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Sterols and Sterol Glucoside from *Phyllanthus* Species

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Abstract

Stigmasterol, stigmasta-5,22-dien-3-*O*-*β*-D-glucoside, *β*-sitosterol, and sitosterol-3-*O*-*β*-D-glucoside were obtained from the organic solvent extractives of different parts of *Phyllanthus species P. niruri, P. reticulatus, P. emblica, and P. acidus*, through chromatographic separation. The structures of isolated compounds have been elucidated with the help of physical and spectroscopic studies. Medicinal importances of these species have also been discussed.

I. Introduction

The *Phyllanthus* species (Euphorbiaceae) available in Bangladesh are *P. niruri, P. reticulatus, P. emblica and P. acidus*. *P. reticulatus* is known to contain *β*-sitosterol[1] and *β*-sitosterol-3-*O*-*β*-D-glucoside[2] while *P. emblica* was recognized to contain *β*-sitosterol-3-*O*-*β*-D-glucoside[3]. On the other hand, *P. niruri*[4,5,6,7,8,9] and *P. acidus*[10,11,12] were reported neither to contain sterol nor sterol glucoside. All the *Phyllanthus* species of Bangladesh origin were scrupulously studied. Stigmasterol and stigmasterol glucoside were isolated for the first time from both the leaves and stem bark of *P. reticulatus* in contrast to the previous report of the occurrence of only *β*-sitosterol-3-*O*-*β*-D-glucoside[2]. There is also a report[3] of isolation of *β*-sitosterol-3-*O*-*β*-D-glucoside from *P. emblica,* whereas the present studies achieved a success of the isolation and characterization of stigmasta-7,22-dien-3-*O*-*β*-D-glucoside for the first time. The literature survey reveals that there is no report of occurrence of any sterols in the fruits of *P. acidus*. However, the present work further reports here the isolation and identification of sitosterol-3-*O*-*β*-D-glucoside for the first time.from the fruits of *P. acidus.*

The common sterols occurring in plants are usually sitosterol, stigmasterol and campesterol. These are also predominantly supplied by vegetable oils. The nutritional value derives from the fact that the sterols have a similar structure to cholesterol and have the capacity to lower plasma cholesterol and LDL cholesterol. Since the morbidity and mortality from cardiovascular disease have been dramatically reduced by the use of cholesterol lowering drugs (statins), the interest in plant sterols lies in their potential to act as a natural preventive dietary product[13]. It is being thought that the leaf juice or fruits of *P. reticulatus, P. emblica* and *P. acidus* can be taken for reduction of blood plasma cholesterol. Isolation and characterization of sterols and sterol glucosides along with their medicinal importance are being reported here.

II. General Methods

All evaporations were carried out under reduced pressure at around 40ºC. Distilled solvents were always used throughout this investigation. Silica gel $60GF_{254}$ was used for thin layer chromatography (TLC) and vacuum liquid chromatography (VLC). IR spectra were recorded in a Shimadzu IR-470 spectrometer using KBr pellet. ^{1}H and ^{13}C NMR spectra were recorded in a BRUKER (400 MHz) spectrometer in deuterated chloroform using TMS as an internal standard. Mass spectrum was taken by gas chromatography-mass spectrometer.

III. Materials and Methods

Plant Materials: The whole plants of *P. reticulatus* was collected from Curzon Hall campus of the University of Dhaka. The leaves of *P. emblica* was collected from the garden of Dhaka Nagor Bhaban campus and the fruits of *P. emblica* and *P. acidus* were collected from Dhaka New market. The leaves, stembark and pulp of the fruits were individually cleaned, dried and powdered by a cyclotec grinder (200mesh) and finally extracted in a soxhlet apparatus with petroleum ether followed by ethyl acetate.

IV.Extraction and Isolation

The extracts were filtered, concentrated in *vacuo* and were subjected to VLC. The column was first eluted with 100% pet.ether (40º-60ºC) and then eluted with mixtures of pet.ether and increasing amount of ethyl acetate and finally with methanol. Each eluate was monitored by TLC and the fractions of similar TLC behavior were combined together and designated. The fraction F_3 (pet.ether extract) of the stem bark of *P. reticulatus* gave white crystalline substance (10mg) and marked as PR1. From the fraction F_4 (ethyl acetate extract) of the leaves of *P. reticulatus* white crystalline substance (8mg) was obtained and designated as PR2. The fraction F_5 (ethyl acetate extract) of the leaves of *P. emblica* was concentrated and left over-night to yield white crystals (5mg) and marked as PE3 while the fraction F_6 (ethyl acetate extract) of the fruit pulp of *P. acidus* gave white crystalline substance (10mg) and designated as PA4.

V. Physical and Spectral Properties of the Isolated Compounds

Compound PR1: White crystals, m.p: 168º-169ºC, soluble in chloroform, R_f value 0.60 (pet.ether: ethyl acetate, 4:1 as mobile phase), **IR, γ KBrmax cm^{-1}:** 3450, 2900, 2850, 1630, 1450, 1095, 720. **GC-MS, m/e :** 412.4 (**M$^+$,** $C_{29}H_{48}O$), 396.4, 382.0, 354.3, 329.3, 303.3, 273.2, 255.2, 231.2, 213.1, 185.1, 163.1, 145.1, 125.1, 107.1, 81.1, 70.1, 55.1, 43.1.

Compound PR2: White crystals, m.p: 290º-292ºC, soluble in a mixture of chloroform and methanol, R_f value 0.49 (chloroform: methanol, 4:1 as mobile phase), **IR, γ KBr$_{max}$ cm^{-1}:** 3450-3350, 2920 & 2850, 1680, 1610, 1450, 1350, 1280, 1150, 1120 & 1050, 970 & 955.

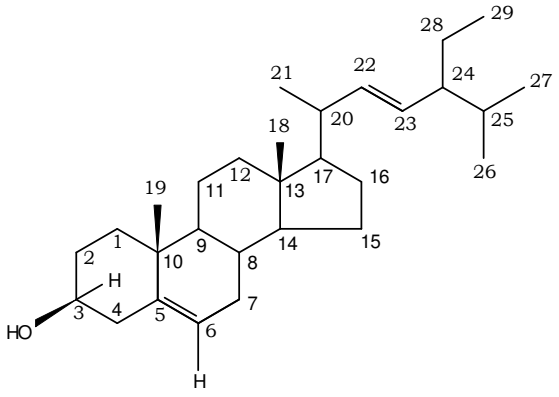
Compound PE3: White crystals, m.p: 288º-290ºC, soluble in a mixture of chloroform and methanol, R_f value 0.50 (chloroform: methanol, 4:1 as mobile phase), **IR γ KBr$_{max}$ cm^{-1}:** 3510, 1690, 1605 & 1570, 1190, 1110 & 1060, 980, 805.

Compound PA4: White crystals, m.p: 272º-274ºC, soluble in a mixture of chloroform and methanol, R_f value 0.48 (chloroform: methanol, 4:1 as mobile phase), **IR γ KBr$_{max}$ cm^{-1}:** 3400, 2920 & 2850, 1705 and 1620, 1445, 1360, 1257, 1160, 1105 & 1020.

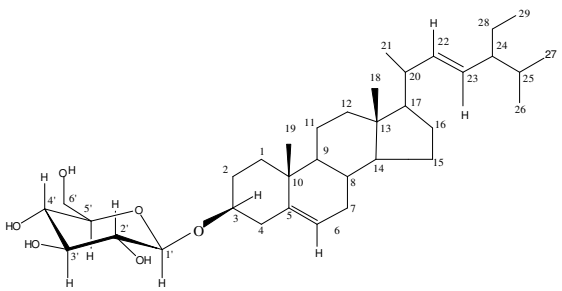
VI. Results and Discussion

Compound PR1 was isolated as white crystals from pet.ether extract having m.p. 168^0-169^{0}C. The IR spectrum showed an absorption band at 3450cm^{-1} due to the presence of –OH stretching. The bands at 1630 cm^{-1} was suggestive of a >C=C< moiety. The absorption bands at 1095 cm^{-1} was indicative of the presence of –C-O-C- structural feature in the compound[14]. The absorption bands at 920 cm^{-1} supported its steroidal nature[15]. The ^{1}H NMR spectrum showed a downfield 1H intensity at δ_H 5.34 ppm, indicative of olefinic proton (H-6)[16]. It also showed olefinic protons at δ_H 5.14 ppm and at δ_H 5.04 ppm (H-22 and H-23). The spectrum had a multiplet at δ_H 3.51 ppm indicative of an oxymethine proton (H-3)[16]. The spectrum showed the presence of six methyl protons at δ_H 0.690 (H-18), 1.00 (H-19), 0.82 (H-27), 0.84 (H-26), 0.86 (H-21) and 0.82 (H-29) respectively. ^{13}C NMR spectrum revealed the presence of 29 carbons suggestive of a steroidal compound. The signals at δ_C 141.01 (C-5), 42.41 (C-13) and 36.17 (C-10) were assigned to three quarternary carbons. The signal at δ_C 71.85 ppm was for oxymethine carbon (C-3). Two olefinic carbons signals at δ_C 141.0 and 121.73 ppm were for (C-5 and C-6) and signals at δ_C 138.00 and 128.95 ppm for (C-22 and C-23)[16]. The mass spectral data gave a molecular formula $C_{29}H_{48}O$, m/e 412 (M^+) and base peak at m/e 43.1. These m/e values, ^{1}H NMR and ^{13}C NMR spectral data are in well agreement with the reported spectral data[16,17,18,19] and thus PR1 is characterized as stigmasterol. Although the compound PR1 is a known compound but this compound constitute its first isolation from *P. reticulatus* .

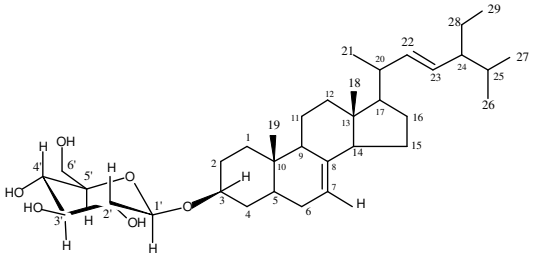
Compound PA4 was isolated as white crystalline solid from ethyl acetate extract of the fruits of *P. acidus*. ^{1}H NMR spectrum showed the olefinic protons resonated as a broad singlet at δ_H 5.09 ppm indicative of the presence of a >C=C< in the ring system. A number of multiplet signals either at or between δ_H 1.12 -2.14 ppm were informative of different methylene and methine protons in the structure of PA4 which does not contain double bond between C-22 and C-23 whereas the compounds PR1, PR2 and PE3 were found to contain double bond in the side chain between C-22 and C-23. So, the compound PA4 has been identified as sitosterol-*β*-D-glucoside based on its m.p (272-274^{0}C). IR, ^{1}H NMR and ^{13}C NMR spectral data and a close agreement with the reported values [1,2,3,16,22,23].



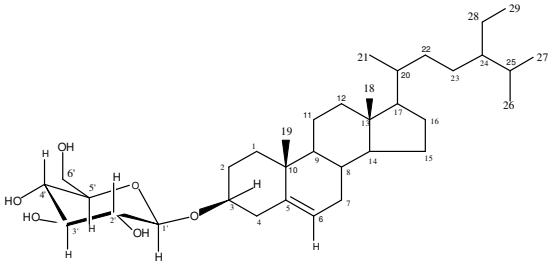
PR1



PR2



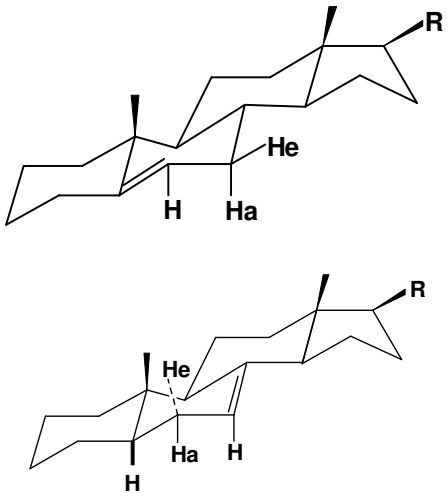
PE3



PA4

Compound PR2 was isolated from ethyl acetate extract of the leaves of *P. reticulatus* and compound PE3 was also isolated from ethyl acetate extract of the leaves of *P. emblica*. ^{1}H NMR spectrum of PE3 showed the olefinic protons resonated as two doublets of doublet at δ_H 5.01 (C-22) and 4.76 (C-23) ppm (J=16,8Hz) exhibitive of *trans* olefinic protons plus adjacent methine proton. The doublet of doublet at δ_H 4.76 ppm seems to be of higher intensity compared to the doublets of doublet at δ_H 5.01 ppm. This higher intensity was only possible if there were less interaction from the adjacent groups. The protons of its sugar moiety resonated at 3.13-4.39 ppm.The compound PR2 almost completely corresponded to the data for stigmasterol (PR1) with the exception of the signals between δ_H 2.33 - 4.33 ppm typical for a sugar moiety.

It is important to mention here that olefinic proton at C_6 (H-6) of compound PR2 resonated at δ_H 5.29 ppm as a broad singlet because the two equivalent adjacent protons are in rapid exchange between 'a' and 'e' whereas that at C_7 (H-7) of compound PE3 appeared as a signal at δ_H 5.35 ppm with a doublet like pattern at its apex[16] possibly because of either/both the slow exchange between the 'a' & 'e' or/and the change of the position of Ha due to its close proximity of 19-CH_3 group.



The TLC examination of PR2, R_f=0.49 (CH_3Cl:MeOH, 4:1) and PE3 R_f =0.50 (CH_3Cl:MeOH, 4:1), m.p of PR2 290^0-292^0C and PE3 288^0-290^0C, showed that these compounds are different. The m.p. of this compound are of almost similar magnitude. However, the compound PE-3 with an olefinic double bond at C-7 in contrast to that at C-6 undergoes more steric interference between 6- methylene groups and 19- CH_3 group. As a result, there originates internal energy weaken the intercrystalline force suggesting its lower m.p.

Moreover, the IR spectrum of PR2 showed carbon double bond absorption band at 1610 cm^{-1} whereas PE3 showed carbon carbon double bond absorbption band at 1605 cm^{-1}. All of these sterols showed absorption bands between 920-980 cm^{-1} typical for sterols[15]. A scrupulous study of the IR spectra confirmed that the compounds PR2 and PE3 are different. In addition to these, their ^{1}H & ^{13}C spectral data are also different (Table 1 & 2). All these considerations suggest that PR2 must be different from PE3.The anomeric proton and carbon in PR2, PE3 and PA4 appears a sharp singlet at δ_H 4.11 -4.39 ppm and δ_C 100.74- 100.98 ppm respectively.

VII. Significance of the Sterols and Sterols Glucoside

Sterols are known to reduce the blood plasma cholesterol[20]. These sterols are believed to interfere the esterification of cholesterol. There is a report[20] that these sterols usually bind with the intestinal mucosal cell and interfere the flow of cholesteryl ester through an interaction with lipoprotein towards the blood vessel. This idea prompts us to think of the biological activity of stigmasterol and stigmasterol glucoside. Since these sterol glucoside contain more hydrophilic part (glucose moiety), this part of these glucosides is being thought to create effective hindrance to the esterification of cholesterol and thus resulting in the inhibition of entry of cholesterol into the blood vessel.

Table. 1. ^{1}H NMR of compound PR1, PR2, PE3 and PA4 and comparison with those of published data[21,22,23]

Hydrogen atom	Ref. Compound[21]	PR1	PR2	PE3	PA4
1	1.25 (m,2H)	1.15 (m,2H)	1.18 (m,2H)	1.28 (m,2H)	1.25 (m,2H)
2	1.44 (m,2H)	1.44 (m,2H)	1.40 (m,2H)	1.51 (m,2H)	1.33 (m,2H)
3	3.51 (m,1H)	3.51 (m,1H)	3.77 (m,1H)	3.84 (m,1H)	3.13 (m,1H)
4	2.22 (m,2H)	1.98 (m,2H)	1.93 (m,2H)	2.14 (m,2H)	2.14 (m,2H)
5	--	--	--	1.93 (m,2H)	--
6	5.35 (bs,1H)	5.34 (bs,1H)	5.29 (bs, 1H)	1.58 (m,2H)	5.09 (bs,1H
7	1.85 (m,2H)	1.85 (m,2H)	1.81 (m,2H)	5.35 (d,1H)	1.73 (m,2H)
8	1.44 (m,1H)	1.44 (m,1H)	1.42 (m,1H)		1.22 (m,1H)
9	1.44 (m,1H)	1.44 (m,1H)	1.42 (m,1H)	1.51 (m,1H)	1.22 (m,1H)
10	--	--	--	--	--
11	1.44 (m,2H)	1.44 (m,2H)	1.42 (m,2H)	1.51 (m,2H)	1.33 (m,2H)
12	1.44 (m,2H)	1.44 (m,2H)	1.42 (m,2H)	1.51 (m,2H)	1.33 (m,2H)
13	--	--	--	--	--
14	1.44 (m,1H)	1.44 (m,1H)	1.42 (m,1H)	1.51 (m,1H)	1.22 (m,1H)
15	1.53 (m,2H)	1.53 (m,2H)	1.77 (m,2H)	1.58 (m,2H)	1.73 (m,2H)
16	1.53 (m,2H)	1.53 (m,2H)	1.77 (m,2H)	1.58 (m,2H)	1.73 (m,2H)
17	1.54 (m,2H)	1.53 (m,2H)	1.77 (m,2H)	1.58 (m,2H)	1.73 (m,2H)
18	0.69 (s,3H)	0.69 (s,3H)	0.61 (s,3H)	0.70 (s,3H)	0.62 (s,3H)
19	1.00 (s,3H)	1.00 (s,3H)	1.02 (s,3H)	1.06 (s,3H)	0.94 (s,3H)
20	2.27 (m,1H)	2.27 (m,1H)	2.19(m,1H)	2.14 (m,1H)	1.32 (m,1H)
21	1.08 (d,3H)	0.86 (d,3H)	0.84 (d,3H)	1.01 (d,3H, J=6.4Hz)	0.84 (d,3H, J=6.3Hz)
22	5.15 (dd,1H, J=12&8Hz)	5.14 (dd,1H, J=12&8Hz)	5.08 (dd,1H, J=12&8Hz)	5.01 (dd,1H, J=12&8Hz)	1.73 (m,2H)
23	5.03 (dd,1H, J=12&8Hz)	5.04 (dd,1H, J=12&8Hz)	4.96 (dd,1H, J=12&8Hz)	4.76 (dd,1H, J=12&8Hz)	1.73 (m,2H)
24	2.23 (m,1H)	2.22 (m,1H)	2.21 (m,1H)	2.14 (m,1H)	1.12 (m,1H)
25	1.85 (m,1H)	1.85 (m,1H)	2.16 (m,1H)	1.98 (m,1H)	2.14 (m,1H)
26	0.68 (d,3H, J=6.8Hz)	0.84 (d,3H, J=6.8Hz)	0.75 (d,3H, J=6.8Hz)	10.93 (d,3H, J=6.4Hz)	0.75 (d,3H, J=7.7Hz)
27	0.84 (d,3H, J=6.8Hz)	0.82 (d,3H, J=6.8Hz)	0.74 (d,3H, J=6.8Hz)	0.84 (d,3H, J=7.1Hz)	0.73 (d,3H, J=1.6Hz)
28	1.25 (m,2H)	1.25 (m,2H)	1.18 (m,2H)	1.28 (m,2H)	1.33 (m,2H)
29	0.82 (t,3H, J=6.9Hz)	0.82 (t,3H, J=6.9Hz)	0.77 (t,3H, J=6.9Hz)	0.88 (t,3H, J=6.9Hz)	0.77 (t,3H, J=6.9Hz)
1′	--	--	4.33 (d,1H, J=7.8Hz)	4.39 (d,1H, J=7.8Hz	4.11 (d,1H, J=7.8Hz
2′	--	--	3.31 (m,1H)	3.31 (m,1H)	3.14 (m,1H)
3′	--	--	3.31 (m,1H)	3.51 (m,1H)	3.14 (m,1H)
4′	--	--	3.31 (m,1H)	3.31 (m,1H)	3.14 (m,1H)
5′	--	--	3.39 (m,1H)	3.35 (m,1H)	3.06 (m,1H)
6′	--	--	2.33 (m,1H)	3.13 (m,1H)	2.94 (m,1H)

Table. 2. ^{13}C NMR of compound PR1, PR2, PE3 and PA4 and comparison with those of published dat[21, 22, 23]

Carbon atom	Reference[21]	PR1	PR2	PE3	PA4
C-1	37.15	37.31	37.15	37.41	36.85
C-2	31.56	31.72	31.78	33.50	29.12
C-3	71.71	71.85	79.13	79.83	78.61
C-4	42.19	42.37	42.24	41.32	42.12
C-5	140.81	141.01	140.14	131.63	139.98
C-6	121.62	121.73	122.13	31.95	121.54
C-7	31.56	31.72	31.72	123.20	31.41
C-8	31.79	31.79	31.78	1142.31	31.46
C-9	50.02	50.19	49.84	51.71	49.83
C-10	36.16	36.17	36.63	38.25	36.27
C-11	21.12	21.11	19.81	21.72	20.21
C-12	39.57	40.10	38.65	40.11	38.20
C-13	42.10	42.41	42.24	41.80	41.88
C-14	56.76	56.12	56.66	56.19	56.36
C-15	24.27	23.12	24.19	24.81	23.79
C-16	28.83	29.72	28.14	29.92	27.76
C-17	55.84	56.12	55.96	57.71	55.66
C-18	12.15	12.01	11.74	11.55	11.27
C-19	19.88	19.42	18.66	18.88	19.10
C-20	40.40-40.51	41.10	39.66	37.79	35.70
C-21	20.99	21.10	20.96	20.35	18.69
C-22	138.23	138.00	138.04	138.09	33.51
C-23	129.16-129.60	128.95	129.30	129.18	25.64
C-24	51.13-51.30	50.15	51.16	51.17	45.49
C-25	31.94	31.72	31.83	30.75	28.74
C-26	21.23	21.30	19.21	21.30	18.69
C-27	19.01	19.06	18.90	20.51	18.35
C-28	25.40-25.50	25.38	24.87	24.72	22.60
C-29	12.25-25.30	12.28	12.26	12.25	12.29
C-1′		-------	100.98	103.94	100.74
C-2′		-------	74.26	76.71	73.21
C-3′		-------	76.91	76.19	76.18
C-4′		-------	70.06	70.05	69.90
C-5′		-------	76.75	77.01	75.62
C-6′		-------	62.22	62.15	61.36

VIII. Acknowledgement

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