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Research Article

ISOLATION AND CHARACTERIZATION OF STIGMASTEROL AND *β*-SITOSTEROL-*D*-GLYCOSIDE FROM ETHANOLIC EXTRACT OF THE STEMS OF *SALVADORA PERSICA* LINN.

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ABSTRACT

Objective: Miswak is a member of the Salvadoraceae family has been used by many Islamic communities as toothbrushes and has been scientifically proven to be very useful in the prevention of tooth decay, even when used without any other tooth cleaning methods. The objective of the research was to isolate and characterize the stigmasterol and *β*-sitosterol-*D*-glycoside from ethanolic extract of the stems of *Salvadora persica* Linn. *Methods:* About 800 g of the air dried coarse powdered defatted plant material was percolated with alcohol (95%) for 16 hrs. This extraction process was repeated three times until exhaustion. The structures of isolated compounds were elucidated with the help of UV, IR, NMR and elemental analysis data. *Results: β*-Sitosterol-D-glycoside and Stigmasterol were isolated from the ethanolic extract of the stems of *Salvadora persica* L (Salvadoraceae). This compound has not been previously isolated or reported from the stems of this species. *Conclusion:* In present work five compounds were isolated by column chromatography out of that β-sitosterol, Stigmasterol and β-sitosterol-D-glucoside were not reported earlier.

Keywords: *Salvadora persica*, Phytochemistry, Miswak, Oral hygiene, Salvadoraceae.

INTRODUCTION

Oral hygiene is the practice of keeping the mouth clean and healthy by brushing and flossing to prevent tooth decay and gum diseases. Oral hygiene can also be referred to as the general mouth cleanliness and there are various methods of cleaning to make it hygienic. A number of plants are used as chewing Sticks *Citrus aurantafolia, Citrus sinensis, Cassia vinnea, Cassia sieberianba* and *Azadirachta indica*[1].The toothbrush tree, *Salvadora persica*, L., locally called Miswak, is a member of the Salvadoraceae family has been used by many Islamic communities as toothbrushes and has been scientifically proven to be very useful in the prevention of tooth decay, even when used without any other tooth cleaning methods[2]. It is a small genus of evergreen trees or shrubs distributed in tropical Africa and Asia extending up to Mascarene Island and China[3]. Leaves of *Salvadora persica* have carminative, antiseptic, anti-fungal[4], antiscorbutic, deobstruent, liver tonic, diuretic, analgesic, anthelmintic, astringent properties , hypoglycaemic[5], anti-microbial[6], anti-bacterial[7], anti-plasmodial[8], anti-microbial[9], anti-caries, anti-spasmodial[10], anticonvulsant and also used in hepatic disorders[11].Because of the presence of fluoride in stems these are used as oral hygiene tool[12]. Stems also show anti-plaque[13], anti-microbial[14]. Fruits are used as carminative, diuretic, stomachic, and in rheumatism[15]. Chlorine, trimethylamine and sulphur compounds in aqueous extract of roots of Miswak tree shows anti-mycotic effect[16]. Antimicrobial activity of both Glucosinolates: glucotropeolin and sinigrin were investigated against tooth decay microorganisms and bacterial species[17]. A new indole alkaloid Salvadorocine has been isolated from the leaves of *Salvadora persica*[18].

Volatile oil extracted from *Salvadora persica* leaves, identified as benzyl nitrile, eugenol, thymol, isothymol, eucalyptol, isoterpinolene, and β-caryophyllene[19]. Leaves also possess the Flavanoids and flavanoid glycosides[20]. Stem also contains β-sitosterol, β-sitosterol-3-*o*-*β*-*D*-glucopyranoside, octacosanol and 1-tricontanol[21]. A Sulfated glycoside: Salvadoside (Sodium 1-o-benzyl-*β*-*D*-glucopyranoside-2-sulfate) isolated from *S.persica*[22]. Seed oil shows the presence of lauric acid, myristic acid, palmitic acid, stearic acid, oleic acid, linolic acid, malvalic acid and sterculic acid[23]. An aerial part contains β-amyrin, betulin, ursolic acid and lupeol[24].

MATERIAL AND METHODS

Plant materials

The fresh stems were collected in the month of July-August 2007 from Agricultural University Campus, Bikaner and authenticated by Dr. Shekhar Bhargava, Head of the Botany Department, Rajasthan University. After the collection, stems dried in shade at temperature 25-30 °C for 10 days and were crushed to obtain coarse powder which could pass through sieve number 40.

Spectroscopic investigation

Melting point was determined by determined in open-glass capillaries on Stuart SMP10 melting point apparatus and were uncorrected. The IR (KBr) spectrum was recorded on a Shimadzu UV-168A and Perkin-Elmer 1600 FTIR spectrometer, respectively. The 1H-NMR and 13C-NMR spectra were recorded on a Bruker R-32 (400 MHz) in DMSO-d_6 with TMS as an internal standard (chemical shifts in δ, ppm). TLC was performed with silica gel 60G F254 and spots were visualized by iodine vapors or ultraviolet light. The electron spray ionization mass spectrum (ESI-MS) was acquired on a Bruker Daltronics Esquire 3000 plus ion trap mass spectrometer. All solvents were analytical reagent grade.

Extraction and isolation

About 800 g of the air dried coarse powdered defatted plant material was percolated with alcohol (95%) for 16 hrs. This extraction process was repeated three times until exhaustion. The alcoholic extract was evaporated by Rotatory evaporator at about 50 °C (44.12g). Phytochemical screening of extract showed the presence of different chemical constituents. For their isolation dried alcoholic extract (30g) was taken and dissolved in the minimum quantity of petroleum ether and adsorbed on silica gel (80g) of 100-200 mesh particle size. The slurry formed was allowed to dry to get free flowing material. A neat and dried column (95 cm X 6 cm) was taken. A cotton plug was put at the base of column and petroleum ether with silica gel (150 g) was poured into the column and gradually added the slurry. The adsorbed extract was charged into the column. The column was first eluted with petroleum ether and then with the solvent by gradually increasing the percentage of ethyl acetate in petroleum ether. Fraction of 250 ml was collected and concentrated on water bath.

Compound A eluted in 5% ethyl acetate in petroleum ether, which on concentration and filtration yielded a residue, which was recrystallized in chloroform. Compound B eluted with 10% ethyl acetate in petroleum ether which on concentration and filtration yielded a residue, which was recrystallized in chloroform. Compound C eluted in 5% chloroform in methanol showed same TLC pattern with one major spot were pooled and concentrated to minimum volume resulted in crystallization. Crystals were filtered

out and further recrystallized in methanol. Compound D eluted in 10% chloroform in methanol showed same TLC pattern with three major spot were pooled and concentrated to minimum volume resulted in crystallization. Crystals were filtered out and further recrystallized in methanol. Compound E eluted in 15% chloroform in methanol showed same TLC pattern with one major spot were pooled and concentrated to minimum volume resulted in crystallization. Crystals were filtered out and further recrystallized in methanol.

Phytochemical screening of alcoholic extract and TLC analysis of alcoholic extract of stems of *S. persica* has been shown in Table 1 and 2.

Table 1: Phytochemical screening of alcoholic extract

Chemical constituents	Alcoholic extract
Alkaloids	+
Glycosides	+
Saponins	-
Steroids	+
Flavonoids	+
Tannins	-
Sugars	-
Triterpenoids	+

Table 2: TLC analysis of *Salvadora persica*

Compd.	Solvent system	Spraying reagent	No of spots	R_f values
Compound A	Ethyl acetate Pet. Ether (5:95)	1% cerric ammonium sulfate	01	0.56
Compound B	Ethyl acetate Pet. Ether (10:90)	1% cerric ammonium sulfate	01	0.62
Compound C	Chloroform Methanol (5:95)	Vanillin Boric Acid	01	0.73
Compound D	Chloroform Methanol (10:90)	Vanillin Boric Acid	01	0.46
Compound E	Chloroform Methanol (15:85)	Vanillin Boric Acid	01	0.39

Identification Test

Tests for alcohol

4g of cerric ammonium nitrate was dissolved in 10ml of 2N HNO_3, on mild heating. A few crystals of isolated compound were dissolved in 0.5ml of dioxane. The solution was added to 0.5ml of cerric ammonium nitrate reagent and diluted to 1ml with dioxane and shaken well. The developed yellow to red color indicates the presence of an alcoholic hydroxyl group.

Tests for steroid:

Salkowski reaction: A few crystals 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 were dissolved in chloroform and a few drops of concentrated sulfuric acid were added to it followed by addition of 2-3 drops of acetic anhydride.

Solution turned violet blue and finally green.

Characterization of the compounds

The structure of compound B according to spectroscopic data was found to be as follows:

IR 3373.6 cm^{-1} (O-H stretching.); 2940.7 $cm^{-1}$and 2867.9cm^{-1} (aliphatic C-H stretching); 1641.6 cm^{-1} (C=C absorption peak); other absorption peaks includes 1457.3 cm^{-1} (CH_2); 1381.6 cm^{-1}(OH def), 1038.7cm^{-1} (cycloalkane) and 881.6 cm^{-1}; 1H-NMR (400 Mz, DMSO-d_6): 3.2 (1H, m, H-3), 5.26 (1H, m, H-6), 5.19 (1H, m, H-23), 4.68 (1H, m, H-22), 3.638 (1H, m, H-3), 2.38 (1H, m, H-20), 1.8-2.0 (5H, m), 0.76-0.89 (m, 9H), 0.91-1.05 (m, 5H), 1.35-1.42 (m, 4H), 0.69-0.73 (m, 3H), 1.8-2.00 (m, 5H), 1.07-1.13 (m, 3H), 1.35-1.6 (m, 9H); 13C-NMR (400 Mz, DMSO-d_6): 150.98, 145.2 (C-5), 139.8 (C-22), 121.7, 118.89 (C-6), 79.03 (C-3), 55.3 (C-14), 55.18(C-17), 50.45 (C-9), 48.3 (C-9), 40.8 (C-20), 40.1(C-12), 39.2 (C-13), 38.9 (C-4), 38.6 (C-12), 37.18 (C-1), 37.12 (C-10), 36.3 (C-8), 35.59 (C-20), 34.29 (C-22), 34.24 (C-7), 32.66 (C-8), 29.86 (C-25), 29.71 (C-16), 28.41 (C-2), 28.1 (C-15), 27.4 (C-28), 26.1 (C-11, 26), 21.6 (C-27), 19.32 (C-19), 17.71 (C-21), 15.6 (C-18, 29).

FAB-MS spectroscopy showed the molecular ion peaks at 414 that correspond to molecular formula, $C_{29}H_{50}O$. Ion peaks were also observed at m/z 367, 271, 255, 229,189, 175, 161, 133, 121, 105, 107, 95, 81, 69, 55, 41.

The structure of compound C according to spectroscopic data was found to be as follows:

IR (3600-3400), 2900, 2850, 1720, 1640, 1450, 1240, 900, (830-800) cm-1; 1H-NMR (400 Mz, DMSO-d_6): 7.25 (s, 1-H, –OH), 6.67-6.84 (m, 1H proton of sugar moiety), 5.47 (s, 1H, H-6), 5.35 (dd, 1H, J=12.5 and 8.5 Hz, H-23), 5.03-5.08 (dd, 1H, J= 12.5 and 8.5 Hz, H-22), 4.96 (s, 1H, proton of sugar moiety), 4.85 (s, 1H, anomeric proton), 3.86 (m, 1H, H-3) 2.03 3.31 (m 3H, proton of sugar moiety), 1.24 (s, 3H, H-19), 1.0 (d, 3H, J=6.5 Hz, H-21), 0.97 (t, 3H, J=7.1 Hz, H-29), 8.8 (s, 3H, H-27), 8.7 (s, 3H, H-26), and 0.85 (s, 3H, H-18) ; 13C-NMR (400 Mz, DMSO-d_6): 39.9 (C-1), 29.9 (C-2), 77.3 (C-3), 39.8 (C-4), 55.8 (C-5), 21.6 (C-6),39.2 (C-7), 29.7 (C-8), 48.7 (C-9), 29.4 (C-10), 21.6 (C-11), 27.2 (C-12), 50.8 (C-13), 30.2 (C-14), 62.1 (C-15), 77.3 (C-16), 124.3 (C-17), 118.26 (C-18), 130.2 (C-19), 151.87 (C-20), 178.91 (C-21), 146.47 (C-22), 29.2 (C-23), 14.1 (C-24), 62.12 (C-25), 76.7 (C-26), 63.75 (C-27), 184.9 (C-28), 111.14 (C-29), 121.2(C-30) and the chemical shifts (210.3, 209.45, 130.24, 130.0 and 143.96) ppm are due to the carbon of the sugar moiety.

FAB-MS spectroscopy showed the molecular ion peaks at 302 that correspond to molecular formula, $C_{35}H_{60}O_6$. Ion peaks were also observed at m/z 357, 261, 245, 219,179, 165, 141, 123, 111, 95, 87, 77, 51, 39.

RESULT AND DISCUSSION

From the positive tests for steroids and alcohols given by B, it is assumed to be a compound containing steroidal nucleus. The B is white crystalline needles like substance with melting point 144-146 °C. On subjection to IR spectroscopic analysis, the observed absorption bands are 3373.6 cm^{-1} that is characteristic of -OH stretching. Absorption at 2940.7 cm^{-1} and 2867.9 cm^{-1} is due to aliphatic -CH stretching. Other absorption frequencies include 1641.6 cm^{-1} as a result C=C stretching however this band is weak, at 1457.3 cm^{-1} is a bending frequency for cyclic $(CH_2)_n$ and 1381.6 cm^{-1} for $-CH_2(CH_3)_2$. The absorption frequency at 1038 cm^{-1} signifies cycloalkane. The out of plane -CH vibration of unsaturated part was observed at 881cm^{-1}. These absorption frequencies resemble the absorption frequencies observed for Stigmasterol. The proton NMR showed the proton of H-3 appeared as a multiplet at δ 3.2 and revealed the existence of signals for olefinic proton at δ 5.19(m), 4.68(m), 4.6(m) and 2.38(m). Angular methyl proton at 0.69(s), 0.80(s) and 1.02(s) corresponds to C_{18} and C_{19} proton respectively.

The 13C NMR has shown recognizable signals 145.2 and 121.7 ppm, which are assigned C_5 and C_6 double bonds respectively as in spirostene. The value at 19.32 ppm corresponds to angular carbon atom (C_{19}). Spectra show twenty nine carbon signal including six methyls, nine methylenes, eleven methane and three quaternary carbons. The alkene carbons appeared at 145.2, 139.8, 121.7 and 118.89. The weak molecular ions were given at m/z 414 and the

characteristic peaks were given at m/z 367 that corresponds, to (M-45) or loss of $HO^{+}=CH\text{-}CH_3$. These suggest that the sample B contains two compounds with molecular weight of 414 and 412. Other ion peaks are m/z 271, 273 due to the formation of carbocation by β bond cleavage of side chain leading to the loss of $C_{10}H_{21}$ and $C_{10}H_{23}$ that corresponds to the M 141and M 143. The molecular weight and fragmentation pattern indicate that the compounds β-sitosterol and Stigmasterol respectively. The dehydration of fragment at m/z 273 would yield m/z 255, which on successive dealkylation would yield ions at m/z 188, 189, 175, 161, 148, 135, 121, 108, 95, 82, 69, 55, 41. The above IR, 1HNMR, 13C NMR and MS spectral data and their comparison with those described in the literatures showed the structure of B to be the mixture of β-sitosterol and Stigmasterol in which may have maximum portion of Stigmasterol. The only difference between the two compounds is the presence of $C_{22}=C_{23}$ double bond in Stigmasterol and $C_{22}\text{-}C_{23}$ single bond in β-sitosterol hence, the lack of practical difference in their R_f despite the use of several solvent systems. Furthermore, literatures have shown that β-sitosterol is difficult to be obtained in pure state. Compound B was identified and established as mixture of β-sitosterol and Stigmasterol as shown in Fig. 1.

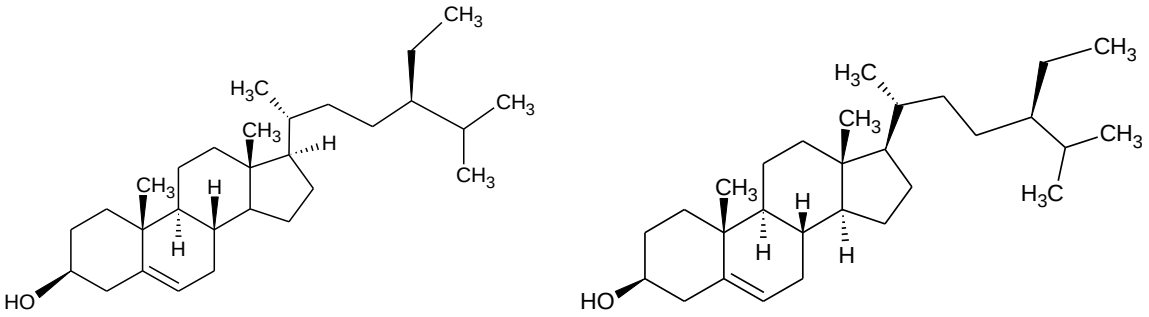


Fig. 1: *β*-Sitosterol and Stigmasterol

Compound C was obtained as a buff colour amorphous solid. Its IR spectrum showed an absorption peak in the region (3600-33400) cm^{-1} indicating the presence of a hydroxyl group (-OH) and the absorption bands at 2900-2850 cm^{-1} indicated the presence of –CH aliphatic asymmetric stretching of $–CH_3$, $–CH_2–$ and > CH_2 groups. The absorption band at 1720 cm^{-1} indicated the presence of (>C=O) stretching of normal aliphatic ester. The absorption band at 1240 cm^{-1} indicated the presence of C–N stretching. The absorption peak at 900 cm^{-1} indicated the aromatic stretching (out of plane bending). Finally, the absorption band at 830 and 800 cm^{-1} indicated the –CH stretching of >C=C-H group. The 1H-NMR spectrum showed the chemical shift at δ 0.85 and 1.24 indicated the presence of two angular methyl signals. The proton NMR spectrum also exhibited one olefinic double bond proton as a doublet at δ 5.35, along with the two up field signals at δ 0.87 and 0.88 respectively, due to the presence of two secondary methyl groups at position 26 and 27 of the skeleton, i.e., the presence of an isopropenyl group of the molecular structure. The very up field chemical shift at δ 0.97 as a triplet with the intensity of 3H and coupling constant of J=7.1 Hz was assigned for the terminal methyl group of 29. Similarly,

The other up field chemical shift at δ 1.0 with the coupling constant J=6.5 Hz of 3H intensity was assigned the secondary methyl group at position 21 of the molecular structure. The chemical shifts in the region δ 2.03-3.31 as a multiplet was assigned the presence of five protons of the sugar moiety and the very downfield chemical shift at δ 7.25 was assigned for the proton of OH group of glycoside. The 13C-NMR spectra of the compound C revealed the presence of 29 carbons, the chemical shift at δ 76.7 and 63.8 were assigned for the two separate terminal methyl groups linked at position 25 of the molecular structure. The three downfield chemical shifts at δ 128.3, 130.2 and 178.9 respectively, were assigned for the angular methyl carbons linked at C-18, C-19 and C-21 position. The up field signals at δ 29.7, 29.4 and 30.4 were assignable to the carbon at positions 8, 10 and 14 that was fused in the proposed â-Sitosteryl-D-glycoside derivative. Similarly, the relative down field chemical shifts at δ 48.7, 50.8 and 55.8, respectively, were assigned for the carbon that was fused at positions C-9, C-13 and C-5, respectively, in the proposed skeleton. The up field chemical shift at δ 39.9, 29.9 77.3, 39.8,21.6, 39.2, 21.6, 27.2, 62.1 and 77.3 were appropriate for the cyclohexyl and cyclopentyl carbons at positions 1, 2, 3, 4, 6, 7, 11, 12, 15 and 16, respectively. The other shifts at δ 151.87, 146.47, 29.2, 14.1, 62 12 and 184.9 were assigned for the carbon numbers 20, 22, 23, 24, 25 and 28, respectively, which constitute the side chain of six carbons which were linked at position 17 of the cyclopentyl ring.

The chemical shift at δ 124.3 was assigned for the carbon number 17 which was the point of link of a side chain to the cyclopentyl ring. The very down field chemical shift at 210.3, 209.45, 130.24, 130.0 and 143.96 were assigned for the carbon of the sugar moiety.

On the basis of IR, 1H-NMR, 13C-NMR, spectral data and the other physical properties the isolated pure compound C was identified and established as β-sitosterol-D-glycoside as shown in Fig. 2.

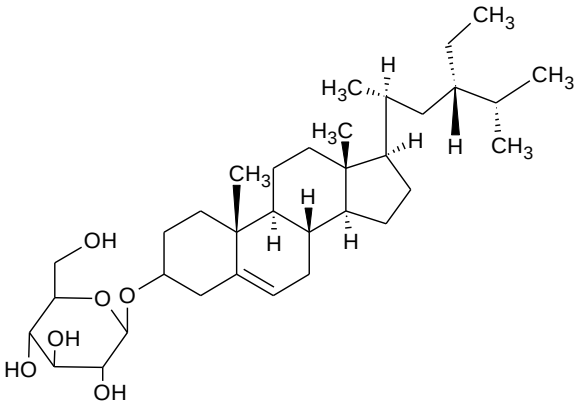
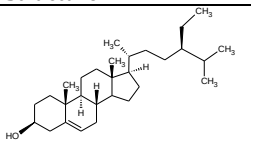
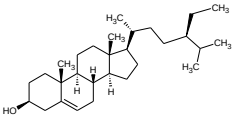
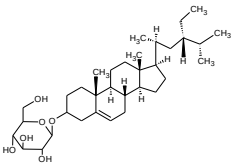
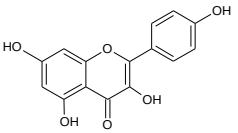
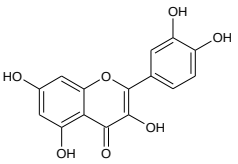


Fig. 2: *β*-Sitosteryl-D-glycoside

Table 3: List of the isolated compounds from *Salvadora persica* L

Compd. No.	Name/Mol. formula	Mol. Wt.	Melting Point (°C)	Category	Color of compound	Structure
A	β-sitosterol $C_{29}H_{50}O$	414.6	140	Steroid	Cream	
B	Stigmasterol $C_{29}H_{48}O$	412.6	170	Steroid	White colour	
C	β-sitosterol-D-glucoside $C_{35}H_{60}O_6$	302.2	314	Steroidal glycoside	Buff colour	
D	Kaempferol $C_{15}H_{10}O_6$	286.2	276-278	Flavonoid	Light yellow	
E	Quercetin $C_{15}H_{10}O_7$	576.8	282	Flavonoid	Light yellow	

CONCLUSION

In the research work five compounds were isolated by column chromatography out of that β-sitosterol, Stigmasterol and β-sitosterol-D-glucoside were not reported earlier. Research has indicated that these compounds may be responsible for the therapeutic action of Miswak tree.

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