Trends in Phytochemical Research (TPR)

Journal Homepage: https://sanad.iau.ir/journal/tpr

Original Research Article

Evaluation of pharmacological properties using *in vitro* and *in vivo* (Swiss albino male mice) model followed by isolation of ergosterol from the stem bark extract of *Delonix regia* (Bojer ex Hook.) Raf. (Fabaceae family)

Samia Nahar Tahiti[1], A.H.M. Nazmul Hasan[1]✉*, Sumaia Akter[1], Ramisa Anjum[1], Md. Khokon Miah Akanda[1], Afsana Akter Khuku[1], Akash Majumder[1], Fayad Bin Abdus Salam[2], Tania Binte Wahed[3], And Sadia Noor[1]

[1]Department of Pharmacy, University of Asia Pacific, 74/A Green Road, Farmgate, Dhaka 1205, Bangladesh
[2]Pharmacy Discipline, Khulna University, Khulna 9208, Bangladesh
[3]Department of Pharmacy, Jahangirnagar University, Savar, Dhaka-1342, Bangladesh

ABSTRACT

This study investigated the pharmacological properties of stem bark extracts of *Delonix regia* (Bojer ex Hook.) Raf. (Fabaceae family), followed by the isolation of phytoconstituents. The methanolic extract exhibited significantly higher antioxidant activity (68.96 ± 1.82 mg/g AAE *vs.* 46.4 ± 2.3 mg/g AAE) and DPPH free radical scavenging potential (IC_{50} = 22.387 µg/mL *vs.* 154.88 µg/mL) compared to the petroleum ether extract. Additionally, it demonstrated superior thrombolytic activity (59.62% *vs.* 51.38%) and significantly reduced the number of defecated pellets by 17.29% and 45.86% at doses of 200 and 400 mg/kg BW ($p < 0.01$). Both extracts dose-dependently and significantly reduced blood glucose levels ($p < 0.001$). The methanolic extract also exhibited stronger analgesic, anti-inflammatory, and antipyretic effects compared to the petroleum ether extract in mice. The structure of the isolated ergosterol was confirmed using ^{1}H-NMR spectroscopy. This study may guide future research and the isolation of additional bioactive compounds, while in silico studies could help elucidate the mechanisms underlying the observed activities.

ARTICLE HISTORY

Received: 01 January 2025
Revised: 02 March 2025
Accepted: 27 March 2025
ePublished: 30 March 2025

KEYWORDS

Anti-diabetic
Anti-diarrheal
Anti-inflammatory
Antioxidant
Delonix regia (Bojer ex Hook.) Raf. (Fabaceae)
Ergosterol
Thrombolytic activity

1. Introduction

The vast majority of people on earth rely exclusively on medicinal plants to meet their medical needs. Plant-based medications serve as the primary foundation of therapeutic practices in impoverished countries, and their use has recently expanded on a global scale. Due to their rich composition of minerals, primary metabolites, and diverse secondary metabolites with antioxidant properties, medicinal plants have garnered increasing therapeutic interest. Their bioactive compounds, such as γ-pyrones, coumarins, and polyphenols provide antioxidant, antibacterial, antifungal, and vasodilatory benefits, contributing to traditional medicine and offering promising applications in pharmaceuticals, cosmetics, and the food industry (Ebada et al., 2023; El Jabboury et al., 2023). Some natural compounds involving coumarins and their derivatives, widely found in plant families, exhibit notable antioxidant, anti-inflammatory, and antimicrobial properties, making them valuable in disease prevention and treatment. Likewise, some plant genera are recognized for their essential oils and bioactive compounds, demonstrating significant anticancer, antifungal, anti-inflammatory and ethnopharmacological benefits, offering remedies for ailments like malaria, diabetes, and gastrointestinal disorders. They have been well-documented for their antimicrobial and antioxidant effects, which account for their potential use as natural preservatives in the food industry. Additionally, their phytochemical diversity holds pharmacological significance and contributes to various medicinal applications (Mohammadhosseini et al., 2019; Mohammadhosseini et al., 2021; Kazeminia et al., 2022; Olaoluwa et al., 2022; Sharif and Jabeen, 2024). The stem bark of medicinal plants, such as

✉ Corresponding author: A.H.M. Nazmul Hasan
Tel: +8801756199375; Fax: +8802-58157097
E-mail address: nazmul_0155@yahoo.com, **https://doi.org/10.71596/tpr.2025.195281**

Lannea coromandelica, contains bioactive compounds, including ursolic acid and palmitoleic acid, and exhibits significant antioxidant, antidiabetic, anti-diarrheal, and analgesic activities (Anjum et al., 2024). Consequently, the exploration of medicinal plants highlights their potential in modern medicine, paving the way for future research and drug development.

A species of the flowering plants known as *Delonix regia* (Bojer ex Hook.) Raf. belongs to the Fabaceae (pea) family and the subfamily Caesalpinioideae. Commonly referred to as the Royal Poinciana, Flamboyant, and locally as is widely cultivated as an ornamental tree in tropical regions (Wang et al., 2016). *D. regia* can reach heights of up to 40 feet. Its flowers feature five petals, with a large reddish-orange petal accompanied by four spreading scarlet or orange-red petals, each measuring up to 8 cm in length. One of the petals is significantly larger than the others and displays white streaks. The seeds are firm, glossy, grayish, and rectangular, approximately the size of date seeds, measuring about 2 cm long, and are transversely speckled with a bony testa. The wood is soft and white in color. The flowering season occurs from April to July, while the fruiting season spans from August to October (Özbek et al., 2004; Singh et al., 2014). Vivek et al. (2013) assessed the antioxidant potential of leaf and flower extracts of the plant and found that the free radical scavenging capacity of the leaves is greater than that of the flower extract. Hepatoprotective activities of the flower extracts and gastroprotective properties of 70% ethanolic flower extracts have also been reported (El-Sayed et al., 2011; Shiramane et al., 2011). Additionally, hypoglycemic, analgesic, anti-inflammatory, and anti-arthritic properties of leaf extracts of this plant have been documented in animal models (Chitra et al., 2010; Rahman et al., 2011; Shewale et al., 2012).

To the best of our knowledge, there are only a few limited studies on the stem bark of this plant. Therefore, the current study was designed to explore the therapeutic potential of the stem bark and to isolate bioactive compounds from this part.

2. Experimental

2.1. Collection of stem bark of *D. regia*

Firstly, identification of the plant (Accession No: UAP_Herb/1054_23) was done by a taxonomist of the Department of Botany from Jahangirnagar University, situated in Savar, Dhaka, Bangladesh, and then the stem bark of *D. regia* was collected accordingly from the Jahangirnagar University campus.

2.2. Extraction process

Stem barks were ground into a coarse powder, sun-dried, and dissolved in 2.5 L of methanol and petroleum ether separately for 15 days. Cotton filtering was used to obtain filtrate, and finally the solvent was evaporated at room temperature to obtain the final concentrated extract (El Sawi et al., 2014).

2.3. Phytochemical screening

According to the standard protocols, phytochemical screening of the extracts was done to find the presence of different substances such as carbohydrates, glucosides, saponins, steroids, tannins, flavonoids, and alkaloids (Trease and Evans, 1989; Tiwari et al., 2011).

2.4. Antioxidant activities

2.4.1. Total phenolic content estimation

Phenols completely ionize in an alkaline environment and are rapidly oxidized once the Folin-Ciocalteu reagent (FCR) is added to the ionized phenolic solution (Meena et al., 2012). Each test tube contained 1.0 mL of either the sample or reference standard solution, to which 5 mL of a tenfold diluted reagent solution was added. Subsequently, 4 mL of Na_2CO_3 solution was mixed into the test tubes. The samples were then incubated at 20 °C for 30 minutes for the reference standard and 1 hour for the plant extract. Absorbances of both the sample and standard solutions were taken at 765 nm against a blank. A standard curve was generated using solutions of varying concentrations of gallic acid, and the phenolic content was measured in grams equivalent to gallic acid (GA) (Ghanem et al., 2024).

2.4.2. Determination of flavonoid content

Flavonoids were identified using a colorimetric method with aluminum chloride, employing quercetin as the reference standard. A solution was prepared by combining aluminum chloride (0.2 mL, 10% *w/v*), potassium acetate (0.2 mL, 1.0 M), distilled water (5.6 mL), methanol (3 mL), and the sample (0.2 mL) to achieve a total volume of 1 mL. Absorbance was measured after 30 minutes at 415 nm using a UV/Visible spectrophotometer. A calibration curve was established by preparing quercetin solutions in methanol at various concentrations. The flavonoid content in the sample was quantified in mg/g QE (Li et al., 2016).

2.4.3. Determination of total antioxidant

This process depends on reducing molybdenum (VI) to molybdenum (V) and creating a compound of phosphate and molybdenum (V). 0.3 mL of extracts and 3 mL of reagent solution consisting of H_2SO_4 (0.6 M), Na_3PO_4 (28 mM), and ammonium molybdate (4 mM) were mixed together and heated to 95 °C for 1 hour and 30 minutes. Then, the absorbances were taken at 695 nm against a blank after the solution had completely cooled. The extract's total antioxidant potential was measured from an equation derived from the ascorbic acid concentrations against optical density plot (Mahfuz et al., 2019).

2.4.4. DPPH (1,1-Diphenyl-2-picrylhydrazyl) scavenging capacity assay

1,1-Diphenyl-2-picrylhydrazyl radical (DPPH) is commonly used to assess an antioxidant's ability to scavenge free radicals present in the reaction medium. The DPPH free radical interacts with hydrogen donors, transforming into hydrazine, which allows for the determination of the antioxidant's antiradical power at 517 nm (Braca et al., 2002). In test tubes, 1 mL of sample or standard solutions at various diluted concentrations were mixed with 2 mL of freshly prepared DPPH solution (0.004%) to achieve a final volume of 3 mL. After 30 minutes of incubation at room temperature, the optical density of the solution was measured at 517 nm using the UV-spectrophotometric method. The percentage of inhibition was calculated using the following equation, and the anti-radical efficacy (IC_{50}) was calculated accordingly.

Inhibition (%) = $[(A_0 - A_1)/A_0] \times 100$ (Eqn .1)

Where A_0 and A_1 represent the absorbance of the control and the absorbance of the test sample, respectively.

2.5. Determination of *in vitro* thrombolytic activity

An *in vitro* method, as described in the literature, was employed to evaluate thrombolytic activity of the prepared extracts (Prasad et al., 2006). Accordingly, 100 mg of each extract was placed in separate vials and dissolved in 10 mL (10,000 µL) of distilled methanol, petroleum ether, and sterile water, respectively. 5 mL venous blood was drawn from healthy adult male volunteers with no hematological disorders or history of anticoagulant therapy. Aliquots of blood were then transferred to pre-weighed sterile Eppendorf tubes and incubated at 37 °C for 45 minutes to allow clot formation. The serum was carefully removed using a sterile cotton swab after clot formation, taking care not to disturb the clot. Each Eppendorf tube was weighed again after serum removal to determine the clot weight. 100 µL solutions containing different extracts were added separately to each Eppendorf tube containing the pre-weighed clot. One Eppendorf tube contained only 100 µL of streptokinase (30,000 I.U.), while another contained only 100 µL of sterile distilled water. All Eppendorf tubes were then incubated at 37 °C for approximately 90 minutes and subsequently observed for clot lysis. The released fluid was removed from all Eppendorf tubes after incubation, followed by weighing to determine the difference in weight after clot disruption.

2.6. Determination of membrane stabilizing activity

In the experiment, hemolysis was induced using a stock erythrocyte suspension (30 mL) combined with plant extracts (1.0 mg/mL) or acetyl salicylic acid (0.10 mg/mL) and 5 mL of a hypotonic solution. After incubating the solutions at room temperature for 10 minutes, they were centrifuged at 1500 rpm for the same duration. The absorbance of the supernatant at 540 nm was measured using a UV spectrophotometer. Membrane stabilization was determined using the equation provided below.

Inhibition (%) of hemolysis = $100 \times [(OD_1 - OD_2)/OD_1]$ (Eqn. 2)

Where OD_1 represents the optical density of the hypotonic-buffered saline solution by itself (the control) and OD_2 equals the optical density of the sample being tested.

Tubes received 30 mL of erythrocyte suspension, which was mixed gently by inversion as a control. One set of tubes was incubated in a water bath at 54 °C for 20 minutes. The second pair was placed in an ice bath, centrifuged, and the absorbance was measured at 540 nm. The following equation was used to determine whether hemolysis in the tests was slowed down or accelerated by a specific proportion:

Inhibition (%) of hemolysis = $100 \times [1 - (OD_2 - OD_1/ OD_3 - OD_1)]$ (Eqn. 3)

Where OD_1, OD_2 and OD_3 represent an unheated test sample, a heated test sample, and a heated control sample, respectively (Omale et al., 2023).

2.7. Brine shrimp lethality bioassay: cytotoxicity

The cytotoxicity of the extract was assessed using lethal concentration estimation with *Artemia salina* (brine shrimp) (Meyer et al., 1982; Omale et al., 2023). A sufficient number of brine prawns (*Artemia salina*) eggs were hatched in a glass tank filled with artificial seawater (38 g/L sea salt) and properly ventilated for two days. To prepare the sample solutions, the test materials were dissolved in a predetermined amount of dimethyl sulfoxide. In each test tube containing 5 mL of simulated sea water, ten nauplii were added. Samples of varying concentrations were then combined. After 24 hours, the number of surviving nauplii was counted, and lethality was determined from the log concentration versus percent mortality graph.

2.8. Evaluation of antimicrobial activity

The disc diffusion method, first described by Bauer et al. (1966) using several bacterial strains, is a technique for assessing antibiotic susceptibility. In this method, filter paper discs with a diameter of 6 mm are soaked in the test substance and then placed on Mueller-Hinton agar media. The test material diffuses into the media, creating zones of inhibition where bacterial growth is suppressed, depending on the solubility and size of the substance. This same principle can also be applied to measure antifungal activity.

2.9. *In vivo* pharmacological activities

2.9.1. Experimental animal

Swiss albino male mice weighing between 25 and 30 g and aged 4 to 5 weeks were used in this investigation. All animals required for this study were provided by the Animal Resources Facility of the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR, B). The experiments were conducted in accordance with the ARRIVE guidelines.

2.9.2. Dose administration

Mice were divided into experimental and control groups, each consisting of four mice. Both plant extracts were administered orally in two doses: the methanolic extract at 200 mg/BW (DRM 200) and 400 mg/kg BW (DRM 400), as well as the petroleum ether extract at 200 mg/kg BW (DRP 200) and 400 mg/BW (DRP 400).

2.9.3. Evaluation of anti-diarrheal activity by castor oil induced method

Oral doses of loperamide hydrochloride (3 mg/kg BW) and stem bark extracts (200 mg/kg and 400 mg/kg BW) were administered to mice. Following a 60-minute interval, castor oil (0.5 mL) was given orally. The mice were monitored for 4 hours to evaluate the consistency and frequency of fecal matter, and the percentage of inhibition of defecation pellets was measured (Umer et al., 2013).

2.9.4. Evaluation of peripheral analgesic activity by acetic acid induced writhing method

The acetic acid-induced writhing test in mice, as described by Koster (1959), was conducted to evaluate analgesic activity. Six groups of four mice were randomly assigned to receive different treatments. The first group received Tween 80 (1.0%) orally, while the second group received diclofenac sodium. After 30 minutes, 0.7% acetic acid was administered intraperitoneally, and the mean abdominal writhing was calculated and recorded.

2.9.5. Evaluation of hypoglycemic activity

Mice were fasted overnight, and blood glucose levels were estimated using the glucose oxidase method (Mahfuz et al., 2019). Six groups, each consisting of four mice, were formed from a total of 24 fasting animals. Group I received a DMSO and Tween-80 suspension, while Group II received 5 mg/kg of glibenclamide orally. Groups III to VI received crude extracts at doses ranging from 200 to 400 mg/kg BW orally. After 60 minutes of extract administration, mice from all groups were given a glucose solution at a dosage of 200 mg/kg BW. Blood samples were taken from the tail vein, and glucose levels were measured using glucometers and glucose-oxidase-peroxidase reactive strips (Barham and Trinder, 1972).

2.9.6. Antipyretic activity study

Pyrexia was induced via a subcutaneous injection of a 15.0% *w/v* solution of Brewer's yeast in distilled water at a dosage of 10 mL/kg BW. The study used 50 mg/kg BW of paracetamol and Tween-80 (1.0%) as a control to examine mice whose temperature rose by 0.6 °C after 18 hours of receiving a Brewer's yeast injection. Methanolic preparations from the stem bark of *D. regia* were given orally. Mice in Groups III to VI received oral doses of 200 and 400 mg/kg BW dose of *D. regia* methanol and petroleum ether extract of stem bark, respectively, and were then compared before and after treatment (Braca et al., 2002).

2.9.7. Tail immersion test for analgesic activity

Tail immersion method investigates analgesic mechanism using thermal stimulation in animals' painful reactions (Braca et al., 2002; Özbek et al., 2004; Kumar and Shankar, 2009). Oral administration of diclofenac, control, and test samples was conducted, with a 30-minute break for proper absorption. Mice tail was submerged in hot water, and time was compared with the standard and control groups. Extracts were administered in same dose as mentioned in previous studies.

2.9.8. Acute toxicity study

Acute toxicity refers to the harmful effects caused by a substance following one or more exposures within a 24-hour period (Omale et al., 2023). In this study, Group I received 10 mL/kg of regular saline water as a control. The methanolic extract was administered orally to Groups II to VI at doses of 500, 1000, 2000, 4000, and 8000 mg/kg BW, respectively. Meanwhile, the petroleum ether extract was administered orally to Groups VII to XI at the same doses. Both animal mortality rates and behavioral changes were observed.

2.10. Isolation of bioactive compounds

Column chromatography can be used to isolate phytoconstituents from plant extracts, followed by thin-layer chromatography (TLC) and preparative TLC (PTLC), and ultimately nuclear magnetic resonance (NMR) spectroscopy to determine the structures of the recovered compounds. The underlying principle of silica column chromatography is that a mixture of different molecules is placed onto the stationary phase, through which solvents of varying compositions are passed. Compounds are separated based on their differing polarities (Walum, 1998; Coskun, 2016). The methanol extract of the plant was then applied to the top of the stationary phase and eluted using a mobile phase composed of *n*-hexane, chloroform, and ethyl acetate in various ratios, ranging from non-polar to polar. The eluted samples were subsequently analyzed on TLC plates using a specified solvent system as part of the initial screening procedure to identify test tubes containing similar compounds. The contents of test tubes with the same R_f values were combined for further separation by TLC. Following this procedure, samples from test tubes numbered 100 to 165 were isolated using ethyl acetate (4.0%) in chloroform, separated by preparative TLC, and the compounds were spotted under a UV lamp. They were then eluted and labeled as DR-01-4 EAT-01 and referred for ^{1}H-NMR analysis to the Bangladesh Council of Scientific and Industrial Research (BCSIR). After comparing the NMR data with reference materials, the structures of the isolated compound was

determined.

2.11. Statistical analysis

The data were expressed using the standard error of the mean (SEM). The results were statistically evaluated using Microsoft Excel 2010. T-test of two equal variances was done and the results less than $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ were considered statistically significant. The LC_{50} values for cytotoxicity as well as the IC_{50} values for scavenging free radicals were calculated using the dosage response curve.

3. Results and Discussion

3.1. Phytochemical screening

Initial phytochemical group analysis of stem bark extracts of *D. regia* confirmed a number of secondary metabolites, including carbohydrates, glycosides, steroids, flavonoids, and alkaloids, summarized in Table 1. Our preliminary experiments revealed that both of methanolic and petroleum ether extracts of the stem bark in the current study provided positive results for tests of carbohydrates, alkaloids, flavonoids, and glycosides. The study subject contained phytochemicals with potential therapeutic benefits, including antioxidant, anticancer, antibacterial, antidiarrheal, analgesic, and antipyretic properties.

3.2. Determination of *in vitro* antioxidant activities

3.2.1. Total phenolic content

The calibration curve ($y = 0.011x + 0.1418$, $R^2 = 0.991$) of gallic acid was used to estimate the total phenolic content of the sample extracts, expressed as mg/g GAE. This study found that the methanolic extract contained a higher amount of phenolic content (12.1036 ± 0.40 mg/g GAE) compared to the petroleum ether extract (11.1581 ± 0.65 mg/g GAE) (Table 2).

3.2.2. Total flavonoid content

Petroleum ether stem bark extract of *D. regia* was found to contain higher amount of flavonoid (16.27 ± 1.35 mg/g QE) than the methanolic extract (10.95 ± 0.3mg/g QE) as estimated from the calibration curve of standard quercetin ($y = 0.0124x - 0.0705$, $R^2 = 0.9$) and expressed as mg/g QE (Table 2).

3.2.3. Total antioxidant capacity

Both methanol and petroleum ether extracts of the stem bark of *D. regia* have demonstrated a considerable level of total antioxidant content in this study, with the methanolic extract exhibiting a higher concentration than the petroleum ether fraction. The total antioxidant content of the methanolic fraction was 68.96 ± 1.82 mg/g AAE, while the petroleum ether fraction was 46.4 ± 2.3 mg/g AAE (Table 2). These values were estimated using the calibration curve ($y = 0.005x + 0.1095$, $R^2 = 0.9934$) of the reference standard ascorbic acid.

3.2.4. DPPH free radical scavenging capacity assay

According to this study, the methanol extract demonstrated greater potency in scavenging DPPH compared to the petroleum ether extract of the plant (see Table 2 and Fig. 1). The IC_{50} value for the methanol extract was 22.387 µg/mL, while the petroleum ether extract had an IC_{50} value of 154.88 µg/mL. In comparison, the standard ascorbic acid exhibited an IC_{50} of 13.458 µg/mL.

Antioxidants play a crucial role in slowing cellular aging. Diets rich in vegetables and fruits are beneficial for health; however, supplements have proven ineffective in preventing illness. Shabir et al. (2011) reported that the methanolic extract of *D. regia* leaves exhibits excellent antioxidant properties. In their study, they found that the total phenolic content was 3.63 g/g GAE, the total flavonoid content was 1.19 g/g GAE, and the DPPH scavenging capacity had an IC_{50} value of 8.89 µg/mL. The methanolic extract of *D. regia* stem bark used in this investigation had a total phenolic content of 12.10 ± 0.40 mg/g GAE, a total flavonoid content of 10.95 ± 0.3 mg/g GAE, and an antioxidant content of 68.96 ± 1.82 mg/g AAE. Additionally, the petroleum ether extract of *D. regia* stem bark exhibited a total phenolic content of 11.15 ± 0.65 mg/g GAE, a total flavonoid content of 16.27 ± 1.35 mg/g GAE, and an antioxidant content of 46.4 ± 2.3 mg/g AAE. Dose-dependent scavenging of the DPPH free radical was observed for both extracts, with the methanolic extract demonstrating a higher inhibitory potential. Our current study also supports the antioxidant properties of the stem bark of *D. regia*.

3.3. Thrombolytic activity

In this study, the methanolic stem bark extract demonstrated a clot lysis percentage of 59.62 ± 1.36%, while the petroleum ether extract exhibited a clot lysis percentage of 51.38 ± 0.37%. In comparison, the reference standard, streptokinase, showed a significant clot lysis percentage of 66.38 ± 0.896% (Table 3). These findings indicate that both extracts possess considerable potential for clot lysis.

Thrombus formation in the blood vessel can lead to elevated blood pressure, heart attack, and oxygen deprivation. In contrast to the drawbacks of thrombolytic drugs, medicinal plants and their constituents exhibit antithrombotic properties. A study by Rahman et al. (2020) demonstrated that the methanolic extract of flowers from *D. regia* exhibited 35.5% thrombolytic activity. In our current study, both methanolic and petroleum ether extracts showed significant thrombolytic activity compared to the streptokinase standard (66.38%), with respective values of 59.62% and 51.38%. This suggests that the stem bark of this plant contains a substantial concentration of thrombolytic compounds.

Table 1
Phytochemical screening of *Delonix regia*.

Phytochemical Tests	Methanolic extract	Petroleum ether extract
Carbohydrate test	+	+
Glycoside test	+	-
Glucoside test	+	+
Saponin test	+	+
Steroid test	+	+
Tannin Test	+	-
Flavonoid test	+	+
Alkaloid test		
Hager's reagent	+	+
Wagner's reagent	+	+
Dragendroff's reagent	+	+

Table 2
Antioxidant potential of stem bark extract of *Delonix regia*.

Sample	Total phenol content (mg/g GAE ± STD)	Total flavonoid content (mg/g QE± STD)	Total antioxidant capacity (mg/g AAE± STD)	DPPH free radical scavenging capacity assay (IC_{50}, µg/mL)
Methanolic extract	12.1036+ 0.40[a]	10.95 ± 0.3	68.96±1.82[a]	22.387
Petroleum ether extract	11.1581+ 0.65[a]	16.27± 1.35	46.4± 2.3[a]	154.88
Ascorbic acid		-		13.458

[Note: Values are represented as mean ± SEM, t-test of two equal variance was done to analyze the data set. Values in the same column with different superscripts are significantly different from another, p < 0.05,; **GAE:** Gallic Acid Equivalent; **QE:** Quercetin Equivalent; **AAE:** Ascorbic Acid Equivalent].

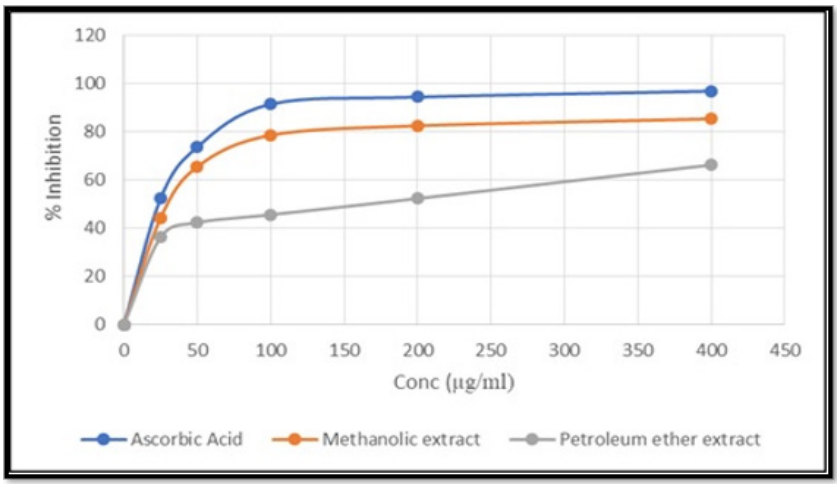

Fig. 1. Comparison of DPPH scavenging of different extracts of *Delonix regia* against ascorbic acid.

3.4. Membrane stabilizing activity

Standard acetyl salicylic acid (ASA) reduced red blood cell hemolysis by 55.71% in a hypnotic solution-induced hemolysis model, while methanol and petroleum ether extracts inhibited hemolysis by 49.54% and 48.63%, respectively. Although the effect of petroleum ether is noteworthy, the results indicate that the methanol component of the stem bark has substantial activity as a membrane stabilizer (see Table 3). In the case of heat-induced hemolysis, standard acetylsalicylic acid demonstrated a percent inhibition of hemolysis of 69.59%. On the other hand, the methanol and petroleum ether extracts exhibited 22.74% and 50.17% inhibition of hemolysis, respectively (see Table 3).

Current conventional therapies, such as nonsteroidal anti-inflammatory drugs (NSAIDs), can have adverse effects. However, there is potential for plants to serve as sources of novel calming medications. Recent studies indicate modest effectiveness in preventing red blood cell hemolysis in hypnotic solutions.

Table 3
Thrombolytic, membrane stabilizing and cytotoxic activity of stem bark extract of *Delonix regia*.

Extract/Standard	Thrombolytic activity	% Inhibition of hemolysis ± STD		Brine shrimp lethality bioassay LC_{50}, µg/mL
	% of clot lysis	Hypotonic solution	Heat induced	
Methanolic stem bark extract	59.62 ± 1.36	49.54%	22.74%	269.153
Petroleum ether stem bark extract	51.38 ± 0.37	48.63%	50.17%	141.25
Streptokinase	66.38 ± 0.896			
Distilled water	20.38 ± 0.21			
Aspirin		55.71%	69.59%	
Vincristine				0.394

3.5. Brine shrimp lethality bioassay

The cytotoxic effects of the plant extracts were assessed by counting the surviving nauplii after 24 hours. The LC_{50} values for the methanolic extract (y = 53.882x - 81.438, R^2 = 0.8966) and the petroleum ether extract (y = 74.149x - 109.95, R^2 = 0.884) were determined using regression equations. This study found that both extracts exhibited limited cytotoxic activity compared to the standard vincristine; however, the petroleum ether extract demonstrated greater potency than the methanolic extract (Table 3).

3.6. Evaluation of antimicrobial activity

The antimicrobial activity of *D. regia* extracts was tested against two Gram-positive bacteria (*Staphylococcus aureus* and *Bacillus* sp.), two Gram-negative bacteria (*Salmonella typhi* and *Pseudomonas aeruginosa*), and two fungal species (*Aspergillus niger* and *Aspergillus flavus*) at three different concentrations (100, 200, and 300 µg/disc). None of the extracts exhibited any antimicrobial activity against the tested microorganisms. Ciprofloxacin and ketoconazole were used as standard antibacterial and antifungal agents at concentrations of 5 and 30 µg/disc, respectively. However, according to Shabir et al. (2011), *D. regia* flower extracts demonstrated stronger antibacterial efficacy against *Pseudomonas stutzeri*, *Escherichia coli*, and *Aspergillus niger*.

3.7 Evaluation *in vivo* pharmacologic activities

3.7.1 Evaluation of antidiarrheal activity

The quantity of defecated pellets over time decreased in a dose-dependent manner when *D. regia* stem bark extract was used to treat castor oil-induced diarrhea in male Swiss albino mice. The highest average percentage decrease in defecated pellets was observed with methanolic extracts (Fig. 2-A), which reduced the number of pellets by 45.86% at a dose of 400 mg/kg BW (DRM 400), a result that was statistically significant ($p < 0.01$). In contrast, the petroleum ether extract at the same dose of 400 mg/kg BW exhibited lower efficacy in inhibiting defecation, resulting in a decrease of only 25.56% ($p < 0.05$).

3.7.2 Evaluation of peripheral analgesic activity

At the doses of 200 and 400 mg/kg BW, the methanol extract of the stem bark of *D. regia* demonstrated a writhing inhibition of 53.97% and 79.37% ($p < 0.001$) (Fig. 2-B). In comparison, the petroleum ether extract exhibited 58.73% and 76.19% ($p < 0.001$) inhibition of writhing at the same doses. The positive control, diclofenac sodium, achieved an inhibition percentage of 73.02% at a dose of 50 mg/kg BW.

3.7.3 Evaluation of antipyretic activity

The methanolic extract of *D. regia* stem bark possessed higher efficacy in reducing body temperature compared to the petroleum ether extract, with the maximum effect observed at the fourth hour of administration in a dose-dependent manner (Fig. 3-A), which was statistically significant.

3.7.4 Evaluation of hypoglycemic activity

Both extracts exhibited anti-diabetic efficacy in a dose-dependent manner, with the highest activity observed at the third hour following glucose administration, which was statistically significant. The methanolic and petroleum ether stem bark extracts of *D. regia* reduced blood glucose levels by 59.1% and 54.3%, respectively (Fig. 3-B, $p < 0.001$), in mice model at a dosage of 400 mg/kg BW after three hours of glucose administration. This study indicates that both extracts possess strong anti-diabetic effectiveness.

3.7.5 Evaluation of analgesic activity by tail immersion test

To assess the analgesic activity, diclofenac sodium was used as the reference standard. The analgesic efficacy of the stem bark extract of *D. regia* is expressed as a percentage of analgesia and measured by the duration of time the tail remains submerged in hot water, as shown in Fig. 3-C. According to this study,

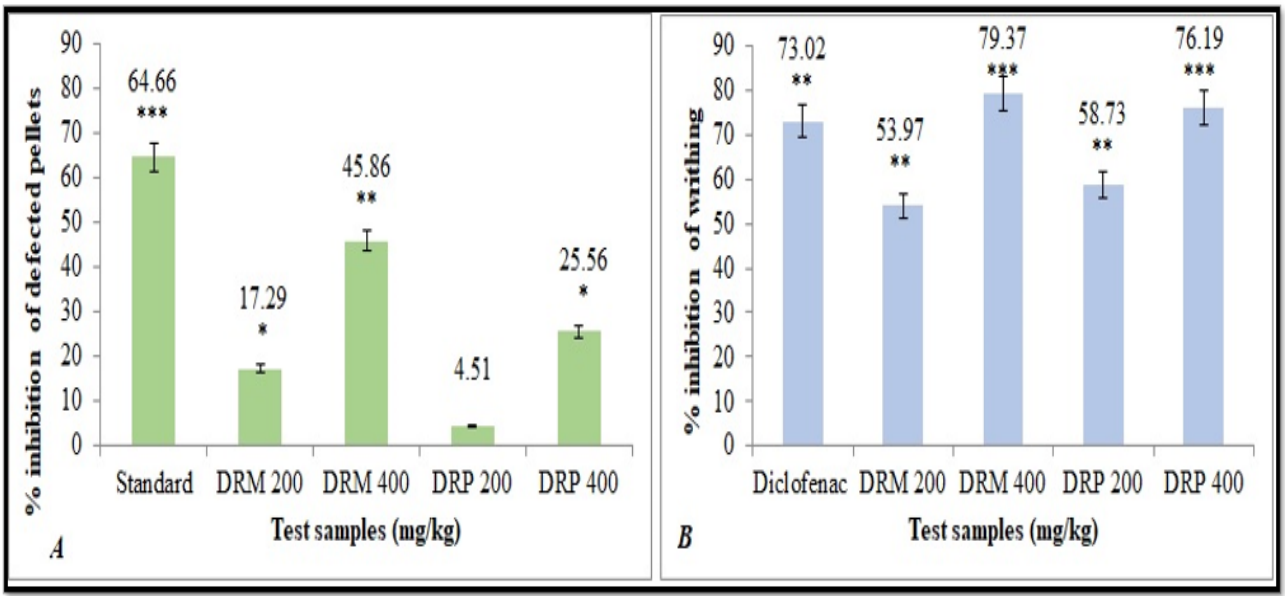

Fig. 2. Percent inhibition of (A) defected pelleted and (B) writhing by stem bark extracts of *Delonix regia*.

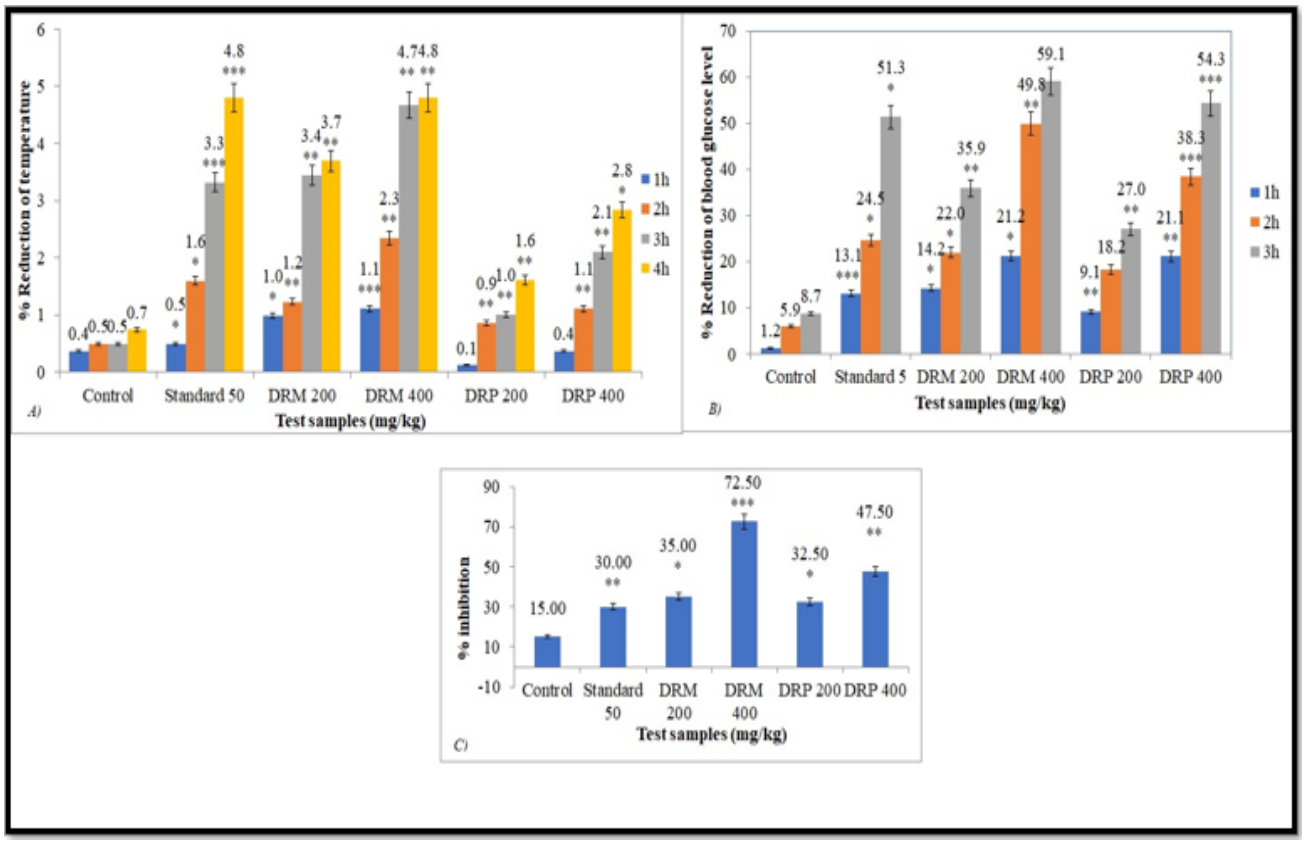

Fig. 3. Percentage reduction of (A) body temperature, (B) blood glucose and (C) percent of analgesia by *Delonix regia* extracts.

the methanolic stem bark extract demonstrated the highest percentage of analgesia (72.50%) at a dose of 400 mg/kg BW dose compared to the petroleum ether extract, and this result was found to be statistically significant ($p < 0.001$).

3.7.6 Acute toxicity study

To estimate the level of toxicity, mice were administered increasing dosages (500, 1000, 2000, 4000, and 8000 mg/kg BW) of plant extracts, while the control group received distilled water. Consequently, even at considerably higher doses of up to 8000 mg/kg BW, no mortality or other behavioral changes were observed.

3.8 Compound isolation

After performing column chromatography, TLC and PTLC technique, using 4.0% ethyl acetate in chloroform, a compound was isolated and designated as DR-01-4EAT-01. This compound was analyzed using ^{1}H-NMR spectroscopy (Fig. 4). Previous phytochemical reports indicate that the compound DR-01-4EAT-01 corresponds to the data for ergosterol (Nowak et al., 2016). Therefore, the isolated compound from the methanolic extract of *D. regia* stem bark, DR-01-4EAT-01, is identified as ergosterol, with the spectral data summarized in Table 4. The structure of ergosterol is depicted in Fig. 4.

The methanolic extract of *D. regia* stem bark contains ergosterol, a provitamin derived from vitamin D2, as well as a unique polyphenolic compound characterized by its diverse structure and hydrophilic properties. Notably, ergosterol has been shown to inhibit the growth of human colon cancer cells (Merdivan and Lindequist, 2017). This effect is associated with altered mitochondrial activity and reduced DNA synthesis, suggesting its potential cytotoxicity. Additionally, this remarkable compound exhibits analgesic and anti-inflammatory properties. The *in vivo* results of the current study further indicate that ergosterol may possess significant analgesic and anti-inflammatory effects. In one study, delphinidin and cyanidin at 15 µg/mL were identified as the primary compounds in the red pigment constituents of *D. regia* pigment extracts, as determined by HPLC analysis. The pigment extract from *D. regia* contained significant amounts of anthocyanins (26.33 mg/g), phenolic compounds (64.7 mg/g), and flavonoids (10.30 mg/g). These compounds contributed to the extract's antioxidant activity, which effectively scavenged 92% of DPPH free radicals. Additionally, the extract demonstrated inhibitory effects on pancreatic cancer cell lines and exhibited antibacterial activity (Ebada et al., 2023). Another study employed three different extraction methods to obtain leaves from *D. regia*. Their GC-MS study confirmed that the oil extracted from hydrodistilled, steam-distilled, and macerated air-dried leaves yielded 0.32%, 0.54%, and 1.25% oil, respectively. Gas chromatography identified a total of 22, 22, and 12 compounds in the hydrodistilled, steam-distilled, and macerated air-dried plant materials, respectively. The GC-MS analysis of the hydrodistilled oil revealed the presence of thymol (40.09%), *cis*-muurola-4,5-diene (4.93%), δ-cadinene (4.04%), and α-copaene (3.61%) (Li et al., 2016). The primary constituents of the steam-distilled oil included caryophyllene (41.64%), thymol (20.42%), β-selinene (8.02%), isocaryophyllene (7.86%), γ-gurjunene (3.45%), and δ-cadinene (3.44%). In contrast, the main constituents of the cold-macerated oil were benzene (1,1-dimethoxylethoxy) (61.90%), *epoxy* linalool oxide (17.10%), β-lonone (8.23%), and 2,7-octadiene-1,6-diol-2,6-dimethyl- (6.21%) (Njoku et al., 2022).

Table 4
Comparison of ^{1}H-NMR (400 MHz) spectral data of DR-01-4EAT-01 and Ergosterol (chemical shift in ppm,ref-TMS).

1H-NMR of DR-01-4EAT-01	Ergosterol
1.715, 3.659 (m), 6.990, 1.221 (m), 1.276 (m), 1.584 (m), 1.407 (m), 1.324 (m), 0.837 (s), 2.040 (m), 1.001 (d), 5.284 (dd), 1.866 (m), 1.604(m), 0.819, 0.923	1.73 (j =13.8, 3.4), 3.98 (m), 6.52 (j = 8.6), 1.23 (m), 1.27(m), 1.59(m), 1.42 (m), 1.33 (m), 0.83(s), 2.05 (m), 1.00 (d), 5.16 (dd), 1.86 (m), 1.6 (m), 0.82 (d, j = 6.8), 0.91 (d, j = 6.8)

4. Concluding remarks

The current study on the methanolic and petroleum ether extracts of the bark of *D. regia* elucidated the role and contribution of these extracts in the treatment of various ailments and diseases. The *in vitro* assessment of *D. regia* highlighted its significance as one of the most potent sources of cytotoxic, and antioxidative agents. High doses of both plant extracts demonstrated strong analgesic, antipyretic, and hypoglycemic activities with a favorable margin. The findings of the current report revealed the potential analgesic and anti-inflammatory properties of *D. regia*. Moreover, a valuable natural compound, ergosterol, was isolated from this plant. However, future research should focus on elucidating the precise molecular mechanisms underlying these pharmacological effects through advanced *in vivo* and *in silico* studies. Further investigations are warranted to explore other bioactive compounds present in the plant, identify potential synergistic interactions with standard drugs, and develop novel pharmaceutical formulations to enhance bioavailability and therapeutic efficacy. Although promising pharmacological activities have been demonstrated *in vitro* and *in vivo* (using mice model), the lack of clinical validation remains a significant limitation; therefore, preclinical and clinical trials are necessary to assess its efficacy and safety in human subjects. Its therapeutic potential should also be more thoroughly established through comparative studies with traditional therapies. This study is novel as

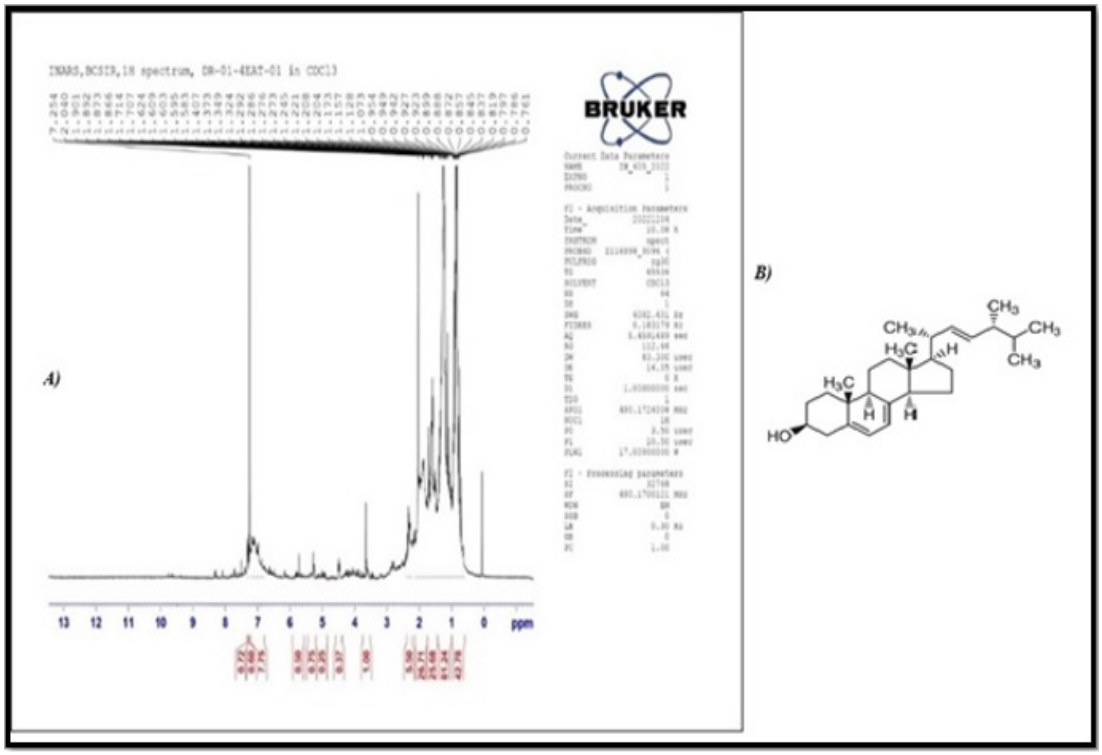

Fig. 4. A) ^{1}H-NMR spectrum of isolated DR-01-4EAT-01 from the stem bark extract of *Delonix regia* and **B)** structure of ergosterol.

it is the first to isolate ergosterol from the stem bark extracts of *D. regia* and to conduct a comprehensive pharmacological evaluation. These findings also pave the way for future drug discovery research. Given its potential, further research on *D. regia* could significantly impact the pharmaceutical and medical sectors, particularly in the development of plant-based therapeutic agents.

Author contribution

Conceptualization was conducted by Samia Nahar Tahiti, A.H.M. Nazmul Hasan, and Sumaia Akter. Data curation was handled by Samia Nahar Tahiti. Formal analysis was carried out by Samia Nahar Tahiti, Sumaia Akter, Md. Khokon Miah Akanda, Fayad Bin Abdus Salam, Tania Binte Wahed. The investigations were performed by Samia Nahar Tahiti, Ramisa Anjum, Sumaia Akter, Akash Majumder, Afsana Akter Khuku and Sadia Noor. Methodology was developed by A.H.M. Nazmul Hasan, Tania Binte Wahed and Sadia Noor. Supervision was provided by A.H.M. Nazmul Hasan. The original draft was written by A.H.M. Nazmul Hasan and Samia Nahar Tahiti, while the review and editing were completed by A.H.M. Nazmul Hasan and Ramisa Anjum.

Acknowledgments

The authors are thankful to ICDDR'B for their cooperation to provide Swiss albino mice and guidance in handling laboratory animals. The authors are also thankful to BCSIR (Bangladesh Council of Scientific and Industrial Research) for the NMR study of isolated plant compounds.

Ethical approval

A research proposal was submitted to the Research Ethics Committee (REC) prior to the study for approval and approved in a meeting (UAP/REC/2023/112) by the REC of the Department of Pharmacy, University of Asia Pacific.

Funding

The authors receive no external funding for this study.

Conflict of interest

The authors declare that there is no conflict of interest

References

Anjum, R., Hasan, A.H.M.N., Hawa, M.A.A., Rahman, M.N., Akanda, M.K.M., Wahed, T.B., Salam, F.B.A., Islam, M.R., Shahriar, M., 2024. Characterization of pharmacological properties and isolation of two bioactive compounds (ursolic acid and palmitoleic acid) from the stem bark extract of *Lannea coromandelica*. Trends Phytochem. Res. 8(2),106-121.

Barham, D., Trinder, P., 1972. An improved colour reagent for the determination of blood glucose by the

oxidase system. Analyst 97(1151), 142-145.
Bauer, A.W., Kirby, W.M.M., Sherris, J.C., Turck, M., 1966. Antibiotic susceptibility testing by a standardized single disk method. Am. J. Clin. Pathol. 45(4), 493-496.
Braca, A., Sortino, C., Politi, M., Morelli, I., Mendez, J., 2002. Antioxidant activity of flavonoids from *Licania licaniaeflora*. J. Ethnopharmacol. 79(3), 379-381.
Chitra, V., Ilango, K., Rajanandh, M.G., Soni, D., 2010. Evaluation of *Delonix regia* Linn. flowers for antiarthritic and antioxidant activity in female Wistar rats. Ann. Biol. Res. 1(2), 142-147.
Coskun, O., 2016. Separation techniques: Chromatography. North Clin. Istanb. 3(2), 156.
Ebada, D., Hefnawy, H.T., Gomaa, A., Alghamdi, A.M., Alharbi, A.A., Almuhayawi, M.S., Alharbi, M.T., Awad, A., Al Jaouni, S.K., Selim, S., Eldeeb, G.S., 2023. Characterization of *Delonix regia* flowers' pigment and polysaccharides: Evaluating their antibacterial, anticancer, and antioxidant activities and their application as a natural colorant and sweetener in beverages. Molecules 28(7), p.3243.
El-Sayed, A. M., Ezzat, S. M., Salama, M. M., Sleem, A. A. 2011. Hepatoprotective and cytotoxic activities of *Delonix regia* flower extracts. Pharmacog. J. 3, 49-56.
El-Jabboury, Z., Bentaib, R., Stevanovic, Z. D., Ousaaid, D., Benjelloun, M., EL Ghadraoui, L. 2023. *Ammi visnaga* (L.) Lam.: An overview of phytochemistry and biological functionalities. Trends Phytochem. Res. 7, 141-155.
El Sawi, I., Fawaz, Z., Zitoune, R., Bougherara, H., 2014. An investigation of the damage mechanisms and fatigue life diagrams of flax fiber-reinforced polymer laminates. J. Mater. Sci. 49, 2338-2346.
Ghanem, K.Z., Ramadan, M.M., Mohammed, A.T., Mahmoud, A.E., Babintsev, K., Elmessery, W.M., El-Messery, T.M., 2024. Enhancing the antioxidant properties of functional herbal beverages using ultrasonic-assisted extraction: Optimized formulation and synergistic combinations of taurine and vit. C. Heliyon 10(15), e35685.
Kazeminia, M., Mehrabi, A., Mahmoudi, R., 2022. Chemical composition, biological activities, and nutritional application of Asteraceae family herbs: A systematic review. Trends Phytochem. Res. 6, 187-213.
Koster, R., 1959. Acetic acid analgesic screen. In Fed. Proc. Fed. Am. Soc. Exp. Biol. 18, 418-420.
Kumar, J.P., Shankar, N.B., 2009. Analgesic activity of *Mollugo pentaphylla* Linn. by tail immersion method. Asian J. Pharm. Clin. Res. 2(1), 61-63.
Li, Y., Zhang, J.J., Xu, D.P., Zhou, T., Zhou, Y., Li, S., Li, H.B., 2016. Bioactivities and health benefits of wild fruits. Int. J. Mol. Sci. 17(8), 1258.
Mahfuz, A., Salam, F.B.A., Deepa, K.N., Hasan, A.N., 2019. Characterization of *in-vitro* antioxidant, cytotoxic, thrombolytic and membrane stabilizing potential of different extracts of *Cheilanthes tenuifolia* and stigmasterol isolation from *n*-hexane extract. Clin. Phytosci. 5, 1-10.
Meena, H., Pandey, H.K., Pandey, P., Arya, M.C., Ahmed, Z., 2012. Evaluation of antioxidant activity of two important memory enhancing medicinal plants *Baccopa monnieri* and *Centella asiatica*. Indian J. Pharmacol. 44(1), 114-117.
Merdivan, S., Lindequist, U., 2017. Ergosterol peroxide: A mushroom-derived compound with promising biological activities– A review. Int. J. Med. Mushrooms. 19(2), 93-105.
Meyer, B.N., Ferrigni, N.R., Putnam, J.E., Jacobsen, L.B., Nichols, D.E.J., McLaughlin, J.L., 1982. Brine shrimp: A convenient general bioassay for active plant constituents. Planta Med. 45(05), 31-34.
MohammadhosseinI, M., Frezza, C., Venditti, A., Akbarzadeh, A., 2019. Ethnobotany and phytochemistry of the genus *Eremostachys* Bunge. Curr. Org. Chem. 23, 1828-1842.
Mohammadhosseini, M., Venditti, A., Frezza, C., Serafini, M., Bianco, A., Mahdavi, B. 2021. The genus *Haplophyllum* Juss.: Phytochemistry and bioactivities—A review. Molecules 26, 4664.
Njoku, I.S., Rahman, N.U., Khan, A.M., Otunomo, I., Asekun, O.T., Familoni, O.B., Ngozi, C., 2022. Comparison of three different extraction methods for the determination of the quality of the leaf volatile oils of *Delonix regia* (Bojer ex Hook. Raf). Trop. J. Nat. Prod. Res. 6(1), 71-75.
Nowak, R., Drozd, M., Mendyk, E., Lemieszek, M., Krakowiak, O., Kisiel, W., Rzeski, W., Szewczyk, K., 2016. A new method for the isolation of ergosterol and peroxyergosterol as active compounds of *Hygrophoropsis aurantiaca* and *in vitro* antiproliferative activity of isolated ergosterol peroxide. Molecules 21(7), 946.
Olaoluwa, O., Taiwo, O., Nahar, L., Sarker, S. D. 2022. Ethnopharmacology, phytochemistry and biological activities of the African species of the genus *Ficus* L. Trends Phytochem. Res. 6, 46-69.
Omale, S., Amagon, K.I., Johnson, T.O., Bremner, S.K. and Gould, G.W., 2023. A systematic analysis of anti-diabetic medicinal plants from cells to clinical trials. Peer J. 11, 14639.
Özbek, H., Ceylan, E., Kara, M., Özgökçe, F., Koyuncu, M., 2004. Hypoglycemic effect of *Rheum ribes* roots in alloxan induced diabetic and normal mice. Scand. J. Lab. Anim. Sci. 31(2), 113-115.
Prasad, S., Kashyap, R.S., Deopujari, J.Y., Purohit, H.J., Taori, G.M., Daginawala, H.F., 2006. Development of an *in vitro* model to study clot lysis activity of thrombolytic drugs. Thromb J. 4, 14.
Rahman, F.B., Ahmed, S., Noor, P., Rahman, M.M.M., Huq, S.A., Akib, M.T.E. and Shohael, A.M., 2020. A comprehensive multi-directional exploration of phytochemicals and bioactivities of flower extracts from *Delonix regia* (Bojer ex Hook.) Raf., *Cassia fistula* L. and *Lagerstroemia speciosa* L. Biochem. Biophys. Rep. 24, 100805.
Rahman, M., Hasan, N., Das, A.K., Hossain, T., Jahan, R., Khatun, A., Rahmatullah, M., 2011. Effect of *Delonix regia* leaf extract on glucose tolerance in glucoseinduced hyperglycemic mice. Afr. J. Tradit. Complement. Altern Med. 8(1), 34-36.
Shabir, G., Anwar, F., Sultana, B., Khalid, Z.M., Afzal, M., Khan, Q.M., Ashrafuzzaman, M., 2011. Antioxidant and antimicrobial attributes and phenolics of different

solvent extracts from leaves, flowers and bark of Gold Mohar [*Delonix regia* (Bojer ex Hook.) Raf.]. Molecules. 16(9), 7302-7319.

Sharif, N., Jabeen, H. 2024. Natural sources for coumarins and their derivatives with relevance to health-promoting properties: A systematic review. Trends Phytochem. Res. 8(3), 149-162.

Shewale, V.D., Deshmukh, T.A., Patil, L.S., Patil, V.R., 2012. Anti-Inflammatory activity of *Delonix regia* (Boj. Ex. Hook). Adv. Pharmacol. Pharm. Sci. 2012(1), 789713.

Shiramane, R.S., Biradar, K.V., Chivde, B.V., Shambhulingayya, H.M., Goud, V., 2011. *In-vivo* antidiarrhoeal activity of ethanolic extract of Delonix regia flowers in experimental induced diarrhoea in Wistar albino rats. Int. J. Res. Pharm. Chem. 1(3), 2231-2781.

Singh, A.K., Attrey, D.P., Deep, P.R.A.K.A.S.H., Dubey, S.U.C.H.I.T.A., Naved,T., Roy, B., 2014. Acute and sub-acute toxicity studies of pharmacologically active seabuckthorn leaf extract. Int. J. Pharm. Sci. 6, 415-419.

Tiwari P, Kumar B, Kaur M, Kaur G, Kaur H., 2011. Phytochemical screening and extraction: A review. Int. Pharm. Sci. 1, 103-104.

Trease GE, Evans WC. 1989. Pharmacognosy. 13th Ed. London: ELBS/Bailliere Tindall. 345-3466. pp. 535-536, pp. 772-7703.

Umer, S., Tekewe, A., Kebede, N., 2013. Antidiarrhoeal and antimicrobial activity of *Calpurnia aurea* leaf extract. BMC Complement. Altern. Med. 13, 21.

Vivek, M.N., HC, S.S., Manasa, M., Pallavi, S., Kambar, Y., Asha, M.M., Chaithra, M., TR, P.K., Mallikarjun, N., Onkarappa, R., 2013. Antimicrobial and antioxidant activity of leaf and flower extract of *Caesalpinia pulcherrima*, *Delonix regia* and *Peltaphorum ferrugineum*. J. Appl. Pharm. Sci. 3(8), 064-071.

Walum, E., 1998. Acute oral toxicity. Environ. Health. Perspect. 106(2), 497-503.

Wang, L.S., Lee, C.T., Su, W.L., Huang, S.C., Wang, S.C., 2016. *Delonix regia* leaf extract (DRLE): A potential therapeutic agent for cardioprotection. PLOS ONE 11(12), 0167768.