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Phenolic and Terpenoid Constituents from the Sri Lankan Medicinal Plant *Osbeckia aspera*

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Abstract

A crude aqueous acetone extract of *Osbeckia aspera* Blume (Melastomataceae), a plant from Sri Lanka used traditionally to treat liver disease, was fractionated by column and preparative paper chromatography, and the fractions were analyzed by high-performance liquid chromatography (HPLC) using diode array and mass spectrometric detection. Phenolic acids (gallic, protocatechuic, and ellagic acid), flavonol glycosides [quercetin 3-*O*-β-galactopyranoside, quercetin 3-*O*-β-glucopyranoside, kaempferol 3-*O*-β-glucopyranoside, and kaempferol 3-*O*-(6″-*O*-*p*-coumaroyl-β-glucopyranoside) (tiliroside)] and flavonol aglycones (quercetin and kaempferol) were identified by comparison of their retention times, UV and MS spectra with those of authentic standards. Five compounds from a methanol extract were identified by NMR spectroscopy as the flavonol glycosides, quercetin 3-*O*-(3″-*O*-acetyl-β-galactopyranoside) and kaempferol 3-*O*-[2″,6″-di-*O*-(*E*, *E*)-*p*-coumaroyl-β-glucopyranoside], and the norsesquiterpenoids 6,9-dihydroxy-4,7-megastigmadien-3-one, 9-hydroxy-4,7-megastigmadien-3-one and 9-hydroxy-4-megastigmen-3-one. A crude water extract, 50% acetone extract and fractions from this extract, a 100% methanol extract, and three of the phenolic acids in the fractions were tested for *in vitro* hepatoprotective activity against bromobenzene and 2,6-dimethyl-*N*-acetyl *p*-quinoneimine toxicity to HepG2 liver-derived cells. The crude water extract showed protective activity against both liver toxins, whereas the fractions and compounds were more protective against 2,6-dimethyl-*N*-acetyl *p*-quinoneimine than bromobenzene. Of the three phenolic acids present in the extracts that were tested, gallic and protocatechuic acids were more active at protecting the liver cells from the two toxic compounds than ellagic acid.

Keywords: Flavonoids, hepatoprotection, megastigmane derivatives, melastomataceae, *Osbeckia aspera*, phenolic acids.

Introduction

For many centuries, extracts of plants have been used in traditional medicine for the treatment of liver diseases, including viral hepatitis, chronic cholecystitis, alcohol-related liver disorders, as well as mushroom poisoning (Thabrew & Hughes, 1996). Plant constituents associated with the hepatoprotective activity of these extracts include various flavonoids. For example, the flavan-3-ol (+)-catechin is thought to be the active constituent in *Uncaria gambier* Roxb. (Rubiaceae), a species used to treat acute and chronic hepatitis, and the flavonolignan mixture, silymarin, from the milk thistle, *Silybum marianum* Gaertn. (Asteraceae), is used to treat toxic and metabolic liver damage (Valenzuela et al., 1986). Crude aqueous extracts of two closely related species, *Osbeckia octandra* DC. and *O. aspera* Blume (Melastomataceae), have traditionally been used as a treatment for viral hepatitis by Ayurvedic and other traditional practitioners in Sri Lanka (Jayaweera, 1982). These extracts exhibited protective effects against galactosamine and *tert*-butylhydroperoxide-induced hepatocyte damage *in vitro* (Thabrew et al., 1995a), and against carbon tetrachloride (Thabrew et al., 1987) and paracetamol-induced liver injury *in vivo* (Thabrew et al., 1995b). The immunomodulatory

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effects of crude extracts from these species have been studied previously (Thabrew et al., 1991; Nicholl et al., 2001), but the active constituents have not been determined. However, another species of *Osbeckia, O. chinensis* L., which has long been used as an anti-inflammatory agent and antipyretic in China, has been chemically analyzed and a number of antioxidant flavonoids and hydrolyzable tannins were isolated and identified (Su et al., 1987a, 1988). In addition, osbeckic acid, a furan-carboxylic acid, was obtained from this species and shown to be an antioxidative synergist (Su et al., 1987b). Antioxidant and free radical scavenging properties are considered to be an important mechanism by which plants may exert their liver-protective properties (Valenzuela et al., 1986; Navarro et al., 1992). A crude aqueous extract of *O. aspera* was shown to have significant activity against the xanthine oxidase generated l,l-diphenyl-2-picrylhy-drazyl free radical in a dose-dependent manner and demonstrated a scavenging effect on hydroxyl radical mediated damage to deoxyribose (Thabrew et al., 1998).

As antioxidant activity is often associated with the phenolics in plants (Valenzuela et al., 1986; Navarro et al., 1992; Grayer et al., 2003), we have now analyzed extracts and fractions of *O. aspera* for flavonoids and phenolic acids. This article describes the fractionation of crude aqueous acetone and methanol extracts of *O. aspera* by column and paper chromatography and the analysis of the fractions by high-performance liquid chromatography coupled to diode array detection (HPLC-DAD) and mass spectrometry (LC-MS). Compounds that could not be identified by co-chromatography with authentic standards were purified further by semipreparative HPLC and analyzed by NMR spectroscopy. The hepatoprotective activities of the crude extracts, fractions, and some pure compounds were determined using an *in vitro* cytotoxicity assay with HepG2 human liver-derived cells exposed to bromobenzene (BB) and 2,6-dimethyl *N*-acetyl *p*-quinoneimine (2,6-diMeNAPQI).

Materials and Methods

Plant material

Mature leaves of *O. aspera* were collected in the wild at Meetiyagoda, southern Sri Lanka. The plant material was identified by Dr. A.H.M. Jayasuriya, Curator, Botanical Gardens (Peradeniya, Sri Lanka), and a voucher specimen (BI 12647) has been deposited of the herbarium, Royal Botanic Gardens (Kew, UK). The material was air-dried in Sri Lanka and then freeze-dried on arrival in The United Kingdom, ground and stored at 4°C.

Extraction and fractionation

Three different crude extracts of *Osbeckia aspera* were prepared: (1) a water extract (by boiling 5 g of ground freeze-dried leaves with 50 ml of H_2O for 30 min), (2) a 50% acetone extract [by steeping 10 g of ground freeze-dried leaves twice in 100 mL of acetone/water (1:1, v/v) for 24 h at room temp] and (3) a methanol extract (by extracting 50 g of the same leaf material twice in 500 mL of 100% MeOH for 24 h at room temp). After filtration, the water extract was freeze-dried and the aqueous acetone and methanol extracts were concentrated by rotary evaporation at 40°C. The water extract was used for activity tests and a sample (equivalent to 1 g of dried material) was analyzed by HPLC and LC-MS. A small portion of the 50% acetone extract was used for activity tests and another was analyzed by HPLC. The remainder of this extract was used for fractionation on a Sephadex LH-20 column [130 × 15 (i.d.) mm]. The extract on the column was eluted first with 100% H_2O and then with increasing amounts of MeOH in H_2O (50 mL each of 100% H_2O, 20% MeOH, 40% MeOH, 60% MeOH, 80% MeOH, and 100% MeOH). The eluted fractions were collected in volumes of 50 mL (fractions A–F), which after concentration were further fractionated by preparative paper chromatography using 15% aqueous HOAc as the solvent. After chromatographic development, the papers were inspected under UV light and cut into three or four bands, which were eluted in 50% aq. acetone. The paper chromatographic fractions were numbered according to the R_f value of the eluted bands of the chromatograms (e.g., A12 is the fraction prepared from fraction A after elution of the chromatographic band with an R_f value of 0.12). All these fractions were tested for liver protective activity *in vitro.*

The methanol extract was used for isolation of the compounds, especially the less polar ones. After evaporation, the residue was redissolved in MeOH and used for fractionation on a silica gel column [460 × 25 (i.d.) mm]. The concentrated extract on the column was eluted first with petroleum ether b.p. 40–60°C (100 mL), then 100 mL each of petroleum etherethyl acetate mixtures with an increasing proportion of ethyl acetate (7:3 and 3:7), followed by elution with 100% ethyl acetate, subsequently ethyl acetate-methanol mixtures with increasing proportions of methanol (7:3 and 3:7), and finally 100% methanol. Fractions of 50 mL were collected and monitored by HPLC-DAD, so that those containing the same compounds could be combined. These combined fractions were used for the isolation of the compounds by semipreparative HPLC.

Spectral and HPLC analysis

^{1}H NMR (400 MHz) spectra were recorded in CD_3OD (30°C) or DMSO-d_6 (37°C) on either Bruker Avance 400 MHz or Varian 600 MHz instruments. Residual solvent resonances were used as internal references relative to TMS at 0.00 ppm. Standard pulse sequences and parameters were used to acquire 1D ^{1}H, 1D ^{13}C, ID selective Rotating Frame Overhauser Enhancement (ROE), Double Quantum Filtered-Correlation (DQF-COSY), Heteronuclear Single Quantum Correlation (HSQC), and Heteronuclear Multiple Bond Correlation (HMBC) data as required. Positive ion atmospheric pressure chemical ionization (APCI) MS were obtained with a quadrupole ion-trap instrument (Finnigan LCQ, Cheshire, UK) using a vaporizer temperature of

550°C, sheath and auxiliary nitrogen gas pressures of 80 and 20 psi, a needle current of 5 μA, and a heated capillary temperature of 150°C. Samples were introduced by direct infusion, or via an HPLC fitted with a Merck (Darmstadt, Germany) LiChrospher 100RP-18 (250 × 4.0 mm i.d.; 5 μm particle size) column using a 20-min linear gradient of 25–100% MeOH in 1% aq. HOAc at 1 mL/min. The system for analytical and semipreparative HPLC consisted of a Waters LC600 (Manchester, UK) pump and a 996 photodiode array detector. For analytical HPLC, the column and gradient program were as described for LC-MS described above, but the eluting solvents were 2% aq. HOAc and MeOH:HOAc:H_2O (18:1:1). An identical LiChrospher column but with 10 mm i.d. was used for semipreparative HPLC with H_2O (A) and MeOH (B) as solvents. The column temperature was maintained at 30°C for both analytical and semipreparative HPLC.

The following chemicals and standards were used: bromobenzene, 2,6-dimethyl-*N*-acetyl *p*-quinoneimine, gallic acid, protocatechuic acid, quercetin, (+)-catechin, and silymarin from Sigma Chemical Co. (Poole, Dorset, UK); ellagic acid, kaempferol, quercetin 3-*O*-galactoside, quercetin 3-*O*-glucoside, quercetin 3-*O*-rutinoside (rutin), kaempferol 3-*O*-glucoside, and tiliroside from Apin Chemicals Ltd. (Abingdon, Oxon, UK).

Hepatoprotective properties

HepG2 cells (ECACC, Porton Down, Hampshire, UK) were plated in Dulbecco's modified Eagle medium (DMEM) containing 10% (v/v) fetal calf serum at approximately 30,000 cells/well in 96-well microtiter plastic plates (Nunclon D; Invitrogen, Paisley, UK) and cultured for 24 h at 37°C in a humidified atmosphere of 5% CO_2/95% air. Stock solutions of BB and 2,6-diMeNAPQI, an analogue of the active metabolite of paracetamol, in DMSO were diluted in DMEM immediately before use. The cells were then incubated with either BB (10 mM) or 2,6-diMeNAPQI (0.2 mM) with and without the plant material for 1 h as described by Thabrew et al. (1997).

Cytotoxicity was assessed by estimation of the viability of the HepG2 cells by means of the Cell Titre 96 aqueous non-radioactive cell proliferation assay (Promega, Southampton, Hampshire, UK) based on the metabolism of the tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) in the presence of the electron coupling reagent phenazine methosulphate (PMS). After 1 h incubation, the medium in each well was removed by aspiration and replaced by the same volume (200 μL) of fresh medium before addition of 20 μL of the MTS/PMS solution. After a further 2 h incubation at 37°C, the absorbance at 490 nm was determined with an ELISA plate reader (Dynatech MR 5000; Billingshurst, West Sussex, UK). Silymarin and (+)-catechin were used as plant-derived positive controls as they have been shown to have hepatoprotective properties (Valenzuela et al., 1986).

Results and Discussion

Identification of the compounds in extracts of *O. aspera*

Table 1 lists the compounds detected in the various extracts of *Osbeckia aspera* and Sephadex LH-20 fractions of the acetone/water extract with the HPLC retention times, UV maxima and mass spectral data, relative concentrations and identifications. Compounds **1–3** were only detected in the water and acetone/water extracts, compounds **4**, **5**, **7**, and **10** in all three extracts (water, acetone/water, and MeOH), compounds **6**, **8**, **9**, and **11** only in the acetone/water and MeOH extracts, and compounds **12–14** only in the MeOH extract. Using HPLC-DAD, compounds **1–3** were identified in polar Sephadex LH-20 fractions from their UV spectra and retention times, which were the same as those of authentic standards of gallic acid (**1**), protocatechuic acid (**2**), and ellagic acid (**3**) (see Table 1). Compounds **4–6** showed the typical UV spectra of quercetin 3-*O*-glycosides (λ_{max} at 256 and 355 nm; and shoulders at 269 and 299 nm; Mabry et al., 1970). The protonated aglycone $[A + H]^+$ at m/z 303 (Table 1) in the APCI-MS spectra (positive mode) of **4–6** also suggested that these compounds were based on quercetin. Moreover, the fragmentation of the $[A + H]^+$ ions in the MS^3 product ion analysis matched the MS^2 of quercetin under identical collision energies and isolation widths (Grayer et al., 2000). The protonated molecules $[M + H]^+$ of **4** and **5** at m/z 465 indicated that a loss of 162 amu was required to produce the corresponding protonated aglycones. This corresponds with the loss of a hexose sugar (e.g., galactose or glucose). Co-chromatography with authentic standards indicated that **4** was quercetin 3-*O*-β-galactopyranoside, which had a slightly shorter retention time than **5** in the solvent system used (Table 1). The latter co-chromatographed with quercetin 3-*O*-β-glucopyranoside. A mixture of **4** and **5** analyzed by ^{1}H NMR spectroscopy confirmed the presence of both the 3-*O*-β-galactopyranoside and 3-*O*-β-glucopyranoside of quercetin (Agrawal, 1992; Markham & Geiger, 1994). The protonated molecule $[M + H]^+$ of **6**, a third quercetin 3-*O*-glycoside, was at m/z 507, with a loss of 204 amu required to produce the protonated aglycone observed at m/z 303. This suggested that the sugar moiety was an acetylated hexose (162 + 42 amu). One- and two-dimensional NMR spectroscopic analysis of a semipurified fraction in which **6** was the major component confirmed its identification as quercetin 3-*O*-(3″-*O*-acetyl-β-galactopyranoside). The coupling constants of the sugar resonances H-1″ to H-4″ assigned from the DQF-COSY spectrum were as expected for β-galactopyranoside (Duus et al., 2000). A long-range connectivity detected in the HMBC spectrum from H-3″ of β-Gal to the carbonyl carbon of the acetyl group at δ169.7 confirmed the site of acylation on the sugar as C-3″. Quercetin 3-*O*-(3″-*O*-acetyl-β-galactopyranoside) has been reported only once, as a constituent of *Ledum palustre* L. (Ericaceae) (Zapesochnaya & Pangarova, 1980). The following NMR data from the current study are therefore included for reference.

Table 1. HPLC retention times, UV spectral data, and APCI-MS fragment ions of identified constituents from *O. aspera,* their relative concentrations in the crude extracts, and the fractions in which they were present.

Comp. no.	t_R (min)[a]	Identification[b]	Fractions of the acetone/water extract in which the compound was present[c]	UV λ_{max} (nm)	APCI-MS $[A+H]^+$ $[M+H]^+$	Rel. conc.[d]
1	2.9	Gallic acid	C40, C77	273	—	+
2	4.9	Protocatechuic acid	C77, E75	260, 295	—	±
3	13.1	Ellagic acid	B13, **C13**, D12, E15	253, 367	—	±
4	12.5	Quercetin 3-*O*-*β*-galactopyranoside	**D37, D62, E40**	256, 355	303 465	++
5	12.7	Quercetin 3-*O*-*β*-glucopyranoside	**D37, D62, E40**	256, 355	303 465	++
6	14.4	Quercetin 3-*O*-(3″-*O*-acetyl-*β*-galactopyranoside)	**D62, E40**	256, 355	303 507	++
7	13.9	Kaempferol 3-*O*-*β*-glucopyranoside	**D37, D62, E40**	264, 349	287 449	+
8	17.2	Kaempferol 3-*O*-[6″-*O*-(*E*)-*p*-coumaroyl-*β*-glucopyranoside] (tiliroside)	**E40**	265, 314	287 595	++
9	19.5–20.2	Kaempferol 3-*O*-[2″, 6″ -di-*O*-(*E*, *E*)-*p*-coumaroyl-*β*-glucopyranoside][e]	**E40, F28**	267, 313	287 741	++
10	16.6	Quercetin	**F07**	255, 372	303 303	+
11	18.6	Kaempferol	**F07**	265, 366	287 287	±
12	10.3	6,9-Dihydroxy-4,7-megastigmadien-3-one (vomifoliol)	Present in 100% MeOH extract only	241	225	++
13	17.0	9-Hydroxy-4,7-megastigmadien-3-one	Present in 100% MeOH extract only	242	209	+
14	16.9	9-Hydroxy-4-megastigmen-3-one	Present in 100% MeOH extract only	—	211	+

[a] HPLC retention times. For composition of the solvents and other details, see "Materials and Methods" section.
[b] Compounds **1–3** were detected in the water and acetone/water extracts, compounds **4**, **5**, **7** and **10** in all three extracts, compounds **6**, **8**, **9**, and **11** in the acetone/water and MeOH extracts, and compounds **12–14** in the MeOH extract.
[c] Fractions with liver protective activity in the BB test are printed in bold face, see "Materials and Methods" section for details about the fractions.
[d] Relative concentrations of the compounds in the crude extracts. ++means a relatively high concentration in the extracts; +medium concentration; ± low concentration.
[e] The corresponding (*E*,*Z*), (*Z*,*E*), and (*Z*,*Z*) isomers of this compound were detected as minor components of the sample by NMR.

*Quercetin 3-O-(3″-O-acetyl-β-galactopyranoside) (**6**)*

^{1}H NMR (400 MHz, DMSO-d_6): δ12.57 (1H, br s, 5-OH), 7.65 (1H, dd, J = 8.5, 2.3 Hz, H-6′), 7.53 (1H, d, J = 2.3 Hz, H-2′), 6.83 (1H, d, J = 8.5 Hz, H-5′), 6.40 (1H, d, J = 2.0 Hz, H-8), 6.20 (1H, d, J = 2.1 Hz, H-6), 5.46 (1H, d, J = 7.6 Hz, Gal H-1″), 4.61 (1H, dd, J = 10.1, 3.3 Hz, Gal H-3″), 3.88 (1H, br d, J = 3.2 Hz, Gal H-4″), 3.82 (1H, dd, J = 10.0, 7.6 Hz, Gal H-2″), 3.45 (1H, m, Gal H-5″), 3.45, 3.30 (2 × 1H, 2 × m, Gal CH_2-6″), 2.07 (3H, s, $OCOCH_3$); ^{13}C NMR (DMSO-d_6; nonquaternary carbon atoms by HSQC): δ 121.5 (C-6′), 115.6 (C-2′), 114.7 (C-5′), 101.2 (Gal C-1″), 98.3 (C-6), 93.1 (C-8), 75.6 (Gal C-3″), 74.9 (Gal C-5″), 68.0 (Gal C-2″), 64.5 (Gal C-4″), 59.4 (Gal C-6″), 20.7 ($OCOCH_3$).

Compound **7** had a UV spectrum typical of kaempferol 3-*O*-glycosides (λ_{max} at 264 and 349 nm). The protonated aglycone $[A + H]^+$ ion at m/z 287 (Table 2) and its product ion spectrum was consistent with kaempferol. The protonated molecule $[M + H]^+$ of **7** at m/z 465 indicated a monohexoside, and co-chromatography with an authentic standard confirmed that **7** was kaempferol

Table 2. Percentage of liver cell protection against bromobenzene (BB) and 2,6-dimethyl-*N*-acetyl *p*-quinoneimine (2,6-diMe-NAPQI) toxicity of phenolic acid and flavonol standards.

	% Protection against BB toxicity[a]		% Protection against 2,6-diMeNAPQI toxicity[a]	
Pure compound concentration (μg/mL)	125	250	125	250
Gallic acid (**1**)	26	41	48	56
Protocatechuic acid (**2**)	19	24	40	48
Ellagic acid (**3**)	3	6	−13	−10
Quercetin 3-*O*-rutinoside (rutin)	7	15	8	N/D

N/D, not determined.
[a] Mean of two determinations against either BB or 2,6-diMeNAPQI.

3-*O*-β-glucopyranoside (astragalin). APCI-MS of **8** and **9** indicated that these compounds were also kaempferol derivatives, but their long wavelength maxima in UV spectra were ca. 314 nm instead of 349 nm as expected for kaempferol 3-*O*-glycosides (Table 1). This suggested that they were acylated with *p*-coumaric acid (Marin et al., 2004). The protonated molecule and aglycone in the APCI-MS spectrum of **8** suggested that it was a kaempferol mono-*p*-coumaroylhexoside (losses of 162 amu for a hexose and 146 amu for one molecule of *p*-coumaric acid). This was identified as kaempferol 3-*O*-(6″-*O*-*p*-coumaroyl-β-glucopyranoside) (tiliroside) by co-chromatography with an authentic standard.

Analysis of APCI-MS data for **9** suggested that it was a kaempferol hexoside acylated by two *p*-coumaroyl residues. However, following scale-up by semipreparative HPLC to provide material for further spectroscopic analysis, the purified sample proved to be a mixture of the four possible *E*,*Z*-isomers. The structure of the most abundant of these was determined to be kaempferol 3-*O*-(2″,6″-di-*O*-(*E*,*E*)-*p*-coumaroyl-β-glucopyranoside) by NMR spectroscopy. The coupling constants and chemical shift values of the sugar resonances of this compound confirmed that it was a β-glucopyranoside acylated at C-2″ and C-6″. The specific ^{1}H NMR resonance assignments of the *p*-coumaroyl residues at C-2″ and C-6″ were determined using long-range connectivities in the HMBC spectrum from δ 5.06 (Glc H-2″) and 4.21 (Glc CH_2-6″$_B$) to the carbonyl carbons at δ 168.5 and 168.8, respectively. The remaining assignments were confirmed using connectivities in HMBC spectra and by use of an ROE connectivity detected between H-α and H-2,6 in both cases. Although Skaltsa et al. (1994) presented a partial set of ^{1}H NMR resonance assignments for this compound, they were unable to obtain either a full assignment of the sugar protons or the specific assignments of the protons of the 2″-and 6″-*O*-(*E*)-*p*-coumaroyl residues, which are given below for the first time.

*Kaempferol 3-O-[2″,6″-di-O-(E, E)-p-coumaroyl-β-glucopyranoside] (**9**)*

^{1}H NMR (600 MHz, CD_3OD): Aglycone, δ6.07 (1H, d, J = 2.1 Hz, H-6), 6.26 (1H, d, J = 2.1 Hz, H-8), 7.95 (2H, d, J = 8.9 Hz, H-2′,6′), 6.86 (2H, d, J = 8.9 Hz, H-3′,5′); Sugar, 5.62 (1H, d, J = 8.1 Hz, Glc H-l″), 5.06 (1H, dd, J = 9.6, 8.1 Hz, Glc H-2″), 3.66 (1H, t, J = 9.2 Hz, Glc H-3″), 3.41 (1H, dd, J = 9.7, 9.0 Hz, Glc H-4″), 3.53 (1H, ddd, J = 9.7, 6.7, 2.2 Hz, Glc H-5″), 4.35 (1H, dd, J = 11.8, 2.1 Hz, Glc CH_2-6″$_A$), 4.21 (1H, dd, J = 11.8, 6.7 Hz, Glc CH_2-6″$_B$); 2″-*O*-(*E*)-*p*-coumaroyl, 6.41 (1H, d, J = 15.9 Hz, H-α), 7.72 (1H, d, J = 15.9 Hz, H-β), 7.48 (2H, d, J = 8.7 Hz, H-2,6), 6.82 (2H, d, J = 8.7 Hz, H-3,5); 6″-*O*-(*E*)-*p*-coumaroyl, 6.06 (1H, d, J = 15.9 Hz, H-α), 7.40 (1H, d, J = 15.9 Hz, H-β), 7.31 (2H, d, J = 8.6 Hz, H-2,6), 6.81 (2H, d, J = 8.7 Hz, H-3,5). ^{13}C NMR (CD_3OD; non quaternary carbon atoms by HSQC and selected quaternary carbon atoms by HMBC): Aglycone, δ100.1 (C-6), 94.8 (C-8), 132.2 (C-2′,6′), 116.2 (C-3′,5′); Sugar, 100.4 (Glc C-l″), 75.6 (Glc C-2″), 76.2 (Glc C-3″), 72.2 (Glc C-4″), 76.1 (Glc C-5″), 64.1 (Glc C-6″); 2″-*O*-(*E*)-*p*-coumaroyl, 168.5 (CO), 115.4 (C-α), 147.0 (C-β), 127.3 (C-l), 131.3 (C-2,6), 116.8 (C-3,5), 161.4 (C-4); 6″-0-(*E*)-*p*-coumaroyl, 168.8 (CO), 114.7 (C-α), 146.7 (C-β), 127.2 (C-l), 131.3 (C-2,6), 116.8 (C-3,5), 161.3 (C-4).

Two minor compounds present in fraction F07, **10** and **11**, were identified from their UV and APCI mass spectra as the flavonols, quercetin (**10**) and kaempferol (**11**) (Table 1). This was confirmed by co-chromatography with authentic standards.

Compounds **12–14** were obtained from nonpolar silica gel fractions of the methanol extract (**13** and **14** as an inseparable mixture). The λ_{max} of compounds **12** and **13** was ca. 240 nm, whereas compound **14** did not absorb UV light with a wavelength greater than 200 nm (Table 1). NMR analysis of **12** revealed that it was 6,9-dihydroxy-4,7-megastigmadien-3-one, a compound with four possible stereoisomers at C-6 and C-9. The (6*S*,9*R*)-stereoisomer (vomifoliol or blumenol A) is the most commonly encountered in plants, although the (6*S*,9*S*)-(corchoionol C) and (6*R*,9*R*)-stereoisomers have also been reported, the latter only in glycosidic form (Dictionary of Natural Products, 2006). The absolute configuration of **12** was not determined in the current study. The remaining sample was found to be a 5:4 mixture of two related compounds, 9-hydroxy-4,7-megastigmadien-3-one **(13)** (Aasen et al., 1971; Ito et al., 2001) and 9-hydroxy-4-megastigmen-3-one **(14)** (Galbraith & Horn, 1972).

*6,9-Dihydroxy-4,7-megastigmadien-3-one (**12**)*

^{1}H NMR (400 MHz, CD_3OD): δ5.87 (1H, m, H-4), 5.79 (1H, m, H-8), 5.78 (1H, m, H-7), 4.32 (1H, m, H-9), 2.51 (1H, d, J = 17.0 Hz, CH_2-2$_A$), 2.16 (1H, d, J= 17.0 Hz, CH_2-2$_B$), 1.92 (3H, d, J = 1.3 Hz, CH_3-13), 1.24 (3H, d, J = 6.4 Hz, CH_3-10), 1.04, 1.01 (2 × 3H, 2 × s, CH_3-11 and CH_3-12); ^{13}C NMR (100 MHz, CD_3OD): δ201.3 (C-3), 167.5 (C-5), 137.0 (C-8), 130.2 (C-7), 127.2 (C-4), 80.0 (C-6), 68.8 (C-9), 50.8 (C-2), 42.5 (C-l), 24.5, 23.5 (CH_3-11 and CH_3-12), 23.9 (CH_3-10), 19.6 (CH_3-13).

Megastigmane derivatives such as **12–14** and the corresponding glycosides have been recorded sporadically from many different plant families, e.g., Apocynaceae, Aquifoliaceae, Betulaceae, Cupressaceae, Euphorbiaceae, Lamiaceae, Leguminosae, Magnoliaceae, Pinaceae, Podocarpaceae, Rhamnaceae, Rosaceae, Simaroubaceae, Solanaceae, and Vitaceae (Hegnauer, 1986, 1989, 1990; Schwab & Schreier, 1990, Pabst et al., 1992; Takeda et al., 1997a,b; Champavier et al., 1999; Mohamed et al., 1999), but this is the first report of their occurrence in the Melastomataceae. They are thought to be the products of the oxidative cleavage of carotenoids (Galbraith & Horn, 1972;

Aasen et al., 1973), and according to Maier et al. (2000), they are formed via the methylerythritol phosphate pathway. Megastigmane glycosides are accumulated in roots of tobacco and tomato when colonized by arbuscular mycorrhizal fungi (Maier et al., 2000) and contribute to the flavour of tobacco, wine, and fruits (Sefton et al., 1992).

Hepatoprotective activity of crude extracts and fractions of *O. aspera*

Incubation of the hepatocytes with the toxin 10 mM BB resulted on average in a ca. 35% decrease in cell viability and with the toxin 0.2 mM 2,6-diMeNAPQI there was on average a ca. 50% decrease in comparison with the control (100%). The percentage of protection by an extract, fraction or compound was the percentage of decrease in cell viability in comparison with the control after addition of a toxin and the plant extract, fraction, or compound at the same time to the hepatocytes, minus the percentage determined after addition of the toxin alone. For example, after addition of both 125 μg/mL of gallic acid and BB to the hepatocytes, the cell viability was on average 63% of the control, and the average cell viability after addition of BB alone during that experiment was 37%, so the percentage of protection was calculated as $63\% - 37.0\% = 26\%$.

The crude water extract of leaves of *O. aspera* when tested at 1000 ppm showeda greater level of protection against BB and 2,6-diMeNAPQI toxicity than the hepatoprotective milk thistle derived-product, silymarin 455 (Table 3). Because of the frothing of the water extract during concentration and difficulties redissolving the freeze-dried extract, samples of leaves were extracted with 50% acetone to prepare fractions for activity test-sand with 100% methanol for the isolation of compounds, especially the less polar ones. Although neither of these two extracts was as active at 1000 ppm as the water extract against BB induced toxicity, they both showed 12% protection against BB. Fractions were prepared from the 50% acetone extract by means of CC on Sephadex LH20 using water/MeOH mixtures of decreasing polarity and subsequent PPC using 15% aq. HOAc as a solvent. The 20 fractions obtained were tested against BB and 2,6-diMeNAPQI toxicity on the hepatocytes at a concentration of 250 ppm. At the same time, three authentic standards of the phenolic acids occurring in the crude aqueous acetone extract were also tested, gallic (**1**), protocatechuic (**2**), and ellagic (**3**) acids, as well as the quercetin glycoside rutin, which was not present in the extract, but is structurally closely related to quercetin glycosides **4** and **5**. We chose rutin for activity tests rather than **4** and **5**, as large amounts of rutin were available and at that stage of the investigations it was only known that the extracts contained quercetin glycosides as the compounds had not been isolated and fully identified. At 125 and 250 ppm, gallic acid (**1**) gave the greatest levels of protection against BB and 2,6-diMe NAPQI, followed by protocatechuic acid (**2**) (Table 2). Rutin was less active, whereas ellagic acid (**3**) showed very little activity against BB and negative values were recorded when it was tested against 2,6-diMe NAPQI (Table 2).

Four of the 20 fractions had activity greater than or equal to 7% in both tests and these are presented in Table 3. The activity of these fractions was not associated with the presence of the identified phenolic acids, for although the active compounds gallic acid (**1**) and protocatechuic acid (**2**) were present in the active water extract and the aqueous acetone extracts, they were not present in the active fractions (Table 1) and were present in some inactive fractions. The lack of activity in the polar fractions could be explained by the fact that although they contained **1** and **2** they probably

Table 3. Percentage of protection against bromobenzene (BB) and 2,6-dimethyl-*N*-acetyl *p*-quinoneimine (2,6-diMeNAPQI) toxicity of crude extracts of *Osbeckia aspera* and active fractions.

Extract or active fraction	Conc. (μg/mL)	% Protection against BB toxicity[a]	% Protection against 2,6-diMeNAPQI toxicity[a]	Main compounds present[b] (major constituents in each fraction are underlined)
Osbeckia crude water extract	1000	38	37	**1–5**, **7**, **10**
Osbeckia crude aq. 50% acetone extract	1000	12	N/D	**1–11**
Fraction C13 (C: Sephadex; 40% MeOH)	250	9	8	**3** Unidentified compounds
Fraction D62 (D: Sephadex; 60% MeOH)	250	7	32	**4**, **5**, **7**, Unidentified compounds
Fraction E40 (E: Sephadex; 80% MeOH)	250	7	10	**4–6**, **7–9**
Fraction F28 (F: Sephadex; 100% MeOH)	250	13	22	**8**, **9**, Unidentified compounds
Osbeckia crude 100% MeOH extract	1000	12	N/D	**4–14**
(+)-Catechin[c]	1000	69	64	
Silymarin[c]	1000	25	25	

N/D, not determined.

[a] Mean of two determinations.

[b] For the identification of the compounds, see Table 1. The unidentified compounds in fractions C13 were an inseparable mixture of polar compounds with very short retention times (2.2 min).

[c] Positive controls.

contained a high proportion of compounds that do not absorb UV light, such as sugars and aliphatic amino acids, so that the concentration of **1** and **2** in these fractions might have been low. Ellagic acid (**3**) was present in one active fraction, C13, and as a minor constituent in three inactive fractions (Table 1). In addition to compound **3**, fraction C13 contained a complex mixture of extremely polar non-flavonoid constituents, which had very short retention times (ca. 2.2 min) and were not separable. C13 was the only active fraction that did not contain flavonoids; the other three active fractions (D62, E40, and F28) contained at least three different flavonoids (Table 3) and none of these flavonoids occurred in inactive fractions. The UV-absorbing compounds in fraction E40 were exclusively flavonoids, but in fractions D62 and F28, unidentified compounds were present as well as the flavonoids.

To conclude, gallic (**1**) and protocatechuic (**2**) acids could contribute to the liver protective activity of the crude extracts of *O. aspera,* as could one or more of the constituents in the polar mixture present in fraction C13, and unidentified compounds in active fractions D62 and F28. However, the flavonol glycosides are most likely associated with liver-protective activity of the *O. aspera* extracts, as the activity was mainly associated with fractions that contained flavonol glycosides, such as quercetin 3-*O*-β-galactopyranoside (**4**), quercetin 3-*O*-β-glucopyranoside (**5**), and kaempferol 3-*O*-β-glucopyranoside (**7**). Inactive fractions did not contain these flavonoids. Traditionally, patients with liver problems take hot infusions of the leaves of *O. aspera* daily for a period of time. Thus, if the levels of liver protection recorded in these *in vitro* experiments reflect the sisuation when infusions are taken traditionally, then the extracts could provide some protection to liver cells exposed to toxins. Further research is needed to isolate and characterize the other compounds present in the active extracts together with *in vivo* experiments using standardized extracts that contain a profile of known compounds. These experiments would help confirm whether flavonoids play a role in the traditional use of *O. aspera* for the treatment of liver disorders.

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