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Isolation of three metabolites from the plant *Cajanus cajan* (L.) Huth and evaluation of antibacterial and cytotoxic potentials of extract of *Cajanus cajan* (L.) Huth available in Bangladesh

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Abstract

The chemical and biological aspects of the Fabaceae plant *Cajanus cajan* (L.) Huth are the focus of this investigation. Some research has been done on these species, but Bangladeshi species has not been substantially explored. The leaf of *Cajanus cajan* (L.) Huth was cold extracted with methanol and then fractionated using column chromatography with silica gel as the stationary phase, successively with *n*-hexane, mixtures of *n*-hexane/CH_2Cl_2, CH_2Cl_2 itself, mixtures of CH_2Cl_2/CH_3OH and CH_3OH respectively. The crude methanolic extract and column fractions were phytochemically analysed to identify active components and biologically tested for pharmacological properties. The two stilbene metabolites: cajaninstilbene acid and longistylin A and one sterol, stigmasterol were isolated and identified by a combination of repeated column chromatography and solvent treatment. Some column fractions of methanol leaf extract exhibited significant antibacterial activity at a concentration of 200µg/disc against both Gram-positive and Gram-negative bacteria using the disc diffusion method. Moreover, most of the column fractions revealed notable cytotoxicity in brine shrimp lethality bioassay.

Keywords: Cajaninstilbene acid; Longistylin A; Stigmasterol; Antibacterial activity; Cytotoxicity

Introduction

A variety of secondary metabolites derived from plant origin have been discovered to provide substantial health benefits including hepatoprotective, cytotoxic, antidepressant, and antioxidant properties (Adebesin *et al.* 2019). The rise of antibiotic resistance is presented as a significant global health risk, with both mortality rates and economic strain being increased. New therapeutic approaches, particularly natural antimicrobial agents, are needed to be explored to combat multi-drug-resistant bacteria (Saha *et al.* 2022) These compounds including flavonoids, cyclitols, alkaloids, phenolic compounds, and tannins exert pharmacological effects on the human body (Pang *et al.* 2021) due to their notable biological activities. As a result, there has been an increase in interest in the extraction, isolation, and purification of these plants' secondary metabolites with various techniques being widely employed for these purposes (Al-Suod *et al.* 2019).

Medicinal plants are gaining mainstream acceptance due to improvements in analysis and quality control, along with advancements in clinical research that have demonstrated the efficacy of traditional medicine in treating and preventing diseases (Abeloff *et al.* 2008).

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Cajanus cajan (L.) Huth (Family: Fabaceae), commonly known as Pigeon pea, is one of the most important perennial or annual leguminous food crops throughout Asia, Africa, and some parts of South America (Luo *et al.* 2008; Orni *et al.* 2018; Ezike *et al.* 2010). In traditional Chinese medicine, *Cajanus cajan* (L.) Huth has been employed for pain management and as a sedative (Ahsan *and Islam* 2009). It has also been extensively used over the years to treat diabetes, skin sores, bedsores, measles, jaundice, dysentery, and other ailments, as well as to expel bladder stones and regulate menstrual cycles (Yuan-gang *et al.* 2010). Additionally, the leaves and seeds are also used as a poultice on the breast to stimulate lactation (Upadhya *et al.* 2010). In recent years, *Cajanus cajan* (L.) Huth has also been explored for its potential in treating ischemic necrosis of the caput femoris, aphthous ulcers, bedsores, and wound healing. Chemical studies have identified two globulins, cajanin and concajanin, within the plant. The present study reports the isolation of a total three compounds from the plant and the evaluation of antibacterial activity and cytotoxicity of different fractions of the crude extracts.

Materials and methods

Plant collection and extraction of Cajanus cajan (L.) Huth

Cajanus cajan (L.) Huth plant specimen was retrieved from the garden of BCSIR in Bangladesh. After a few days of air drying, the leaves of this plant were oven dried for one day before being milled into a coarse powder. After the plants were air-dried and powdered, 1.25 kg of the material was suspended in 3.0 litres of methanol and left to sit for five days to fulfill the purpose of cold extraction. Once the extracts had passed through a fresh cotton bed, they were filtered using Whatman No. 1 filter paper. At low temperatures (40-50°C) and low pressure, the filtrate's volume was concentrated using a rotary evaporator.

Chemical studies of the extract

Column chromatography of methanolic plant extract

Column chromatography was used to fractionate the 6.72 grams of *Cajanus cajan* (L.) Huth leaf methanolic extract. A silica gel (Kieselgel 60, mesh 70-230, Merck, Germany) column was prepared, with the silica gel slurry mixed in *n*-hexane, and packed into a glass column with dimensions of 63 cm in length and 12.5 cm in diameter. The sample was loaded to a height of 1.5 cm, while the adsorbent height was 29.0 cm. The column was then eluted sequentially with *n*-hexane, followed by increasing polarity mixtures of *n*-hexane and dichloromethane of increasing polarity, then by dichloromethane and finally with dichloromethane and methanol mixtures of increasing polarity.

Initial screening of the fractions by TLC (Thin layer chromatography)

The obtained column fractions were first screened using thin layer chromatography in various solvent systems. In order to identify the presence of colored compounds, the produced chromatogram was visually inspected. It was then placed under UV light at 254 nm and 365 nm to identify any spots or bands containing quenching or fluorescent substances. Spray reagent was used to identify the kind of compounds that were anticipated to be present in the extracts (such as 1% vanillin in concentrated sulphuric acid)

Compound isolation and purification

Total three compounds have been isolated and purified from the column fractions adopting various techniques. Compound 1, 2 and 3 were isolated by repeated column chromatography from the column fractions by elution with *n*-hexane/90-95% CH_2Cl_2, CH_2Cl_2/5-10% MeOH and CH_2Cl_2/50-100% MeOH, respectively.

Antibacterial activity

By using the disc diffusion method, the antibacterial activity of plant column fractions was tested against two Gram negative bacteria i.e. *Pseudomonas aeruginosa* and *Escherichia coli*, and three Gram positive bacteria i.e. *Bacillus megaterium*, *Micrococcus*, and *Staphylococcus aureus* (Bauer *et al*. 1966).

The concentration of the column fractions were 200 µg/ disc. Sterile filter paper disc of 6 mm in diameter were loaded with sample using micropipette and was air dried. Kanamycin (30 µg/disc) was used as standard.

Brine shrimp lethality bioassay

Column fractions of the plant were evaluated for possible cytotoxicity using brine shrimp lethality bioassay following the protocol proposed by Meyer (Meyer *et al*.1982). After 24 hours, by counting the number of brine shrimps died the LC_{50} values for all the fractions were calculated manually.

Results and discussion

Characterization of isolated compounds from Cajanus cajan (L.) Huth

Characterization of Compound 1 as Longistylin A

Compound 1(Fig.1) was found as yellow needles. It appeared as a deep blue spot on TLC plate (R_f=0.27, toluene/5% EtOAc) under UV light at 254 nm and showed a blue fluorescence at 365 nm. It is soluble in dichloromethane and methanol and insoluble in *n*-hexane and pet-ether. Spraying the developed plate with a vanillin-sulfuric acid reagent followed by heating produced orange color.

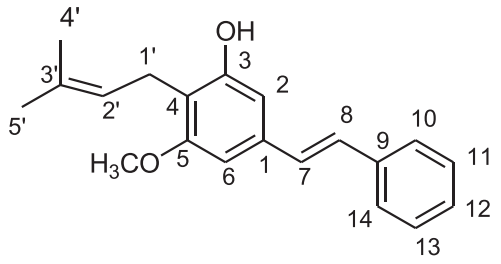


Fig.1. Structure of compound 1 as longistylin A

^{1}H NMR (400 MHz, $CDCl_3$): δ =1.67 (3H, s, H-5'), 1.80 (3H, s, H-4'), 3.42 (2H, brs, H-1'), 3.77 (3H, s, OMe-5), 5.07 (1H, d, *J*=29.6Hz, H-2'),6.36 (1H, s, H-2), 6.68 (1H, s, H-6), 6.91 (1H, d, *J*= 16 Hz, H-8), 7.27 (1H, brs, H-7), 7.29 (1H, brs, H-12), 7.34 (1H, brs, H-11,H-13), 7.46 (1H, brs, H-10,H-14).

^{13}C NMR (100 MHz, $CDCl_3$): δ= 18.0 (C-4′), 24.4 (C-1′), 25.8 (C-5′), 55.7 (O**C**H_3), 98.5 (C2),104.1 (C6),121.1 (C4),123.4 (C-2′), 126.6 (C11, C13),127.6 (C12), 128.7 (C10, 14),130.5 (C7), 137.5 (C3), 137.9 (C5).

The ^{13}C NMR spectrum (100 MHz, $CDCl_3$) of Compound 1 displayed 20 carbon resonances while the DEPT-135 experiment structured these signals into 2 methyls, 1 methylene, 1 methoxy group,10 methines and 6 quaternary carbons, i.e. 14 out of the 20 carbons were linked to protons. The doublet at δ 5.07 ppm in ^{1}H NMRand at δ 123.4 ppm in the ^{13}C NMR spectra, in conjunction with the DEPT-135 spectrum attributed to one olefinic proton. Resonance at δ 6.91 ppm and δ 7.27 ppm in the ^{1}H NMR and at δ 131.0 ppm and δ 130.5 ppm in the ^{13}C NMR spectra, could be connected to two olefinic protons. The resonance at δ 6.36 ppm in the ^{1}H NMR and at δ 98.5 ppm in ^{13}C NMR spectra indicated the presence of an additional aromatic proton. Compound 1 was confirmed by comparing the ^{13}C NMR data which were reported for the compound longistylin A, isolated from the plant *Cajanus cajan.* (George Duker-Eshunwith *et al*. 2004).

Characterization of Compound 2 as Stigmasterol

Compound 2 (Fig. 2) was obtained as needle shaped crystals with R_f= 0.27, toluene/10%EtOAc. It was determined to be soluble in both ethyl acetate and chloroform.

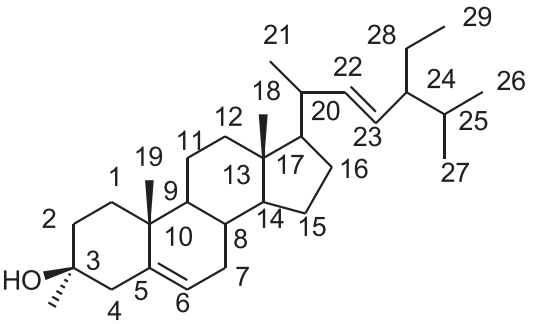


Fig. 2. Structure of compound 2 as Stigmasterol

^{1}H NMR (400 MHz, $CDCl_3$): δ= 0.67 (3H, s, Me-13), 0.83 (3H, s, H-26), 0.91 (3H, s, Me-20), 1.00 (3H, s, Me-10), 3.51 (1H, m, H-3), 5.02 (1H, m, H-23), 5.13 (1H, m, H-22), 5.34 (1H, m, H-6).

Compound 2's ^{1}H NMR spectrum (400 MHz, $CDCl_3$) showed that H-3 had a proton multiplet at δ 3.51 ppm and that the steroidal skeleton's olefinic H-6 had a signal at δ 5.34. The characteristic downfield signals at δ 5.13 ppm and δ 5.02 ppm, respectively, were observed in the 1H NMR spectra for the olefinic protons H-22 and H-23. On the basis, the identity of compound 2 was confirmed as stigmasterol (Kaur *et al*. 2011; Khan *et al* 2018).

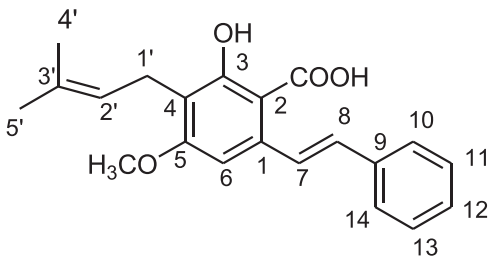


Fig. 3. Structure of compound 3 as cajaninstilbene acid

Characterization of Compound 3 as Cajaninstibene Acid

Compound 3 (Fig. 3) was attained as white amorphous crystals. It become visible as a sky blue spot on TLC plate (R_f= 0.33, CH_2Cl_2/1% MeOH) under UV light at 254 nm and displayed a blue fluorescence at 365 nm. Spraying the resulting plate with vanillin-sulfuric acid spray reagent, then heating, produced a yellow colour.

^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.69 (3H, s, H-5′), 1.80 (3H, s, H-4′),3.38 (2H, brs, H-1′), 3.95 (3H, s, OMe-5),5.22 (1H, brs, H-2′), 6.66 (1H, s, H-6), 6.83 (1H, d, *J*= 15.5 Hz, H-8),7.30 (1H, brs, H-12), 7.39 (2H, brs, H-11, H-13), 7.53 (2H, brs, H-10, H-14), 7.85 (1H, d, *J*= 15.5 Hz, H-7), 11.53 (1H, s, OH-3).

^{13}C NMR (100 MHz, $CDCl_3$): δ = 17.9 (C-4′),22.1 (C-1′),25.9 (C-5′), 55.8 (OMe-5), 103.1 (C-6), 103.4 (C-2),116.8 (C-4), 121.9 (C-2′), 126.9 (C-10, C-14), 127.9 (C-12), 128.8 (C-11, C-13), 130.4 (C-7), 130.9 (C-8), 132.0 (C-3′), 137.3 (C-9), 142.0 (C-1), 162.3 (C-3), 162.5 (C-5), 175.9 (COOH).

The ^{13}C NMR spectrum (100 MHz, $CDCl_3$) of compound 3 revealed 21 carbon resonances while the DEPT-135 experiment sorted these signals into 2 methyls, 1 methylene, 1 methoxy group, 9 methines and 8 quaternary carbons, ie., 13 out of the 21 carbons were attached to protons. The broad singlet at δ 5.22 ppm in ^{1}H NMR and at δ 121.9 ppm in the ^{13}C NMR spectra, attributed to one olefenic proton and the resonance at δ 6.83 ppm and δ 7.85 ppm in the ^{1}H NMR and at δ 130.9 ppm and δ 130.4 ppm in the ^{13}C NMR spectra, in conjunction with the DEPT-135 spectrum, could be attributed to two olefinic protons. The ^{1}H NMR spectra showed the signals for aromatic protons in broad singlet at δ 7.53 ppm, δ 7.39 ppm and δ 7.30 ppm. The resonance at δ 175.9 ppm in the ^{13}NMR spectra and one proton sharp singlet at δ11.53 ppm indicated the presence of carboxylic group and one phenolic hydroxyl group.This hydroxyl group comparatively deshielded state suggested that it might create an intramolecular

Table I. Antimicrobial activity of the twelve column fractions of methanol leaf extract of *Cajanus cajan*

Bacterial strain	Diameter of Zone of inhibition (mm) (200 µg/Disc)												Kanamycin (30 µg/Disc)
	BC-1	BC-2	BC-3	BC-4	BC-5	BC-6	BC-7	BC-8	BC-9	BC-10	BC-11	BC-12	
Gram Positive bacteria													
Bacillus megaterium (BTCC-18)	…	7	…	…	…	…	…	…	13	28	20	12	29
Staphylococcus aureus (BTCC-43)	9	8	…	…	7	…	…	…	14	19	18	15	33
Micrococcus (ATCC 9341)	…	…	7	…	…	…	…	8	10	20	14	9	33
Gram negative bacteria													
Escherichia coli (ATCC 8739)	…	7.5	…	…	…	…	…	…	12	25	21	9.5	34
Pseudomonas aeruginosa (ATCC 27833)	…	…	7.6	…	8	…	…	…	10	21	15	12	35

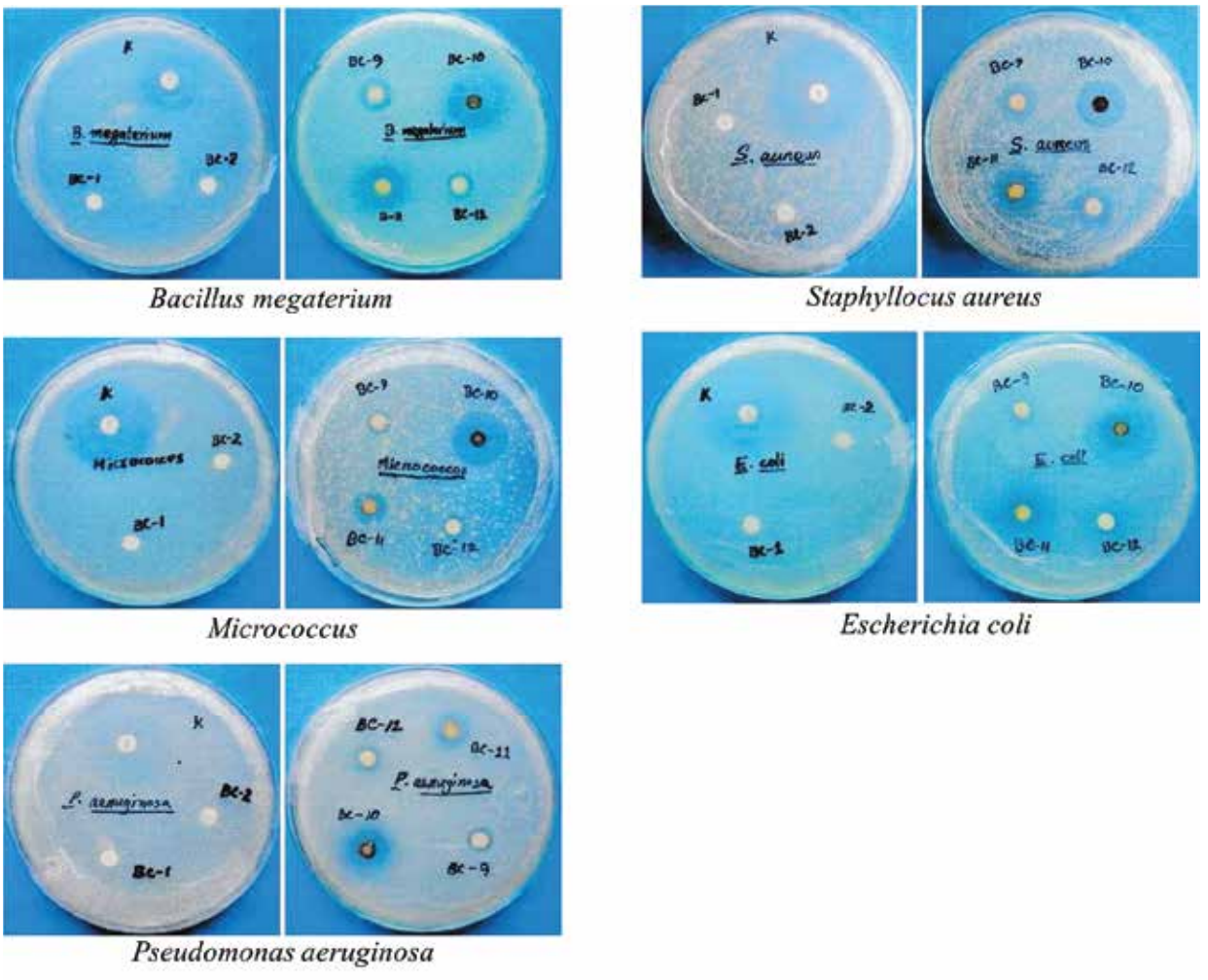
"…" Indicates 'No activity'.

hydrogen bond with a functional group's lone pairs. Therefore, it is possible to argue that carboxylic and hydroxyl groups are vicinal. It became apparent that the ^{1}H NMR and ^{13}C NMR data of Compound 3 were remarkably comparable with the data that had been reported for the compound cajaninstilbene acid that was obtained from the plant *Cajanus-cajan*. (Kong *et al*. 2010).

Antibacterial activity

Using the disc diffusion method, twelve column fractions of methanol leaf extract were assessed for potential antibacterial activity against a variety of Gram-positive and Gram-negative bacteria. The results obtained were compared with kanamycin, standard antibiotics. The twelve column fractions exhibited poor to significant antibacterial activity against most of the test organisms (Table I). The zone of inhibition produced by twelve column fractions of methanol leaf extract was 07 -28 mm at concentration of 200 µg/disc (Figure 4). The column fractions BC-9, BC-10, BC-11 and BC-12 showed mild to significant activity towards five test bacteria. Among them BC-10 showed prominent activity against *Bacillus megaterium* (28 mm), *Escherichia coli* (25 mm) and *Pseudomonas aeruginosa* (21 mm) and also showed good activity against *Micrococcus* (20 mm) and *Staphylococcus aureus* (19 mm). *Escherichia coli* (21 mm) showed the highest sensitivity and *Micrococcus* (14 mm) showed the lowest sensitivity to column fraction BC-11. Fraction BC-9 showed the highest zone of inhibition against *Staphylo-*

Fig. 4. Antimicrobial activity of column fractions of methanol leaf extract of *Cajanus cajan*



coccus aureus (14 mm) and the lowest zone of inhibitionagainst *Micrococcus* (10 mm) as well as *Pseudomonas aeruginosa* (10 mm) and BC-12 demonstrated the highest activity against *Staphylococcus aureus* (15 mm) and lowest activity was 9 mm which was against *Micrococcus*.

Brine shrimp lethality bioassay

Twelve column fractions of methanol leaf extract were evaluated for possiblecytotoxic activities using brine shrimp lethality bioassay (Meyer *et al*. 1982). From the bioassay, it is evident that almost all of the column fractions were lethal to the brine shrimp nauplii which indicating that the test samples are biologically active (Table II). Column fraction BC-10 is more active with minimum LC_{50} value (11.22 µg/mL), while BC-5 is less active with maximum LC_{50} value (27.44 µg/mL). On the other hand columnfraction BC-3, BC-6, BC-7 and BC-8 showed no lethality to brine shrimp nauplii. These fractions may not contain cytotoxic metabolites.

Table II. Cytotoxic activities using brine shrimp lethality bioassay

Sl no.	Sample code	LC_{50} (µg/mL)	Sl no.	Sample code	LC_{50} (µg/mL)
1	BC-1	22.89	7	BC-7	--
2	BC-2	24.97	8	BC-8	--
3	BC-3	--	9	BC-9	16.24
4	BC-4	22.69	10	BC-10	11.22
5	BC-5	27.44	11	BC-11	12.07
6	BC-6	--	12	BC-12	19.05

C. cajan is rich in phytochemicals and three compounds were isolated and identified. The column fractions showed strong effectiveness against various pathogenic bacteria, likely due to natural coumarins like Cajanuslactone, which has demonstrated high antibacterial properties, particularly against S. aureus (Luo *et al.* 2008). Previous research has highlighted the effectiveness of *C. cajan* leaf extracts against pathogens, including Salmonella typhi, the causative agent of typhoid, a major concern in many developing countries. The major compounds of this plant, stilbenes and flavonoids were shown to inhibit the growth of *S. typhi, S. aureus,* and E. coli (Orni *et al.* 2018).

This study evaluated the cytotoxic activities of the column fraction. From the bioassay, it is evident that almost all of the column fractions were lethal to the brine shrimp nauplii indicating the test samples are biologically active possessing cytotoxic metabolites. Further investigation is required to explore the pure bioactive compounds from this plant.

Conclusion

The leaf of *Cajanus cajan* (L.) Huth was cold extracted with methanol and subjected to column chromatography for fractionation. The column fractions were analyzed phytochemically to isolate the active compounds as well as screened biologically to explore the medicinal values of the plant.In the present study, two stilbene metabolites longistylin A (compound 1) and cajaninstilbene acid (Compound 3) and one sterol, stigmasterol (compound 2) were identified by comparing their spectral data with those reported in the literature. The column fractions were tested for antibacterial activity by disc diffusion methodand cytotoxic activity using the brine shrimp lethality bioassay. In conclusion, our results suggested that medicinal plants of Bangladesh would be a great place to discover bioactive compounds for novel drug development.

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