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Ethnopharmacological investigations of methanolic extract of *Pouzolzia Zeylanica* (L.) Benn

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Abstract

Background: Phytochemical derived from plant consisting of phenols and flavonoids possess antioxidant properties, which are useful to scavenge reactive oxygen species (ROS). The aim of the work is to ascertain the level of antioxidant, cytotoxic, anti-microbial properties and in vivo analgesic and anti-inflammatory activity of methanolic extracts of *Pouzolzia zeylanica* (L.) Benn.

Methods: The present study was conducted to evaluate antioxidant, cytotoxic, anti-microbial potential, analgesic and anti-inflammatory activity of methanol extracts of *Pouzolzia zeylanica* (L.) Benn. using DPPH (1,1-diphenyl-2-picrylhydrazyl) scavenging assay, cupric reducing antioxidant capacity, nitric oxide scavenging assay, ferric reducing antioxidant power, total antioxidant capacity, determination of total phenol and flavonoid contents and cytotoxic activity test using brine shrimp lethality bioassay whereas antimicrobial activity was evaluated against some potent Gram positive and Gram negative bacteria by disc diffusion method, anti-inflammatory and analgesic activity were determined by Xylene-induced ear edema and acetic acid induced writhing method respectively.

Results: Preliminary phytochemical study revealed the presence of alkaloid and flavonoids in the extract. The tested fraction showed significant antioxidant activities in the assay compared to the reference ascorbic acid in a dose dependent manner. In DPPH radical scavenging assay, the IC₅₀ value of the crude methanol extract was 485.223 µg/mL, whereas IC₅₀ value for the reference ascorbic acid was 34.88 µg/mL. In case of nitric oxide scavenging assay, the IC₅₀ value of the extract was 76.737 µg/mL, whereas IC₅₀ value for the reference ascorbic acid was 51.078 µg/mL. Moreover, at 200 µg/mL extract concentration, lower grade total antioxidant activity (160.813 ± 0.653 mg/g equivalent to ascorbic acid) was observed. Furthermore, extract showed good cupric reducing power and ferric reducing power capability. In addition, significant amount of flavonoids and low phenols content were obtained from the extract. In case of cytotoxicity test the crude extract showed a significant activity compared to the standard vincristine sulfate. In essence the tested extract exhibited moderate antibacterial activity which reveals that *Pouzolzia zeylanica* (L.) Benn. might be a source of antimicrobial agent. In case of anti-inflammatory and analgesic activity the tested extracts showed dose dependent significant inhibition (78.86 and 21.95 % at 500 mg/kg doses).

Conclusions: Further study on different solvent extracts of *Pouzolzia zeylanica* (L.) Benn. would be carried out to elucidate the active principles for its outmost activity.

Keywords: *Pouzolzia zeylanica*, Antioxidant, DPPH, Cytotoxicity, Disc diffusion, Antimicrobial activity, Analgesic, Anti-inflammatory

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Background

Free radicals can lead to cell and tissue damage in the body which is reported as oxidative damage [1]. This damage in turn can lead to many degenerative diseases, such as brain dysfunction, cancer, and heart diseases, age-related degenerative conditions, declination of the immune system, cancer, coronary arteriosclerosis, ageing processes, carcinogenesis, gastric ulcer and DNA damage arise [2–7].

Antioxidants have been used for the prevention and treatment of free radical-related disorders [8]. Recent study shows, a number of natural compounds such as polyphenols and flavonoids have been reported as antioxidants, which entrap and deactivate damaging ROS and RNS [9, 10]. Furthermore, they also possess anti-inflammatory, antiviral and anticancer properties [11–13].

Many medicinal plants contain large amounts of antioxidants such as polyphenols, which have an important role in preventing a variety of stress-related diseases and aging which are closely related to the active oxygen and lipid peroxidation [14]. *Pouzolzia zeylanica* (L.) Benn. (Family: Urticaceae), a perennial herb, height 15–30 cm. It is found all over in Bangladesh. The local name of this plant is Bishkatali, mostly found in Betbunia, Rangamati.

Leaves of this plant are anthelmintic and vulnerary; used as a cicatrizant for gangrenous ulcers, in syphilis and gonorrhoea. Leaf juice is used as galactagogue. Poultice of the herb is applied to sores, boils and to relieve stomachache [15]. Moreover, this plant contains flavones, flavonoids, tannin, carotene, carotenoids, ascorbic, tartaric, malic and pectic acids, gum, minerals and their salts [16]. The present research was aimed to investigate the phytochemical constituents, cytotoxic, antibacterial and antioxidant activities of *P. zeylanica*. To evaluate the antioxidant potential of the tested extract of *P. zeylanica*, several assay strategies have been implemented in this study. These consists of various in vitro and in vivo assay systems, such as the DPPH (1,1-diphenyl-2-picrylhydrazyl) scavenging assay, cupric reducing antioxidant capacity, ferric reducing antioxidant power, NO scavenging activity, total antioxidant capacity and determination of total phenol and flavonoid content, analgesic activity and anti-inflammatory activity in order to understand the usefulness of this plant as a functional food as well as in medicine.

Methods

Chemicals

DPPH (1, 1-diphenyl, 2-picrylhydrazyl) was purchased from Sigma Chemical Co., USA, potassium fericyanide [K₃Fe(CN)₆] from Loba Chemie Pvt. Ltd., Mumbai, India, Ascorbic acid from SD Fine Chem. Ltd., Biosar, India, Vincristine sulphate from Jayson Pharmaceuticals

Ltd, Bangladesh and neocaproin (C₁₄H₁₂N₂), ammonium molybdate, folin-ciocalteu phenol reagent, gallic acid (C₇H₆O₅.H₂O), quercetin were purchased from Merck, Germany.

Microorganisms

The antimicrobial activity assay of the extracts was carried out on both Gram-positive and Gram-negative bacteria.

Gram positive	Gram negative
<i>Bacillus cereus</i>	<i>Escherichia coli</i>
<i>Bacillus subtilis</i>	<i>Serratia spp.</i>
<i>Staphylococcus aureus</i>	<i>Salmonella typhi</i>
	<i>Pseudomonas spp.</i>
	<i>Proteus mirabilis</i>

These strains were obtained from Department of Microbiology, Jahangirnagar University, Savar, Dhaka, Bangladesh.

Selection of plant

The plant *Pouzolzia zeylanica* (L.) Benn. was selected for study. The plants were collected from Jahangirnagar University campus, Savar, Dhaka, Bangladesh in August, 2013. The collected plants were identified and authenticated by experts in Bangladesh National Herbarium, Mirpur, Dhaka, where a Voucher specimen has been deposited for future reference.

Methanol extract

The whole plants were sun dried and then crushed through grinding machine. Then 100 g of dried whole plant powder were taken in a separate container. To this 250 ml of methanol was added and kept for 24 h at room temperature with periodic shaking. Then filtered with Whatman No. 1 filter paper and the filtrate was collected. The procedure was repeated three times with fresh volume of methanol. The filtrates were pooled.

Preliminary phytochemical screening

The freshly prepared crude extract was qualitatively tested for the presence of chemical constituents. Phytochemical screenings of the extracts were performed using the following reagents and chemicals: alkaloids with Dragendorff's reagents, flavonoids with the use of Mg and HCl; tannins with ferric chloride and potassium dichromate solutions and saponins with ability to produce stable foam and steroids with Libermann- Burchard reagent. Gum was tested using Molish reagent and concentrated sulfuric acid; reducing sugars with Benedict's reagent. These were

identified by characteristic color changes using standard procedures by Ghani A., 2005 [17].

Tests for antioxidant activity

DPPH free radical scavenging activity

The free radical scavenging activity of the extracts, based on the scavenging activity of the stable 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical, were determined by the method described by Braca et al., 2001 [18]. The method is based on the reduction of ethanolic DPPH⁻ solution in the presence of a hydrogen donating antioxidant, due to the formation of the non-radical form DPPH-H by reaction. Plant extract (0.1 mL) of each concentrations were added to 3 mL of a 0.004 % ethanol solution of DPPH. Absorbance at 517 nm was determined after 30 min and the percentage inhibition activity was calculated from $[(A_0 - A_1)/A_0] \times 100$, where A_0 is the absorbance of the control (DPPH solution) and A_1 is the absorbance of the extract/standard. The inhibition curves were prepared and IC₅₀ values were calculated.

Ferric reducing antioxidant power (FRAP)

The ferric reducing antioxidant power was determined according to the method previously described by Oyaizu, 1986 [19]. According to this method, the reduction of Fe³⁺ to Fe²⁺ is determined by measuring the absorbance of Perl's Prussian blue complex. Briefly, different concentrations of extracts (5–200 µg) in 1 mL of distilled water were mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide [K₃Fe(CN)₆] (2.5 mL, 1 %). The mixture was incubated at 50 °C for 20 min. An aliquot (2.5 mL) of trichloroacetic acid (10 %) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The supernatant (2.5 mL) was mixed with distilled water (2.5 mL) and FeCl₃ (0.5 mL, 0.1 %) and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. Ascorbic acid was used as the reference.

Cupric reducing antioxidant capacity

The cupric reducing antioxidant activity of the methanol extract was determined by the method described by Resat et al., 2004 [20]. Different concentrations of the extract (5–200 µg) in 0.5 mL of distilled water were mixed with Cupric Chloride (1 mL, 0.01 M), Ammonium acetate buffer (1 mL, pH 7.0), Neocapronin (1 mL, 0.0075 M) and finally distilled water (0.6 mL). The mixture was incubated for 1 h at room temperature. Then the absorbance of the solution was measured at 450 nm against blank. Distilled water (0.5 mL) in the place of extract is used as the blank. The molar absorptivity of the Cuprac method for each antioxidant was found from the slope

of the calibration line concerned. Ascorbic acid was used as the standard solution.

Nitric oxide scavenging assay

Based on the principle that the compound Sodium Nitroprusside (SNP) is known to decompose in aqueous solution at physiological pH (7.2) producing NO. Under aerobic conditions, NO reacts with oxygen to produce stable products: nitrate and nitrite, the quantities of nitrate and nitrite can be determined using Griess reagent. The scavenging effect of *P. zeylanica* extract on nitric oxide was measured according to the method of Alisi and Onyeze, 2008 [21]. Briefly 4 ml of extracts solution at different concentrations were added (in the test tubes) to 1 ml of Sodium Nitroprusside (SNP) solution (25 mM) and the tubes incubated at 29 °C for 2 h. A 2 ml aliquot of the incubation solution was diluted with 1.2 ml Griess Reagent (1 % sulfanilamide in 5 % H₃PO₄ and 0.1 % naphthylethylenediamine-dihydrochloride). The absorbance of the chromophore that formed during diazotization of the nitrite with sulfanilamide and subsequent coupling with naphthylethylenediamine dihydrochloride was immediately read at 550 nm. The percentage (%) inhibition activity was calculated from the following equation $\{(A_0 - A_1)/A_0\} \times 100$. Where, A_0 is the absorbance of the Control and A_1 is the absorbance of the extract or standard. IC₅₀ was calculated by linear regression method.

Determination of total antioxidant capacity

The antioxidant activity of the extract was evaluated by the phosphomolybdenum method according to the procedure describe by Prieto et al, 1999 [22]. A 0.3 mL extract was combined with 3 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes containing the reaction solution were incubated at 95 °C for 90 min. Then the absorbance of the solution was measured at 695 nm using a spectrophotometer (UV visible spectrophotometer, Shimadzu, 1601) against blank after cooling at room temperature. Methanol (0.3 mL) in the place of extract is used as the blank. The antioxidant activity is expressed as the number of equivalents of ascorbic acid.

Determination of total phenol content

The total phenolic content of plant extract was determined using Folin–Ciocalteu reagent [23]. Plant extract (100 µL) was mixed with 500 µL of the Folin–Ciocalteu reagent and 1.5 mL of 20 % sodium carbonate. The mixture was shaken thoroughly and made up to 10 mL using distilled water. The mixture was allowed to stand for 2 h. Then the absorbance at 765 nm was determined. These data were used to estimate the phenolic contents

using a standard curve obtained from various concentration of Gallic acid.

Determination of total flavonoid content

The content of flavonoids compounds in the extract was determined by the method described by Chang et al., 2002 [24]. One milliliter of extract was mixed with methanol (3 mL), aluminium chloride (0.2 mL, 10 %), potassium acetate (0.2 mL, 1 M) and distilled water (5.6 mL) and incubated the mixture for 30 min at room temperature. Then the absorbance was measured at 415 nm against blank. Methanol (1 mL) in the place of extract was used as the blank and Quercetin was used as the standard solution. All determinations were carried out in triplicates. The amount of flavonoids in plant extracts in quercetin equivalents (QE) was calculated by the following formula: $X = (A \times m_0)/(A_0 \times m)$, where X is the flavonoid content, mg/mg plant extract in QE, A is the absorption of plant extract solution, A_0 is the absorption of standard rutin solution, m is the weight of plant extract in mg and m_0 is the weight of quercetin in the solution in mg.

Cytotoxicity test

Brine shrimp lethality bioassay was used for probable cytotoxic action [25, 26]. The eggs of brine shrimp (*Artemia salina* Leach) were collected and hatched in a tank at a temperature around 37 °C with constant oxygen supply. Two days were allowed to hatch and mature the nauplii. Stock solution of the extract sample was prepared by dissolving required amount of extract in specific volume of pure dimethyl sulfoxide (DMSO). 10 living nauplii were taken to each of the vial containing different concentrations of test sample with Pasteur pipette. Then specific volumes of sample were transferred from the stock solution to the vials to get final sample concentration. In the control vials same volumes of DMSO (as in the sample vials) were taken. Vincristine sulphate was used as the positive control. After 24 h the vials were observed and the number of nauplii survived in each vial was counted. From this, the percentage of mortality of brine shrimp nauplii was calculated for each concentration of the extract.

Antibacterial activity by disc diffusion method

The antibacterial activity of the plant extract was performed by the well accepted Bauer-Kirby disc diffusion

method [27, 28]. The inoculums of microorganisms were spread over nutrient agar plates with a sterile swab. One hundred milligram of the test sample was dissolved in 1 ml of methanol to obtain the concentration 100 µg/µl in an aseptic condition. Sterilized metrical filter paper discs (Whatman No. 1, 6 mm diam.) were soaked with 30, 20 and 10 µl of solutions of test sample. Then the discs were placed on the previously marked agar plate and dried. The extract was tested in triplicate and the plates were inoculated at 37 °C for 24 h. Antibacterial activities were evaluated by measuring inhibition zone diameters. Amoxicillin was used as positive control.

In-vivo pharmacological activity evaluation

Acute toxicity study

Overnight-fasted Swiss albino mice (39–45 g) of either sex were used for the study. The animals were divided into seven groups of five animals each. Groups A to F received orally 250, 500, 1000, 2000, 3000, 4000 mg/kg of the extract, respectively, while the control (group G), received distilled water (3 mL/kg) by the same route. General symptoms of toxicity and mortality in each group were observed within 24 h. Animals that survived after 24 h were observed for any signs of delayed toxicity for 2 weeks.

Anti-inflammatory activity evaluation

Xylene induced ear edema

Swiss albino mice were divided into four groups of five animals each. Animals were treated orally with the extract (250 and 500 mg/kg), diclofenac (1 mg/kg) and distilled water (3 mL/kg). Thirty minutes later, edema was induced in each mouse group by applying a drop of xylene to the inner surface of the right ear. After 15 min, the animals were sacrificed under ether anesthesia and both ears cut off, sized and weighed. The anti-inflammatory activity was expressed as the percent-age inhibition of edema in the treated mice in comparison with the control mice [29].

Analgesic activity evaluation

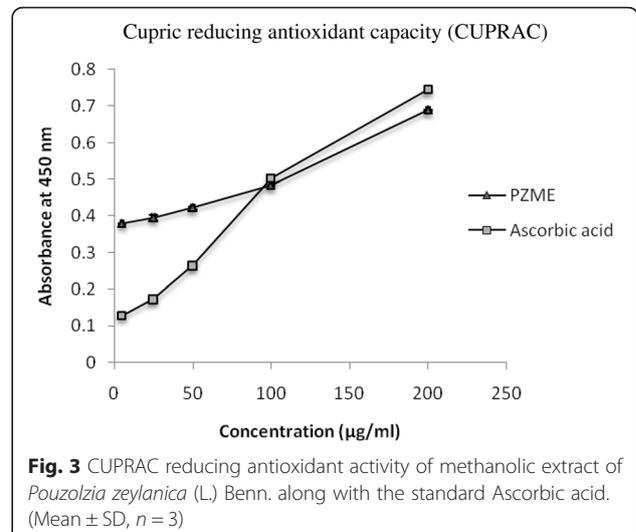
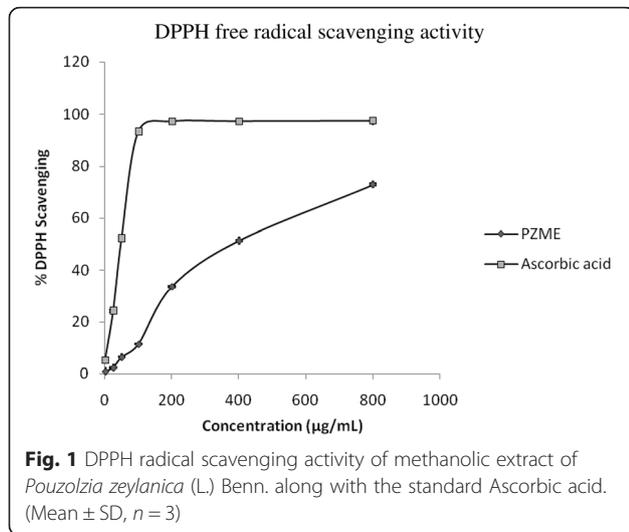
Acetic acid induced writhing

The method according to (Koster et al., 1959) [30] was employed for this test. Four groups of five mice each were pretreated with extracts (250 and 500 mg/kg),

Table 1 Results of phytochemical screening of methanol extract of *Pouzolzia zeylanica* (L.) Benn

Extract	Alkaloid test			Carbohydrate test	Flavonoid test	Glucoside test	Glycoside test	Saponin test	Steroid test	Tanin test
PZME	Hagers	Wagners	Dragendorffs	+	+	-	+	-	+	+
	Test	Test	Test							
	+	+	+							

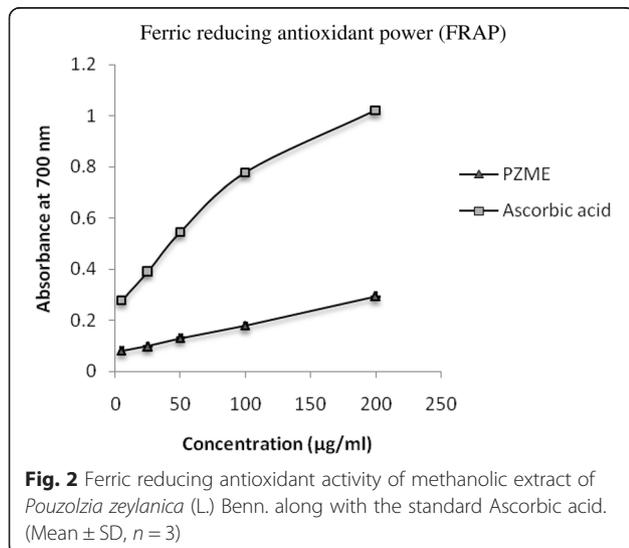
PZME *Pouzolzia zeylanica* (L.) Benn. Methanol, Values are the average of triplicate experiments and represented as mean ± SD; (+): Present; (-): Absent



diclofenac (1 mg/kg) and distilled water (3 mL/kg). Forty five minutes later each mouse was injected intraperitoneally with 0.7 % acetic acid at a dose of 10 ml/kg body weight. The number of writhing responses was recorded for each animal during a subsequent 5 min period after 15 min i.p. administration of acetic acid and the mean abdominal writhing for each group was obtained. The percentage inhibition of writhing was calculated by comparison with the control mice.

Statistical analysis

The results were expressed as mean ± standard deviation (SD) from triplicate experiments and evaluated with the analysis of student's *t*-test. Differences were considered significant at a level of *P* < 0.05. IC50 was calculated using Sigma Plot 11.0 software.



Results and discussion

Preliminary qualitative phytochemical screening

The methanolic extract of *P. zeylanica* (L.) Benn. has been shown to possess phytoconstituents including carbohydrates, alkaloids, glycosides, steroids, tannins, flavonoids and saponin etc. (Table 1). These phytoconstituents present in the extracts may account for their various pharmacological activities shown in other investigations [17].

DPPH[•] radical scavenging activity

In DPPH radical scavenging assay, as shown in Fig. 1, here the extract exhibited a concentration dependent antiradical activity by inhibiting DPPH[•] radical. Ascorbic acid, which is a well-known antioxidant, showed higher degree of free radical-scavenging activity than that of the plant extract at each concentration points. The IC50 value of the methanol extract was 485.223 µg/mL, while the IC50 value for the reference ascorbic acid was 34.88 µg/mL. The extracts were able to reduce DPPH

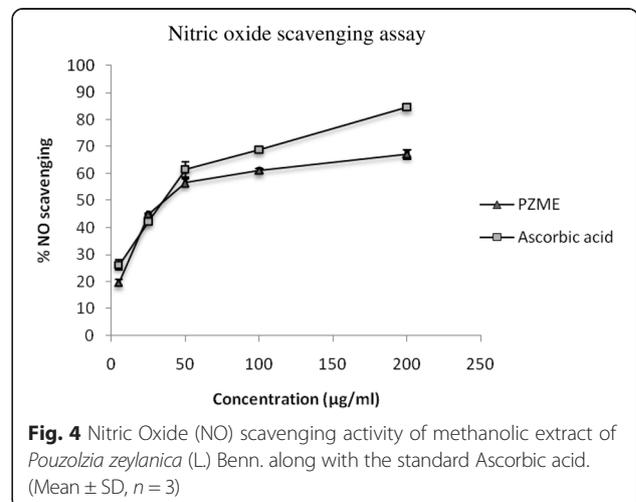


Table 2 Total antioxidant capacity, total phenol and total flavonoid contents of methanol extracts of the bark and leaf of *Pouzolzia zeylanica* (L.) Benn

Extract	Total antioxidant capacity equivalent to ascorbic acid mg/g plant extract	Total phenol (in mg/g, gallic acid equivalents)	Total flavonoid (in mg/g, quercetin equivalents)
PZME	160.813 ± 0.653	82.428 ± 1.02	89.235 ± 1.082

PZME *Pouzolzia zeylanica* (L.) Benn. Methanol, Values are the average of triplicate experiments and represented as mean ± SD

radical (visible deep purple color) to the yellow-coloured diphenylpicrylhydrazine. It has been found that cysteine, glutathione, ascorbic acid, tocopherol, polyhydroxy aromatic compounds (e.g. hydroquinone, pyrogallol, gallic acid), and aromatic amines (e.g. p-phenylenediamine, p-aminophenol), reduce and decolorise 1,1-diphenyl-2-picrylhydrazyl by their hydrogen donating ability [31]. This is concluded that the plant might have active principles that can be used as antioxidants.

Ferric reducing antioxidant power (FRAP)

Figure 2 shows the reducing power capabilities of the plant extract compared to ascorbic acid. The extract displayed moderate reducing power which was found to rise with increasing concentrations of the extract. In reducing power assays, the presence of antioxidants in the root can reduce the oxidized form of iron (Fe³⁺) to its reduced form (Fe²⁺) by donating an electron. Thus, it can be assumed that the presence of reductants (i.e. antioxidants) in *P. zeylanica* (L.) Benn. extract causes the reduction of the Fe³⁺/ferricyanide complex to the ferrous form which reveals the antioxidative nature of the

extract. Therefore, the Fe²⁺ complex can be monitored by measuring the formation of Per's Prussian blue at 700 nm. A higher absorbance indicates greater reducing power ability [32].

Cupric reducing antioxidant capacity (CUPRAC)

The reducing ability of a compound generally depends on the presence of reductants [33], which have been reported to exhibit antioxidative potential by breaking the free radical chain, donating a hydrogen atom [32].

According to changed concentration trend, we concluded that the reducing power of the extract was lower than that of ascorbic acid but it was significant (Fig. 3). The probable mechanism of Cupric reducing power of extracts, would be the resultant of having a good number of polyphenolics and flavonoids, as the reactive hydroxyl groups of polyphenolics, oligomeric flavonoids, is oxidized with the CUPRAC reagent to the corresponding quinines [20].

Nitric oxide scavenging assay

Nitric Oxide (NO) scavenging assay is based on the scavenging ability of the extracts as well as ascorbic acid, which is used as standard. The scavenging of the NO generated from sodium nitroprusside in vitro indicates the possibility of preventing the peroxynitrite formation in the cell in vivo [34]. Reducing the nitric oxide generation in the digestive tract was reported to be effective in preventing the reactions of nitrate with amines and amides to form carcinogenic nitrosamines and nitrosamides [35, 36]. The scavenging of NO was found to increase in dose dependent manner (Fig. 4). The IC₅₀ value of the crude extract was 76.737 µg/mL, while the IC₅₀ value for the

Table 3 % Mortality of Brine shrimp produced by *Pouzolzia zeylanica* (L.) Benn. and the standard Vincristine sulphate

Test sample	Concentration (µg/ml)	Log concentration	% Mortality	Corrected % mortality	LC ₅₀ (µg/ml)	LC ₉₀ (µg/ml)
PZME	12.5	1.09691	40	33.33	46.24	363.07
	25	1.39794	40	33.33		
	50	1.69897	50	44.44		
	100	2	60	55.55		
	200	2.30103	80	77.55		
	400	2.60206	100	100		
	800	2.90309	100	100		
VS	0.06	-1.22185	10	0	0.0699	6.33
	0.125	-0.90309	20	11.11		
	0.25	-0.60206	30	22.22		
	0.5	-0.30103	40	33.33		
	1	0	50	44.44		
	5	0.69897	90	88.88		
	10	1	100	100		

[PZME *Pouzolzia zeylanica* (L.) Benn. Methanol, VS vincristine sulfate]

reference ascorbic acid was 51.078 $\mu\text{g}/\text{mL}$. Based on these we speculate that nitric oxide scavenging activity of *Pouzolzia zeylanica* (L) Benn. may have great relevance in the prevention and control of disorders where NO is thought to play a key role.

Determination of total antioxidant capacity

The total antioxidant capacity of the methanol extract of *P. zeylanica* (L) Benn. is given in Table 2. Significant amount of total antioxidant activity was obtained from the methanol extract (160.813 ± 0.653 mg/g ascorbic acid equivalent) at 200 $\mu\text{g}/\text{mL}$ extract concentration. The phosphomolybdenum method was based on the reduction of Mo (VI) to Mo(V) by the antioxidant compound and the formation of a green phosphate/Mo(V) complex with a maximal absorption at 695 nm. The assay is successfully used to quantify vitamin E in seeds and, being simple and independent of other antioxidant measurements commonly employed, it was decided to extend its application to plant extracts [22]. Moreover, it is a quantitative one, since the antioxidant activity is expressed as the number of equivalents of ascorbic acid.

Determination of total phenol content

It has been conclusively shown close relationship between total phenolic content and antioxidative activity of the fruits and vegetables based on several reports. Phenolic compounds, as natural antioxidants exhibit therapeutic potential in multiple diseases including cardiovascular disease, aging and cancer [33]. It has been reported that phenolic compounds with *ortho*- and *para*-dihydroxylation or a hydroxyl and a methoxy group are more effective than simple phenolics [37]. Moreover, the antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides [33]. However, the methanol extract of the *P. zeylanica* (L) Benn. was found to contain little amount of phenolics 82.428 ± 1.02 mg/g Gallic acid equivalent (GAE) using Folin-Ciocalteu method. The result was presented in Table 2.

Determination of total flavonoids content

It has been reported that flavonoids play an important role in antioxidant system in plants. The antioxidative properties of flavonoids are due to several different mechanisms, such as scavenging of free radicals, chelation of metal ions, such as iron and copper, and inhibition of enzymes responsible for free radical generation [38]. However, total flavonoid content of *P. zeylanica* (L) Benn. extract is shown in Table 2. The result was

Table 4 Zone of Inhibition produced by Methanolic Extract of *Pouzolzia zeylanica* (L.) Benn. against some Gram positive and Gram negative bacteria

Test microorganism	Zone of inhibition in mm			
	Extract Conc. $\mu\text{l}/\text{disc}$ <i>Pouzolzia zeylanica</i> (L.) Benn.			Standard amoxicillin
	10	20	30	
<i>Bacillus subtilis</i>	7.5	7.5	8.0	28.5
<i>Bacillus cereus</i>	7.0	7.5	8.5	9.5
<i>S. aureus</i>	8.5	8.5	9.5	12.5
<i>P. mirrabilis</i>	7.5	8.0	9.0	9.5
<i>E. coli</i>	7.0	7.0	8.5	18.0
<i>Serratia spp.</i>	8.0	8.5	10.0	10.5
<i>S. typhi</i>	7.5	8.0	8.5	12.0
<i>Pseudomonas spp.</i>	7.0	7.0	8.0	20.5

exhibited as quercetin equivalent of flavonoids per gm of extracts of the sample. For the claimed extract, the total flavonoid content was found to be 89.235 ± 1.082 mg/g Quercetin equivalent. These results suggested that flavonoid contents might be responsible for the antioxidant activities of *P. zeylanica* (L) Benn.

Cytotoxic activity test

The cytotoxicity of the crude extracts to brine shrimp was determined on *Artemia salina* [26]. Table 3 shows the results of the brine shrimp lethality testing after 24 h of exposure to the samples and the positive control, vincristine sulphate. The LC50 obtained from the semi log plot were found 0.0699 and 46.24 $\mu\text{g}/\text{mL}$ for vincristine sulphate (positive control) and DMSO soluble fraction of methanolic crude extract, respectively whereas the LC90 for vincristine sulphate and the tested extract was 363.07 and 6.33 $\mu\text{g}/\text{mL}$. The results of this study indicate that there might be presence of potent bioactive principles in this crude extract which might be very useful as antitumor, pesticidal and other cytotoxic use.

Table 5 Effect of methanolic extract of *Pouzolzia zeylanica* (L.) Benn. in Xyline Induced Ear Edema Test

Group	Doses (mg/kg)	Ear Wt difference (mg)	% inhibition
Control	1 % Tween 80 in Water (10 ml/kg)	1.23 ± 0.015	-----
Diclofenac	100	9.5 ± 0.213	82.02
PZME	250	11.83 ± 0.143	76.34
PZME	500	$11.17 \pm 0.032^*$	78.86

Values of ear weight are mean \pm S.E.M, ($n = 5$), * $P < 0.05$, significantly different from control; done by independent sample t-test, (PZME = *Pouzolzia zeylanica* (L.) Benn. Methanol)

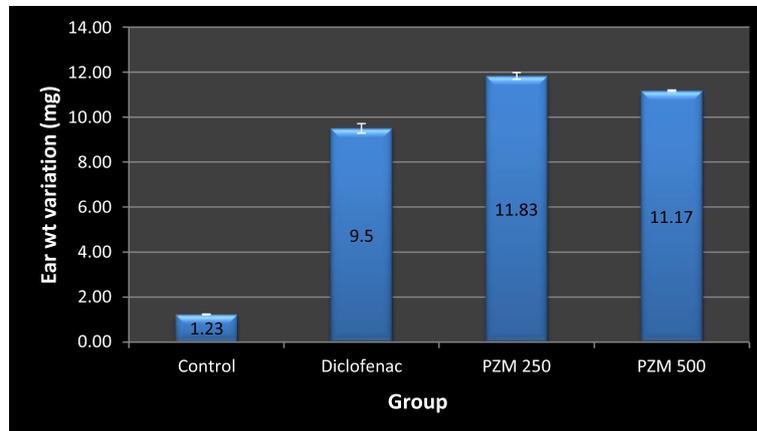


Fig. 5 Variation of ear weight of different groups in Xylene Induced Ear Edema test

Antimicrobial activity

Plants are important source of potentially useful sources for the development of new chemotherapeutic agents. The first step towards this goal is the in vitro antibacterial activity assay [39]. Table 4 reveals the antibacterial activity of the extract. Significant zone of inhibition was observed against all the studied pathogens by methanol extracts of *Pouzolzia zeylanica* (L) Benn. Which concludes that tested fraction might have active compounds that can be a source of potential antimicrobial agent though further study is require.

Acute toxicity study

After the acute toxicity studies no mortality was observed up to dose as high as 4000 mg/kg for the extract. So no LC₅₀ could be obtained and extracts were considered to be safer with broad therapeutic range. Therefore, two comparative doses (250 and 500 mg/kg) for the extract was taken for the in-vivo models.

The Xylene induced ear edema method

The xylene ear edema model is a useful method that can be utilized to evaluate the anti-inflammatory steroids and is less sensitive to non-steroidal anti-inflammatory agents [40]. Histopathologically, severe vasodilation, edematous changes of skin and infiltration of inflammatory cells are detected as signs of acute inflammation after topical application of xylene [41].

The increase in weight and inflammation exerted by xylene application is due to neutrophil accumulation which plays a critical role in cutaneous inflammatory diseases such as dermatitis, and is related to the pathological mechanism of the disease [42]. In the present study the extracts reduced the ear edema in dose dependent manner (Table 5). The extract at higher dose (500 mg/kg) showed marked ($p < 0.05$) inhibition whereas at low dose extract it was promising (76.34 %). Figure 5 showed the variation of ear weight of different groups in Xylene Induced Ear Edema test in a graphical manner.

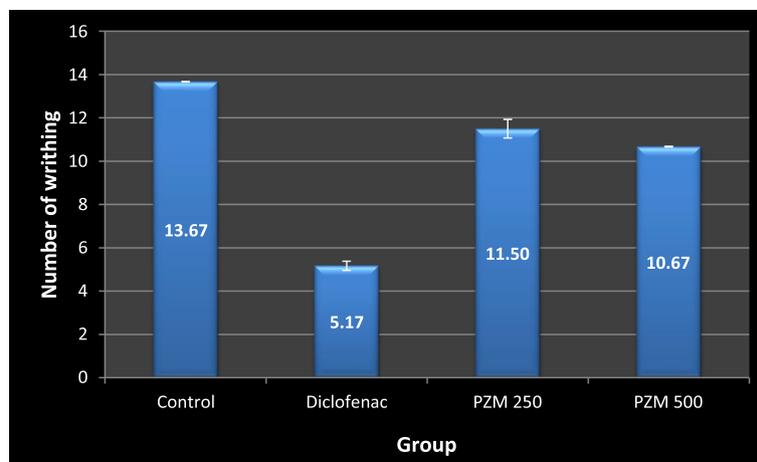


Fig. 6 Number of writhing observed in different groups in Acetic Acid Writhing test

Table 6 Effect of methanolic extract of *Pouzolzia zeylanica* (L.) Benn. In Acetic Acid Induced Writhing test

Group	Doses (mg/kg)	Number of writhing	% Inhibition
Control	1 % Tween 80 in Water (10 ml/kg)	13.67 + 0.012	-----
Diclofenac	100	5.17 + 0.213*	62.20
PZME	250	11.5 + 0.432	15.85
PZME	500	10.67 + .013	21.95

Number of writhing values are mean \pm S.E.M. ($n = 5$), * $P < 0.05$ significantly different from control; done by independent t -test, PZME = *Pouzolzia zeylanica* (L.) Benn. Methanol

Acetic acid induced writhing method

Acetic acid induced writhing model represents pain sensation by triggering localized inflammatory response. Such pain stimulus leads to the release of free arachidonic acid from tissue phospholipids [43]. The acetic acid induced writhing response is a sensitive procedure to evaluate peripherally acting analgesics. The response is thought to be mediated by peritoneal mast cells [44] acid sensing ion channels [45] and the prostaglandin.

Figure 6 showed the number of writhing observed in different groups in Acetic Acid Writhing test while Table 6 represents the effect of the extract of in Acetic Acid Induced Writhing test. The methanol extract inhibited writhes in a dose dependent manner (15.85 and 21.95 % respectively) while the standard Diclofenac showed a significant inhibition (62.20 %).

Conclusions

The current study clearly indicates that the methanolic extract contains significant amount of antioxidants. This might be rationale behind the using of this plant extract as folk medicine. Since the chemical composition and structures of active extract components are important factors governing the efficacy of natural antioxidants, the extracts of *P. zeylanica* (L.) Benn. need their characterization and quantification of the bioactive components. Therefore, further research is necessary for elucidating the active principles e.g. alkaloids and phenolics compound and also in- vivo studies are needed for understanding their mechanism of action as an antioxidant. Furthermore in vitro and in vivo studies are to be conducted to elucidate the potentiality of the extract as medicine as it shows significant activity against the studied organisms. Whereas more in vivo studies are necessary to examine the underlying mechanism of anti-inflammatory and analgesic effects.

Competing interests

Md. Solayman Hossain, M. Sohanur Rahman, A.H.M. Rahmatullah Imon, Sinthia Zaman, A.S.M. Badrul Alam Siddiky, Milon Mondal, Abeer Sarwar, Tamanna Binte Huq, Bikash Chandra Adhikary, Taslima Begum, Asma Tabassum, Sumaiya Alam, Mst. Marium Begum declare that they have no competing interest.

Authors' contributions

MSH, SZ, ASMBAS, MM performed all of the experiments in the laboratory. Data collection, analysis, graphical representation and interpretation were done by AHMRI and MSR. Article was written by AS and TBH. Critical revision of the article was done by BCA, TB, AS and SA. Critical statistical analysis was done by AHMRI. MSR made the necessary corrections in the write up. Conception, experiment design, overall monitoring and final approval of the article was done by MMB. It was a self funded research initiatives. All authors take full responsibility for the content of the paper. All Authors read and approved the final manuscripts.

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