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Chemical constituents of *Citrus sinensis* var. Shukri from Pakistan

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NOTE

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The variety ‘Shukri’ is a new hybrid of *Citrus sinensis* and is frequently grown for its sweet edible fruits in the southern part of Pakistan. The leaves of this hybrid variety have been investigated in search of secondary metabolites for the first time. As a result of chromatographic analysis of the methanolic extract, a new ceramide along with a flavonone glycoside and two steroids has been isolated, which were spectroscopically characterized as (*E*)-*N*-(1,3,4,5-tetrahydroxyhexadecan-2-yl)dec-4-enamide (**1**), atripliside B (**2**), β-sitosterol (**3**), and β-sitosterol-3-*O*-β-D-glucopyranoside (**4**), respectively. Compound **1** was found to be a new addition in the list of natural products, whereas, to the best of our knowledge, compounds **2–4** have been isolated for the first time from this source.

Keywords: *Citrus sinensis*; ceramide; flavonoid; steroids

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1. Introduction

The orange, specifically the sweet orange (*Citrus sinensis*) is a hybrid of two *Citrus* species; *C. maxima* and *C. reticulata* [1]. The plant is a small tree with a height of about 10 m having evergreen leaves, which are arranged alternately. The *C. sinensis* var. (Shukri) is widely cultivated in the Punjab, Peshawar, and Sindh regions of Pakistan. It is one of the most popular *Citrus* fruits, extensively consumed as fresh fruit, juices, squashes, etc. *C. sinensis* has been reported to contain a wide range of active ingredients which are used as appetizer, blood purifier, carminative, miscellany, skin tonic, etc. Vitamin C, flavonoids, fatty acids, volatile oils, and coumarins are the main metabolites of this plant [2]. This species is also used as a source of antioxidants and chemical exfoliants in specialized cosmetics, and the fresh rind is rubbed on the face to cure acne [2]. The dried peels are used in the treatment of anorexia, colds, coughs, etc. [3]. Various varieties of *Citrus* have been intensively investigated but the variety Shukri growing in Pakistan has never been explored for its chemicals. In this paper, we report on the isolation and characterization of a new ceramide, (*E*)-*N*-(1,3,4,5-tetrahydroxyhexadecan-2-yl)dec-4-enamide (**1**) together with atripliside B (**2**), [4] β-sitosterol (**3**) [5,6], and β-sitosterol-3-*O*-β-D-glucopyranoside (**4**) [7] from *C. sinensis* (Figure 1).

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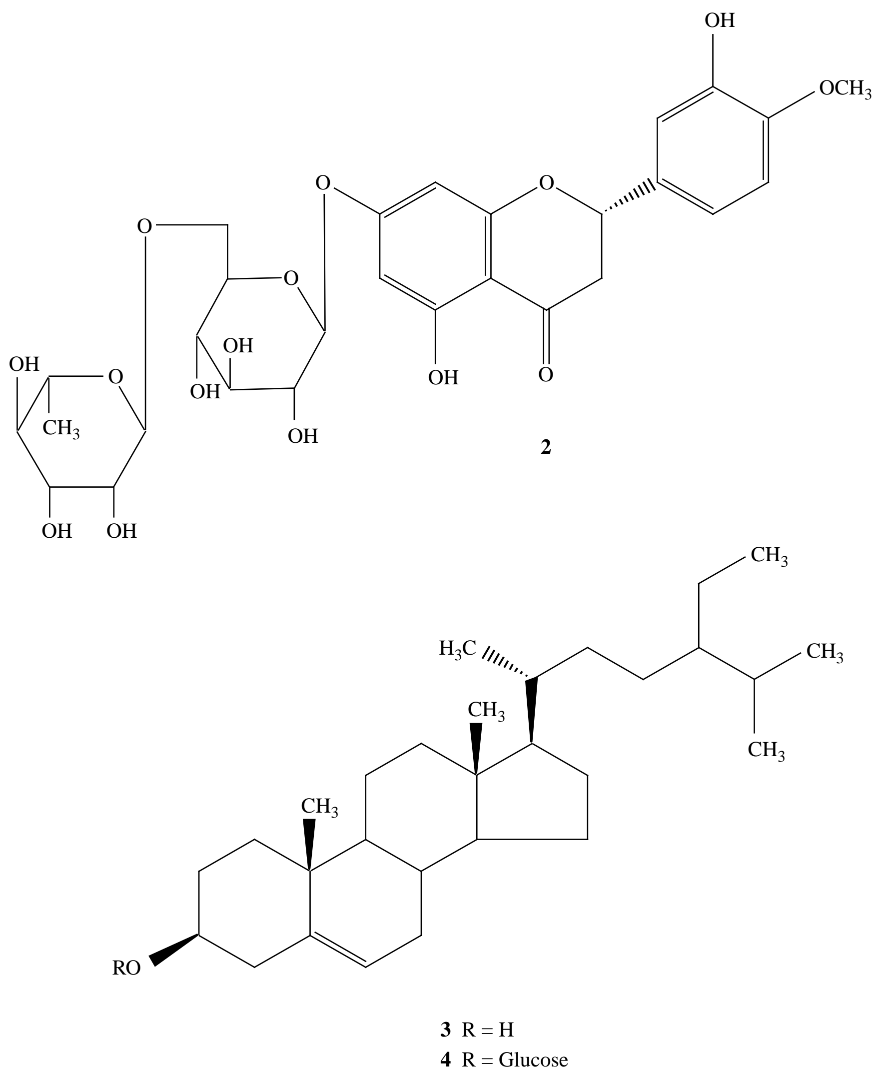

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Figure 1. Structures of compounds **2–4**.

2. Results and discussion

Compound **1** was isolated as a colorless amorphous powder. The molecular formula $C_{26}H_{51}NO_5$ was established with the help of HR-FAB-MS (positive ion mode), which showed a pseudo-molecular ion at *m/z* 458.3836 $[M + H]^+$. The EI-MS exhibited a fragment ion at *m/z* 439 after the loss of H_2O from the molecule. The formula showed two double bond equivalents (DBEs). The IR spectrum displayed characteristic absorption bands at 3510–3460 (O—H + N—H), 3105–2920 (C—H), 1660 (C═O), and 1630 (C═C) cm^{-1}. These data helped conclude that the two DBEs could be due to a carbonyl and a double bond, which means the compound must be an open-chain aliphatic one. This hypothesis was further supported due to the resonance of various proton multiplets between δ 1.23 and 1.30 with few diagnostic signals at δ 8.58 (1H, d, $J = 8.5$ Hz, CO—NH), 5.50 (2H, br m, $W_{1/2} = 19.8$ Hz, CH═CH), 5.11 (1H, m),

4.60 (1H, dd, $J = 10.6, 6.3$ Hz), 4.40 (1H, dd, 10.6, 3.4), 4.62, 4.49, and 4.44 (3H, m, for three oxygenated methines), and 0.85 (6H, t, $J = 6.2$ Hz) for a sphingolipid-type skeleton [8,9]. The corresponding carbons of the above-discussed H-signals appeared in ^{13}C NMR spectrum at δ 130.8, 130.6 (CH=CH), 76.8, 72.9, 72.4 ($3 \times$ OCH), and 61.9 (OCH_2), 52.9 (CH—NH) and 14.2 ($2 \times CH_3$). The signal resonating at δ 172.1 was attributed to an amide carbonyl. The magnitude of the $W_{1/2}$ of the ^{1}H-signal at δ 5.50 indicated the *trans* nature of the double bond. The positions of the OH groups were ascertained by the mass fragmentation pattern (Figure 2), ^{1}H,^{1}H-COSY (Figure 3), and HMBC spectra (Table 1). Cross-peaks in ^{1}H,^{1}H-COSY were observed between an amide H–N (δ 8.58) and H-2 (δ 5.11), which, in turn, was coupled to H-1a (δ 4.60), H-3 (δ 4.62), and H-1b (δ 4.40). H-3 (δ 4.62) showed correlations with H-2 (δ 5.11) and H-4 (δ 4.49). Furthermore, H-4 (δ 4.49) showed correlations with H-3 (δ 4.62) and H-5 (δ 4.44). COSY spectrum further helped to fix the position of C=C, as a methylene signal of a fatty acid chain resonating at δ 2.19 showed correlation with another methylene at δ 1.75, which in turn was found to couple with olefinic signal (δ 5.50), which further suggested the presence of a fatty acid chain without hydroxyl group at C-2′ [10]. The positions of the OH groups and the C=C bond were further confirmed from HMBC correlations (Table 1). The length of the fatty acid was determined by the characteristic ions (Figure 3) at *m/z* 153 ([Me(CH_2)$_6$

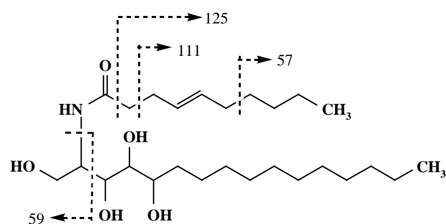

Figure 2. EI-MS fragmentation of **1**.

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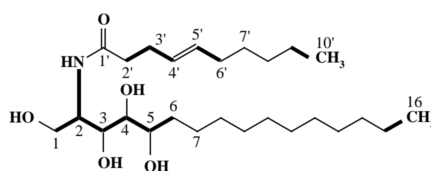

Figure 3. Structure and key COSY correlations of **1**.

(CH=CH)CO]$^+$), 170 ([Me(CH_2)$_6$(CH=CH)CONH$_2$ + H]$^+$), and 244 ([Me(CH_2)$_6$ (CH=CH)C(OH)=NC(CH)CH_2OH]$^+$). The length of the base unit was also determined by the characteristic ion peaks at *m/z* 212 ([M-Me(CH_2)$_{10}$CHOH)$_3$]$^+$), 245 ([Me(CH_2)$_{10}$(CHOH)$_3$]$^+$), and 215 ([Me(CH_2)$_{10}$CHOH)$_2$]$^+$) in the HR-EI-MS [10]. Methanolysis [11] of **1** with methanolic HCl gave the base and the methyl ester of the long-chain acid. After acetylation [11] and subsequent GC/MS, the two fragments could be identified as methyl dec-4-enoate (*m/z* 194) and 2-acetamino-1,3,4,5-tetraacetoxyhexdecane (*m/z* 531).

In natural ceramides from higher plants, the stereochemistries at C-2, C-3, and C-4 are already established as 2*S*, 3*S*, and 4*R*, [12] and many have been reported to have double bonds at C-5 [8,13]. In biosynthesis, the hydroxylation of these double bonds usually introduces hydroxyl function in anti-fashion. Few ceramides have been reported from higher plants with additional hydroxyl groups at C-5 [14–16], and the C-5 stereochemistry of hydroxyl groups in these compounds has been determined to be anti with reference to C-4 stereocenter, which is in agreement to the biosynthetic rules. Although the cited compounds have sugar moieties which may vary the optical rotation data from simple ceramides, the data suggested that compound **1** could have the same stereochemistry at C-2, C-3, C-4, and C-5.

On the basis of this evidence, the compound was elucidated as (*E*)-*N*-(1,3,4,5-tetrahydroxyhexadecan-2-yl)dec-4-enamide (**1**). This compound is a new addition in the list of natural products.

Table 1. ^{1}H and ^{13}C NMR spectral data and HMBC correlations of **1** ($CDCl_3/CD_3OD$).

Position	δ_H[a] (J in Hz)	δ_C[b]	HMBC[c] (H → C)
1	4.60 (1H, dd, 10.6, 6.3) 4.40 (1H, dd, 10.6, 3.4)	61.9	2, 3
2	5.11 (1H, m)	52.9	1, 3, 4, 1′
3	4.62 (1H, m)	72.9	1, 2, 4, 5
4	4.49 (1H, m)	76.8	2, 3, 5, 6
5	4.44 (1H, m)	72.4	3, 4, 6, 7
6	1.35 (1H, m)	32.1	4, 5, 7, 8
7	1.27 (2H, m)	22.9	5, 6, 8
8–14	1.29 (14H br s)	29.6–32.1	6, 7, 15, 16
15	1.23 (2H, m)	22.9	14, 16
16, 10′,	0.85 (6H, t, 6.3),	14.2	14, 15, 9′
NH	8.58 (1H, d, 8.5)	–	1, 2, 3, 1′, 2′
1′	–	172.1	–
2′	2.19 (2H, m)	35.7	1′, 3′, 4′
3′	1.75 (2H, m),	32.1	1′, 2′, 4′, 5′
4′	5.50 (1H, m, $W_{1/2} = 19.8$),	130.6	2′, 3′, 5′, 6′
5′	5.50 (1H, m, $W_{1/2} = 19.8$)	130.8	3′, 4′, 6′, 7′
6′	1.97 (2H, m),	33.8	4′, 5′, 7′
7′–9′	1.23 (6H, m).	29.8, 32.1, 22.9	5′,6′, 10′

Notes: [a] ^{1}H NMR spectra were recorded at 400 MHz.
[b] ^{13}C NMR spectra were recorded at 100 MHz.
[c] HMBC spectra were recorded at 400 MHz.

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The structures of the known compounds **2–4** were determined by comparing the spectral data with reported values in literature (Figure 1) [4–7].

3. Experimental

3.1 General experimental procedures

Optical rotations were measured on a Jasco DIP-360 digital polarimeter. IR spectra were recorded on Shimadzu IR-460 spectrophotometer (ν in cm^{-1}). FAB-MS and HR-FAB-MS spectra were recorded on Jeol JMS-HX 110 spectrometer with data system. The ^{1}H NMR spectra were recorded on Bruker AMX-400 MHz instrument using TMS as an internal reference. The chemical shift values are reported in ppm (δ) units and the scalar coupling constants (J) are in Hz. The ^{13}C NMR spectra were recorded at 100 MHz on the same instrument. Column chromatography was carried out using silica gel of 70–230 and 230–400 mesh. Aluminum sheets precoated with silica gel 60 F_{254} (20 × 20 cm, 0.2 mm thick; E-Merck; purchased from a local authorized dealer of E-Merck) were used for TLC to check the purity of the compounds and were visualized under UV light (254 and 366 nm), followed by ceric sulfate as spraying reagent.

3.2 Plant material

The leaves of *C. sinensis* var. were collected from the District Lodhran (Pakistan), and identified by Dr Muhammad Arshad (Plant Taxonomist), Cholistan Institute of Desert Studies (CIDS), The Islamia University of Bahawalpur, where a voucher specimen (CS-CIDS-29/09) is deposited. The plant material was dried under shade for 15 days and crushed to semi-powder (3 kg).

3.3 Extraction and isolation

The chopped plant was soaked in methanol (12 l) for 5 days twice. The extract was

concentrated under reduced pressure and dried to powder (40 g), which was suspended in water and extracted with *n*-hexane (9 g) and ethyl acetate (5 g). The concentrated ethyl acetate fraction (5 g) was further divided into eleven fractions by vacuum liquid chromatography (VLC) and by thin layer chromatography using silica gel as stationary phase and eluted with various compositions of *n*-hexane, chloroform, ethyl acetate, and methanol. The fraction obtained from VLC with 100% ethyl acetate was further cleaned by silica gel column chromatography eluted with chloroform, chloroform:methanol. A subfraction obtained with 4% methanol in chloroform yielded an amorphous powder which was further cleaned by washing with methanol to get compound **1** (15 mg). The fraction from VLC obtained by 25% chloroform in *n*-hexane after concentrating was washed with methanol to get **3** (55 mg), whereas the fraction eluted from VLC by 5% methanol in chloroform was further purified by silica gel column chromatography to get compound **4** (35 mg). The fraction eluted from VLC by 10% methanol in chloroform was further purified by silica gel column chromatography to get compound **2** (15 mg).

3.3.1 Compound 1

A white amorphous powder (15 mg), $[\alpha]_D + 21.7$ (*c* 0.0025, MeOH); IR ν_{max} (KBr): 3510–3460, 3105–2920, 1660, 1630, 1290 cm^{-1}; The ^1H and ^{13}C NMR spectral data (see Table 1); HR-FAB-MS (positive ion mode) *m*/*z*: 458.3836 $[M + H]^+$ (calcd for $C_{26}H_{52}NO_5$, 458.3845); EI-MS fragmentation pattern (see Figure 2).

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