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Isolation of Stigmasterol and β-Sitosterol from the dichloromethane extract of *Rubus suavissimus*

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ABSTRACT

Purification of the dichloromethane (CH_2Cl_2) fraction of the aqueous extract of *Rubus suavissimus* resulted in the isolation of two sterols namely stigmasterol and β-sitosterol. The structures of the isolated compounds were characterized on the basis of extensive spectral data (1D and 2D NMR; and MS) and in comparison with their literature data.

Key Words: Rosaceae, Sterols, Purification, NMR, MS, Structure elucidation.

INTRODUCTION

Rubus suavissimus S. Lee belongs to the genus *Rubus* belongs to the flowering plants in the rose family, Rosacea (subfamily Rosoideae). Raspberries, blackberries, and dewberries are widely distributed members of this genus. *R. suavissimus* is a perennial shrub grows widely grown in Guang-xi and Guang-dong, China (Koh *et al*., 2009). The leaves of *R. suavissimus* are used to make beverage leaf tea by the local residents of China due to its intensely sweet flavor, which is known as tiancha in Chinese or Chinese sweet tea. Previous phytochemical studies of this plant mainly showed the presence of diterpene and triterpene glycosides as well as phenolic compounds (Gao *et al*., 1985; Wang and Lu, 2007; Sugimoto *et al*., 2001). The major constituent of this plant is the sweet diterpenoid glycoside rubusoside with an aglycone moiety belongs to the class of the diterpene, *ent*-13-hydroxykaur-16-en-19-oic acid, known as steviol (Brandle *et al*., 1998). As a part of our research to discover natural sweeteners, we have recently reported several ditepene glycosides from *S. rebaudiana* and *R. suavissimus* (Chaturvedula *et al*., 2011 a-g), triterpene glycosides from *Siraitia grosvenorii* (Chaturvedula and Prakash., 2011 h) and phenolic glycosides from *R. suavissimus* (Chaturvedula *et al*., 2012).

This paper describes the isolation and structure elucidation of the two sterol components **1**-**2** (Figure 1) on the basis of extensive spectroscopic and in comparison of their physical and spectral properties reported from the literature.

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MATERIALS AND METHODS

Melting points were measured using a SRS Optimelt MPA 100 instrument and are uncorrected. Optical rotations were recorded using a Rudolph Autopol V at 25°C and NMR spectra were acquired on a Varian Unity Plus 600 MHz instrument using standard pulse sequences at ambient temperature. Chemical shifts are given in δ (ppm), and coupling constants are reported in Hz. HRMS data was generated with a Thermo LTQ Orbitrap Discovery mass spectrometer in the positive positive ion mode electrospray. Instrument was mass calibrated with a mixture of Ultramark 1621, MRFA [a peptide], and caffeine immediately prior to accurate mass measurements of the samples. Samples were diluted with water:acetonitrile:methanol (1:2:2) and prepared a stock solution of 50µl concentration for each sample. Each sample (25µl) was introduced via infusion using the onboard syringe pump at a flow injection rate of 120µl/min. Low pressure chromatography

Table 1: ^{1}H and ^{13}C NMR chemical shift values for Stigmasterol (1) and β-Sitosterol (2) recorded in $CDCl_3$[a-c].

Position	1		2	
	^{1}H	^{13}C	^{1}H	^{13}C
1		37.6		37.5
2		32.1		31.9
3	3.51 (tdd, 1H, *J* = 4.5, 4.2, 3.8 Hz)	72.1	3.53 (tdd, 1H, *J* = 4.5, 4.2, 3.8 Hz)	72.0
4		42.4		42.5
5	5.31 (t, 1H, *J* = 6.1 Hz)	141.1	5.36 (t, 1H, *J* = 6.4 Hz)	140.9
6		121.8		121.9
7		31.8		32.1
8		31.8		32.1
9		50.2		50.3
10		36.6		36.7
11		21.5		21.3
12		39.9		39.9
13		42.4		42.6
14		56.8		56.9
15		24.4		26.3
16		29.3		28.5
17		56.2		56.3
18		40.6		36.3
19	0.91 (d, 3H, *J* = 6.2 Hz)	21.7	0.93 (d, 3H, *J* = 6.5 Hz)	19.2
20	4.98 (m, 1H)	138.7		34.2
21	5.14 (m, 1H)	129.6		26.3
22		46.1		46.1
23		25.4		23.3
24	0.83 (t, 3H, *J* = 7.1 Hz)	12.1	0.84 (t, 3H, *J* = 7.2 Hz)	12.2
25		29.6		29.4
26	0.82 (d, 3H, *J* = 6.6 Hz)	20.2	0.83 (d, 3H, *J* = 6.4 Hz)	20.1
27	0.80 (d, 3H, *J* = 6.6 Hz)	19.8	0.81 (d, 3H, *J* = 6.4 Hz)	19.6
28	0.71 (s, 3H)	18.9	0.68 (s, 3H)	19.0
29	1.03 (s, 3H)	12.2	1.01 (s, 3H)	12.0

[a]assignments made on the basis of COSY, HMQC and HMBC correlations; [b]Chemical shift values are in δ (ppm); [c]Coupling constants are in Hz.

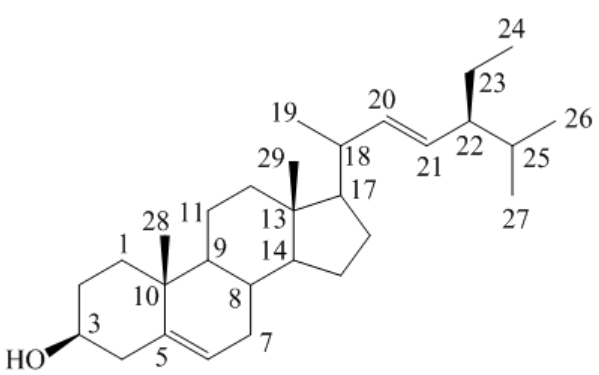
was performed on a Biotage Flash system using a C-18 cartridge (40+ M, 35-70μm). TLC was performed on Baker Si-$C_{18}F$ plates and identification of the spots on the TLC plate was carried out by spraying 10% H_2SO_4 in EtOH and heating the plate at about 80°C.

Plant Material

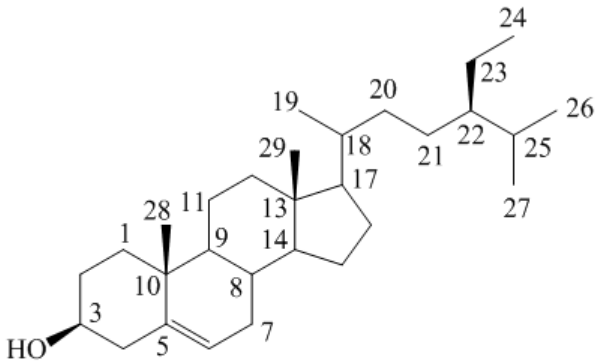
The commercial sample consisting of the aqueous extract of the leaves of *R. suavissimus* was purchased from Chengdu Biopurify Phytochemicals, China. The plant material was identified by Professor Weiping He, Natural Plant Scientific Institute, Guangdong Ocean University, Guangxi, China and a voucher specimen was deposited at The Coca Cola Company, No. VSPC-3166-68.

Isolation

The aqueous extract of the leaves of *R. suavissimus* (10 g) was suspended in 100 ml water and extracted successively with *n*-hexane (3 x 100 ml), CH_2Cl_2 (3 x 100 ml) and *n*-BuOH (2 x 100 ml). The CH_2Cl_2 layer was concentrated under vacuum furnished a residue (1.5 g) which was purified on a Biotage flash chromatography system using C-18 (100 g) column (solvent system: gradient from 80-20 MeOH-water to 100% MeOH at 60 ml/min. detection at UV 210 nm) for 40 min. Fractions 48-52 and 55-60 were combined to get residues 0.25 g and 0.32 g respectively, which on repeated purification using the gradient 80-100% MeOH-water at 30 ml/min for 40 min resulted stigmasterol (**1**, 65 mg), and β-sitosterol (**2**, 70 mg), respectively.



Stigmasterol (1)



β-Sitosterol (2)

Figure 1: Structure of Stigmasterol (1) and β-Sitosterol (2).

Identification of Stigmasterol and β-Sitosterol

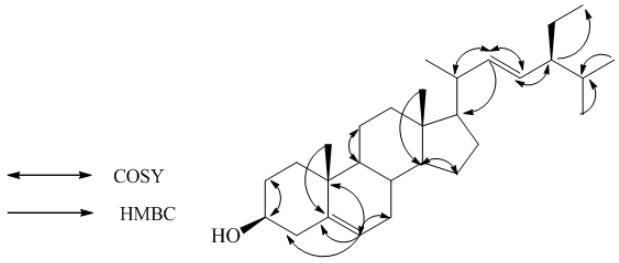
Stigmasterol (1): White powder (65 mg); mp: 174-176 °C; ^{1}H NMR ($CDCl_3$, 600 MHz): see Table 1; ^{13}C NMR ($CDCl_3$, 150 MHz): see Table 1; MS (*m*/*z*): 412 [M+], 394, 351, 314, 300, 271, 229, 213, 55.

β-Sitosterol (2): White powder (70 mg); mp: 134-135°C; ^{1}H NMR ($CDCl_3$, 600 MHz): see Table 1; ^{13}C NMR ($CDCl_3$, 150 MHz): see Table 1; MS (*m*/*z*): 414(M+), 396, 339, 325, 310, 298, 257, 227, 140, 139, 125, 97, 71, 57.

RESULTS AND DISCUSSION

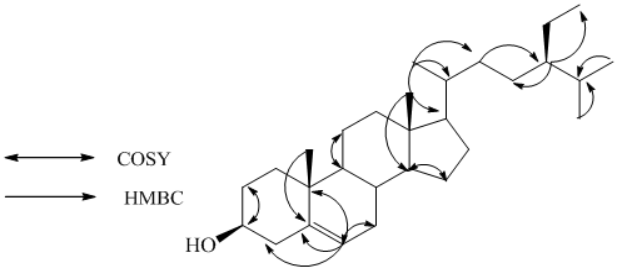
Compound **1** was isolated as a white powder. The mass spectral data of the compound gave a molecular formula $C_{29}H_{48}O$, which was supported by the ^{13}C NMR spectral data. ^{1}H NMR spectra of Compound **1** showed the presence of two methyl sinlgets at δ 0.71, and 1.03; three methyl doublets that appeared at δ 0.80, 0.82, and 0.91; and a methyl triplet at δ 0.83. Compound **1** also showed protons at δ 4.98, 5.14, and 5.31 suggesting the presence of three protons corresponding to that of a trisubstituted and a disubstituted olefinic bond. Liebermann-Burchard reaction indicated compound **1** is having a sterol skeleton (Kandati *et al*., 2012; Raju *et al*., 2012). The proton corresponding to the H-3 of a sterol moiety was appeared as a triplet of doublet of doublets at δ 3.51. The ^{1}H and ^{13}C NMR values for all the protons and carbons were assigned on the basis of COSY, HMQC and HMBC correlations and were given in Table 1. The above spectral data supported the presence of sterol skeleton having a hydroxyl group at C-3 position with two double bonds at C-5/C-6 and C-20/C-21 with six methyl groups which was supported by the key COSY and HMBC correlations as shown in Figure 2. Thus, the structure of **1** was assigned as the known compound stigmasterol. The physical and spectral data are consistent to the reported literature values (Habib *et al*., 2007; Jamal *et al*., 2009; Moghaddam *et al*., 2007) of stigmasterol.

Compound **2** was also isolated as a white powder and its mass spectral data suggested the molecular formula as $C_{29}H_{50}O$. Compound **2** also showed positive Liebermann-Burchard reaction indicated its sterol nature as in **1**. The ^{1}H NMR spectra of compound **2** showed the presence of six methyl signalss that appeared as two methyl sinlgets at δ 0.68, and 1.01; three methyl doublets that appeared at δ 0.81, 0.83, and 0.93; and a methyl triplet at δ 0.84; same as **1**. The ^{1}H NMR spectra of compound **2** also showed one olefinic proton at δ 5.36 instead of three in **1**. The absence of protons corresponding to the double bond between C-20/C-21 in compound **2** together with the appearance of mass spectral data which showed 2 amu more than **1** suggested the presence of a trisubstituted double bond at C-5/C-6 in its structure. The ^{1}H NMR spectra of compound **2** showed a proton corresponding to the proton connected to the C-3 hydroxy group which appeared as a triplet of doublet of doublets at δ 3.53. The ^{13}C NMR together with COSY, HMQC and HMBC showed twenty nine carbon signal including six methyls, eleven methylenes, ten methane and



Stigmasterol (1)

Figure 2: Key COSY and HMBC Correlations of Stigmasterol (1).



β-Sitosterol (2)

Figure 3: Key COSY and HMBC Correlations of β-Sitosterol (2).

three quaternary carbons. Thus, the structure of **2** was assigned as β-sitosterol that was consistent to the reported literature values (Habib *et al*., 2007; Jamal *et al*., 2009) and was further supported by the key COSY and HMBC correlations as shown in Figure 3.

CONCLUSION

Two sterols were isolated from the commercial extract obtained from the leaves of *R. suavissimus* obtained from Chengdu Biopurify Phytochemicals Limited, China. The structures of the isolated new compounds were identified as stigmasterol (**1**), and β-sitosterol (**2**) on the basis of spectroscopic and by comparing their physical properties reported in the literature. The complete ^{1}H and ^{13}C NMR spectral assignments of the two isolated compounds were made based on COSY, HSQC, HMBC, and MS/MS spectroscopic data.

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