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EXPLORATION OF CHEMICO-BIOLOGICAL POTENTIALS OF *POLYGONUM PLEBEIUM* R. BR.

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Abstract

In this study, *Polygonum plebeium* R. Br. underwent extensive chemical and biological investigations. Repeated chromatographic separation and spectroscopic analysis afforded caffeic acid (**1**) and β-sitosterol (**2**) from *P. plebeium* for the first time. Methanolic extract of *P. plebeium* (MEPP) and its different Kupchan fractions were evaluated for *in vitro* antioxidant, antibacterial and *in vivo* antidiarrheal, analgesic activities in mice model. The n-hexane fraction showed DPPH scavenging activity with IC_{50} value 24.2 µg/ml. Both n-hexane and ethyl acetate fractions exhibited activity against *Pseudomonas aeruginosa* with a zone of inhibition of 20.0 and 14.0 mm, respectively. Oral administration of the MEPP (200 mg/kg bw) effectively ($p<0.01$) reduced castor oil-induced diarrhea by 58.70% at 4 hrs of observation period. In the acetic acid induced writhing study, MEPP at 400 mg/kg bw demonstrated a significant ($p < 0.001$) inhibition of writhing responses by 50.0% where the standard diclofenac-Na (10 mg/kg) showed 77.91% inhibition in mice. During tail immersion method, the MEPP demonstrated a substantial ($p < 0.001$) reduction in pain sensation in mice. The promising bioactivities observed in this study underscore the need for further investigations to isolate and identify the compounds along with their mode of action responsible for these effects.

Introduction

Polygonum plebeium R. Br., commonly known as knotweed, locally referred to as "Chemti Sakh," "Dubia Sakh," and "Anjaban," is a low-growing, prostrate herb with diffuse branching belongs to Polygonaceae. *Polygonum plebeium* is found in various parts of the world, including Pakistan, India, Bangladesh, and Sri Lanka. This plant is rich in a variety of phytochemicals, including essential oils, alkaloids, tannins, and flavonoids (Ahsan *et al*. 2021). Traditionally, the plant has been employed for the treatment of various conditions like liver ailments, inflammation, dysentery, eczema, and ringworm infections (Hasan *et al*. 2015). The powdered herb of the plant has been employed in the treatment of pneumonia, while the rootstock is used to address bowel complaints (Hasan *et al.* 2015). It contains vitamin C and showed anti-oxidant activity. Additionally, the plant has been found effective in treating dysentery when combined with the bark of *Butea superba* and the adventitious roots of *Ficus benghalensis* (Rehman *et al*. 2018). In some countries, *P. plebeium* is known as a blood purifier and has been extensively used to alleviate liver disorders such as jaundice and hepatitis (Rehman *et al*. 2018).

Insufficient research has been conducted to provide a comprehensive account of the specific phytochemical components found in *P. plebeium*. This suggests that there is a lack of detailed scientific investigation into the precise chemical compounds present in this plant. This study was designed primarily for the phytochemical investigation of the methanolic extract of the selected plant and its organic soluble fractions. Subsequently, a comprehensive assessment of the plant extracts was also conducted to ascertain their antioxidant, antimicrobial, antidiarrheal, analgesic, anti-depressant, and antidiabetic properties.

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Materials and Methods

All reagents and chemicals such as DPPH (SRL, India), butylated hydroxytoluene (BHT) (Sigma-Aldrich), Tween-80 (Sigma-Aldrich), castor oil (local source) and acetic acid (Daejung, South Korea) were of analytical grade and obtained from reliable sources in Bangladesh. Loperamide, morphine and diclofenac-Na were the gift samples from Square Pharmaceuticals Ltd.

The whole plants of *P. plebeium* were collected from Gazipur, Bangladesh, and identified at the Bangladesh National Herbarium with accession number DSCB-65129. Stems and roots were separated from the aerial parts of the plants, cleaned and dried for two weeks in the shade followed by several days in the sun. The plant parts were then ground into approximately 1000 grams of coarse powder using a high capacity grinding machine.

The powdered materials (800 gm) were placed in a clean amber-colored bottle and soaked in methanol (2.5 L) for 14 days. Then the crude extract was filtered using Whatman filter paper. Subsequently, the concentrated crude extract was obtained using a Buchi Rotavapour, with careful control of temperature (below 40°C). About 5 gm of crude methanol extract was subjected for modified Kupchan partitioning (VanWagenen *et al.* 1993) to yield *n*-hexane (1.5 g), dichloromethane soluble (2.0 g), ethyl acetate soluble (1.0 g) and aqueous soluble (0.5 g) materials.

The n-hexane soluble fraction was subjected to size exclusion chromatography over Sephadex LH-20 (Sigma-Aldrich, USA) using mixture of *n*-hexane, dichloromethane and methanol (2:5:1) as the eluting solvent. Preparative thin layer chromatography over silica gel of Sephadex column fractions 22 and 25 yielded compounds **1** and **2**, respectively.

For pharmacological study, male and female Swiss Albino mice were procured from International Center for Diarrheal Disease Research, Bangladesh (icddr,b). These mice were housed in polypropylene cages within a controlled environment (Humidity: 60-70%, temperature: 24 ± 2ºC) at the State University of Bangladesh. They were provided with rodent feed specially formulated by icddr,b and had access to water *ad libitum*. Before conducting any animal experiments, the ethical clearance (Fa.Ph.E/002-A/22) from the Ethical Review Committee of the Faculty of Pharmacy, University of Dhaka, Bangladesh.

Antioxidant activity was determined by total phenolic content analysis (Harbertson and Spayd 2006) and DPPH radical quenching assay (Brand-Williams *et al.* 1995). During DPPH assay, the plant extract at various concentrations was mixed with 3.0 ml of a methanol solution of DPPH. The assessment of antioxidant activity was carried out by observing the change in color of the purple DPPH methanol solution caused by the plant extract. This color change was quantified using a UV spectrophotometer at 517 nm.

$$\% \text{ Inhibition of DPPH radical} = \left(1 - \frac{\text{Absorbance of sample}}{\text{Absorbance of the control reaction}}\right) \text{x } 100$$

For anti-diarrheal activity test (Shoba and Thomas 2001), four groups were formed, each consisting of three albino mice, which were subjected to a 24 hrs fasting period. Group 1 served as the control and received 1% Tween-80 in distilled water. Group 2, the standard group was administered loperamide (2 mg/kg bw) intraperitoneally. Groups 3 and 4 were given 200- and 400 mg/kg bw of MEPP, respectively. One hour after administering the test substances, all mice were given 0.5 ml of castor oil and individually placed in a cage containing a surface covered with transparent paper for observation. The plant extract was found to have a percentage reduction in diarrhea based on the number of diarrheal feces emitted by the mice, which was monitored for up

to 4 hrs of experiment. The percentage reduction in diarrhea was determined using the following formula:

$$\% \text{ Reduction of diarrhea} = \frac{D_{Control} - D_{Test}}{D_{Control}} \times 100\%$$

D = number of diarrheas in respective group.

During tail immersion test (Hasan *et al*. 2023), Group 1 was provided with 1% Tween-80 dissolved in distilled water while Group 2 received morphine solution (2 mg/kg bw) subcutaneously. Groups 3 to 4 were administered two doses (200 and 400 mg/kg bw) of the plant extract, respectively. At the outset (zero hour), the experiment involved immersing approximately 1-2 cm of the mouse's tail into warm water kept at a constant temperature of 55°C. A latency period of 20 s was established as the point at which complete analgesia occurred, and the measurements were promptly discontinued to prevent any harm to the mice. The response was assessed both before the administration of any drugs and at various time intervals following drug administration, specifically at the start and at 30-, 60- and 90-min post-administration.

Acetic acid-induced writhing test (Hasan *et al*. 2023) was carried out to evaluate the peripheral analgesic activity of *P. plebeium*. The control group received only the vehicle, consisting of 1% Tween 80 in distilled water, while the second group was treated with the standard drug diclofenac-Na at a dose of 10 mg/kg administered orally. Groups 3 and 4 orally received the plant extract at doses of 200 and 400 mg/kg bw, respectively, 30 min before intraperitoneal injection of a 0.6% acetic acid solution (10 ml/kg). Subsequently, each mouse was monitored for a 30 min period to monitor the number of writhing responses, serving as an indicator of the anti-nociceptive activity of the treatments.

Results from various assays have been presented as mean ± SEM. Data analysis was carried out using one-way ANOVA followed by Dunnett's t-test using IBM SPSS 27. Statistical significance was determined at a threshold of $p \leq 0.05$.

Caffeic acid (**1**): Colorless amorphous powder soluble in chloroform; ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.26 (1H, d, J = 16.0 Hz, H-8), 6.83 (1H, d, J = 8.0 Hz, H-5), 6.99 (1H, dd, J = 2.0, 8.0 Hz, H-6), 7.06 (1H, d, J = 2.0 Hz, H-2), 7.52 (1H, d, J = 16.0 Hz, H-7).

β-sitosterol (**2**): White crystalline mass soluble in chloroform; ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.33 (1H d, J = 4.8 Hz, H-6), 3.52 (1H, m, H-3), 1.00 (3H, s, H-19), 0.91 (3H, d, J = 7.6 Hz, H-21), 0.84 (3H, t, J = 7.6 Hz, H-29), 0.82 (3H, d, J = 6.5 Hz, H-26), 0.80 (3H, d, J = 6.5 Hz, H-27), 0.67 (3H, s, H-18).

Results and Discussion

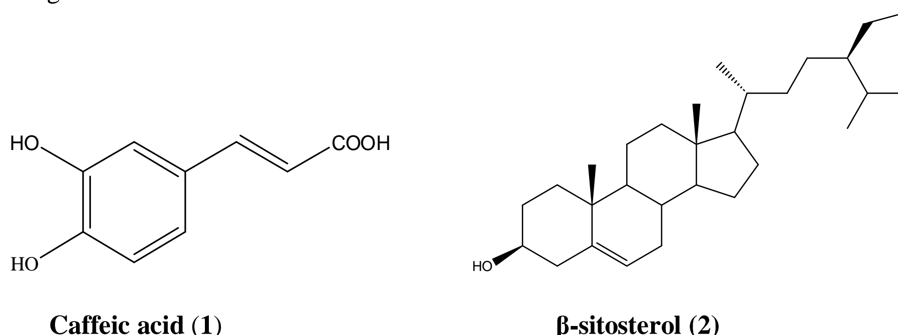
In this experiment, a total of two compounds have been successfully isolated from the *n*-hexane soluble fraction of *P. plebeium* which were characterized as caffeic acid (1) and *β*-Sitosterol (2) (Fig. 1). The structures of these compounds were determined through an extensive analysis of ^{1}H NMR spectroscopy including a comparative study with published data (Islam *et al*. 2022, Yu *et al*. 2024).

The ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of compound 1 displayed a doublet (J = 16.0 Hz) centered at δ 7.50 and 6.41 that could be ascribed to the *trans* coupled protons H-7 and H-8, respectively. The relatively low field resonance of H-7 could easily be explained by its beta (*β*) position to the carbonyl group, in the form of a carboxylic acid. In the aromatic region, the signals at δ 7.06 (1H, d, J = 2.0 Hz, H-2), 6.99 (1H, dd, J = 8.0, 2.0 Hz, H-6) and 6.83 (1H, d, J = 8.0 Hz, H-5) are characteristic of 1,3,4-trisubstituted benzene ring. Considering all the spectral data and

comparing with published values (Yu *et al*. 2024) the structure of compound 1 was identified as caffeic acid.

The ^{1}H NMR spectrum of compound 2 displayed a multiplet of one proton intensity at δ 3.52, the position and multiplicity of which was suggestive of H-3 of a steroid nucleus. The presence of a doublet at δ 5.33 indicates the presence of olefinic H-6 of the steroidal skeleton. The spectrum also displayed two singlets at δ 0.67 (H-18) and 1.00 (H-19), three doublets centered at δ 0.91 (H-21), 0.82 (H-26) and 0.80 (H-27) and one triplet of three proton intensity at δ 0.84 that could be assigned to the primary methyl group (H-29) at C-28. Based on the spectral data, compound 2 was characterized as β-sitosterol (Morales *et al*. 2003) (Fig. 1) that was previously isolated by Islam *et al*. (2022). The identification of compound 2 was further confirmed by co-TLC with authentic samples.

In the DPPH assay, various fractions of *P. plebeium* were examined for their ability to scavenge DPPH free radicals. The hexane fraction of the methanolic extract exhibited the highest capacity to scavenge DPPH free radicals with IC_{50} values of 24.2 µg/ml, followed by the dichloromethane fraction (IC_{50} = 32.09 µg/ml) and the ethyl acetate fraction (IC_{50} = 58.86 µg/ml) compared to the standard BHT (IC_{50} = 23.16 µg/ml) (Fig. 2). Antioxidant activity exhibited by the plant extracts may be due to the bioactive compounds capable of hydrogen donating and neutralizing harmful free radicals.



Caffeic acid (1) **β-sitosterol (2)**

Fig. 1. Compounds isolated from *Polygonum plebeium*.

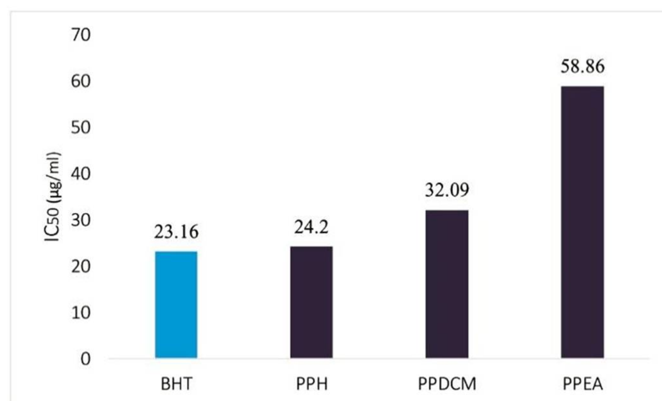


Fig. 2. Comparison of IC_{50} values of standard BHT and different fractions of *P. plebeium*. BHT: Butylated Hydroxy Toluene, PPH: n-hexane soluble fractions, PPDCM: Dichloromethane soluble fractions and PPEA: Ethyl Acetate soluble fraction.

Table 1. Antidiarrheal properties exhibited by loperamide and methanolic extracts of *Polygonum plebeium* at different intervals.

Sample code	1 hour		2 hour		3 hour		4 hour	
	Mean ± SEM	% Reduction of diarrheal feces	Mean ± SEM	% Reduction of diarrheal feces	Mean ± SEM	% Reduction of diarrheal feces	Mean ± SEM	% Reduction of diarrheal feces
Control	1.25±0.68	--	4±0	--	7±0.58	--	11.5±0.92	--
Loperamide	0	100.00*	0.25±0.36	93.75**	1±0.58	85.71**	2.5±0.71	78.26**
MEPP 200	0	100.00*	1±1	75.00**	1.5±1.23	78.57**	4.75±2.12	58.70**
MEPP 400	0.25±0.36	80.00	1.75±1.21	56.25*	3±1.53	57.14**	5.75±1.68	50.00**

Significance levels are denoted as $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ when compared to the control group.

Table 2. Central analgesia exhibited by diclofenac sodium and methanolic extracts of *Polygonum plebeium* at different intervals.

Sample code	0 hr		30 min		60 min		90 min	
	Mean ± SEM	Elongation (%)	Mean ± SEM	Elongation (%)	Mean ± SEM	Elongation (%)	Mean ± SEM	Elongation (%)
Control	1.88±0.15	--	2.42±0.24	--	2.21±0.14	--.	2.46±0.14	--
Morphine	1.98±0.18	--	5.47±0.09	126.43***	9.69±0.29	339.62***	16.35±0.15	566.57***
MEPP 200	1.76±0.05	--	3.47±0.25	43.84***	4.45±0.11	101.82***	6.32±0.23	157.5***
MEPP 400	1.71±0.11	--	3.95±0.11	63.74***	5.54±0.12	151.42***	7.55±0.38	207.85***

Significance levels are denoted as $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ when compared to the control group.

The antimicrobial activity of test samples was determined by the disc diffusion method (Bauer *et al.* 1966). Here, the hexane and ethyl acetate fractions of *P. plebeium* showed significant activity against only *Pseudomonas aeruginosa* with the zone of inhibition is 20 and 14 mm, respectively. The dichloromethane fraction demonstrated activity against *Shigella dysenteriae* (Zone of inhibition = 15.0 mm) as compared to standard ciprofloxacin which exhibited potent antimicrobial activity with the zone of inhibition ranging from 25 to 40 mm against all microorganisms used in this study.

In the castor oil induced-antidiarrheal test, methanol extract of *P. plebeium* (200 mg/kg bw) exhibited notable effectiveness in diminishing diarrhea, as evident at both the 1 and 2 hrs after administration when compared to a control group. The reduction in fecal output was 100, 75%, and 78.57% at 1, 2 and 3 hrs, respectively. In addition, at the 3 and 4 hrs time points post-administration, all doses of the methanolic extract displayed a significant reduction in diarrhea (Table 1). Standard loperamide demonstrated 78.26% drop in diarrheal feces at 4 hrs. The antidiarrheal effect may be attributed to various mechanisms, including a decrease in the secretion of prostaglandins, modulation of COX-1, COX-2 as well as lipoxygenase (LOX) production that can impede the synthesis of prostaglandins and autacoids (Mishra *et al.* 2016), or restricting intestinal motility and hydro-electrolytic secretions.

Table 3. Peripheral analgesic activity of diclofenac sodium and methanolic extracts of *Polygonum plebeium.*

Sample Code	Mean ± SEM	% Inhibition
Control	21.5±1.88	--
Diclofenac-Na	4.75±0.68	77.91***
MEPP 200	13±0.58	39.54***
MEPP 400	10.75±0.68	50***

Significance levels are denoted as *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ when compared to the control group.

Plant extract demonstrated an excellent analgesic effect in both tail immersion and acetic acid-induced writhing assays ($p<0.05$), indicating its potential for peripheral and central pain inhibition. When acetic acid is administered intraperitoneally, it activates the production of prostaglandins leading to increased levels of these prostanoids and lipoxygenase products in the peritoneal fluid of mice (Hasan *et al.* 2023). In the tail immersion test, the methanolic extract of *P. plebeium* at the doses of 200- and 400 mg/kg bw showed significant central analgesic effects at 30, 60 and 90 min as compared to standard morphine (Table 2). When compared to the negative control group, the 200 mg/kg dose of MEPP extended the tail immersion time by 43.84, 101.82, and 157.5% at these respective time points while the extract at the dose of 400 mg/kg bw increased the tail immersion time by 63.74, 151.42, and 207.85%.

The reduction in writhing episodes is typically indicative of analgesic behavior by reducing either prostanoids or lipoxygenase products or both (Tasleem *et al*. 2014). Methanol extract at the doses of 200 and 400 mg/kg bw demonstrated 39.53 and 50.0% reduction of writhing count, respectively while standard diclofenac-Na showed 77.91% writhing inhibition in mice. Likewise central analgesic effects, all these two doses of plant extract significantly ($p < 0.05$) reduced peripheral pain compared to the control in animals (Table 3). Phytochemicals such as alkaloids, glycosides, flavonoids and saponins are reported to play role in analgesic activity both (Tasleem *et al*. 2014).

In this study, two isolated compounds were characterized as caffeic acid and β-sitosterol from *P. plebeium*, employing a series of chromatographic techniques and purification steps. All the solvent fractions demonstrated significant antioxidant activities. The methanol extract of the plant exhibited significant analgesic effects along with substantial antidiarrheal activities in mice. Given the promising bioactivity observed in this study, further in-depth investigations are warranted to isolate and identify the specific phytochemicals responsible for these effects.

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