Phytochemical and Biological Activity Studies on *Pouzolzia Zeylanica* (Linn.) Benn.

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Abstract: Two steroids, namely, Stigmasterol (**1**) and β-Sitosterol (**2**) and one triterpenoid, namely, Friedelin (**3**) were isolated from the n-hexane extract of the aerial parts of *Pouzolzia zeylanica* and identified by an extensive spectroscopic evidence. These compounds (**1-3**) were isolated for the first time from this species. The cytotoxic potential of the different solvent extracts (n-hexane, ethyl acetate and n-butanol) and pure compounds (**1-3**) were examined by using brine shrimp lethality bioassay. However, ethyl acetate extract and n-butanol extract as well as isolated compound **3** exhibited quite potent activity in brine shrimp lethality bioassay with LC_{50} 3.32, 3.44 and 2.80 µg/ml, respectively. These results suggested that they might contain antitumor or pesticidal activity. Further, no antioxidant activity was shown by the test samples. On the other hand, the n-butanol extract and compound **3** demonstrated moderate antibacterial activity with a zone of inhibition of 7-10 mm against the tested pathogenic microorganisms.

Keywords: Bioactive phytoconstituents, cytotoxicity, free radical scavengers, antibacterial screening, *Pouzolzia zeylanica* (Linn.) Benn.

Introduction

General bioassays that exhibit capable of detecting broad spectrum of bioactivity present in crude extracts are brine shrimp lethality bioassay (BSLT) and free radical scavenging activity test (FRST). Both techniques are easily mastered, low cost, and needs small amount of test material. BSLT is predictive cytotoxicity and pesticidal activity [9]. This test has been introduced in 1982 [13] and employed for bioassay-guide fractionation of active cytotoxic and antitumor agents such as trilobacin from the bark of *Asimina triloba* [20] and cis-annonacin from *Annona muricata* [15]. FRST is also predictive antioxidant activity and introduced in 1958 [5] and employed for the detection of active free radical scavengers like vitamin C, vitamin E, flavonoids, carotenes, phenolic acids, phytate and phytoestrogens have been recognized as having the potential to reduce disease risk [4]. There are a number of clinical studies suggesting that the antioxidants in fruits, vegetables, tea and red wine are the main factors for the observed efficacy of these foods in reducing the incidence of chronic diseases including heart disease and some cancers [14].

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Pouzolzia zeylanica (Common name-Kulluruki; Synonym-*Plumbago zeylanica*; Family – Urticaceae) is a herb: native to tropical and subtropical regions worldwide [21]. In Bangladesh, *P. zeylanica* widely distributed commonly on roadsides and waste place throughout the country. This plant is administered as a remedy for diarrhea, leucorrhoea & gonorrhea, indestion, infantile malnutrition, urination difficulties and injuries from falls. Moreover, it is especially useful in conditions such as acute mastitis & pyogenic infections [22, 23]. Previous phytochemical investigations resulted in the isolation of flavonoids, flavones, tannin, carotene, carotenoids, ascorbic, tartaric, malic and pectic acids [25]. Flavonoids (Quercetin, Vitexin, Isovitexin & Phylanthin), methyl stearate and daucosterol was isolated from this herb [24].

Therefore, the present study was undertaken to isolate and elucidate the structure of some pure compounds from the aerial parts of *P. zeylanica* by using extensive spectroscopic techniques and also to study the antioxidant, antibacterial and cytotoxic activities of the crude extracts as well as pure compounds.

Experimental

General experimental procedures

The NMR spectra were recorded using a Bruker DBX-400 MHz instrument (Bruker BioSpin GmbH, Silberstreifen 4, 76287 Rheinstetten, Germany), with chemical shift (δ) data reported in ppm relative to the solvent used. The spectras were taken by using $CDCl_3$ and CD_3OD with tetra methyl silane (TMS) as standard reference. A Shimadzu FT-IR, 8400S, IR-prestige spectrometer (Shimadzu, Japan) was used for recording infrared spectrum. Major bands (ν_{max}) were recorded in wave number (cm^{-1}) as potassium bromide (KBr) pellets. The UV absorbance was performed with a PerkinElmer Shelton, CT 06484 USA, Lambda 25 UV/VIS spectrometer. Vacuum rotary evaporator (BUCHI, Rotavapor R-210 Switzerland) was used for evaporating solvents. Melting points (m.p.) were determined by using an electro-thermal melting point apparatus SMP3 (Stuart Scientific, UK) and are uncorrected. All solvents were of analytical grade and obtained from commercial sources (Sigma-Aldrich, St. Louis, MO, USA).

Plant material

Fresh aerial parts of *P. zeylanica* were collected from the roadsides of Gazipur, Bangladesh in October, 2009 and identified by the taxonomist of Bangladesh National Herbarium, Dhaka, where a voucher specimen (No. 34106) has been deposited.

Extraction and isolation of compounds

Freshly collected aerial stems of *P. zeylanica* were dried in an oven at 38˚C and crushed into tiny pieces. The crushed powder (290.0 g) was extracted with ethanol for 5 days. The extract was concentrated to gummy mass (11.8 g) using Buchi Rotary Evaporator. The ethanol extract (11.0 g) was then partitioned by separatory funnel by using n-hexane, then by ethyl acetate and finally by n-butanol. Then these extract were concentrated by using rotary vacuum evaporator to provide n-hexane (5.0 g), ethyl acetate (1.2 g), n-butanol (2.0 g) and water (2.1 g) extracts.

The concentrated crude n-hexane extract (5.0 g) was subjected to vacuum liquid chromatography (VLC) by using n-hexane and then eluted with mixtures of n-hexane and ethyl acetate increasing the polarity of the solvents and finally with the mixtures of ethyl acetate and methanol to afford a total of 18 fractions (each 200 ml). VLC fractions 3-4 (389·4 mg) shows similar spots in TLC analysis; they were got mixed and dried by rotary evaporator and then fractionated by column chromatography. As they contained chlorophyll, so they were done column using Sephadex LH-20 as stationary phase and $CHCl_3$-MeOH as mobile phase. Among the fractions, the TLC analyses of test tubes (5-10) were similar. So they got mixed and had done further column which was packed with silica gel-60 and sample was applied in the top of the column bed. Elution with n-hexane and the polarity of the mobile phase was increased gradually by adding more polar solvent such as DCM and MeOH. The eluted sample was collected in 20 test tubes. The test tubes 10-12 afforded white niddle shaped crystal **3** (15.8 mg, soluble in $CHCl_3$). It's m.p. 262-265˚C (Uncorr.). VLC fractions 8-9 (239·5 mg) shows similar spots in TLC analysis; they were got mixed and dried by rotary evaporator and then fractionated by column chromatography. The column was packed with silica gel-60 and sample was applied in the top of the column bed. Elution with n-hexane and the polarity of the mobile phase was increased gradually by adding more polar solvent such as DCM and MeOH. The eluted sample was collected in 40 test tubes. According to TLC pattern 9 different fraction were obtained. The eluted samples (20-25) were mixed and dried. Then it is dissolved in $CHCl_3$ and carried out a pipette column by silica gel-60 using 100% $CHCl_3$ as a mobile phase. The eluted sample was collected in 10 test tubes. According to TLC pattern the test tubes (4-5) and (8-10) afforded colorless needle **1** (15.5 mg, soluble in $CHCl_3$) and **2** white crystalline needle (12.5 mg, soluble in $CHCl_3$), respectively.

Bioassays

Cytotoxicity bioassays

The cytotoxic activity was performed as described previously [13] by brine shrimp lethality bioassay method. The test samples for crude extracts as well as pure isolated compounds were dissolved in DMSO and serial dilution were made as 100, 50, 25, 12.5, 6.25, 3.125, 1.563, 0.781 and 0.3095 µg/ml. On the other hand, vincristine sulphate (positive control) was dissolved in DMSO and serial dilution were made as 20, 10, 5, 2.5, 1.25, 0.625, 0.3125, 0.15625, 0.0781 µg/ml. Then each of these test solutions was added to test tubes containing 12 shrimps in simulated brine water (5 ml) and incubated at room temperature for 24 h. After 24 h, the median lethal concentration (LC_{50}) of the test samples was determined by a plot of percentage the shrimps against the logarithm of the sample concentrations (Finney method). Vincristine sulphate (LC_{50}= 0.57µg/ml) was used as positive control in this assay to compare the cytotoxicity of the test samples. Each treatment was replicated thrice.

Antibacterial screening

The test samples were dissolved separately in specific volume of chloroform or methanol depending their solubility. The antibacterial screening was then carried out by the disc

diffusion method [2, 3]. The diluted samples were applied on to sterile blank discs (Oxoid, UK) at a concentration of 100 μg/disc for this test where Streptomycin (10 μg/disc, Oxoid, UK) used as a standard.

Free radical scavenging activity

The free radical scavenging activity was assayed spectrophotometrically by DPPH method [5]. The DPPH (2,2-Diphenyl-1-picrylhydrazyl) radical has a deep violet color due to its unpaired electron, and radical scavenging activity can be followed spectrophotometrically by a loss of absorbance at 525 nm. Sample stock solutions (1 mg/ml) were diluted to final concentrations of 100, 50, 10, 5 and 1 μg/ml in 70% ethanol or DMSO. DPPH ethanol solution (0.2 mM, 0.5 ml) was added to 1 ml of sample solutions of different concentrations, shaken well by vortex, and allowed to react at room temperature. The absorbance values were measured after 10 min at 525 nm by UV/Vis spectrophotometer. The free radical scavenging activity of samples was calculated according to the formula:

DPPH radical scavenging activity (%)= $[1-(Abs_{sample}-Abs_{blank})/Abs_{control}] \times 100$

where Abs_{sample} is the absorbance of the experimental sample, Abs_{blank} is the absorbance of the blank, $Abs_{control}$ is the absorbance of the control.

As a blank, 70% EtOH or DMSO solvent (0.5 ml) and sample solution (1.0 ml) were used. DPPH solution (0.5 ml, 0.2 mM) and 70% EtOH or DMSO solvent (1.0 ml) was used as a negative control. The ascorbic acid (vitamin C) (IC_{50}=4.01μg/ml) was used as a positive control in this assay to compare the free radical scavenging activity of the test samples. Each treatment was replicated thrice.

Results and Discussion

Three pure compounds (**1-3**) (**Fig. 1**) were isolated from the aerial parts of *P. zeylanica* with the help of different chromatographic techniques and elucidated their structure by various spectroscopic analyses as well as by comparison of their spectral data with previously available in the literature.

Compound (**1**): Colorless needles; m.p. 144-146˚C (Uncorr.) (Lit. 145-146˚C) [17]; It gave positive steroid test. IR ν_{max} (KBr) cm^{-1}: 3290 (m, H-bonded O-H str.), 2910 (s, C-H str. sp^3), 1673 (m-w, C=C str. sp^2), 1565 (m, C-H bend. CH_2 gr.), 1445 (m, C-H bend. CH_3 gr.), 1275 (s, C-O str. due to alcohol) and 870 (alicyclic gr.); ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.35 (1H, m, H-6, olefinic proton), 5.18 (1H, dd, J=15.2, 8.8 Hz, H-22, olefinic proton, trans-configuration), 5.04 (1H, dd, J=15.2, 8.4 Hz, H-23, olefinic proton, trans-configuration), 3.51 (1H, m, H-3, oxymethine proton), 1.0 (3H, s, CH_3-10), 0.69 (3H, d, J=7.2 Hz, CH_3-13), 1.02 (3H, d, J=8.0 Hz, CH_3-20), 0.83 (3H, d, J=8.0 Hz, CH_3-25), 0.79 (3H, d, J=15.6 Hz, CH_3-25), 0.92 (3H, d, J=6.4 Hz, CH_3-28). ^{13}C NMR: δ_C: 37.3 (C-1), 31.7 (C-2), 71.8 (C-3, oxymethine carbon), 42.3 (C-4), 140.8 (C-5, olefinic carbon), 121.7 (C-6, olefinic carbon), 31.9 (C-7), 31.9 (C-8), 51.2 (C-9), 36.5 (C-10), 21.1 (C-11), 39.7 (C-12), 42.2 (C-13), 56.8 (C-14), 24.3 (C-15), 28.2 (C-16), 56.1 (C-17), 11.8 (C-18),

19.8 (C-19), 40.4 (C-20), 21.2 (C-21), 138.3 (C-22, olefinic carbon), 129.3 (C-23, olefinic carbon), 50.2 (C-24), 31.7 (C-25), 21.0 (C-26), 19.0 (C-27), 25.4 (C-28), 12.0 (C-29). These spectral evidences were identical to the reported data of Stigmasterol [1, 17]. Therefore, the compound (**1**) was identified as Stigmasterol.

Compound (**2**): White crystalline needle; m.p. 137-138˚C (Uncoor.) (Lit. 138-139˚C) [26]; It gave positive steroid test. IR ν_{max} (KBr) cm^{-1} : 3390 (m, H-bonded O-H str.), 2918 (s, C-H str. sp^3), 1678 (m-w, C=C str. sp^2), 1558 (m, C-H bend. CH_2 gr.), 1450 (m, C-H bend. CH_3 gr.), 1275 (s, C-O str. due to alcohol) and 870 (olefinic >=< gr.). ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.34 (1H, t, 5.2 Hz, H-6, olefinic proton), 3.51 (1H, m, H-3, oxymethine proton), 0.99 (3H, s, CH_3-10), 0.67 (3H, s, CH_3-13), 0.82 (3H, d, J=1.2 Hz, CH_3-20), 0.85 (3H, d, J=7.0 Hz, CH_3-25), 0.79 (3H, d, J=7.0 Hz, CH_3-25), 0.83 (3H, d, J=6.4 Hz, CH_3-28). ^{13}C NMR: δ_C: 37.3 (C-1), 28.3 (C-2), 71.8 (C-3, oxymethine carbon), 42.4 (C-4), 140.8 (C-5, olefinic carbon), 121.7 (C-6, olefinic carbon), 31.7 (C-7), 31.9 (C-8), 50.2 (C-9), 36.5 (C-10), 21.1 (C-11), 39.8 (C-12), 42.4 (C-13), 56.8 (C-14), 24.3 (C-15), 26.2 (C-16), 56.1 (C-17), 11.9 (C-18), 19.4 (C-19), 40.5 (C-20), 19.1 (C-21), 34.0 (C-22), 26.2 (C-23), 45.9 (C-24), 31.9 (C-25), 19.8 (C-26), 21.1 (C-27), 24.3 (C-28), 12.0 (C-29). These spectral evidences were identical to the reported data of β-sitosterol [1, 26]. Therefore, the compound (**2**) was identified as β-sitosterol.

Compound (**3**): White crystalline needle; m.p. 262-265˚C (Uncorr.) (Lit. 264-265˚C) [19]; It gave positive terpenoid test. IR ν_{max} (KBr) cm^{-1}: 2915 (s, C-H str. sp^3), 1715 (s, C=O, aliphatic ketone), 1465 (m, C-H bend. CH_2 gr.), 1425 (m, C-H bend. CH_3 gr.), 1250 (m, C-C, alkane). ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.28 (1H, m, H-4), 2.35 & 2.39 (2H, m, H-2a & 2b), 0.87 (3H, d, 6.4 Hz, CH_3-4), 0.71 (3H, s, CH_3-5), 0.86 (3H, s, CH_3-9), 0.99 (3H, s, CH_3-13), 1.03 (3H, s, CH_3-14), 1.16 (3H, s, CH_3-17), 0.94 (3H, s, CH_3-20) and 0.98 (3H, s, CH_3-20). ^{13}C NMR: δ_C: 22.3 (C-1), 41.5 (C-2), 213.2 (C-3, saturated C=O), 58.2 (C-4), 42.1 (C-5), 41.3 (C-6), 18.2 (C-7), 53.1 (C-8), 37.4 (C-9), 59.5 (C-10), 35.6 (C-11), 30.5 (C-12), 39.7 (C-13), 38.3 (C-14), 32.4 (C-15), 36.0 (C-16), 30.0 (C-17), 42.8 (C-18), 35.3 (C-19), 28.1 (C-20), 32.7 (C-21), 39.2 (C-22), 6.8 (C-23), 14.6 (C-24), 17.9 (C-25), 20.2 (C-26), 18.6 (C-27), 32.1 (C-28), 35.0 (C-29), 31.8 (C-30). These spectral evidences were identical to the reported data of Friedelin [19]. Therefore, the compound (**3**) was identified as Friedelin.

The cytotoxic activity of the solvent extracts as well as pure isolated compounds (**1-3**) were determined by using brine shrimp lethality bioassay. The LC_{50} for crude vincristine sulphate (positive control), n-hexane, ethyl acetate and n-butanol extract and three pure compounds (**1-3**) obtained from Finney method were found to be 0.57, 6.95, 3.32, 3.44, 32.6, 10.32 and 2.80 µg/ml, respectively (**Table 1**). In comparison with the positive control (vincristine sulphate), it can be mentioned that all the test samples were lethal to brine shrimp nauplii. However, ethyl acetate extract and n-butanol as well as one pure compound **3** demonstrated quite potent activity in brine shrimp lethality bioassay. These positive results suggested that they may contain antitumor or pesticidal activity.

Further, the antibacterial activity of all solvent extracts and three pure compounds (**1-3**) were subjected to screening at 100µg/disc by using disc diffusion method. The moderate antibacterial activity exhibited by n-butanol extract and compound **3** against almost all tested pathogenic microorganisms having the zone of inhibition of 9±1 mm each (**Table 2**).

The free radical scavenging activity of the solvent extracts as well as three pure compounds (**1-3**) were assayed by using DPPH method. It can be mentioned that all the solvent extracts as well as three isolated compounds (**1-3**) may not be used as scavengers due to the absence of phenolic character (**Table 3**).

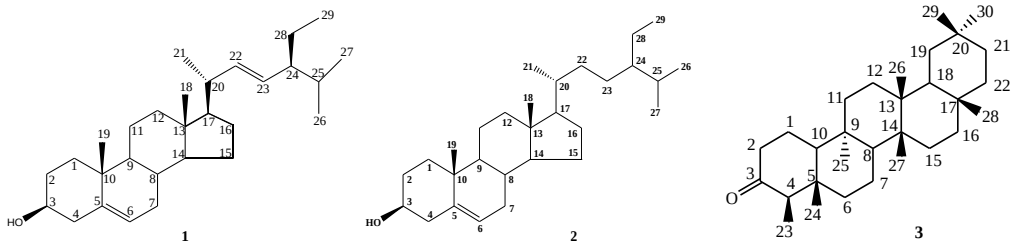

Fig. 1: *The structure of compounds **1-3**.*

Table 1: *LC_{50} values of solvent extracts and pure compounds from P. zeylanica.*

Samples	LC_{50} (µg/ml)	Samples	LC_{50} (µg/ml)
VS	0.57	B-1	32.6
HPz	6.95	B-2	10.32
EPz	3.32	B-3	2.80
BPz	3.44	-	-

VS: Vincristine sulphate (Std.); HPz: n-hexane extract; EPz: Ethyl acetate extract; BPz: n-butanol extract; B-1, B-2 and B-3 for pure compounds **1**, **2** and **3**, respectively.

Table 2: *Antibacterial screening of solvent extracts (100 µg/disc) and pure compounds (100 µg/disc) from P. zeylanica.*

Sample	Diameter of zone of inhibition (mm)						
	B.cereus	*B.megaty-erium*	*B. subtilis*	*S.aureus*	*E.coli*	*S.sonnei*	*S.dysenteriae*
HPz	NA	NA	7	NA	7	7	7
EPz	NA	NA	7	NA	NA	7	7
BPz	7	7	8	NA	7	8	9
B-1	NA	8	9	NA	NA	7	9
B-2	NA	NA	9	7	NA	NA	10
B-4	8	NA	9	10	8	9	NA
Streptomycin	22	23	17	17	17	15	27

HPz: n-hexane extract; EPz: Ethyl acetate extract; BPz: n-butanol extract; B-1, B-2 and B-3 for pure compounds **1**, **2** and **3,** respectively; NA: no activity observed; Streptomycin (Std.) (10.0 µg/disc).

Table 3: *IC_{50} values of solvent extracts and pure compounds from P. zeylanica.*

Samples	IC_{50} (µg/ml)	Samples	IC_{50} (µg/ml)
VC	4.01	B-1	-
HPz	-	B-2	-
EPz	-	B-4	-
BPz	-	-	-

VC: Vitamin C (Ascorbic acid, Std.); HPz: n-hexane extract; EPz: Ethyl acetate extract; BPz: n-butanol extract; B-1, B-2 and B-3 for pure compounds **1**, **2** and **3,** respectively.

Conclusions

It is concluded that three compounds (**1-3**) were isolated for the first time from the n-hexane extract of the aerial parts of *P. zeylanica* and characterized by an extensive spectroscopic evidence. The antioxidant, antibacterial and cytotoxicity screening of the different solvent extracts as well as three pure isolated compounds (**1-3**) were found to be consistent with the folk uses of *P. zeylanica* by local people.

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