Namibian Chewing Stick, *Diospyros lycioides*, Contains Antibacterial Compounds against Oral Pathogens

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The twigs of *Diospyros lycioides*, a plant commonly known as "muthala", are frequently used as chewing sticks for the cleaning of teeth by rural and urban people in Namibia. Preliminary studies showed that a methanol extract of *D. lycioides* inhibited growth of selected oral pathogens. Subsequent bioassay-guided fractionation led to the isolation of four novel bioactive naphthalene glycosides, diospyrosides A, B, C, and D (**1**−**4**), and two known bioactive naphthoquinones, juglone (**5**) and 7-methyljuglone (**6**). The structures of the new compounds were elucidated using spectroscopic techniques including 1D and 2D NMR. These compounds inhibited the growth of oral cariogenic bacteria (*Streptococcus mutans* and *Streptococcus sanguis*) and periodontal pathogens (*Porphyromonas gingivalis* and *Prevotella intermedia*) at minimum inhibitory concentrations ranging from 0.019 to 1.25 mg/mL. Juglone exhibited the strongest inhibitory activity among these compounds.

Keywords: *Diospyros lycioides; Ebenaceae; African chewing sticks; "Miswak"; "Muthala"; diospyrosides A−D; juglone; 7-methyljuglone; naphthalene glycosides; oral bacteria; periodontal pathogens; oral hygiene; natural oral antimicrobial compounds; antiplaque agents*

INTRODUCTION

It is generally accepted that oral hygiene maintenance through regular removal of dental plaque and food deposits is an essential factor in the prevention of dental caries and periodontal disease (Greene, 1963; Mandel, 1988). Methods for oral hygiene vary from country to country and from culture to culture. In many Middle Eastern, African, and Asian countries chewing sticks have been extensively used for the cleaning of teeth over thousands of years (Homer et al., 1990; Hardie and Ahmed, 1995). The World Health Organization (WHO) has recommended and encouraged the use of these sticks as an effective tool for oral hygiene (Al Lafi and Ababneh, 1995). Chewing sticks are made from either the roots, stems, or twigs of local trees and shrubs. The wood is cleaned, the bark is removed, and the wood is cut into suitable lengths, made into bundles, and sold in the local market. *Diospyros lycioides* Desf. (Ebenaceae), known commonly as "muthala", is a popular chewing stick used for tooth cleaning in Namibia (Addo-Yobo et al., 1991). A natural oral health survey in Namibia involving 2394 subjects (12−44 years) revealed that ∼20% of the subjects used *D. lycioides* twigs (Schier, 1993). In general, these muthala users had a relatively low caries rate (Schier and Cleaton-Jones, 1995). Although it is generally believed that these sticks provide benefits to oral health by both mechanical and therapeutic means, scientific evidence of actual active components present in these chewing sticks is limited.

Our laboratory has focused on the search for plant-derived oral antimicrobial agents, especially chewing sticks. We have previously reported two new antimicrobial binaphthalenone glycosides from *D. lycioides* twigs, namely, 1′,2-binaphthalen-4-one-2′,3-dimethyl-1,8′-epoxy-1,4′,5,5′,8,8′-hexahydroxy-8-*O*-*β*-glucopyranosyl-5′-*O*-*β*-xylopyranosyl(1→6)-*β*-*O*-glucopyranoside and 1′,2-binaphthalen-4-one-2′,3-dimethyl-1,8′-epoxy-1,4′,5,5′,8,8′-hexahydroxy-5′,8-di-*O*-*β*-xylopyranosyl(1→6)-*β*-glucopyranoside (Li et al., 1998). The present study was conducted in an attempt to isolate and identify additional antibacterial compounds from the MeOH extract. Guided by antimicrobial assays, four novel naphthalene glycosides and two known naphthoquinones have been isolated and identified. Their structures were established using a variety of spectroscopic techniques. In this paper, the isolation, identification, and antimicrobial activity of these compounds are reported.

MATERIALS AND METHODS

General. ^{1}H and ^{13}C NMR spectra were recorded on Bruker DRX-500 and DPX-300 spectrometers at 500.16 and 300.13 MHz for proton and 125.76 and 75.47 MHz for carbon spectra, respectively. Compounds were analyzed in MeOD-d_4 with TMS as internal standard. Fast atom bombardment (FAB) mass spectra were obtained using a Finnigan MAT-90 (Bremen, Germany) mass spectrometer. Thin-layer chromatographic (TLC) analysis was performed on Kieselgel 60 (Merck, Darmstadt, Germany) plates (silica gel F_{254} glass or aluminum-backed sheets). Compounds were visualized by spraying with 10% (v/v) H_2SO_4 followed by charring at 110 °C for 10 min. Silica gel (Merck 60A, 230−400 mesh ASTM), C_{18} reversed-phase silica gel (HPLC sorbent, 40−63 μm, Sigma Chemical Co., St. Louis, MO), and polymeric adsorbent resin (Diaion HP-20, 20−60 mesh, Supelco Inc., Bellefonte, PA) were used for column chromatography.

Plant Material. Authentic twigs of *D. lycioides* Desf. were collected in Namibia. A voucher specimen of this plant has

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been deposited at the Oral and Dental Research Institute, Faculty of Dentistry, University of Stellenbosch, Tygerberg, South Africa.

Extraction and Isolation Procedure. The ground dried twigs of *D. lycioides* (300 g) were refluxed with MeOH (1.5 L × 3) in a Soxhlet extraction apparatus. Removal of the solvent yielded an MeOH extract (33.3 g), which demonstrated growth inhibitory activity against the oral pathogens *Streptococcus mutans* and *Porphyromonas. gingivalis* at 1.25 mg/mL. The MeOH extract was dissolved in H_2O (100 mL). The solution was loaded on a Diaion HP-20 column (500 g of resin, 10 × 100 cm) and the column eluted with H_2O (1.5 L) and MeOH (2 L), successively. The MeOH eluate was concentrated under reduced pressure to yield a residue (6.9 g), which demonstrated growth inhibitory activity against *S. mutans* [minimum inhibitory concentration (MIC) = 1.25 mg/mL)] and *P. gingivalis* (MIC = 0.625 mg/mL). This residue was chromatographed on a silica gel column (200 g) and eluted with $CHCl_3$/MeOH (7:1, 2 L) and $CHCl_3$/MeOH/H_2O (50:10:1, 3 L), (40:10:1, 3 L), (30:10:1, 2 L), and (20:10:1, 1.5 L). Eighty fractions were collected, which were combined into 20 pooled fractions based on their TLC profiles. The active fractions were further separated in the following manner: Fractions 31−33 (221 mg) yielded compound **1** (30 mg) by passage through a silica gel column (22 g, 2.5 × 50 cm) eluted with $CHCl_3$/MeOH/H_2O (50:10:1, 2 L) and further chromatography on a reversed-phase silica gel RP-18 column (10 g, 1 × 30 cm) eluted with 40% MeOH (200 mL). Fractions 25−27 yielded compound **2** (1.6 mg) through a silica gel column (22 g, 2.5 × 50 cm) eluted with $CHCl_3$/MeOH/H_2O (50:10:1, 1.5 L). Fractions 19−24 produced compounds **3** and **4** from a silica gel column (22 g, 2.5 × 50 cm) eluted with $CHCl_3$/MeOH/H_2O (50:10:1, 2 L). Fraction 1 (166 mg) gave compounds **5** (7.2 mg) and **6** (5.2 mg) from a silica gel column (10 g, 1 × 30 cm) chromatograph eluted with 3% MeOH in $CHCl_3$ (1 L).

Acidic Hydrolysis of 1−4. MeOH solutions of the above compounds **1**−**4** together with standard sugar samples were applied at a point 1 cm from the bottom of silica gel TLC plates and hydrolyzed with HCl vapor for 1 h at 50 °C. The plate was then heated at 60 °C for 2 h to remove residual HCl, developed using $CHCl_3$/MeOH/AcOH/H_2O (14:6:2:1) as eluate, sprayed with 10% H_2SO_4, and charred at 110 °C for 10 min to visualize the sugars. Glucose and xylose were detected, having R_f values of 0.28 and 0.42, respectively, in a ratio of 2:1 for compound **1** and in a ratio of 1:1 for compounds **2**−**4**.

*Diospyroside A [1,4,5-trihydroxy-7-methylnaphthalene-4-O-β-xylopyranosyl(1→4)-β-xylopyranosyl(1→6)-β-glucopyranoside (**1**)]* was obtained as a purple amorphous powder: FABMS (negative), *m/z* 616 $[M]^-$, 615 $[M - H]^-$, 189 [aglycon − H]$^-$; FABMS (positive), *m/z* 639 $[M + Na]^+$, 617 $[M + H]^+$, 507 [M + Na − Xyl^{132}], 190 $[aglycon]^+$; ^1H and ^{13}C NMR spectra, see Table 1.

*Diospyroside B [5,8-dihydroxy-2-methyl[1,4]naphthoquinone-5-O-β-xylopyranosyl(1→6)-β-glucopyranoside (**2**)]* was obtained as a purple amorphous powder: FABMS (negative), *m/z* 498 $[M]^-$, 497 $[M - H]^-$, 203 [aglycon − H]$^-$; FABMS (positive), *m/z* 521 $[M + Na]^+$, 499 $[M + H]^+$, 389 [M + Na − Xyl^{132}], 204 $[aglycon]^+$; ^1H and ^{13}C NMR spectra, see Table 1.

*Diospyroside C [8,6′-binaphthalene-1′,4′-one-7,7′-dimethyl-1,4,5,5′-tetrahydroxy-4-O-β-xylopyranosyl(1→6)-β-glucopyranoside (**3**)]* was obtained as a purple amorphous powder: FABMS (negative), *m/z* 670 $[M]^-$, 669 $[M - H]^-$, 359 [aglycon − H]$^-$; FABMS (positive), *m/z* 693 $[M + Na]^+$, 671 $[M + H]^+$, 561 [M + Na − Xyl^{132}], 360 $[aglycon]^+$; ^1H and ^{13}C NMR spectra, see Table 1.

*Diosproside D [6,8′-binaphthalene-1′,4′-one-7,7′-dimethyl-1,4,5,5′-tetrahydroxy-4-O-β-xylopyranosyl(1→6)-β-glucopyranoside (**4**)]* was obtained as a purple amorphous powder: FABMS (negative), *m/z* 670 $[M]^-$, 669 $[M - H]^-$, 359 [aglycon − H]$^-$; FABMS (positive), *m/z* 693 $[M + Na]^+$, 671 $[M + H]^+$, 360 $[aglycon]^+$; ^1H and ^{13}C NMR spectra, see Table 1.

*7-Methyljuglone (**5**)* was obtained as a yellow amorphous residue: mp 150−152 °C (lit. mp 155 °C); structure assigned by comparing MS and NMR data with those found in the literature (Budzianowski, 1995).

*Juglone (**6**)* was obtained as a yellow amorphous residue: mp 150−152 °C (lit. mp 155 °C); structure assigned by comparing MS and NMR data with those found in the literature (Hedin, 1980).

Determination of Antibacterial Activity against Oral Pathogens. Growth inhibitory effects of the four isolated novel compounds were tested against cariogenic oral streptococci including *S. mutans* strain Ingbritt and *Streptococcus sanguis*, and the periodontal pathogens most frequently associated with periodontitis, *P. gingivalis* and *Prevotella intermedia*. The procedures employed were as described previously (Cai and Wu, 1996). Sterile 96-well microtiter plates were used. Each well contained test bacteria [5×10^5 colony forming units (CFU)/mL for *S. mutans* and *S. sanguis* and 5×10^6 CFU/mL for *P. gingivalis* and *P. intermedia*], serially diluted test compounds, and the respective growth medium. Three samples were used for each test concentration, and the experiments were performed in triplicate. The controls included inoculated growth medium without test compounds. Sample blanks contained uninoculated medium only. All plates were incubated at 37 °C under appropriate atmospheric conditions with growth estimated spectrophotometrically (650 nm) after 48 h using a microtiter plate reader (PowerWave 200 microplate scanning spectrophotometer, Bio-Tec Instruments, Winooski, VT). The MIC for each test bacterium was defined as the minimum concentration of test compound limiting turbidity to <0.05 absorbance at 650 nm. For a positive control, the plant alkaloid sanguinarine with documented antimicrobial activity was used (Sigma Chemical Co.).

RESULTS AND DISCUSSION

The crude methanolic extract of dried twigs of *D. lycioides* demonstrated preferential growth inhibitory activity against the oral pathogens *S. mutans* and *P. gingivalis* at 1.25 mg/mL. It was subjected to Diaion HP-20 column chromatography and eluted with water followed by methanol. The methanolic eluate was further separated on a silica gel column and yielded compounds **1**−**6**. Compounds **5** and **6** were established as juglone (5-hydroxy-1,4-naphthoquinone) and 7-methyljuglone (5-hydroxy-7-methyl-1,4-naphthoquinone), which have been previously described in the literature (Budzianowski, 1995; Hedin et al., 1980).

Compound **1** was obtained as a purple amorphous powder. It produced a deep yellow color with ferric chloride and a dark blue color with sulfuric acid on TLC. The molecular formula was deduced as $C_{27}H_{36}O_{16}$ from an $[M - H]^-$ peak observed at *m/z* 615 and an $[M + Na]^+$ peak observed at *m/z* 639 in the FABMS, in conjunction with the analysis of the NMR data. The acid hydrolysis of **1** yielded only glucose and xylose in a ratio of 1:2. The ^1H NMR spectrum of compound **1** displayed three anomeric proton signals at δ 4.96 (d, $J = 7.5$ Hz), 4.38 (d, $J = 7.4$ Hz), and 4.29 (d, $J = 7.5$ Hz), suggesting that all three of the sugars possessed β-pyranosyl configurations. The presence of the three sugars was also supported by the anomeric carbon signals at δ 105.1, 105.0, and 104.0 in the ^{13}C NMR spectrum. The portion of the ^{13}C NMR spectrum of compound **1** due to the aglycon moiety demonstrated 11 resonance signals (Table 1), representing 10 aromatic carbon signals and 1 characteristic methyl signal at δ 22.0 in the upfield region. The ^1H NMR spectrum due to the aglycon moiety of **1** displayed two pairs of coupled aromatic doublets and one methyl singlet (Table 1). These spectroscopic characteristics suggested that the aglycon of compound **1** was a 7-methyljuglone derivative.

Further information on the structure of **1** was obtained from 2D NMR experiments. The HMQC spectrum of compound **1** established one-bond C−H connec-

Table 1. ^{13}C and ^{1}H NMR Data for Compounds 1−4[a,b]

	1 (in MeOD-d_4)			**2** (in pyr-d_5)			**3** (in MeOD-d_4)			**4** (in MeOD-d_4)		
C/H	δ_C	δ_H (J, Hz)	HMBC corrre-lation (C no.)	δ_C	δ_H (J, Hz)	HMBC corre-lation (C no.)	δ_C	δ_H (J, Hz)	HMBC corre-lation (C no.)	δ_C	δ_H (J, Hz)	HMBC corre-lation (C no.)
aglycon												
1	150.0			190.8			151.3			150.0		
2	108.1	6.67 d (8.3)	1, 4, 9	146.2			110.4	6.58 d (8.5)	1, 4, 9	110.4	6.72 d (8.3)	1, 4, 9
3	112.1	7.15 d (8.3)	1, 10	138.5	6.69 d (1.4)	1, 10, 11	112.8	7.24 d (8.5)	1, 4, 10	112.8	7.21 d (8.3)	1, 4, 10
4	148.6			184.1			148.9			148.7		
5	154.5			154.1			154.7			151.4		
6	113.9	6.68 d (1.6)	5, 8, 10, 11	130.4	8.36 d (9.4)	8, 10	114.9	6.84 s	5, 7, 8, 10, 11	123.9		
7	137.3			126.9	7.47 d (9.4)	5, 9	136.0			135.6		
8	114.0	7.44 d (1.2)	1, 6, 9, 10, 11	158.3			121.7			115.1	7.65 s	6, 7, 8, 10, 11
9	128.9			114.7			126.8			127.9		
10	116.1			119.6			117.5			116.4		
11	22.0	2.40 brs	6, 7, 8,	15.2	1.98 br s	1, 2, 3	21.0	2.00 br s	6, 7, 8	21.0	1.99 br s	6, 7, 8
1′							186.6			186.3		
2′							141.6	6.76 d (10.2)	4′, 9′	140.5	6.94 d (10.2)	4′, 9′
3′							140.1	6.97 d (10.2)	1′, 10′	138.5	6.96 d (10.2)	1′, 10′
4′							192.2			191.7		
5′							160.5			162.5		
6′							141.8			125.8	7.30 s	7′, 8′, 10′, 11′
7′							149.8			147.3		
8′							121.4	7.51 s	6′, 7′, 10′, 11′	133.7		
9′							130.7			130.4		
10′							113.5			115.2		
11′							20.7	1.98 br s	6′, 7′, 8′	20.2	1.97 br s	6′, 7′, 8′
sugar												
Glc-1	105.1	4.96 d, (7.5)	4, Glc-3 and -5	104.7	5.42 d (7.3)	5, Glc-5	105.2	5.02 d (6.7)	4, Glc-3 and -5	104.7	4.98 d (7.3)	4, Glc-3 and -6
Glc-2	75.1	3.41 m		75.1	4.08 dd (7.3, 7.8)		75.1	3.52 m		75.0	3.51 m	
Glc-3	77.8[a]	3.67 m		78.1	4.15 m		77.8	3.70 m		77.7	3.68	
Glc-4	71.5	3.40 m		71.4[a]	4.23 m		71.5	3.43 m		71.5	3.42 m	
Glc-5	78.0	3.31 m		78.4	4.19 m		78.1	3.41 m		78.1	3.40 m	
Glc-6	69.8	4.13 dd (11.5, 1.5)		70.1	4.93 d (9.8)		70.1	4.18 dd (12.0, 1.8)		70.0	4.14 dd (12.0, 1.8)	
		3.81 dd (11.5, 6.4)			3.29 m			3.84 dd (12.0, 5.0)			3.82 dd (12.0, 6.3)	
Xyl-1	105.0	4.38 d (7.4)	Glc-1 and -6, Xyl-5	106.1	5.02 d (7.3)	Glc-6, Xyl-5	105.6	4.39 d (7.6)	Glc-6, Xyl-3	105.5	4.37 d (7.5)	Glc-6, Xyl-3 and 5
Xyl-2	74.7	3.23 m		74.9	4.38 m		75.0	3.23 m		75.0	3.22 m	
Xyl-3	75.7	3.52 m		77.9	4.32 m		77.7	3.26 m		77.7	3.25 m	
Xyl-4	78.3	3.47 m		71.2[a]	4.36 m		71.3	3.45 m		71.3	3.44 m	
Xyl-5	64.4	3.97 dd (11.7, 5.2)		67.2	4.37 m		66.9	3.87 dd (11.5, 5.1)		66.9	3.86 dd (11.3, 5.0)	
		3.56 dd (11.7, 3.6)			3.67 t (10.4)			3.19 dd (11.5, 1.0)			3.17 dd (11.3, 1.0)	
Xyl-1′	104.0	4.29 d (7.5)	Xyl 1 and -5′									
Xyl-2′	74.3	3.21 m										
Xyl-3′	77.6[a]	3.30 m										
Xyl-4′	71.0	3.38 m										
Xyl-5′	67.1	3.89 dd (11.3, 5.3)										
		3.52 dd (11.3, 3.4)										

[a] Interchangeable assignments within the spectrum. [b] Assignments confirmed by HMQC, HMBC, DQF-COSY, ROESY, and TOCSY experiments.

tivity, whereas $^1H-^1H$ COSY determined each spin-coupling system. In the HMBC spectrum of compound **1**, the methyl signal at δ 2.40 (s) displayed cross-peaks with the carbon signals at δ 137.3 (s), 114.0 (d), and 113.9 (d) (Table 1). The proton signal at δ 7.44 (d, J = 1.2 Hz) correlated with five carbon signals at δ 150.0 (s), 128.9 (s), 116.1 (s), 113.9 (d), and 22.0 (q). The proton signal at δ 6.68 (d, J = 1.2 Hz), which showed long-range coupling with the signal at δ 7.44 (d) in the $^1H-^1H$ COSY, displayed cross-peaks with four carbon signals at δ 154.5 (s), 116.1 (s), 114.0 (d), and 22.0 (q). The proton signal at δ 7.15 (d, J = 8.3 Hz) displayed cross-peaks with the carbon signals at δ 150.0 (s), 148.6 (s), and 116.1 (s). The proton signal at δ 6.67 (d, J = 8.3 Hz), which showed coupling with the signal at δ 7.15 (d) in the $^1H-^1H$ COSY spectrum, had cross-peaks with three carbon signals at δ 150.0 (s), 148.6 (s), and 128.9 (s). The above evidence led to the assignment of the aglycon of **1** as 1,4,5-trihydroxy-7-methylnaphthalene. The anomeric proton signal at δ 4.96 (d) showed a long-range correlation with the carbon signals at δ 148.6 (s), 78.0 (d), and 77.8 (d) in the HMBC spectrum. This sugar was identified as glucose by a comprehensive analysis of $^1H-^1H$ COSY, TOCSY, HMQC, and HMBC spectra. The linkage sites and sequence of three saccharide and aglycon moieties were established by the HMBC spectrum. It was observed that two xylose anomeric proton signals at δ 4.38 (d) and 4.29 (d) showed long-range correlations with carbon signals at δ 75.1, 69.8, and 64.4 and δ 78.3 and 67.0, respectively. The glycosidic coupling of the first xylose to the hydroxyl group of C-6 of the glucose resulted in downfield shifts for C-6 (~8−9 ppm) and H-6 (~0.3 ppm) (Table 1). The linkage of the second xylose to the hydroxyl group of C-4 of the first one was confirmed by the downfield shift of C-4 (~7 ppm) of the first xylose and the upfield shift of C-5 and C-3 (~2 ppm). Therefore, the structure of compound **1** was determined to be 1,4,5-trihydroxy-7-methylnaphthalene-4-*O*-β-xylopyranosyl(1→4)-β-xylopyranosyl (1→6)-β-glucopyranoside and named diospyroside A.

Compound **2** was a purple amorphous powder. It produced a molecular ion peak at m/z 498 $[M]^-$ in the negative ion FABMS and a quasi-molecular ion peak at m/z 521 $[M + Na]^+$ in the positive ion FABMS, consistent with having a molecular formula of $C_{22}H_{26}O_{13}$. Acid hydrolysis of compound **2** produced glucose and xylose in a ratio of 1:1. Anomeric carbon signals at δ 106.1 and 104.7 in the ^{13}C NMR spectrum supported the presence of these two sugars in their β-pyranosyl configurations. The ^{13}C NMR spectrum of compound **2** demonstrated 11 aglycon moiety carbon signals (Table 1), among which there were 2 carbonyls resonating at δ 190.8 and 184.1, and 1 characteristic methyl signal at δ 15.2 in the upfield region. The ^1H NMR spectrum displayed one pair of ortho-coupled (J = 9.4 Hz) aromatic doublets, one aromatic doublet with long-range coupling, and one methyl singlet (Table 1). These spectroscopic characteristics suggested that, by a comprehensive analysis of $^1H-^1H$ COSY, TOCSY, HMQC, and HMBC spectra, the aglycon of compound **2** was 5,8-dihydroxyl-2-methyl-1,4-naphthoquinone. Glycosidation of the hydroxyl at C-5, not at C-8, was confirmed by the analysis of long-range coupled peaks of the HMBC spectrum. The linkage position of the xylose to C-6 of the inner glucose was confirmed by the downfield glycosylation shifts ($\Delta\delta \sim 8$ ppm for C-6) (Budzianowski, 1997). Thus, the structure of compound **2** was established as 5,8-dihydroxy-2-methyl[1,4]naphthoquinone-5-*O*-β-xylopyranosyl (1→6)-β-glucopyranoside and named diospyroside B.

Compound **3** was a purple amorphous powder. The molecular formula was deduced as $C_{33}H_{34}O_{15}$ from an $[M - H]^-$ peak observed at m/z 669 and an $[M + Na]^+$ peak observed at m/z 693 in the FABMS, in conjunction with the analysis of the NMR data. The acid hydrolysis of compound **3** yielded only glucose and xylose in a ratio of 1:1. The ^1H NMR spectrum of **3** displayed two anomeric proton signals at δ 5.02 (d, J = 6.7 Hz) and 4.39 (d, J = 7.6 Hz), suggesting that all of the two sugars possessed β-pyranosyl configurations. Additionally, the presence of the two sugars was also supported by the anomeric carbon signals at δ 105.2 and 105.6 in the ^{13}C NMR spectrum. The ^{13}C NMR spectrum of compound **3** due to its aglycon moiety demonstrated 22 resonance signals (Table 1), among which there were 2 characteristic methyl signals at δ 20.7 and 21.0 in the upfield region. The ^1H NMR spectrum due to the aglycon moiety displayed two pairs of coupled aromatic doublets and two methyl singlets (Table 1). These spectroscopic characteristics suggested that the aglycon of compound **3** was a dimer of a 7-methyljuglone derivative. Further information was obtained from 2D NMR experiments. In the HMBC spectrum of compound **3**, the methyl signal at δ 2.00 (s) displayed cross-peaks with the carbon signals at δ 136.0 (s), 121.7 (s), and 114.9 (d). The proton signal at δ 6.84 (s) correlated with five carbon signals at δ 154.7 (s), 136.0 (s), 121.7 (s), 117.5 (s), and 21.0 (q). The proton signal at δ 7.24 (d, J = 8.5 Hz) displayed cross-peaks with the carbon signals at δ 151.3 (s), 148.9 (s), and 117.5 (s). The proton signal at δ 6.58 (d, J = 8.5 Hz), which showed coupling with the signal at δ 7.24 (d) in the $^1H-^1H$ COSY spectrum, had cross-peaks with four carbon signals at δ 151.3 (s), 148.9 (s), and 126.8 (s). Considering the above evidence, it was concluded that 1,4,5-trihydroxy-7-methylnaphthalene was a part of the dimer aglycon. Another methyl signal at 1.98 (br s) revealed cross-peaks with the carbon signals at δ 149.8 (s), 141.8 (s), and 121.4 (d). The proton signal at δ 7.51 (s) had cross-peaks with four carbon signals at δ 149.8 (s), 141.8 (s), 113.5 (s), and 20.7 (q). The proton signal at δ 6.97 (d, J = 10.2 Hz) displayed cross-peaks with the carbon signals at δ 186.6 (s) and 113.5 (s). The proton signal at δ 6.76 (d, J = 10.2 Hz), which showed coupling with the signal at δ 6.97 (d) in the $^1H-^1H$ COSY spectrum, had cross-peaks with signals at δ 192.2 (s) and 130.6 (s). All of this information demonstrated that the other part of the dimer aglycon was 7-methyljuglone. It was noticed that there were only six aromatic protons in the ^1H NMR spectrum of this naphthalene dimer. Meanwhile, quaternary carbon signals δ 121.7 and 141.8 (s) were assigned to C-8 and C-6′, respectively, supported by DEPT, HMQC, and HMBC spectral data. Therefore, the linkage position of these two 7-methyljuglone derivatives was identified at C-8/C-6′ by comprehensive analysis of the NMR data. Glycosylation of the hydroxyl at C-4 and the linkage position of this xylose to C-6 of the inner glucose were assigned by analyses similar to those described for compounds **1** and **2**. Thus, the structure of compound **3** was as postulated as 8,6′-binaphthalene-1′,4′-one-7,7′-dimethyl-1,4,5,5′-tetrahydroxy-4-*O*-β-xylopyranosyl (1→6)-β-glucopyranoside (diospyroside C).

Compound **4** had the same molecular formula as compound **3**, $C_{33}H_{34}O_{15}$, from its FABMS and NMR

Table 2. MIC (Milligrams per Milliliter) of Compounds Isolated from *D. lycioides* against Oral Pathogens

compound	*S. mutans*	*S. sanguis*	*P. gingivalis*	*P. intermedia*
diospyroside A	1.25	0.039	0.078	0.039
diospyroside B	0.625	0.039	0.078	0.156
diospyroside C	0.156	0.625	0.312	0.039
diospyroside D	0.156	0.312	0.156	0.156
juglone	0.078	0.039	0.039	0.019
7-methyljuglone	0.156	0.078	0.039	0.078

data. It also displayed spectroscopic characteristics very similar to those of **3** and hence could be tentatively assigned as a glycosidic dimer of a 7-methyljuglone derivative. The difference between these two compounds is the linkage position of the two aglycon monomers. In the HMBC spectrum, the H-8 signal at δ 7.65 (d, $J = 1$ Hz) had cross-peaks with 123.9 (s) (C-6) and 116.4 (s) (C-10). The proton signal at δ 7.30 (H-6′) showed cross-peaks with 162.5 (s) (C-5′), 133.7 (s) (C-8′), and 115.2 (s) (C-10′). These clearly indicated that the linkage position of the monomer units was C-6/C-8′. Glycosylation of the hydroxyl at C-4 and the linkage position of this xylose to C-6 of the inner glucose were assigned by analyses similar to those described for compounds **1** and **2**. Thus, the structure of compound **4** was identified as 6,8′-binaphthalene-1′,4′-one-7,7′-dimethyl-1,4,5,5′-tetrahydroxy-4-*O*-*β*-xylopyranosyl (1→6)-*β*-glucopyranoside (diospyroside D).

As shown in Table 2, the six antimicrobial compounds identified from *D. lycioides* demonstrated inhibitory activity against the Gram-positive cariogenic oral streptococci, *S. mutans* and *S. sanguis*, and the Gram-negative, anaerobic rods, *P. gingivalis* and *P. intermedia*, frequently associated with human periodontitis (gum disease). Their MIC values ranged from 0.019 to 1.25 mg/mL. Among the six substances, juglone demonstrated the strongest activity against test bacteria with MIC values ranging from 0.019 to 0.078 mg/mL. Substitution of the methyl group at C-7 of juglone (7-methyljuglone) did not appear to alter its antimicrobial activity because comparable MIC values were noted for both compounds. Diospyrosides A and B were assigned as glycosidated derivatives of 7-methyljuglone (Figure 1). Although the MIC values of diospyrosides A and B against *S. mutans* were higher compared to those of 7-methyljuglone, all four glycosidated derivatives inhibited growth of *S. sanguis*, *P. gingivalis*, and *P. intermedia*, with MIC values comparable to those obtained from 7-methyljuglone. It appears that the glycosidation of juglone or 7-methyljuglone did not drastically affect the antimicrobial activities of these compounds.

Chewing sticks made from the roots of selected plants have been used for centuries as oral hygiene tools in many parts of the world, especially in Middle Eastern and African countries, for example, “miswak” from *Salvadora persica* (Farooql and Srlvastava, 1968; El Said, 1969). It has been reported that the incidence of caries among users of chewing sticks in these areas is low despite a high-carbohydrate diet and a lack of modern dental prophylactic measures (Elvin-Lewis et al., 1980). In a comprehensive survey of several thousand Saudi school children, Guile et al. (1988) reported that the low incidence of periodontal disease noted appeared to be related to the practice of using miswak for oral hygiene. Several studies have demonstrated equal plaque-removing effects of toothbrushes and chewing sticks (Olsson, 1978; Sote, 1987). No differences in plaque and gingival bleeding scores were found

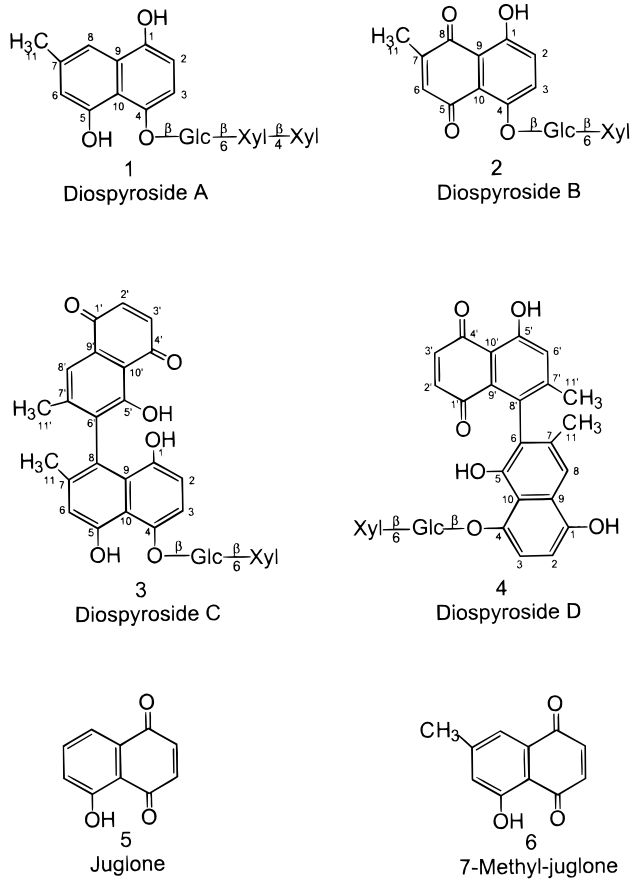

Figure 1. Structures of diospyrosides A−D, juglone, and 7-methyljuglone isolated from *D. lycioides*.

between toothbrush and chewing stick users among 7−15-year-old children in Tanzania (Normark and Mosha, 1989). Not only do chewing sticks remove plaque effectively in a mechanical fashion, but they may also contain antimicrobial compounds that inhibit oral pathogens that cause dental caries and periodontal disease.

Several studies have been performed to determine the biological activity of substances present in the chewing sticks, and a number of active compounds have been detected. Extracts from *Fagara zanthoxyloides* demonstrated antisickling activity of red blood cells (Sofowora and Isaacs, 1971), and an alkaloid, fagaronine, has been shown to possess tumor-inhibiting (Messmer et al., 1972) and antimicrobial (Asuquo and Montefiore, 1977) properties. Asuquo and Montefiore (1977) reported a substance isolated from *Terminalia glaucescens* that had activity against *Staphylococcus aureus*. However, relatively few studies have been performed on oral pathogens. Wolinsky and Sote (1983) examined the effect of aqueous extracts from eight Nigerian chewing sticks against growth and adherence of the cariogenic bacterium *S. mutans*. They found that an extract from *Serindeia warnecki* demonstrated the strongest inhibitory activity and speculated that tannin-like, heat stable substances might be involved in the microbial inhibitory activity; however, no chemical evidence was provided. A few investigations reported the inhibitory effect of chewing stick extracts on the growth of black-pigmented *Bacteroides* species (Rotimi and Masadomi, 1983; Rotimi et al., 1987), but none of these studies identified the nature of the active constituents. Most researchers speculated that tannin-like substances might have contributed to the observed antimicrobial activity.

We have previously (Li et al., 1998), as well as in the current paper, reported the isolation and identification of selected known and novel antimicrobial compounds from the Namibian chewing sticks, *D. lycioides*, capable of inhibiting oral disease-causing bacteria. These data have not been previously reported and contribute greatly to the understanding of the antimicrobial properties of the chewing sticks. Although it is difficult to extrapolate from these in vitro results to the likely effects in the mouth, these data may also provide explanations for the rarity of dental caries and gingivitis among the users of chewing sticks (Manley et al., 1975). Because chewing sticks are readily available, inexpensive, and culturally acceptable in many developing countries, they may represent an effective alternative tool to toothbrushes/toothpastes for the prevention and control of oral diseases.

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