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Isolation of steroids from n-hexane extract of the leaves of *Saurauia roxburghii*

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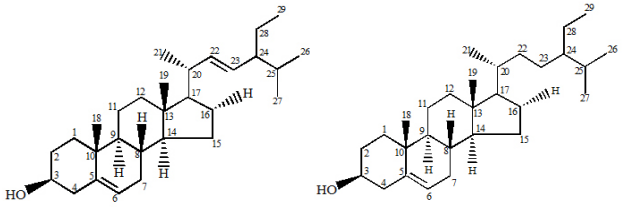
Abstract

General phytochemical screening of the leaves of *Saurauia roxburghii* (*Actinidiaceae*) revealed the presence of alkaloids, flavonoids, glycosides, O-glycosides, terpenoids, carbohydrates, steroids, reducing sugar, tannins, phlobatannins and saponin are present in this plant whereas cardiac glycosides are absent. Two steroid compounds were isolated from the n-hexane extract of the leaves from *S. roxburghii.* Based on the spectral evidence IR, ^{1}H-NMR and ^{13}C-NMR, structures were determined to be stigmasterol (1) and *β*-sitosterol (2) This is the first report so far of occurrence and details spectroscopic description of these compounds from *S. roxburghii.*

Introduction

Plants have formed the basis of sophisticated traditional medicine systems that have been used for thousands of years in countries, such as China (Chang and But, 1986) and India (Kapoor, 1990).The use of plants in the traditional medicine of many other cultures has been extensively documented. These plant based systems continue to play an important role in health care and it has been estimated by the World Health Organization that approximately 80% of the world's inhabitants rely mainly on traditional medicines for their primary health care, while plant products also play an important role in the health care systems of the remaining 20% of the population mainly residing in developed countries (Farnsworth *et al.*, 1985).

Saurauia roxburghii is known as Singkrang, Sing khau (Bengali name: Pannikomari, Pahari Kadam or Bhola Kadam) (Sarder Nasir Uddin, 2006) is an evergreen tree belonging to the family *Actinidiaceae.* Familiy synonyms: *Saurauiaceae*. The synonyms of this plant are *Saurauja roxburghii* and *Ternstroemia serrata*. The plant is widely distributed in the coastal forest and hill tracts of Bangladesh and also found outside of Bangladesh such as Vietnam, Nepal, India, Myanmar, Thailand, Cambodia, Laos, China (including Taiwan), Japan, Malaysia and the species less frequently occurs in the greater districts of Sylhet, Chittagong and Chittagong Hill Tracts in Bangladesh (Sarder, 2006) and the stems and leaves of the plants are extensively used as herbal medicines against a large number of severe diseases (Rizwana *et al*., 2010) like asthma, bronchitis, hepatitis B, ulcers, and central nerves depression and also in the treatment of boils, Eczema, Epilepsy, Fever, Gout and Piles (Sarder, 2006).



Stigmasterol (1) *β*-sitosterol (2)

Figure 1

As the plant is being used extensively in our country as an herbal medicine, it is necessary to have knowledge of the constituents of the plant of our native species. Previous phytochemical investigations resulted in the isolation of ursolic acid, corosolic acid, 24-hydroxy corosolic acid, maslinic acid and 3b, 7b, 24-trihydroxy-urs-12-en-28-oic acid (Mazumder *et al.*, 2011). In this study, the n-hexane extract of its leaves were subjected to chromatographic separation to afford two steroids, including stigmasterol (1) and *β*-sitosterol (2) (Figure 1). Both compounds were isolated for the first time from this species.

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Materials and Methods

Collection, identification and preparation of plant materials

Fresh leaves of *S. roxburghii* were collected from Chittagong University campus, Chittagong in the month of January, 2011. It was identified by a Scientific officer, Sardar Nasir Uddin, Bangladesh National Herbarium, Dhaka, where a voucher specimen (DACB 32567) has been deposited.

Extraction and isolation

The powdered leaves (1.0 kg) of *S. roxburghii* were soaked in 3 liter of ethanol and then 2 liter ethanol for 7 days. The whole mixture was then filtered through filter paper and the filtrate was then evaporated under reduced pressure at (40-50)^{o}C using a Buchii Rotary Evaporator to provide 130 gm of a gummy concentrate of the crude extract. A portion of the ethanol extract (10.5 gm) was dissolved in 90% ethanol. It was partitioned with n-hexane, then with chloroform ($CHCl_3$) and finally with ethylacetate (EA). All the extracts were filtered through a cotton plug followed by Whatman filter paper number 1 and then concentrated by using a rotary vacuum evaporator to provide n-hexane (2.5 gm), then with chloroform ($CHCl_3$) (2.0 gm) and finally with ethylacetate (1.0 gm) extractives.

Chromatographic separation

The column was packed with fine TLC grade silica gel (Kiesel gel 60H) was used as the packing material. A column having 40 cm length and 3 cm in diameter was packed with the silica gel (70 gm) up to a height of 23 cm under reduced pressure. The column was washed with n-hexane to facilitate compact packing. The sample was prepared by adsorbing 3.5 gm of n-hexane soluble extract onto silica gel (Kiesel gel 60H, mesh 70-230), allowed to dry and subsequently applied on top of the adsorbent layer. The column was then eluted with n-hexane followed by mixtures of n-hexane and dichloromethane and then dichloromethane and methanol (Stahl, 1969). The polarity was gradually increased by adding increasing proportions of dichloromethane and methanol. A total of 30 fractions were collected each in 100 ml beakers.

The Fractions 15 to 17 of the crude n-hexane extract was subjected to Sub-Column Chromatography) (Kieselgel 60, mesh 70-230) for further fractionation. The column was eluted with n-hexane, ethylacetate and methanol mixtures of increasing polarities to provide 27 fractions. Fraction-10 was found to yield crystals on the wall of the beaker. The crystals were washed with n-hexane carefully. As a result mother solution was obtained leaving back the needle shape crystals which were isolated as compound 1.

The column fractions 12-13 of crude n-hexane extract were bulked together as they showed similar TLC feature. The mixed fraction was found to yield crystals on the wall of the beaker. The crystals were washed with n-hexane carefully. As a result mother solution was obtained leaving back the colorless sharp crystals. These sharp crystals provide the compound 2.

Test for alcohol

Four grams of ceric ammonium nitrate was dissolved in 10 mL of 2 N HNO_3, on mild heating. A few crystals of compounds 1 and 2 were dissolved in 0.5 mL of dioxane. The solution was added to 0.5 mL of ceric ammonium nitrate reagent and diluted to 1 mL with dioxane and shaken well. Both compounds 1 and 2 developed yellow to red color indicating the presence of an alcoholic hydroxyl group (Harborne, 1998).

Test for steroid

Salkowski reaction

A few crystals of compounds 1 and 2 were dissolved in chloroform and a few drops of concentrated sulphuric acid were added to the solution, both compounds 1 and 2 formed a reddish color in the upper chloroform layer (Harborne, 1998) indicating presence of steroids.

Liebermann-Burchard reaction

A few crystals of compounds 1 and 2 were dissolved in chloroform and few drops of concentrated sulfuric acid were added to it followed by the addition of 2-3 drops of acetic anhydride. In this case both compounds 1 and 2 turned to violet blue and finally formed green color which indicates the presence of steroids (Harborne, 1998).

Spectroscopic characterization

Different spectroscopic methods were used to elucidate the structure of isolated compounds 1 and 2. Among the spectroscopic techniques IR, ^{1}H and ^{13}C-NMR were carried out. The infrared spectrum was recorded on Shimadzu affinity-1, ^{1}H and ^{13}C-NMR spectra were recorded using $CDCl_3$ as solvent on BRUKER NMR DBX-400 MHz spectrometer, Analytical research division, Bangladesh Council of Scientific and Industrial Research (BCSIR), Dhaka.

Stigmasterol (1) : White crystalline needle. IR (KBr) υ_{max}/cm^{-1}: 3446.79, 2935.66, 2866.22, 1653.0, 1458.18, 1375.25, 1055.06, 883.4. ^{1}HNMR (400

MHz, $CDCl_3$) δ: 5.34 (^{1}H, d, J 5.2 Hz, H6), 5.16 (1H, dd, J 15.0, 8.4 Hz, H22), 5.03 (1H, dd, J 15.0, 8.4 Hz, H23), 3.52 (1H, dd, J 9.6, 4.8 Hz, H3), 1.00, 0.67 (3H, s, H19 and H18), 0.92 (3H, d, J 6.0 Hz, H21), 0.85 (3H, d, J 8.0 Hz, H29), 0.81 (3H, d, J 7.2 Hz, H26) and 0.79 (3H, d, J 7.2 Hz, H27). ^{13}CNMR (100 MHz, $CDCl_3$) δ: 37.29 (CH_2,C1), 28.28 (CH_2,C2), 71.85 (CH,C3), 42.36 (CH_2,C4), 140.81 (Cq,C5), 121.74 (CH,C6), 31.72 (CH_2,C7), 34.01 (CH,C8), 50.2 (CH,C9), 36.54 (Cq,C10), 26.17 (CH_2,C11), 39.83 (CH_2,C12), 42.37 (Cq,C13), 56.82 (CH,C14), 24.33 (CH_2,C15), 29.23 (CH_2,C16), 56.12 (CH,C17), 12.02 (CH_3,C18), 19.41 (CH_3,C19), 40.50 (CH_2,C20), 21.13 (CH_3, C21), 138.33 (CH,C22), 129.0 (CH,C23), 51.28 (CH,C24), 45.91 (CH,C25), 19.42 (CH_3, C26), 19.84 (CH_3,C27), 24.34 (CH_2,C28), 12.26 (CH_3,C29).

β-sitosterol (2): White crystal. IR (KBr) υ_{max}/ cm^{-1}: 3421.72, 2935.66, 2866.22, 1653.00, 1458.18, 1375.25, 1062.78, 883.40, 800.46. ^{1}HNMR (400 MHz,$CDCl_3$) δ: 5.34 (1H, d, J 5.2 Hz, H6), 3.51 (1H, m, H3), 1.00, 0.67 (3H, s, H19 and H18), 0.92 (3H, d, J 6.0 Hz, H21), 0.85 (3H, d, J 8.0 Hz, H29), 0.83 (3H, d, J 7.2 Hz, H26) and 0.79 (3H, d, J 7.2 Hz, H27). ^{13}CNMR (100 MHz, $CDCl_3$) δ: 37.29 (CH_2,C1), 31.95 (CH_2,C2), 71.84 (CH,C3), 42.36 (CH_2,C4), 140.80 (Cq,C5), 121.73 (CH,C6), 31.71 (CH_2,C7), 31.95 (CH,C8), 50.19 (CH,C9), 36.18 (Cq,C10), 21.12 (CH_2,C11), 39.82 (CH_2,C12), 42.36 (Cq,C13), 56.81 (CH,C14), 24.33 (CH_2,C15), 28.26 (CH_2,C16), 56.11 (CH,C17), 11.88 (CH_3,C18), 19.41 (CH_3,C19), 36.54 (CH,C20), 19.07 (CH_3,C21), 34.00 (CH_2,C22), 26.16 (CH_2,C23), 45.89 (CH,C24), 29.23 (CH,C25), 19.83 (CH_3,C26), 18.81 (CH_3,C27), 23.12 (CH_2,C28), 12.01 (CH_3,C29).

Results and Discussion

The compound 1 is a white needle shaped crystal with melting point 138-140^{o}C which gave positive Salkowski and Lieberman-Burchard test for steroid. The IR spectrum (in KBr) of compound 1 exhibit characteristic absorption band at 3446.79 cm^{-1} that is characteristic of O-H stretching. Absorption at 2935.66 and 2866.22 cm^{-1} is due to aliphatic C-H stretching. Other frequencies include 1653.00 cm^{-1} as a result C=C stretching however this band was weak at 1458.18 cm^{-1} was a bending frequency for cyclic (CH_2)n and 1375.25 cm^{-1} for CH bending. The absorption frequency at 1055.06 cm^{-1} due to CO stretching. The out of plane C-H vibration of unsaturated part was observed at 883.4 cm^{-1}. These assignments were good agreements with reported values (Kamboj and Saluja, 2011; Muhit *et al*., 2010; Ian *et al*., 1976). The ^{1}H NMR spectrum showed two one proton multiplets at δ 3.53 and δ 5.36 typical for H3 and H6 of a steroidal nucleus. Two olefinic protons appeared as characteristic downfield signals at δ 5.16 (1H, dd, J = 15.0, 6.5 Hz) and 5.03 (1H, dd, J = 15.0, 9.0 Hz) in the ^{1}H NMR spectrum which were identical with the chemical shift of H22 and H23 respectively of stigmasterol (Habib *et al*., 2007; Jain *et al*., 2009). The spectrum also displayed two three proton singlets at δ 1.00 and δ 0.67 assignable for H19 and H18 respectively. In addition, two doublets at δ 0.82 (3H, d, 7.2 Hz) and 0.80 (3H, d, 7.2 Hz) could be ascribed to the two methyl groups at H26 and H27 and another three-proton doublet at δ 0.91 (3H, d, 6.8 Hz) for H21. On the other hand, one three-proton triplet at δ0.85 (3H, t, 7.2 Hz) could be assigned to the primary methyl group attached H29 (Ahmed *et al*., 2010). The ^{13}C NMR spectrum showed 29 carbons including an oxymethine carbon at δ 71·85, was characteristics of spirostene (Agarwal *et al*., 1985) and two olefinic carbons appeared at δ 138.33 and 129.0 which were identical with the chemical shift of C22 and C23 respectively of stigmasterol. If we compare DEPT 90 and 135 experiments for 1 then we confirmed that this compound was having six methyl (CH_3) groups, nine methylene (CH_2), eleven methine (CH) and three quaternary carbons (Cq) groups. The physical and spectral data of the compound was in complete agreement to the reported data in literature value (Kamboj and Saluja, 2011; Ahmed *et al*., 2010; Jain and Bari, 2010; Hartati *et al*., 2008). The compound 1 was identified as stigmasterol.

Compound 2 was obtained as a white crystalline compound with melting point 135-137^{o}C which gave positive Salkowski and Lieberman-Burchard test for steroids. The IR spectrum (in KBr) of compound 2 exhibit characteristic absorption band at 3421.72 cm^{-1} that is O-H stretching. Absorption at 2935.66 and 2866.22 cm^{-1} is due to aliphatic C-H stretching. Other frequencies include 1653.00 cm^{-1} as a result C=C stretching however this band was weak at 1458.18 cm^{-1} was a bending frequency for cyclic (CH_2)n and 1375.25 cm^{-1} for CH bending. The frequency at 1062.78 cm^{-1} due to CO stretching. These absorption frequencies resemble the absorption frequencies observed for *β*-sitosterol (Patra *et al*., 2010). Compound 2 was identified as *β*-sitosterol by comparison the ^{1}H and ^{13}C NMR spectra with those of 1. The NMR data were very similar to those of the compound 1 except two olefinic protons were absent while two methylene signals were present for H22 and H23 and showed two one-proton multiplet at δ 3.51 ppm and δ 5.34 ppm typical for H3 and H6 of a steroidal nucleus. The ^{13}C NMR spectrum showed 29 carbons including an oxymethine carbon

signal at δ 71·84 and two olefinic carbons at δ 140·80 and δ 121·73. The double bonded unsaturation at δ 140.80 and δ 121.73 was characteristics of spirostene (Agarwal *et al.*, 1985) and two olefinic carbons were absent at 138.33 and 129.0 while two methylene carbon signals were present at 34.0 and 26.16 for C22 and C23. If we compared DEPT 135 & DEPT 90 experiments for 2 then we confirmed that this compound was having six methyl (CH_3) groups, eleven methylene (CH_2) groups, nine methine (CH) groups and three quaternary carbons (C) groups. These assignments are in good agreement for the structure of *β*-sitosterol (Habib *et al.*, 2007; Pateh *et al*., 2009; Patra *et al*., 2010; Ahmed *et al*., 2010; Trivedi and Choudhrey, 2011).

Conclusion

From the above findings, stigmasterol and *β*-sitosterol were isolated from n-hexane extract of the leaves of S.roxburghii and chemical structures elucidated respectively. It was carried out by means of various physical (solvent extraction, TLC, CC) and spectral techniques.

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