely due to the presence of abundant phenolics identified in the HPLC analysis [16]. The antioxidant efficacy can be attributed to the presence of bioactive polyphenols, particularly (-) epicatechin (1) and myricetin (3), both of which are well-documented DPPH scavengers [33].

The comparison between total antioxidant capacity (TAC) vs DPPH radical scavenging activity are presented in **Figure 5**. Compare to ascorbic acid the extract and subfractions showed promising radical scavenging potential.

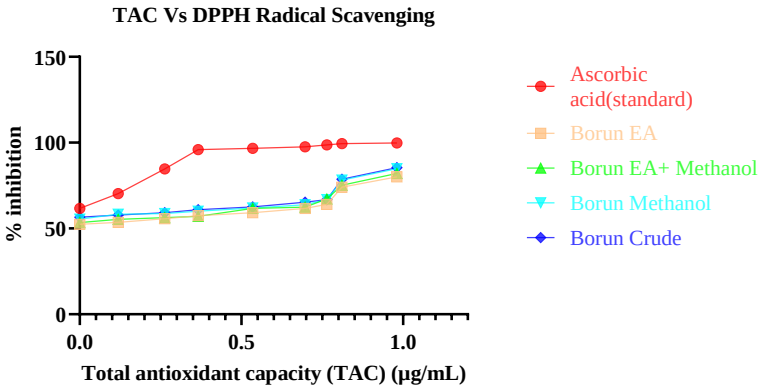


Figure 5: Comparison between Total antioxidant capacity (TAC) and DPPH free radical scavenging activity of the ethanolic bark extract of *Crateva nurvala* and its different fractions.

3.4 NO Scavenging Activity of Crateva nurvala bark crude ethanol extract and its fractions with ascorbic acid (standard).

The Nitric Oxide (NO) scavenging activity of *Crateva nurvala* bark crude ethanol extract and its fractions with ascorbic acid (standard). The extract showed the strong NO scavenging activity compared to the ascorbic acid. The IC_{50} value for ascorbic acid was found 2.979 µg/mL while for the *Crateva nurvala* extract possesses IC_{50} value of 7.865 µg/mL (**Table 2**)

In the NO radical scavenging assay, ascorbic acid showed an IC_{50} of 2.979 µg/mL. Among the test samples, the crude extract exhibited an IC_{50} of 7.865 µg/mL, with slightly reduced activity in the methanol (8.371 µg/mL), ethyl acetate: methanol (9.413 µg/mL), and ethyl acetate (10.05 µg/mL) fractions. This high level of NO inhibition indicates a substantial ability to neutralize reactive nitrogen species. Earlier studies reported IC_{50} values >20 µg/mL for *Crateva nurvala* leaf extracts [34]. The observed efficacy is further supported by the presence of myricetin (3) and (-) epicatechin (1), both of which are capable of neutralizing reactive nitrogen species (RNS) and preventing peroxynitrite formation [35].

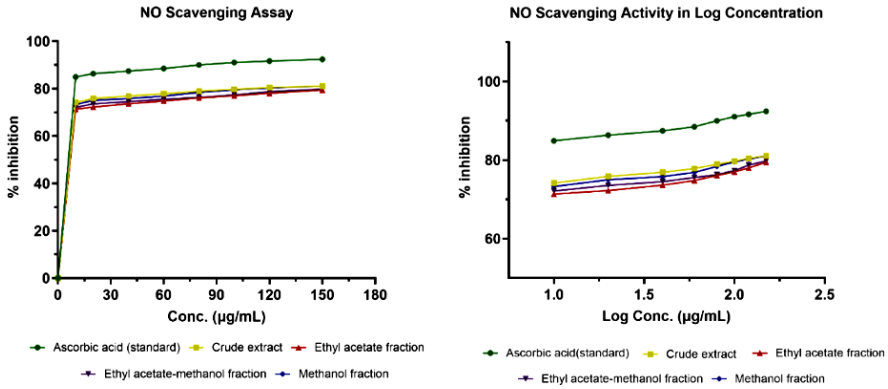


Figure 6: NO scavenging activity of *Crateva nurvala* bark crude ethanol extract and its fractions with ascorbic acid (standard)

3.5 Hydroxyl radical scavenging activity

Hydroxyl radical scavenging was the least potent among all assays, with ascorbic acid showing an IC_{50} of 105.8 µg/mL. The *Crateva nurvala* crude extract (IC_{50} = 156.5 µg/mL) was slightly more effective than the methanol fraction (157.5 µg/mL) and ethyl acetate: methanol fraction (171.6 µg/mL). The ethyl acetate fraction (190.8 µg/mL) showed the least activity. The hydroxyl radical (•OH) scavenging activity indicated moderate-to-strong inhibition. The hydroxyl radical is among the most reactive and damaging ROS, and its neutralization is a key indicator of antioxidant strength. Studies on similar polyphenols show that epicatechin and myricetin effectively inhibit •OH-mediated damage, with IC_{50} values of 12.3 µM and 15.8 µM, respectively [9]. These findings corroborate the effectiveness of the tested extract in mitigating oxidative insults associated with hydroxyl radicals.

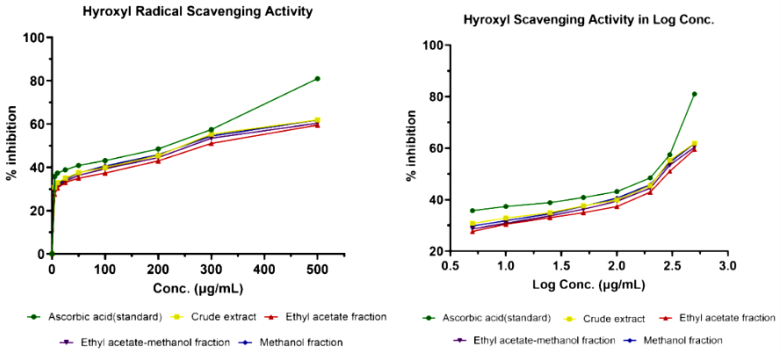


Figure 7: Hydroxyl radical scavenging activity of *Crateva nurvala* crude extract and its fractions with ascorbic acid (standard)

3.6 Ferric Reducing Activity Assay

The ferric reducing power assay evaluates the electron donating capacity of antioxidants, reflecting their potential to reduce Fe^{3+} to Fe^{2+} [36]. The increasing absorbance at 700 nm with sample concentration indicates a dose-dependent enhancement of reducing capacity. Among the tested fractions, the methanol and crude extracts exhibited the highest absorbance values, suggesting a stronger reducing ability. The mechanism behind this activity is typically linked to the presence of phenolic hydroxyl groups that can donate electrons to oxidized intermediates, thus terminating free radical chain reactions. Given the presence of (-) epicatechin (2) and myricetin (3), which have strong redox potentials, the observed FRAP activity aligns well with the HPLC findings. This result is also in agreement with previous studies, where high phenolic content was associated with superior ferric reducing ability in medicinal plant extracts [37]. These findings reinforce the role of *Crateva nurvala* fractions as effective electron donors capable of stabilizing oxidative intermediates.

Collectively, the results from all antioxidant assays and HPLC profiling confirm the strong antioxidant capacity of *Crateva nurvala* bark extract, with activities mediated primarily by phenolic compounds such as p-coumaric acid (2), (-) epicatechin (1), and myricetin (3). Based on IC_{50} values across all antioxidant assays, the general order of radical scavenging activity for the tested fractions was:

Ascorbic acid > *Crateva nurvala* crude extract > Methanol fraction > Ethyl acetate: methanol fraction > Ethyl acetate fraction.

All the fractions showed considerable antioxidant activity, because the ethyl acetate and methanol fractions can retain phenolic compounds due to their higher polarity and better solubility for phenolic components present in plant materials [38]. This trend was consistent for DPPH, nitric oxide, and hydroxyl radical assays, highlighting the superior efficacy of the crude and methanol fractions, likely due to their higher concentration of bioactive phenolics such as epicatechin (1) and myricetin (3). These findings substantiate the therapeutic relevance of *Crateva nurvala* in mitigating oxidative stress and validate its traditional use as a medicinal plant.

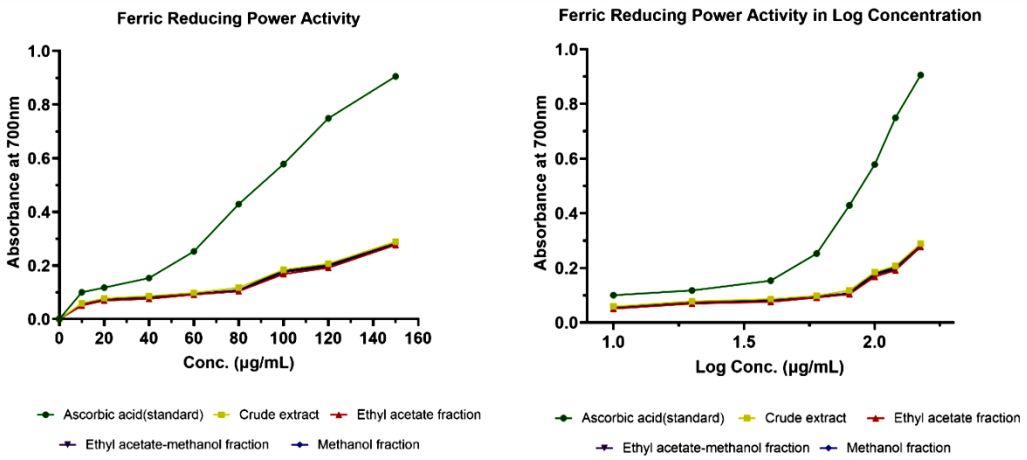


Figure 8: Ferric reducing power activity of *Crateva nurvala* crude extract and its fractions with ascorbic acid (standard)

3.7 IC_{50} values of Crateva nurvala bark extract and its fractions in different antioxidant assays

Table 2: IC_{50} values of *Crateva nurvala* bark extract and its fractions in different antioxidant assays,

Sample	DPPH Scavenging Method (µg/mL)	NO Scavenging Method (µg/mL)	H_3O Scavenging Activity (µg/mL)
Ascorbic Acid	0.8579	2.979	105.8
Crateva nurvala Crude	3.276	7.865	156.5
Ethyl acetate fraction	4.841	10.05	190.8
Ethyl acetate: methanol fraction	4.291	9.413	171.6
Methanol fraction	3.436	8.371	157.5

4. Conclusion:

The growing concerns over their long-term toxicity and the rising cost of synthetic pharmaceuticals have intensified global interest in traditional, plant-based medicine. The profuse growth of *Crateva nurvala* ensures the availability of this plant in this subcontinent region and its use as an herbal complementary medicine. *Crateva nurvala*, long valued in ethnomedicine, offers a promising natural alternative with its rich reservoir of bioactive phytochemicals. In this study, comprehensive HPLC-DAD analysis identified key antioxidant compounds- p-coumaric acid (2), (-) epicatechin (1), and myricetin (3)- which demonstrated strong radical scavenging and reducing power across multiple in vitro assays. Among the tested samples, the methanol fraction and crude extract exhibited the most potent antioxidant effects, with low IC_{50} values and high total antioxidant capacity. These results not only validate the traditional therapeutic claims of *Crateva nurvala* but also address a critical scientific gap by providing quantified biochemical evidence of its efficacy. Given their potential for low toxicity, affordability, and therapeutic effectiveness, plant-derived antioxidants like those in *Crateva nurvala* are increasingly vital in modern healthcare. This study underscores the importance of bioprospecting and phytopharmacological evaluation in discovering safe, natural agents for managing oxidative stress and related metabolic disorders. Thus, *Crateva nurvala* fractions may hold promise as cost-effective nutraceuticals or complementary therapies in the prevention of chronic diseases.

Author Contributions: Conceptualization, MA and NS; methodology, MA, SS, and HCG; software, MA and SS; validation, MA, NS, and HH; formal analysis, MA, MAA, and NS; investigation, ASMS, NMS, SAE, SM and NJ; resources, NJ and RB; data curation, MA and KSA; writing—original draft preparation, SS, HCG; writing—review and editing, MA; visualization, MA, NS; supervision, MA and NS project administration, MA; funding acquisition, MA. All authors have read and agreed to the published version of the manuscript.

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Original Article

Evaluation of Analgesic, and CNS Depressant Activities of Ethanolic Extract of Roots of *Heritiera fomes* Buch.- Ham.

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Abstract: The purpose of the study was to examine analgesic and neuropharmacological effects of ethanolic extract of root of *Heritiera fomes* Buch.-Ham (RHF). All the tests were conducted on Swiss albino mice in three distinct doses (100, 200, 300 mg/kg body weight). The peripheral and central analgesic actions in rodents were investigated using the acetic acid-induced writhing test and the tail immersion test, respectively. The extract significantly ($p < 0.05$, $p < 0.01$) prolonged the latency period to the thermal stimuli in the tail immersion method in a dose-dependent manner which was comparable to the reference standard Morphine. The extract (300 mg/kg) produced a maximum of 68.08% inhibition ($p < 0.01$) of the writhing reflex in the acetic acid-induced writhing test, compared to the reference medicine Diclofenac-Na (10 mg/kg) (76.45%). Hole cross, open field, and thiopental sodium-induced sleeping time tests were carried out to assess its CNS depressant action. According to statistical analyses, the extract makes mice sleep longer when they are exposed to thiopental sodium. RHF showed a significant ($P<0.05$, $P<0.01$) diminution in locomotion of rodents in both open field and hole cross methods at utmost dose in comparison with control group. Taken together, these findings imply that *H. fomes* root extract has strong analgesic and central nervous system depressive qualities. Advanced investigations are required for extensively phytochemical screening and establishing precise mechanisms of action.

Keywords: *Heritiera fomes* Buch.-Ham; analgesics; CNS depressant; thiopental sodium-induced sleeping time test, hole cross, open field

1. Introduction

Since the dawn of human civilization, medicinal plants that contain bioactive compounds have served as a crucial source of therapeutic drugs. Some estimates claim that about 80% of modern medications are either directly or indirectly derived from medicinal herbs. They act as significant natural resources for the production of traditional and modern healing agents [1, 2, 3]. Due to their antidiabetic, anti-inflammatory, antimicrobial, anti-carcinogen, sedative, depressive, anxiolytic, anticonvulsant, antispasmodic, antimalarial, antidiarrheal and

anti-HIV characteristics, medicinal plants with a high concentration of bioactive phytochemicals are still popular as remedies in developing countries [4].

Mangroves are halophile plants with important economic and environmental ecosystem [5]. Scientists have become interested in them over time due to their unique morphology, ecology, and applications. The local traditional healers and the rural residents who live in these areas frequently employ them for the treatment of leprosy, elephantiasis, tuberculosis, malaria, dysentery, ulcers and some skin diseases. The numerous ways that various mangrove plant species have been used to relieve human suffering provide persuasive evidence of their medicinal value and urge further study. However, there is a dearth of information on mangroves because of the rarity of these species as well as the difficulties in collecting and identifying them. Additionally, there is a lack of relevant knowledge regarding their ethnomedicinal usage [6, 7].

Heritiera fomes Buch. Ham. is an imperative moderate-sized mangrove plant that grows mainly in the world's single largest mangrove forest, Sundarbans, which extends across Bangladesh and West Bengal of India. It is also noticed in coastal regions of India, Myanmar, Thailand, and Northern Malaysia. Over the years traditional medicine practitioners extensively used these plant parts to treat a variety of ailments including diarrhea, dysentery, constipation, indigestion, diabetes, cancer, skin disorders like dermatitis, rash, eczema, boils, itch, scabies, sores, and infections, as well as hepatic disorders like jaundice and hepatitis. Additionally, the plant showed anti-inflammatory, anti-nociceptive, antimicrobial, wound-healing, antioxidant, and insect-repelling properties in previous investigations [8, 9, 10, 11, 12]. The earlier studies revealed the presence of valuable bioactive components in *H. fomes*, such as alkaloids, glycosides, flavonoids, saponins, polysaccharides, phenols, gums, sterols, and procyanidins. trimeric, pentameric, hexameric procyanidins, β-Sitosterol, stigmasterol and stigmast-4-en-3-one partially of which were further confirmed in our phytochemical screening [13, 14, 15].

Despite the high concentration of bioactive components, the literature study showed that the root of *H. fomes* has received very little scientific attention. This inspired us to conduct a thorough investigation into the ethanolic extractive and analgesic and neuropharmacological qualities of the aforementioned plant's root, bearing in mind the worldwide need for natural mineral supplements and bioactive substances derived from roots.

2. Materials and Methods

2.1 Plant sample collection, identification, and processing technique

Fresh root of *H. fomes* were collected from Sundarbans, Bagerhat district, Bangladesh in 2023. They were kept in our lab for future reference after being identified and verified by a taxonomist from the Bangladesh National Herbarium in Mirpur, Dhaka (DACB Accession No: 50664). To eliminate dirt, the collected plant roots were carefully washed in running tap water, followed by a rinsing in distilled water. Then the roots were cut into small pieces, sorted, and dried in the shade for 14 days prior to getting pulverized into a coarse powder in a lab electric grinder. 700 g root powder of the plant was taken into a clean glass container and submerged in 2.0 liters of ethanol for two weeks with frequent shaking and agitation. The liquid was then filtered into a different clean beaker with the number 1Whatman filter paper [16]. For future assessment, the powdered plant material was kept apart in airtight containers in a cool, dark, and dry environment.

2.2 Chemicals and reagents

We obtained the following drugs from Incepta Pharmaceuticals Ltd. in Bangladesh: morphine, diclofenac sodium, thiopental sodium, and diazepam. All chemicals and reagents, including acetic acid, and tween 80, were of analytical grade (Merck, Darmstadt, Germany).

2.3 Phytochemical Analysis

The freshly prepared crude extract was qualitatively analyzed for the existence of bioactive phytochemicals to relate the activities of the *in vivo* pharmacological tests by using standard techniques [17].

2.4 Animals

Swiss-albino healthy adult male mice (weighing 35–45 g and aged 4-5 weeks) were used for the *in vivo* studies. They were bought from the Animal Resource Branch of the International Centre for Diarrheal Diseases and Research, Bangladesh (ICDDR, B). Because of the high vulnerability of the animals to environmental divergence, rodents were accommodated in plastic cages with free access to pelleted food and tap water under typical animal house conditions (temperature: 28 to 31°C, photoperiod: approximately 12h of natural light per day, and relative humidity: 55–60%) for at least 3 - 4 days for acclimation. The study was conducted in conformity with State University of Bangladesh's Institutional Ethical Review Committee's (SUB-IERC) norms and procedures, as well as generally accepted worldwide standards for the use and care of animals. All surgical interventions were accomplished under ether anesthesia via aseptic techniques [2, 18].

2.5 Animal experimental design

Rodents were divided into the following groups each with six animals. The vehicle (1% tween 80 in normal saline, 10 mL/kg) was administered to Group I as the negative control, whilst the reference drug for the specific test was administered to Group II as the positive control. Groups III–V received RHF (100, 200, and 300 mg/kg, respectively).

2.6 Analgesic activity

2.6.1 Peripheral analgesic activity

The peripheral analgesic activity of roots of *H. fomes* was estimated by acetic acid-induced writhing test [2, 19]. Similar to the Central analgesic test design, both standard diclofenac sodium and tested plant sample were administered to the experimental mice by oral route (Group I- Group V). After 45 minutes, 0.7% (v/v) acetic acid was intraperitoneally injected to each rodent at a dose of 10 mL/kg body weight. Fifteen minutes after the administration of acetic acid, the number of writhing (contraction of the abdomen) responses was counted for each animal during a 5-min period. Besides, the mean abdominal writhing for each group was calculated. The percent inhibition of writhing was computed with the following formula:

$$\% \text{ inhibition of writhing} = \frac{N_{Control} - N_{Test}}{N_{Control}} \times 100$$

In the above formula, N denotes the mean number of abdominal writhing for each group.

2.6.2 Central analgesic activity

Central analgesic activity was evaluated by following the previous protocol [19]. A total of 36 mice were used in this investigation; each group consisted of six animals and included the control group (a vehicle), reference group (morphine, 2 mg/kg body weight), and test group (RHF at 100, 200, and 300 mg/kg body weight). Here, the thermal stimulation caused by dipping the tip of the tail in warm water-induced painful reactions in rodents. Mice were grouped and treated as described earlier. Morphine was used as the reference drug. After the treatment of each group, the basal response time was measured by submerging the last 1-2 cm of the mice's tail tips of the mice in hot water of (55 ± 1) °C. The flick reaction of mice, i.e., time taken (in second) to remove it from the hot water source was calculated and outcomes were compared with the control group. To avoid rodents agitation a fifteen-second latency period was chosen as the cut-off threshold. The latent period of the tail-flick response was recorded at 0 min and after 30, 60 and 90 min of drug and extract administration.

2.7 Evaluation of neuropharmacological activity

Neuropharmacological activity of plant extract was evaluated by open-field, hole-cross and thiopental sodium induced sleeping time methods.

2.7.1. Open field method

The open field behavioral test is widely used to assess the emotional state and locomotor activity of rodents [20]. The test was conducted using the approach described in Sultana et al., 2018, with a few minor adjustments. Three test groups, each with five mice, were formed from the animals, along with a control and a positive control. Oral extract was administered to the test groups at doses of 100, 200, and 300 mg/kg body weight, whereas distilled water (0.1 mL/mouse, p.o.) was given to the control group. Using i.p., diazepam (1 mg/kg) served as the positive control group. There were squares all throughout the vast field. Black and white are applied separately to each square. The equipment featured a 40 cm-tall wall. At intervals of 0, 30, 60, 90, and 120 minutes, the animals were brought into the squares, and the number of squares visited was counted for three minutes.

2.7.2. Hole cross method

The strongest alterations in behavior come from a strong emotional reaction to a new environment. The prior technique was followed in order to conduct the hole cross test [20] a 30 cm by 20 cm by 14 cm cage with a partition in the center. The device is made up of hardwood planks. At a height of 7.5 cm, a hole with a diameter of 3 cm was drilled in the middle of the cage. A mouse was positioned in the center of the cage, and oral treatments (vehicle, extracts, and standard) were given. At 0, 30, 60, 90, and 120 minutes, the number of mice that passed through the aperture connecting one chamber to the next was counted for three minutes.

2.7.3. Thiopental sodium induced sleeping time test

The animals in this experiment were divided into five groups, each with five mice. When the control group was given distilled water (0.1 ml/mouse, p.o.), the test groups anticipated the extract at doses of 100, 200, and 300 mg/kg. As a positive control, conventional medication diazepam (1 mg/kg, intraperitoneally) was employed. Thirty minutes later, thiopental sodium (40 mg/kg, i.p.) was administered to each mouse in order to put them to sleep. The rodents were kept under observation by positioning them in separate chambers throughout the duration of their sleep and the latent period, which is the interval between the loss and recovery of the righting reflex after administering thiopental sodium [20].

2.8 Statistical analysis

The results were expressed as mean ± SEM (Standard Error of Mean). For statistical comparisons, we used one-way analysis of variance (ANOVA) followed by Dunnett's t-test. $P< 0.05$ was considered as the threshold for statistical significance in all tests, which were conducted using SPSS (Statistical Package for Social Sciences) version 28.0.

3. Results

3.1. Phytochemical screening

The experimented extract of root of *H. fomes* revealed the tested positive for alkaloid, carbohydrate, tannins, flavonoid, saponin, glycoside, steroid, phenol and resin (**fig 1**).

Table 1. Screening of bioactive phytocompounds in different extracts of *H. fomes*

Phytocompounds	RHF
Carbohydrate	+
Glycoside	+
Tannin	+
Alkaloid	+
Saponin	+
Resin	+
Phenol	+

Phytocompounds	RHF
Flavonoid	+
Steroid	+
Fixed oil	+

Here, RHF= ethanolic extract of root of *H. fomes*, respectively; + = present; - = negative

3.2 Analgesic Tests

3.2.1 Peripheral Analgesic Activity

According to **Table 2**, the oral administration of the ethanolic root extract of *H. fomes* (100–300 mg/kg) significantly inhibited (P <0.05) the nociception brought on by acetic acid. The extract produced maximal protection at a dose of 300 mg/kg (65.56% writhing inhibition) compared to standard (76.45% writhing inhibition). Furthermore, the findings showed that lower dosages have modest to moderate peripheral analgesic activity.

Table 2. Peripheral analgesic effect of *H. fomes* extract on mice by writhing test

Group	Treatment	Number of writhing	% of Inhibition
Control (I)	Tween 80 solution	19.83 ± 1.47	---
Standard (II)	Diclofenac sodium 10 mg/kg	4.67 ± 1.51**	76.45
III	RHF 100 mg/kg	12.33 ± 1.03	37.82
IV	RHF 200 mg/kg	9.00 ± 0.89*	54.61
V	RHF 300 mg/kg	6.83 ± 0.41*	65.56

Each value represents mean ± SEM for n = 6, ** p < 0.01, and * p < 0.05 vs. control.

3.2.2.Central Analgesic Activity

Table 3 displays the outcomes of the central analgesic effect of *H. fomes* extract in the tail immersion method. In comparison to the reference drug, diclofenac sodium, all extracts significantly (p<0.05) exhibited a strong escalation in pain reaction time (PRT) in a dose-dependent manner. At 90 minutes after loading plant extracts, root extract demonstrated an increased pain reaction time of 9.17±0.90 at the largest dose in comparison to the standard 18.25±0.33.

Table 3. Central analgesic effect of *H. fomes* extract on mice by tail immersion method.

Group	Treatment	Average Time of Tail Immersion of Mice			
		Time (in Sec) after Loading the Plant extracts/Drug Standard			
		0 min	30 min	60 min	90 min
Control	Tween 80 solution	2.63 ± 0.22	2.55 ± 0.26	2.98 ± 0.54	3.39 ± 0.24
Standard (II)	Morphine 2 mg/kg	1.65 ± 0.04*	6.41 ± 0.23***	10.73 ± 0.15**	18.25 ± 0.33***
III	RHF 100 mg/kg	2.31 ± 0.10	4.42 ± 0.16*	5.48 ± 0.94	8.16 ± 0.69*
IV	RHF 200 mg/kg	2.06 ± 0.8*	3.40 ± 0.19*	5.88 ± 0.54*	7.27 ± 0.82
V	RHF 300 mg/kg	1.88 ± 0.08*	5.31 ± 0.19	6.39 ± 0.12*	9.17 ± 0.90*

Each value represents mean ± SEM for n = 6, *** p < 0.001, ** p < 0.01, and * p < 0.05 vs. control.

3.3 Neuropharmacological activity

3.3.1 Open Field Method

At 100, 200, and 300 mg/kg body weight, the extract dramatically reduced the locomotor activity in mice ($p<0.05$; $p < 0.01$), and this effect was visible from 60 to 120 minutes (**Table 4**). As anticipated, mice given 1 mg/kg, i.p. of diazepam demonstrated a discernible reduction in movement from 30 to 120 minutes of observation.

Table 4. Neuropharmacological effect of *H. fomes* root extract on mice in Open Field method

Groups	**Treatment**	**Number of movements**				
		0 min	**30 min**	**60min**	**90 min**	**120 min**
Negative Control (I)	Tween 80 solution	158.80 ± 0.83	155.00 ± 0.63	155.20 ± 0.74	150.40 ± 0.66	152.60 ± 0.33
Positive control (Standard) (II)	Diazepam 1 mg/kg,i.p.	142.00 ± 0.84*	98.20 ± 0.56**	70.20 ± 2.3**	60.20 ± 0.88**	9.20 ± 0.56**
III	RHF 100 mg/kg	141.33 ± 1.92	130.88 ± 0.54*	93.00 ± 1.64*	86.33 ± 0.89**	70.62 ± 1.58*
IV	RHF 200 mg/kg	128.00 ± 1.53*	87.2 ± 2.86**	71.4 ± 1.14**	60.2 ± 1.65*	48.0 ± 1.88**
V	RHF 300 mg/kg	110.66 ± 0.89*	76.8 ± 0.83	62.20 ± 1.33	56.88 ± 1.58**	39.0 ± 0.56*

The values are revealed as mean±SD (n=5); One-Way Analysis of Variance (ANOVA) followed by Dunnet's test. *P<0.05, **P<0.01 significant compared to the negative control.

3.3.2. Hole cross method

At doses of 100, 200, and 300 mg/kg body weight, the RHF extract demonstrated a substantial decrease in movement from its initial value at 0 min to 120 min ($p < 0.05$; $p < 0.01$). Mice given the usual medication diazepam (1 mg/kg, i.p.) had fewer holes spanned between chambers after 0 to 120 minutes (**Table 5**). The extract exhibited dose-dependent action, with the fifth observation period showing the greatest depressive effect.

Table 5. Neuropharmacological effect of *H. fomes* root extract on mice in hole cross test.

Groups	**Treatment**	**Number of movements**				
		0 min	**30 min**	**60min**	**90 min**	**120 min**
Negative Control (I)	Tween 80 solution	30.00 ± 1.64	29.60 ± 0.88	29.00 ± 0.75	29.00 ± 0.56	29.80 ± 0.58
Positive control (Standard) (II)	Diazepam 1 mg/kg,i.p.	24.80 ± 0.75*	18.00 ± 1.94*	9.80 ± 0.65**	3.00 ± 0.00**	1.00 ± 0.00**
III	RHF 100 mg/kg	28.40 ± 0.49*	25.2 ± 0.52*	23.2 ± 1.89*	18.60 ± 0.86	16.00 ± 0.89*
IV	RHF 200 mg/kg	23.20 ± 1.02*	17 ± 1.66*	14.0 ± 0.97*	11.40 ± 0.49	9.40 ± 0.80**
V	RHF 300 mg/kg	20.00 ± 0.38*	12.63 ± 0.53*	10.80 ± 0.48*	8.00 ± 0.88**	4.0 ± 0.63*

The values are revealed as mean±SD (n=5); One-Way Analysis of Variance (ANOVA) followed by Dunnett's test. *P<0.05, **P<0.01 significant compared to the negative control.

3.3.3 Thiopental induced hypnosis test

The extract demonstrated a significant reduction in the time of onset of sleep and an increase in sleep duration in the thiopental-induced hypnosis test at dosages of 100, 200, and 300 mg/kg. The findings were deemed statistically significant ($p< 0.05$). In comparison to controls, all doses of the extract prolonged the test animals' thiopental sodium-induced sleep period. The dose-dependent activity of RHF was clear from the results of dosages between 100 and 300 mg/kg. The effects of diazepam were most pronounced ($p<0.001$) during onset of sleep and during the peak of sleep (**Table 6**).

Table 6. Effects of *H. fomes* extract on thiopental sodium induced sleeping time test in mice.

Groups	Treatment	Thiopental sodium induced sleeping time test	
		Onset of sleep (Min)	**Duration of sleep (Min)**
Control (I)	Tween 80 solution	27 ± 1.79	57.67 ± 6.31
Standard (II)	Diazepam 1 mg/kg,i.p.	11.50 ± 1.05**	249.33 ± 3.4**
III	RHF 100 mg/kg	14.83 ± 1.47	70.33 ± 1.41
IV	RHF 200 mg/kg	11.33 ± 1.20*	109.67 ± 1.57
V	RHF 300 mg/kg	10.33 ± 1.03*	200.67 ± 1.03*

Each value represents mean ± SEM for n = 6, ** $p < 0.001$, and * $p < 0.05$ vs. control.

4. Discussion

The use of medicinal plants has emerged as a fascinating avenue for the development of traditional and modern medications, and research has demonstrated the true medical benefits of herbal medicines [21, 22, 23]. Our current study's objective was to look into *in vivo* analgesic and CNS depressant bioactivities of *H. fomes* root. Biological effectiveness of medicinal plants is largely dependent on their phytochemical content. A key factor in the discovery of novel, uncommon, and active chemicals is phytochemical analysis. The existence of secondary metabolites in plants is associated with their biological significance [22, 24]. The crude root extract of the experimented plant exhibited the presence of numerous valuable secondary metabolites such as alkaloids, glycosides, tannins, reducing sugars, steroids, fixed oil, terpenoids, flavonoids, and phenols (**Table 1**). *H. fomes* plant parts are already reported as an ailment for various diseases in the traditional system [13, 14]. The seed of the plant is reported to have a diverse nature of compounds including anthocyanins, complex polyphenols, leucoanthocyanidin, catechin, ellagic acid, geraniin, corilagin etc. [14, 15].

The central pain mechanism heavily relies on the brain and spinal cord. A variety of inhibitory pain-targeting biomolecules, including prostaglandins, somatostatins, bradykinins, and others, are abundant in the dorsal portion of the spinal cord. Pain, or algesia, is invariably an unpleasant experience. Usually, it is brought on by irritating stimuli, either internal or external. These often cause the phospholipids in the afflicted tissues to release arachidonic acid. Consequently, numerous intracellular components start to secrete. The sense of pain has been attributed to secreted prostacyclin (PGI2), leukotrienes, cytokines, and PGE2, PGF2α [24]. The acetic acid-induced writhing response technique is a widely used method to evaluate the peripheral analgesic activity of any plant component, as acetic acid is the major inducer of pain in an animal model [25]. Because of its sensitivity and ability to detect antinociceptive effects of natural products and test compounds at dose levels that are inert for other procedures, the acetic acid-induced writhing test is a valuable paradigm for assessing the peripheral analgesic potentials of test compounds [26]. A variety of endogenous inflammatory mediators, such as histamine, serotonin, bradykinin substance P, and PGs, are produced and released when acetic acid is injected intraperitoneally because it stimulates and irritates the peritoneal cavity. The body lengthens and the forelimbs extend while the abdominal muscles contract as a result of the chemically induced visceral discomfort caused by these various endogenous inflammatory mediators. Moreover, this model has been connected to increased PGE and PGF2α levels. By activating primary afferent nociceptors and expanding capillary permeability, raising PG levels in the peritoneal cavity intensifies inflammatory pain [26, 27, 28]. All three dosages of RHF extract showed substantial ($p < 0.05$ and $p < 0.01$) peripheral analgesic effects by reducing the number of writhing (**Table 2**),

with respective values of 37.82%, 54.61%, and 65.56%, when compared to the negative control. In animal models where thermal stimuli are utilized to generate pain, the tail immersion method is frequently used to assess central analgesic action. These techniques highlight the alterations above the level of the spinal cord, which provide a useful example of centrally mediated anti-nociceptive responses. Due to its great selectivity for analgesics derived from opioids, the approach is preferred [25]. Using the tail immersion method, the RHF extract demonstrated a strong and dose-dependent anti-nociceptive effect (**Table 3**). A potential suppression or alteration of pain induction via a spinal reflex is indicated by the inhibition of nociceptive activity. Since peripherally acting drugs are ineffective in response to certain kinds of heat stimulation, this technique is specific to central analgesia [29]. Spinal reflexes, which may be assessed by the tail immersion method, are a means by which the μ2, κ1, and δ2 opioid receptors contribute to nociception [25, 30, 31]. This approach also shown an increase in anti-nociceptive activity that was dose-dependent. The results of both techniques point to the anti-nociceptive properties of *H. fomes* root extract being mediated by spinal and supraspinal receptors. The combined analgesic effects of RHF extracts are thought to be caused by the reduction of prostaglandin synthesis and its impact on the central and peripheral analgesic mechanisms. Plant extracts might have a stronger analgesic effect.

Three neuropharmacological models- the open field, hole cross, and thiopental sodium induced sleeping time tests- were used to investigate the CNS depressive impact of *H. fomes* root extract. These models are commonly employed classical models for the purpose of neuropharmacological activity screening [20]. Locomotor activity serves as a gauge for the central nervous system's excitability, and a decline in this activity may be specifically linked to sedation brought on by central nervous system depression [32]. The plant extract's CNS depressive action may be the cause of this drop in spontaneous motor activity. The primary inhibitory neurotransmitter in the central nervous system is gamma amino butyric acid [20, 32, 33]. It has a role in physiological processes associated with neurological and psychological illnesses include epilepsy, depression, Parkinson's disease syndrome, and Alzheimer's disease [33]. A variety of medications have the ability to alter the GABA system at the level of its manufacture by increasing GABA-mediated postsynaptic inhibition via altering the allosteric properties of GABA receptors. It either directly increases chloride conductance or indirectly, similar to barbiturates, by potentiating GABA-induced chloride conductance while concurrently depressing voltage-activated Ca^{2+} channel [20]. Hence, it is foreseeable that the extract may function by directly activating GABA receptors or by potentiating GABAergic inhibition in the central nervous system via membrane hyperpolarization, which lowers the firing rate of important brain neurons. A longer GABA-gated channel opening duration or increased affinity for GABA could possibly be the cause [20, 33]. According to prior phytochemical research, neuroactive steroids and flavonoids bind to GABA-A receptors in the central nervous system, suggesting that they have benzodiazepine-like properties [20, 32, 33]. Furthermore, several flavonoids have been shown to bind to the GABA-A receptor's benzodiazepine region with great affinity.

Thiopental sodium is a barbiturate that puts rodents and people to sleep. The sedative-hypnotic medications were examined using the thiopental sodium induced sleeping time test in mice. This test is a traditional method used in behavioral pharmacology to look into the hypnotic and sedative effects of extract from medicinal plants [34, 35]. The drug binds to the GABA receptor complex and causes postsynaptic neurons to become hyperpolarized by GABA [35]. By extending the time of the chloride channel opening, it increases GABA activity and allows chloride to enter the neuron. However, thiopental has the ability to inhibit glutamate receptors that are excitatory. In the current investigation, varying concentrations of the extract markedly reduced the latency to induce sleep while lengthening the thiopental sodium-induced hypnosis duration (**Table 4**). Our findings indicate a possible correlation between the CNS depressive action of roots of *H. fomes* and diazepam in this test. Thus, the phytoconstituents in the RHF extract may be the cause of the CNS depressive action. It has been observed that triterpenoids and saponins exhibit agonistic actions at the GABA-A receptor complex [32, 35, 36]. These phytoconstituents might have played a role in the mice's CNS depressive effects. The precise compounds causing the CNS depressing effects are not well demonstrated.

5. Conclusions

Traditional herbal medicines have been used to treat and prevent a wide range of illnesses throughout human history. By considering the medicinal properties of herbs, researchers are allegedly evaluating the development of plant-based pharmaceuticals as a key and demanding topic of focus. Large amounts of glycosides, alkaloids, tannins, flavonoids, terpenoids, resin, and other secondary bioactive metabolites present in *H. fomes* Buch root have been demonstrated to have potent analgesic and CNS depressant properties. Every experimental dosage examined results in an immediate, pervasive, and statistically significant effect. Our findings suggest that pharmaceutical companies could be able to lower the cost of healthcare by using the roots of *H. fomes* to generate new, safer, more effective, and less toxic candidate medications. Further research will be conducted to determine the precise molecular pathways and identify the bioactive compound(s) in order to establish a safe and effective dosage and validate the possibility of using it for the prevention and treatment of different illnesses.

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Original Article

In vitro Antioxidant Activities and Comparison of Different Fractions of the Ethanolic Extract of *Ficus racemosa* Fruits

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Abstract: Oxidative stress, a major contributor to chronic diseases like diabetes and cardiovascular disorders, demands safer and more effective solutions than synthetic antioxidants. Natural alternatives, such as *Ficus racemosa*, have gained attention for their therapeutic potential. This study investigates the antioxidant and enzyme-inhibitory properties of *Ficus racemosa* extracts through comprehensive *in vitro* analyses. Ethanolic extracts and fractions were assessed using DPPH radical scavenging, nitric oxide (NO) scavenging, and ferric reducing power assays. High-performance liquid chromatography (HPLC) identified bioactive polyphenols, including gallic acid, quercetin, and caffeic acid, while molecular docking demonstrated strong binding affinities to oxidative stress-related enzymes. The ethyl acetate fraction exhibited the highest antioxidant activity, with an IC_{50} value of 0.194µg/mL for DPPH scavenging and 12.49µg/mL for NO scavenging. For the DPPH and the NO scavenging methods, IC_{50} value for other fractions are as followed, the ethyl acetate-methanol fraction (1.095 µg/mL, 13.25 µg/mL), the n-hexane-ethyl acetate fraction (1.521 µg/mL, 23.95 µg/mL), the crude extract (1.748 µg/mL, 16.18 µg/mL), ascorbic acid (2.024 µg/mL), the methanol fraction (2.411 µg/mL, 23.60 µg/mL), and the n-hexane fraction (2.738 µg/mL, 24.79 µg/mL). Docking results revealed quercetin as the most potent compound, with a binding affinity of –9.5 kcal/mol. These findings validate the traditional use of *Ficus racemosa* in folk medicine and highlight it's potential as a natural, safe, and effective alternative to synthetic antioxidants, paving the way for their application in functional foods and therapeutic formulations.

Keywords: antioxidant; *Ficus racemosa;* dumur; molecular docking; oxidative stress

1. Introduction

The widespread impact of oxidative stress on human health has become a growing concern. It disrupts the delicate balance of biological systems, tipping the scale between reactive molecules and the body's defense

mechanisms. The modern lifestyle, with its processed diets, environmental pollution, and high levels of stress, fuels an excess production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) [1]. While these molecules play essential roles in immune defense and cellular signaling [2], their uncontrolled accumulation disrupts biological stability. Lipids, proteins, and DNA become primary targets [3], leading to irreversible damage that accelerates aging and contributes to chronic diseases such as diabetes [4, 5], cardiovascular disorders, and cancer [6]. Left unchecked, oxidative stress weakens cellular integrity, paving the way for disease progression.

To counteract this, the body relies on antioxidants- compounds that neutralize reactive molecules and restore balance [7]. They are classified as endogenous, produced by the body (e.g., SOD, catalase, and glutathione peroxidase), or exogenous, obtained from diet and supplements [8]. While endogenous antioxidants provide a primary defense, excessive ROS and RNS can overwhelm their capacity, necessitating support from dietary antioxidants. Recent studies have increasingly focused on functional food ingredients and their potential health benefits [9, 10, 11]. The precision of *in vitro* assays offers unparalleled insights into their antioxidant potential, enabling researchers to dissect mechanisms at a molecular level. Techniques such as the DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging assay, ferric reducing power assay, and nitric oxide (NO) scavenging assay form the cornerstone of antioxidant evaluation [12, 13]. These assays illuminate the multi-faceted roles of antioxidants, from radical neutralization to electron donation, providing a window into their functional dynamics. High-performance liquid chromatography (HPLC) adds depth to this analysis by identifying and quantifying the bioactive compounds responsible for these effects.

Synthetic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) have been extensively utilized for their free radical-scavenging abilities; however, multiple studies have raised concerns about their possible carcinogenicity and adverse metabolic effects [14, 15]. Toxicological studies have indicated that BHT and BHA, when administered at high doses (typically 0.1–0.5% in food), can induce liver toxicity, promote tumorigenesis, and disrupt endocrine signaling pathways [15]. These limitations highlight the urgent need for natural, biocompatible alternatives that not only combat oxidative stress effectively but also provide additional health benefits. Plant-derived antioxidants, including polyphenols, flavonoids, carotenoids, and vitamins, have emerged as powerful and safer alternatives [16]. These bioactive molecules not only exhibit superior antioxidant activity but also contribute to a range of additional health benefits [17]. Polyphenols, a diverse class of compounds found abundantly in fruits, vegetables, herbs, and medicinal plants, possess significant free radical-scavenging properties [16]. For instance, polyphenolic compounds such as resveratrol and quercetin (6) demonstrate DPPH radical scavenging activity with IC_{50} values ranging from 5–25μM [18]. In addition to scavenging ROS and RNS, polyphenols modulate key cellular signaling pathways, including NF-kB [19], MAPK [20], and SIRT1 [21], which regulate inflammation, apoptosis, and oxidative stress responses. These effects are critical in preventing the onset and progression of chronic diseases, including cardiovascular diseases, cancer, and neurodegenerative disorders [22, 23]. Flavonoids, a subclass of polyphenols, further enhance the antioxidant defense system by chelating metal ions, such as iron and copper, and inhibiting the formation of ROS. The flavonoid quercetin, for example, has been shown to chelate metal ions with a binding constant of 3.8×10^5 M^{-1}, thereby preventing Fenton-type reactions that generate highly reactive hydroxyl radicals [24]. Additionally, flavonoids exert anti-inflammatory, anti-cancer, and neuroprotective effects, making them valuable candidates in therapeutic strategies [25, 26]. Other notable dietary antioxidants include vitamins C and E, beta-carotene, and minerals such as selenium and zinc, which play essential roles in maintaining oxidative balance and supporting overall health [27].

Ficus racemosa, commonly known as “dumur” in certain regions, are a member of the *Moraceae* family and are widely recognized for their nutritional and therapeutic properties. They are rich in bioactive compounds such as phenolics, flavonoids, anthocyanins, and dietary fibers, contributing to their pharmacological effects [28, 29]. Traditionally, *Ficus racemosa* have been used in folk medicine for their antioxidant, anti-inflammatory, and antimicrobial properties [29]. It also helps to regulate blood pressure and blood cholesterol due to the presence of potassium, omega-6, and omega-3 fatty acids [30, 31]. *Ficus racemosa* is rich in trace and macro elements, along with a diverse array of biologically active phytochemicals, such as anthocyanins, carotenoids, flavonoids, polyphenols, phenolic acids, triterpenoids, glycosides, polysaccharides, reducing agents, and vitamins C, K, and

E [27]. Its active components play a vital role in combating oxidative stress and improving metabolic health through metal ion chelation, metal reduction, and free radical scavenging [32]. Phytochemical analyses of *Ficus racemosa* have also revealed the presence of ceramides such as zeaxanthin, lutein, *a*-carotene, lycopene, *β*-carotene, and cryptoxanthin [33]; also cerebrosides, steroids, pentacyclic triterpenes, flavonoids, and phenolic compounds [34, 35].

Some scientific studies confirm the antioxidant potential of *Ficus racemosa*. Methanolic extracts of *Ficus racemosa* show strong DPPH radical scavenging activity, demonstrating their ability to neutralize free radicals [36, 37]. Similarly, ferric reducing power assays highlight their electron-donating potential, while NO scavenging studies reveal their effectiveness in reducing reactive nitrogen species [37]. These findings validate the use of *Ficus racemosa* in traditional medicine for managing conditions related to oxidative stress. However, few reports have been published on studies isolating the bioactive from *Ficus racemosa* and focusing on their individual antioxidant effects and contents. This study focuses on the antioxidant potential of *Ficus racemosa* extracts and their fractions, aiming to explore their role in mitigating oxidative stress and their application in therapeutic formulations.

2. Materials and Methods

2.1 Plant Material Collection

As a plant sample, the ripe fruits of *Ficus racemosa* were collected from Tangail, Bangladesh. An experienced taxonomist then recognized the sample and identified it (Accession Number of DACB87264) at the Mirpur National Herbarium.

2.2 Extraction of plant materials

The plant material was washed, air-dried, and powdered. The powdered material was subjected to maceration in ethanol (70% v/v) for 7 days at room temperature with occasional stirring. The extract was filtered and concentrated using a rotary evaporator under reduced pressure to yield a crude ethanolic extract.

2.3 Different fraction preparation

2.3.1 Column Chromatography

Preparation of reagent:

The crude extract was fractionated via column chromatography on silica gel, employing a gradient solvent system (hexane, ethyl acetate, and methanol). Initially, 1 gram of crude extract was processed through the column. Subsequently, a total of 8 grams of crude extract was fractionated in successive runs using the solvent gradient system. This process yielded 13 sub-fractions, which were later grouped into 5 major fractions based on their thin-layer chromatography (TLC) profiles and stored at 4°C for further analysis.

Solvent gradient system:

The gradient solvent ratio to be applied in column chromatography is summarized in the following table.

Table 1: Gradient solvent system to be used in the column chromatography

Solvent system	Ratio	Volume	Fraction No.
Hexane	100%	60 ml	1
Hexane: Ethyl acetate	10:1	60 ml	2
Hexane: Ethyl acetate	5:1	60 ml	3
Hexane: Ethyl acetate	1:1	60 ml	4

Solvent System	Ratio	Volume	Fraction No.
Hexane: Ethyl acetate	1:5	60 ml	5
Hexane: Ethyl acetate	1:10	60 ml	6
Ethyl acetate	100%	60 ml	7
Ethyl acetate: Methanol	10:1	60 ml	8
Ethyl acetate: Methanol	5:1	60 ml	9
Ethyl acetate: Methanol	1:1	60 ml	10
Ethyl acetate: Methanol	1:5	60 ml	11
Ethyl acetate: Methanol	1:10	60 ml	12
Ethyl acetate: Methanol	100%	60 ml	13

2.4 Quantitative and Qualitative Analysis through HPLC

2.4.1 Chemicals

Gallic acid, 3,4-dihydroxybenzoic acid, catechin-hydrate, catechol, (-) epicatechin, caffeic acid, vanillic acid, syringic acid, rutin hydrate, p-coumaric acid, trans-ferulic acid, rosmarinic acid, myricetin, quercetin, trans-cinnamic acid, and kaempferol were purchased from Sigma–Aldrich (St. Louis, MO, USA). Acetonitrile (HPLC), methanol (HPLC), acetic acid (HPLC), and ethanol were obtained from Merck (Darmstadt, Germany). α-amylase, 2,2-diphenyl-1-picrylhydrazyl (DPPH), and naphthyl ethylenediamine dihydrochloride were obtained from Sigma-Aldrich Chemical Co. (USA). Starch soluble (extra pure) was obtained from J.T. Baker Inc., Phillipsburg, USA. Other chemicals and reagents used were of analytical grade.

2.4.2 RP-HPLC-DAD Analysis

2.4.2.1 Preparation of standard solutions

Sixteen phenolic compounds were dissolved in methanol to produce stock standard solutions. The stock solution concentrations ranged from (4.0-50 µg/ml). Each stock solution's appropriate volumes were mixed, then diluted serially to prepare the working standard solutions. All solutions were stored under refrigeration for further analysis.

Methanol stock solutions containing Gallic acid (20 µg/ml), 3,4-Dihydroxybenzoic acid (15 µg/ml), Catechin (1) hydrate (50 µg/ml), Catechol, (-) Epicatechin (2), rosmarinic acid (30 µg/ml each), Caffeic acid, vanillic acid, Syringic acid, rutin (4) hydrate, p-coumaric acid, trans-ferulic acid, quercetin (6) (10 µg/ml each), myricetin (5), kaempferol (8 µg/ml each), and trans-cinnamic acid (7) (4 µg/ml) were prepared and diluted to appropriate concentrations for the construction of calibration curves. The calibration curves were constructed by plotting the peak areas under the curve versus the amount of the analytes.

2.4.2.2 Chromatographic protocol development

Ethanolic extract of *Ficus racemosa* fruits was thawed, filtered through 0.22 µm membrane filters, and then separated by RP-HPLC-DAD analysis as described by Ahmed et al. (2021) [38] with some modifications to obtain chromatograms. Detection and quantification of selected polyphenolic compounds were performed on a Shimadzu (LC-20A, Japan) equipped with a binary solvent delivery pump (LC-20AT), an auto sampler (SIL-20A HT), column oven (CTO-20A), a photodiode array detector (SPD-M20A) and Phenomenex Luna C18 column (4.6 x 250 mm, 5µm) at ambient temperature. The RP-HPLC-DAD machine was controlled by the LC Lab Solution software. The mobile phase comprises A (1% acetic acid in acetonitrile) and B (1% acetic acid in water), and the flow rate was set at 0.5 mL/min. The gradient program was set as follows: 0-20 min, eluent A was kept at 5-25%; 21-30 min, eluent A was increased from 25% to 40%; then in the next 5 min, eluent A was

increased to 60%; but next 5 min (36-40 min), eluent A decreased to 60-30%; this down fall was continued for next 5 min, and eluent A was reached from 30 to 5% and the final 5 min eluent A was kept at 5%. The sample injection volume was 20µL. The UV detector was set at 270 nm and applied to validate the method and analysis. The mobile phase was filtered through a 0.45 µm Nylon 6, 6 membrane filter (India) and degassed under vacuum.

2.5 In Vitro Antioxidant Assays

2.5.1 DPPH Radical Scavenging Assay

In HPLC grade methanol, a DPPH solution (0.004% w/v) was prepared [39]. To make the stock solution (500 µg/mL), each of the five fractions (A, B, C, D & E) and the *Ficus racemosa* crude extract was combined individually with Milli-Q water. 1mL extracts were taken to the test tubes of serially diluted different concentrations (12.5 µg/mL to 500 µg/mL), and then a freshly made 1mL DPPH solution (0.004% w/v) was added. So, the total volume was 2 mL, and after 10 minutes of incubation in a dark place, the absorbance was measured at 515 nm using a spectrophotometer (HACH 4000 DU UV-visible spectrophotometer). As a reference standard, ascorbic acid was dissolved in Milli-Q water to create a stock solution with the same strength (500 mg/mL). The percent scavenging of the DPPH free radical activity was measured by using the following equation:

% of inhibition = [(absorbance of the control – absorbance of the test sample) / absorbance of the control] X 100

Lower absorbance of the reaction mixture indicates higher free radical-scavenging activity. All the tests were performed in triplicate.

2.5.2 Nitric Oxide (NO) Radical Scavenging Assay

For the NO radical scavenging assay, a previously described method was used [40]. At a physiological pH, sodium nitroprusside solution produced nitric oxide (NO) radicals. In phosphate buffer, 1 ml of sodium nitroprusside (10 mM) was combined with 1 ml of ethanolic extract and fractions of various concentrations (12.5 - 150 µg/ml) (pH 7.4). 150 minutes were spent incubating the mixture at 25°C. Griess' reagent (1% sulfanilamide, 2% o-phosphoric acid, and 0.1% naphthyl ethylene diamine dihydrochloride) was added to 1ml of the incubated solution. The absorbance was measured at 546 nm, and the % inhibition was measured by using the following equation:

% of inhibition = [(absorbance of the control – absorbance of the test sample) / absorbance of the control] X 100

2.5.3 Ferric Reducing Power Assay

The reducing power of the fractions was evaluated by mixing 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of potassium ferricyanide (1%) with 1 mL of each fraction. The mixture was incubated at 50°C for 20 minutes, followed by the addition of 2.5 mL of trichloroacetic acid (10%). After centrifugation at 3000 rpm for 10 minutes, 2.5 mL of the supernatant was mixed with 2.5 mL of distilled water and 0.5 mL of ferric chloride (0.1%). The absorbance was measured at 700 nm.

2.6 Molecular Docking

2.6.1 Receptor Preparation

The proteins/receptors, Human milk xanthine Alpha amylase (PDB ID: 1PPI), iNOS (PDB ID: 4NOS), NADPH oxidase (PDB ID: 5VOH), in PDB format, were downloaded from the Protein Data Bank. (https://www.rcsb.org/). By using PyMol, Water molecules and original ligands were deleted [41, 42]. AutoDock tools 1.5.7 were used to prepare the protein; Addition of polar hydrogen and Kollman charge. The receptors/proteins were saved in PDBQT format [43, 44].

2.6.2 Ligand preparation

The 3D structure of Ligands Caffeic acid (PubChem ID: 689043), Gallic acid (PubChem ID: 370), and Quercetin (PubChem ID: 5280343) were downloaded from PubChem as SDF format. (https://pubchem.ncbi.nlm.nih.gov/). PDB format is required for using AutoDock tools; therefore, using PyMol, SDF files were converted into PDB format. By using AutoDock tools 1.5.7, the ligands were prepared for docking and saved as a PDBQT file [43, 44].

2.6.3 Grid preparation

The grid menu of AutoDock tools was used to calculate the AutoGrid parameters [43]. The proteins were uploaded in the PDBQT format, and from the grid menu, the grid box was selected, and a box appeared. The default centers and the default dimensions of X, Y, and X coordinate were taken. The grid file was saved in the GPF format [43, 44].

2.6.4 Docking

AutoDock Vina is a complete computational docking method based on a quick conformational search and a basic scoring system [45]. The default techniques in AutoDock and AutoDock Vina have been extensively utilized for applications like virtual screening since they are quite efficient for typical drug-like ligands [46]. AutoDock Vina was run using the command prompt [45], and the docked file was saved in the PDBQT format [44].

2.6.5 Visualization

For visualizing the 2D and 3D structure of docked protein and ligand, Biovia Discovery Studio client 2021 is used [47].

3. Results

This section may be divided by subheadings. It should provide a concise and precise description of the experimental results, their interpretation, as well as the experimental conclusions that can be drawn.

3.1 RP-HPLC-DAD Analysis

HPLC, the most remarkable chromatographic technique, is a powerful and widely used technique that is used to analyze plant extracts because this technique can systematically profile the composition of samples, and it focuses on the identification and consistency assessment of the components. Ethanolic extract of *Ficus racemosa* fruits and selected standards for HPLC analysis were carried out. HPLC analysis provides accurate quantitative precision and accuracy to allow the identification of the chemicals in the selected ethanolic extract. The chromatogram revealed that *Ficus racemosa* fruit extract depicts the presence of phenolic acid, phenolics, such as flavonoids. It was observed that the extract contains catechin hydrate (10.12±0.22 mg/100 g dry extract), epicatechin (92.41±0.32 mg/100 g dry extract), caffeic acid (6.22±0.25 mg/100 g dry extract), rutin hydrate (8.24±0.03 mg/100 g dry extract), myricetin (132.69±0.63 mg/100 g dry extract), quercetin (22.81±0.37 mg/100 g dry extract), and trans-cinnamic acid (1.85±0.21 mg/100 g dry extract), which is shown in **Figure 1** and **Table 2** respectively.

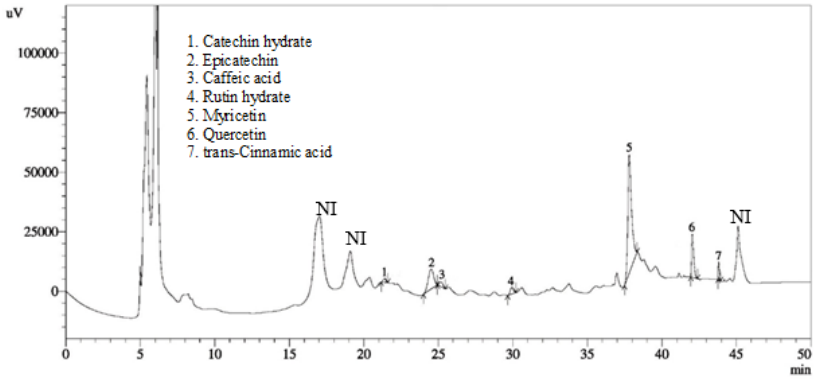

Figure 1. HPLC Chromatographic profiles of the ethanolic extract of *Ficus racemosa*. The peak labelled NI was not identified. The phenolic compound found in the *Ficus racemosa* extract was calculated from the corresponding standard curve and was presented as the mean±SD as shown in **Table 2.**

Table 2: Identification and quantification of phenolic compounds in *Ficus racemosa*.

Peak no.	Name of phenolic compounds	Retention time (min)	Concentration (mg/100 g dry extract)
1.	Catechin hydrate	21.37	10.12±0.22
2.	(-) Epicatechin	24.52	92.41±0.32
3.	Caffeic acid	25.15	6.22±0.25
4.	Rutin hydrate	29.91	8.24±0.03
5.	Myricetin	37.8	132.69±0.63
6.	Quercetin	42.03	22.81±0.37
7.	trans-Cinnamic acid	43.8	1.85±0.21

3.2 DPPH Radical Scavenging Activity

The DPPH radical contains an odd electron responsible for the visible deep purple color. Antioxidant compounds can donate an electron, and DPPH accepts this electron. When DPPH accepts an electron, then decolorizes the purple color, which can be quantitatively measured from the changes in absorbance. The antioxidant activity of the extract and its fractions is shown in **Figure 2**. Ascorbic acid was also used as a standard.

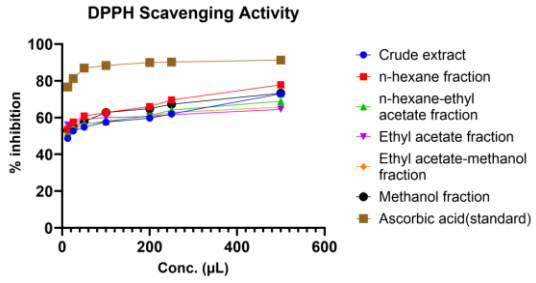

Figure 2: Dose-response curve of DPPH scavenging activity of the crude ethanol extract of *Ficus racemosa* fruit and its five different fractions.

3.3 NO Scavenging Activity:

The dose-response curve of nitric oxide (NO) scavenging activity of the crude ethanol extract of *Ficus racemosa* fruit and its five different fractions are given in the **Figure 3**. It represents the ethyl acetate fraction as the most effective in mitigating nitrosative stress, with an IC_{50} of 12.49 µg/mL found in **Table 2**.

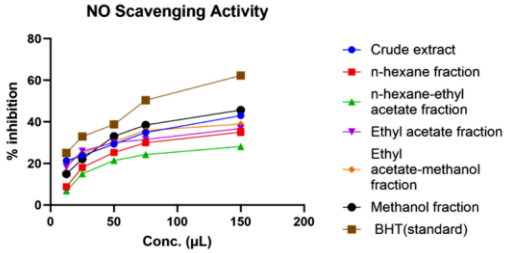

Figure 3: Dose-response curve of NO scavenging activity of the crude ethanol extract of *Ficus racemosa* fruit and its five different fractions.

3.4 Ferric Reducing Power Activity

The dose-response curve of reducing power of the crude ethanol extract of *Ficus racemosa* fruit and its five different fractions are shown in the **Figure 4**. It established a concentration-dependent increase in activity, with the ethyl acetate fraction exhibiting the highest reducing potential.

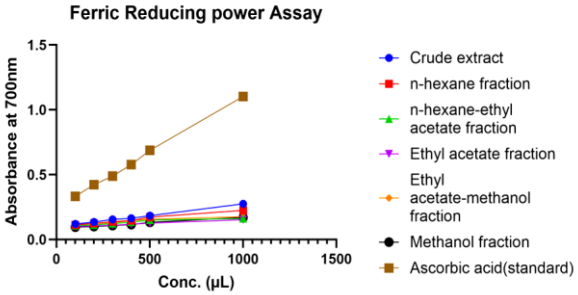

Figure 4: Dose-response curve of reducing power of the crude ethanol extract of *Ficus racemosa* fruit and its five different fractions.

Table 3: IC_{50} values of *Ficus racemosa* extract and its five fractions in different antioxidant assays such as DPPH and NO Scavenging Method.

Sample	**DPPH Scavenging Method (µg/mL)**	**NO Scavenging Method (µg/mL)**
Ascorbic Acid	2.024	-
BHT	-	21.36
Crude Extract	1.748	16.18
n-hexane Fraction	2.738	24.79
n-hexane - Ethyl acetate Fraction	1.521	23.95
Ethyl acetate Fraction	0.1940	12.49

Sample	DPPH Scavenging Method (µg/mL)	NO Scavenging Method (µg/mL)
Ethyl acetate - Methanol Fraction	1.095	13.25
Methanol Fraction	2.411	23.60

Values are expressed as IC_{50} values of triplicate analysis. Ascorbic Acid is used as a DPPH standard, and BHT is used as a NO Scavenging.

3.5 Molecular docking of caffeic acid, gallic acid, and quercetin present in Ficus racemosa fruit extract.

Docking studies revealed strong binding affinities of gallic acid, quercetin, and caffeic acid with key oxidative stress-related enzymes. Quercetin exhibited the highest binding affinity with iNOS (−9.5 kcal/mol), followed by NADPH oxidase (−8.4 kcal/mol). Hydrogen bonds and hydrophobic interactions were the primary forces stabilizing these complexes. These results provide a molecular basis for the antioxidant efficacy observed *in vitro* and suggest potential therapeutic applications for *Ficus racemosa* bioactive in oxidative stress-related diseases.

Compounds from *Ficus racemosa*, such as Gallic acid, Caffeic acid, and Quercetin, strongly bind with Alpha amylase, iNOS, and NADPH oxidase. (**Figure: 4, 5, 6**) Their binding affinity is shown in **Table 3**. Ligands form several types of bonds, such as hydrogen bonds, hydrophobic bonds, and salt bridges with proteins/receptors. (**Table 2, 3, and 4)**

Table 4: Binding properties of caffeic acid, gallic acid, and quercetin present in *Ficus racemosa* fruit extract.

Ligand	Receptor	Affinity (Kcal/mol)	rmsd
Caffeic acid	Alpha amylase (1ppi)	-7.3	0.000
	iNOS (4nos)	-6.3	
	NADPH ox (5voh)	-6.8	
Gallic acid	Alpha amylase (1ppi)	-5.8	0.000
	iNOS (4nos)	-5.7	
	NADPH ox (5voh)	-5.6	
Quercetin	Alpha amylase (1ppi)	-8.9	0.000
	iNOS (4nos)	-9.5	
	NADPH ox (5voh)	-8.4	

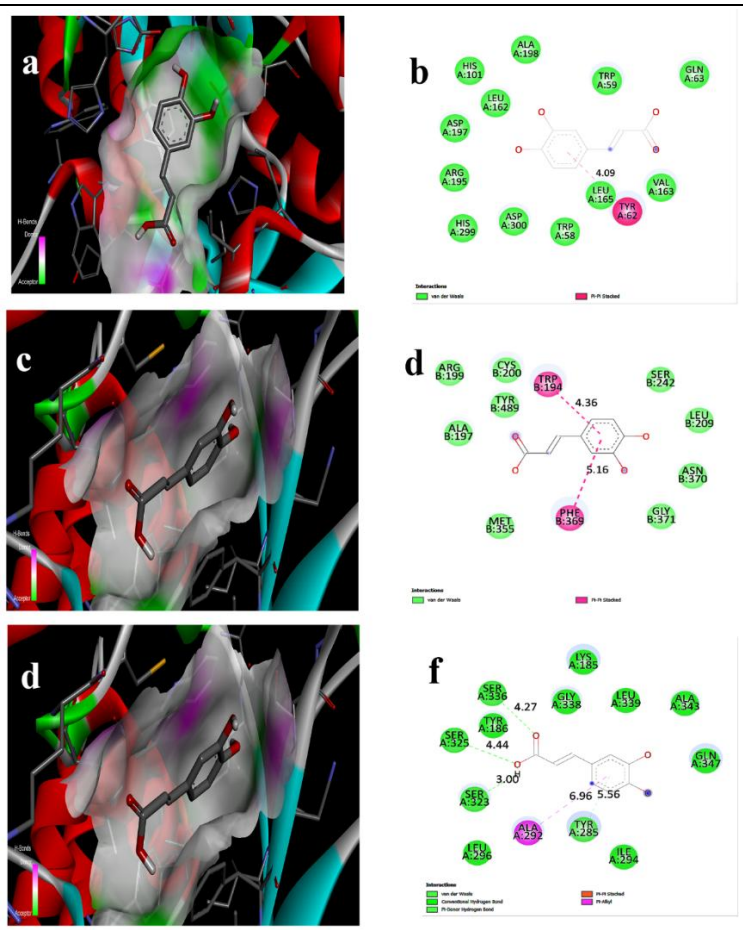

Figure 4: Best rank imposes of caffeic acid and alpha amylase 3D (a), 2D (b); caffeic acid and iNOS 3D (c), 2D (d); caffeic acid and NADPH ox 3D (e), 2D (f).

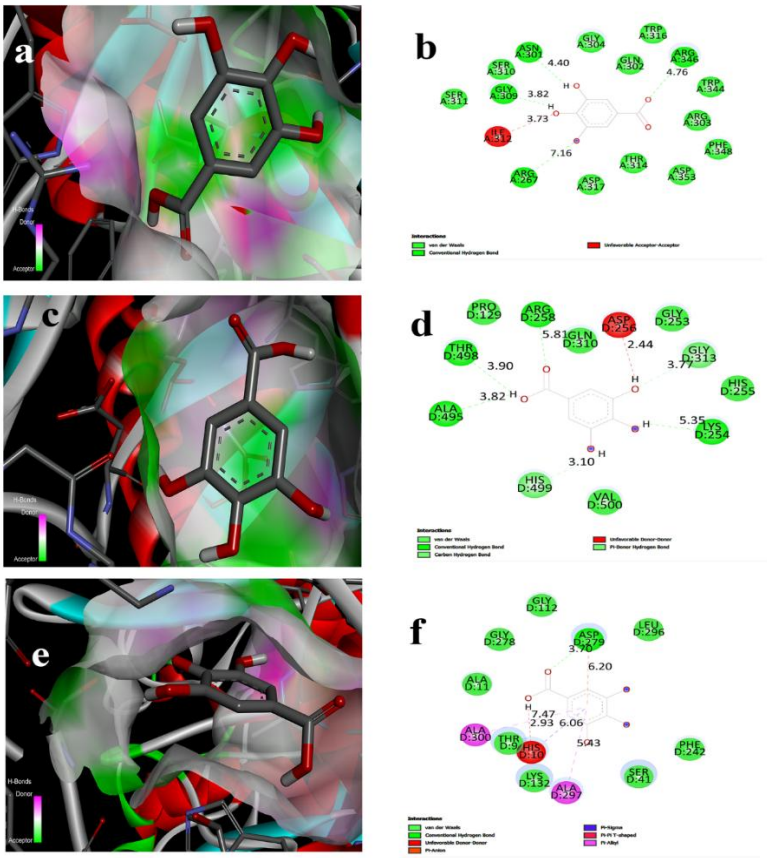

Figure 5: Best rank imposes of gallic acid and alpha amylase 3D (a), 2D (b); gallic acid and iNOS 3D (c), 2D (d); gallic acid and NADPH ox 3D (e), 2D (f).

3.6 Ficus racemosa extract component caffeic acid interaction with protein target 1ppi

The caffeic acid interaction with protein target 1ppi (the complex of a pancreatic alpha-amylase with a carbohydrate inhibitor refined to 2.2-A resolution). This table summarizes key non-covalent interactions stabilizing the binding of caffeic acid to the protein 1ppi. It is divided into two sub-tables:
Hydrogen bonds significantly enhance ligand-protein specificity and affinity through directional electrostatic interactions. **Table 4.1** catalogs hydrogen bonds formed between caffeic acid and the protein 1ppi. Metrics include distances (H-A: hydrogen-acceptor; D-A: donor-acceptor), donor angle geometry, and roles of protein donor groups/side chains. Notably, GLN63A and ARG195A act as key donors, with strong angles (153°–174°) and short H-A distances (2.22–2.89 Å), indicating robust interactions critical for ligand anchoring. Together, these interactions elucidate structural mechanisms driving ligand-protein affinity and specificity.

Table 4.1: Hydrogen bonds interaction data between caffeic acid and the target protein 1ppi

Index	Residue	AA	Distance H-A	Distance D-A	Donor Angle	Protein donor?	Side chain	Donor Atom	Acceptor Atom
1	63A	GLN	2.22	3.13	153.01	Y	Y	521 [Nam]	3922 [O3]
2	195A	ARG	2.89	3.87	174.11	Y	Y	1526 [Ng+]	3924 [O3]

AA: Amino acid residue; Distance H-A: Distance between hydrogen and acceptor atom; Distance D-A: Distance between donor and acceptor atom; Donor Angle: Angle between donor, acceptor, and hydrogen atom; Protein donor?- Does protein provide the donor group?; Side chain: Is the hydrogen bond formed with the amino acid side chain?; Donor Atom: ID of donor atom; Acceptor Atom: ID of acceptor atom.

Hydrophobic interactions are critical non-covalent forces that stabilize ligand-protein binding by excluding water from non-polar interfaces. **Table 4.2** details key hydrophobic contacts between the ligand and residues in the target protein 1ppi. Entries include the residue index, amino acid type (AA), distance (Å) between interacting carbon atoms, and identifiers for the ligand and protein atoms involved. Distances ≤4.0 Å highlight residues like TYR62A and LEU165A that contribute to binding stability through van der Waals forces and desolvation effects.

Table 4.2: Hydrophobic Interactions between ligand caffeic acid and protein target 1ppi

Index	Residue	AA	Distance	Ligand Atom	Protein Atom
1	58A	TRP	3.78	3915	466
2	62A	TYR	3.45	3914	506
3	62A	TYR	3.66	3915	507
4	62A	TYR	3.83	3917	508
5	63A	GLN	3.91	3920	519
6	165A	LEU	3.94	3920	1286

AA: Amino acid residue; Distance: Distance between interactions' carbon atoms; Ligand Atom: ID of ligand carbon atom; Protein Atom: ID of protein carbon atom.

3.7 Ficus racemosa extract component caffeic acid interaction with protein target 4nos

The protein target human inducible nitric oxide synthase with inhibitor (4nos) is used for the interaction with Caffeic Acid as the ligand. The results have been given below in **Table 4.3** and **Table 4.4.**

Through directed electrostatic interactions, hydrogen bonds significantly improve the specificity and affinity of ligands for proteins. Here in **Table 4.3**, it shows the hydrogen bond between ligand caffeic acid and protein target 4nos. Here ASN370B act as key donor, with a strong angle of 160.43° and short H-A distance of 2.82 Å, demonstrating a strong interaction that is essential for ligand anchoring.

Table 4.3: Hydrogen bonds interaction data between caffeic acid and the target protein 4nos

Index	Residue	AA	Distance H-A	Distance D-A	Donor Angle	Protein donor?	Side chain	Donor Atom	Acceptor Atom
1	370B	ASN	2.82	3.75	160.43	No	No	13684 [O3]	5740 [O2]

AA: Amino acid residue; Distance H-A: Distance between hydrogen and acceptor atom; Distance D-A: Distance between donor and acceptor atom; Donor Angle: Angle between donor, acceptor, and hydrogen atom; Protein donor?- Does protein provide the donor group?; Side chain: Is the hydrogen bond formed with the amino acid side chain?; Donor Atom: ID of donor atom; Acceptor Atom: ID of acceptor atom.

Since hydrophobic interactions keep water out of non-polar surfaces, they are essential non-covalent forces that maintain ligand-protein binding. In **Table 4.4**, the hydrophobic interactions between the ligand and residues in the target protein 4nos has been described. Residues like PHE369B and TRP194B, which support binding stability through van der Waals forces and desolvation effects, are highlighted at distances ≤4.0 Å.

Table 4.4: Hydrophobic Interactions between ligand caffeic acid and protein target 4nos

Index	Residue	AA	Distance	Ligand Atom	Protein Atom
1	194B	TRP	3.87	13686	4310
2	194B	TRP	3.62	13689	4304
3	194B	TRP	3.69	13690	4306
4	369B	PHE	3.55	13692	5734
5	369B	PHE	3.91	13690	5730
6	489B	TYR	3.83	13692	6712
7	489B	TYR	3.58	13689	6714

AA: Amino acid residue; Distance: Distance between interactions carbon atoms; Ligand Atom: ID of ligand carbon atom; Protein Atom: ID of protein carbon atom.

3.8 Ficus racemosa extract component caffeic acid interaction with protein target 5voh

The caffeic acid interaction with protein target 5voh (Crystal structure of engineered water-forming NADPH oxidase (TPNOX) bound to NADPH). This table describes critical non-covalent interactions that stabilize the binding of caffeic acid to the protein 5voh. It's separated into two sub-tables.

In **Table 4.5**, hydrogen bonds between the ligand caffeic acid and protein target 5voh has been shown. Here, residue- GLN347A has shown strong donor angle of 162.45° and short H-A distance of 4.05 Å, demonstrating a strong interaction that is essential for ligand anchoring.

Table 4.5: Hydrogen Bonds interaction data between caffeic acid and the protein target 5voh

Index	Residue	AA	Distance H-A	Distance D-A	Donor Angle	Protein donor?	Side chain	Donor Atom	Acceptor Atom
1	323A	SER	2.21	3.02	139.59	-	-	13719 [O.co2]	2462 [O3]
2	336A	SER	2.40	2.88	109.88	-	-	2558 [O3]	13718 [O.co2]
3	347A	GLN	3.10	4.05	162.45	-	-	2633 [Nam]	13721 [O3]

AA: Amino acid residue; Distance H-A: Distance between hydrogen and acceptor atom; Distance D-A: Distance between donor and acceptor atom; Donor Angle: Angle between donor, acceptor, and hydrogen atom; Protein donor?- Does protein provide the donor group?; Side chain: Is the hydrogen bond formed with the amino acid side chain?; Donor Atom: ID of donor atom; Acceptor Atom: ID of acceptor atom.

In **Table 4.6**, the hydrophobic interactions between the ligand and residues in the target protein 4nos have been described. Residues like TRY285A, which support binding stability through van der Waals forces and desolvation effects, are highlighted at distances ≤4.0 Å.

Table 4.6: Hydrophobic Interactions between ligand caffeic acid and protein target 5voh

Index	Residue	AA	Distance	Ligand Atom	Protein Atom
1	285A	TYR	3.62	13713	2170
2	285A	TYR	3.91	13709	2171
3	294A	ILE	3.96	13713	2245

AA: Amino acid residue; Distance: Distance between interactions' carbon atoms; Ligand Atom: ID of ligand carbon atom; Protein Atom: ID of protein carbon atom.

3.9 Ficus racemosa extract component gallic acid interaction with protein target 1ppi

Molecular interactions between gallic acid and human pancreatic α-amylase (PDB: 1PPI). The 1PPI structure (resolved at 2.2 Å) depicts the enzyme co-crystallized with a carbohydrate inhibitor, providing a proven active site for docking analysis.

Hydrogen bonds increase ligand affinity and specificity via directional interactions. GLY309A and ARG346A serve as the leading hydrogen donors to gallic acid in key bond interactions. Near-ideal geometry, characterized by bond angles of 150°–152° and H-A distances ranging from 1.86 Å to 2.93 Å, suggests significant stability. This demonstrating geometrically complementary bonding that inhibits starch substrate access through competitive mechanisms.

Table 5.1: Hydrogen Bonds interaction data between ligand gallic acid and target protein 1ppi

Index	Residue	AA	Distance H-A	Distance D-A	Donor Angle	Protein donor?	Side chain	Donor Atom	Acceptor Atom
1	267A	ARG	2.76	3.23	110.26	y	y	2095 [Ng+]	3918 [O3]
2	301A	ASN	2.37	3.05	126.61	x	x	3924 [O3]	2373 [O2]
3	304A	GLY	3.53	3.98	110.17	y	x	2398 [Nam]	3924 [O3]
4	309A	GLY	1.86	2.75	151.80	x	x	3910 [O2]	2428 [O2]
5	346A	ARG	2.93	3.81	149.62	y	y	2737 [Ng+]	3921 [O3]

AA: Amino acid residue; Distance H-A: Distance between hydrogen and acceptor atom; Distance D-A: Distance between donor and acceptor atom; Donor Angle: Angle between donor, acceptor, and hydrogen atom; Protein donor?- Does protein provide the donor group?; Side chain: Is the hydrogen bond formed with the amino acid side chain?; Donor Atom: ID of donor atom; Acceptor Atom: ID of acceptor atom.

3.10 Ficus racemosa extract component gallic acid interaction with protein target 4nos

The interactions at the molecular level between gallic acid and human inducible nitric oxide synthase (iNOS) (PDB: 4NOS). The 4NOS structure, co-crystallized with a selective inhibitor, offers a validated binding site for evaluating the interactions of gallic acid, highlighting key residues within the catalytic domain.

Hydrogen bonds stabilizing gallic acid within the inhibitor-binding site of human inducible nitric oxide synthase (PDB: 4NOS). Donor residues ASP256D and VAL500D establish essential hydrogen bonds with gallic acid, demonstrating near-linear geometry (bond angles: 162°–165°) and short H-A distances (2.03–3.42 Å). The interactions indicate significant electrostatic complementarity in the catalytic domain, implying competitive displacement of the native inhibitor.

Table 5.2: Hydrogen Bonds interaction data between ligand gallic acid and protein target 4nos

Index	Residue	AA	Distance H-A	Distance D-A	Donor Angle	Protein donor?	Side chain	Donor Atom	Acceptor Atom
1	256D	ASP	2.03	3.00	165.21	y	x	11651 [Nam]	13692 [O3]
2	256D	ASP	3.04	3.75	131.35	x	y	13692 [O3]	11658 [O.co2]
3	313D	GLY	2.36	3.00	122.78	x	x	13684 [O3]	12116 [O2]
4	495D	ALA	2.64	3.15	112.75	x	x	13696 [O.co2]	13611 [O2]
5	498D	THR	2.57	3.07	112.69	y	y	13642 [O3]	13695 [O.co2]
6	499D	HIS	2.39	3.21	140.90	x	y	13698 [O3]	13650 [N2]
7	500D	VAL	2.46	3.42	162.49	y	x	13653 [Nam]	13698 [O3]

AA: Amino acid residue; Distance H-A: Distance between hydrogen and acceptor atom; Distance D-A: Distance between donor and acceptor atom; Donor Angle: Angle between donor, acceptor, and hydrogen atom; Protein donor?- Does protein provide the donor group?; Side chain: Is the hydrogen bond formed with the amino acid side chain?; Donor Atom: ID of donor atom; Acceptor Atom: ID of acceptor atom.

Table 5.3 presents the recognized salt bridges detected within the protein-ligand complex, mentioning the interacting residues, their distances, and the nature of the ligand groups present. This table also assumes that the protein residue aiding in the interaction carries a positive charge, concurrent with enumerating the specific ligand atoms participating in the salt bridge formation.

Table 5.3: *Ficus racemosa* extract ligand with protein target salt bridge

Index	Residue	AA	Distance	Protein positive?	Ligand Group	Ligand Atoms
1	258D	ARG	4.23	Yes	Carboxylate	13696, 13695

3.11 Ficus racemosa extract component gallic acid interaction with protein target 5voh

The gallic acid interaction with protein target 5voh (Crystal structure of engineered water-forming NADPH oxidase (TPNOX) bound to NADPH). This table describes critical non-covalent interactions that stabilize the binding of gallic acid to the protein 5voh.

High-affinity hydrogen bonds within the iNOS-gallic acid complex (PDB: 4NOS). Residues ALA11D and ASP279D establish geometrically optimized hydrogen bonds with gallic acid, demonstrating near-linear donor angles (168°–173°) and notably short H-A distances (2.01–2.04 Å). The interactions illustrate electrostatic complementarity essential for competitive inhibition in the heme-containing catalytic domain.

Table 5.4: Hydrogen Bonds interaction data between ligand gallic acid and target protein 5voh

Index	Residue	AA	Distance H-A	Distance D-A	Donor Angle	Protein donor?	Side chain	Donor Atom	Acceptor Atom
1	10D	HIS	2.28	3.12	142.69	Y	X	10340 [Nam]	13719 [O.co2]
2	11D	ALA	2.04	3.02	172.85	Y	X	10350 [Nam]	13719 [O.co2]
3	41D	SER	2.67	3.22	116.50	Y	Y	10591 [O3]	13707 [O3]
4	41D	SER	2.47	3.22	134.25	X	Y	13707 [O3]	10591 [O3]
5	132D	LYS	2.88	3.25	102.27	Y	Y	11278 [N3+]	13721 [O3]
6	279D	ASP	2.01	2.98	168.48	Y	X	12398 [Nam]	13718 [O.co2]

AA: Amino acid residue; Distance H-A: Distance between hydrogen and acceptor atom; Distance D-A: Distance between donor and acceptor atom; Donor Angle: Angle between donor, acceptor and hydrogen atom; Protein donor?- Does protein provide the donor group?; Side chain: Is the hydrogen bond formed with the amino acid side chain?; Donor Atom: ID of donor atom; Acceptor Atom: ID of acceptor atom.

3.12 Ficus racemosa extract component quercetin interaction with protein target 1ppi

Quercetin interaction with protein target 1ppi (the complex of a pancreatic alpha-amylase with a carbohydrate inhibitor refined to 2.2-A resolution).

Hydrogen bonds facilitate the binding of quercetin to pancreatic α-amylase (PDB: 1PPI). Donor residues GLU233A and ARG195A establish geometrically optimized hydrogen bonds with quercetin, demonstrating near-linear angles (152°–155°) and short H-A distances (2.12–2.23 Å). The interactions stabilize the flavonoid core within the catalytic pocket.

Table 6.1: Hydrogen Bonds interaction data between ligand quercetin and target protein 1ppi

Index	Residue	AA	Distance H-A	Distance D-A	Donor Angle	Protein donor?	Side chain	Donor Atom	Acceptor Atom
1	63A	GLN	3.47	4.01	117.97	X	Y	3935 [O3]	522 [O2]
2	195A	ARG	2.12	3.03	151.52	Y	Y	1525 [Ng+]	3929 [O3]
3	195A	ARG	2.25	3.12	147.03	Y	Y	1526 [Ng+]	3929 [O3]
4	233A	GLU	2.23	3.14	155.29	X	Y	3927 [O3]	1836 [O3]
5	305A	HIS	2.22	2.98	132.93	Y	Y	2409 [Nar]	3931 [O3]

AA: Amino acid residue; Distance H-A: Distance between hydrogen and acceptor atom; Distance D-A: Distance between donor and acceptor atom; Donor Angle: Angle between donor, acceptor, and hydrogen atom; Protein donor?- Does protein provide the donor group?; Side chain: Is the hydrogen bond formed with the amino acid side chain?; Donor Atom: ID of donor atom; Acceptor Atom: ID of acceptor atom.

Hydrophobic interactions stabilize quercetin within the substrate-binding site of pancreatic α-amylase (PDB: 1PPI). Hydrophobic interactions improve ligand binding by promoting desolvation-induced entropy

increases, thereby removing water from nonpolar surfaces. Key residues TYR62A (3.8 Å) and LEU165A (3.6 Å) establish significant van der Waals interactions with the flavonoid rings of quercetin, thereby competitively obstructing the catalytic triad from starch substrates.

Table 6.2: Hydrophobic Interactions between ligand quercetin and target protein 1ppi

Index	Residue	AA	Distance	Ligand Atom	Protein Atom
1	59A	TRP	3.94	3920	477
2	62A	TYR	3.64	3925	510
3	163A	VAL	3.99	3918	1275
4	165A	LEU	3.45	3918	1286

AA: Amino acid residue; Distance: Distance between interactions' carbon atoms; Ligand Atom: ID of ligand carbon atom; Protein Atom: ID of protein carbon atom.

Table 6.3 briefly states the salt bridge interactions discovered within the analyzed protein-ligand complex, referring to the residue information, interatomic distances, and the charge status of the interacting protein residues. It also denotes the ligand groups involved and lists the ligand atom IDs that participate in the formation of these salt bridges.

Table 6.3: *Ficus racemosa* extract ligand with protein target salt bridge

Index	Residue	AA	Distance	Protein positive?	Ligand Group	Ligand Atoms
1	305A	HIS	5.10	Yes	Carboxylate	3910, 3911

3.13 Ficus racemosa extract component quercetin interaction with protein target 4nos

The protein target 4nos (human inducible nitric oxide synthase with inhibitor) is used for the interaction with Quercetin as the ligand. The results have been given below in **Table 6.4** and **Table 6.5**

Hydrogen bonds facilitating the interaction of quercetin with human inducible nitric oxide synthase (PDB: 4NOS). Key bonds include ALA197C (H-A: 2.98 Å), ARG199C (H-A: 2.50 Å), and SER242C (H-A: 3.53 Å), with donor angles ranging from 115° to 125°, suggesting geometrically constrained interactions. The minimal H-A distance for ARG199C (2.50 Å) indicates significant electrostatic complementarity in proximity to the heme cofactor, effectively displacing the native inhibitor.

Table 6.4: Hydrogen Bonds interaction data between ligand quercetin and target protein 4nos

Index	Residue	AA	Distance H-A	Distance D-A	Donor Angle	Protein donor?	Side chain	Donor Atom	Acceptor Atom
1	197C	ALA	2.98	3.63	125.68	x	x	13701 [O3]	7756 [O2]
2	199C	ARG	2.50	3.16	124.17	y	x	7765 [Nam]	13701 [O3]
3	242C	SER	3.53	4.05	115.47	x	y	13707 [O3]	8127 [O3]

AA: Amino acid residue; Distance H-A: Distance between hydrogen and acceptor atom; Distance D-A: Distance between donor and acceptor atom; Donor Angle: Angle between donor, acceptor, and hydrogen atom; Protein donor?- Does protein provide the donor group?; Side chain: Is the hydrogen bond formed with the amino acid side chain?; Donor Atom: ID of donor atom; Acceptor Atom: ID of acceptor atom.

Hydrophobic interactions that stabilize quercetin within the catalytic domain of human inducible nitric oxide synthase (iNOS) (PDB: 4NOS). Critical contacts include TRP194C (3.50–3.58 Å), LEU209C (3.52 Å), PHE369C (3.64–3.79 Å), and TYR489C (3.30 Å), with distances of ≤4.0 Å signifying high-affinity van der Waals interactions. The interactions competitively displace the native inhibitor by occluding the heme cofactor and limiting substrate access to the active site.

Table 6.5: Hydrophobic Interactions between ligand quercetin and target protein 4nos

Index	Residue	AA	Distance	Ligand Atom	Protein Atom
1	194C	TRP	3.50	13694	7727
2	194C	TRP	3.58	13693	7725
3	209C	LEU	3.52	13694	7856
4	369C	PHE	3.79	13692	9153
5	369C	PHE	3.64	13700	9155
6	489C	TYR	3.30	13692	10135

AA: Amino acid residue; Distance: Distance between interactions' carbon atoms; Ligand Atom: ID of ligand carbon atom; Protein Atom: ID of protein carbon atom. (1)

3.14 Ficus racemosa extract component quercetin interaction with protein target 5voh

The quercetin interaction with protein target 5voh (Crystal structure of engineered water-forming NADPH oxidase (TPNOX) bound to NADPH).

High-fidelity hydrogen bonds stabilize quercetin within the NADPH-binding site of engineered TPNOX (PDB: 5VOH). Significant interactions involve GLU161A (H-A: 2.17 Å; 169°; carbonyl acceptor) and GLY326A (H-A: 2.29 Å; 174°; backbone amide), demonstrating near-optimal geometry that competitively displaces the NADPH cofactor via electrostatic mimicry.

Table 6.6: Hydrogen Bonds interaction data between ligand quercetin and target protein 5voh

Index	Residue	AA	Distance H-A	Distance D-A	Donor Angle	Protein donor?	Side chain	Donor Atom	Acceptor Atom
1	45A	ALA	2.72	3.16	107.98	y	x	327 [Nam]	13732 [O3]
2	131A	CYS	2.74	3.16	106.98	x	x	13730 [O3]	990 [O2]

Index	Residue	AA	Distance H-A	Distance D-A	Donor Angle	Protein donor?	Side chain	Donor Atom	Acceptor Atom
3	161A	GLU	2.17	3.13	169.03	x	y	13724 [O3]	1220 [O.co2]
4	326A	GLY	2.29	3.27	174.37	y	x	2475 [Nam]	13726 [O3]
5	422B	PHE	2.40	3.24	144.73	x	x	13726 [O3]	6629 [O2]

AA: Amino acid residue; Distance H-A: Distance between hydrogen and acceptor atom; Distance D-A: Distance between donor and acceptor atom; Donor Angle: Angle between donor, acceptor, and hydrogen atom; Protein donor?- Does protein provide the donor group?; Side chain: Is the hydrogen bond formed with the amino acid side chain?; Donor Atom: ID of donor atom; Acceptor Atom: ID of acceptor atom.

Stabilization of quercetin through hydrophobic interactions within the NADPH-binding cleft of engineered TPNOX (PDB: 5VOH). Key residues ILE158A (3.69 Å), ILE44A (3.96 Å), and TYR157A (3.99 Å) establish complementary van der Waals interactions with the flavonoid scaffold of quercetin, working in conjunction with hydrogen bonds (**Table 6.6**) to obstruct cofactor access via competitive desolvation.

Table 6.7: Hydrophobic Interactions between ligand quercetin and target protein 5voh

Index	Residue	AA	Distance	Ligand Atom	Protein Atom
1	44A	ILE	3.96	13716	323
2	157A	TYR	3.99	13719	1186
3	158A	ILE	3.89	13713	1201
4	158A	ILE	3.69	13717	1200

AA: Amino acid residue; Distance: Distance between interactions carbon atoms; Ligand Atom: ID of ligand carbon atom; Protein Atom: ID of protein carbon atom.

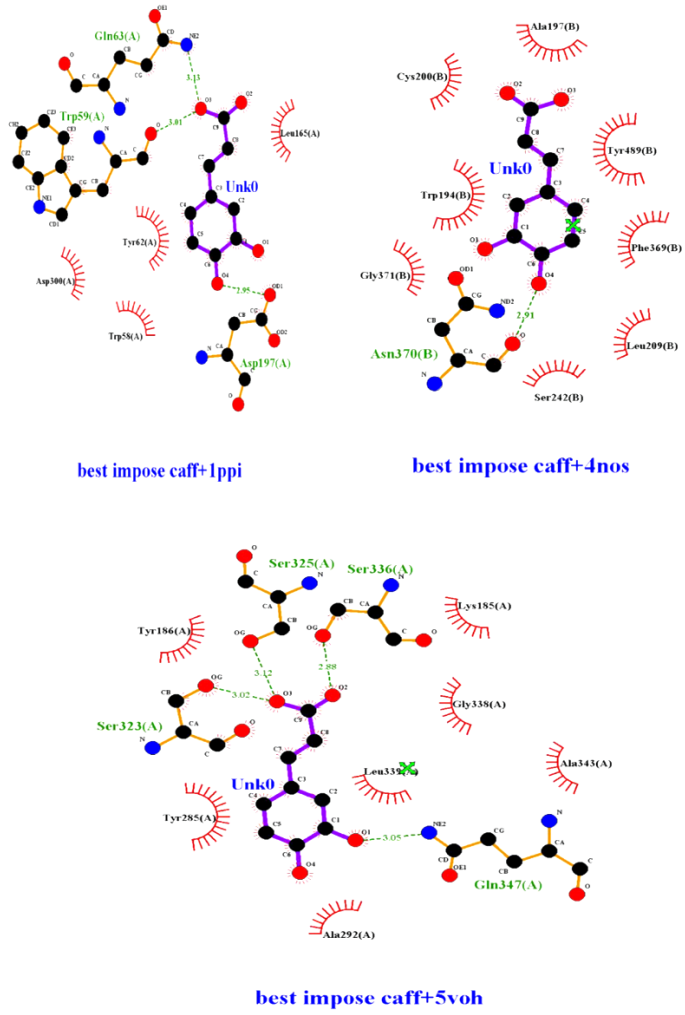

Figure 6: LigPlot demonstration of Caffeic acid and receptors.

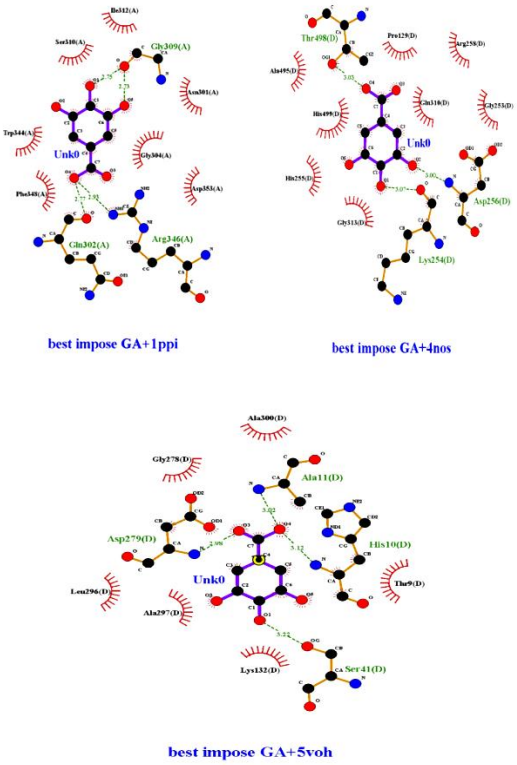

Figure 7: LigPlot demonstration of Gallic acid and receptors.

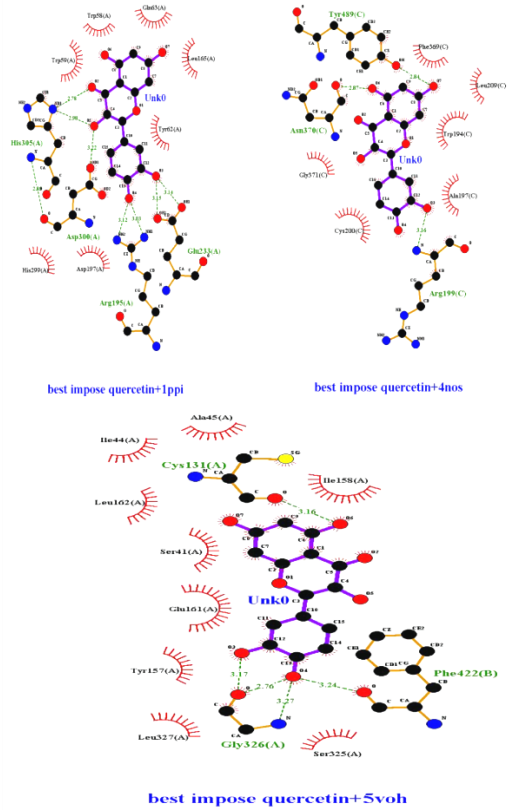

Figure 8: LigPlot demonstration of Quercetin and receptors.

4. Discussion:

The findings of this study highlight the importance of natural antioxidants, especially those derived from *Ficus racemosa*, in reducing oxidative stress. Phytochemicals such as phenolics and flavonoids, which are abundant in plant-based foods, are well-known for their ability to scavenge free radicals, neutralize reactive oxygen species (ROS), and prevent oxidative damage to biomolecules [48, 49, 50]. These bioactive compounds not only play a crucial role in the plant's defense mechanisms but also offer significant health benefits to humans when consumed.

The DPPH assay results demonstrated that the ethyl acetate fraction exhibited the highest free radical scavenging activity, with an IC_{50} value of 0.194 µg/mL. This value surpasses standard antioxidants such as ascorbic acid, which typically falls within the 0.5–20 µg/mL [48, 51]. Extracts with IC_{50} values below 50 µg/mL are considered potent antioxidants, and the ethyl acetate fraction's significantly lower IC_{50} value underscores its remarkable antioxidant efficacy. Hexane hardly carries phenolic compounds in extraction. However, n-hexane acetate fraction has shown greater antioxidant activity than the methanol fraction, which may be due to the presence of acetate solvent or other chemicals present in this selected fraction. The order of radical scavenging activity was as follows:

Ethyl acetate fraction > ethyl acetate-methanol fraction > n-hexane-ethyl acetate fraction > crude extract > methanol fraction > n-hexane fraction

The nitric oxide (NO) scavenging assay further validated these findings, highlighting the ethyl acetate fraction as the most effective in mitigating nitrosative stress, with an IC_{50} of 12.49 µg/mL**.** Given that strong NO scavengers like gallic acid and quercetin typically exhibit IC_{50} values in the range of 10–50 µg/mL [52, 53], the ethyl acetate fraction's activity is well within this range, indicating its strong nitrosative stress-reducing potential. The reduction in nitrite levels is critical, as excessive NO production leads to the formation of reactive peroxynitrite species, exacerbating cellular damage and inflammatory responses. The ranking of NO scavenging activity was as follows:

Ethyl acetate fraction > ethyl acetate-methanol fraction > crude extract > Methanol fraction > n-hexane-ethyl acetate fraction > hexane fraction.

The ferric-reducing power assay demonstrated a concentration-dependent increase in activity, with the ethyl acetate fraction displaying the highest reducing potential. This result aligns with previous studies linking ferric-reducing ability to polyphenolic content [54, 55]. Since phenolics and flavonoids are known to donate electrons to neutralize free radicals, the correlation between HPLC-detected polyphenolic content and reducing power further supports their antioxidant capacity.

The HPLC analysis of the ethanolic extract was carried out to identify the phenol and flavonoid molecules that have important antioxidant activity, and they are used to reduce the risk of cell damage and cell death [1]. Seven natural polyphenolic compounds, such as catechin hydrate (1), (-) epicatechin (2), caffeic acid (3), rutin (4) hydrate, myricetin (5), quercetin (6), and trans-cinnamic acid (7), were identified in the fruit extract of *Ficus racemosa* **Figure 9**. The antioxidant property of phenolic compounds is attributed to their ability to prevent the formation of reactive species, neutralizing (scavenging) free radicals, forming chelate complexes with pro-oxidizing metals, and also the number of hydroxyl groups in the molecule correlates positively with the antioxidant activity [2]. Catechin (1) (structurally an isomer of epicatechin) and epicatechin (2), flavanols, have strong antioxidant activity, which can donate one electron of the phenolic OH group, thus reducing free radicals, and are responsible for deactivating free radicals [3]. Phenolic acids are the most prominent and well-characterized phenolic compounds in plants [4]. Hydroxycinnamic acids such as caffeic acid (3) and trans-cinnamic acid (7) were found in the extract, which are structurally simple and widely distributed in plants. Normally, this kind of phenolic has antioxidant activity and interesting pharmacological properties, though their activity decreases due to their difficulty in penetrating cells because of their high polarity [5]. Other phenolic

compounds like rutin (4) hydrate, myricetin (5), and quercetin (6) have a long history of different pharmacological activities [6, 7, 8, 9]. Rutin or hydrated rutin, a flavanol, has demonstrated excellent antioxidant, anti-inflammatory, and anti-diabetic properties [10]. Myricetin (5) and quercetin (6) are representatives of flavonols subgroups, which have high prevalence in the plant kingdom, and are important components of a healthy diet [11]. Previous literatures explain that the greater the number of hydroxyl substituents present in the B ring Figure **9**; the compound can give the stronger the antioxidant properties [9]. Myricetin (5), which was identified in our sample, has the highest number of hydroxyl groups present, and it has evidence of antioxidant, anti-hyperglycemic, and renoprotective effects [12]. Quercetin (6), though it has one hydroxyl group absent in the B ring, also exhibits a similar kind of pharmacological effect like myricetin [13]. The HPLC and various antioxidant assay results indicate that phenolic and flavonoid compounds are key contributors to the antioxidant activity of the selected plant extract and its fraction. A significant positive correlation has been found in the crude extract of *Ficus racemosa*, and the ethyl acetate fraction due to the presence of phenolic compounds. Methanol fraction comes after ethyl acetate fraction, for this, the phenolic compound presence is at a minimum range, so as their antioxidant activity.

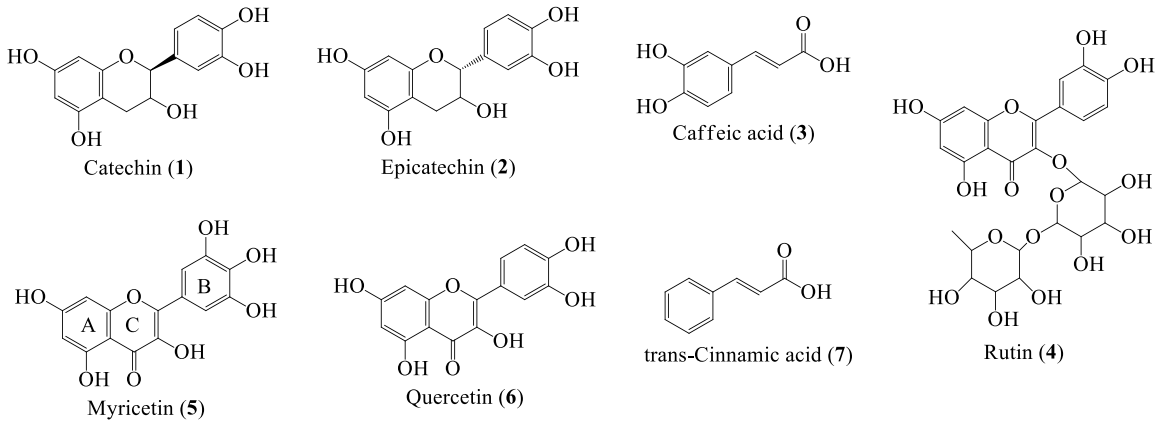

Figure 9: Phenolic compounds found in the ethanolic extract of *Ficus racemosa*.

Molecular docking studies revealed strong binding affinities of key bioactive compounds to oxidative stress-related enzymes. Quercetin (6) exhibited the highest binding affinity with inducible nitric oxide synthase (iNOS) at -9.5 kcal/mol, followed by caffeic acid (3) (-7.3 kcal/mol) and gallic acid (-5.8 kcal/mol). Given that docking scores below -6 kcal/mol indicate strong interactions [56, 57]. Quercetin's high affinity suggests a potential inhibitory role against iNOS, which is implicated in oxidative and inflammatory responses. These interactions, stabilized by hydrogen bonding and hydrophobic forces, suggest that the bioactive compounds in *Ficus racemosa* can inhibit enzyme activity, thereby reducing ROS and reactive nitrogen species (RNS) production, contributing to its therapeutic potential.

Overall, these findings provide strong evidence that *Ficus racemosa* exhibits significant antioxidant properties, with the ethyl acetate and methanol fractions showing the highest efficacy across different assays. The potent free radical scavenging, nitrosative stress mitigation, and enzyme inhibition activities highlight the potential application of these extracts in preventing oxidative stress-related diseases.

5. Conclusion:

The findings of this study reinforce the role *of Ficus racemosa* as a promising natural antioxidant source. Its rich composition of polyphenols, flavonoids, and other bioactive compounds contributes to significant free radical scavenging, nitrosative stress mitigation, and redox balance restoration. The strong antioxidant potential observed across various assays suggests its potential therapeutic application. These findings validate the traditional use of *Ficus racemosa* in folk medicine and position them as promising candidates for functional food development and therapeutic formulations. Given the concerns associated with synthetic antioxidants, the exploration of plant-derived alternatives like *Ficus racemosa* is crucial for developing safer and more effective

health-promoting interventions. The comparative analysis of antioxidant assays and docking results also suggests that *Ficus racemosa* extracts could serve as lead candidates for developing therapeutic agents targeting oxidative stress-related diseases.

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