

Cytotoxic glucosphingolipid from *Celtis Africana*

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ABSTRACT

Background: Literature survey proved the use of the powdered sun-dried bark and roots of *Celtis africana* for the treatment of cancer in South Africa. **Objective:** The aim of this study was to do further isolation work on the ethyl acetate fraction and to investigate the cytotoxic activities of the various fractions and isolated compound. **Materials and Methods:** Cytotoxicity of petroleum ether, chloroform, ethyl acetate, *n*-butanol fractions and compound 1 were tested on mouse lymphoma cell line L5178Y using the microculture tetrazolium assay. **Results:** One new glucosphingolipid 1 was isolated from the aerial parts of *C. africana*. The structure of the new compound was determined by extensive analysis by one-dimensional and two-dimensional nuclear magnetic resonance spectroscopy and mass spectrometry. The ethyl acetate fraction and compound 1 showed strong cytotoxic activity with an EC₅₀ value of 8.3 µg/mL and 7.8 µg/mL, respectively, compared with Kahalalide F positive control (6.3 µg/mL). **Conclusion:** This is the first report of the occurrence of a cytotoxic glucosphingolipid in family *Ulmaceae*.

Key words: *Celtis africana*, glucosphingolipid, microculture tetrazolium assay, *Ulmaceae*

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INTRODUCTION

Celtis africana Burm f. (*Ulmaceae*) is common and widespread in South Africa where the leaves are used as a traditional human and veterinary medicine for the treatment of indigestion and edema.^[1] The sun-dried bark and roots are powdered and infused in water or milk and taken orally every day by the patients for the treatment of cancer in South Africa.^[2,3] In our previous research on this plant, we reported the isolation and characterization of several new and known compounds from chloroform and *n*-butanol fractions.^[4,5] In continuation of our work on *C. africana*, we have examined the ethyl acetate fraction. In this paper, we describe the isolation and structure elucidation of the new glucosphingolipid by different spectroscopic analysis. This is the first report to isolate sphingolipid glucoside from the family *Ulmaceae*. The ethyl acetate fraction and isolated compound 1 were evaluated for their cytotoxic activity against L5178Y mouse lymphoma cells.

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MATERIALS AND METHODS

General experimental procedures

Optical rotation $[\alpha]_D^{25}$ was determined using a Jasco-DIP-360 digital polarimeter. The infrared (IR) spectrum was recorded on Jasco 320-A spectrometer. The hydrogen-1 nuclear magnetic resonance (¹H NMR), carbon-13 NMR (¹³C NMR), and two-dimensional NMR spectra were recorded on a Bruker AMX-400, 100 spectrometers. Chemical shifts are in ppm (δ), relative to tetramethylsilane as an internal standard and scalar coupling constants (J) reported in Hertz. Electrospray ionisation mass spectrometry (ESI-MS) were recorded on an Agilent Triple Quadrupole 6410 QQQ LC/MS mass spectrometer with ESI ion source (gas temperature 350°C, nebulizer pressure is 60 psi and gas flow rate 12 L/min), operating in the negative and positive scan modes of ionization through direct infusion method using CH₃OH-H₂O (1:1 v/v) at a flow rate of 0.4 ml/min. FAB and HRFABMS: (neg. ion mode, matrix: Glycerol) are performed on JEOL JMS-HX110 and JMS-DA-500 mass spectrometers. The gas chromatography (GC) was performed on a Shimadzu (GC-9A) (3% OV-1 silanized chromosorb W, column temperature 180°C, injection port

and detector temperature 275–300°C, flow rate 35 ml/min, flame-ionization detector). Column chromatography was carried out on silica gel 230–400 mesh (E. Merck, Darmstadt, Germany). Thin layer chromatography (TLC) was performed on precoated silica gel F₂₅₄ plates (E. Merck, Darmstadt, Germany); the detection was done at 254 nm and by spraying with ceric sulphate reagent. All chemicals were purchased from Sigma Chemical Company (St. Louis, MO, USA).

Plant material

The aerial parts of *C. africana* (1.2 kg) were collected from Riyadh (Saudi Arabia) and air-dried. The identity of the plant was verified by Dr. M. Atiqur Rahman, plant taxonomist, College of Pharmacy, King Saud University. A voucher specimen (No. 44) has been deposited in the herbarium of Department of Pharmacognosy, King Saud University.

Extraction and isolation

The aerial parts of *C. africana* were shade-dried, ground and extracted at room temperature with ethanol-water (8:2, thrice). The combined ethanol extract (65 g) was divided into petroleum ether (13.0 g), chloroform (7.0 g), ethyl acetate (8.0 g) and *n*-butanol (10.0 g) soluble sub-fractions. A part of the ethyl acetate soluble sub-fraction (6.0 g) was loaded on a silica gel column and the elution was successively carried out with mixture of *n*-hexane-ethyl acetate, and mixture of ethyl acetate-methanol in increasing order of polarity leading to two major sub-fractions I-II. The sub-fraction II obtained from *n*-hexane-ethyl acetate (1:1) showed one major spot on TLC along with little impurities and was further purified on silica gel column using *n*-hexane-ethyl acetate (4:6), to afford compound 1 (15 mg).

Cytotoxic activity

From the results, it could be seen that the ethyl acetate extract and compound 1 exhibited the most potent cytotoxic activity, with an EC₅₀ of 8.3 µg/mL and 7.8 µg/mL, respectively compared to Kahalalide F positive control (EC₅₀: 6.3 µg/mL). In general glucosphingolipids (GSL) exhibit a wide range of biological functions that might be related to the amphipathic nature of the molecule. Several GSL and other various glycolipids (GL) have been isolated from a number of marine sources, mainly including sponges and echinoderms. GL are known to possess immunomodulating and antitumor activity, in particular, those isolated from sponges. Previous literature reported the cytotoxic activity of various structurally related cerebrosides.^[6-10] The previous results suggested that the nature of the 2-hydroxylated fatty acyl chain (chain length and possible double bonds) seems to be important

for the cytotoxic activities of cerebrosides. Recently, it was shown that the nature of the sugar residue may be relevant for the biological activity of this type of GSL; those with glucopyranosides showing a stronger cytotoxicity than those with galactocerebrosides.^[11]

Cell cultures and microculture tetrazolium colorimetric assay

The cytotoxicity was tested against L5178Y mouse lymphoma cells using the microculture tetrazolium (MTT) assay. L5178Y mouse lymphoma cells were grown in Eagles minimal essential medium supplement with 10% horse serum in roller tube culture. The medium contained 100 units/ml penicillin and 100 µg/ml streptomycin. The cells were maintained in a humidified atmosphere at 37°C with 5% CO₂.^[12]

Of the test samples, stock solutions in ethanol 96% (v/v) were prepared. Exponentially growing cells were harvested, counted and diluted appropriately. Of the cell suspension, 50 µl containing 3750 cells were pipetted into 96-well microtiter plates. Subsequently, 50 µl of a solution of the test samples containing the appropriate concentration was added to each well. The concentration range was 3 and 10 µg/ml. The small amount of ethanol present in the wells did not affect the experiments. The test plates were incubated at 37°C with 5% CO₂ for 72 h. A solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was prepared at 5 mg/ml in phosphate buffered saline (PBS; 1.5 mM KH₂PO₄, 6.5 mM Na₂HPO₄, 137 mM NaCl, 2.7 mM KCl; pH 7.4) and from this solution, 20 µl was pipetted into each well. The yellow MTT penetrates the healthy living cells and in the presence of mitochondrial dehydrogenases, MTT is transformed to its blue formazan complex. After an incubation period of 3 h 45 min. at 37°C in a humidified incubator with 5% CO₂, the medium was centrifuged with 200 µl DMSO, the cells were lysed to liberate the formed formazan product. After thorough mixing, the absorbance was measured at 520 nm using a scanning microtiter-well spectrophotometer. The color intensity is correlated with the number of healthy living cells. Cell survival was calculated using the formula:

$$\text{Survival \%} = \frac{100 \times \text{absorbance of treated cells} - \text{absorbance of culture medium}}{\text{absorbance of untreated cells} - \text{absorbance of culture medium}}$$

All experiments were carried out in triplicates. As controls, media with 0.1% EGMME/DMSO were included in the experiments. The depsipeptide Kahalalide F isolated from *Elysia grandifolia* was used as positive control [Table 1].^[13]

RESULTS AND DISCUSSION

The ethyl acetate fraction of the ethanol extract of *C. africana* was subjected to column chromatography on silica gel to obtain one new glucocerebroside 1.

Compound 1 was obtained as white amorphous solid. The molecular formula was deduced as $C_{40}H_{75}NO_{10}$ by negative HRFABMS, which showed a molecular ion peak $[M - H]^-$ at m/z 728.5287 (calcd. for $C_{40}H_{74}NO_{10}$, 728.5312) indicating four degrees of unsaturation. The IR spectrum showed absorption bands for hydroxyl and amide functionalities (3250–3500/cm), an olefinic group (1665/cm) and an amide carbonyl group (1664/cm). Compound 1 gave a positive result to Molish test, suggesting its glycosidic nature.

The 1H NMR spectrum of 1 [Table 2] showed the presence of three oxymethines at δ 3.62 (1H, m), 3.53 (1H, m), 4.20 (1H, m), an oxymethylene at δ 4.07 (1H, m), 3.82 (1H, m), aliphatic methylenes δ 1.31 (24H, brs) and two terminal methyls at δ 0.91 (6H, t, $J = 7.0$ Hz) indicated that one could be a sphingolipid. The identity of one as a sphingolipid was also confirmed from its 1H -NMR spectrum by the presence of a characteristic amide NH doublet at δ 8.55 (1H, d, $J = 9.0$ Hz). In the 1H -NMR spectrum, the signal appeared at δ 4.28 (1H, d, $J = 9.0$ Hz) was assigned to nonoxygenated methine proton which is connected to amide NH.^[14] All of the above spectral data revealed that one was a phytosphingosine-type sphingolipid.

It further showed two trans-olefinic bonds at (δ 5.44 [1H, dd, $J = 15.5, 8.0$ Hz], 5.71 [1H, dd, $J = 15.6, 8.3$ Hz],

5.40 [2H, m]). The signals for hexose moiety at δ 4.30 (1H, d, $J = 7.5$ Hz), 3.20 (1H, t, $J = 8.0$ Hz), 3.30 (1H, m), 3.53 (1H, m), 3.38 (1H, m), 3.88 (1H, brd, $J = 11.0$ Hz) and 3.68 (1H, brd, $J = 11.0$ Hz) suggesting one could be a glucosphingolipid.^[15] The ^{13}C NMR spectrum of 1 [Table 2] was in full agreement with that of 1H NMR data as it showed the signal for an amide carbonyl group (δ 177.1), two double bonds (δ 131.5, 136.1, 130.7, 130.9), oxygenated carbons (δ 75.5, 72.9, 87.5), secondary amine (δ 51.6), aliphatic chain (δ 30.8) and sugar moiety (δ 104.6, 78.0, 77.8, 75.0, 71.5, 62.6). The long chain hydrocarbon skeleton and the substitutions at various positions were confirmed by 1H - 1H COSY and long-range heteronuclear multiple bond (HMBC) correlations. The carbon and proton spectra confirmed the signals of methylene and terminal methyl groups of the fatty acyl/alkyl chains present in compound 1.

The point of attachment of the glucose moiety was confirmed by HMBC experiments in which anomeric proton H-1'' (δ H 4.30) showed 3J correlation with C-1 (δ C 69.9). The sugar was identified as D-glucose by acid hydrolysis and co-TLC with an authentic sample. The coupling constant of the anomeric proton at δ H 4.30 (1H, d, $J = 7.3$ Hz, H-1'') showed the β -configuration of the glycoside linkage unit [Table 2].

The above spectral data, suggested that compound 1 was a glucosphingolipid and it was further supported by the mass fragmentation pattern [Figure 1], showing prominent peaks at m/z 565 due to the loss of the hexose moiety. The length of the hydroxy fatty acyl moiety was established to be C_{18} from the presence of a fragment ion at m/z 297 [$C_{18}H_{35}NO_2$] and 279 [$C_{18}H_{33}NO$]. The typical fragment ion at m/z 71 was formed by elimination of pentene from $[M]^+$ through McLafferty rearrangement. Methanolysis of 1 yielded methyl 2-hydroxyocta-6-enoate detected by GC-MS (GC-MS). The presence of a methyl 2-hydroxyocta-6-enoate moiety was also confirmed by the characteristic ion at m/z 312 in the EI-MS. The aqueous layer from the above methanolysis of 1 was neutralized and subjected to column chromatography (silica gel) to yield

Table 1: Cytotoxic activities of *Celtis africana* extracts and compound 1

Sample tested	L5178Y growth in percentage	EC ₅₀ (μ g/mL)
Petroleum ether extract	105.5	
Chloroform extract	62.3	
Ethyl acetate extract	10.3	8.3
<i>n</i> -butanol extract	80.5	
Compound 1	7.0	7.8
Kahalalide F (positive control)		6.3

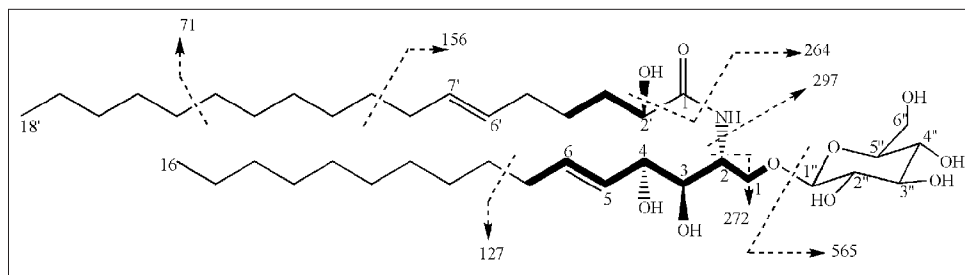


Figure 1: Structure, mass fragmentation pattern, and COSY (\rightarrow) correlations of compound 1

methyl D-glucoside, based on MS analysis (m/z 194) and optical rotation $[\alpha]_D^{25} = 76.5$.

The presence of a trihydroxy unsaturated C_{16} long-chain base was deduced from the 1H - 1H COSY and HMBC. In the 1H - 1H COSY spectrum, two methylene protons (H-1) at δ 4.07 and 3.82 correlated with the methine proton at δ 4.28 (H-2), the methine proton at δ 4.28 correlated with the methine proton at δ 3.62 (H-3), the methine proton at δ 3.62 correlated with the methine proton at δ 3.53 (H-4), which suggested the presence of three hydroxy groups at C-1, C-3 and C-4.^[6] A careful analysis of the HMBC and 1H - 1H COSY spectra enabled us to determine the position of a double bond at C-5 [Figure 1].

The double bonds were determined to be trans on the grounds of the allylic chemical shifts of C -5/C -6 and C -6'/C -7' at δ 33.1 (C -7) δ 33.1 (C -5') and δ 32.9 (C -8').^[16] The carbon chemical shifts at δ 69.9 (C -1), δ 51.6 (C -2), δ 75.5 (C -3), δ 72.9 (C -4), δ 177.1 (C -1') and δ 87.5 (C -2') in compound 1 were virtually identical with those of other (2S, 3S, 4R, 2'R) -phytosphingosine moieties.^[17,18] Based on these evidences, one could be identified as 1-O-(β -D-glucopyranosyl)-(2S, 3S, 4R, 5E)-2N-([2'R, 6'E]-2'-hydroxyoctadeca-6'-enoilamino)-5-pentadecaene-1, 3, 4-triol.

Compound 1

White amorphous powder from hexane-ethyl acetate (4:6), 14 mg, $[\alpha]_D^{25}$ 26.5 (C 0.05, MeOH); ESI-MS (m/z): 729; FABMS (negative mode, m/z): 728.5287; HRFABMS: m/z [M-H]⁻ calcd for $C_{40}H_{74}NO_{10}$, 728.5312; EIMS m/z : 565, 297, 279, 264, 222, 156, 138, 127, 71; IR (KBr) ν_{max} : 3250–3500 (hydroxyl), 1668 (olefinic group), 1664 (amide C = O)/cm; 1H and ^{13}C NMR data [Table 2].

Methanolysis of compound 1

Compound 1 (5 mg) was treated with 5% methanolic HCl at 80°C for 1 h. The fatty acid methyl ester produced was extracted with n-hexane and analyzed by GC-MS. A single peak was obtained (m/z 312, $C_{19}H_{36}O_3$ for of methyl 2-hydroxyocta-6-enoate from 1. The identity was further confirmed through Co-TLC with authentic samples. The MeOH layer was then neutralized by addition of Ag_2CO_3 . After centrifugation, the supernatant was evaporated to dryness under a nitrogen stream at room temperature. The residue was subjected to column chromatography on silica gel using $CHCl_3$ -MeOH- H_2O 95:3:0.5) to give a methyl glycoside and a long chain base. The former was treated with N-trimethylsilylimidazole, and the derivative was analyzed by GC (fused silica capillary column Bonded Supelcowax 10, 0.53 mm, 330 m). The peaks were identical with those of authentic methyl glycoside derivatives.

Table 2: 1H , ^{13}C -NMR and important HMBC correlations for compound 1 (CD_3OD)

1			
Position	δ (H)	δ (C)	HMBC (H→C)
Long chain base			
1	4.07 (m) 3.82 (m)	69.9	2, 1'', 3
2	4.28 (d, 9.0)	51.6	1, 1', 3, 4
3	3.62 (m)	75.5	1, 2, 4, 5
4	3.53 (m)	72.9	2, 5, 6
5	5.44 (dd, 15.5, 8.0)	131.5	3, 6, 7
6	5.71 (dd, 15.6, 8.3)	136.1	4, 5, 8
7	2.07 (m)	33.1	5, 6, 8
8	1.27 (brs)	30.5	6, 7, 10
9	1.28 (brs)	30.3	
10-14	1.31 (m)	30.8	
15	1.33 (m)	23.7	16
16	0.91 (t, 7.0)	14.5	15
NH	8.55 (d, 9.0)*		
N-acyl moiety			
1'	-	177.1	-
2'	4.20 (m)	87.5	1', 3', 4'
3'	1.78 (m)	35.7	1', 2', 4', 5'
4'	1.28 (m)	30.3	2', 5', 6'
5'	2.07 (m)	33.1	3', 6', 7'
6'	5.40 (m)	130.7	4', 5', 7', 8'
7'	5.40 (m)	130.9	5', 6', 8'
8'	2.01 (m)	32.9	6', 7', 9'
9'	1.31 (brs)	30.5	7', 10'
10'-16'	1.31 (brs)	30.8	
17'	1.33 (m)	23.7	18'
18'	0.91 (t, 7.0)	14.5	17'
Sugar moiety			
1''	4.30 (d, 7.3)	104.6	1, 2'', 3''
2''	3.20 (m)	75.0	
3''	3.30 (m)	77.8	
4''	3.53 (m)	71.5	
5''	3.38 (m)	78.0	
6''	3.88 (brd, 11.0) 3.68 (brd, 11.0)	62.6	

*This signal found in pyridine- d_6 . HMBC: Heteronuclear multiple bond correlation; NMR: Nuclear magnetic resonance

CONCLUSION

The present study reported the isolation of compound 1 from the ethyl acetate soluble sub-fraction of *C. africana*. This adds sphingolipids glucoside 1 to the list of genus *Celtis*, and there is no report found for the isolation of any sphingolipid glucoside from this family *Ulmaceae*. The isolation and identification of compound 1 from *C. africana* represents a contribution to the phytochemical analysis of the components of the plant, and it could be a potential chemical marker and chemotaxonomic constituent for *C. africana*.

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