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RESEARCH ARTICLE

New Potential Allelochemicals from *Crotalaria Medicaginea* Lam.

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Abstract: Two new potential allelochemicals have been isolated from methanolic extract of the stems of *Crotalaria medicaginea* Lam. Along with three known compounds Quercitrin, Acacetin and Isorhamnetin. The structure of two new allelochemicals were characterized as 3,5,7,3′,4′-pentahydroxy-6-methoxyflavone-3-*O*-*α*-*L*-rhamnopyranosyl-7-*O*-*β*-*D*-glucopyranosyl-(1→4)-*O*-*β*-*D*-xylopyranoside **(1)** and 3, 5, 7-trihydroxy-8,4′-dimethoxyflavone-5-*O*-*β*-D-galactopyranosyl-7-*O*-*α*-*L*-rhamnopyranosyl-(1→3)-*O*-*α*-*L*-arabinopyranoside **(2)** by various colour reactions, spectral analysis and chemical degradations.

Keywords: *Crotalaria medicaginea* Lam., Leguminosae, Allelochemical, Antimicrobial activity

Introduction

Crotalaria medicaginea Lam.[1-3] belongs to family leguminosae which is commonly known as "Gulabi" in Hindi. It is found throughout India from the W. Himalaya to Ceylon and Burma. The plant is used in medicine in Punjab. A variety of this species, var. Luxurians, common in western U.P. is considered to be a good camel fodder. The seeds may be used as cattle feed after cooking with common salt. Earlier workers[4-8] has reported various chemical constituents from this plant. In the present paper we report the isolation and structural elucidation of two new allelochemicals 3, 5, 7, 3′, 4′-pentahydroxy-6-methoxyflavone-3-*O*-*α*-*L*-rhamnopyranosyl-7-*O*-*β*-*D*-glucopyranosyl-(1→4)-*O*-*β*-*D*-xylopyranoside **(1)** and 3, 5, 7-trihydroxy-8, 4′-dimethoxyflavone-5-*O*-*β*-*D*-galactopyranosyl-7-*O*-*α*-*L*-rhamnoopyranosyl-(1→3)-*O*-*α*-*L*-arabinopyranoside (**2**) along with three known compounds Quercitrin **(3),** Acacetin **(4)** and Isorhamnetin (**5**) from methanolic extract of stems of this plant.

Experimental

All of the melting points were determined on a thermoelectrical melting point apparatus and are uncorrected. The IR spectra were recorded in KBr disc on Perkin Elimer spectrum RX1 (4000-450 cm^{-1}). ^{1}H NMR and ^{13}C NMR spectra were recorded at 90 MHz using solvent DMSO-d_6 and TMS as internal standard on Bruker DRX-300 spectrometer. UV spectra were

recorded in MeOH (Shimadzu UV 1800 spectrophotometer) and mass spectra on a Jeol SX-300 mass spectrometer.

Plant material

The stems of the plant were collected locally around Sagar region and were taxonomically authenticated by taxonomist, Department of Botany, Dr. H.S. Gour Central University, Sagar (M.P.) India. A voucher specimen has been deposited in the Natural Products Laboratory, Department of Chemistry of this university.

Extraction and isolation

Air dried and powdered stems (3 kg) of the plant were extracted with methanol in a Soxhlet apparatus for 74 h. The methanolic extract of stems of the plant was further exhaustively partitioned with chloroform, ethyl acetate and acetone. The acetone soluble fraction was further concentrated under reduced pressure to yield brown viscous mass (2.50 g), which was subjected to TLC examination using nBAW (4:1:5) as solvent and I_2 vapours as visualizing agent. It gave five spots indicating it to be mixture of five compounds **1**, **2, 3, 4** and **5**. These compounds were separated by TLC and purified by column chromatography over silica gel using $CHCl_3$: MeOH as eluents and studied separately.

*Study of compound **1***

It was crystalysed from acetone to yield 1.45 g. It has m.p. 224-225 °C, m.f. $C_{33}H_{40}O_{21}$, $[M]^+$ 772 (FABMS); found(%): C 51.18, H 5.26, calcd.(%) for m.f. $C_{33}H_{40}O_{21}$:C 51.30, H 5.18; UV λ_{max} MeOH (nm): 260, 358, 393; IR: ν_{max}^{KBr} (cm^{-1}), 3400, 2924, 2848, 1730, 1465, 1375, 1269, 1025, 746, 584, 537, 428; ^{1}H NMR (90 MHz, DMSO-d_6), δ (ppm); 6.43 (1H, s, H-8), 9.28 (1H, s, 3-OH), 11.40 (1H, s, 5-OH), 10.74 (1H, s, 7-OH), 3.81 (3H, s, 6-OCH_3), 7.70 (1H, d, J = 2.0 Hz, H-2′), 6.82 (1H, d, J= 8.0 Hz, H-5′), 7.63 (1H, dd, J = 8.3 , 2.0 Hz, H-6′), 8.88 (1H, s, 3′-OH), 8.94 (1H, s, 4′-OH), 5.32 (1H, br, s, H-1″), 4.20 (1H, m, H-2″), 3.21-3.74 (3H, m, H-3″, H-4″, H-5″), 0.90 (3H, d, J = 5.3 Hz, CH_3-6″), 4.72 (1H, d, J = 7.1 Hz, H-1‴), 3.21-3.48 (3H, m, H-2‴, H-3‴, H-4‴), 3.16 (1H, dd, J= 8.9, 8.9 H-5a‴), 3.78 (1H, dd, J =10.6, 5.1 H-5b‴), 4.62 (1H, d, J= 7.6 Hz, H-1⁗), 3.15-3.78 (6H, m, H-2⁗, H-3⁗, H-4⁗, H-5⁗, H-6⁗). ^{13}C NMR (90 MHz, DMSO-d_6), δ(ppm): 157.23(C-2), 136.4 (C-3), 179.42 (C-4), 153.54 (C-5), 132.68 (C-6), 157.16 (C-7), 94.78 (C-8), 152.24 (C-9), 105.82 (C-10), 122.64 (C-1′), 116.24 (C-2′), 145.92 (C-3′), 148.85 (C-4′), 116.54 (C-5′), 120.96 (C-6′), 61.26 (6-OCH_3), 102.0 (C-1″), 70.8 (C-2″), 70.6 (C-3″), 70.3 (C-4″), 70.0 (C-5″), 16.2 (C-6″), 106.3(C-1‴), 75.2 (C-2‴), 77.24 (C-3‴), 70.72 (C-4‴), 66.73 (C-5‴), 102.4 (C-1⁗), 83.2 (C-2⁗), 76.2 (C-3⁗), 69.3 (C-4⁗), 77.5 (C-5⁗), 60.8 (C-6⁗) and $[M]^+$ 772 (FABMS).

*Acid hydrolysis of compound **1***

Compound **1** (50 mg) was dissolved in ethanol (15 mL) and refluxed with 20 mL of H_2SO_4 on water bath for 6-8 h. The reaction mixture was concentrated and allowed to cool and residue was extracted with diethyl ether (Et_2O). The ether layer was washed with water and evaporated to dryness. The residue was subjected to column chromatography over silica gel column using $CHCl_3$: MeOH (3:6) to give compound **1-A**, identified as 3, 5, 7, 3′, 4′-pentahydroxy-6-methoxy flavone by comparison of its spectral data with reported literature values. The aqueous hydrolysate was neutralized with $BaCO_3$ and $BaSO_4$ filtered off. The filtrate was concentrated and subjected to paper chromatography examination using nBAW (4:1:5) solvent and aniline hydrogen phthalate as spraying reagent, showed the presence of *L*-rhamnose (R_f 0.36), *D*-glucose (R_f 0.19) and *D*-xylose (R_f 0.27) (Co-PC).

*Study of compound **1-A***

It has m.f. $C_{16}H_{12}O_8$, m.p. 242-245 °C, $[M]^+$ 332 (EIMS); found(%): C 57.64, H 3.54, calcd (%) for m.f. $C_{16}H_{12}O_8$, C 57.83, H 3.61; UV: λ_{max} (nm): (MeOH) 263, 355, 390; IR: ν_{max}^{KBr} (cm^{-1}), 3404, 2926, 2850, 1734, 1463, 1372, 1266, 1028, 742, 586, 534, 426; ^{1}H NMR (90 MHz, DMSO-d_6), δ(ppm): 6.45 (1H, s, H-8), 9.32 (1H, s, 3-OH), 12.36 (1H, s, 5-OH), 10.76 (1H, s, 7-OH), 3.84 (3H, s, 6-OCH_3), 7.72 (1H, d, J = 2.1 Hz, H-2′), 6.84 (1H, d, J = 8.1 Hz, H-5′), 7.61(1H, dd, J = 8.4, 2.1 Hz, H-6′), 8.92 (1H, s, 3′-OH), 8.96 (1H, s, 4′-OH). ^{13}C NMR (90 MHz, DMSO-d_6), δ(ppm) : 147.24 (C-2), 135.92 (C-3), 176.45 (C-4), 151.93 (C-5), 131.14 (C-6), 157.46 (C-7), 93.72 (C-8), 152.53 (C-9), 103.95 (C-10), 120.78 (C-1′), 115.06 (C-2′), 145.14 (C-3′), 147.74 (C-4′), 115.21 (C-5′), 123.15 (C-6′), 60.12 (6-OCH_3).

*Permethylation of compound **1***

Compound **1** (30 mg) was refluxed with MeI (10 mL) and Ag_2O (20 mL) in DMF (25 mg) for two days and then filtered. The filtrate was hydrolyzed with 10% ethanolic H_2SO_4 for 6-7 h, to give methylated aglycone identified as 3, 7-dihydroxy-5, 6, 3', 4'-tetramethoxy flavone and methylated sugars, which were identified as 2, 3, 4 -tri-*O*-methyl- L-rhamnose (R_G 1.02), 2, 3, 4, 6- tetra-*O*-methyl-*D*-glucose (R_G 1.00) and 2, 3-di-*O*-methyl-*D*-xylose (R_G 0.73).

*Enzymatic hydrolysis of compound **1***

Compound **1** (25 mg) was dissolved in MeOH (20 mL) and hydrolysed with equal volume of takadiastase enzyme. The reaction mixture was allowed to stay at room temperature for 2 days and filtered. The proaglycone and hydrolysate were studied separately. The hydrolysate was concentrated and subjected to paper chromatography examination using nBAW (4:1:5) as solvent, which showed the presence of *L*-rhamnose (R_f 0.36) (Co-PC). The proaglycone was dissolved in MeOH (25 mL) and further hydrolysed with equal volume of almond emulsin enzyme at room temperature as usual procedure yielded aglycone, identified as 3, 5, 7, 3', 4'-pentahydroxy-6-methoxy flavone and sugars were identified as *D*-glucose (R_f 0.82) and *D*-xylose (R_f 0.27) (Co-PC).

*Study of compound **2***

It was crystalysed from acetone to yield 950 mg. It has m.p. 234-235 °C, m.f. $C_{34}H_{42}O_{20}$, $[M]^+$ 770 (FABMS); found(%): C 52.36, H 5.24, calcd.(%) for m.f. $C_{34}H_{42}O_{20}$:C 52.99, H 5.45; UV: λ_{max} (nm): (MeOH) 264, 364; (+$AlCl_3$) 268, 425; (+$AlCl_3$/HCl) 270, 426; (+NaOAc) 266, 370; IR: ν_{max}^{KBr} (cm^{-1}), 3484, 2906, 2873, 1656, 1623, 1591, 926, 852; ^{1}H NMR (90 MHz, DMSO-d_6), δ (ppm); 6.15 (1H, d, J = 2.0 Hz, H-6), 9.44 (1H, s, 3-OH), 12.48 (1H, s, 5-OH), 10.86 (1H, s, 7-OH), 3.90 (3H, s, 8-OCH_3), 8.15 (2H, d, J =8.8 Hz, H-2′, H-6′), 7.12 (1H, d, J= 8.8 Hz, H-3′, H-5′), 3.86 (3H, s, 4′-OCH_3), 5.23 (1H, d, J = 7.5, H-1″), 3.36-3.72 (4H, m, H-2″, H-3″, H-4″, H-5″), 3.80 (1H, dd, J = 11.1, 7.1 Hz, H-6_a″), 4.23 (1H, dd, J = 11.2, 4.2 Hz, H-6_b″), 4.96 (1H, d, J = 6.9 Hz, H-1‴), 3.46-4.42 (4H, m, H-2‴, H-3‴, H-4‴, H-5‴), 4.54 (1H, d, J 1.21 Hz, H-1⁗), 3.10-3.68 (4H, m, H-2⁗, H-3⁗, H-4⁗, H-5⁗), 1.08 (3H, d, J 6.0 Hz, 6⁗- CH_3). ^{13}C NMR (90 MHz, DMSO-d_6), δ(ppm): 144.6 (C-2), 137.2 (C-3), 175.3 (C-4), 155.2 (C-5), 100.6 (C-6), 156.4 (C-7), 132.4 (C-8), 152.8(C-9), 106.7 (C-10), 123.2 (C-1′), 129.3 (C-2′), 114.0 (C-3′), 160.4 (C-4′), 114.7 (C-5′), 129.8 (C-6′), 62.0 (8-OCH_3), 55.8 (4′-OCH_3), 104.6 (C-1″), 73.5 (C-2″), 75.6 (C-3″), 70.2 (C-4″), 77.6 (C-5″), 62.5 (C-6″), 103.6 (C-1‴), 73.3 (C-2‴), 74.6 (C-3‴), 69.6(C-4‴), 67.7 (C-5‴), 100.44 (C-1⁗), 70.18 (C-2⁗), 69.44 (C-3⁗), 71.95 (C-4⁗), 68.21 (C-5⁗), 17.67 (C-6⁗) and $[M]^+$ 770 (FABMS).

Acid hydrolysis of compound ***2***

Compound **2** (50 mg) was dissolved in ethanol (15 mL) and refluxed with 20 mL of H_2SO_4 on water bath for 6-8 h. The reaction mixture was concentrated and allowed to cool and residue was extracted with diethyl ether (Et_2O). The ether layer was washed with water and evaporated to dryness. The residue was subjected to column chromatography over silica gel column using $CHCl_3$: MeOH (3:6) to give compound **2-A**, identified as 3, 5, 7-trihydroxy-8, 4′-dimethoxy flavone. The aqueous hydrolysate was neutralized with $BaCO_3$ and $BaSO_4$ filtered off. The filtrate was concentrated and subjected to paper chromatography examination using nBAW (4:1:5) solvent and aniline hydrogen phthalate as spraying reagent, showed the presence of L-rhamnose (R_f 0.36), *L*-arabinose (R_f 0.22) and *D*-galactose (R_f 0.15) (Co-PC).

Study of compound ***2-A***

It has m.f. $C_{17}H_{14}O_7$, m.p. 256-257 °C, $[M]^+$ 330 (EIMS); found(%): C 60.28, H 4.10, calcd (%) for m.f. $C_{17}H_{14}O_7$, C 61.82, H 4.24; UV: λ_{max} (nm): (MeOH) 262, 366; (+$AlCl_3$) 266, 427; (+$AlCl_3$/HCl) 272, 424; (+NaOAc) 268, 372; IR: ν_{max}^{KBr} (cm^{-1}), 3486, 2904, 2876, 1654, 1626, 1594, 924, 854; ^{1}H NMR (90 MHz, DMSO-d_6), δ(ppm): 6.18 (1H, d, *J* = 2.1 Hz, H-6), 9.46 (1H, s, 3-OH), 12.45 (1H, s, 5-OH), 10.86 (1H, s, 7-OH), 3.88 (3H, s, 8-OCH_3), 8.13 (2H, d, *J* = 8.9 Hz, H-2′, H-6′), 7.10 (2H, d, *J* = 8.9 Hz, H-3′, H-5′), 3.84 (3H, s, 4′-OCH_3).^{13}C NMR (90 MHz, DMSO-d_6), δ (ppm) : 146.4 (C-2), 136.3 (C-3), 176.2 (C-4), 156.9 (C-5), 99.4 (C-6), 157.6 (C-7), 128.4 (C-8), 150.3(C-9), 104.4 (C-10), 123.5 (C-1′), 129.6 (C-2′), 114.3 (C-3′), 160.8 (C-4′), 114.3 (C-5′), 129.6 (C-6′), 61.8 (8-OCH_3), 55.6 (4′-OCH_3).

Permethylation of compound ***2***

Compound **2** (20 mg) was refluxed with MeI (10 mL) and Ag_2O (20 mL) in DMF (25 mg) for two days and then filtered. The filtrate was hydrolyzed with 10% ethanolic H_2SO_4 for 6-7 h, to give methylated aglycone, identified as 5,7-dihydroxy- 3, 8, 4'-trimethoxy flavone and methylated sugars, which were identified as 2, 3, 4, 6 -tetra-*O*-methyl-*D*-galactose (R_G 0.89), 2, 3, 4- tri-*O*-methyl-*L*-rhamnose (R_G 1.02) and 2, 4-di-*O*-methyl-*L*-arabinose (R_G 0.63).

Enzymatic hydrolysis of compound ***2***

Compound **2** (15 mg) was dissolved in MeOH (20 mL) and hydrolysed with equal volume of takadiastase enzyme. The reaction mixture was allowed to stay at room temperature for 2 days and filtered. The proaglycone and hydrolysate were studied separately. The hydrolysate was concentrated and subjected to paper chromatography examination using nBAW (4:1:5) as solvent, which showed the presence of *L*-rhamnose (R_f 0.36) and *L*-arabinose (R_f 0.22) (Co-PC). The proaglycone was dissolved in MeOH (25 mL) and further hydrolysed with equal volume of almond emulsin enzyme at room stemperature as usual procedure yielded aglycone, identified as 3, 5, 7-trihydroxy-8, 4'-dimethoxy flavone and sugar was identified as *D*-galactose (R_f 0.15) (Co-PC).

Antimicrobial activity of compounds 1 and 2

The antibacterial activity of compounds **1** and **2** was determined by filter paper disc diffusion method[9]. The various gram (+ve) and gram (-ve) bacterial species were first incubated at 48 °C for 42 h. The sterile filter paper discs (6 mm) were soaked with standard antibacterial agent and various test samples and were dried at 50 °C. The discs were then

placed on soft nutrient agar (2%) petri plates previously seeded with suspension of each bacterial species. The diameters of zone of inhibition were measured at 35±1 °C after 24 h. The results are recorded in Table 1.

The antifungal activity of compounds was measured by PDA (Potato Dextrose Agar) with 4% agar for the preparation of plates and incubated with spores and mycelium suspension of fungi obtained from one week old culture. The diameters of zone of inhibition were measured at 26±1 °C after 46 h. The results are recorded in Table 2.

Table 1. Antibacterial activity of compound **1** and **2**

S. No	Bacterial species	Diameters of zone of inhibition mm*								Std,**
		Concentration of compound **1** %				Concentration of compound **2** %				
		100	80	60	40	100	80	60	40	
1.	*Escherichia coli*	-	-	-	-	6.54	3.56	-	-	11.25
2.	*Staphylococcus aureus*	-	-	-	-	-	-	-	-	34.33
3.	*Bacilius subtilis*	-	-	-	-	-	9.51	-	-	30.25
4.	*Micrococcus luteus*	-	-	-	4.66	-	-	3.12	-	10.5

**The zone of inhibition (mm) taken as average of four determination direction. **Ampicillin (10 mg/mL) used as standard antibacterial agent*

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

S. No	Fungal species	Diameters of zone of inhibition mm*								Std,***
		Concentration of compound **1** %				Concentration of compound **2** %				
		100	80	60	40	100	80	60	40	
1.	*Candida albicans*	-	-	4.88	-	-	-	-	3.84	13.00
2.	*Rhizopus oryzae*	-	-	-	-	-	-	-	-	32.25
3.	*Aspergillus niger*	-	-	-	-	5.85	-	-	-	13.75
4.	*Mucor indicus*	-	-	-	-	-	-	4.24	-	10.00

The zone of inhibition (mm) taken as average of four determination direction. ***Ketocozole (100 mg/mL) used as standard antifungal agent. Study of compound **3*

It was crystallized from acetone to give 600 mg. It has m.f. $C_{21}H_{20}O_{11}$, m.p. 192-194 °C, $[M]^+$ 448 (EIMS); found(%), C 56.10, H 4.68, calcd(%) for m.f. $C_{21}H_{20}O_{11}$, C 56.25, H 4.46; UV:λ_{max} (nm): (MeOH) 215, 258, 352; IR: ν_{max}^{KBr} (cm^{-1}), 3404, 1662, 1516. ^{1}H NMR (90 MHz, DMSO-d_6), δ(ppm): 6.18 (1H, d, J = 2.0 Hz, H-6), 6.40 (1H, d, J = 2.0 Hz, H-8), 7.65 (1H, d, J = 2.2 Hz, H-2′), 6.83 (1H, d, J = 8.1 Hz, H-5′), 7.52 (1H, dd, J = 8.4, 2.0 Hz, H-6′), 4.10 (1H, d, J =1.3 Hz, H-1″), 3.53(1H, m, H-2″), 3.64 (1H, m, H-3″), 3.33 (1H, m, H-4″), 4.26 (1H, m, H-5″),1.20 (1H, d, J = 1.3 Hz, H-6″). ^{13}C NMR (90 MHz, DMSO-d_6), δ(ppm): 156.60 (C-2), 134.36 (C-3), 177.84 (C-4), 161.40 (C-5), 98.90 (C-6), 164.36 (C-7), 93.94 (C-8), 157.35 (C-9), 104.28 (C-10), 121.26(C-1′), 115.62 (C-2′), 145.30 (C-3′), 148.52 (C-4′), 115.86 (C-5′), 121.28(C-6′),101.92 (C-1″), 70.56 (C-2″), 70.72 (C-3″), 71.33 (C-4″), 70.15 (C-5″), 17.64 (C-6″).

*Study of compound **4***

It was crystalysed from acetone to give 450 mg. It has m.f. $C_{16}H_{12}O_5$, m.p. 260-262 °C, $[M]^+$ 284 (EIMS); found(%), C 67.20, H 3.85, calcd(%) for m.f. $C_{16}H_{12}O_5$, C 67.61, H 4.23, UV: λ_{max} nm: (MeOH) 265, 308, 324; IR: ν_{max}^{KBr} (cm^{-1}), 3610, 2980, 2248, 2128, 1652, 1598, 1436;

^{1}H NMR (90 MHz, DMSO-d_6), δ(ppm): 6.76 (1H, s, H-3), 6.88 (1H, d, J = 2.4 Hz, H-6), 6.48 (1H, d, J = 2.4 Hz, H-8), 12.86 (1H, s, 5-OH), 8.07 (2H, d, J = 8.8 Hz, H-2′, 6′), 7.14 (2H, d, J = 8.8 Hz, H-3′, 5′), 3.93 (3H, s, OCH_3-4′).^{13}C NMR (90 MHz, DMSO-d_6), δ(ppm): 162.4(C-2), 103.4 (C-3), 181.9 (C-4), 162.7(C-5), 99.95 (C-6), 163.5 (C-7), 94.3 (C-8), 156.5 (C-9), 105.6 (C-10), 122.2 (C-1′), 128.9 (C-2′, 6′), 114.2 (C-3′, 5′), 161.4 (C-4′), 55.8 (OCH_3-4′).

Study of compound ***5***

It was crystallized from acetone to give 350 mg. It has m.f. $C_{16}H_{12}O_7$, m.p. 186-188 ^{0}C, $[M]^+$ 316 (EIMS); found (%), C 60.36, H 3.64, calcd (%) for m.f. $C_{16}H_{12}O_7$, C 60.76, H 3.80; UV: λ_{max} (nm): (MeOH) 262, 366; (+$AlCl_3$**)** 266, 436; **(**+$AlCl_3$/HCl) 264, 420; (+NaOAc) 288, 372; IR: ν_{max}^{KBr} (cm^{-1}), 3402, 2976, 1688, 1638. ^{1}H NMR (90 MHz, DMSO-d_6) : δ(ppm) 6.22 (1H, d, J = 2.1 Hz, H-6), 6.46 (1H, d, J= 2.1 Hz, H-8), 9.43 (1H, s, 3-OH), 12.48 (1H, s, 5-OH), 10.86 (1H, s, 7-OH), 7.74 (1H, d, J =1.6 Hz, H-2′), 6.91 (1H, d, J = 8.4 Hz, H-5′), 7.66 (1H, dd, J = 8.6, 1.4 Hz, H-6′), 9.75 (1H, s, 4′-OH), 3.19 (3H, s, 3′-OCH_3). ^{13}C NMR (90 MHz, DMSO-d_6), δ(ppm): 146.5 (C-2), 136.3 (C-3), 176.5(C-4), 161.4(C-5), 98.7(C-6), 164.6 (C-7), 93.6 (C-8), 156.8 (C-9), 103.7 (C-10), 122.6 (C-1′), 111.7 (C-2′), 149.5 (C-3′), 147.9(C-4′), 115.4(C-5′), 122.3(C-6′), 56.2 (OCH_3).

Results and Discussion

Chemical examination of methanolic extract of stems of *Crotalaria medicaginea* Lam., gave two new allelochemicals **1** and **2**. Compound **1** (Figure 1) has molecular formula $C_{33}H_{40}O_{21}$, m.p. 224-225 °C, $[M]^+$ 772 (FABMS). It gave Molisch and Shinoda tests[10] showing its flavonoidal glycosidic nature. Its IR spectra showed strong absorption bands at 3400, 2924, 2848, 1730, 1465, 1375, 1269, 1025, 746, 584, 537 and 428 cm^{-1}. In UV spectrum two bands at 260 nm and 358 nm showed its flavonoidal skeleton. In ^{1}H NMR spectrum of compound **1** three singlets at δ 9.28, δ 11.40 and δ 10.74 suggested the presence of –OH groups at C-3, C-5 and C-7 positions respectively. A singlet at δ 3.81 confirmed the presence of –OMe group at C-6 position. Two singlets at δ 8.88 and δ 8.94 confirmed the presence of –OH groups at C-3' and C-4' positions. In ^{1}H NMR spectrum of the aglycone **1-A,** a singlet at δ 6.48 was assigned to H-8 of **A** ring. A doublet at δ 6.84 (1H, d, J = 8.1 Hz) was assigned to H-5' in ring **B**. A doublet at δ 7.72 (1H, d, J = 2.1 Hz) and a double doublet δ 7.61 (1H, dd, J = 8.4, 2.1 Hz) were assigned to H-2' and H-6' in ring **B** respectively. The anomeric proton signals at δ 5.32 (1H, br, s), δ 4.72 (1H, d, J = 7.1 Hz) and δ 4.62 (1H, d, J = 7.6 Hz) were assigned for H-1", H-1'" and H-1"" of *L*-rhamnose, D-xylose and D-glucose respectively.

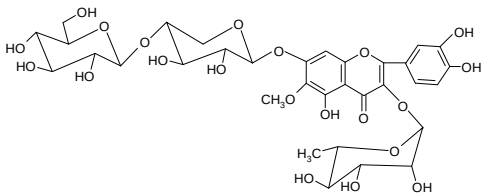

Figure 1. Compound **1**

In the mass spectrum of the compound **1**, characteristic ion peaks at *m/z* 772 $[M]^+$, 626 [M^+-*L*-rhamnose], 464 [M^+-*D*-glucose] and 332 [M^+-*D*-xylose, aglycone] were found by subsequent losses from the molecular ion of each molecule of *L*-rhamnose, *D*-glucose and *D*-xylose showing *D*-glucose as terminal sugar, *D*-xylose was linked at C-7 position and *L*-rhamnose was attached at C-3 position of the aglycone.

Acid hydrolysis of compound **1** with 10% ethanolic H_2SO_4 gave aglycone **1-A** (Figure 2), m.p. 242-245 °C, m.f. $C_{16}H_{12}O_8$, $[M]^+$ 332 (EIMS) and sugar moiety (ies).These were separated and studied separately. The aglycone **1-A** was identified as 3, 5, 7, 3', 4'-pentahydroxy-6-methoxy flavone by comparison of its spectral data with reported literature values[11].

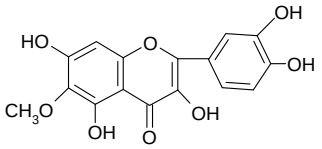

Figure 2. Compound **1-A**

The aqueous hydrolysate after the removal of aglycone was neutralized with $BaCO_3$ and $BaSO_4$ filtered off. The filtrate was concentrated and subjected to paper chromatography examination and sugars were identified as *L*-rhamnose (R_f 0.36), *D*-glucose (R_f 0.19) and *D*-xylose (R_f 0.27) (Co-PC)[12]. Periodate oxidation of compound **1,** confirmed that all the sugars were present in the pyranose form[13].

The positions of sugar moieties in compound **1** were determined by permethylation[14] followed by acid hydrolysis yielded methylated aglycone identified as 3, 7-dihydroxy-5, 6, 3', 4'-tetramethoxy flavone showed that glycosydation was involved at C-3 and C-7 positions of the flavanone and methylated sugars were identified as 2, 3, 4 -tri-*O*-methyl- *L*-rhamnose (R_G 1.02), 2, 3, 4, 6-tetra-*O*-methyl-*D*-glucose (R_G 1.00) and 2, 3-di-*O*-methyl-*D*-xylose (R_G 0.73) indicating that C-1''-OH of *L*-rhamnose was linked at C-3 position of the aglycone, C-1''''-OH of *D*-glucose was linked to C-4'''-OH of *D*-xylose and C-1'''-OH of *D*-xylose was attached with C-7 position of the aglycone. Therefore it was concluded that interlinkage (1→4) was found between *D*-glucose and *D*-xylose which was further confirmed by ^{13}C NMR spectra (*c.f.* Experimental section).

Enzymatic hydrolysis of compound **1** with takadiastase enzyme liberated *L*-rhamnose (R_f 0.36) and proaglycone identified as 3, 5, 7, 3', 4'- pentahydroxy-6-methoxyflavone-7-*O*-*β*-*D*-glucopyranosyl-(1→4)-*O*-*β*-*D*-xylopyranoside showed the presence of *α*-linkage between *L*-rhamnose and proaglycone. The proaglycone on further hydrolysis with almond emulsion enzyme liberated *D*-glucose (R_f 0.19) followed by *D*-xylose (R_f 0.27) and aglycone suggesting the presence of *β*-linkage between *D*-glucose and *D*-xylose as well as *D*-xylose and aglycone.

On the basis of above evidences the structure of compound **1** was characterized as 3, 5,7,3',4′-pentahydroxy-6-methoxyflavone-3-*O*-*α*-*L*-rhamnopyranosyl-7-*O*-*β*-*D*-glucopyranosyl-(1→4)-*O*-*β*-*D*-xylopyranoside .

Compound **2** (Figure 3) has molecular formula $C_{34}H_{42}O_{20}$, m.p. 234-235 °C, $[M]^+$ 770 (FABMS). It gave Molisch and Shinoda tests[10] showing its flavonoidal glycosidic nature. Its IR spectra showed strong absorption bands at 3484, 2906, 2873, 1656, 1623, 1591, 926 and 852 cm^{-1}. In UV spectrum two bands at 264 nm and 364 nm showed its flavonoidal skeleton. The bathochromic shift of 23 nm with $AlCl_3$ and 35 nm with NaOAc in band I relative to methanol showed the presence of –OH groups at C-5 and C-7 positions in the aglycone **2-A**[15,16]. In ^1H NMR spectrum a singlet at δ 9.44 confirmed the presence of –OH group at C-3 position. Two singlets at δ 3.90 and δ 3.86 confirmed the presence of –OMe groups at C-8 and C-4' positions. A doublet at 6.15 (1H, d, *J* = 2.0 Hz) was assigned to H-6 of **A** ring. Two doublets at δ 8.15 (2H, d, *J* = 8.8 Hz) were assigned to H-2' and H-6' and two doublets at δ

7.12 (2H, d, *J* = 8.8 Hz) were assigned to H-3' and H-5' in ring **B**. The anomeric proton signals at δ 5.23 (1H, d, *J* = 7.5), δ 4.96 (1H, d, *J* = 6.9 Hz) and δ 4.54 (1H, d, *J* = 1.21 Hz) were assigned for H-1", H-1"" and H-1"" of *D*-galactose, *L*-arabinose and *L*-rhamnose respectively.

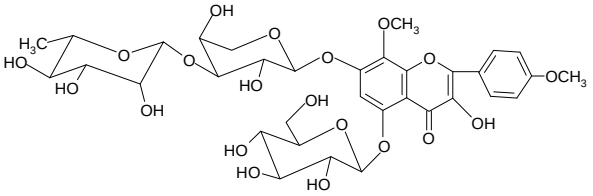

Figure 3. Compound **2**

In the mass spectrum of the compound **2**, characteristic ion peaks at *m/z* 770 [M]+, 608 [M^+-*D*-galactose], 462 [M^+-*L*-rhamnose] and 330 [M^+-*L*-arabinose, aglycone] were found by subsequent losses from the molecular ion of each molecule of *D*-galactose, *L*-rhamnose and *L*-arabinose revealing *L*-rhamnose as terminal sugar, *L*-arabinose was linked to aglycone at C-7 position and *D*-galactose was attached at C-5 position of aglycone.

Acid hydrolysis of compound **2** with 10% ethanolic H_2SO_4 gave aglycone **2-A** (Figure 4), m.p. 256-257°C, m.f. $C_{17}H_{14}O_7$, $[M]^+$ 330 (EIMS) and sugar moiety(ies). These were separated and studied separately. The aglycone **2-A** was identified as 3, 5, 7-trihydroxy-8, 4'-dimethoxy flavones (*c.f.* Experimental section).

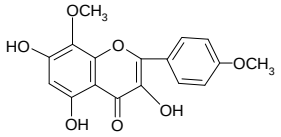

Figure 4. Compound **2-A**

The aqueous hydrolysate after the removal of aglycone was neutralized with $BaCO_3$ and $BaSO_4$ filtered off. The filtrate was concentrated and subjected to paper chromatography examination and sugars were identified as *D*-galactose (R_f 0.15), *L*-rhamnose (R_f 0.38) and *L*-arabinose (R_f 0.23) (Co-PC)[12]. Periodate oxidation of compound **2**, confirmed that all the sugars were present in the pyranose form[13].

The positions of sugar moieties in compound **2** were determined by permethylation[16] followed by acid hydrolysis yielded methylated aglycone identified as 5, 7-dihydroxy-3, 8, 4'-trimethoxy flavone showed that glycosydation was involved at C-5 and C-7 positions of the flavone and methylated sugars were identified as 2, 3, 4, 6 -tetra-*O*-methyl-*D*-galactose (R_G 0.89), 2, 3, 4-tri-*O*-methyl-*L*-rhamnose (R_G 1.02) and 2, 4-di-*O*-methyl-*L*-arabinose (R_G 0.65) indicating that C-1"-OH of *D*-galactose was linked to C-5 position of the aglycone, C-1""-OH of *L*-rhamnose was linked to C-3'"-OH of *L*-arabinose and C-1'"-OH of *L*-arabinose was attached with C-7 position of the aglycone. Therefore, it was concluded that interlinkage (1→3) between *L*-rhamnose and *L*-arabinose was found which was further confirmed by ^{13}C-NMR spectra (*c.f.* Experimental section).

Enzymatic hydrolysis of compound **2** with takadiastase enzyme liberated *L*-rhamnose (R_f 0.38) followed by *L*-arabinose (R_f 0.22) and proaglycone identified as 3, 5, 7-trihydroxy-8, 4′-dimethoxyflavone-5-*O*-*β*-*D*-galactopyranoside showed the presence of *α*-linkage between *L*-rhamnose and *L*-arabinose as well as between *L*-arabinose and proaglycone. Proaglycone on further hydrolysis with almond emulsin enzyme liberated *D*-galactose (R_f 0.15) and aglycone suggesting the presence of *β*-linkage between *D*-galactose and aglycone.

On the basis of above evidences the structure of compound **2** was characterized as 3, 5, 7-trihydroxy-8,4′-dimethoxyflavone-5-*O*-*β*-*D*-galactopyranosyl-7-*O*-*α*-*L*-rhamnopyranosyl-(1→4)-*O*-*α*-*L*-arabinopyranoside.

Compound **3** (Figure 5) has m.p.192-194 °C, m.f. $C_{21}H_{20}O_{11}$, $[M]^+$ 448 (EIMS). It was characterized as quercitrin by comparison of its spectral data with reported literature values[17-19].

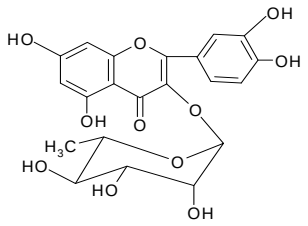

Figure 5. Compound **3**

Compound **4** (Figure 6) has m.p. 260-262 °C, m.f. $C_{16}H_{12}O_5$, $[M]^+$ 284 (EIMS). It was identified as acacetin by comparison of its spectral data with reported literature values[20-21].

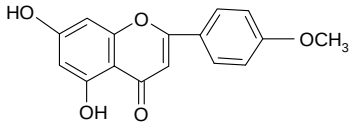

Figure 6. Compound **4**

Compound **5** (Figure 7) has m.p.186-187 °C, m.f. $C_{16}H_{12}O_7$, $[M]^+$ 316 (EIMS). It was identified as isorhamnetin by comparision of its spectral data with reported literature values[22,23].

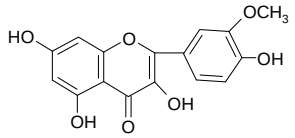

Figure 7. Compound **5**

Compounds **1** and **2** were screened for antibacterial and antifungal activity against various gram (+ve) gram (-ve) bacteria and fungi. The results reported in Table 1 showed that compound **1** was found to be active against gram bacteria *Micrococcus luteus* (+ve) at lower concentration and no activity was found against *E.coli*(-ve), *Staphyloccus aureus* (+ve) and *Bacilius subtilis* (+ve). Compound **2** has also shown less activity against bacteria *E. coli* (–ve), *Bacilius subtilis* (+ve) and *Micrococcus luteus* (+ve). In case of antifungal activity compound **1** showed activity against *Candida albicans* and no activity was found against *Rhizopus oryzae, Aspergillus niger* and *Mucor indicus*. Compound **2** showed less activity against *Candida albicans, Aspergillus niger* and *Mucor indicus* at lower concentration.

Conclusion

The phytochemical analysis of methanolic extract of *Crotalaria medicaginea* Lam. shown the presence of two new allelochemicals. These compounds showed antibacterial and antifungal activity against various gram positive and gram negative bacteria and fungi. The results also suggested the medicinal importance of the plant. The plant could serve as useful sources for new antimicrobial agents.

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