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Isolation of Flaccidine, 3-Sitostenone and β-Sitosterol-3-O-β-D-glucopyranoside from *Polygonum hydropiper* and Evaluation of their Antimicrobial Activities

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Flaccidine, 3-sitostenone and β-sitosterol-3-O-β-D-glucopyranoside were isolated from aerial part of medicinal plant *Polygonum hydropiper*. These compounds were isolated upon repeat column chromatography, HPTLC, RP-18 reverse phase column of dichloromethane and ethyl acetate fractions of the crude methnolic extract. The structures of the isolated compounds were identified by spectroscopic technique. Antimicrobial activities of these compounds against some of bacteria and phyto-pathogenic fungus have been investigated in this study.

Keywords: *Polygonum hydropiper*, Flaccidine, 3-Sitostenone, β-Sitosterol-3-O-β-D-glucopyranoside, Antimicrobial activities.

INTRODUCTION

Polygonum hydropiper is an annual herb, which is important for the medicinal and economic point of view and growing to 2-3 feet high at wet places in tropical region [1]. Medicinal chemistry is the discipline which is concerned in determining the influence of chemical structure on biological activity. Medicinal chemistry developed from an empirical one involving synthesis of organic compounds is based largely on the modification of structure and identification of their biological activity. This chemistry also involves the discovery, development, interpretation and the identification of mechanism of action of biologically active compounds at the molecular level. It was found that *Polygonum hydropiper* is very important considering its antimicrobiological activities [2,3].

In Bangladesh, these plants are also used as an insecticide. In agriculture, it is used as a poison for the insects [1]. The farmer used the dry leaves with the seeds to store food grains; the leaves juice is used to spray on wheat and/or paddy field as insecticidal agent. Different isolated ccompounds of *Polygonum hydropiperare* are used for the treatment of various diseases as well as to kill the harmful insects [4-6]. These harmful insecticides may be replaced by plant-derived active principles which will be environment-friendly and economically profitable [7]. The treatment of infectious diseases still remains an important and challenging problem. The search of novel antimicrobial agents is a field of current and growing interest [8,9].

From the literature review it was found that this plant is very important considering its various biological activities such as antibacterial, antifungal, anti-inflammatory, fertility retarding properties and other medicinal and insecticidal activities [10,11]. *Polygonum hydropiper* grow in marshy lands, where the fish grow side by side. So it is not so much harmful for lower animals and it can be used safely as insecticide [12,13]. It is very much essential to study more extensively on this valuable plant of Bangladeshi origin [1]. This concentrative study may contribute a lot to the treatment of numerous diseases. So, it will be very challenging and interesting to work on isolation, characterization and bioassay studies on constituents of *Polygonum hydropiper* [14,15].

EXPERIMENTAL

Melting points were determined on BUCHI digital melting point apparatus (model- 535). Optical rotations were measured on JASCO polarimeter (model P-360), with a 10 cm cell and it was measured in methanol at specified temperatures and concentrations. Ultraviolet spectra were recorded in methanol on HITACHI spectrophotometer (model U-3200). Infrared spectra were measured as KBr discs on SHIMADZU FTIR

spectrophotometer (model 8900). All the chemicals and solvents used in the reactions were of AR grade and obtained from commercial sources (Merck, Germany). TLC chromatograms were viewed under ultraviolet light at 254 nm for fluorescence quenching spots and at 366 nm for fluorescent spots. ^{1}H NMR, ^{13}C NMR spectra were recorded in deuterated solvents ($CDCl_3$, C_5D_5N) on Bruker Avance spectrometers equipped with 500 & 300 and 150 & 125 MHz, respectively. Residual proton of the solvent were used as an internal standard to measure the chemical shifts (δ) and these were measured in ppm relative to $CDCl_3$ (δ_H 7.25, δ_C 77.2) and C_5D_5N (δ_H 8.62, 7.29, 7.68, δ_C 149.9, 123.7, 135.9) and coupling constants (*J*) are given in Hz. Electron impact mass spectrometry (EI-MS) was scanned on Joel D-300 mass spectrometer. High resolution electron spin ionization mass (HR-ESI-MS) were measured on Bruker (ULTRA FLEX III TOF/TOF) mass spectroscopy. All these analysis were performed in various laboratories of H.E.J. Research Institute of Chemistry, International Center for Chemical and Biological Science (ICCBS), University of Karachi, Pakistan. The structures of the isolated compounds were elucidated with the help of extensive IR, UV, MS, 1D and 2D ^{1}H & ^{13}C NMR spectroscopic techniques.

Collection of plant: The plants *Polygonum hydropiper* were collected from the campus area of Chittagong University of Engineering & Technology (CUET), Raozan, Chattogram, Bangladesh.

Preparation of sample: The dust free air dried plants (13 kg) was pulverized and macerated in methanol (60 L × 3) at room temperature (27-33 ºC) for 3 days. Whatman No.1 filter paper was used to separate the extract of plant. The extracts were concentrated to dryness. The dried filtrates (crude extract) were combined and used for further phytochemical analysis.

Isolation and characterization: The crude methanol extract was suspended in distilled water and fractionated by solvent-solvent extraction with *n*-hexane, dichloromethane, ethyl acetate and *n*-butanol, sequentially. The dichloromethane fraction (98.0g) from crude methanolic extract was subjected to repeated column chromatography, HPTLC, RP-18 reverse phase column and by applied these techniques where flaccidin (**1**) and 3-sitostenone (**2**) were isolated from this fraction. The ethyl acetate soluble fraction (24 g) was also subjected to column chromatography and isolated a well-known compound β-sitesterol-3-O-β-D-glucopyranoside (**3**).

Antimicrobial activity: The compounds, flaccidine, 3-sitostenone and β-sitosterol-3-O-β-D-glucopyranoside were tested for their antibacterial and antifungal activities by filter paper disc method using nutrient broth medium (contained g/L: beef extract 30 g; casein hydrolyzate 17.5 g; soluble starch 1.5 g; pH 7.4). The Gram-positive and Gram-negative bacteria utilized in this study consisted of *S. aureus*, *E. coli* and fungus *Candida albicans*. In the filter paper disc method, sterile paper discs (0.5 mm) impregnated with compound dissolved in dimethyl formamide (DMF) at concentration 200 µg/mL were used. Then, the paper disc impregnated with the solution of compound tested was placed on the surface of media inoculated with the microorganism. The plates were incubated at 37 ºC for sufficient period of time for proper incubation and noted.

RESULTS AND DISCUSSION

Flaccidine (1): Dichloromethane fraction of methanolic extract was subjected to column chromatography over flash silica gel column chromatography with ethyl acetate/dichloromethane (1:19) as mobile phase followed by reverse phase RP-18 silica gel with MeOH/H_2O (7:3) eluent and further purified by Sephadex LH 20 column with MeOH afforded a pure known compound flaccidine (**1**) as yellowish gammy materials.

Compound **1** gave a deep yellow spot on TLC after spraying with ceric sulphate reagent, which indicated compound **1** might be flavonoid. The IR spectrum of compound **1** displayed absorption at 3636, 3520 (OH), 1741 (ester), 1665 (C=O), 1620, 1600 (β-pyrone) [15] and UV spectrum showed the absorption band at 348, 284 nm indecated the presence of a flavone skeleton [16,17]. The molecular formula of compound **1** deduced as $C_{23}H_{22}O_9$ by EI-MS at *m/z* 442 and confirmed by HREI-MS which showed peak at *m/z* 442.1275 (calcd. 442.1264 for $C_{23}H_{22}O_9$). The FAB-MS (positive ion mode) showed ion peak at *m/z* 443 $[M + H]^+$ consisted with the formula $C_{23}H_{22}O_9$. Moreover, the fragment ion at *m/z* 360 $[M\text{-}C_5H_6O]^+$, 345 $[360\text{-Me}]^+$, 331, 316, 83 $[C_4H_7CO]^+$, 55 $[83\text{-CO}]^+$ revealed the presence of angelate moiety group.

The ^{13}C NMR ($CDCl_3$, 125 MHz) BB and DEPT spectrum showed resonances for 23 carbons at which five methyl, five methine and thirteen quaternary carbons (Table-1). The ^{13}C NMR spectrum showed two resonance at down field region at δ_C 155.3 (C-2) and 131.1 (C-3) for olefinic carbon, were characteristic of flavone skeleton. The downfield chemical shift at δ_C 175.9 assigned for the C-4 carbonyl carbon indicated the presence of chelated hydroxyl functionality at δ_C 157.6 (C-5). The other resonance at δ_C 98.9, 155.8, 126.8, 148.9 and 105.1 were assigned to the C-6, C-7, C-8, C-9 and C-10. In ^{1}H NMR ($CDCl_3$, 500 MHz) spectrum appeared as a singlet at δ_H 6.42 for an aromatic proton, a double doublet at δ_H 7.52 (*J* = 2.0 and 9.0 Hz), and two doublets at δ_H 6.97 (*J* = 2.0 Hz) and 7.40 (*J* = 9.0 Hz) were assigned to H-6, H-6′, H-2′ and H-5′ respectively. Three singlet appeared at δ_H 3.90 (s), 3.96 (s), 4.00 (s) assigned for three methoxy groups and two signal at δ_H 2.07 (dq, *J* = 7.0 & 1.50) and 2.06 (dq, *J* = 1.5 & 1.5) were assigned to H_3-4″ and H_3-5″ methyl protons. With the help of HSQC and HMBC spectrum, compound **1** (Fig. 1) was identified as flaccidine and all the spectral data (Table-1) and physical data were compared and found almost identical with the reported data isolated from *Polygonum flaccidum* [15,16].

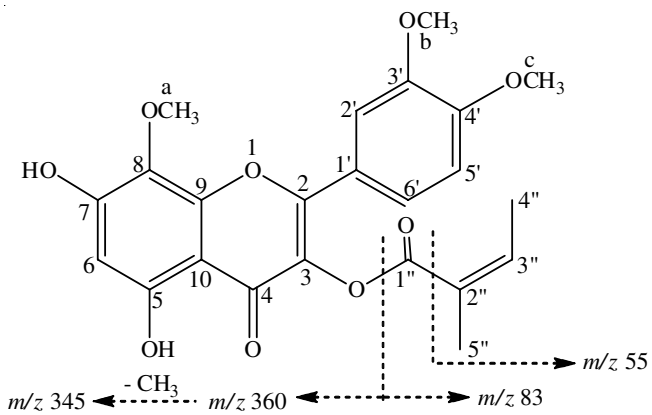

Fig. 1. Mass fragmentation of compound **1**

TABLE-1
^{13}C NMR AND ^{1}H NMR CHEMICAL SHIFT AND MULTIPLICITY OF COMPOUND **1** AND REFERENCE

S. No.	Mult	Compound **1**		[Ref. 15]	
		δ_C	δ_H (m, *J* in Hz)	δ_C	δ_H (m, *J* in Hz)
1	–	–	–	–	–
2	C	155.3	–	155.31 s	–
3	C	131.1	–	131.04 s	–
4	C	175.9	–	175.94 s	–
5	C	157.6	–	157.50 s	–
6	CH	98.9	6.42 (s)	98.89 d	6.43 s
7	C	155.8	–	157.50 s	–
8	C	126.8	–	126.74 s	–
9	C	148.9	–	148.85 s	–
10	C	105.1	–	105.08 s	–
1'	C	122.1	–	122.01 s	–
2'	CH	111.1	6.97 (d, 9.0)	110.99 d	7.43 d(2)
3'	C	148.1	–	148.11 s	–
4'	C	151.9	–	151.78 s	–
5'	CH	110.9	7.40 (d, 2.0)	110.73 d	7.00 d(9)
6'	CH	122.0	7.52 (dd, 2,9)	122.00 d	7.56 dd(2,9)
1'	C	167.6	–	164.56 s	–
2'	C	126.1	–	126.04 s	–
3'	CH	142.6	6.33 (qq, 7.0, 1.5)	142.86 s	6.35 qq (7, 1.5)
4'	CH_3	16.2	2.07 (dq 7.0, 1.5)	20.66 q	2.10 dq (7, 1.5)
5'	CH_3	20.6	2.06 (dq, 1.5,1.5)	16.19 q	2.06 dq (1.5, 1.5)
a*	-OCH_3	61.9	3.90 (s)	61.30 q	3.91 s
b*	-OCH_3	56.0	3.96 (s)	56.03 q	3.96 s
c*	-OCH_3	55.9	4.00 (s)	55.83 q	4.00 s
OH-5	–	–	12.04 (s)	–	12.04 s

*Interchangeable

3-Sitostenone (2): Dichloromethane fraction was subjected to column chromatography over flash silica gel with ethyl acetate/hexane (1:9) followed by Sephadex LH 20 column and HPTLC run two times with dichloromethane yielded a pure known compound 3-sitostenone (**2**).

The EI-MS of molecular ion peak $[M]^+$ at *m/z* 412 and confirmed by HR-EI-MS, peak at *m/z* 412.3716 (calcd. 412.3705 for $C_{29}H_{48}O$) was consisted with the molecular formula of $C_{29}H_{48}O$ with six degrees of unsaturation. The ^{13}C NMR ($CDCl_3$, 150 MHz) showed 29 resonances established for a steroid compound (Table-2). Two low field resonances at δ_C 123.7 and 171.9 assigned for olifinic carbon C-4 and C-5, respectively. Another low field resonance at δ_C 199.8 assigned for a ketone carbonyl carbon C-3. The ^{1}H NMR ($CDCl_3$, 500 MHz) showed the steroid and tri-terpenoied characteristic resonance with six methyl signals in high field region. A characteristic low field resonance at δ_H 5.70 as a singlet was assigned for olefinic proton to the C-4 methine proton. Compound **2** (Fig. 2) was identified as sigmast-4-en-3-one. The spectral data of the compound were found similar with the reported values of 3-sitostonone, which was previously isolated from the sponge *Geodia cydonium* [18].

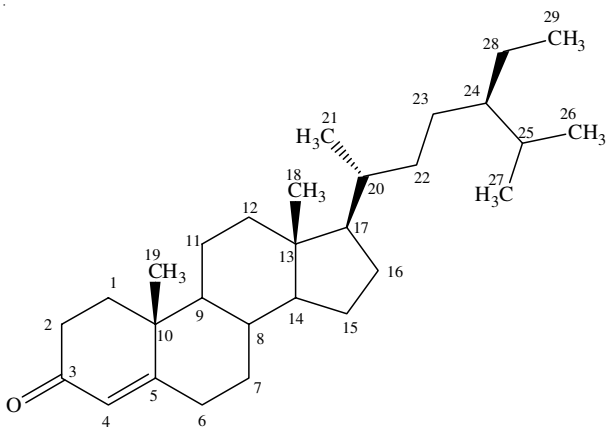

Fig. 2. 3-Sitostenone (**2**)

β-Sitosterol-3-O-β-D-glucopyranoside (3): The ethyl acetate soluble fraction was subjected to repeated column chromatography over silica gel eluted with ethyl acetate/hexane (1:6) followed by Sephadex LH 20 column yielded a known compound β-sitosterol-3-O-β-D-glucopyranoside (**3**) as colourless amorphous solid. The FAB-MS (negative-ion mode) showed the $[M-H]^+$ at *m/z* 575.0205 corresponded to the molecular formula $C_{35}H_{60}O_6$ (calcd. 575.0199) with six degrees of unsaturation. The ^{13}C NMR (C_5D_5N, 125 MHz) showed a downfield resonance at δ_C 102.5 was assigned to anomaric carbon C-1′ and another signal appeared at δ_C 78.5 was assigned to the oxygenated carbon C-3 (Table-2). A group of resonances for six carbons appeared at δ_C from 62.7 to 78.6 indicating the presence of a glucose moiety. The ^{1}H NMR spectrum (C_5D_5N, 500 MHz) showed a downfield resonance at δ_H 5.33 (d, $J_{1',2'}$ = 7.7 Hz) assign for an anomeric proton H-1′ indicating the presence of a β-sugar. A broad triplet at δ_H 5.05 was assigned to an olefinic proton H-6. A resonance as double doublet at δ_H 3.99 (*J* = 10.5, *J* = 5.4 Hz) identified for H-3 proton. Six 3H singlets appeared at δ_H 0.99, 0.94, 0.92, 0.89, 0.84 and 0.67 were corresponding to the H_3-21, H_3-19, H_3-29, H_3-26, H_3-27 and H_3-18 methyl protons, respectively. Finally, compound **3** (Fig. 3) was identified as β-sitosterol-3-O-β-D-glucopyranoside by comparing with the reported data [19].

TABLE-2
NMR DATA OF COMPOUNDS **2** AND **3**

S. No.	Compound **2**			Compound **3**		
	Mult.	δ_C	δ_H	Mult.	δ_C	δ_H
1	CH_2	35.7	2.11 (o)	CH_2	37.4	2.72 (dd, 11.5, 2.5Hz)
2	CH_2	33.9	2.00, 1.68 (o)	CH_2	30.1	2.21,1.72 (d,11.5Hz)
3	C	199.8	–	CH	78.5	4.29, 3.99 (dd, 8.0Hz)
4	CH	123.7	5.70 (s)	CH_2	39.3	2.46 (dd, 11.0 Hz)
5	C	171.9	–	C	140.8	-
6	CH_2	33.0	1.42 (o)	CH	138.8	5.05 (tbr, 2.5Hz)
7	CH_2	32.0	1.36 (o)	CH_2	32.1	1.52, 1.88 (overlap)
8	CH	35.6	1.34 (o)	CH	32.0	1.35 (overlap)
9	CH	53.8	0.89 (m)	CH	50.2	0.89 (s)
10	C	38.6	–	C	36.9	-
11	CH_2	21.0	1.34 (o)	CH_2	21.2	1.38,1.05 (overlap)
12	CH_2	39.6	1.71 (o)	CH_2	39.9	1.94,1.05 (overlap)
13	C	42.4	–	C	42.4	-
14	CH	55.9	1.03 (m)	CH	56.7	0.92 overlap
15	CH_2	24.2	1.43 (o)	CH_2	24.5	1.55, 1.03 (overlap)
16	CH_2	29.7	1.72(o)	CH_2	28.5	1.81, 1.23 (overlap)
17	CH	56.0	1.00 (o)	CH	56.1	1.07
18	CH_3	11.9	0.69 (s)	CH_3	11.9	0.63 (s)
19	CH_3	17.4	1.61 (s)	CH_3	19.4	0.88 (d)
20	CH	36.1	1.33 (o)	CH	36.3	1.36(overlap)
21	CH_3	19.0	0.88 (o)	CH_3	18.9	0.92 (d, 6.5Hz)
22	CH_2	34.0	1.34 (o)	CH_2	34.1	1.38,1.06 (o)
23	CH_2	25.9	1.11 (o)	CH_2	26.2	1.21 (o)
24	CH	45.8	0.90 (m)	CH	45.9	0.97 (o)
25	CH	29.0	1.62 (o)	CH	29.3	1.70 (o)
26	CH_3	19.9	0.77 (s)	CH_3	19.1	0.83 (s)
27	CH_3	18.7	0.79 (d)	CH_3	19.9	0.89(s)
28	CH_2	23.0	1.11 (o)	CH_2	23.3	1.26(o)
29	CH_3	12.0	0.81 (t)	CH_3	12.1	0.87 (o)
1'	–	–	–	CH	102.5	5.33 (d, 7.5Hz)
2'	–	–	–	CH	78.6	4.31 (d, 9.0Hz)
3'	–	–	–	CH	78.0	3.99 (o)
4'	–	–	–	CH	71.6	4.29 (d, 8.0Hz)
5'	–	–	–	CH	75.3	4.06 (t, 8.0Hz)
6'	–	–	–	CH_2	62.7	4.41, 4.56 (d, 9.5, 5.0Hz)

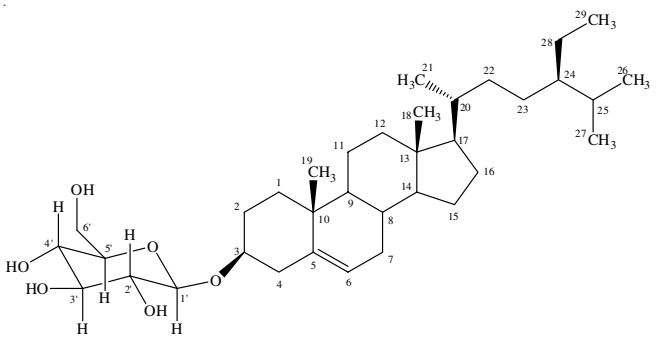

Fig. 3. β-Sitosterol-3-*O*-β-D-glucopyranoside (**3**)

Antimicrobial activity: The development of antimicrobial resistance in many pathogenic microbes possesses one of the most serious problems in the control of infectious diseases. All the test organisms are phytopathogenic, for that all steps of the work were done with high precaution and aseptic condition. The percentage inhibition of mycelia growth of the test fungus/bacteria was calculated by using following equation [16,20]:

$$\text{Inhibition (\%)} = \frac{C - T}{C} \times 100$$

where, C = Diameter of fungal/bacterial colony in control, and T = Diameter of fungal/bacterial colony in treatment.

To measure antibacterial activity, *Escherichia coli* and *Staphylococcus aureus* are selected as test organisms. Antimicrobial activities of flaccidine, 3-sitostenone and β-sitosterol-3-O-β-D-glucopyranoside are summarized in Table-3. The results showed that flaccidine, 3-sitostenone and β-sitosterol-3-O-β-D-glucopyranoside shows different antibacterial activities to a measurable extent. Screenings were conducted against phytopathogenic fungus, *Candida albicans*. This fungus is phytopathogens of important crop plants such as jute, chilli, brinjal, *etc*. Control of such pathogens by non-hazardous fungicides has been a major concern, especially as fungi gradually develop resistance to known fungicides. It is evident from the results presented in Table-3 that these compounds showed some antifungal activity. It was observed that different compound had different effects on these organisms. These observations suggested that these compounds played a significant role in the inhibition of micelial growth. It is found that this plant is very important considering its medicinal and insecticidal activities. However, for a clear understanding of the functions responsible for antibacterial activities of these compounds, more studies

TABLE-3
ANTIMICROBIAL ACTIVITIES OF FLACCIDINE, 3-SITOSTENONE AND
β-SITOSTEROL-3-O-β-D-GLUCOPYRANOSIDE (DIAMETER OF THE ZONE OF INHIBITION IN mm)

Compounds	Antibacterial activity		Antifungal activity
	Escherichia coli	*Staphylococcus aureus*	*Candida albicans*
Flaccidine	13	13	10
3-Sitostenone	14	13	8
β-Sitosterol-3-O-β-D-glucopyranoside	14	15	12
Standard	19	19	14
Control (DMF)	0	0	0

are needed to be performed with a series of analogous compounds against a series of bacteria.

Conclusion

From the results obtained in this study, it could be concluded that flaccidine, 3-sitostenone and β-sitosterol-3-O-β-D-glucopyranoside acts possesses remarkable antimicrobial activity. According to these findings, it could be said that the extract of *Polygonum hydropiper* acts as antifungal and antibacterial agents.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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