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Inhibition of MMP-2 secretion from brain tumor cells suggests chemopreventive properties of a furanocoumarin glycoside and of chalcones isolated from the twigs of *Dorstenia turbinata*

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Abstract

A furanocoumarin glycoside new named turbinatocoumarin (**1**) was isolated from the twigs of *Dorstenia turbinata*. The structure of turbinatocoumarin (**1**) was assigned as 5-methoxy-3-[3-(β-glucopyranosyloxy)-2-hydroxy-3-methylbutyl]psoralen by means of spectroscopic analysis. Known compounds have also been isolated from this genus and identified as (2′S, 3′R)-3′-hydroxymarmesin (**2**), 5-methoxy-3-(3-methyl-2,3-dihydroxybutyl)psoralen (**3**), psoralen (**4**), kanzonol C (**5**) which was isolated for the first time from this genus, 4-hydroxylonchocarpin (**6**), umbelliferone, 4-hydroxy-3-methoxybenzaldehyde and 4-methoxyphenol. As part of our continuing search for potential naturally-occurring antitumor drug candidates, the inhibition of matrix metalloproteinase (MMP)-2 secretion from brain tumor-derived glioblastoma cells by the isolated compounds **1**, **3**, **5**, and **6** was evaluated by zymography and compared to the documented naturally-occurring MMP secretion inhibitors chlorogenic acid (**CHL**) and epigallocatechin-3-gallate (**EGCg**). Among the compounds tested, the inhibiting MMP secretion concentrations ranged from 0.025 to 250 μM with up to 80% inhibition. The inhibitory activities of compounds **5** and **6** were found comparable to the common reference compounds **CHL** and **EGCg**. This suggests that alternate sources can be explored and exploited for the availability of chemopreventive molecules.

Keywords: *Dorstenia turbinata*; Moraceae; Chalcones; Furanocoumarin glucoside; Turbinatocoumarin; 4-Hydroxylonchocarpin; Psoralen; Kanzonol; Chlorogenic acid; Epigallocatechin-3-gallate; Matrix metalloproteinase; Glioblastoma cells

1. Introduction

Tumour progression is often correlated with an overexpression of matrix metalloproteinases (MMP). These enzymes are produced and secreted as inactive zymogens in the extracellular matrix (ECM) by the tumour cells themselves or by surrounding stromal cells (Seiki and Yana, 2003; Folguera et al., 2004). Several proteases, such as MMP and furin-like serine proteases, are responsible for the specific activation of the latent proMMP forms. Among these, MMP-2, MMP-9, and the membrane-associated type (MT)-1 MMP are thought to play a significant role in several degenerative processes (Annabi et al., 2002a; Desrosiers et al., 2006), and are directly involved in metastatic tumour dispersion and angiogenesis (Itoh et al., 1999; John and Tuszynski, 2001; Lafleur et al., 2001; Annabi et al., 2005). More recently, MMP-2 has also been shown to play important roles in tumour cells

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resistance to apoptosis, in the activation of EGF receptors and in cellular proliferation (Cowan et al., 1999; Eguchi et al., 2001; Ahonen et al., 2003). Taken together, these data indicate that MMP-2, MMP-9 and MT1-MMP may represent important targets against which new potential anticancer drugs could be developed (Giannelli and Antonaci, 2002).

Scientists continuously survey plants, particularly of ethnobotanical significance, for their complex spectrum of biological and medicinal activities, which range from antibiotic to antitumor properties. The genus *Dorstenia* (Moraceae) consists of approximately 170 mainly tropical species and is indigenous to many countries in Africa, and in Central and South America (Mabberley, 1987). It is made up of largely herbaceous perennials with succulent scrambling rhizomes (Berg et al., 1989), many of them are used as anti-snakebite, anti-infection, and anti-rheumatic remedies (Abegaz et al., 2000). Among the bioactive molecules previously identified within the genus *Dorstenia*, furanocoumarins have been ascribed analgesic, anti-inflammatory, antibacterial, antiviral, anticoagulant, and photosensitizing activities (Huang et al., 1997; Parrish et al., 1974; Abegaz et al., 2004). Specifically, the presence of sugar moieties in furanocoumarins glycosides is thought, in many cases, to participate in the molecular recognition of its many cellular targets (Dhanasekaran and Polt, 2005; Weymouth-Wilson, 1997; Méndez and Salas, 2001). Alternate plant constituents such as prenylated chalcones were also identified within the genus (Ngameni et al., 2004). Interestingly, previous studies have suggested that these chalcones may have anticarcinogenic (Gerhauser et al., 2002) and antiproliferative properties (Miranda et al., 1999).

In this study, we first report the isolation and structural elucidation of a new furanocoumarin glycoside (**1**), renamed herein turbinatocoumarin, from the twig constituents of *D. turbinate*. In addition, we report the isolation and identification of eight previously known compounds: four coumarins, umbelliferone (Cussans and Huckerby, 1975), (2′S, 3′R)-3′-hydroxymarmesin (**2**) (Vilegas and Pozetti, 1993), 5-methoxy-3-(3-methyl-2,3-dihydroxybutyl)psoralen (**3**) (Franke et al., 2001), and psoralen (**4**) (Elgamal et al., 1979); two flavonoids, kanzonol C (**5**) (Fukai et al., 1994), and 4-hydroxylonchocarpin (**6**) (Ngadjui et al., 1999, 2000) and two phenolic compounds, 4-hydroxy-3-methoxybenzaldehyde (Klinck and Stothers, 1962) and 4-methoxyphenol (Paterson and Tipman, 1962). Based upon their structural similarities (Fig. 1), the capacity of the glycosylated (**1**) and native (**3**) furanocoumarins, and of the two prenylated chalcones, (**5**, **6**), to inhibit MMP secretion from human brain tumor-derived glioblastoma cells, a crucial step involved in ECM degradation and tumor development, was evaluated *in vitro*. The potency of these isolated molecules was further compared to those of the phenolic compounds **CHL** and **EGCg**, both well documented for their chemopreventive properties and for the inhibition of MMP secretion (Annabi et al., 2002b; Belkaid et al., 2006). To the best of our knowledge, no previous phytochemical or pharmacological studies have been reported on this taxon.

2. Results and discussion

2.1. Isolation and structure elucidation

The powder of *D. turbinata* twigs was successively extracted with *n*-hexane, EtOAc, and MeOH as described in Section 3. The EtOAc and MeOH extracts were separately applied to silica gel column chromatography, followed by size exclusion chromatography and preparative TLC to obtain compounds **1**–**6** (Fig. 1) along with umbelliferone (Cussans and Huckerby, 1975), 4-hydroxy-3-methoxybenzaldehyde (Klinck and Stothers, 1962), and 4-methoxyphenol (Paterson and Tipman, 1962).

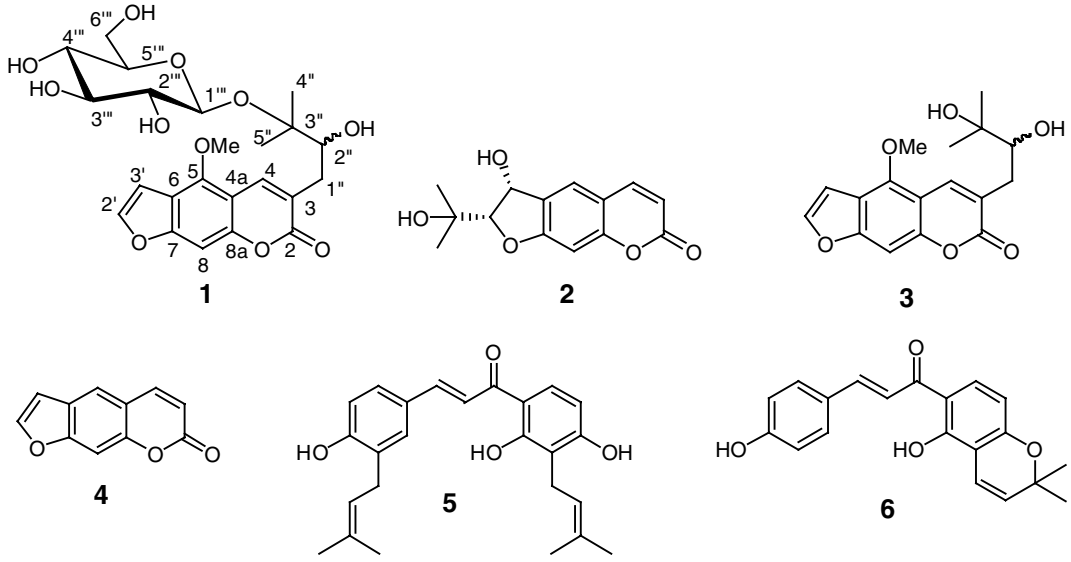

Fig. 1. Structures of furanocoumarins and prenylated chalcones isolated from *Dorstenia turbinata*.

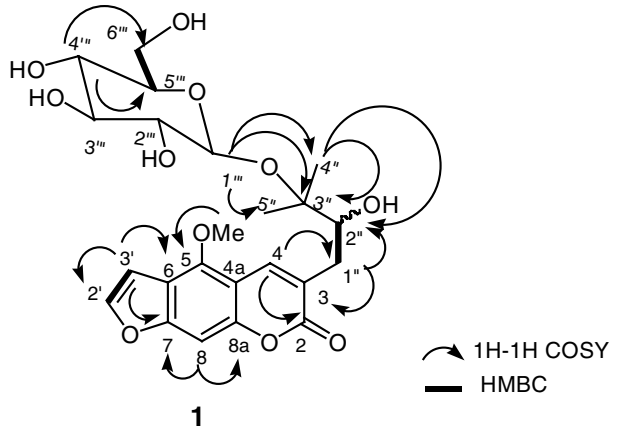

Fig. 2. Selected ^{1}H–^{1}H COSY and HMBC correlations in furanocoumarin glycoside (**1**).

Compound **1** was obtained as an amorphous solid and identified as $C_{23}H_{28}O_{11}$ from the ESI-MS ($[M + Na]^+$: 503) and NMR data. The ^{1}H NMR spectrum of **1** showed characteristic signals for a disubstituted furan ring at δ 6.85 (1H, *d*, $J = 1.5$ Hz) and 7.41 (1H, *d*, $J = 2.4$ Hz), two aromatic proton signals at δ 7.92 (1H, *br s*) and 6.88 (1H, *br s*), one aromatic O-methyl resonance at δ 4.04 (3H, *s*), three aliphatic proton signals at δ 2.25 (1H, *dd*, $J = 10.3$, 14.0 Hz), 2.65 (1H, *d*, $J = 13.8$ Hz), and 3.59 (1H, *dd*, $J = 1.7$, 11.9 Hz) and two methyl signals at δ 1.09 (3H, *s*) and 1.14 (3H, *s*), which were similar to that of furanocoumarin, 5-methoxy-3-(3-methyl-2,3-dihydroxybutyl)psoralen (**3**) (Franke et al., 2001). The ^{1}H NMR spectra further displayed a doublet of one proton at δ 4.31 (1H, *d*, $J = 7.8$ Hz) and complex signals between δ 3.00–3.60 ppm which suggested protons on the glucopyranosyl moiety. The foregoing spectral data further confirmed that compound **1** was a furanocoumarin glycoside. The position of the sugar moiety was determined using HMBC correlations (Fig. 2). The anomeric proton at δ 4.31 showed correlations with the signals at δ 82.1 (C-3″), 24.2 (C-4″), and 24.0 (C-5″), which indicated that the sugar moiety was located at the C-3″ position. The anomeric configuration of the sugar moiety was established to be β from the coupling constant $J_{1'''-2'''} = 7.8$ Hz of the anomeric proton. The foregoing analysis led to the definition of the structure of **1** as 5-methoxy-3-[3-(β-glucopyranosyloxy)-2-hydroxy-3-methyl-butyl]psoralen for which the trivial name turbinatocoumarin is proposed. This structure is supported by the ^{13}C NMR and important HMBC correlations given in Table 1.

Table 1
^{1}H (500 MHz) and ^{13}C NMR (125 MHz) spectroscopic data of turbinatocoumarin (**1**) in $CDCl_3$

Position	δ_H (J/Hz)[a]	δ_C	^{1}H–^{1}H COSY	HMBC (C)[b]
2	–	165.14 (*s*)[c]	–	–
3	–	124.18 (*s*)	–	–
4	7.92 (*br s*)	139.43 (*d*)	–	2, 5, 8a, 1″
4a	–	108.93 (*s*)	–	–
5	–	156.08 (*s*)	–	–
6	–	114.89 (*s*)	–	–
7	–	159.71 (*s*)	–	–
8	6.88 (*br s*)	94.99 (*d*)	–	7, 8a, 4a
8a	–	153.65 (*s*)	–	–
2′	7.41 (*d* 2.4)	146.81 (*d*)	3′	6, 7
3′	6.85 (*d* 1.5)	106.88 (*d*)	2′	6, 7, 2′
5-OMe	4.04 (*s*)	61.82 (*q*)	–	5
1a″	2.65 (*d* 13.8)	35.04 (*q*)	1b″	3, 4
1b″	2.25 (*dd*, 10.3, 14.0)	–	1a″, 2″	2, 3, 4, 2″
2″	3.59 (*dd*, 1.7, 11.9)	76.23 (*d*)	1b″	–
3″	–	82.11 (*s*)	–	–
4″	1.14 (*s*)	24.21 (*q*)	–	2″, 3″, 5″
5″	1.09 (*s*)	23.99 (*q*)	–	2″, 3″, 4″
1‴	4.31 (*d* 7.8)	98.62 (*d*)	2‴	3″, 4″, 5″
2‴	3.01 (*t*, 8.3)	75.74 (*d*)	1‴, 3‴	1‴, 3‴, 4‴
3‴	3.18 (*m*)	78.61 (*d*)	1‴	4‴
4‴	3.15 (*m*)	72.17 (*d*)	–	5‴
5‴	3.06 (*m*)	77.93 (*d*)	6b‴	3‴
6a‴	3.59 (*dd*, 1.7, 11.9)	63.65 (*t*)	6b‴	
6b‴	3.47 (*dd*, 5.1, 12.0)		5‴, 6a‴	

[a] ^{1}H NMR chemical shift values (δ ppm from TMS) followed by multiplicity and then the coupling constants (J/Hz) in parentheses.
[b] Long-range ^{1}H–^{13}C correlations from H to C observed in the HMBC experiment.
[c] Letters in parentheses indicate the multiplicities of carbons, assigned by DEPT.

2.2. Inhibition of MMP-2 secretion from human U87 glioblastoma cells

Based on structural similarities, increasing concentrations of isolated compounds **1**, **3**, **5**, and **6** were tested on brain tumor-derived U87 glioblastoma cells, and their effect on MMP-2 secretion in the conditioned media evaluated using zymography. The extent of MMP-mediated gelatinolytic activity resulting from the tested compounds was further compared to that of documented antioxidant, anticancer, and naturally-occurring compounds **CHL** and **EGCg** (Fig. 3) (Annabi et al., 2002b; Belkaid et al., 2006). Among the tested compounds, a concentration dependent inhibition of MMP-2 secretion (Fig. 4a) was observed and their potencies were found comparable to that of **CHL** and **EGCg** (Fig. 4b). Structurally, the substitution pattern on ring A and B appeared to play an important role in the activity of the chalcones since, when compared to **CHL** and **EGCg**, compounds **5** and **6** showed potent MMP-2 secretion inhibition with close to 80%

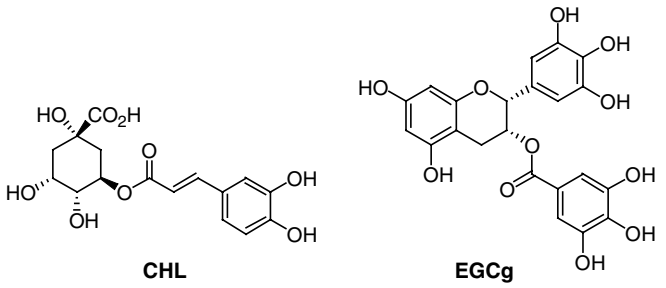

Fig. 3. Chemical structures of MMP secretion inhibitors **EGCg** and **CHL**.

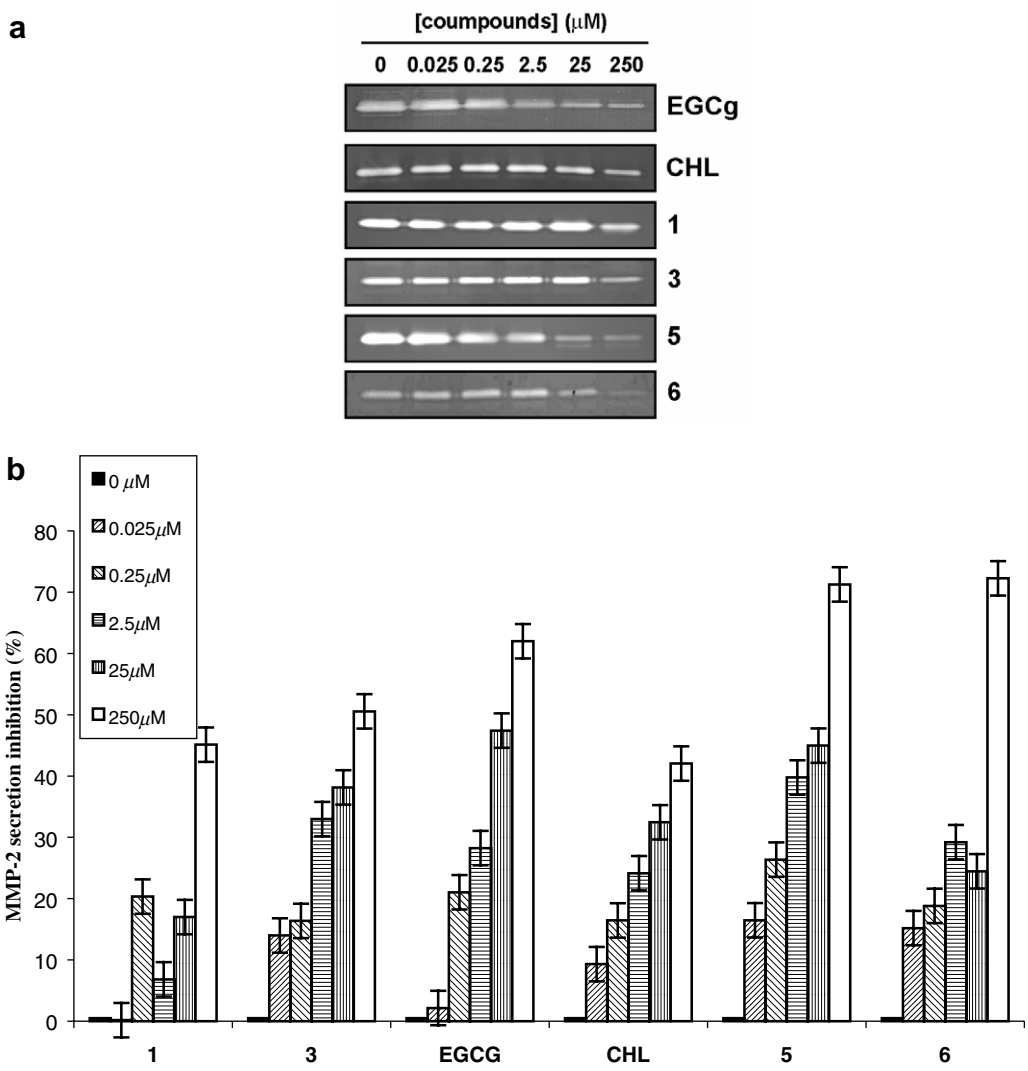

Fig. 4. Effects of compounds **1**, **3**, **5**, and **6** on MMP-2 secretion from U87 glioblastoma cells. (a) U87 glioblastoma cells were treated with increasing concentrations of compounds **1**, **3**, **5** and **6** and gelatin zymography of the conditioned media performed as described in Section 3. (b) The bar graphs present the relative inhibitory effect of the compounds tested. The extent of gelatin hydrolysis was quantified by densitometry. Each value indicates the mean ± SD from three experiments.

inhibition at 250 μM. In light of these effects, one may safely conclude that the prenyl and hydroxyl groups on chalcone A or B ring to be important for the inhibition of MMP-2 secretion. Moreover, the diprenylated compound **5**, in which a hydroxyl group is involved in the chromene formation, indicates that the prenylation with free hydroxy system lead to improved activity in comparison with compound **6**. Our biological assay further suggests that the presence of the tertiary hydroxyl group in compound **3** potentiates its inhibitory effect upon MMP-2 secretion. As demonstrated with compound **1**, the glycosylation at this position did not contribute much to the inhibition of MMP-2 secretion, while the tertiary alcohol appeared to be crucial for its biological activity. These results suggest that the two chalcones were more potent than the furanocoumarin derivatives.

The effects of compounds **5** and **6**, the most potent inhibitors of MMP-2 secretion observed, were further tested on the MT1-MMP-mediated activation of proMMP-2 (Sato et al., 1994). U87 glioblastoma cells were cultured with concanavalin (ConA) in the presence or absence of our compounds. ConA is a well documented inducer of proMMP-2 activation in various kinds of cells through MT1-MMP expression (Yamamoto et al., 1996; Gingras et al., 2000). While ConA treatment induced the appearance of active MMP-2 (Fig. 5), **EGCg** but not compounds **5**, **6**, or **CHL** antagonized proMMP-2 activation (Fig. 5). These results suggest that compounds **5** and **6** specifically inhibit MMP-2 secretion processes rather than MT1-MMP-mediated proMMP-2 activation.

In summary, we have isolated and characterized a new furanocoumarin glycoside, hitherto not reported as a plant secondary metabolite from twig constituents of *D. turbinata* and screened for its in vitro inhibition of MMP-2 secretion along with a few previously isolated prenylated chalcones and a non-glycosilated furanocoumarin to pro-

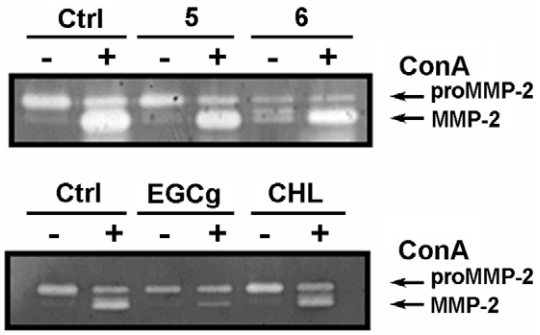

Fig. 5. Effects of compounds **5** and **6** on MT1-MMP-mediated proMMP-2 activation. Serum-starved U87 glioblastoma cells were treated or not with 10 μg/ml concanavalin-A (ConA) for 18 h, and the extent of proMMP-2 activation in the conditioned media assessed by gelatin zymography as described in Section 3. The concentration of compounds **5**, **6**, **EGCg**, and **CHL** was 250 μM.

vide a scientific rational for the inhibition of MMP secretion. Further studies about the characteristics of our isolated compounds structures and on the mechanisms involved in the inhibition of MMP-2 secretion may yield information that will allow an evaluation of new synthetic analogues. The studies on the prenylated chalcones are currently under investigation in our laboratory.

3. Experimental methods

3.1. General experimental procedures

Optical rotations were measured on a Perkin–Elmer 241 polarimeter. NMR spectra were recorded on a Bruker AMX-500 spectrometer or Bruker AV-300 spectrometers. ESI-MS spectra were recorded on Kratos Concepts IIH mass spectrometer. Precoated silica gel plates (Merck, Kieselgel 60 F-254, 0.5 mm) were used for PTLC. Thin layer chromatography (TLC) was performed on silica gel F_{254} (Merck) precoated aluminium sheets and spots were visualized under UV and by spraying with molybdenum solution and heating.

3.2. Cell culture

The U87 glioblastoma cell line was purchased from American type culture collection and maintained in Eagle's minimum essential medium (MEM) containing 10% (v/v) fetal bovine serum (FBS) (HyClone Laboratories, Logan, UT), 2 mM glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. Cell culture was performed at 37 °C under a humidified atmosphere containing 5% CO_2.

3.3. Gelatin zymography

To assess the extent of MMP-2 secretion in the conditioned media, gelatin zymography was used as described previously (Belkaid et al., 2006). Briefly, serum-starved U87 glioblastoma cells were treated with the appropriate compounds for 18 h at 37 °C and an aliquot (20 μl) of the culture medium was subjected to SDS–PAGE in a gel containing 0.1 mg/ml gelatin. The gels were then incubated in 2.5% Triton X-100 and rinsed in nanopure distilled H_2O. Gels were further incubated at 37 °C for 20 h in 20 mM NaCl, 5 mM $CaCl_2$, 0.02% Brij-35, 50 mM Tris–HCl buffer, pH 7.6, then stained with 0.1% Coomassie Brilliant blue R-250 and destained in 10% acetic acid, 30% MeOH in H_2O. MMP-2 gelatinolytic activity was detected as unstained bands on a blue background.

3.4. Plant material

The twigs of *D. turbinata* were collected from Mbalmayo (Cameroon) in March 2004, and identified by Mr. Victor Nana of the National Herbarium in Yaounde (Cameroon) where a voucher specimen (No. 28158/SRF/Cam) was deposited.

3.5. Extraction and isolation

Powder twigs of *D. turbinata* (4.2 kg) was successively extracted with *n*-hexane, EtOAc, and MeOH in a soxhlet apparatus. The solvent was removed under reduced pressure to provide *n*-hexane extract (45.0 g), EtOAc extract (81.0 g), and MeOH extract (75.0 g). The EtOAc extract was chromatographed over silica gel using *n*-hexane-acetone as eluent with a continuous gradient (from 95:5 to 60:40, and finally with pure acetone), followed by gel filtration chromatography over Sephadex LH-20 using CH_2Cl_2: MeOH (9:1) as eluent and by preparative TLC to afford 4-methoxyphenol (8 mg), 4-hydroxy-3-methoxy-benzaldehyde (10 mg), kanzonol C (**5**, 89 mg), 4-hydroxylonchocarpin (**6**, 98 mg), umbelliferone (15 mg), psoralen (**4**, 10 mg), and (2′S, 3′R)-3′-hydroxymarmesin (**2**, 15 mg). The MeOH extract was chromatographed over silica gel using solvents of increasing polarity from CH_2Cl_2, CH_2Cl_2–MeOH mixtures to MeOH as eluent, followed by gel filtration chromatography over Sephadex LH-20 using CH_2Cl_2: MeOH (3:2) as eluent and by preparative TLC to afford (2′S, 3′R)-3′-hydroxymarmesin (**2**, 10 mg), 5-methoxy-3-(3-methyl-2,3-dihydroxybutyl)psoralen (**3**, 12 mg) and 5-methoxy-3-[3-(β-glucopyranosyloxy)-2-hydroxy-3-methylbutyl]psoralen (1, 20 mg).

3.6. 5-Methoxy-3-[3-(β-glucopyranosyloxy)-2-hydroxy-3-methylbutyl]psoralen (turbinatocoumarin) (*1*)

Amorphous solid; $[\alpha]_D$ −10.6° ($c = 1$, $CHCl_3$ + MeOH (1:1)); ^{1}H NMR ($CDCl_3 + CD_3OD$, 500 MHz) see Table 1; ^{13}C NMR ($CDCl_3 + CD_3OD$, 125 MHz) see Table 1; ESI-MS *m/z* (rel. int.): 503 $[M + Na]^+$ (7, calcd. for $C_{23}H_{28}O_{11}$, 480.1632), 481 $[M + H]^+$ (43), 321 (3), 320 (18), 319 (100), 302 (8), 301 $[C_{17}H_{17}O_5]^+$ (45).

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