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Note

New biologically active allelochemical from seeds of *Cassia absus* Linn.

R N Yadava* & Umesh Kumar Vishwakarma

Natural Products Laboratory, Department of Chemistry
Dr. H. S. Gour University, Sagar 470 003, India

E-mail: rnyadava@rediffmail.com

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A new bioactive allelochemical **1**, m.p. 235-36°C, m.f. $C_{34}H_{42}O_{20}$, $[M]^+$ 770 (FABMS), has been isolated from methanolic extract of the seeds of *Cassia absus* Linn. alongwith two known compounds 3, 5, 7, 4′-tetrahydroxy-2′, 5′-dimethoxy flavone and Luteolin. The structure of a new compound has been characterized as 5, 7, 4′-trihydroxy-8,3′-dimethoxyflavone-5-O-α-L-rhamnopyranosyl-7-O-β-D-xylopyranosyl-(1→4)-O-β-D-galactopyranoside by various colour reactions, spectral analysis and chemical degradations. All the secondary plant metabolites are called allelochemicals.

Keywords: *Cassia absus* Linn., Leguminosae, allelochemical

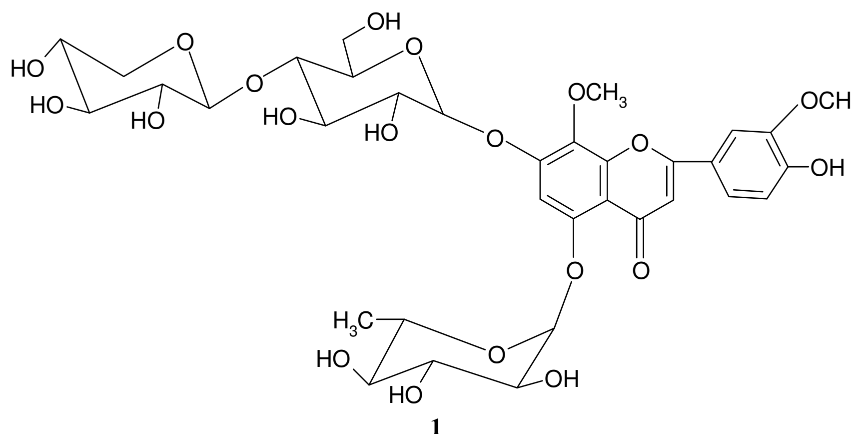
Cassia absus Linn.[1-3] belongs to family Leguminosae, which is commonly known as “Chaksi” or “Chaksu” in Hindi. It is found in Tropical Asia, Australia, Africa and throughout India. Its leaves are hot, bitter and acrid; astringent to the bowels. It is used in the treatment of vata and kapha, tumours, cough, disease of nose, hiccough and asthma. According to Ayurvedic system of medicine its seeds are alexipharmic, astringent to the bowels; heal ulcers and good in diseases of eyes, piles, pains, itching and bronchitis. Its seeds possess diuretic and stimulant properties. Its seeds are used in the treatment of ringworm, opthalmia and skin affections.

Earlier workers[4-8] have reported various chemical constituents from this plant. In the present paper we report the isolation and structural elucidation of a new allelochemical 5, 7, 4′-trihydroxy-8, 3′-dimethoxy flavone-5-O-α-L-rhamnopyranosyl-7-O-β-D-xylopyranosyl-(1→4)-O-β-D-galactopyranoside, **1** along with two known compounds 3, 5, 7, 4′-tetrahydroxy-2′, 5′-dimethoxy flavone **2** and Luteolin **3** from methanolic extract of the seeds of this plant.

Results and Discussion

A new compound **1** has molecular formula $C_{34}H_{42}O_{20}$, m. p. 235-36°C, $[M]^+$ 770 (FABMS). It gave Molisch and Shinoda tests[9] showing its flavanoidal glycosidic nature. Its IR spectrum showed strong absorption bands at 3354, 1650, 1626, 1560, 1512, 1432, 1348, 1298, 1200, 1168, 1028, 992, 860, 829, 792, 756 cm^{-1}. In UV spectrum bands at 268 nm and 340 nm showed its flavonoidal skeleton. A bathochromic shift of 24 nm with $AlCl_3$ and 34 nm with NaOAc in band I revealed the presence of –OH groups at C-5 and C-7 positions in the aglycone **1a** (Ref. 10, 11).

In ^{1}H NMR spectrum, two singlets at δ 3.76 and 3.83 integrating each of three protons intensity suggested the presence of –OMe groups at C-8 and C-3' positions. A singlet at δ 9.92 confirmed the presence of –OH group at C-4' position. Formation of 4-hydroxy-3-methoxy benzoic acid on alkaline degradation also confirmed the presence of –OH



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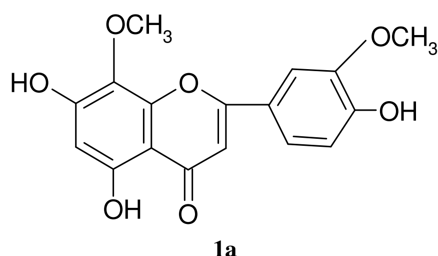
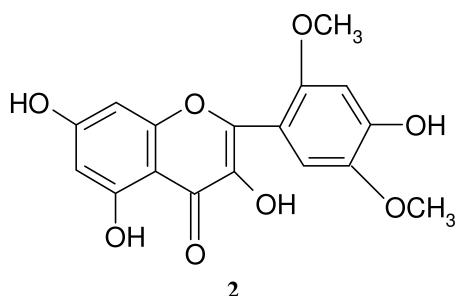


1a



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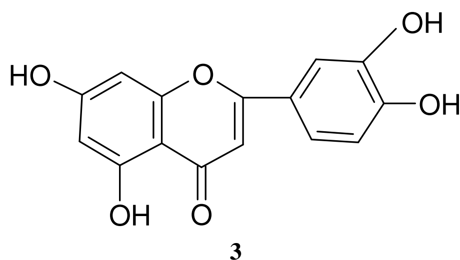

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group at C-4' position and –OMe group at C-3' position in aglycone. In ^{1}H NMR spectrum, a singlet at δ 6.92 of one proton and a meta-coupled doublet of one proton at δ 6.18 (1H, d, J = 2.3 Hz) were assigned to H-3 and H-6 respectively in ring C and A in the aglycone **1a**. Two broad singlets at 7.52 were assigned to H-2' and H-6'. A doublet at δ 6.96 (1H, d, J = 8.1 Hz) was assigned to H-5'. Three anomeric proton signals at δ 5.18 (1H, d, J = 2.1 Hz), δ 5.16 (1H, d, J = 7.4 Hz) and δ 5.02 (1H, d, J = 6.7 Hz) were assigned for H-1'', H-1''' and H-1'''' of L-rhamnose, D-galactose and D-xylose respectively.

In the mass spectrum of the compound **1**, characteristic ion peaks at *m/z* 770 $[M]^+$, 624 [M^+-L-rhamnose], 492 [M^+-D-xylose] and 330 [M^+-D-galactose, aglycone] were found by subsequent losses from the molecular ion of each molecule of L-rhamnose, D-xylose and D-galactose revealing D-xylose as terminal sugar at C-7 position, D-galactose was linked to aglycone at C-7 position and L-rhamnose was attached at C-5 position of aglycone.

Acid hydrolysis of compound **1** with 10% ethanolic H_2SO_4 gave aglycone **1a**, m.p. 259-60°C, m.f. $C_{17}H_{14}O_7$, $[M]^+$ 330 (EIMS) and sugar moiety(ies). These were separated and studied separately. The aglycone **1a** was identified as 5, 7, 4'-trihydroxy-8, 3'-dimethoxy flavones. (See details in experimental section).

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-xylose (R_f 0.29) and D-galactose (R_f 0.15) (Co-PC) (Ref. 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 5,7-dihydroxy-8, 3', 4'-trimethoxy flavone showed that glycosylation was involved at C-5 and C-7 positions of the flavone and methylated sugars were identified as 2, 3, 4 -tri-O-methyl- L-rhamnose (R_G 1.03), 2, 3, 4-tri-O-methyl-D-xylose (R_G 0.92) and 2, 3, 6-tri-O-methyl-D-galactose (R_G 0.70), indicating that C-1'' of L-rhamnose was linked to C-5 position of the aglycone, C-1''' of D-galactose was linked to C-4'''' of D-xylose and C-1'''' of D-galactose was attached with C-7 position of the aglycone. Therefore interlinkage (1→4) between D-xylose and D-galactose was confirmed, which was further confirmed by ^{13}C NMR spectra. (See in experimental section)

Enzymatic hydrolysis of compound **1** with takadiastase liberated L-rhamnose (R_f 0.36) and proaglycone identified as 5, 7, 4'-trihydroxy-8, 3'-dimethoxy flavone-7-O-β-D-xylopyranosyl-(1→4)-O-β-D-galactopyranoside confirming the presence of α-linkage between L-rhamnose and proaglycone. Proaglycone on further hydrolysis with almond emulsin librated D-xylose (R_f 0.29) followed by D-galactose (R_f 0.15) suggesting the presence of β-linkage between D-xylose and D-galactose as well as D-galactose and aglycone.

On the basis of above evidences, the structure of compound **1** was characterized as 5, 7, 4′-tri-hydroxy-8, 3′-dimethoxy flavone-5-O-α-L-rha-mnopyranosyl-7-O-β-D-xylopyranosyl-(1→4)-O-β-D-galactopyranoside.

Table I — Antibacterial activity of the compound 1

Sr.No.	Bacterial species	Diameters of zone of inhibition (mm)* Conc of compd **1** (%)				Std.**
		100	80	60	40	
1.	*Staphylococcus aureus*	15.5	10.2	7.5	4.3	19.5
2.	*Bacillus coagulans*	11.5	8.4	4.5	1.6	20.8
3.	*Pseudomonas aeruginosa*	18.5	14.3	10.4	7.5	22.2

*The zone of inhibition (mm) taken as average of four determination direction.
**Streptomycin (1000 ppm) used as standard antibacterial agent.

Table II - Antifungal activity of the compound 1

Sr.No.	Fungal species	Diameters of zone of inhibition (mm)* Conc of compd **1** (%)				Std.***
		100	80	60	40	
1.	*Penicillium digitatum*	17.5	13.7	10.5	7.5	22.6
2.	*Trichoderma viride*	10.5	6.4	3.2	-	22.5
3.	*Fusarium oxysporum*	14.5	11.2	9.4	5.6	21.4

*The zone of inhibition (mm) taken as average of four determination direction.
***Griseofulvin (1000 ppm) used as standard antifungal agent.

Compound **2** has m.p. 289-90°C, m.f. $C_{17}H_{14}O_8$, $[M]^+$ 346 (EIMS). It was characterized as 3,5,7,4′-tetrahydroxy-2′, 5′-dimethoxy flavone by comparision of its spectral data with reported literature values[15].

Compound **3** has m.p. 326-28°C, m.f. $C_{15}H_{10}O_6$, $[M]^+$ 286 (EIMS). It was identified as Luteolin by comparision of its spectral data with reported literature values[16,17].

Compound **1** was screened for antibacterial and antifungal activity against various gram (+ve) and gram (-ve) bacteria and fungi. The results reported in **Table I** and **Table II** revealed wide variations of antibacterial and antifungal activity. The antibacterial activity of compound **1** was found to be highly active against gram (-ve) bacteria *Pseudomonas aeruginosa* and gram (+ve) bacteria *Staphylococcus aureus*. Compound **1** also showed highest antifungal activity against *Penicillium digitatum* and *Fusarium oxysporum* even on very dilute concentrations. Hence it was concluded that compound **1** may potentially be used as active agent diseases caused by these microorganisms.

Experimental Section

General experimental procedure

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 FTIR spectrometer Shimadzu 8201 PC (4000-400 cm^{-1}). ^1H NMR and ^{13}C NMR spectra were recorded at 300 MHz using solvent $CDCl_3$ 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 D-300 mass spectrometer.

Plant material

The seeds 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 seeds (3kg) of the plant were extracted with pet. ether (40-60°C) in a Soxhlet apparatus for 4 days. The defatted seeds of the plant were further exhaustively partitioned with chloroform, ethyl acetate, acetone and methanol. The methanol soluble fraction was concentrated under reduced pressure to yield brown viscous mass (1.90 g), which was subjected to TLC examination using nBAW (4:1:5) as solvent and I_2 vapours as visualizing agent, gave three spots indicating it to be mixture of three compounds **1**, **2** and **3**. These

compounds were separated by TLC and purified by column chromatography over silica gel using $CHCl_3$: MeOH (4:8) as eluent and studied separately.

Compound 1

It was crystalysed from acetone to yield 1.30 g. It has m.p. 235-36°C, m.f. $C_{34}H_{42}O_{20}$, $[M]^+$ 770 (FABMS); Found: C, 52.23; H, 5.28; Calcd. for m.f. $C_{33}H_{40}O_{19}$: C, 52.99; H, 5.45%; UV (λ_{max} MeOH): 268, 340, 348; (+$AlCl_3$) 266, 300, 368, 386; (+$AlCl_3$-HCl) 270, 300, 354, 390; (+NaOAc) 276, 352, 396 nm; IR (KBr): 3354, 1650, 1626, 1560, 1512, 1432, 1348, 1298, 1200, 1168, 1028, 992, 860, 829, 792, 756 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) : δ 6.90 (1H, s, H-3), 6.16 (1H, d, *J* = 2.2 Hz, H-6), 3.76 (3H, s, 8-OCH_3), 7.50 (1H, br, s, H-2′, H-6′), 6.94 (1H, d, *J* = 8.1 Hz, H-5′), 9.92 (1H, s, 4′-OH), 3.83 (3H, s, 3′-OCH_3), 5.18 (1H, d, *J* = 2.1 Hz, H-1″), 3.1-4.3 (4H, m, H-2″, H-3″, H-4″, H-5″), 1.21 (3H, d, *J* = 6.1 Hz, 6″- CH_3), 5.16 (1H, d, *J* = 7.4 Hz, H-1‴), 3.42-3.72 (4H, m, H-2‴, H-3‴, H-4‴, H-5‴), 3.86 (1H, dd, *J* = 11.2, 7.2 Hz, H-6_a‴), 4.16 (1H, dd, *J* = 11.2, 4.3 Hz, H-6_b‴), 5.02 (1H, d, *J* = 6.7 Hz, H-1⁗), 3.24-3.68 (4H, m, H-2⁗, H-3⁗, H-4⁗, H-5⁗); ^{13}C NMR (300MHz, $CDCl_3$) : δ 163.52 (C-2), 103.64 (C-3), 181.68 (C-4), 157.23 (C-5), 98.64 (C-6), 164.12 (C-7), 127.10 (C-8), 161.36 (C-9), 103.15 (C-10), 120.24 (C-1′), 111.08 (C-2′), 150.68 (C-3′), 147.96 (C-4′), 115.62 (C-5′), 121.42 (C-6′), 101.2 (C-1″), 70.4 (C-2″), 70.3 (C-3″), 71.6 (C-4″), 69.8 (C-5″), 17.6 (C-6″), 102.8 (C-1‴), 71.6 (C-2‴), 73.7 (C-3‴), 68.8 (C-4‴), 75.8 (C-5‴), 60.1 (C-6‴), 106.4 (C-1⁗), 73.5 (C-2⁗), 76.9 (C-3⁗), 67.3 (C-4⁗), 66.4 (C-5⁗) and $[M]^+$ 770 (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 hr. 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 (4:10) to give compound **1a**, identified as 5, 7, 4′-trihydroxy-8, 3′-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), D-xylose (R_f 0.29) and D-galactose (R_f 0.15) (Co-PC).

Compound 1a

It has m.f. $C_{17}H_{14}O_7$, m.p. 259-60°C, $[M]^+$ 330 (EIMS); Found: C, 61.20; H, 3.90; Calcd. for m.f. $C_{17}H_{14}O_7$: C, 61.82; H, 4.24%; UV (λ_{max}) (MeOH): 271, 342, 350; (+$AlCl_3$) 270, 302, 370, 390; (+$AlCl_3$/HCl) 270, 302, 360, 392; (+NaOAc) 278, 354, 400 nm; IR (KBr): 3356, 1652, 1628, 1563, 1516, 1436, 1352, 1302, 1206, 1174, 1030, 998, 865, 832, 796, 760 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ 6.92 (1H, s, H-3) 6.18 (1H, d, *J* = 2.3 Hz, H-6), 3.80 (3H, s, 8-OCH_3), 7.52 (1H, br, s, H-2′, H-6′), 6.96 (1H, d, *J* = 8.2 Hz, H-5′), 9.95 (1H, s, 4′-OH), 12.93 (1H, s, 5-OH), 10.80 (1H, s, 7-OH), 3.86 (3H, s, 3′-OCH_3); ^{13}C NMR (300MHz, $CDCl_3$): δ 163.54 (C-2), 103.66 (C-3), 181.71 (C-4), 157.25 (C-5), 98.67 (C-6), 164.16 (C-7), 127.60 (C-8), 161.42 (C-9), 103.20 (C-10), 120.28 (C-1′), 110.13 (C-2′), 150.72 (C-3′), 148.0 (C-4′), 115.65 (C-5′), 121.48 (C-6′).

Permethylation of compound 1

Compound **1** (30 mg) was refluxed with MeI (5mL) and Ag_2O (15mL) in DMF (25 mg) for two days and then filtered. The filtrate was hydrolyzed with 10% ethanolic H_2SO_4 for 5-6 hr, to give methylated aglycone, identified as 5,7-dihydroxy-8, 3', 4'-trimethoxy flavone and methylated sugars were identified as 2, 3, 6-tri-O-methyl-D-galactose (R_G 0.70), 2, 3, 4-tri-O-methyl-D-xylose (R_G 0.92) and 2, 3, 4-tri-O-methyl-L-rhamnose (R_G 1.03).

Enzymatic hydrolysis of compound 1

Compound **1** (20 mg) was dissolved in MeOH (25 mL) and hydrolysed with equal volume of takadiastase enzyme. The reaction mixture was allowed to stay at RT for two 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) solvent system, which showed the presence of L-rhamnose (R_f 0.36) (Co-PC). The proaglycone was dissolved in MeOH (35 mL) and further hydrolysed with equal volume of almond emulsin enzyme at room temperature as usual procedure yielded aglycone, identified as 5, 7, 4'-trihydroxy-8, 3′-dimethoxy flavone and sugars were identified as D-galactose (R_f 0.15) and D-xylose (R_f 0.29) (Co-PC).

Antimicrobial activity of the compound 1

The stock solution of the compound **1** was prepared of 1000 ppm in methanol at different concentrations. For antibacterial activity, Filter Paper Disc

Diffusion[18] method was employed. The sterile filter paper disc (6 mm) soaked with the standard antibacterial agent with various test samples of compound **1** was placed on seeded agar plates and were kept in incubator at 36±1°C for 34 hrs. The diameters of zone of inhibition were recorded and reported in **Table I**.

For the antifungal activity of compound **1**, Saboraud's[19] broth media with 4% agar was used for the preparation of plates and inoculated with the spore and mycilium suspension of fungi found from 7 days old culture. The plates after inoculation were incubated at RT for 48 hr and the diameters of zone of inhibitions were reported in **Table II**.

Compound 2

It has m.f. $C_{17}H_{14}O_8$, m.p. 289-90°C, $[M]^+$ 346 (EIMS); Found: C, 58.80; H, 3.98%. Calcd. for m.f. $C_{17}H_{14}O_8$: C, 58.96; H, 4.05%. UV (λ_{max}) (MeOH): 266, 315; (+$AlCl_3$) 274, 330; (+$AlCl_3$ / HCl) 272, 326; (+NaOAc) 272, 396 nm; ^{1}H NMR (300 MHz, $CDCl_3$): δ 6.60 (1H, s, H-6), 6.64 (1H, s, H-8), 7.10 (1H, s, H-3′), 7.46 (1H, s, H-6′), 3.83 (6H, d, 2′-OCH_3, 5′-OCH_3).

Compound 3

It has m.f. $C_{15}H_{10}O_6$, m.p. 326-28°C, $[M]^+$ 286 (EIMS); Found: C, 62.30; H, 3.26%. Calcd. for m.f. $C_{15}H_{10}O_6$: C, 62.94; H, 3.50%. UV (λ_{max}) (MeOH): 206, 258, 266, 348 nm; IR (KBr): 3425, 2926, 1652, 1618, 1506, 1362, 1260, 1168, 1037, 834, 568 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ 6.62 (1H, s, H-3), 6.20 (1H, d, J = 2.1 Hz, H-6), 6.42 (1H, d, J = 2.2 Hz, H-8), 12.92 (1H, s, 5-OH), 10.64 (1H, br, s, 7-OH), 7.36 (1H, d, J = 2.0 Hz, H-2′), 6.84 (1H, d, J = 8.2 Hz, H-5′), 7.40 (1H, dd, J = 8.1, 2.0 Hz, H-6′), 9.90 (1H, s, 3′-OH), 9.64 (1H, br, s, 4′-OH); ^{13}C NMR (300 MHz, $CDCl_3$): δ 163.4 (C-2), 102.6 (C-3), 181.2 (C-4), 161.8 (C-5), 98.2 (C-6), 164.5 (C-7), 93.3 (C-8), 157.6 (C-9), 103.2 (C-10), 121.9 (C-1′), 113.7 (C-2′), 145.3 (C-3′), 149.2 (C-4′), 116.6 (C-5′), 118.3 (C-6′).

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