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CYTOTOXIC NAPHTHOQUINONES, MOLLUSCICIDAL SAPONINS AND FLAVONOLS FROM *DIOSPYROS ZOMBENSIS*

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Key Word Index—*Diospyros zombensis*; Ebenaceae; naphthoquinones; saponins; flavone glycosides; molluscicides; cytotoxins.

Abstract—7-Methyljuglone, isodiospyrin and three glucuronides of oleanolic acid were isolated from the lipophilic and the methanol extracts, respectively, of the root bark of *Diospyros zombensis*. Cytotoxic activities of the naphthoquinones and strong molluscicidal activity of 3-*O*-[*O*-α-L-rhamnopyranosyl-(1 → 3)-β-D-glucuronopyranosyl]-oleanolic acid were shown. In addition hyperoside, quercitrin and quercetin-3-*O*-β-D-glucuronopyranoside were isolated from a methanol extract of the leaves.

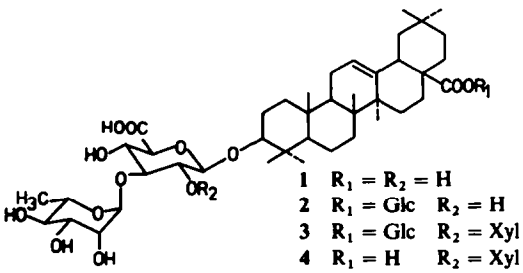
INTRODUCTION

Numerous *Diospyros* species are used in Africa as a chewing stick [1]; several of these plants have been reported to contain molluscicidal [2], fungicidal [2], antibiotic [3], termiticidal [4], immunostimulant [5] and antitumoral [6] naphthoquinones. The root bark of *D. zombensis* White, a plant which has not been phytochemically investigated previously, is used by the traditional healers of Malawi (southeast Africa) for the treatment of schistosomiasis. The petrol and chloroform extracts were molluscicidal (*Biomphalaria glabrata*), fungicidal (*Cladosporium cucumerinum*) and cytotoxic against human colon carcinoma cells. Thin layer chromatography analysis of the methanolic extract showed the presence of saponins, which could not be detected in other *Diospyros* species such as *D. usambarensis*, *D. whyteana* and *D. lycioides*. In the present paper, we report, in addition to flavonoid glycosides and biologically active naphthoquinones, for the first time structure determination of molluscicidal saponins in the Ebenaceae.

RESULTS AND DISCUSSION

The petrol and chloroform extracts of the root bark of *Diospyros zombensis* were submitted to flash and low-pressure liquid chromatography on silica gel yielding the known naphthoquinones isodiospyrin and 7-methyljuglone. The identity of these compounds was established by comparison of their spectral data (UV, MS (EI), ^{1}H NMR) with those from the literature [7–9], by mixed melting points and HPLC co-chromatography with authentic samples. On-line HPLC/UV spectroscopy on a CN-chemically bonded column with *n*-hexane–acetic acid (99:1) was used. In addition to the known molluscicidal and fungicidal activities of 7-methyljuglone and the fungicidal activity of isodiospyrin [2], both naphthoquinones showed cytotoxic activities against human colon carcinoma cells. 7-Methyljuglone had a LD_{50} of 7.0×10^{-2} μg/ml and isodiospyrin a LD_{50} of 3.8×10^{-2} μg/ml, whereas the known antitumour compounds vinblastine and 5-fluoruracil exhibited LD_{50} values of 0.55×10^{-2} μg/ml and 6.5×10^{-2} μg/ml respectively [10].

The methanol extract of the root bark was subjected to DCCC, reversed-phase chromatography and gel filtration (Sephadex LH 20) providing saponins **1**, **2** and **3**. Hydrolysis of the three saponins, under acidic conditions, afforded the same aglycone, which was identified as oleanolic acid by comparison with an authentic sample (TLC, ^{13}C NMR, MS). The sugars obtained from the saponin hydrolysates were rhamnose and glucuronic acid from **1**, rhamnose, glucuronic acid and glucose from **2** and rhamnose, glucuronic acid, glucose and xylose from **3**. Basic hydrolysis of **2** afforded saponin **1** and glucose, whereas **3** yielded compound **4** and glucose. This suggested that **2** and **3** are bidesmosidic saponins with a glucosyl unit attached through an ester bond to C-28 of oleanolic acid. The M_r and the sugar sequence of the saponins were established by fast atom bombardment mass spectrometry (FABMS). The FABMS (thioglycerol matrix, negative ion mode) of **1** showed a quasimolecular ion $[M-H]^-$ at *m/z* 777 and signals at *m/z* 631 $[(M-H)-\text{rhamnosyl}]^-$ and *m/z* 455 $[(M-H)-(\text{rhamnosyl})-(\text{glucuronyl})]^-$, indicating that rhamnose was the terminal sugar. The FABMS of **2** presented a quasimolecular ion $[M-H]^-$ at *m/z* 939 and signals at *m/z* 793 $[(M-H)-\text{rhamnosyl}]^-$, *m/z* 777 $[(M-H)-\text{glucosyl}]^-$, *m/z* 631 $[(M-H)-(\text{rhamnosyl})-(\text{glucosyl})]^-$ and *m/z* 455 $[(M-H)-(\text{rhamnosyl})-(\text{glucosyl})-(\text{glucuronyl})]^-$.



1 $R_1 = R_2 = H$
2 $R_1 = Glc$ $R_2 = H$
3 $R_1 = Glc$ $R_2 = Xyl$
4 $R_1 = H$ $R_2 = Xyl$

The FABMS of 3 exhibited a quasi-molecular ion at m/z 1071 $[M-H]^-$ and signals at m/z 939 $[(M-H)-\text{xylosyl}]^-$, m/z 909 $[(M-H)-\text{glucosyl}]^-$, m/z 925 $[(M-H)-\text{rhamnosyl}]^-$, m/z 793 $[(M-H)-(\text{rhamnosyl})-(\text{xylosyl})]^-$, m/z 777 $[(M-H)-(\text{glucosyl})-(\text{xylosyl})]^-$, m/z 763 $[(M-H)-(\text{glucosyl})-(\text{rhamnosyl})]^-$ and m/z 455 $[(M-H)-(\text{glucosyl})-(\text{rhamnosyl})-(\text{xylosyl})-(\text{glucuronosyl})]^-$. The FABMS of 4, the prosapogenin of 3, showed a quasi-molecular ion at m/z 909 $[M-H]^-$ and signals at m/z $[(M-H)-\text{xylosyl}]^-$, m/z 763 $[(M-H)-\text{rhamnosyl}]^-$, m/z 631 $[(M-H)-(\text{xylosyl})-(\text{rhamnosyl})]^-$ and m/z 455 $[(M-H)-(\text{xylosyl})-(\text{rhamnosyl})-(\text{glucuronosyl})]^-$. This indicates that xylose and rhamnose are terminal sugars, attached to the glucuronic acid moiety. The glycosylation of the C-3 hydroxyl group of the aglycone was confirmed by ^{13}C NMR. Carbons C-3 of 1-4 appeared between 89 and 90 ppm, whereas in oleanolic acid this C-atom was observed at 78.8 ppm [11].

The free COOH-group on C-28 of 1 and 4 appeared at 180.3 and 180.1 ppm, whereas when esterified with a glucosyl moiety, the chemical shift was 176.4 ppm. The β-D-configuration of glucose, xylose and glucuronic acid and the α-L-configuration of rhamnose were established by comparison of the ^{13}C chemical shifts of the anomeric carbons of the sugars with published data [12].

The interglycosidic linkage of compounds 1 and 4, isolated previously in our laboratory from *Swartzia madagascariensis* (Leguminosae) [13] and *S. simplex* [14] respectively, were determined by GC/MS of their methylated alditolacetate derivatives [13].

Thus, the structure of 1 is established as 3-*O*-[*O*-α-L-rhamnopyranosyl-(1 → 3)-*O*-β-D-glucuronopyranosyl]-oleanolic acid, that of 2 as β-D-glucopyranosyl 3-*O*-[*O*-α-L-rhamnopyranosyl-(1 → 3)-*O*-β-D-glucuronopyranosyl] oleanolate and that of 3 as β-D-glucopyranosyl 3-*O*-[(*O*-β-D-xylopyranosyl-(1 → 2)) (*O*-α-L-rhamnopyranosyl (1 → 3))β-D-glucuronopyranosyl]oleanolate.

Saponins 1 and 2 have been previously identified by chemical methods from seed coats of *Putranjiva roxburghii* (Euphorbiaceae) [15] and by spectroscopic methods from aerial parts of *Zexmentia buphthalmiflora* (Compositae) [16]. Saponins 3 and 4 have been isolated previously from fruits of *Swartzia simplex* (Leguminosae) [14]. The monodesmosidic saponin 1 exhibited strong molluscicidal activity (3 ppm) against *Biomphalaria glabrata* snails [17] whereas the bidesmosidic saponins 2 and 3 were inactive. The methanol extract exhibited no molluscicidal activity at 400 ppm. This is perhaps due to the low concentration of 1 in the plant.

Three known flavone glycosides were isolated from the methanol extract of the leaves of *D. zombensis*. The identity of quercitrin, hyperoside and quercetin-3-*O*-β-D-glucuronopyranoside [18] was established by chemical (acidic hydrolysis) and spectral methods (UV with shift reagents [19], D/CIMS, ^1H NMR) and comparison with authentic samples. Quercetin-3-*O*-β-D-glucuronopyranoside was confirmed by FABMS and ^{13}C NMR.

EXPERIMENTAL

Plant material. The root bark and leaves of *D. zombensis* were collected in 1984 at Zomba, Malawi (southeast Africa) and identified by M. Banda, Herbarium, University of Malawi, Zomba. A voucher specimen is deposited at this Herbarium.

General techniques. TLC was carried out on precoated silica gel 60-F 254 Al sheets (Merck) with $CHCl_3$–MeOH–H_2O (65:40:10) (saponins, flavone glycosides) (system 1), petrol–EtOAc (3:1) (naphthoquinones), EtOAc–H_2O–MeOH–HOAc (65:15:15:20) (sugars) and on RP-8 pre-coated glass plates (HPTLC, Merck) with MeOH–H_2O in different proportions. Detection was by UV (254 nm, 366 nm), Godin reagent [20] (saponins), diphenylboryloxyethylamine [21] (flavone glycosides) and *p*-anisidine phthalate (sugars). Droplet counter-current chromatography (DCCC) was carried out using DCCC-A (Tokyo Rikakikai, Japan) and DCCC Chromatograph 670 (Büchi, Switzerland) apparatus, with 316 (2 × 400 mm) and 283 (2.7 × 400 mm) tubes, respectively. CC and flash chromatography were achieved on silica gel 60 (63–200 μm, Merck) and Sephadex LH 20 (Pharmacia Fine Chemicals). Lobar LiChroprep RP-8 columns (40–63 μm; 2.5 × 27 cm; Merck) equipped with a Duramat-80 pump (Chemie und Filter, Regensdorf) were used; the flow rate was 3 ml/min. On-line HPLC/UV spectroscopy was achieved on a μ-Bondapak CN column (10 μm; 3.9 × 300 mm; Waters) coupled with a photodiode array detection system HP 1040 A (Hewlett Packard); detection was at 254 nm.

^1H NMR were recorded in $CDCl_3$ (naphthoquinones), DMSO-d_6 (flavone glycosides), ^{13}C NMR in C_5D_5N (saponins) and DMSO-d_6 (flavone glycosides) with TMS as int. standard. FABMS were obtained on a ZAB-1S spectrometer (VG instruments), EIMS on a Kratos MS-30, D/CI MS on a Ribermag R-10-10B (Nermag). Bioassays were made with *Biomphalaria glabrata* snails [22]. Acidic and basic hydrolyses as described in ref. [22].

Isolation and identification. The dried powdered root bark of *D. zombensis* (137 g) was extracted successively with petrol (3 × 11), $CHCl_3$ (3 × 11), MeOH (3 × 11) and finally with MeOH 70% (3 × 11) yielding 0.7 g, 1.2 g, 11.9 g and 7.3 g of extracts, respectively. The lipophilic extracts were submitted to flash CC with petrol–EtOAc (7:1). Isodiospyrin was recrystallized from *n*-heptane–CH_2Cl_2 in yields of 25 mg and 10 mg. The fractions containing 7-methyljuglone were purified on a Lobar silica gel column with toluene–EtOAc (99:1). 10 mg were recrystallized from *n*-heptane–CH_2Cl_2. The methanol extract suspended in H_2O (300 ml) was partitioned with *n*-BuOH (3 × 300 ml). The butanol extract (7.9 g) was separated on CC with $CHCl_3$–MeOH–H_2O (65:30:0.5 → 65:60:5) with a flow of 50 ml/hr, affording fractions 1–4. Fraction 2 (200 mg) was filtered on Sephadex LH 20 (MeOH). The saponin containing fractions were evaporated, injected on a Lobar RP-8 column and eluted with MeOH–H_2O (70:30), yielding 40 mg of saponin 1. Fraction 3 (900 mg) was filtered on Sephadex LH 20 (MeOH), the fractions were evaporated (600 mg) and chromatographed in three portions on a Lobar RP-8 column with MeOH–H_2O (60:40). The resulting saponin fraction was submitted to DCCC with *n*-BuOH–Me_2CO–H_2O (33:10:50) in the descending mode (316 columns, 20 ml/hr) yielding 12 mg of 2. Fraction 4 (1.2 g) was injected onto a DCCC apparatus (283 columns, 50 ml/hr) using the same system and mode as before. The mobile phase, containing saponin 3 was evaporated (200 mg) and chromatographed on silica gel CC with $CHCl_3$–MeOH–H_2O (65:45:10). The main fraction (130 mg) was separated on a Lobar RP-8 column with MeOH–H_2O (60:40). Filtration on Sephadex LH 20 (MeOH) yielded 70 mg of saponin 3.

The dried powdered leaves (200 g) were extracted with petrol, $CHCl_3$, MeOH and MeOH 70%. The methanol extract (30 g) was suspended in H_2O (400 ml) and partitioned successively with $CHCl_3$ (3 × 400 ml) and *n*-BuOH (3 × 400 ml). The butanol extract (20 g) was separated on silica gel CC with $CHCl_3$–MeOH–H_2O–HOAc (80:20:2:1 → 80:50:5:1). Fraction 1 (500 mg) was injected in three portions onto a Lobar RP-8 column and eluted with MeOH–H_2O–HOAc (53:47:1). The eluate was neutralized (conc NH_3), evaporated and filtered on

Sephadex LH 20 (MeOH) yielding 100 mg of quercetrin. Fraction 2 (300 mg) was injected onto a Lobar RP-8 column, eluted with MeOH–H_2O–HOAc (50:50:1), neutralized (conc NH_3) and filtered on Sephadex LH 20 (MeOH), yielding 50 mg of hyperoside. Fraction 3 (10 g) was filtered on Sephadex LH 20 (MeOH). The flavone glycoside-containing fraction (1 g) was injected in six portions onto a Lobar RP-8 column, eluted with MeOH–H_2O–HOAc (48:52:1), neutralized (conc NH_3), evapd and filtered on Sephadex LH 20 (MeOH), yielding 80 mg of quercetin-3-*O*-β-D-glucuronopyranoside.

3-O-[O-α-L-*Rhamnopyranosyl*-(1 → 3)-O-β-D-*glucuronopyranosyl*]-*oleanolic acid* (1). $C_{42}H_{66}O_{13}$; M_r 778; powder; R_f 0.35 (system 1); ^{13}C NMR (50.29 MHz, C_5D_5N): δ's of the aglycone correspond to those of oleanolic acid [11]; rhamnose: 102.9 (C-1); 72.5 (C-2); 72.8 (C-3); 74.2 (C-4), 69.8 (C-5); 18.5 (C-6); glucuronic acid: 107.0 (C-1); 75.9 (C-2); 82.7 (C-3); 71.7 (C-4); 77.7 (C-5); 172.8 (C-6).

β-D-*Glucopyranosyl* 3-O-[O-α-L-*rhamnopyranosyl*-(1 → 3)-β-D-*glucopyranosyl*] *oleanolate* (2). $C_{48}H_{76}O_{18}$; M_r 940; powder; R_f 0.23 (system 1).

β-D-*Glucopyranosyl* 3-O-[(O-β-D-*xylopyranosyl*-(1 → 2))(O-α-L-*rhamnopyranosyl* (1 → 3)) β-D-*glucuronopyranosyl*] *oleanolate* (3); $C_{53}H_{84}O_{22}$; M_r 1072; powder; R_f 0.2 (System 1); ^{13}C NMR (100.61 MHz; C_5D_5N): δ's of the aglycone correspond to those of oleanolic acid [11]; rhamnose: 103.1 (C-1); 72.6 (C-2); 72.8 (C-3); 74.1 (C-4); 70.2 (C-5); 18.3 (C-6); xylose: 104.7 (C-1); 75.7 (C-2); 77.0 (C-3); 71.2 (C-4); 67.2 (C-5); glucuronic acid: 105.1 (C-1); 79.2 (C-2); 84.2 (C-3); 71.2 (C-4); 78.5 (C-5); 176.4 (C-6); glucose: 95.8 (C-1); 74.2 (C-2); 78.9 (C-3); 72.1 (C-4); 79.4 (C-5); 62.2 (C-6).

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