Phytochemical Analysis, Antioxidant and Cytotoxic Activities of *Mucuna pruriens* Leaves

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ABSTRACT: *Mucuna pruriens* is a tropical legume native to Africa, India and Bangladesh and is widely cultivated in tropical countries. In this study, a crude methanolic extract of the leaves of *M. pruriens* was investigated for its chemical constituents and to explore the phenolic and flavonoid content, antioxidant, cytotoxic and antimicrobial activities using established protocols. From the ethyl acetate soluble fraction of the crude methanol extract, three known compounds namely ferulic acid (**1**), 2-(5-methoxy-1-benzofuran-3-yl)-*N*-ethylethanamine (**2**) and stizolamine (**3**) were isolated and their structures were elucidated by the analysis of NMR spectral data. The crude extract was found to possess phenolic content of 216.16 µg/g whereas the concentration of flavonoid was found to 214.8 µg/g expressed in quercetin standard. Free radicals generated through DPPH were neutralized by crude methanolic extract and the IC_{50} value was obtained as 19.63 µg/ml. Regression analysis during brine shrimp lethality test enumerated LC_{50} value of crude methanolic extract at 10.72 µg/ml and was significant compared to the positive standard. The crude methanolic extract of leaf of *M. pruriens* did not show any significant antimicrobial activity against the organisms used in our test.

Keywords: *Mucuna pruriens*, Ferulic acid, Fabaceae, DPPH free radical, Total phenolic content, Brine shrimp lethality bioassay

INTRODUCTION

Mucuna pruriens (Velvet Bean) belonging to the family Fabaceae, is a topical legume plant found all over in Bangladesh.[1] Different parts of this plant have been traditionally used against several diseases worldwide.[2] This plant is a natural source of L-3,4-dihydroxyphenyl alanine (L-DOPA), a neurotransmitter precursor which is effective against Parkinson's disease.[3] The seeds of *M. pruriens* are considered as astringent, aphrodisiac, nervine and have a high nutritional value which showed trypsin inhibitory activity[4], hepatoprotective activity and antioxidant properties.[5] Anti-inflammatory, diuretic and antibacterial activities of the aerial parts of *M. pruriens* have been already reported.[6] In another study, it has been reported that *M. pruriens* seed increased sperm concentration and motility in healthy infertile adults.[7] Previous phytochemical investigation revealed an array of alkaloids and flavonoids in the seed such as the alkaloids bufotenin, dimethyltryptamine, tetrahydroquinolone alkaloids, stizolamine, mucunine, mucunadine, prurienidine, nicotine and the flavonoids namely genistein, medicarpin, kievitone, cajanol etc.[8,9] Other chief constituents have also been reported such as arachidic acid, behenic acid, betacarboline, beta-sitosterol, 5-hydroxytryptamine, isoleucine and different kinds of fatty acids such as palmitic acid, myristic acid, oleic acid, palmitoleic acid and phenylalanine etc.[10]

A tremendous effort has already been given to explore the phytochemical and biological activity of the seed extracts of *M. pruriens*. However, very few

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reports are available with the leaf extract of this plant. With a view to this consideration, the present study was undertaken to explore the antioxidant and cytotoxic potential of the crude methanolic extract of leaves of *M. pruriens* and also to isolate the secondary metabolites from leaf extract of this plant.

MATERIALS AND METHODS

General experimental procedures. Bruker Avance-400 instrument was used to collect the ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra and the chemical shifts (δ) were reported with respect to the internal standard, tetramethylsilane (TMS). Silica gel (Keiselgel 60, 220-400 mesh, Germany) was used in the column chromatographic technique. Preparative TLC was carried out in glass plates previously coated with silica gel (Keiselgel 60, Merck Co., Japan). The developed chromatogram was visualized under UV light operating at short wavelength (254 nm) and long wavelength (365 nm). The plates were also sprayed with dil. H_2SO_4 and heated until the color developed to know the identity of the constituents.

Chemicals. All the chemicals used in the experiment were purchased from Sigma Chemical Co. (USA). Brine shrimp eggs were collected from the local market. Muller-Hinton agar, Muller-Hinton broth, media were collected from TM media Ltd. India. Standard antibiotic discs were purchased from Himedia (India) and blank discs were prepared by perforating Whatman filter paper No. 1. All other analytical grade reagents were collected from BDH Ltd. and Merck Co.

Plant material. The fresh leaves of *M. pruriens* were collected from the Natore district in the year 2015 and were identified by Dr. Mr. Mustafizur Rahaman, Associate Professor, Department of Botany, University of Rajshahi.

Extraction and isolation. About 1 kg of the dried and coarsely powdered leaf was macerated using 2.5L of methanol for 7 days with occasional stirring. Whatman filter paper No. 1 was used to filter the extract to separate particulate matters. It was concentrated in a rotary vacuum evaporator to yield a semisolid residue (30.45 g). Solvent-solvent partitioning was done by at first dissolving the crude extract (29.08 g) in a sufficient amount of 10% aqueous methanol and then extracted in a separating funnel with ethyl acetate to provide aqueous (11.3 g) and ethyl acetate (8.4 g) soluble materials. These were kept in a refrigerator for future analysis.

A portion of the ethyl acetate soluble (5.0 g) was subjected to silica gel column chromatography starting with the nonpolar mobile phase which was prepared with 7 parts of n-hexane and 3 parts of previously mixed chloroform (95): methanol (5). The column was then eluted with the same solvent mixture of different ratio of increasing polarities and finally, the column was washed with chloroform - methanol (1:1). From the total 18 fractions collected, fraction F [1.3 g; eluted with equal parts of n-hexane and chloroform (95)- methanol (5)] was subjected to preparative TLC using chloroform-methanol (98:2) and compound **1** with a R_f value of 0.6 was purified (5.6 mg). Purification of fraction L (0.42 g) eluted with chloroform-methanol (99:1) by preparative TLC using chloroform-methanol (95:5) gave compound **2** (9.07 mg) with a R_f value of 0.5 and compound **3** (6.0 mg) with a R_f value of 0.4.

Determination of total phenolic content: With a slight modification, Folin-Ciocalteu method was used to determine the total amount of phenolic compound (TPC) in the methanolic fraction.[11] Briefly 0.2 mL of the sample (1 mg/mL), 1.8 mL of distilled water, 0.2 mL of FC reagents were mixed in a test tube. 2 mL of sodium carbonate (7.5%) was added after 5 minutes and sufficient water was added to make the volume 5 mL. After 90 minutes of incubation, absorbance was measured at 760 nm. As a reference standard, gallic acid was used and the result has been expressed as mg gallic acid equivalent (mg GAE)/g of extract. Total phenolic content was calculated from the following equation:

$Y = 0.006 X + 0.039$, $R2 = 0.997$

Here X is the Gallic acid equivalent and Y is the absorbance at 760 nm.

Determination of total flavonoid content: Aluminum chloride colorimetric method was used to

determine the total flavonoid content of the crude extract.[12] 1.5 mL of the sample (1 mg/mL), 0.1 mL of $AlCl_3$ (10%), 0.1 mL of sodium acetate (1M) were mixed and made the volume 5 mL with water. After 15 minutes of incubation, absorbance was measured at 415 nm. A calibration curve of quercetin (50, 100, 150, 200 and 250 µg/mL) was used to measure the total flavonoid compound and it was expressed as quercetin equivalent per gram dry weight of the extract. The following equation was used to measure the flavonoid content:

$Y = 0.0067 X + 0.0132, R^2 = 0.993$

Here X is the Quercetin equivalent and Y is the absorbance.

Antioxidant activity. Free radical neutralizing capacity was determined using DPPH method as described earlier.[13] In a brief, 0.2 mM of DPPH solution was added to the pre-marked test tubes containing different concentrations of the crude extract and the colour change was measured at 517 nm using a UV-Vis spectrophotometer. Ascorbic acid was used as standard. Inhibition of DPPH radical scavenging was determined using the following equation:

$I\% = (1 - A_{sample}/A_{control}) \times 100$

Where, $A_{control}$ stands for the absorbance of the DPPH solution, and A_{sample} stands for the absorption of the test sample. The concentration required for 50% inhibition (IC_{50}) was calculated from a plot drawn in the MS Excel office 2010.

Cytotoxic activity. Brine shrimp lethality bioassay is a rigorous, convenient and popular method to determine the preliminary cytotoxic activity of any crude extract and the process has been described earlier.[14] Anticancer alkaloid, vincristine sulfate (VS) was used as positive standard. The LC_{50} values were calculated from a plot drawn in the MS Excel office 2010.

Antimicrobial activity. Antimicrobial activity was evaluated using Muller-Hinton (MH) agar plate disc diffusion technique.[15] Five gram (+)ve bacteria *B. cereus*, *B. megaterium*, *B. subtilis*, *S. aureus*, *S. lutea* and eight Gram (-)ves *E coli*, *P. aeruginosa*, *S. paratyphi*, *S. typhi*, *Shigella boydii*, *S. dysenteriae*, *Vibrio mimicus*, *V. parahemolyticus*, and three unicellular fungi were *C. albicans*, *A. niger*, *S. cerevacae* were cultured in a petridish with Muller-Hinton agar medium. Standard kanamycin (30 µg/disc) discs were used as positive control and sterile blank discs were used as negative. The crude methanolic extract of *M. pruriens* was loaded on the discs at a concentration of 400 µg/disc to observe the antimicrobial potential.

Properties of isolated compounds

Ferulic acid (**1**): Reddish yellow powder; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.75 (1H, d, *J* = 15.0 Hz, H-7), 7.14 (1H, dd, *J* = 8.0, 1.5 Hz, H-6), 7.09 (1H, br. s, H-2), 6.97 (1H, d, *J* = 8 Hz, H-5), 6.34 (1H, d, *J* = 15.0 Hz, H-8), 3.98 (3H, s, H-10); ^{13}C NMR (100 MHz, $CDCl_3$): 127.7 (C-1), 109.5 (C-2), 146.8 (C-3), 140.8 (C-4), 121.7 (C-5), 122.7 (C-6), 147.7 (C-7), 114.9 (C-8), 168.7 (C-9), 56.0 (C-10).

2-(5-Methoxy-1-benzofuran-3-yl)-*N*-ethyle-thanamine (**2**): Yellowish white powder; ^{1}H NMR (400 MHz, CD_3OD): δ 6.94 (1H, s, H-2), 6.71 (1H, d, overlapped, H-4), 6.65 (1H, dd, overlapped, H-6), 6.64 (1H, overlapped, H-7), 4.2 (2H, m, H-8), 3.81 (3H, s, OMe), 3.80 (2H, m, H-9), 3.55 (2H, m, H-10), 1.92 (3H, t, *J* = 6.5 Hz, H-11).

Stizolamine (**3**): Yellowish white crystals; ^{1}H NMR (400 MHz, CD_3OD): δ 6.65 (1H, s, H-3), 4.31 (2H, s, H-7), 3.31 (3H, s, H-8).

RESULTS AND DISCUSSION

Isolation and structure identification. A crude methanolic extract of the dried leaves of *M. pruriens* was subjected to polarity dependent partitioning between EtOAc and H_2O. The EtOAc soluble fractionate was further purified by column and thin layer chromatography over silica gel to yield three compounds (Figure 1). The isolated compounds were characterized by analysis of their NMR data as well as comparison with published values.

Compound **1** appeared as a light brownish yellow spot on TLC when the plate was sprayed with dil. sulphuric acid and was heated at 110°C for 2-3

minutes. The ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of compound **1** showed several peaks at aromatic regions specifically ABX-type aromatic proton signals [δ 7.14 (1H, dd, *J* =8, 1.5 Hz, H-6), 7.09 (1H, br. S, H-2) and 6.97 (1H, d, *J* =8 Hz, H-5)] along with two olefinic protons at δ 7.75 (1H, d, *J* = 15 Hz, H-7) and 6.34 (1H, d, *J* =15 Hz, H-8), resembling a feruloyl moiety in its structure. Besides, the coupling constant between these two olefinic protons was calculated 15 Hz, recommending the *E*-configuration. The ^{13}C NMR spectrum of the compound displayed 8 aromatic carbons among which six carbons can be assigned for an aromatic ring. The signals δ 114.7 and 147.7 could be assigned for two olefinic carbons among which one of them was highly deshielded due to the presence of a carboxyl moiety at beta position. Among the six aromatic carbons, two signals were appeared in δ 146.8 and 140.8 due to oxygenation of those positions. This was further confirmed by the presence of a methoxy group signal δ 3.98 which was correlated with the carbon resonance at δ 56.0. According to the above spectral data, compound **1** was identified as ferulic acid and its spectral data were in accordance with the published values.[16]

Compound **2** was found as a brownish spot on TLC when the chromatographed plate was sprayed with dil. sulphuric acid followed by heating at 110°C for 2-3 minutes. The ^{1}H NMR spectrum (400 MHz, CD_3OD) of compound **2** revealed three aromatic protons at δ 6.71 (1H, d, overlapped, H-4), 6.65 (1H, dd, overlapped, H-6), 6.64 (1H, overlapped, H-7) and 6.94 (1H, s, H-2) directing a benzofuran ring in its structure. The spectrum also showed three methylene protons δ 4.2 (2H, m, H-8), 3.8 (2H, m, overlapped, H-9) and 3.55 (2H, m, H-10) concluding the presence of branched aliphatic chain with nitrogenous base. A methyl proton at δ 1.92 (3H, t, *J* = 6.5 Hz, H-11) might be attached to the aliphatic chain, corresponding to the ethylamine structure. On the other hand, the methyl protons at δ 3.81 (3H, s, OMe) could be assigned for the position of the oxygenated aromatic ring. Extensive literature search revealed that this kind of ethylamine structured alkaloidal compound (bufotenine) has been previously isolated from this plant.[17] The spectral data of compound **2** were moderately consistent with the reported data of 2-(5-methoxy-1-benzofuran-3-yl)-*N*-ethylethanamine acquired in $CDCl_3$, which is reported here first time from this plant.[18] Because of the limitations of instrumental facilities, we could not get the ^{13}C and MS spectra to confirm the structure. Thus, compound **2** was tentatively identified as 2-(5-methoxy-1-benzofuran-3-yl)-*N*-ethylethanamine.

Compound **3** was obtained as a brownish spot on TLC when the plate was sprayed with dil. sulphuric acid and heated until the spot appeared. The ^{1}H NMR spectrum (400 MHz, CD_3OD) of compound **3** revealed only three signals among which one appeared in δ 6.65 which could be assigned to H-6. The spectrum also revealed signal for an oxymethylene protons at δ 4.31 (2H, s, H-7) and a three proton singlet at 3.31 (3H, s, H-8) attributable to the methyl group attached to the nitrogen atom. The values were compared with the published data of stizolamine, which has previously been isolated from this plant.[19] On this basis, compound **3** was tentatively identified as stizolamine {N-[3,4-dihydro-5-(hydroxymethyl)-4-methyl-3-oxo-2-pyrazinyl] guanidine}, which could not be confirmed due to the lack of ^{13}C and MS data.

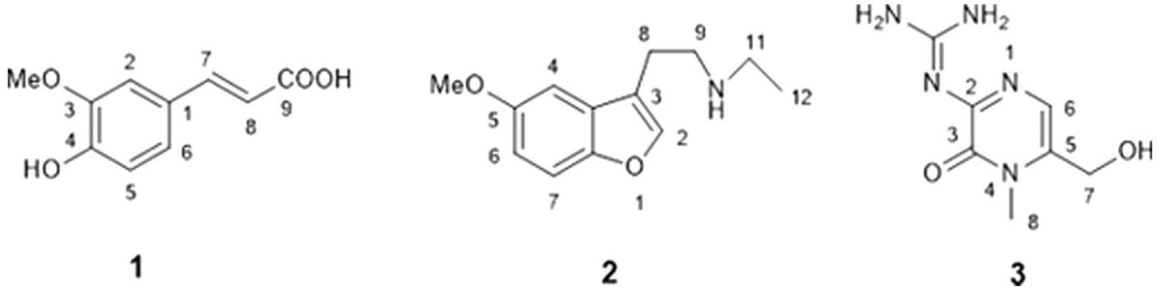

Figure 1. Phytoconstituents (**1**-**3**) isolated from *Mucuna pruriens.*

Total phenolic and flavonoid content. Hydroxyl group containing compounds such as tannins, catechins, anthraquinones and flavonoids are phenolic in nature and they show antioxidant properties both *in vitro* and *in vivo*.[20] To determine the presence of phenolic contents, FC reagents were mixed with the crude extracts and the result was compared with gallic acid, a common phenolic compound found in nature. A calibration curve (R^2=0.997) was developed with the different concentrations of gallic acid and the tested extract was found to possess 216.16 ± 1.20 mg of gallic acid equivalents/g. Flavonoids are a class of natural compounds that possesses phenolic structures too and display different biological activities. For this reason, the amount of flavonoid content was also determined by the reaction mixture of crude extracts with aluminum chloride. The total flavonoid content of the crude methanolic extract was found at 214.8±1.45 mg of quercetin equivalents/g (Table 1).

Antioxidant activity. The ability to donate a hydrogen atom or electron of the tested sample was measured by the bleaching of a purple coloured methanolic DPPH solution. Wherein the bleaching tendency of DPPH was carefully measured by the absorbance at 517 nm. In its radical form, DPPH showed maximum absorbance at 517 nm when it is in radical form but upon reduction by any antioxidants, its absorption decreases. Crude methanolic leaf extract of *M. pruriens* showed IC_{50} values of 19.63 µg/mL whereas ascorbic acid revealed IC_{50} values of 4.36 µg/mL (Table 1). Previous study reported that aqueous and ethanolic extract of *M. pruriens* leaf inhibited the DPPH in a concentration dependent manner.[21] Aqueous extract of *M. pruriens* seeds showed DPPH free radical inhibition and prevented lipid peroxidation in a dose dependent manner.[22] So, these results are in accordance with our findings.

Cytotoxic activity. Brine shrimp lethality bioassay is based on the toxicity of test compounds exerted on simple *Artemia salina* larvae hatched in the brine solutions. LC_{50} values were calculated from a plot drawn by the log value of the concentrations versus percentage lethality. The LC_{50} value of the *M. pruriens* extract was found 10.72 µg/mL (Table 1). Ethyl acetate and methanol extracts of *M. pruriens* inhibited the proliferation of hepatoma cells *in vitro* with a concentration at 1 µg/mL.[23] However, another study reported that the aqueous and ethanolic extracts of *M. pruriens* seed has no toxicity to human cervix adenocarcinoma (HeLa) cells when tested using 50 µg/mL of it.[24]

Table 1. Antioxidant and cytotoxic activities of the crude methanolic extracts of *M. pruriens*.

Test procedure	*M. pruriens*	Standard
Total phenolic content (in mg/g GAE)	216.16 ± 1.20	-
Total flavonoid content (in mg/g QE)	214.8±1.45	-
IC_{50} value at antioxidant assay (µg/mL)	19.63	4.36 (AA)
LC_{50} value at cytotoxicity assay (µg/mL)	10.72	1.76 (VS)

Values are expressed as mean±SD; n = 3, AA = Ascorbic acid, VS = Vincristine sulfate.

Reactive oxygen and nitrogenous species generated from cellular metabolism are detrimental to our health. Over production of them causes damage to the biological macromolecules such as DNA, RNA, proteins, membranes etc. and responsible for aging as well as the pathogenesis of several diseases.[25] Endogenous antioxidants such as superoxide dismutase, catalase, peroxidase may not be sufficient to minimize or prevent these deleterious effects. Plant derived phenolics can play a major role in alleviating these conditions. The antioxidants can prevent oxidative stress and can terminate chain reactions triggered by free radicals. Antioxidant capacity of *M. pruriens* leaf extract has shown quite promising result which has also been reflected in the total phenolic and flavonoid content of the extract. Similar results have also been reported in earlier taking the whole plant.[26] Although the seed extracts

of *M. pruriens* showed antibacterial activity against clinical isolates[27], negligible antimicrobial activity was observed in the variety of pathogens in our studies (data not shown). Phytochemical analysis of the ethyl acetate fraction of *M. pruriens* revealed three compounds among which two have free hydroxyl groups in their structures that can attribute the antioxidant property of the plant.

CONCLUSION

Extensive phytochemical investigation of ethyl acetate soluble fraction of a methanol extract of leaf of *M. pruiens* has provided three previously reported compounds (**1-3**). The crude methanolic extract showed notable antioxidant activity in DPPH free radical scavenging assay and it was found to be a rich source of total phenolic and flavonoid contents. These bioactivities support the traditional use of *M. pruriens* as neuroprotective, nerve tonic and aphrodisiac. Therefore, *M. pruriens* can be further explored for the development of new therapeutic agents.

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