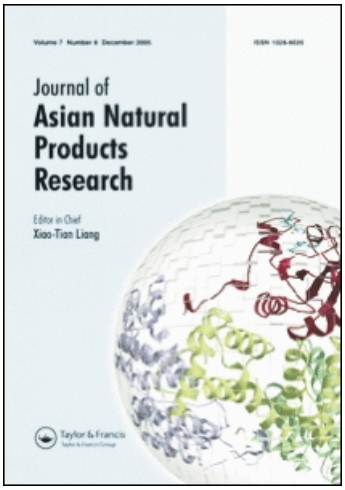

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A new bioactive flavone glycoside from the seeds of *Melilotus indica* All.

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Melilotus indica All. [Chopra, R.N., Nayar, S.L. and Chopra, I.C. (1956) *Glossary Indian Med. Plants*, 164 C.S.I.R. Publication New-Delhi; Kirtikar, K.R. and Basu, B.D. (1935) *Indian Medicinal Plants*, 2nd Ed., **Vol. I**, pp. 703–704 Lalit Mohan Basuan Co. Allahabad. The Wealth of India (1962) *A Dictionary of Raw Materials and Industrial Products*, **Vol. VI**, pp. 329–331 (C.S.I.R. Publication: New-Delhi)] belongs to family Leguminosae, which is commonly known as 'Banmethi' in Hindi. It is found in North India, extending into S. Persia, S. Europe and the Tropical zone of India. The seeds are used as an anthelmintic, an antipyretic, for curing heart diseases, bronchitis, leprosy, bowel complaints and infantile diarrhea. The plant has also been used as a discutient, emollient, and as a fomentation. It is also useful in a plaster for swelling. It is considered astringent and narcotic. Earlier workers have reported the presence of C-glycosides [Sayed, E.L., Ishak, M.S. and Mabry, T.J. (1997) *Asian J. Chem.*, **9**, 551], methylene-dioxypterocarpan (MIS_6) [Saxena, V.K. and Nigam, S. (1997) *Fitoterapia*, **68**, 343–345], pterocarpane (MIS_2) [Saxena, V.K. and Nigam, S. (1996) *J. Institution Chem.* **68**, 122–125] and prenylated pterocarpan [Saxena, V.K. and Nigam, S. (1997) *Fitoterapia*, **68**, 403–407] from this plant. Here, we report the isolation of the new flavone glycoside 5,7,4′-trihydroxy-6,3′-dimethoxyflavone-7-*O*-α-L-arabinopyranosyl(1 → 6)-*O*-β-D-galactopyranoside (**1**) from the seeds of this plant.

Keywords: *Melilotus indica* All; Leguminosae; Flavonoid; Flavone glycoside; Antimicrobial activity

1. Results and discussion

The new compound **1** was isolated from the ethyl acetate-soluble fraction of the 95% ethanolic extract of the seeds of *M. indica* All. It has an mp of 210–212°C, $C_{28}H_{32}O_{16}$, $[M]^+$ 624 (EIMS) and gave a positive response to the Molisch test for glycosidic nature and to the Shinoda test [1] for its flavonoid nature. A bathochromic shift of 48 nm (MeOH + NaOMe) showed that the hydroxy group is at C-4′ and a bathochromic shift of 25 nm (MeOH + $AlCl_3$) in band I with MeOH suggested the presence of an OH group at C-5 [2]. Its IR spectrum showed absorption bands at 3504 (–OH), 2952 (–CH), 2869 ($-OCH_3$), 1626 (C═O), 1616 (aromatic ring system), 1125 (–O–gly) and 826 cm^{-1}.

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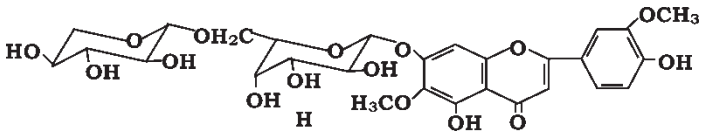
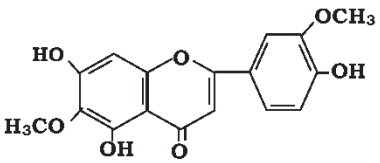
On acid hydrolysis with 7% ethanolic H_2SO_4 **1** gave an aglycone **2**, mp 194–196°C, m.f. $C_{17}H_{14}O_7$, $[M]^+$ 330 (EIMS), and sugars that were identified as L-arabinose (0.24) and D-galactose (0.16) (by Co-PC and Co-TLC). The aglycone was identified as 5,7,4′-trihydroxy-6,3′-dimethoxyflavone by comparison of its spectral data with that reported in the literature [3].



1



2

The ^{1}H NMR spectrum of compound **1** has three aromatic protons as three singlets at δ 7.43 (1H), 7.54 (1H) and 7.12 (1H), which were assigned to H-2′, 5′, and 6′, respectively, and two singlets at δ 3.95 and 3.89 due to OMe-6 and OMe-3′ and two singlets at δ 6.63 and 7.34 due to H-3 and H-8 protons. Signals for anomeric protons observed at δ 5.56 (1H, d, $J = 7.9$, H-1″) and 4.49 (1H, d, $J = 6.4$, H-1‴) are assigned to D-galactose and L-arabinose.

The position of sugar moiety in compound **1** was established by permethylation of **1** [4] followed by acid hydrolysis, which afforded 2,3,4-tri-*O*-methyl-D-galactose, 2,3,4-tri-*O*-methyl-L-arabinose and 5,4′,3′,6-tetramethoxy-7-hydroxy flavone, showing that the C-1‴ of arabinose was linked with C-6″ of galactose and that the C-7 position of the aglycone (**2**) was originally involved in glycosylation. The inter-linkage (1 → 6) between the sugars was further confirmed by the ^{13}C NMR spectrum (see "Experimental" Section).

Periodate oxidation [5] of compound **1** consumed 3.01 moles of periodate with the liberation of 1.16 moles of formic acid, suggesting that both sugars are in the pyranose form.

Enzymatic hydrolysis of **1**, with almond emulsion liberated D-galactose (Co-PC and Co-TLC) revealed a β-linkage with aglycone **2**, and hydrolysis with Takadiastase suggested an α-linkage between L-arabinose and D-galactose.

From the above, compound **1** was identified as 5,7,4′-trihydroxy-6,3′-dimethoxy flavone-7-*O*-α-L-arabinopyranosyl-(1 → 6)-*O*-β-D-galactopyranoside.

Compound **1** was tested for antimicrobial activity against various plant pathogenic fungi and bacteria.

2. Experimental section

2.1 General experimental procedures

Melting points were obtained on a Reichert microscope hot-stage apparatus and are uncorrected, UV spectra were determined in MeOH, FDMS using carbon emitters, with an accelerating voltage

of 3 kV and an emitter current of 15–29 mA; chamber at room temperature, MS (70 eV); IR spectra were recorded in KBr discs. ^{1}H NMR spectra were run at 300 MHz using TMS as internal standard and $CDCl_3$ as solvent. ^{13}C NMR spectra were run at 75 MHz using $CDCl_3$ as solvent.

2.2 Plant material

The seeds (3 kg) of *M. indica* were procured from M/s United Chemicals and Allied Products, Calcutta and were taxonomically-authenticated by the Botany Department of Dr. H.S. Gour University. A voucher specimen (no. 22) has been deposited in the Natural Products Laboratory, Department of Chemistry, Dr. H.S. Gour University, Sagar, (M.P.), India.

2.3 Extraction and isolation

The air-dried and powdered seeds (2.5 kg) of *Melilotus indica* were extracted with 95% EtOH in a Soxhlet extractor. The total extract was concentrated under reduced pressure to yield a brown viscous mass, which was successively partitioned with light petroleum ether (60–80°C), n-hexane, acetone, chloroform and ethyl acetate.

The ethyl acetate-soluble fraction of the ethanolic extract was concentrated under reduced pressure to give a light yellow syrupy mass that produced a single spot on TLC examination using the solvent system n-BuOH–AcOH–H_2O (4:1:5) and I_2 vapour as visualising agent. It was therefore purified by column chromatography over Si-gel-G and eluted with C_6H_6–$CHCl_3$ in various proportions. The fractions collected from C_6H_6–$CHCl_3$ (9:6) gave light yellow needles of compound **1**. (95 mg), mp 210–212°C (from EtOH), $C_{28}H_{32}O_{16}$, $[M]^+$ *m/z* 624; UV_{max} (MeOH) λ (nm): 245 (sh), 274, 349, (NaOMe + MeOH) 285, 287, 397, (+$AlCl_3$) 262, 285, 297, 374, (+$AlCl_3$/HCl) 254, 279, 265, 356; FABMS *m/z* (rel. int.), 624 $[M]^+$, 492 [M^+ − arabinose], $[M]^+$ 330, [M^+ sugar moieties], 329 [$M^+ - H$], 315 [$M^+ - CH_3$], 287 [$M^+ - CH_3CO$], 183 $[A_2]^+$, 182 $[A_1]^+$, 154 [A_1^+–CO], 151 $[B_1]^+$, 148 $[B_2]^+$, 124 [B_1^+–CO]. IR bands (KBr) ν (cm^{-1}): 3504, 2869, 2952, 1626, 1616, 1165, 826; ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 6.63 (1H, s, H-3), 7.34 (1H, s, H-8), 7.43 (1H, d, $J = 2.1$ Hz, H-2′), 7.54 (1H, d, $J = 9.1$ Hz, H-6′), 7.12 (1H, d, $J = 9.0$ Hz, H-5′), 3.95 (3H, s, 6-OMe), 3.89 (3H, s, 3′-OMe), 2.48 (3H, s, 5-OAc), 2.34 (3H, s, 4′-OAc], 5.56 (1H, d, $J = 7.9$ Hz, H-1″), 4.49 (1H, d, $J = 6.4$ Hz, H-1‴); ^{13}C NMR (75 MHz, DMSO-d_6) δ (ppm): 165.8 (C-2), 105.2 (C-3), 182.9 (C-4), 155.1 (C-5), 128.9 (C-6), 166.0 (C-7), 98.0 (C-8), 160.1 (C-9), 106.2 (C-10), 122.0 (C-1′), 105.39 (C-2′), 147.10 (C-3′), 133.9 (C-4′), 117.1 (C-5′), 107.9 (C-6′), 102.1 (C-1″), 71.5 (C-2″), 73.4 (C-3″), 67.9 (C-4″), 73.8 (C-5″), 64.9 (C-6″), 106.1 (C-1‴), 70.9 (C-2‴), 74.8 (C-3‴), 69.1 (C-4‴), 66.3 (C-5‴).

2.4 Acid hydrolysis of compound 1

Acid hydrolysis of **1** with 10% H_2SO_4 yielded aglycone **2** and sugar moieties, which were separated by filtration. The aglycone (**2**) was recrystallised from EtOH as yellowish needles, mp 194–196°C; m.f. $C_{17}H_{14}O_7$, $[M]^+$ *m/z* 330; UV_{max} (MeOH) λ (nm): 246 (sh), 274, 345, (NaOMe + MeOH) 284, 288, 393, (+$AlCl_3$) 285, 299, 370, (+$AlCl_3$/HCl) 287, 269, 356; IR bands (KBr) ν (cm^{-1}): 3510, 2875, 2956, 1635, 1620, 1170, 830; ^{1}H NMR (300 MHz, $CDCl_3$); δ (ppm): 6.61 (1H, s, H-3), 7.31 (1H, s, H-8), 7.41 (1H, d, $J = 2.1$ Hz, H-2′), 7.52 (1H, d, $J = 9.1$ Hz, H-6′), 7.17 (1H, d, $J = 9.1$ Hz, H-5′), 3.93 (3H, s, 6-OMe),

3.88 (3H, s, 3′-OMe), 2.47 (3H, s, 5-OAc), 2.33 (3H, s, 4′-OAc], 2.38 (3H, s, 7-OAc); 2 was then identified as 4′,5,7-trihydroxy-3′,6-dimethoxyflavone.

The aqueous hydrolysate obtained after acid hydrolysis of **1** was neutralised with $BaCO_3$ and the resultant $BaSO_4$ was filtered off. The filtrate was then concentrated and subjected to PC examination (n-BuOH–AcOH–H_2O 4:1:5), which revealed L-arabinose (R_f 0.24) and D-galactose (0.16) (by Co-PC and Co-TLC).

2.4.1 Permethylation of 1 followed by acid hydrolysis. Compound **1** was treated with Mel and Ag_2O in DMF at room temperature for 24 h and then filtered. The filtrate was dried *in vacuo* and hydrolysed with 20% ethanolic H_2SO_4 for 7 h. The usual work-up yielded aglycone **2** and methylated sugars identified as 2,3,4-tri-*O*-methyl-L-arabinose and 2,3,4-tri-*O*-methyl-D-galactose according to Petek [6].

2.4.2 Periodate oxidation of compound 1. Compound **1** was dissolved in MeOH and treated with sodium metaperiodate for two days. The amount of formic acid liberated and periodate consumed was estimated by Jones' method [5] and revealed that both sugars are in the pyranose form.

2.4.3 Enzymatic hydrolysis of Compound 1. Compound **1** (50 mg) was dissolved in MeOH (20 ml) and on hydrolysis with an equal volume of Takadiastase at room temperature yielded L-arabinose, confirming the α-linkage between L-arabinose and D-galactose, and on hydrolysis with almond emulsin liberated D-galactose, showing the β-linkage between D-galactose and the aglycone.

2.4.4 Quantitative estimation of sugars. A quantitative estimation of the sugars in the glycoside was performed using the procedure of Mishra and Rao [7], which revealed that two sugars were present in a 1:1 ratio.

2.4.5 Microbial activity of Compound 1. The ethyl acetate-soluble fraction of the ethanolic extract of the plant was tested for antibacterial and antifungal activity at different dilutions using ethylene glycol as solvent, at a concentration of 6 mg ml^{-1} of phosphate buffer's saline (w/v). The zone of inhibition was recorded at 37 ± 1°C after 48 h of incubation for bacterial species and at 28°C after 2–5 days incubation for fungal species.

The antimicrobial activity was determined using Whatman No. 1 filter paper discs (6 mm) [8,9]. Paper discs were soaked with various samples tested and dried at 50°C. The discs were then placed on soft nutrient Agar Petri plates previously spread with a suspension of each bacterial species. Then the plates were incubated at 37 ± 1°C for 48 h.

For the fungal species, Sabouraud's agar medium was used and the fungal species were inoculated; the plates were then incubated at 28°C for 2–5 days. The zones of inhibition were expressed as an average of the maximum diameter in four different directions. The various results are recorded in tables 1 and 2.

Tables 1 and 2 show that the antibacterial activity of the extract of the plant is fairly good against both Gram positive bacteria, *e.g. Bacillus anthracis*, *Bacillus cereus*, *Escherichia coli*, and Gram negative bacteria, *e.g. Proteus vulgaris*, *Salmonella newport*, and that the activity was retained even at 1:16 dilution. The plant extract showed greater antifungal activity against *Penicillium notatum*, *Penicillium purpurogenus* and *A. fumigatus*.

Table 1. Antibacterial activity of compound **1**.

		*Diameters of zone of inhibition (mm)**				
S. No.	*Bacterial species*	*Ethyl acetate soluble*	*1:4*	*1:8*	*1:12*	*1:16*
1	[+] *Streptococus pyogenes*	20.9	17.9	14.5	0	0
2	[+] *Bacillus coreus*	8.0	7.8	7.1	7.1	6.3
3	(−) *Klebsiella pneumeniae*	9.7	7.9	6.9	0	0
4	[−] *Pseudomonas aeruginosa*	8.7	7.4	6.7	7.7	0
5	[+] *Streptococus agalactiae*	20.9	17.8	15.2	0	0
6	[−] *Salmonella newport*	25.0	19.6	18.1	18.1	12.1
7	[+] *Bacillus anthracis*	8.6	8.0	7.1	7.1	6.4
8	[−] *Proteus vulgaris*	18.4	12.6	11.4	10.3	10.1
9	[+] *Escherichia coli*	8.5	7.9	7.0	7.0	6.2

* Zone of inhibition (mm) taken as average of four determinations in four different directions; Whatman No. 1 (6 mm) were soaked with each sample tested at a concentration of 6 mg ml^{-1} of PBS (w/v).

Table 2. Antifungal activity of compound **1**.

		*Diameters of zone of inhibition (mm)**				
S. No.	*Fungal species*	*Ethyl acetate soluble*	*1:4*	*1:8*	*1:12*	*1:16*
1	*Aspergillus niger*	9.7	6.8	0	0	0
2	*Fusarium oxysporum*	3.6	2.1	2.1	0	0
3	*Microspermum canis*	7.9	7.6	0	0	0
4	*Penicilliumnotatum*	8.9	8.6	7.4	7.4	6.4
5	*Epidermophyton floccosum*	8.6	7.2	0	0	0
6	*Microspermum gypsum*	8.2	7.7	0	0	0
7	*Aspergillus niger*	9.4	6.6	0	0	0
8	*Penicillium purpurogenus*	9.2	8.3	7.6	7.2	6.3
9	*A. fumigatus*	8.2	7.4	7.4	7.1	7.1

* Zone of inhibition (mm) taken as average of four determinations in four different directions; Whatman No. 1 (6 mm) were soaked with each sample tested at a concentration of 6 mg ml^{-1} of PBS (w/v).

Thus above investigations revealed that the ethyl acetate-soluble fraction of the ethanolic extract of the plant may potentially be useful as a therapeutic agent for diseases caused by these microorganisms.

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