Methanolic extracts and different fractions of whole plants of *Leucas zeylanica* show promising analgesic and antioxidative activities

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Background and objective
Leucas zeylanica is a medicinal plant used traditionally in tropical Asian countries including Bangladesh. This study was conducted to assess the pharmacological activities of whole plants of *L. zeylanica*.
Materials and methods
Methanolic crude extracts (MCEs) and petroleum ether-soluble fractions (PESFs), chloroform-soluble fractions (CSFs), and ethyl acetate-soluble fractions (EASFs) of whole plants were studied for the probable peripheral analgesic, central analgesic, and antioxidant and antimicrobial activities by acetic-acid-induced writhing and radiant heat tail-flick tests in mice, 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging, and disk diffusion method, respectively.
Results and conclusion
The MCEs showed the highest peripheral analgesic activity among the test samples with an inhibition of pain sensation of 40% (at 200 mg/kg body weight dose, MCE_{200}) and 32% (at 100 mg/kg body weight dose, MCE_{100}) in comparison to diclofenac sodium (66.67%, $P<0.001$). In central analgesic activity test, elongation of flicking time was calculated at 30, 60, and 90 min of administration of test samples where morphine was the positive control. All extracts and fractionates showed significant increase in tail-flicking time. However, methanolic extract showed the highest activity (at 60 min) among all test samples (15.44±0.256, $P<0.001$). In antioxidant tests, butylated hydroxytoluene (BHT) was used as the positive control where methanolic extracts exhibited the highest antioxidant potentials among the test samples. IC_{50} (μg/ml) values were 19.61 (BHT), 28.46 (MCE), 65.61 (EASF), 84.75 (PESF), and 97.09 (chloroform-soluble fractions). Additionally, whole plant extracts showed weak antimicrobial activity. This ethnopharmacological investigation suggests that methanolic extracts and different fractions from *L. zeylanica* have strong analgesic and antioxidant potential and can be a significant source of natural medicine.

Keywords:
analgesic, antimicrobial, antioxidant, *Leucas zeylanica*, tail flicking

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Introduction

Plant diversity is a basic source of food and medicine for local Himalayan communities. Natural plants are a trustworthy source of medicinal compounds that have been being used from the ancient times to treat many diseased conditions with or without further modification [1,2]. These medicinal plants show very less side effects with better safety margin and are serving as a great source to develop new drugs [3]. Nowadays, around 80% of world population has trust on herbal plants for their primary health care, which indicates the necessity of extensive study of these plants for detecting the probable phytoconstituents and their potential pharmacological effects [4].

Leucas zeylanica is a small-sized herb and a terrestrial aromatic plant of the subfamily Lamioideae of the family Lamiaceae [5]. It is commonly known as Ceylon Slitwort and in Bangladesh as Kusha or Swetadrone [6]. It usually grows on sandy soils, open grasslands, waste places, roadsides, etc. It is commonly distributed all over the countries of Southeast Asia [7]. In Bangladesh, it is found in Dhaka, Cumilla, Sylhet, Chattogram, and the Chittagong Hill Tracts.

The plants of *L. zeylanica* have been widely used by the traditional healers to cure many diseases such as cough, cold, toothaches, abdominal pain and also as antirheumatics, for thrombolytic activity [8]. A variety of phytoconstituents have been detected in

these plants which include alkaloids, steroids, tannins, flavonoids, glycosides, etc. by using standard procedures [9–11]. These phytoconstituents indicate that *L. zeylanica* has a huge possibility for the discovery of new drugs or lead molecules.

Any medicinal plants that are traditionally used for pain may pave the way to detect one or more phytoconstituents important for developing new analgesic agents. These are the therapeutic agents that may work on the peripheral or central pathway of pain sensation [12].

To get protection against oxidative stress, substances having antioxidative properties are used. They are commonly known as free radical scavengers [13,14]. Free radicals produced in oxidative stress cause potential destruction of many important cellular components such as proteins, DNA, lipids, etc [15]. These type of destructions may result in a number of diseased conditions like carcinogenesis, age-related diseases, diabetes mellitus, neurodegenerative diseases, etc [16].

Phytoconstituents possessing antimicrobial properties are of great importance. In recent times, a significant number of microorganisms have built resistance to many antimicrobial drugs that encourages scientists to develop new approaches [17]. Current experiments tried to explore the ethnopharmacology of the whole plant of *L. zeylanica,* whether it has analgesic, antioxidant, and antimicrobial potentials.

Materials and methods

Collection and identification of plant materials

Whole plants of *L. zeylanica* were collected during summer from a hilly area of Chattogram, Bangladesh and were identified properly in Dhaka University Herbarium by an expert taxonomist. The plant samples were washed properly. They were then cut into very small pieces. After that, the plant pieces were dried for few days by air-drying. To facilitate the grinding process, the pieces have undergone oven-drying at low temperatures for 24 h. Finally, the pieces were ground into a coarse powder.

Extraction of the plant material

The powdered material (about 600 g) was taken in a clean, round-bottomed flask (5 l), and soaked in 2.5 l of methanol. A foil was used to seal the flask. Then the flask was kept still for a period of 15 days with occasional shaking and stirring. Finally, the mixture was filtered through a Whatman No.1 filter paper, followed by filtering through a fresh cotton plug. The volume of the filtrate was then reduced by using the evaporator at low temperature and pressure. Finally, the crude methanolic extracts became ready for the next steps of the experiment.

Solvent–solvent partition of crude extract

Solvent–solvent partitioning was done using the protocol designed by Kupchan and modified by Van Wagenen [18]. The crude methanolic extracts (5 gm) were taken in aqueous methanol (10%). It was partitioned with petroleum ether, carbon tetrachloride, chloroform, and ethyl acetate, successively. After the partitioned fractions were dried, the containers carrying different dried fractionates were covered with aluminum foil and preserved in a refrigerator. As we found insignificant amounts of carbon tetrachloride-soluble fractions, we did not use them in the next steps of the experiment.

Chemicals

The chemicals were purchased from different sources – methanol, carbon tetrachloride, chloroform, pet. (petroleum) ether, ethyl acetate, acetic acid, 1,1-diphenyl-2-picrylhydrazyl (DPPH), and tert-butyl-1-hydroxytoluene (BHT) were from Merck (Darmstadt, Germany); Tween 80 and dimethyl sulfoxide (DMSO) were from BDH Chemicals (Leicestershire, UK); morphine, diclofenac sodium and ciprofloxacin were from Renata Ltd (Dhaka, Bangladesh); and normal saline (0.9% NaCl) was from Orion Infusion Ltd (Dhaka, Bangladesh).

Experimental animals

Swiss albino mice (of either sex, aged 4–5 weeks) weighing 25–30 g were collected from the animal house of Jahangirnagar University, Dhaka. The mice were kept in standard polypropylene cages under a temperature of 24±2°C and a relative humidity of 60–70% in a 12 h light–dark cycle in the animal house of the Institute of Nutrition and Food Science, University of Dhaka. A standard diet and water were given to the mice and withdrawn 12 h before the experiment. The experimental works with mice were performed in accordance with the standard guidance recommended by Ethics Committee of the National Research Council, USA (Guide for the Care and Use of Laboratory Animals: Eighth Edition).

Assessment of peripheral analgesic activity

Peripheral analgesic activity of whole plants of *L. zeylanica* was assessed by the acetic-acid-induced

writhing method [19]. In this method, to create pain sensation (squirm or writhing), acetic acid was administered intraperitoneally. Diclofenac sodium at 50 mg/kg and normal saline with DMSO at 0.1 ml/10 g bw (body weight) dose given orally were considered as the positive control and negative control, respectively. Acetic acid 1% (v/v) was administered at a dose of 0.1 ml/10 g to each mouse to induce pain. Crude extracts and different fractionates were administered orally to the Swiss albino mice at two different doses: 200 and 100 mg/kg. Each test group contained five mice. Test samples, and negative and positive controls were administered at zero hour. Acetic acid was administered after a period of 40 min and at a 5 min interval of this, the counting of the number of writhing was taken for 10 min for individual mouse.

Assessment of central analgesic activity

Central analgesic activity of whole plants of *L. zeylanica* was assessed by radiant heat tail-flick method [20]. Pain stimulus was given to the mice by applying a constant heat stress. Morphine was considered as the positive control and administered at 2 mg/kg dose. Normal saline with DMSO at a dose of 0.1 ml/10 g was considered as the negative control. Crude extracts and different fractionates were given at two doses: 200 and 100 mg/kg. There was a total of 10 groups consisting of five mice in each one. Test samples and negative control were administered orally by a feeding needle where morphine was given subcutaneously. Finally, by using an analgesiometer (Medicraft, India), the central analgesic activity (% elongation of flicking time) was assessed at 30, 60, and 90 min of the administration of the aforementioned materials.

Assessment of antioxidant activity

Antioxidant property of –crude extracts and different fractionates of whole plants of *L. zeylanica* was assessed by the method of Brand-Williams *et al.* [21]. In this method, DPPH was used as the free radical and BHT was used as the positive control. By dissolving 2 mg of crude extracts, the fractionates and BHT in methanol, solution of a number of concentrations were prepared by serial dilution process such as 500, 250, 125, 62.5, 31.25, 15.625, 7.813, 3.906, 1.953, and 0.977 µg/ml. Then 2 ml solution of individual concentrations was added to a solution (3 ml) of methanol containing DPPH at 20 µg/ml conc. These solutions were kept at usual room temperature in a place where light is absent for 30 min. After that, the absorbance of individual concentrations was taken by a UV spectrophotometer where methanol played the role of the blank. The wavelength was 517 nm. Finally, inhibition (%) of DPPH was calculated by the following formula:

$$\text{Inhibition}(\%) = \left(1 - A_{\text{sample}}/A_{\text{blank}}\right) \times 100$$

where A_{sample} is the absorbance of the sample and A_{blank} is the absorbance of the blank (methanol).

The IC_{50} value of individual concentrations was calculated from a graph found by plotting inhibition percentages against their respective concentrations.

Assessment of antimicrobial activity

To assess the antimicrobial activity of the whole plant of *L. zeylanica*, disk diffusion method was followed [22]. The positive control was a ciprofloxacin disk (5 µg/disc) and the negative control was a blank disk. The crude extracts and fractionates were used at a dose of 400 µg/disk. The disks soaked with the experimental samples and positive control were dried properly. The disks were placed in the agar plates on the definite marked zones where the microorganisms (bacteria or fungi) were inoculated earlier. Then the disk containing agar plates were reserved upside down in a refrigerator for 24 h at 4°C to allow proper diffusion of the materials to the agar medium. After that, the agar plates were inverted and kept in an incubator at 37°C for about 24 h. Finally, the clear zones of the plates were measured with a scale.

Statistical analysis

Statistical calculations were displayed as mean±SEM. One-way analysis of variance was used for the analysis purpose of the results. Here, P less than 0.05 reflects the statistical significance.

Results

Peripheral analgesic activity

In this study, the methanolic crude extracts (MCEs) at both doses (200 and 100 mg/kg) showed very significant and high peripheral analgesic activity with an inhibition of pain sensation of 40 and 32%, respectively, where the standard drug (diclofenac sodium) showed an inhibition of 66.67% pain sensation. Ethyl acetate-soluble fractions (EASFs) and chloroform-soluble fractions (CSFs) at double dose also showed very significant outcomes with an inhibition of 25 and 27.33% pain sensation, respectively. Other test samples also showed significant results (Table 1).

Central analgesic activity

The radiant heat tail-flick method showed that, at 30 min of administration, the MCEs at both doses (200 and 100 mg/kg), EASFs, PESFs, and CSFs at only double dose (200 mg/kg) exhibited significant central analgesic activity with an elongation of flicking time of 25.85, 23.01, 19.6, 17.61, and 14.2%, respectively. Here, the standard drug (morphine) showed an elongation of flicking time of 55.4%. At 60 min, almost all the test samples showed significant central analgesic activity. The MCEs showed an elongation of flicking time of 25.53% at 200 mg/kg dose and 17.4% at 100 mg/kg dose. EASFs showed an elongation of flicking time of 20.65% at 200 mg/kg dose and 14.8% at 100 mg/kg dose. Other fractionates at only double dose showed significant central analgesic activity compared with the standard drug showing an elongation of flicking time of 82.28%. At 90 min, only MCE at double dose showed statistically significant central analgesic activity where the standard drug showed an elongation of flicking time of 36.8% (Table 2).

Table 1 Peripheral analgesic activity of *Leucas zeylanica* extracts and fractions

Test group	Number of writhing	% of inhibition of writhing
NC	30±1.14	–
PC	10±0.71*,**	66.67
MCE_{200}	18±1.48***	40.00
MCE_{100}	20.4±1.21***	32.00
$EASF_{200}$	22.5±0.45***	25.00
$EASF_{100}$	23.8±0.66**	20.67
$PESF_{200}$	23.4±0.70**	22.00
$PESF_{100}$	24.7±0.44**	17.67
CSF_{200}	21.8±1.02***	27.33
CSF_{100}	23.6±0.91**	21.33

Values are expressed as mean±SEM (n=5). CSF_{100}, chloroform-soluble fractions at 100 mg/kg bw; CSF_{200}, chloroform-soluble fractions at 200 mg/kg bw; $EASF_{100}$, ethyl acetate-soluble fractions at 100 mg/kg bw; $EASF_{200}$, ethyl acetate-soluble fractions at 200 mg/kg bw; MCE_{100}, methanolic crude extract at 100 mg/kg bw; MCE_{200}, methanolic crude extract at 200 mg/kg bw; NC, negative control; PC, positive control; $PESF_{100}$, pet. ether-soluble fractions at 100 mg/kg bw; $PESF_{200}$, pet. ether-soluble fractions at 200 mg/kg bw. $^{*}P<0.05$, significance compared with negative control. $^{**}P<0.01$. $^{***}P<0.001$.

Antioxidant activity

In DPPH free radical scavenging method, the MCEs, EASFs, PESFs, and CSFs showed significant antioxidant potential with an IC_{50} value of 28.46, 65.61, 84.75 and 97.09 μg/ml, respectively. Here, the standard drug (BHT) showed an IC_{50} value of 19.61 μg/ml (Table 3).

Antimicrobial activity

The assessment study of antimicrobial activity by the disk diffusion method was conducted with some common Gram-positive bacteria, Gram-negative bacteria, and fungi. The standard drug

Table 2 Central analgesic activity of *Leucas zeylanica* extracts and fractions (n=5)

Test group	At 30 min		At 60 min		At 90 min	
	Mean±SEM	% Elongation of flicking time	Mean±SEM	% Elongation of flicking time	Mean±SEM	% Elongation of flicking time
NC	7.04±0.367	–	12.3±0.374	–	10.38 ±0.463	–
PC	10.94 ±0.225***	55.40	22.42 ±0.477***	82.28	14.2 ±0.439***	36.80
MCE_{200}	8.86±0.333**	25.85	15.44 ±0.256***	25.53	11.74 ±0.291*	13.10
MCE_{100}	8.66±0.500*	23.01	14.44 ±0.389**	17.40	11.1±0.352	6.94
$EASF_{200}$	8.42±0.379*	19.60	14.84 ±0.232**	20.65	11.36 ±0.199	9.44
$EASF_{100}$	7.98±0.511	13.35	14.12 ±0.183**	14.80	10.96 ±0.446	5.59
$PESF_{200}$	8.28±0.242*	17.61	14.42 ±0.162***	17.24	11.24 ±0.206	8.29
$PESF_{100}$	7.26±0.287	3.13	12.98±0.331	5.53	10.9±0.138	5.01
CSF_{200}	8.04±0.125*	14.20	14.04 ±0.427*	14.15	11.12 ±0.344	7.13
CSF_{100}	7.46±0.163	5.97	13.2±0.420	7.32	10.54 ±0.284	1.54

CSF_{100}, chloroform-soluble fractions at 100 mg/kg bw; CSF_{200}, chloroform-soluble fractions at 200 mg/kg bw; $EASF_{100}$, ethyl acetate-soluble fractions at 100 mg/kg bw; $EASF_{200}$, ethyl acetate-soluble fractions at 200 mg/kg bw; MCE_{100}, methanolic crude extract at 100 mg/kg bw; MCE_{200}, methanolic crude extract at 200 mg/kg bw; NC, negative control; PC, positive control; $PESF_{100}$, pet. ether-soluble fractions at 100 mg/kg bw; $PESF_{200}$, pet. ether-soluble fractions at 200 mg/kg bw. $^{*}P<0.05$, significance compared with negative control. $^{**}P<0.01$. $^{***}P<0.001$.

(Ciprofloxacin) showed a vast zone of inhibition (45–46 mm) which meant the selected microorganisms were highly sensitive to it. In comparison to this result, the MCEs, EASFs, and CSFs of whole plants of *L. zeylanica* produced a very small zone of inhibition (8–9 mm). Here, the PESFs showed the lowest zone of inhibition (7 mm) or no activity (0 mm). The findings are shown in Table 4.

Discussion

Pain, oxidative damages, and microbial infections are well-known terms in scientists' community dealing with drug discovery. In this explorative study, we attempted to help them with new sources of medicinal agents that would be effective against pain stimulus, oxidative stress, and infectious diseases.

To assess the peripheral analgesic potential of the experimental plant, pain-related writhing was induced in mice model by acetic acid and the target samples were studied if they were sufficiently able to block this pain sensation or not. Pain was also applied by holding the tail of the mice in a source of radiant heat and the test samples were observed whether they possessed any activity to block the pathway of pain stimulus in the central nervous system. From the aforementioned outcomes of the analgesic activity (peripheral and central) tests, it is clear that *L. zeylanica* has moderate to high analgesic potential. Here, the MCE of *L. zeylanica* at both doses (200 and 100 mg/kg) were significantly effective where other fractionates showed significant effects only at 200 mg/kg dose. This finding indicates that *L. zeylanica* may have inhibitory potential on the cyclooxygenase (COX) pathway. This may be due to the presence of flavonoids that have inhibitory action on prostaglandin synthesis [23]. Tannins and saponins may also be responsible for this activity [24]. These phytoconstituents of *L. zeylanica* have already been reported. DPPH free radical scavenging test is a well-accepted method that mimic the free radical-related oxidation and associated damages in the human body. This research demonstrates that *L. zeylanica* has moderate to high antioxidative potential. Among the test samples, the MCEs showed the highest antioxidative activity. This property of *L. zeylanica* may be attributed to the presence of the phytoconstituents – flavonoids and polyphenols [25]. Forthcoming exploration may confirm the particular mechanism behind this outcome.

Table 3 Antioxidant activity of *Leucas zeylanica* extracts and fractions

Samples	IC_{50} (μg/ml)
BHT (tert-butyl-1-hydroxytoluene)	19.61
Methanolic crude extracts	28.46
Ethyl acetate-soluble fractions	65.61
Pet. ether-soluble fractions	84.75
Chloroform-soluble fractions	97.09

Table 4 Antimicrobial activity of *Leucas zeylanica* extracts and fractions

Microorganism	Diameter of the zone of inhibition (mm)				
	MCE	EASF	PESF	CSF	Ciprofloxacin
Gram-positive bacteria					
Bacillus cerus	9	9	0	9	46
Bacillus megaterium	9	8	0	8	46
Bacillus subtilis	9	9	0	8	45
Staphylococcus aureus	9	9	0	8	46
Sarcina lutea	9	9	0	9	45
Gram-negative bacteria					
Escherichia coli	9	8	0	8	46
Pseudomonas aeruginosa	9	9	0	9	45
Salmonella paratyphi	9	9	0	9	45
Salmonella typhi	9	9	7	9	45
Shigella boydii	9	9	7	9	46
Shigella dysenteriae	9	9	0	9	45
Vibrio mimicus	8	9	0	9	45
Vibrio parahaemolyticus	8	8	0	8	45
Fungi					
Candida albicans	9	9	0	8	45
Aspergillus niger	9	9	0	8	45
Saccharomyces cerevisiae	9	9	0	8	45

CSF, chloroform-soluble fraction; EASF, ethyl acetate-soluble fraction; MCE, methanolic crude extract; PESF, petroleum ether-soluble fraction.

Additionally, we endeavored to determine the antimicrobial potential of the test samples by the disk diffusion method. In comparison with the standard drug, MCEs and other fractionates of the whole plant of *L. zeylanica* exhibited very mild antimicrobial activity, which may be because of the presence of essential oils in this plant [26,27]. Combinedly all the findings of the methanolic extracts and different fractions of *L. zeylanica* settle it as a scientifically reliable and medicinally resourceful plant.

Conclusion

Based on the findings of this study, it can be claimed that the whole plant of *L. zeylanica* possesses significant peripheral and central analgesic, antioxidative, and very mild antimicrobial potential. These findings rationalize the traditional uses of *L. zeylanica* to some extent. To detect the exact individual phytoconstituents and mechanisms responsible for these activities and for more authentication of these findings, further extensive studies may be required.

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Conflicts of interest

There are no conflicts of interest.

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