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Bioorganic & Medicinal Chemistry Letters 17 (2007) 239–242
Bioorganic & Medicinal Chemistry Letters

New antifungal flavonoid glycoside from *Vitex negundo*☆

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Received 4 July 2006; revised 15 September 2006; accepted 19 September 2006
Available online 5 October 2006

Abstract—Flavonoids are ubiquitous in photosynthesizing cells and are common part of human diet. For centuries, preparations containing these compounds as the principal physiologically active constituents have been used to treat human diseases. Increasingly, this class of natural products is becoming the subject of anti-infective research. Our bioactivity guided fractionation of ethanolic extract of leaves of *Vitex negundo* resulted in the isolation of new flavone glycoside (**4**) along with five known compounds **1**–**3**, **5** and **6**. All the isolated compounds were evaluated for their antimicrobial activities. The new flavone glycoside **4** and compound **5** were found to have significant antifungal activity against *Trichophyton mentagrophytes* and *Cryptococcus neoformans* at MIC 6.25 μg/ml.

Resistance to antimicrobial agents has become a global problem since last three decades. About 2 million people in US acquire bacterial and fungal infections each year, of the 65% patients have resistance to at least one drug.[1,2] A similar cause of concern in other countries including UK that leads to repeated use of antibiotic and insufficient control of the disease.[3] Second adequate treatment of mycotic infections is difficult since fungi are eukaryotic organisms with a structure and metabolism that is similar to those of eukaryotic host. For this reason, substantial researches in the field of anti-infectives are now desperately needed to develop new prototype antimicrobial agents to avert this situation.

Development of new drugs from plants is not a new phenomenon. Plants and plant derived agents have long history to clinical relevance as source of potential chemotherapeutic agents.[4] In our continuing efforts to identify antifungal agents from plant sources, we have studied antimicrobial activity of *Vitex negundo* (Leaves) against a variety of microorganisms including Gram-positive and Gram-negative bacteria and fungi (Fig. 1).

Vitex negundo (Linn.) is a large shrub grown throughout the India. It is one of the common plants used in traditional medicine and reported to have variety of biological activities.[5] The preparation of leaves used in catarrhal fever and applied to sinuses and scrofulous sores.[6] Aqueous extract and oil of seeds possessed anti-oxidant and anti-inflammatory property.[7–9] Antigenotoxic effects were reported from the leaves of *V. negundo* in combination with other constituents.[10] Flavonoid-rich fraction of the seeds showed anti-fertility effect in adult dogs.[11] Plant also exhibited CNS depressant activity[12] and antihistamine release property.[13] Flavonoids,[14–16] iridoids,[17–20] terpenes,[21,22] and steroids[23] are the major classes of compounds isolated from this plant.

In our study, we have found significant antifungal activity in ethanolic extract against *Cryptococcus neoformans* and *Trichophyton mentagrophytes*. The extract of the leaves and twigs was reported to show antibacterial activity against *Micrococcus pyogenes* and *Escherichia coli*.[6] On bioassay guided fractionation of ethanolic extract, the activity was localized in *n*-butanol fraction. Further work led to the isolation of compound (**1**–**6**) including a new flavone glycoside (**4**). All the six compounds were evaluated for antifungal and antibacterial activity.

Powdered leaves of the plant (5.0 kg) were extracted with ethanol by percolation, affording (590 g) extract.

Keywords: *Vitex negundo*; Antimicrobial activity; Fluconazole.
☆ CDRI communication no. 7053.
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doi:10.1016/j.bmcl.2006.09.051

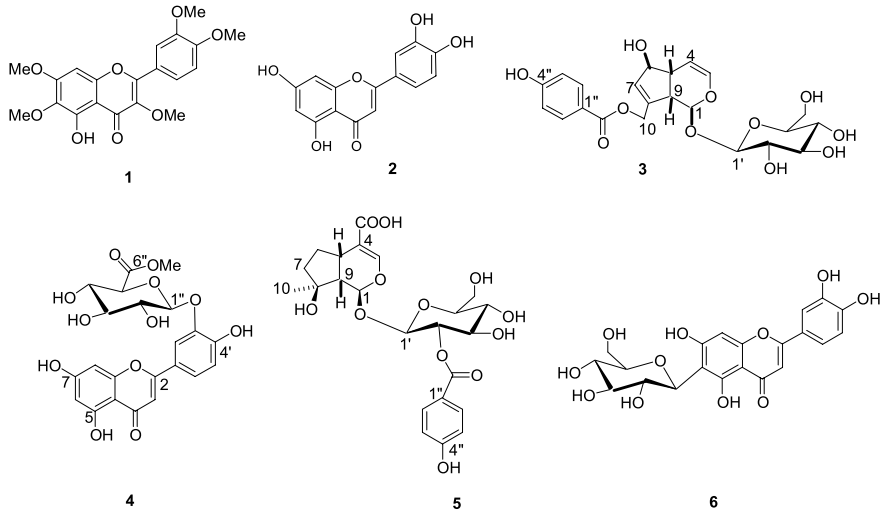

Figure 1. Chemical structure of isolated compounds **1**–**6**.

Ethanolic extract (550 g) was fractionated into *n*-hexane (180 g), $CHCl_3$ (70 g) and *n*-butanol (240 g) soluble fractions. The *n*-butanol soluble fraction was subjected to column chromatography over flash silica gel (230–400 mesh), eluted with a gradient of chloroform/methanol (95:05) to methanol/water (95:05) to afford six fractions (F-1 to F-6). Flash column chromatography of fraction F-1 using $CHCl_3$/MeOH (97:3) afforded compound **1** (50 mg). Purification of F-2 using $CHCl_3$/MeOH (95:5) afforded compound **2** (350 mg) and with $CHCl_3$/MeOH (88:12) gave compound **3** (35 mg). Successive purification of F-3 by reverse-phase flash chromatography, eluted with a gradient of MeOH/H_2O (30:70), afforded **4** (25 mg). Column chromatography purification of F-4, using $CHCl_3$/MeOH (80:20), afforded **5** (500 mg). Repeated column chromatography of fraction F-5, eluted with EtOAc/acetone/H_2O (80:18:2), yielded compound **6** (50 mg).

Compound **4** was obtained as yellow colored solid. Positive Shinoda and Fiegel's test indicated that compound could be a flavonoid glycoside. The FAB-MS exhibited molecular ion peak $[M+H]^+$ at *m/z* 477 corresponding to the molecular formula $C_{22}H_{20}O_{12}$. IR absorption bands at 3250, 1746, 1640, and 1498 cm^{-1} indicated the presence of hydroxyl group, ester moiety, conjugated carbonyl, and aromatic ring, respectively. UV spectrum showed λ_{max} at 336, 329, 266, and 209 nm (MeOH). A bathochromic shift (λ_{max} 329, 266, 216 nm) of 7 nm of the band II with NaOAc indicated the presence of free 7-OH group. Addition of NaOMe resulted in the bathochromic shift (λ_{max} 391, 324, 272 nm) of 55 nm of the band I confirmed the presence of free 4′-OH group. The UV spectrum of **4** was unaffected by the addition of NaOAc + H_3BO_3 (λ_{max} 328, 266, 210 nm) which clearly suggested the absence of *ortho* dihydroxyl system.[24]

^1H and ^{13}C NMR (Table 1) spectrum showed the characteristic signal for H-3 proton at δ_H 6.80 (s); δ_C 104.1. A sharp singlet observed at δ_H 12.8 was assigned to the C-5 chelated hydroxyl proton. The aromatic protons were resolved as one ABX system at δ_H 7.60 (d, J = 1.5 Hz), δ_C 114.7 for H-2′, δ_H 6.97 (d, J = 8.2 Hz); δ_C 117.5 for H-5′ and δ_H 7.62 (dd, J = 8.2, 1.5 Hz) for H-6′. The other AX system at δ_H 6.19 (d, J = 2.0 Hz), δ_C 99.7 and δ_H 6.45 (d, J = 2.0 Hz), δ_C 94.8 was assigned to H-6 and H-8 protons, respectively. A singlet at δ_H 3.67 (δ_C 52.9) was assigned to methoxy group of the esterified sugar. In case of 3′, 4′ dihydroxy flavone system, studies revealed that δ_C value for 4′ appeared downfield[24] than for 3′. Hence δ_C 145.9, 151.6 were assigned to C-3′ and C-4′, respectively. The anomeric proton of the sugar appeared at δ_H 5.21 (d, J = 6.2 Hz; δ_C 101.6). The coupling constant of the anomeric proton (J = 6.2 Hz) confirmed the β-linkage of the sugar.[25] Using anomeric proton as starting point in ^1H–^1H COSY spectrum other sugar protons were assigned, respectively (Table 1). Downfield shift and splitting pattern of H-5 sugar proton with absence of C-6 methylene

Table 1. ^1H and ^{13}C data of compound **4** in DMSO-d_6 (200 MHz)

Position	δ_H (J in Hz)	δ_C	HMBC[a]
2	—	164.1	H-3, H-2′, H-6′
3	6.80 (s)	104.1	—
4	—	182.6	H-3
5	—	162.2	H-6
6	6.19 (d, J = 2.0)	99.7	H-8
7	—	165.0	H-6, H-8
8	6.45 (d, J = 2.0)	94.8	H-6
9	—	158.2	H-8
10	—	104.6	H-3, H-6, H-8
1′	—	122.4	H-2′, H-6′, H-5′
2′	7.60 (d, J = 1.5)	114.7	H-6′
3′	—	145.9	H-1″, H-5′, H-2′
4′	—	151.6	H-2′, H-6′, H-5′
5′	6.97 (d, J = 8.2)	117.5	H-6′
6′	7.62 (dd, J = 8.2, 1.5)	122.8	H-2′, H-5′
1″	5.21 (d, J = 6.2)	101.6	H-3″, H-5″
2″	3.38 (m)	73.7	H-1″, H-3″, H-4″
3″	3.30 (m)	76.1	H-2″, H-3″, H-5″
4″	3.42 (m)	72.2	H-3″, H-5″
5″	4.19 (d, J = 8.9)	75.9	H-1″, H-2″, H-4″
6″	—	170.0	H-5″, 6″-OMe
6″-OMe	3.67 (s)	52.9	—
5-OH	12.8 (s)	—	—

[a] HMBC experiment in DMSO-d_6 (600 MHz).

Table 2. Antimicrobial activity of extract, fractions, and isolated compounds

Extract/fraction/pure compound	Minimum inhibitory concn (MIC) in μg/ml against										
	Bacteria					Fungi					
	1	2	3	4	5	6	7	8	9	10	11
Ethanolic extract	125	250	250	500	250	>500	62.5	>500	125	>500	>500
n-Hexane fraction	>500	—	500	>500	>500	>500	125	>500	>500	>500	>500
Chloroform fraction	250	500	500	>500	>500	>500	>500	>500	>500	>500	>500
n-Butanol fraction	250	250	250	500	125	>500	62.5	>500	250	>500	>500
1*	>50	25	50	50	25	>50	12.5	>50	25	50	25
2*	25	25	50	25	25	>50	25	>50	50	>50	25
3*	>50	50	>50	>50	>50	>50	12.5	>50	12.5	>50	>50
4*	>50	>50	>50	>50	>50	>50	6.25	>50	6.25	>50	>50
5*	>50	25	25	25	50	>50	6.25	>50	6.25	50	>50
6*	25	25	25	>50	25	>50	25	>50	12.5	50	>50
Flu	ND	ND	ND	ND	ND	0.5	1.0	2.0	2.0	2.0	1.0
Amp	0.02	0.09	0.02	50.0	0.09	ND	ND	ND	ND	ND	ND

1, *Streptococcus faecalis*; 2, *Klebsiella pneumoniae*; 3, *Escherichia coli*; 4, *Pseudomonas aeruginosa*; 5, *Staphylococcus aureus*; 6, *Candida albicans*; 7, *Cryptococcus neoformans*; 8, *Sporothrix schenckii*; 9, *Trichophyton mentagrophytes*; 10, *Aspergillus fumigatus*; 11, *Candida parapsilosis* (ATCC-22019)
Flu, fluconazole; Amp, ampicillin; ND, not done; *, pure compound.

of glucose indicated presence of carbonyl group at this position. This observation was well supported with presence of carbonyl carbon at δ_C 170.0 in ^{13}C NMR spectrum. The HMBC spectrum was utilized to identify position of sugar, a long range correlation (Table 1) between H-1″/C-3′ confirmed the attachment of sugar at C-3′, other useful correlation between H-5″/C-6″, -OCH_3/C-6″, H-2′/C-3′,4′, and H-5′/C-1′,3′ confirmed the position of methoxyl and carbonyl group. Further acid hydrolysis of **4** followed by co-TLC with authentic samples glycone (methyl ester of glucuronic acid) and aglycone (luteolin) was confirmed.[26] Further D-configuration of glycone was confirmed with optical rotation of acetate derivative $[\alpha]_D^{30}$ +11.4° ($CHCl_3$) with those reported in the literature.[27] Thus based on the foregoing evidence, the structure of compound **4** was elucidated as 4′, 5, 7-trihydroxy-3′-*O*-β-D-glucuronic acid-6″-methyl ester, a new naturally occurring compound named vitegnoside. Other five known compounds, 5′-hydroxy-3′, 4′, 3, 6, 7-pentamethoxyflavone (**1**)[28], luteolin (**2**)[24], agnuside (**3**)[17], negundoside (**5**)[17,18], and *iso*-orientin (**6**)[29], were characterized by comparing their spectroscopic data with those reported in the literature.

All the isolated compounds **1**–**6** were evaluated for antifungal activity (Table 2). The compounds **4** and **5** showed promising activity against *T. mentagrophytes* and *C. neoformans* of MIC 6.25 μg/ml as compared to standard antifungal drug fluconazole, having MIC of 2 μg/ml against *T. mentagrophytes*. Compounds **1** and **3** showed activity of MIC 12.5 μg/ml against *C. neoformans*, whereas compounds **3** and **6** showed activity against *T. mentagrophytes*. at similar concentration. Compound **2** showed moderate activity (25 μg/ml) against each test organism. The minimum inhibitory concentration (MIC) of standard drug fluconazole and test compounds was determined against test isolates by broth micro-dilution technique as per guidelines of NCCLS using 96-well tissue culture plates using RPMI 1640 media buffered with MOPS (3-[*N*-Morpholino] propanesulfonic acid) (Sigma Chemical Co.).[30–33]

In conclusion, we have isolated five known compounds **1**–**3**, **5** and **6** and a new flavonoid glycoside **4** from *V. negundo* through activity guided fractionation and discovered the potent antifungal activity of isolated compounds. The compounds **4** and **5** were found to be most active at MIC 6.25 μg/ml among all the isolated compounds. The further chemical transformation of compounds **4** and **5** is under progress to improve their biological profile.

Acknowledgments

Authors are thankful to ICMR, New Delhi, for financial assistance. We also thank Dr. Ashish Arora for the HMBC spectral data and S. C. Tiwari for technical assistance.

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