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Antimicrobial constituents from the stem bark of *Feronia limonia*

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Abstract

The stem bark of *Feronia limonia* (Fam. Rutaceae) yielded (−)-(2*S*)-5,3′-dihydroxy-4′-methoxy-6″,6″-dimethylchromeno-(7,8,2″,3″)-flavanone along with several known compounds including an alkaloid, five coumarins, a flavanone, a lignan, three sterols and a triterpene. The structures of these compounds were determined by spectroscopic methods, mainly 1D and 2D NMR. The antimicrobial screening of compounds by a microdilution technique resulted in MICs in the range 25–100 μg/ml. Other biological activities of the known compounds are also discussed. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: *Feronia limonia*; Rutaceae; Alkaloid; Coumarins; Flavanones; Lignan; (−)-(2*S*)-5,3′-Dihydroxy-4′-methoxy-6″,6″-dimethylchromeno (7,8,2″,3″)-flavanone; Antimicrobial

1. Introduction

Feronia limonia Swingle (Syn. *Feronia elephantum* Correa, Fam Rutaceae) is a moderate-sized tree with straight sharp strong spines, 1.2–3.7 cm long, which is widely distributed throughout Bangladesh, India, Ceylon and Java (Hooker, 1875; Kirtikar et al., 1993; Yusuf et al., 1994). The fruits of the plant are edible and considered to be a stomachic, astringent, diuretic, cardiotonic and tonic to the liver and lungs (Kirtikar et al., 1993); the bark is occasionally used for biliousness (Perry, 1980); the leaves are aromatic and carminative and are used for the treatment of indigestion and minor bowel infections of children (Kirtikar et al., 1993); the roots are sometimes given for the treatment of snake-bites (Kirtikar et al., 1993). Previous phytochemical work was mainly focused on the isolation of coumarins (Talapatra et al., 1973; Gupta et al., 1979; Agarwal et al., 1989), steroids and volatile components (Macleod and Pieris, 1981; Ahmad et al., 1989) of the plant. In this paper, we report the isolation and identification of a new pyranoflavanone (**1**) along with some known compounds: a diprenylated flavanone (**4**), a 7-hydroxy coumarin (**5**), an acridone alkaloid (**6**), an epidioxy sterol (**9**) and a lignan (**10**)- as well as the antimicrobial properties of **1**–**6**, **9** and **10**.

2. Results and discussion

VLC fractionation of the petroleum ether extract of the stem bark followed by gel filtration over Sephadex LH20 and preparative TLC led to the isolation of compounds **1**–**3**. The HREIMS of **1** gave a molecular ion at *m*/*z* 368, corresponding to $C_{21}H_{20}O_6$. It showed a strong fragment peak at *m*/*z* 353 due to the loss of a methyl group from the molecule. The fragment ion at *m*/*z* 218 suggested the usual fragmentation of a flavanone (Mabry and Markham, 1975), which further fragmented by losing a methyl group to give the base peak at *m*/*z* 203. The other fragment ion at *m*/*z* 135, due to the loss of a methyl radical from $C_9H_9O_2$, supported the presence of a methoxy and hydroxyl group on ring B. The ^1H NMR spectrum (400 MHz, $CDCl_3$, Table 1) showed a hydrogen bonded C-5 hydroxyl at δ 12.09 and an ABX system with signals at δ 5.34 ($J = 12.9$, 3.1 Hz), 3.05 ($J = 17.1$, 12.9 Hz) and 2.80 ($J = 17.1$, 3.1 Hz)- typical of H-2 and 2×H-3 of a flavanone (Markham and Mabry, 1975). The spectrum also revealed ortho coupled protons at δ 6.54 ($J = 10.0$, 0.7 Hz) and 5.47 ($J = 10.0$ Hz) and gem-dimethyl singlet at δ 1.43, 1.45 indicating the presence of a chromene (pyrano) ring system with the former proton showing long range coupling. Other aromatic protons at δ 6.00 ($J = 0.7$ Hz), 7.06 ($J = 2.1$ Hz), 6.90 (*d*, $J = 8.3$ Hz) and 6.94 (*dd*, $J = 8.3$, 2.1 Hz) were assigned as H-6, H-2′, H-5′ and H-6′, respectively. The COSY90 spectrum also revealed the coupling between H-5′ and H-6′; H-3″ and

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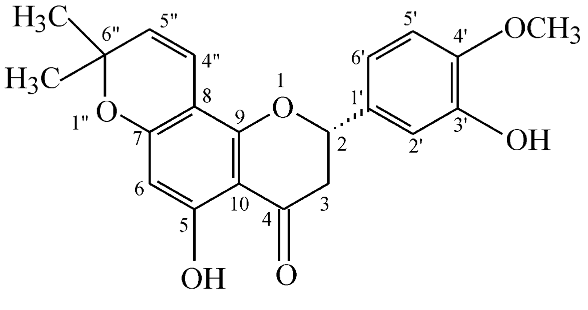
Table 1
^{1}H, ^{13}C and HMBC NMR spectral data of **1** in $CDCl_3$

Position	^{1}H^{a}	^{13}C^{b}	HMBC (C→H)	
			^{2}J	^{3}J
2	5.34 (*dd*, J=12.9, 3.1 Hz)	79.0	C-1′	
3	3.05 (*dd*, J=17.1, 12.9 Hz)	43.4	C-2, C-4	C-1′
	2.80 (*dd*, J=17.1, 3.1 Hz)		C-4	
4	–	196.1	–	
5	–	164.0	–	
6	6.00 (*d*, J=0.7 Hz)	97.8	C-5, C-7	C-8, C-10
7	–	162.5		
8	–	102.2		
9	–	157.0		
10	–	103.1		
1′	–	131.9		
2′	7.06 (*d*, J=2.1 Hz)	118.2	C-3′	C-2,
3′	–	146.1		
4′	–	147.1		
5′	6.90 (*d*, J=8.3 Hz)	112.8		C-1′, C-3′
6′	6.94 (*dd*, J=8.3, 2.1 Hz)	108.8	C-5′	C-2, C-4′
4″	6.54 (*dd*, J=10.1, 0.7 Hz)	115.9		C-6″, C-7, C-9
5″	5.47 (*d*, J=10.1 Hz)	126.6	C-6″	C-8
6″	–	78.4		
5-OH	12.09 (*s*)		C-5	C-6, C-10
3′-OH	5.73 (*s*)			
4′-OMe	3.93 (*s*)	56.3		C-4′
6″-Me	1.43	28.5	C-6″	C-5″, Me-6″
	1.45	28.7	C-6″	C-5″, Me-6″

[a] ^{1}H NMR carried out at 400 MHz.
[b] ^{13}C NMR carried out at 100 MHz.



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H-4″; H-2 and C-3 protons; the latter protons showing mutual coupling among themselves. A one proton broad singlet at δ 5.72 and the three proton singlet at δ 3.94 indicated hydroxyl and methoxy groups in ring B. A J-modulated ^{13}C spectrum (100 MHz, $CDCl_3$, Table 1) showed a total of 21 carbons with one carbonyl group at δ 196.1. The assignment of all carbons was achieved by ^{13}C and HMBC experiments (Table 1). In the HMBC spectrum, the C-5 hydroxyl showed ^{3}J correlation with a methine at δ 97.8 and a quaternary carbon at 103.1 which could be identified as C-6 and C-10, respectively. A ^{3}J correlation of both H-6 and H-5″ with the carbon at 102.2 ppm confirmed its assignment as C-8. Further, C-7 was assigned at 162.5 as it was connected to H-6 and H-4″ by ^{2}J and ^{3}J correlations, respectively. These features of the HMBC experiment confirmed the attachment of the pyrano ring through C-7 and C-8. Another ^{3}J connectivity of H-4″ to a downfield quaternary at 157.0 assigned it as C-9. In the pyrano ring, the gem-dimethyl protons and H-5″ showed a ^{2}J connection to quaternary at 78.4 ppm which was also correlated to H-4″ by ^{3}J; thus, this carbon was assigned as C-6″. In ring B, a ^{3}J correlation by H-2′, H-6′ and methoxyl protons to 147.1 ppm identified it as an oxygenated quaternary attached to methoxyl and favoured its assignment as C-4′. This statement was further proved from a strong interaction between H-5′ and the methoxy protons as evident from the NOESY experiment. The other oxygenated quaternary at 146.1 should, therefore, be recognised as C-3′ since it showed ^{2}J and ^{3}J connectivity by H-2′ and H-5′, respectively. Thus the methoxy group should be placed at C-4′ and the hydroxyl at δ 5.72 must be at position C-3′. Flavanones have one chiral centre at C-2, which translates into two possible orientations-2*S* or 2*R*. The measurement of optical rotation showed the compound to be *laevo*rotatory. Comparison with other flavanones of established absolute configuration supported the dominance of 2*S* isomer (Bohm, 1988; Ahsan et al., 1994; Hanawa et al., 2001; Takahashi et al., 2001). On this basis, the compound was identified as (–)-(2*S*)-5,3′-dihydroxy-4′-methoxy-6″,6″-dimethylchromeno-(7,8,2″,3″)-flavanone which appears to be a novel compound.

Chromatographic separation of the chloroform extract yielded compounds **4**–**10**. Compounds **2**–**10** were identified by comparison of their spectral data (UV, IR, ^{1}H NMR, ^{13}C NMR, EIMS) with literature values as psoralen (**2**) (Gupta et al., 1979), bergapten (**3**) (Banerji et al., 1982), 5,7,4′-trihydroxy-6,8-di-(3-methylbut-2-enyl)-flavanone (**4**) (Roussis et al., 1987), demethylsuberosin (**5**) (Filho et al., 1972), 1-hydroxy-3-methoxy-*N*-methyl-acridan-9-one (**6**) (Takemura et al., 1995), isopimpinellin (**7**) (Banerji et al., 1982), xanthotoxin (**8**) (Razdan et al., 1982), (*E*)-5,8-diepoxy-24(ξ)-methylcholesta-6,22-dien-3-ol (**9**) (Gunatilaka et al., 1981) and 5-(3-acetoxypropenyl)-2-(4-hydroxy-3-methoxyphenyl)-7-methoxy-2,3-dihydroxybenzofuran-3ylmethyl acetate (**10**) (Bohlmann et al., 1982). Among the compounds, **4**–**6**, **9** and **10** were first time reports from this plant and **4**, **9** and **10** were first reports in the Rutaceae. In addition to the above compounds, sitosterol, stigmasterol and lupeol were isolated from this plant. Thus *F. limonia* has been shown to produce a wide range of chemical types in line with many rutaceous taxa.

Antimicrobial screening of the compounds was performed by using a recently developed microdilution titre technique (Drummond and Waigh, 2000) that is economic, relatively quick and based upon the principle of microdilution for the determination of MIC values. The results of the MIC values in μg/ml and μmole are presented in Table 2. In terms of molar concentration, the lignan (**10**) was the most potent against all test organisms

Table 2
MIC values of compounds in μg/ml and μmol

Compound	*Staphylococcus aureus*		*Escherichia coli*		*Enterobacter eloacae*		*Klebsiella erogenes*		*Aspergillus niger*		*Candida albicans*	
	μg/ml	μmol	μg/m	μmol	μg/ml	μmol	μg/ml	μmol	μg/ml	μmol	μg/ml	μmol
1	100	0.272	100	0.272	100	0.272	100	0.272				
2+**3**	50	0.259	25	0.130	50	0.259	50	0.259	50	0.259	50	0.259
4	50	0.123	50	0.123	50	0.123	50	0.123	50	0.123	50	0.123
5	50	0.196	50	0.196	50	0.196	50	0.196	100	0.392	100	0.392
6	50	0.217	50	0.217	50	0.217	50	0.217	100	0.435	100	0.435
9	50	0.117	100	0.234	100	0.234	100	0.234	50	0.117	100	0.234
10	50	0.113	50	0.113	50	0.113	50	0.113	50	0.113	50	0.113
A[a]	3.13	0.008	12.5	0.034	6.25	0.017	6.25	0.017	–	–	–	–
G[a]	–	–	–	–	–	–	–	–	3.13	0.009	3.13	0.009

[a] A = amoxycillin; G = griseofulvin.

with the diprenylated flavanone (**4**) showing slightly less potency. The new flavanone (**1**) was active against both gram positive and gram negative bacteria, but did not show any antifungal activity. The epidioxy steroid (**9**) was active against *Staphylococcus aureus* and *Aspergillus niger*, but less so against the remaining organisms. The remaining compounds revealed moderate activities. However, in comparison to standard antibiotics the activities of the compounds were not so promising, but the presence of this mixture may support the traditional use of the plant to treat minor bowel infections (Kirtikar et al., 1993).

Prenylated flavanones similar to compound (**4**) have been shown (Miyamoto et al., 1998) to have oestrogenic effects and may be a useful treatment for oesteoporosis. It has been suggested (Ingram et al., 1997) that these types of phytoestrogens have anti-oestrogenic effects which may reduce the risks of hormone-related cancers when conventional steroid treatments for osteoporosis are used. The acridone alkaloid (**6**) has shown weak cytotoxicity on Raji cells (Takemura et al., 1995). The epidioxy steroid (**9**) was previously reported from a number of fungi including the edible mushroom *Lepiota americana* (Kim et al., 2000). These authors showed that compound (**9**) was a sulfatase inhibitor with the potential for use as a palliative therapy for hormone-dependant metastatic breast cancer. The dimeric coniferyl acetate (**10**) has also been isolated from brazilian propolis derived from *Baccharis* and *Clusia* species and *Araucaria heterophylla* (Banskota et al., 1998) and shown to have in vitro cytotoxicity towards murine colon 26-LS carcinoma and human HT-1080 fibrosarcoma cells. The presence of a range of hydroxyaromatic compounds such as flavanoids, coumarins, lignan and acridone derivatives would confer significant antioxidant activity (free-radical scavenging capacity) (Akdemir et al., 2001). Flavanoids have exhibited a range of activities including anti-inflammatory, antithrombotic, antiviral and hepatoprotection which may, in some measure, be due to their ability to scavenge free-radicals (Akdemir et al., 2001; Saija et al., 1995). Thus some of the folkloric uses indicated in the introduction, such as "tonic to the liver and lung" (Kirtikar et al., 1993), may be justified.

3. Experimental

3.1. General experimental procedures

Optical rotations were measured on a Perkin Elmer Polarimeter 341. Infrared (IR) spectra were recorded as dry film on a Mattson Galaxy 5000 FT–IR spectrometer. Ultraviolet (UV) spectra were obtained on a Unicam UV 4-100 UV/vis spectrophotometer in MeOH. HREIMS were recorded on a Jeol JMS-AX505HA double-focusing instrument at 70 eV. NMR spectra (both 1D and 2D) were recorded on a Bruker AMX-400 (400 MHz for ^{1}H and 100 MHz for ^{13}C) spectrometer, using the residual solvent peaks as internal standard. *J*-modulated ^{13}C spectra were acquired with a relaxation time (d_1) of 4 s. HMBC spectra were optimized for a long range $J_{\text{H-C}}$ of 7 Hz ($d_6 = 0.07$ s) and the NOESY experiment was carried out with a mixing time of 0.5 s. Vacuum-liquid chromatography (VLC) was carried out using Merck Si gel 60H. Gel filtration was performed using Sephadex LH-20 (Sigma). PTLC was carried out using Merck Si gel 60 PF_{254} on glass plates (20×20 cm) at a thickness of 0.5 mm. TLC was conducted on normal-phase Merck Si gel 60 PF_{254} on plates. Spots on TLC and PTLC plates were visualized under UV light (254 and 366 nm) and spraying with 1% vanillin-H_2SO_4 followed by heating at 110 °C for 5–10 min. The alkaloid was detected by modified Dragendorff's reagent.

3.2. Plant material

The stem bark of *Feronia limonia* was collected from Rajshahi, Bangladesh in August 1999. A voucher specimen (Collection No. RK1900; Accession No. 28085) was deposited in the Bangladesh National Herbarium, Dhaka, Bangladesh.

3.3. Extraction and isolation

The dried and powdered stem barks (700 g) were sequentially extracted with petroleum ether (bp 60–80 °C) and $CHCl_3$ in a Soxhlet apparatus. Evaporation of solvent under reduced pressure produced 5.5 g of petroleum ether extract and 11 g $CHCl_3$ extract as a semisolid mass. VLC fractionation of the petroleum ether extract (5.5 g) on Si gel was performed using as mobile phase petroleum ether, EtOAc and MeOH in order of increasing polarity. The fraction eluting with 18% EtOAc in petroleum ether was further subjected to gel filtration over Sephadex LH20 (10% petroleum ether in $CHCl_3$) and finally to preparative TLC (30% EtOAc in petroleum ether) to yield **1** (11 mg). Similarly, the fraction eluted with 16% EtOAc in petroleum ether was also subjected to gel filtration over Sephadex LH20 (10% petroleum ether in $CHCl_3$) and finally to PTLC (30% EtOAc in petroleum ether) to afford **2** (2.8 mg) and **3** (2.9 mg).

$CHCl_3$ extract (11 g) was fractionated by VLC over Si gel 60H using petroleum ether–EtOAc and EtOAc–MeOH mixtures of increasing polarity. The eluates were combined together on the basis of TLC analysis. The VLC fraction eluting with 15–17% EtOAc in petroleum ether was further subjected to gel filtration over Sephadex LH20 ($CHCl_3$) and finally to PTLC (20% EtOAc in toluene, double development) to yield **4** (10.2 mg) and **5** (7 mg). VLC fraction eluting with 20–25% EtOAc in petroleum ether was further subjected to gel filtration over Sephadex LH20. The eluate from the Sephadex column ($CHCl_3$) was further subjected to preparative TLC (25% EtOAc in toluene) to yield 3.2 mg of **6**, 5 mg of **7** and 3 mg of **8**, whereas compound **9** (5 mg) was obtained from the same Sephadex column (5–10% MeOH in $CHCl_3$) followed by preparative TLC over Si gel PF_{254} (15% EtOAc in toluene). The VLC fraction eluted with 30–35% EtOAc in petroleum ether was further subjected to gel filtration over Sephadex LH20 (5–10% MeOH in $CHCl_3$) and finally to PTLC (20% EtOAc in toluene, double development) to yield **10** (5.3 mg).

3.4. (−)-(2S)-5,3′-Dihydroxy-4′-methoxy-6″,6″-dimethylchromeno-(7,8,2″,3″)-flavanone (*1*)

Yellow waxy amorphous solid. $[\alpha]_D^{20}$ −44.1° ($CHCl_3$; *c* 0.022,). UV λ_{max}^{MeOH} nm (log ε): 229 (3.23), 333 (*sh*) (3.21), 267 (*sh*) (3.51), 271 (3.55), 296 (*sh*) (3.03), 307 (*sh*) (2.87), 361 (2.49). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3434, 2975, 2935, 2843, 1640, 1590, 1517, 1439, 1370, 1274, 1160, 1115, 1090, 1070, 1028, 762, 699. ^{1}H NMR, ^{13}C NMR and HMBC, see Table 1. HREIMS *m*/*z* 368.1255 (calc. for $C_{21}H_{20}O_6$, 368.1260), EIMS 354 (20), 353 (87), 218 (4), 204 (12), 203 (100).

3.5. Antimicrobial screening

A newly developed microdilution titre technique (Drummond and Waigh, 2000) was used to determine the antimicrobial activities as well as MICs of the metabolites against *Staphylococcus aureus* NCTC10788, *Escherichia coli* NCTC9001, *Enterobacter eloacae* NCTC9238, *Klebsiella aerogenes* Welcome Res. Lab. CM345, *Candida albicans* IMI149007 and *Aspergillus niger* NCPF3149. In this test, resazurin is used as a colour indicator for determining the level of activity of the compound. Resazurin being itself blue in colour oxidizes to pink when mixed with the organism. The blue colour is regained if the compound, when added to the mixture of organism and resazurin, is capable of inhibiting the growth of the organism. Briefly, in this test the test organism was cultured in nutrient broth medium and mixed with the colour indicator (resazurin) followed by pouring on 96-Well Microtiter plates. The compounds (Table 2) (200 μg/ml) were added into the first column of wells on the plates. Half of the content from these wells was transferred to the second column of wells and each subsequent well was treated similarly (doubling dilution). Both growth control and sterility control were maintained. Both negative (DMSO) and positive (amoxycillin against bacteria and griseofulvin against fungi) controls were also maintained. Finally, the plates were incubated at 37 °C for 5 h (24 h for fungi) and the results were marked by observing the change of colour from pink to blue. The concentration in the last well showing blue colour is taken as the MIC of a given compound.

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