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Chemical and Biological Investigation of Leaves of *Polygonum plebejum*

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ABSTRACT

The cytotoxic and antimicrobial activity of methanol crude extract and column fractions of the extract of the leaves of *P. plebejum* were examined by brine shrimp lethality bioassay and disc diffusion method respectively. The extracts showed significant cytotoxic as well as antimicrobial activities. Silica gel column chromatography of methanol extract of *P. plebejum* afforded a steroid. The structure was elucidated on the basis of spectral analysis, including ^{1}H NMR and ^{13}C NMR and also by comparing with data in the literature.

Key words: *Polygonum plebejum, Polygonaceae*, Stigmasterol, Cytotoxicity.

INTRODUCTION

The natural products, mainly of plant origin, have been used for treatment of diseases, and an impressive number of drugs have been isolated from natural sources. According to WHO about 4 billion people in the world rely on plant a sources of drugs (N.R. fransworth, 1988). The plant kingdom represents reservoir of new chemical compounds (M. Hamvergur et al., 1991) such as glycosides, steroids, alkaloids, terpenoids, flavones, etc. are mainly responsible for their various therapeutic properties and pharmacological actions. Research studies leading to extraction, isolation and biological study of plant constituents have now formed the major field of study. The plant *Polygonum plebejum* belongs to the family *Polygonaceae*, locally known as Chemti Sag, Small Knotweed in English is a prostrate, densely branched, annual herb (Ghani A., 1998). The main constituents of the leaves of *P. plebejum* are flavonoids (13.03%), saponin (8.9%), steroids (4.8%) and alkaloids (0.7%) (Scalbcrt A. 1997). We investigated this plant and isolated a steroid. The purpose of this study is to identify and characterize the bioactive principles from the leaves of *P. plebejum.*

MATERIALS AND METHODS

Melting point of the compound was determined by using an electrothermal melting point apparatus (Mel-temp, OGAWA SEIKI CO., Japan). Shimadzu IR-470 spectrophotometer was used for recording IR (KBr pellets). The ^{1}H- and ^{13}C- NMR spectra were recorded on 400 MHz (Bruker DPX400) NMR spectrometer using $CDCl_3$ (7.25/77.03) as the internal reference. TLC was carried out on pre-coated silica gel 60 PF_{254} on aluminium sheets. For column chromatography silica gel, G-60 (70-230 mesh) was used.

Extraction and Isolation

The fresh leaves of *Polygonum plebejum* were collected from local market, Dhaka. The leaves were dried under 40ºC and ground into coarse powder. The powdered sample (400 gm) was separately extracted with petroleum ether, dichloromethane and ethyl acetate in Soxhlet apparatus at 50ºC. The extractive free powder was then extracted repeatedly with methanol in a reflux condition for half an hour. The methanol extract (M) was evaporated to dry mass (6.02 g) by using a rotary evaporator at 40ºC and then subjected to coloum chromatography. The column was eluted with 100% pet-ether, dichloromethane and methanol in order of increasing polarity. The eluted sample was monitored by TLC, samples having similar TLC behavior was combined together to give twelve different fractions, C_1 to C_{12} (15, 20, 13, 40, 18, 15, 10, 50, 90, 80, 60

and 900 mg, respectively). Among them the fraction C_6 (15 mg) was found to give single spot on TLC plate. So attempt was taken to characterize this compound.

Test for alcohol

Four gram of ceric ammonium nitrate was dissolved in 10 mL^{-1} of 2N HNO_3 on mild heating. A few crystals of C_6 were dissolved in 0.5 mL^{-1} of dioxane. The solution was added to 0.5 mL^{-1} of ceric ammonium nitrate reagent, diluted to 1 mL^{-1} with dioxane and shaken well. The solution was developed yellow to red color indicating the presence of a alcoholic hydroxyl group.

Test for steroid

Salkowski Reaction: A few crystals of C_6 were dissolved in chloroform and a few drops of concentrated sulfuric acid were added to the solution, a reddish color was seen in the upper chloroform layer.

Liebermann-Burchard reaction: A few crystals of C_6 were dissolved in chloroform and a few drops of concentrated sulfuric acid were added to it followed by the addition of 2-3 drops of acetic anhydride. The solution turned violet, blue and finally green.

Biological screening

Crude extract of methanol, two column fractions of methanol extract (C_3, C_9) and pure compound (C_6) were screened for their possible cytotoxicity by brim shrimp lethality bioassay and antimicrobial activity.

Cytoxicity study

Brine shrimp lethality bilassay (Persoone, 1980) technique was applied for the determination of cytotoxic property of crude extract of methanol, two column fractions (C_3, C_9) and pure compound C_6 of the leaves of the plant *P. plebejum*.

Preparation of positive control group

Vincristine sulphate was used as the positive control. Measured amount of the vincristine sulphate was dissolved in DMSO to get an initial concentration of 20 µg/ml from which serial dilutions are made using DMSO to get 10 µg/ml, 5 µg/ml, 2.5 µg/ml, 1.25 µg/ml, 0.625 µg/ml, 0.3125 µg/ml, 0.15625 µg/ml, 0.078125 µg/ml and 0.0390 µg/ml. Then the positive control solutions are added to the pre-marked vials containing ten living brine shrimp nauplii in 5 ml simulated sea water to get the positive control groups.

Preparation of negative control group

30 µl of DMSO was added to each of three pre-marked glass vials containing 5 ml of simulated seawater and 10 shrimp nauplii to use as control groups. If the brain shrimps in these vials show a rapid mortality rate, then the test is considered as invalid as the nauplii died due to some reason other than the cytotoxicity of the compounds.

Counting of nauplii

After 24 hours, the vials were inspected using a magnifying glass and the number of survived nauplii in each vial were counted. From these data, the percent (%) of lethality of the brine shrimp nauplii was calculated for teach concentration.

Antimicrobial assay

The disc diffusion method (Bauer et al., 1966) was used to test antimicrobial activity against thirteen bacteria and three fungi (Table-2). Solutions of known concentration (mg/ml) of the test samples were made by dissolving measured amount of the samples in calculated volume of solvents. Dried and sterilized filter paper disc (6 mm diameter) were then impregnated with known amounts of the test substances using micropipette. Discs containing the test material were placed on nutrient agar medium uniformly seeded with the test microorganisms. Standard antibiotic discs (kanamycin 30 µg/disc) and blank discs (impregnated with solvents) were used as a positive and negative control. These plates were then kept at low temperature (4°C) for 24 h to allow maximum diffusion. There is a gradual change in concentration in the media surrounding discs. The plates were then incubated at 37°C for 24 h to allow maximum growth of the organisms. The test materials having antimicrobial activity inhibited the growth of the microorganisms and a clear, distinct zone of inhibition was visualized surrounding the medium. The antimicrobial activity of the

test agent was determined by measuring the diameter of zone of inhibition expressed in millimeter. The experiment is carried out three times and the mean of the reading is required (Bauer et al., 1966).

RESULTS AND DISCUSSION

Compound C_6 was a white amorphous solid, soluble in diclromethane and chloroform (Rowshanul MH et al., 2007), m.p. 155-160ºC and showed positive results in alcohol test and in all the tests of steroids**.** IR (KBr) u_{max}: 3450, 2920, 2850, 1655, 1455, 1365 and 1040 cm^{-1}. The ^{1}H-NMR and ^{13}C-NMR data C_6 are summarized in Table-1.

Cytotoxicity study

Following the procedure of Mayer (Larson, 1988), the lethality of M, C_3, C_9 and C_6 to brine shrimp was determined on *A. salina*. The LC_{50} obtained from the best-fit line slope were found to be 2.82 µg/ml, 1.26 µg/ml, 3.98 µg/ml and 1.20 µg/ml for the test samples of M, C_3, C_9 and C_6 respectively.

Antimicrobial assay

The methanol crud extract (M), column fraction C_3, column fraction C_9 and pure compound C_6 of the leaves of *P. plebejum* were screened against thirteen bacteria and three fungi (Table 2) for antimicrobial activities by disc diffusion method.

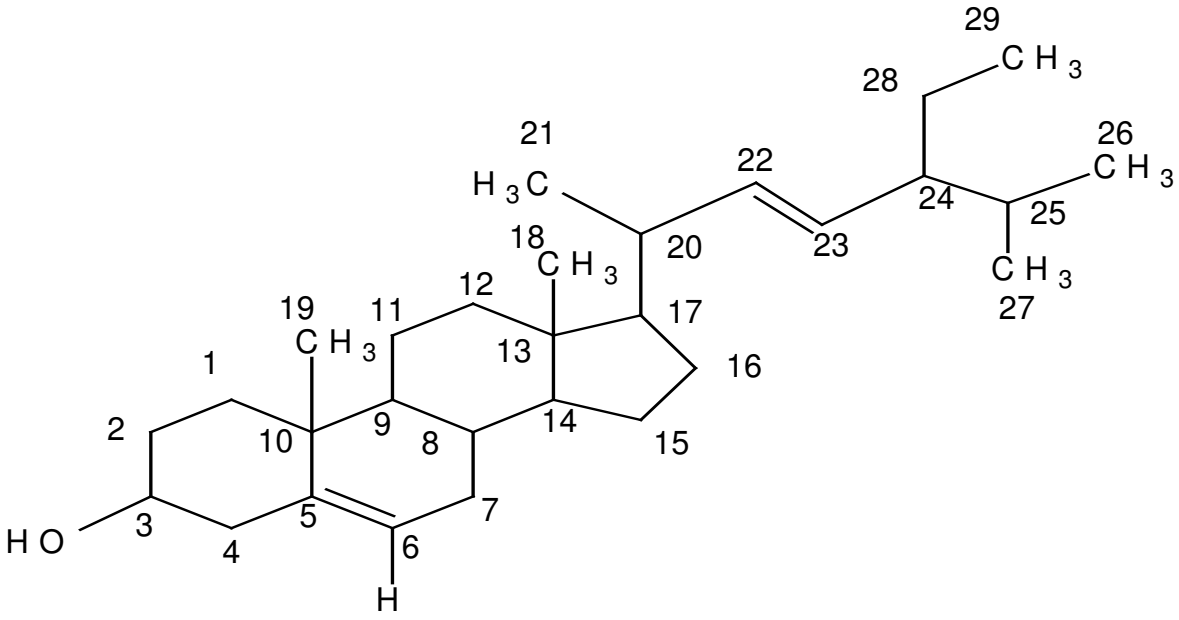

Fig. 1: The chemical structure of C_6.

Table -1: ^{1}H-NMR and ^{13}C-NMR data of C_6

Position	δ_H	δ_C
1		32.9
2		34.5
3	3.25 (tdd j = 4.5 MHz)	79.0
4		42.0
5		154.6
6	5.14 (1H, m)	124.5
7		31.2
8		28.7
9		42.0
10		39.6
11		19.4
12		31.9
13		40.8
14		47.7
15		21.4
16		21.3
17		48.2
18	1.07 (3H, s)	18.3
19	1.26(3H, s)	18.2
20		33.4
21	0.91(3H, s)	17.4
22	4.62 (1H, m)	107.1
23	4.16 (1H, m)	139.5
24		47.7
25		30.6
26	1.01 (3H, s)	20.2
27	1.00(3H, s)	20.2
28		25.4
29	0.97 (3H, s)	12.2

δ_H = chemical shift values in ^{1}H-NMR spectrum; δ_C = chemical shif values in ^{13}C-NMR spectrum

Table 2: Antimicrobial activities of the different parts of methanol extract of the leaves of *Polygonum plebejum*.

Test Bacteria and Fungi	MCE (400 µg/disc)	CF-C_3 (400 µg/disc)	CF-C_9 (400 µg/disc)	PC (400 µg/disc)	Kanamycin (30 µg/disc)
Bacteria					
BC	10	10	9	10	35
BM	10	10	-	10	35
BS	9	10	-	10	36
SA	10	9	8	10	35
SL	10	10	8	9	35
EC	12	10	-	8	35
PA	10	8	-	9	36
SP	8	12	9	12	36
ST	10	10	10	10	36
VM	10	10	9	10	35
VP	8	9	-	8	35
SD	12	12	-	10	36
SB	10	12	10	12	35
Fungi					
CA	10	12	-	12	35
AN	12	12	12	12	35
SC	12	10	9	10	35

BC=*Bacillus cereus (BTCC-19),* BM=*Bacillus megaterium (BTCC-18),* BS=*Bacillus subtilis,* SA=*Staphylococcus aureus,* SL=*Sarcina lutea,* EC=*Escherichia coli (BTCC-172),* PA=*Pseudomonas aureus (BTCC-1252),* SP=*Salmonella paratyphi,* ST=*Salmonella typhi,* VM=*Vibrio mimicus,* VP=*Vibrio parahemolyticus,* SD= *Shigella dysenteriae,* SB= *Shigella boydii* ,CA=*Candida albicans,* AN=*Aspergillus niger,* SC=*Sacharomyces cerevacae,* MCE=Methanol Crud Extract, CF=Column Fraction, PC=Pure Compound, "-" indicates no sensitivity.

From the positive tests of for steroids and alcohols given by the C_6 was assumed to be a sterol. The melting point of C_6 (160ºC) was in a good agreement with the melting point of stigmasterol in the literature (Holland et al., 1976). In ^{1}H-NMR spectrum of C_6, H-3 proton appeared as a triplet of a double of a double doublet (tdd) at δ 3.25 (J = 4.5 and 1.1 MHz) and H-6 olefinic proton showed a multiplet at δ 5.14. Two olefenic protons appeared doenfield at δ 4.14 (m) and δ 4.61 (m) which were identifcal with the chemical shift of H-22 and H-23, respectively of stigmasterol (Li et al., 2006). Six methyl protons also appeared at δ 1.07, δ 1.26, δ 0.91, δ 1.01, δ 1.00 and δ 0.97. ^{13}C-NMR data of C_6 was also quite similar with the data in the literature of sigmasterol (Conolly and Hill, 1994)

Cytrocity study

The comparison with positive control (vincristine sulphate, LC_{50} value 0.33 µg/mL), the pure compound as well as crude extract and column fractions showed potential cytotoxic activities.

Antimicrobial assay

Pure compound C_6 showed poor activity but crude extract and column fractions C_6 and C_9 exhibited moderate to significant activity against most of the test bacteria and fungi.

CONCLUSION

From these physical, chemical and spectral evidences C_6 was confirmed as stigmasterol (Fig. 1). The cytotoxicity exhibited by the pure compound and crude methanol extract of leaves is promising and they might have antitumor or pesticidal compounds (Meyer et al., 1982). However, this can not be confirmed without further higher and specific tests.

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