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Characterization of pharmacological properties and isolation of two bioactive compounds (ursolic acid and palmitoleic acid) from the stem bark extract of *Lannea coromandelica*

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ABSTRACT

The current research aimed to conduct bioassay-guided isolation of bioactive compounds from the stem bark of *Lannea coromandelica*. Using column chromatography and NMR spectroscopy, two compounds -ursolic acid and palmitoleic acid- were isolated from the plant's stem bark. The methanolic stem bark extract possessed higher total phenolic, flavonoid, and antioxidant capacity content than the *n*-hexane extract. The IC_{50} value of the methanol and *n*-hexane stem bark extracts for DPPH free radical scavenging potential was found to be 37.37 and 27.726 µg/mL, respectively, while for nitric oxide, the IC_{50} value was 14.615 and 22.136 µg/mL, respectively. The *n*-hexane extract exhibited higher antidiabetic effectiveness (46% blood glucose reduction) than the methanolic extract (37.8% blood glucose reduction) after 3 hours of glucose administration in mice model at a dose of 400 mg/kg body weight ($p < 0.001$). The methanolic extract demonstrated the highest anti-diarrheal action at a dose of 400 mg/kg body weight (88.70% inhibition of defecation, $p < 0.001$). Furthermore, the *n*-hexane extract showed the highest analgesic activity (70.5% inhibition of writhing, $p < 0.01$).

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1. Introduction

Medicinal plants, often known as herbs, have been utilized to cure a wide range of illnesses since primitive times (Mulat et al., 2019). In order to treat a variety of human diseases, many newly developed therapeutic methods make use of medicinal plants. These natural plant resources are truly valuable for the human beings as they are enriched with beneficial phytochemical constituents, which are economically available and have potential pharmacological effects that may play a pivotal role to treat human illness (Martins and Brijesh, 2018). The therapeutic potential of medicinal plants has shown promising impacts in the treatment of various diseases, offering a natural and effective alternative to conventional medications (Makenzi et al., 2023; Singh et al., 2023). Plant materials are rich in a diverse array of valuable natural bioactive compounds, including phenolic compounds, sesquiterpenoids, coumarins, alkaloids, carotenoids, and dietary fibers, which possess antioxidant, antimicrobial, and anti-inflammatory properties, offering significant potential for applications in food, pharmaceutical, and cosmetic industries (Mohammadhosseini, et al., 2019; Mohammadhosseini, et al., 2021a; Mohammadhosseini, et al., 2021b).

Lannea coromandelica is a topical deciduous tree with broad leaves that is included in the Anacardiaceae family. It is frequently applied by tribal people such as Teli, Pahan, Garo in tropical countries like Bangladesh, India, etc. The juice prepared from the leaves of this plant can be used to treat ulcer-related discomfort. The sap of this plant is applied as a cough and cold remedy, while the bark is used for dysentery, dyspepsia, skin lesions and pain from ulcers and toothache (Alam et al., 2017). Diabetes, hepatitis, heart conditions, and digestive issues can also be treated using its stem bark. According to the literature, the twigs of this plant have

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shown apoptosis induction in the cancerous human liver cells, while the leaves have shown anti-diarrheal, antinociceptive and potential antioxidant capacity (Kaur et al., 2013, Weerapreeyakul et al., 2016). Furthermore, the barks of the plant have shown zoo sporicidal activity, anti-inflammatory, anti-hyperglycemic, hypotensive and antimicrobial activity (Islam et al., 2002). However, there is no extensive research on the pharmacological properties of stem bark of this plant.

In the current report, the stem bark of *L. coromandelica* was tested pharmacologically *in vitro* and *in vivo*, and the bioactive components of the plant were also successfully isolated and characterized.

2. Experimental

2.1. Collection and identification of plant sample

Mr. Abdur Rahim, a taxonomist from the Department of Botany at Jahangirnagar University in Savar, Dhaka, Bangladesh, identified *Lannea coromandelica* (Accession number: UAP_Herb/10541) and collected the plant material from the Khulna district in Bangladesh, which is located at latitude 22.820000 and longitude 89.550003.

2.2. Extraction of the plant sample

The approaches outlined by Shinwari (2011), Saradhajyothi and Subbarao (2011) as well as Welters et al. (2006) were used for the extraction process. Initially, the plant was dried naturally under the sunlight for 15 days, then oven-dried at 40 °C for a week before being ground into a coarse powder. In order to facilitate filtration, 600 g of the obtained powder was dissolved in 2.5 L of methanol and *n*-hexane separately and subjected to repeated shaking for 2 weeks. Next, the supernatant was obtained using cotton filtering. The extract, which included both methanol and *n*-hexane, naturally evaporated at ambient temperature.

2.3. Phytochemical screening

The existence of various phytoconstituent groups in the plant extract, such as carbohydrate, steroids, phenolics, flavonoids, alkaloids, saponin and so on, were determined using the established protocols (Trease and Evans, 1989, Tiwari et al., 2011).

2.4. Antioxidant potentials

2.4.1. Determination of total phenolic content

Using Folin-Ciocalteu reagent (FCR), total phenolic content of the methanolic and *n*-hexane extracts of *L. coromandelica* was calculated. FCR actually determines total phenolic content of a plant extract (Stanojević et al., 2009). Test tubes containing 1 mL extracts and standard were mixed with 5 mL of FCR solution (1.9-2.1 N, diluted tenfold), followed by the addition of 4 mL of sodium carbonate solution (7.5% *w/v*). Then, the test tubes were incubated at 20.0 °C for 30 minutes for standard solutions and one hour at 20.0 °C for the plant extracts. The solutions' absorbances were measured at 765 nm using a spectrophotometer and compared to a control. Gallic acid equivalents (GAE) were used to determine the total amount of phenolic compounds in plant methanol extracts, as described by Harbertson and Spayd (2006).

2.4.2. Determination of total flavonoid content

Flavonoids found in fruits and vegetables may enhance mental and physical performance, while lowering the risk of infection (Davis et al., 2009; Mehjabin et al., 2024). In this report, a colorimetric technique using aluminum chloride was employed to determine the flavonoids, with quercetin serving as the standard (Bao et al., 2005). Briefly, a mixture of 5.6 mL of distilled water, 3 mL of methanol, 0.2 mL of aluminum chloride (10.0%), and 0.2 mL of potassium acetate (1.0 M) was prepared with 1 mL of the sample and the standard at different concentrations. After 30 minutes at room temperature, the reaction mixture's absorbance was measured at 415 nm using a UV/Visible spectrophotometer. For the construction of the relevant calibration curve, various concentrations of quercetin solutions were considered, and the level of flavonoids was expressed as mg/g equivalent of the sample (Lin and Tang, 2007; Mahboubi et al., 2015).

2.4.3. Determination of total antioxidant capacity

According to the steps outlined by Prieto et al. (1999), total antioxidant potential of both extracts was evaluated spectrophotometrically using the phosphomolybdenum technique. The test tubes contained a mixture of the sample extracts and a reagent solution. The reagent solution was prepared by combining 3.3 mL of concentrated H_2SO_4 (98%), 0.381 g of sodium phosphate and 0.494 g of ammonium molybdate and the final volume adjusted upto 100 mL with distilled water in a volumetric flask. The resulting mixture was then incubated at 95 °C for about 90 minutes and the corresponding absorbance was measured at 695 nm. A typical blank solution, consisting of 3 mL of reagent solution, was used in place of the required amount of sample solution (300 µL) of the same solvent.

2.4.4. DPPH free radical scavenging assay

The 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) assay is a commonly used assay to evaluate an antioxidant's ability to scavenge free radicals. This technique enables the measurement of an antioxidant's antiradical power, as described by Choi et al. (2000). Each test tube received 2 mL of the reagent solution (DPPH: 0.004%) and 200 µL of the plant extracts or standards, and was then incubated for half an hour. The solution's absorbance was measured using a spectrophotometer at 517 nm, compared to a blank. The inhibitory activity was estimated as a percentage (%).

$$\text{Inhibition (\%)} = (A_o - A_1)/A_o \times 100 \qquad \text{(Eqn. 1)}$$

Eqn. 1 was used to calculate the percentage (%) inhibitory activity. Next, inhibition percentages were plotted against log concentration, and the IC_{50} was determined from the graph.

2.4.5. Nitric oxide free radical scavenging capacity assay

It is generally known that at physiological pH (7.2), sodium nitroprusside degrades in aqueous solution, producing NO-. Nitric oxide (NO-) and oxygen interact to generate stable products (nitrate and nitrite) in an aerobic environment. The quantities of nitric oxide that react with oxygen to form stable products can be calculated using the Griess reagent. A chromophore was created when nitrite was diazotized with sulfanilamide and subsequently linked with naphthyl ethylene diamine dihydro chloride. At 550 nm, the absorbance of this chromophore was taken (Sreejayan and Rao, 1997; Haenen and Bast, 1999). 1 mL of sodium nitroprusside solution (5 mM) was added to each test tube along with 4 mL of each plant extract or standard solution of various concentrations. To finish the reaction, the test tubes were incubated for two hours at 30 °C. Then, 2 mL of solutions from each test tube containing the standards and extracts were withdrawn and mixed with 1.2 mL of Griess reagent (0.5% *w/v*) and the absorbances of the solutions were measured at 550 nm using a spectrophotometer against blank.

2.5. Determination of *in vitro* thrombolytic activity

Uddin et al. (2016) and Prasad et al. (2006) have described the method used to assess thrombolytic activity *in vitro*. Beforehand, each of the eight vials was weighed. One milliliter of blood was then added to each vial, and the vials were incubated at 37 °C for 45 minutes. After incubation, blood clots remained at the bottom of each vial, and the serum was removed from the upper part. The weight of the vial-containing clots was then measured. Four vials (vials 1, 2, 3, and 4) were filled with 100 µL of sample extract solutions, two vials (vials 5 and 6) were filled with 100 µL of distilled water, and the final two vials (vials 7 and 8) were filled with 100 µL of streptokinase (30,000 IU). After 90 minutes of incubation at 37 °C, the serum was removed from the upper part of each vial, leaving the clots at the bottom. The vials' weight was then measured once more and percent of clot lysis was estimated by using the following equation:

Clot lysis (%) = (Weight of the clot after lysis/Weight of the clot before lysis) × 100 (Eqn. 2)

2.6. Determination of membrane stabilizing potential

The Omale and Shahriar methods were used to test the extracts' hypotonic and heat-induced stabilizing membrane effects on human erythrocytes (Omale and Okafor, 2008; Shahriar et al., 2015). The test sample was made up of a 30 mL stock suspension of erythrocytes (RBCs) in a hypotonic solution containing sodium chloride (50 mM), sodium phosphate saline buffer (10 mM, pH 7.4), plant extracts, and acetyl salicylic acid (0.10 mg/mL, each). After the mixture was centrifuged for 10 minutes at 1500 rpm and preheated for 10 minutes to room temperature, the absorbance was measured at 540 nm. The following calculation was used to compute the percentage inhibition of membrane stabilization or hemolysis:

Hemolysis inhibition (%) = (OD1 - OD2)/OD1 × 100 (Eqn. 3)

Two falcon tubes were filled with isotonic buffer containing plant extracts and erythrocyte suspension along with reagents. The tubes were kept in an ice bath and incubated in water for 20 minutes. Percentage hemolysis inhibition was determined using the following equation (Omale and Okafor, 2008; Shahriar et al., 2015).

Hemolysis Inhibition (%) = [1 - (OD2-OD1/OD3-OD1)] × 100 (Eqn. 4)

2.7. Cytotoxic activity: Brine shrimp lethality bioassay

This investigation utilized *Artemia salina*, or brine shrimp, as the test organism. Cysts were hatched in saline water (38 g/L NaCl) for 48 hours, transforming into living nauplii. Both methanolic and *n*-hexane extracts were prepared in various concentrations using dimethyl sulfoxide (DMSO) as the solvent. Each test tube was inoculated with ten nauplii, and the volume was adjusted with saline water. Vincristine sulfate, a highly cytotoxic alkaloid, served as the positive control, tested at concentrations of 25, 12.5, 5, 1, 0.5, 0.25, 0.125, and 0.06 µg/mL.Three pre-marked test tubes holding 4.9 mL of simulated sea water each received 50 µL of DMSO. Ten shrimp nauplii were added to serve as negative control groups. After a 24-hour rest period, all test tubes were counted to determine the number of living and dead nauplii using a magnifying glass against a black background, as described by Price et al. (1974). The percent of lethality was determined accordingly.

2.8. *In vitro* α-amylase inhibitory activity

This investigation utilized a modified starch-iodine technique, involving different plant extracts or standard dosages. Test tubes were incubated with amylase, starch solution (1.0%), and distilled water. Absorbance was determined at 565 nm and the relevant IC_{50} values were calculated by the use of linear regression analysis. Three experiments were conducted, resulting in a comprehensive understanding of the process (Uddin et al., 2014).

2.9 Antimicrobial activity

A common test for antimicrobial susceptibility is the disc diffusion method. This strategy was first introduced by Bauer et al. (1966), who tested it on a large variety of microorganisms. This technique can also be used to test phytochemicals and plant extracts. In this method, the test material is placed in Mueller-Hinton Agar media after being soaked in 6 mm filter paper discs. There will be obvious places where there is enough of the test agent to stop bacterial growth. A drug's antibacterial activity can be estimated using the clear zone's diameter (Nascimento et al., 2000).

2.10. *In vivo* pharmacological activities

2.10.1. Experimental animal

This study employed male Swiss albino mice that weighed 25-30 g (Akanda and Hasan, 2021). The International Centre for Diarrheal Disease Research, Bangladesh (ICDDRB) provided the animals for all of the experiments. All animals were kept under standard environmental conditions: Relative humidity 55-65%, ambient temperature 22-25 °C, and a 12-hour light-dark cycle. The experiment was conducted in the Phytopharmacology Laboratory of the Department of Pharmacy of University of Asia Pacific as per the guideline.

2.10.2. Evaluation of anti-diarrheal activity

A typical digestive ailment, diarrhea is brought on by bacterial, viral, and parasite species. To test the effectiveness of *L. coromandelica* stem bark extracts as a diarrhea preventative, castor oil-induced diarrhea in mice was used. The control, positive, and test groups each had four mice. Loperamide was administered orally to the positive control group at a dose of 50 mg per kg. *n*-Hexane and methanolic extracts were administered to the test group at doses of 200 and 400 mg/kg body weight, respectively. 60 minutes after obtaining test samples, the mice were also given 0.5 mL of castor oil orally (Amabeoku, 2009; Umer et al., 2013; Araújo et al., 2015; Jabri et al., 2016).

2.10.3. Peripheral analgesic activity

The acetic acid-induced writhing method is Koster and Taber's favored method for determining *in vivo* peripheral analgesic activity (Koster, 1959; Taber et al., 1969). In the current experiment, diclofenac was used as a typical medicine. The pain-relieving potential of two distinct dosages of the crude extracts from the stem bark of *L. coromandelica* was examined to be 200 and 400 mg/kg of body weight (Koster, 1959; Taber et al., 1969).

2.10.4. Antidiabetic activity

This study investigated hypoglycemic action of plant extracts in hyperglycemic mice given glucose. Among six mice groups, group I served as the control and merely got DMSO mixed with regular saline water and Tween-80 (a suspending agent). Glibenclamide was given orally to Group II as the standard group at a dose of 5 mg/kg body weight. Groups III to VI were given oral doses of the crude methanolic and *n*-hexane extract in the amounts of 200 and 400 mg/kg body weight. After 60 minutes, mice were given a glucose solution. Blood samples were collected from the tail vein and tested using a glucometer and reactive strips (Upadhya et al., 2004).

2.10.5. Antipyretic activity

This study assessed the plant extract's ability to reduce fever caused by Brewer's yeast in mice (Tomazetti et al., 2005). A digital thermometer was used to gauge basal rectal temperature at zero hours. Animals with an increase of at least 0.6°C over 18 hours were chosen for the investigation. Pyrexia was produced by administering 10 mL/kg of body weight of a suspension of Brewer's yeast (15 *w/v*%) in distilled water subcutaneously. Four groups were formed, with control being Tween-80 (1.0%), paracetamol administered orally, temperatures were obtained from each group at various intervals, and mice from the control and standard groups were compared (Gupta et al., 2005).

2.10.6. Acute toxicity test

In this investigation, Swiss albino male mice were used to test the acute toxicity of *L. coromandelica* stem bark extracts. The crude extracts in various doses (500, 1000, 2000, and 4000 mg/kg) were given orally to 36 mice divided into 9 groups. After 24 hours, mortality rates were evaluated (Mosnaim et al., 2020).

2.11. Isolation of bioactive substances

Column chromatography can be used to identify bioactive compounds from plant extracts, followed by TLC, and ultimately NMR to determine and characterize the structure of the isolated compounds. The idea behind silica column chromatography is that the solvent (mobile phase) is passed through the stationary phase, while the mixture's molecules are applied to the surface of solid silica (stationary phase) (Coskun, 2016).
The plant's methanol extract was then placed on top of the stationary phase and run by a mobile phase composed of *n*-hexane, chloroform, and ethyl acetate in various ratios from non-polar to semi-polar. Eluted samples were stored in previously numbered (1-300) test tubes, and the eluted samples were then tested over TLC plates with a specified solvent system as part of the initial screening procedure to identify test tubes containing comparable chemicals. The test tube contents which had the same rf value were mixed together for further separation by TLC. Following that, the contents of the test tubes were run over a TLC plate with a mobile phase composed of *n*-hexane, chloroform, and ethyl acetate in various proportions, and the separated compounds were suitably spotted under UV lamps of long- and short wavelength. According to this procedure, the sample of 140-154 numbered test tubes were isolated with ethyl acetate (10.0%) in chloroform separated with preparative TLC, and compounds were spotted under UV lamp, eluted and coded as LC-002-10-EA-LS and sample of 188-200 numbered test tubes are eluted with ethyl acetate (25%) in $CHCl_3$ in a similar way, coded as LC-001-25-EA-L and referred for ^{1}H-NMR and ^{13}C-NMR study to BCSIR (Bangladesh Council of Scientific and Industrial Research). After comparing the NMR data with the references, structure of our isolated compounds was determined.

2.12. 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 results with less than $^*p < 0.05$, $^{**}p < 0.01$

and ***$p < 0.001$ are 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

The plant contains some secondary metabolites like carbohydrate, glucoside, saponin, steroid, flavonoid, and alkaloid, but it also shows the absence of glycoside and tannin. The secondary metabolites glucose, glucoside, saponin, steroid, flavonoid, and alkaloid are all present in the methanolic extract. The *n*-hexane extract contained large amounts of carbohydrates, glycosides, steroids, flavonoids, saponins, and alkaloids, as summarized in Table 1.

The initial phytochemical screening of *L. coromandelica* identified the presence of several crucial secondary metabolites. This finding aligns with previous research, where the methanolic extract of *L. coromandelica* leaves was reported to contain alkaloids, glycosides, cardiac glycosides, saponins, carbohydrates, and flavonoids (Manik et al., 2013).

According to a recent study, it was found that the ethanolic extract of pulp contained tannins, flavonoids, saponins, cyanogenic glycosides, terpenoids, but lacked phenolics, alkaloids, steroids, and anthraquinones (Islam et al., 2022).

3.2. Antioxidant potentials

3.2.1. Determination of total phenolic content

In this study, the methanolic stem bark extract of *L. coromandelica* had a higher total phenolic content than the *n*-hexane fraction, estimated by using the standard curve of gallic acid ($y = 0.0069x + 0.0357$, $R^2 = 0.9958$). The total phenolic content of the methanolic and *n*-hexane fractions were found to be 52.30 ± 0.141 and 23.671 ± 0.101 mg/g of GAE, respectively (Table 2).

3.2.2. Determination of flavonoid content

The equation $y = 0.0084x - 0.1082$, $R^2 = 0.9878$, derived from the reference standard of quercetin, was used to compute the total flavonoid concentration. The flavonoid content for methanolic and *n*-hexane fractions were found to be 47.450 ± 0.636 and 23.475 ± 1.025 mg/g of quercetin equivalent (Table 2). As seen, the total flavonoid content of *n*-hexane stem bark fractions of *L. coromandelica* was lower than the total phenolic content of methanolic fraction.

3.2.3. Determination of total antioxidant capacity

Using the equation $y = 0.0054x-0.0926$, $R^2 = 0.9509$ derived from the reference standard of ascorbic acid, the total antioxidant capacity of various stem bark extracts was computed and expressed as mg/g ascorbic acid equivalent. In this study, the amount of total antioxidant capacity of *L. coromandelica* showed that methanolic stem bark extract was more potent than *n*-hexane extract. Total antioxidant capacity of methanolic and *n*-hexane stem bark extracts of *L. coromandelica* were found to be 12.048 ± 0.102 and 6.582 ± 0.025 mg/g of ascorbic acid equivalent (Table 2).

3.2.4. DPPH free radical scavenging potential

The results of this study showed that the extracts exhibited dose-dependent scavenging activity against DPPH free radicals. Compared to the standard ascorbic acid concentration of 35.854 µg/mL, the IC_{50} values for the methanolic and *n*-hexane fractions of the stem bark extract were 37.37 and 27.726 µg/mL, respectively (Table 2). Both fractions demonstrated scavenging activity comparable to that of ascorbic acid (Fig. 1).

3.2.5. Nitric oxide free radical scavenging capacity assay

According to the experimental findings of this report, both stem bark extracts of *L. coromandelica* inhibited nitric oxide in a dose-dependent way (Fig. 2). This might be attributed to the fact that the extracts contained antioxidant elements that compete with oxygen to react with nitric oxide. According to our current study, methanolic extract was found more potent NO free radical scavenger than *n*-hexane extracts as compared with ascorbic acid (Table 2).

Oxidative stress may give rise to various disorders in the body. Antioxidants may prove to be an inhibitor of oxidative stress (Islam et al., 2022). A method referred as total antioxidant capacity (TAC) is widely applied for the assay of antioxidant properties present in the biological samples. It can also assess the antioxidant property against the free radicals generated in a particular disease (Marques et al., 2014). Compared to the *n*-hexane fraction (23.671 mg/g GAE), the methanolic fraction of the stem bark showed a greater total phenolic content (52.30 mg/g GAE). Comparably, the flavonoid content of the methanolic fraction was greater (47.450 mg/g) than that of the *n*-hexane fraction (23.475 mg/g). The total antioxidant capacity of the methanolic extract was higher (12.048 mg/g ascorbic acid equivalent) than that of the *n*-hexane extract (6.582 mg/g ascorbic acid equivalent).

The review of the literature demonstrates that the total antioxidant capacity of the stem bark of *L. coromandelica* using phosphomolybdenum method was not previously reported and numerous polyphenols and flavonoids are essential for plants overall antioxidant activity (Wahid, 2012).

Free radicals damage cells through reactions with membrane lipids, nucleic acids, proteins, enzymes, contributing to aging and degenerative diseases like cardiovascular and cancer. According to a recent study, methanolic extract of stem bark has a higher potential for nitric oxide scavenging ability than ascorbic acid, which is consistent with the results of the present investigation (Sztanke and Sztanke, 2017). A free radical regarded as DPPH has a deep-violet or purple color and has the capacity to accept an electron or hydrogen

Table 1
Phytochemical screening of *Lannea coromandelica* extractives.

Phytochemical Tests	Methanolic extract	*n*-Hexane extract
Carbohydrate	+	+
Glycoside	-	-
Glucoside	+	+
Saponin	+	+
Steroid	+	+
Tannin	-	-
Flavonoid	+	+
Alkaloid test by different reagent		
Hager's reagent	+	+
Wagner's reagent	+	+
Dragendroff's reagent	+	+

Here, [- = Not present, + = Present].

Table 2
Antioxidant activity of stem bark extract of *L. coromandelica*.

Sample	Total Phenolic Content, mg/g GAE ± STD	Total Falvonoid Content, mg/g QE ± STD	Total Antioxidant Capacity, mg/g AAE ± STD	Scavenging capacity assay, IC_{50} (µg/mL)	
				DPPH	NO
Methanolic extract	52.30 ± 0.141	47.450 ± 0.636	12.048 ± 0.102[a]	37.373[a]	14.615
n-Hexane extract	23.671 ± 0.101	23.475 ± 1.025	6.582 ± 0.025[a]	27.726[a]	22.136
Ascorbic acid	-	-	-	35.854[a]	16.03

[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, $p < 0.05$].

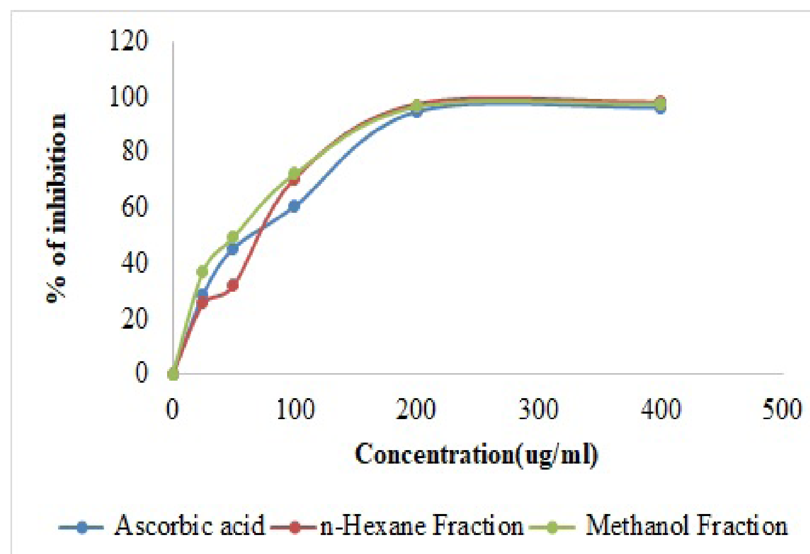

Fig. 1. DPPH free radical scavenging curve of stem bark extracts of *L. coromandelica* at different concentrations.

radical to get stabilized as a diamagnetic molecule that is yellow in color (DPPH) followed by the reaction with antioxidants, whether of natural or synthetic origin (Kedare and Singh, 2011). The DPPH free radical scavenging activity of the methanolic and *n*-hexane extracts was dose-dependent, with IC_{50} values of 37.37 and 27.726 μg/mL, respectively. Ascorbic acid, *n*-hexane and methanolic extract also exhibited dose-dependent scavenging of NO free radicals.

3.3. Determination of *in vitro* thrombolytic activity

A positive control of 100 mL of streptokinase (SK) for fibrinolytic medications (30,000 IU) demonstrated 66.381% clot destruction, whereas a negative control of 50.038% clot lysis was observed using sterile distilled water. Methanolic stem bark extract exhibited 26.196% and *n*-hexane stem bark extract showed 43.46%, respectively, in an *in vitro* thrombolytic activity investigation (Table 3). The results of this investigation demonstrated poor thrombolytic activity of *L. coromandelica* stem bark extract, as *n*-hexane extract was potent than methanolic extract (Table 3).
Streptokinase, a fibrinolytic medication, lowers mortality rates in myocardial infarction patients by triggering a plasminogen enzyme that dissolves fibrin. This medication retains blood flow to the ischemic myocardium and reduces necrosis (Manik et al., 2013). A previous research has shown that the *n*-hexane fraction of *L. coromandelica* bark exhibits weaker activity compared to other extracts, which is consistent with the findings of this study (Manik et al., 2013). It has been documented that the average clot lysis activity of the dichloromethane leaf extract was 23.98 ± 2.71%, while the *n*-hexane leaf extract showed an average activity of 7.19 ± 2.09%. In contrast, the dichloromethane partitionate of *L. coromandelica* bark demonstrated the highest activity, with an average clot lysis value of 34.81 ± 3.02% (Manik et al., 2013).

3.4. Determination of membrane stabilizing potential

In comparison to the standard acetylsalicylic acid (ASA), *L. coromandelica* was tested for the hemolysis of RBC induced by hypnotic solution and heat induced hemolysis. When compared to the standard and test sample, hemolysis is caused by both a hypotonic solution and heat, ASA decreased RBC hemolysis in hypotonic solution-induced cases by 64.235 ± 0.629%, methanolic extract by 31.178 ± 0.291%, and *n*-hexane fraction by 24.674 ± 1.465%. On the other hand, the inhibitions of heat induce hemolysis showed higher compared with inhibition of hypotonic solution hemolysis. The percent of inhibition of heat induce hemolysis standard acetylsalicylic acid (ASA) was 67.591 ± 2.06%, where test sample methanolic fraction of stem bark was 36.177 ± 4.60% and *n*-hexane fraction of the stem bark was found to be 51.729 ± 1.53%, respectively (Table 3). Both extracts exhibited moderated inhibition of percent of hemolysis compared to the standard ASA. The results can be used to hypothesize that the anti-inflammatory activity of *L. coromandelica* may have a mechanism of action involving its ability to stabilize membranes and its suppression of erythrocyte lysis.

3.5. Cytotoxic activity: Brine shrimp lethality bioassay

Percentage of lethality of methanol (y = 53.388x-83.038, R^2 = 0.8959) and *n*-hexane (y = 56.8x-80.9, R^2 = 0.9167) were calculated by monitoring the number of alive nauplii after 24 hours and the corresponding LC_{50} values were determined by using their respective regression equations and compared with the standard cytotoxic, vincristine sulphate (y = 54.48 + 28.5, R^2 = 0.964). Both the methanolic and *n*-hexane extracts exhibited similar cytotoxic properties. The LC_{50} (lethal concentration that kills 50% of cells) values were 2.35 μg/mL for the methanolic extract and 2.303 μg/mL for the *n*-hexane extract, indicating that both extracts possessed potent cytotoxic potential (Table 3). *Artemia larva*e mortality rate indicates potential anticancer activity. Toxicity tests correlate with cytotoxicity of anticancer compounds, but brine shrimp lethality bioassay is not specific. The methanolic fraction of the plant has LC_{50} of 50-60 μg/mL, suggesting potential anticancer, antibacterial, and antiviral properties (Shoeb et al., 2014). In the brine shrimp lethality assays, both the methanolic and *n*-hexane extracts demonstrated strong cytotoxicity, with LC_{50} values of 2.35 and 2.303 μg/mL, respectively. This shows that the stem bark of *L. coromandelica* can have the potential use as an anticancer herb.

3.6. *In vitro* α-amylase inhibitory activity

When compared to the standard acarbose, both of the *L. coromandelica* stem bark extracts displayed almost similar and comparable α-amylase inhibitory potential (Table 3). The methanolic stem bark extract of *L. coromandelica* had an IC_{50} value of 1.743 μg/mL for antidiabetic activity, while the *n*-hexane extract had an IC_{50} of 1.968 μg/mL. In comparison, the standard drug acarbose had an IC_{50} of 1.665 μg/mL. These results indicate that both the methanolic and *n*-hexane extracts of *L. coromandelica* stem bark possess potent antidiabetic activity, comparable to the standard.

3.7. Antimicrobial activity

The antimicrobial activities were evaluated on two-Gram positive bacteria, namely *Staphylococcus aureus*, *Bacillus megaterium,* two-Gram negative bacteria, namely *Escherichia coli, Salmonella typhi* along with a fungus, namely *Aspergillus niger*. No zone of inhibition was found in any of the five species examined in the methanol or *n*-hexane stem bark extract of *L. coromendelica,* as the plant extracts lack antimicrobial activities on the tested microorganisms.
Infectious disease, which accounts for more than 50% of all mortality in tropical countries, is one of the main causes of death worldwide (Shetty and Shetty, 2009). Studies suggest that certain bacteria are susceptible to *L. coromandelica*, with some showing moderate efficacy. The ethanolic extract of *L. coromandelica* bark's aqueous partitionate showed moderate efficacy against several bacteria *e.g.*, *Shigella dysentery* 9 mm (800 g) and 8 mm (400 g), *Pseudomonas aeruginosa* 10 mm

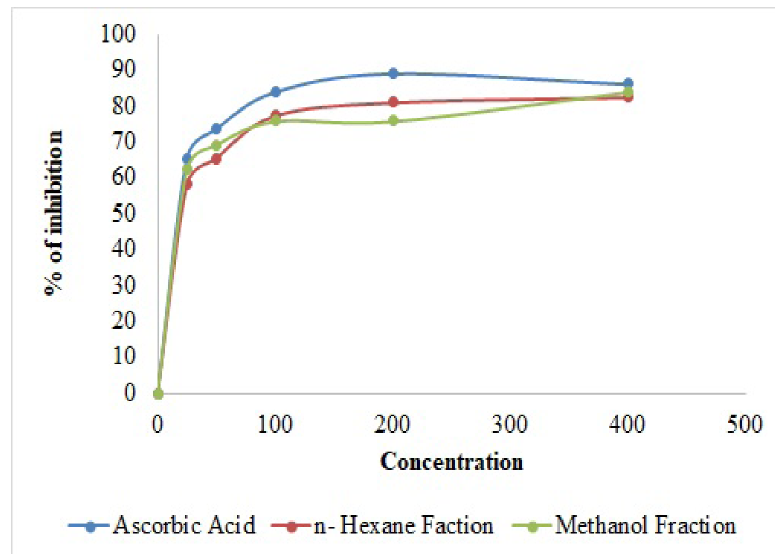

Fig. 2. Nitric oxide free radical scavenging curve of stem bark extracts of *L. coromandelica* at different concentrations.

Table 3
Thrombolytic activity, membrane stabilizing potential, brine shrimp lethality bioassay and α-amylase inhibitory activity of *L. coromandelica* and standard.

Extracts /Std	Thrombolytic activity	% Inhibition of hemolysis ± STD (membrane stabilizing potential)		Brine shrimp lethality bioassay (μg/mL)	α-Amylase inhibitory activity (μg/mL)
	Clot lysis (%)	Hypotonic Solution	Heat Induce	LC_{50}	IC_{50}
Methanol extract	26.196 ± 6.393	31.178 ± 0.291	36.18 ± 4.6	2.35	1.743
n-Hexane extract	43.462 ± 1.507	24.674 ± 1.465	51.729 ± 1.5	2.303	1.968
Streptokinase	66.381 ± 0.896	-	-	-	-
Distilled water	50.038 ± 0.966	-	-	-	-
Acetyl salicylic acid	-	64.235 ± 0.629	67.591 ± 2.0	-	-
Vincristine sulfate	-	-	-	0.392	-
Acarbose	-	-	-	-	1.665

(800 g) and 8 mm (400 g). However, or other examined bacteria, the same extract did not show any zone of inhibition, indicating no antibacterial effect (Ramadhan et al., 2022).

3.8. Evaluation of anti-diarrheal activity

In this study, the defective pellet was reduced by 74.78% and 86.70% ($p < 0.001$) at 200 and 400 mg/kg body weight dose, respectively, from the methanolic extracts (ME). However, the *n*-hexane (*n*-HE) extract showed a statistically significant decrease in defection of 51.30% and 64.35% ($p < 0.001$). The reduction of the defective pellet of the standard loperamide was 73.475% at a dose of 50 mg/kg body weight (Fig. 3-a). The stem bark methanolic extract was more potent than the *n*-hexane extract at the same dosage.

Ricinoleate salts in the intestine cause inflammation and permeability, affecting intestinal absorptive cells. Castor oil inhibits prostaglandin E2 formation, which induces intestinal secretion. This inhibition may contribute to its antidiarrheal effects on stem bark. Reduced ricinoleic acid secretion stimulates Na^+/K^+ ATPase activity, promoting the release of Na^+ and K^+ from the intestinal mucosa. Terpenoids, tannins, and flavonoids in seed and bark extracts may improve colon absorption of water and electrolytes (Mbahi et al., 2018).

3.9. Peripheral analgesic activity

At dosages of 200 and 400 mg/kg body weight, respectively, the methanolic extract achieved 49.02%

and 68.63% (Fig. 3-b) writhing inhibition in the acetic acid-induced writhing model in mice, which is used to evaluate peripheral analgesic activity. *n*-Hexane extract suppressed writhing by 49.02% and 70.59% (Fig. 3-b), respectively at the same dose. The positive standard diclofenac sodium inhibited 60.78% of at a dose of 50 mg/kg body weight. Both extracts exhibited almost similar analgesic activity at higher dose and found statistically significant ($p < 0.01$). Both the extracts exhibited dose-dependent inhibition of percent of writhing.

Analgesic activity was assayed in mice through the application of acetic acid induced writhing model. As the extract concentration was raised, the writhing inhibition grew stronger. The extract caused impressive writhing inhibition at the higher dose tested (400 mg/kg body weight), which was comparable and, in some cases, even greater than the positive standard, diclofenac sodium. Acetic acid-induced writhing has produced algesia by releasing endogenous chemicals that in turn stimulate the pain nerve terminals (Ricciotti and FitzGerald, 2011). Acetic acid administered intraperitoneally has been shown to induce pain, which is associated with elevated levels of PGE2 and PGF2 in peritoneal fluid. Given this result, it is reasonable to assume that a peripheral mechanism may be involved in the mode of action. As alkaloids, flavonoids, and saponins have been proven to be responsible for analgesic and anti-inflammatory effects, the results of phytochemical analysis from previous studies of *L. coromandelica* support the antinociceptive activity (Rajesh and Selvakumar, 2022). The methanolic extract demonstrated marginally greater potency at higher dosages than the other extract, although both showed dose-dependent peripheral analgesic action.

3.10. Antidiabetic activity

Both extracts exhibited antidiabetic efficacy dose-dependently and the highest activity was found at the 3rd hour after glucose administration and found statistically significant. Methanolic (ME) and *n*-hexane (*n*-HE) stem bark extract of *L. coromandelica* reduced 37.8% and 46.0% (Fig. 4-a, $p < 0.001$) of blood glucose in mice model at 400 mg/kg body weight dose after 3 h administration of glucose. According to this study, *n*-hexane extract exhibited higher antidiabetic efficacy than methanolic stem bark extract.

The complex disease known as diabetes mellitus is characterized by a significant breakdown in the metabolism of proteins, carbohydrates, and lipids. It is a long-term metabolic disorder of glucose metabolism that eventually alters the vasculature and has challenging side effects (American Diabetes Association, 2010). In a glucose management trial, insulin production took two to three hours to return blood sugar levels. The plant extracts have antihyperglycemic properties, likely due to peripheral glucose ingestion or increased beta cell sensitivity to glucose (Aziz et al., 2021). In conclusion, our research reveals that the stem bark extract of *L. coromandelica* might contain a number of active phytochemicals that may likely have hypoglycemic and antihyperglycemic effects via a variety of pathways.

3.11. Antipyretic activity

The initial temperature of 6 different groups of Swiss albino mice was recorded. Then, the mice were fed the sample extracts of *L. coromendeica* and temperature was recorded for the next 3 h. It is seen that both the methanolic extract decreased body temperature by 7.5% ($p < 0.05$) and *n*-hexane fraction by 5.7% ($p < 0.01$) at 400 mg/kg BW dose after 3 h (Fig. 4). Hence, it can be concluded that the methanolic stem bark of *L. coromendelica* demonstrated higher anti-pyretic efficacy (Fig. 4-b), and then it could be inferred that this organic extract has moderate anti-pyretic effectiveness. A high body temperature has always been a cause for concern because of its negative consequences, which includes intracranial hemorrhage, sepsis, Kawasaki syndrome, thyroid storm, and serotonin syndrome (Mercier et al., 2021). In conclusion, it can be claimed that *L. coromandelica's* stem bark exhibits some mild antipyretic effect. The antidiabetic effects of both extracts were dose-dependent, with the *n*-hexane extract showing the greatest effectiveness. The methanolic extract was more effective than the *n*-hexane fraction in terms of antipyretic efficacy.

3.12. Acute toxicity test

In the present study, even a higher dose of plant extract which is 4000 mg/kg did not show any signs of toxicity or mortality for animals. Thus, plant extract of stem bark even at 4000 mg/kg may be considered for further investigation. Toxicology studies the adverse effects of substances on organisms, focusing on their potential to harm people and animals. Factors like host characteristics, species, entry point, dose, and exposure length can impact the effects. Yuet Ping et al. (2013) have reported that at a single dose of of 5000 mg/kg, none of the rats observed during the observation period exhibited any signs of acute toxicity or mortality. A dose of 1000 mg/kg significantly reduced the rats' body weight gain during the sub chronic test, The plant extract did not exhibit any toxicity or mortality symptoms, even at a greater dose of 4000 mg/kg, indicating that it is safe for more research.

3.13. Isolation of bioactive substances

The eluted two samples coded as LC-001-25-EA_L and LC-002-10-EA_LS were studied by NMR spectroscopy and NMR data were compared with the phytochemistry report to finally determine their structures. One major molecule has been found from each sample (LC-001-25-EA_L and LC-002-10-EA_LS, respectively), according to the report obtained from BCSIR.

One significant chemical was discovered in the first sample. Sample 1's ^{1}H NMR spectrum (400 MHz, $CDCl_3$) showed a doublet at H 2.09, 0.81, and 0.91, respectively, which corresponds to the locations of H-18, H-29, and H-30 in the structure. The spectra also showed a peak associated with the hydroxyl group, H 3.63, which indicates H-3, along with numerous additional singlets at H 1.58, 1.72, 1.30, 2.04, etc.

All of these indications suggested that LC-001-25-EA_L

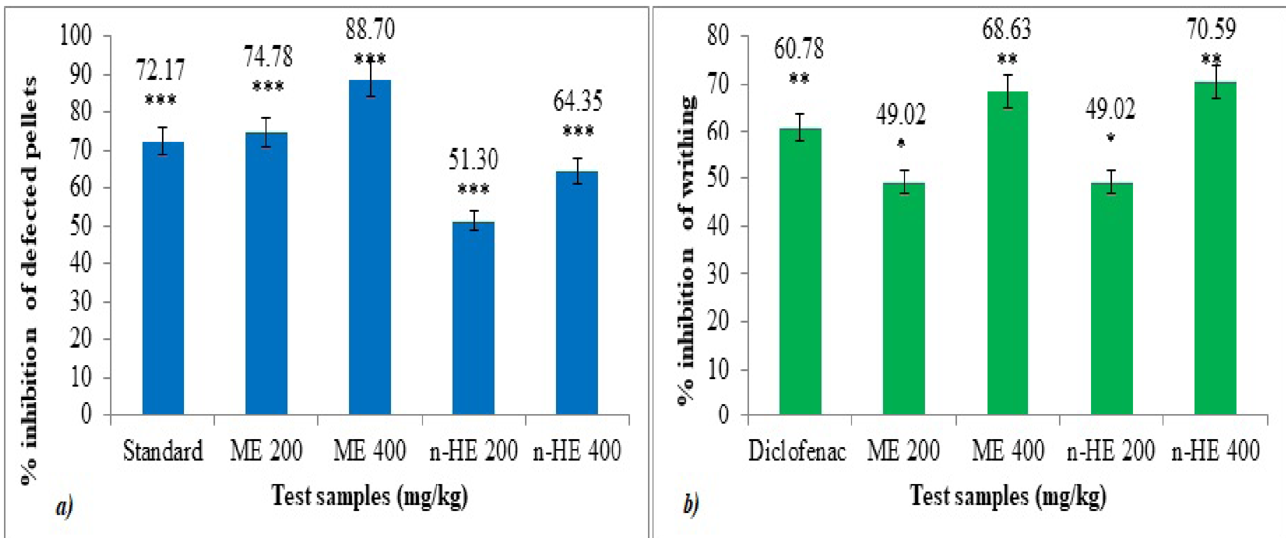

Fig. 3. Inhibition (%) of ***a)*** defecation and ***b)*** % of Inhibition writhing of *L.coromandelica* extractives of plant extract and standard at two different dose.

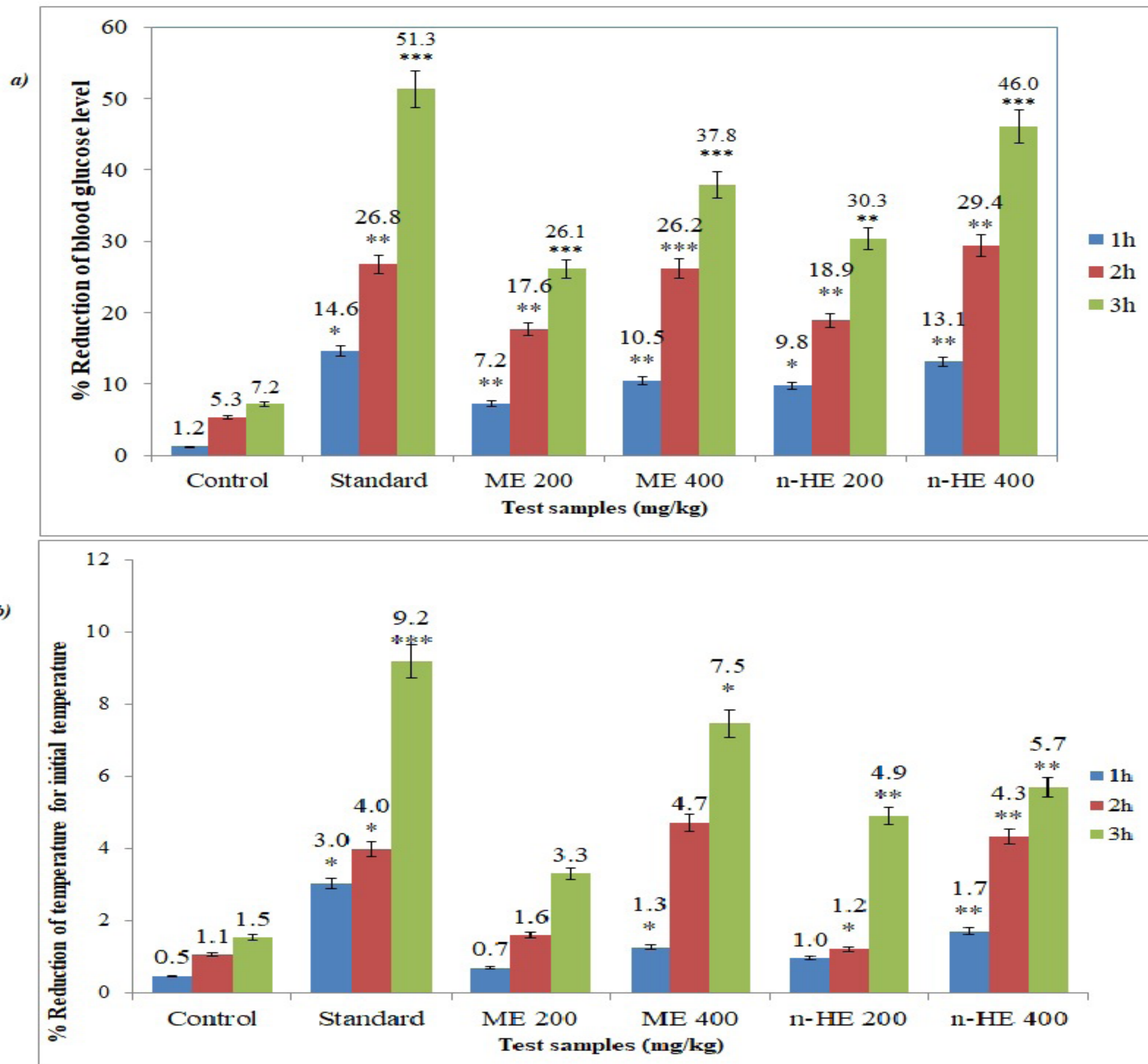

Fig. 4. a) Percent decrease in glucose level and b) Percent decrease in body temperature after three hours of feeding different *Lannea coromendelica* stem bark samples to Swiss albino mice.

includes ursolic acid, a pentacyclic triterpenoid moiety that is a natural triterpene chemical, and when compared to published ursolic acid data, it was determined that the pentacyclic moiety is ursolic acid as shown in Fig. 5 and Fig. 6. The ursolic acid values from were compared to the ^{1}H NMR data (Labib et al., 2016). Table 4 shows the comparison between the standard and the isolated sample 1.

On this basis, the identity of sample 1 was confirmed as ursolic acid shown in Fig. 7(a). The ^{1}H and ^{13}C NMR spectrum of the isolated compound LC-001-25-EA_L is illustrated in Fig. 5.

The positions of the doublet at H 2.338 (d, *J* = *7.5*) in LC-002-10-EA_LS, ^{1}H NMR spectrum (400 MHz, $CDCl_3$) are suggestive of H-4 in the structure. The spectrum also gave rise to a triplet at δ_H 0.882 (3H, t) which indicates Me-18 proton. and multiplate at δ_H 1.684, 1.379, 2.087, 2.043, 2.007, 1.305, 0.906 that revealed H-5, H-9, H-10, H-13, H-15, H-16 and H-17 proton. All of these signals pointed to the presence of a chemical with a monounsaturated fatty acid moiety in sample LC-002-10-EA_LS, and a comparison to previously published data confirmed the compound as palmitoleic acid (Table 5 and Table 6, Fig. 4-b) (Knothe and Kenar, 2004). The ^{1}H and ^{13}C NMR spectrum of isolated compound LC-001-25-EA_L is illustrated in Fig. 5.

Ursolic acid, found in plants like apple peels and holy basil, is a compound used in cosmetics and anticancer drugs. It may increase muscle mass, while reducing fat mass, but no human trials have been conducted. Consuming ursolic acid may reduce fat accumulation, increase muscle growth, promote fat burning, and maintain muscle mass (Saraswati et al., 2013). This acid has been isolated from *L. coromandelica* stem bark for the first time. It is a popular anti-inflammatory, antioxidant, anti-apoptotic, and anti-carcinogenic compound found in fruits and vegetables. Ursolic acid is a crucial component of the human diet, with nearly 700 research articles published in the past decade (Khwaza et al., 2020). It has demonstrated antioxidant and anti-cancer properties. Due to the fact that it scavenges reactive oxygen species, it might play a crucial part in the high glucose-mediated apoptosis. The therapeutic use of ursolic acid affects the growth and death of malignant cells (Saraswati et al., 2013).

Palmitoleic acid, a monounsaturated fatty acid, is found in small amounts in the human diet and blood plasma. Macadamia oil is a significant potential source, making it crucial to understand its importance. *trans*-Palmitoleate is mostly an exogenous source in ruminant fat and dairy products, while it can also be produced in humans. Recent research suggests that palmitoleate is a lipokine that is released from adipose tissue and can influence the metabolism of distant organs. The metabolic effects and mechanisms of palmitoleate have been investigated, which may have potential anti-thrombotic properties in medicinal applications (Bermúdez et al., 2022).

Palmitoleic acid is a monounsaturated omega-7 fatty acid that is primarily found in plants and marine sources (Yang et al., 2019). It has been well documented that palmitoleic acid improves insulin sensitivity, lipid metabolism, and hemostasis. Beta-cell apoptosis that is brought on by glucose or saturated fatty acids may be prevented by palmitoleic acid (Welters et al., 2006). Ultimately, a thorough analysis of *L. coromandelica* extracts has shown a high level of bioactivity, including cytotoxic, anti-diarrheal, antioxidant, and antidiabetic effects. Ursolic acid and palmitoleic acid's separation provides important new insights into the plant's possible bioactive constituents. However, to investigate the molecular processes behind these actions and evaluate the plant's medicinal potential in a range of medical diseases, more research is necessary.

4. Concluding remarks

This research highlights the considerable therapeutic potential of *L. coromandelica* stem bark, which has been confirmed by an extensive analysis that used both *in vitro* and *in vivo* techniques. The plant's medicinal richness is further supported by the extraction of bioactive components, such as ursolic and palmitoleic acid, using the relevant extraction techniques and column chromatography. Although both extracts were effective, the *n*-hexane extract showed slightly stronger antidiabetic effects along with better thrombolytic, membrane stabilizing, and cytotoxic qualities. According to this study's findings, brine shrimp are effectively killed by extracts from *L. coromandelica* that have good to moderate cytotoxic and antioxidant activity. The antioxidant and cytotoxic potential of the plant may be due to its polyphenol components, flavonoids, and other phytochemicals, and it can be an excellent source of new, natural antioxidants. Additionally, it is obvious that variations in the thrombolytic and membrane stabilizing abilities of the various *L. coromandelica* extracts point to a high potential for efficient anti-inflammatory activity both *in vitro* and *in vivo*. Due to the abundance of flavonoids, it may be believed that these extracts are a great source of thrombolytic and membrane-stabilizing substances. Although further research is required to completely understand the underlying mechanisms, it's feasible that the effects of *L. coromandelica* will have a big impact on medicine.

Author contribution statement

Conceptualization and literature search was conducted by Ramisa Anjum, A.H.M. Nazmul Hasan, and Mst. Al Asma Hawa. Data curation was handled by Ramisa Anjum, A.H.M. Nazmul Hasan, and Mst. Al Asma Hawa. Formal analysis was carried out by Md. Khokon Miah Akanda, Fayad Bin Abdus Salam, Tania Binte Wahed, Md. Rabiul Islam, and Mohammad Shahriar. The investigation was performed by Mst. Al Asma Hawa, Ramisa Anjum, Md. Naimur Rahman, and Fayad Bin Abdus Salam. Methodology was developed by Mst. Al Asma Hawa, A.H.M. Nazmul Hasan, and Ramisa Anjum. Supervision was provided by A.H.M. Nazmul Hasan. The original draft was written by A.H.M. Nazmul Hasan and Ramisa Anjum, while the review and editing were completed by A.H.M. Nazmul Hasan and Tania Binte Wahed. All authors read and approved the final manuscript.

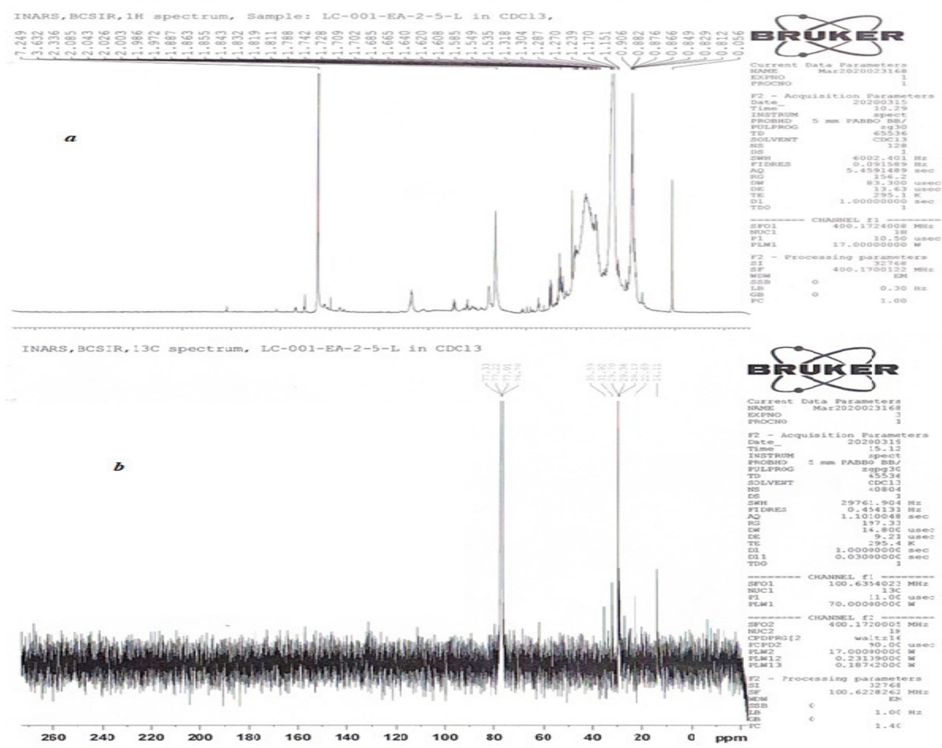

Fig. 5. a) ^{1}H and b)^{13}C NMR spectrum of LC-001-25-EA_L.

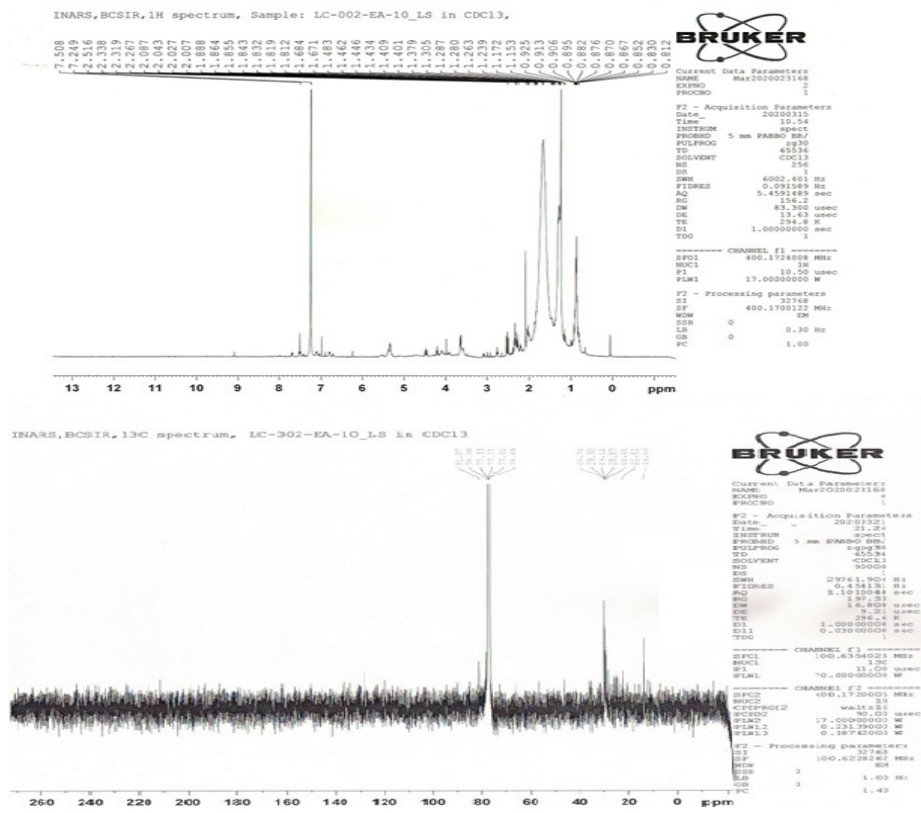

Fig. 6. ^{1}H and ^{13}C NMR spectrum of LC-002-10-EA_LS.

Table 4
^{1}H-NMR (400 MHz) spectral data of LC-001-25-EA_L and Ursolic acid in $CDCl_3$.

Si No.	LC-001		Ursolic acid	
	^{13}C NMR	^{1}H NMR	^{13}C NMR	^{1}H NMR
1		1.58	38.8	1.56
2		1.72	27	1.72
3	76.7	3.63	76.9	3.58 (OH, bs)
4			38.4	
5		1.32	54.8	1.39
6		1.52	18	1.52
7	31.92	1.3	32.7	1.31
8			40.2	
9			47.1	
10			36.6	
11	22.69	2.04	22.9	2.04
12			124.6	5.12 (1H, bs)
13			138.2	
14			41.7	
15			27.6	
16			23.9	
17			46.9	
18		2.09 (1H, d)	52.4	2.10 (1H, d)
19		1.64	38.6	1.63
20		1.61	38.5	1.6
21	29.7	1.27	30.2	1.27
22	35.59		36.4	
23	29.13		28.3	0.67 (3H, s)
24	14.11	0.88 (3H, s)	15.3	0.89 (3H, s)
25		0.87 (3H, s)	16.1	0.86 (3H, s)
26			17	0.74 (3H, s)
27			23.3	1.03 (3H, s)
28			178.3	
29		0.81 (3H, d)	16.9	0.81 (3H, d)
30		0.91 (3H, d)	21.1	0.90 (3H, d)

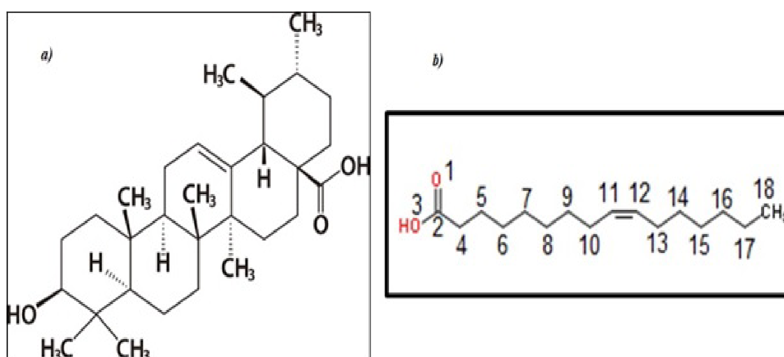

Fig. 7. a) Structure of ursolic acid and b) Palmetoleic acid.

Conflict of interest

The authors declare that there is no conflict of interest.

Acknowledgement

The authors are thankful to ICDDR'B for providing laboratory animals and Analytical Service Division, BCSIR to facilitate NMR study of the isolated compounds.

Ethical guidelines

A research proposal was submitted to the Research Ethics Committee (REC) prior to the study for approval and approved in a meeting (Ref: UAP/REC/20231212) by the REC of the Department of Pharmacy, University of Asia Pacific. Standard protocol was followed in handling of laboratory animal (Guide for the Care and Use of Laboratory Animals, 8th Edition, National Research Council, Washington DC, USA). Blood sample was collected by taking written inform consent from the volunteers and blood was collected by taking all necessary precautions.

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