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NEW AND KNOWN CONSTITUENTS FROM *IRIS UNGUICULARIS* AND THEIR ANTIOXIDANT ACTIVITY

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Abstract - A new compound, 1,3-*O*-diferuloylsucrose (**1**), and a synthetically known compound, 5,7-dihydroxy-6-methoxychromone (**2**), along with several known natural products, irilone (**3**), 4',5,7-trihydroxy-6-methoxyflavanone (**4**), tectorigenin (**5**), kaempferol (**6**), 4',5,7-trihydroxy-3',8-dimethoxyflavanone (**7**), 8-methoxyeriodictyol (**8**), hispidulin (**9**), and mangiferin (**10**) were isolated from the rhizomes of *Iris unguicularis*. Compounds **1**, **6**, **8** and **10** showed a considerable DPPH radicals scavenging activity. Their structures have been deduced through different spectroscopic techniques. The structure of compound **2** was also confirmed by single-crystal X-ray diffraction techniques as well.

Dedicated to Professor Dr. Albert Eschenmoser, ETH Zürich, on his 85th birthday.

The family Iridaceae comprises some 92 genera and has more than 1,800 species,¹ mostly perennial herbs with rhizomes (bulbs). The genus *Iris* includes about 300 species, some of the species being ornamental.

In Pakistan, approximately 16 species are found^{1,2} average about 40 species are found in Turkey.³ Except for the coldest regions, they are found all around the world and especially in South Africa and in tropical America. The roots of *Iris* are used in flavoring dentifrices and perfumes. Phytochemical investigations on various species of *Iris* have resulted in the isolation of more than 250 compounds, which include flavonoids, flavanoids, isoflavonoids and their glycosides, benzoquinones and triterpenoids.⁴⁻⁸ The leaves of *Iris* contain ascorbic acid. The rhizomes contain terpenes, organic acids (undecilene acid, miristic acid, trideclic acid), and glycosides such as iridin. *Iris* species have been reported to contain piscicidal, antineoplastic, antiplasmodial, antioxidant and antituberculosis properties.⁹⁻¹² The flavonoid constituents of red wine and teas have been extensively studied for their antioxidant properties. It was noted that the substitution pattern of flavonoids affects their antioxidant properties.¹³

Iris unguicularis Poir. is closely related to *I. cretensis* and *I. lazica*. It is commonly called the “winter *Iris*” or the “Algerian *Iris*”. It is native to the Eastern Mediterranean regions, with scattered populations in Algeria up through Turkey and the Greek Peloponnese. During the current study, we have isolated a new compound, 1,3-*O*-diferuloylsucrose (**1**), and a synthetically known compound, 5,7-dihydroxy-6-methoxychromone (**2**) along with eight other known compounds **3-10**. The structures of all the compounds were obtained from the spectroscopic analysis, especially 1D and 2D NMR spectral data and comparing them with the reported data. DPPH Radical scavenging activity of new and known compounds was also evaluated. Compound **2** was also subjected to single-crystal X-ray diffraction analysis.

A subfraction of the EtOAc extract was subjected to preparative recycling HPLC using water-methanol (1 : 1) as solvent system to afford compound **1** (10.2 mg) as a yellowish gummy material. Strong IR absorptions at 3401 (OH), 1696 (conjugated ester C=O) and 1598 (C=C) cm^{-1} , and UV bands at 325, 235, 218 and 202 nm indicated conjugated aromatic system. The HRFAB-MS (-ve) of compound **1** exhibited an $[\text{M-H}]^-$ at m/z 693.2019 ($\text{C}_{32}\text{H}_{37}\text{O}_{17}$, calcd. 693.2030).

The $^1\text{H-NMR}$ spectrum suggested that compound **1** contained two feruloyl moieties, represented by two sets of signals for the *trans* olefinic protons [δ_{H} 7.62 (1H, d, $J_{7'',8''} = 15.9$ Hz), 6.46 (1H, d, $J_{8'',7''} = 15.9$ Hz); 7.72 (1H, d, $J_{7''',8'''} = 15.9$ Hz), 6.38 (1H, d, $J_{8''',7'''} = 15.9$ Hz)], two sets of 1,3,4-trisubstituted aromatic ring protons [δ_{H} 7.14 (1H, brs), 6.73 (1H, d, $J_{5'',6''} = 8.5$ Hz), 7.05 (1H, dd, $J_{6'',5''} = 8.1$ Hz, $J_{6'',2''} = 1.2$ Hz); 7.17 (1H, brs), 6.79 (1H, d, $J_{5''',6'''} = 8.5$ Hz), 7.08 (1H, dd, $J_{6''',5'''} = 8.1$ Hz, $J_{6''',2'''} = 1.2$ Hz)], and protons of the two methoxy groups [δ_{H} 3.86 (3H, s); 3.82 (3H, s)]. A doublet at δ_{H} 5.49 ($J_{1',2'} = 3.7$ Hz) indicated the presence of an anomeric proton of an α -linked sugar (Table 1). The broad-band decoupled $^{13}\text{C-NMR}$ spectrum of compound **1** showed resonances for thirty two carbon atoms, including two methyl, three methylene, eighteen methine and nine quaternary carbons (Table 1). Signals at δ_{C} 93.7, 72.9, 74.9,

71.9, 74.5, 62.4; 66.3, 103.3, 79.5, 73.1, 83.9 and 62.9 suggested the presence of a disaccharide moiety. A characteristic doublet with a smaller coupling constant at δ_{H} 5.49 (1H, d, $J_{1',2'} = 3.7$ Hz) was attributed to the anomeric proton of the α -glucopyranose unit.^{14,15} This also supported the presence of a sucrose unit in **1**. Two downfield signals at δ_{C} 56.5 and 56.4 were due to the methoxy carbons of two feruloyl groups. The linkage of the two feruloyl groups, located at C-1 and C-3 of the fructose unit of sucrose, was deduced from the HMBC interactions of the C-3 methine proton (δ_{H} 5.58) with the C-9'' carbonyl carbon (δ_{C} 168.4). The C-1 methylene protons (δ_{H} 4.26, 4.38) showed interactions with the C-9''' carbonyl carbon (δ_{C} 168.5) (Figure 1). On the basis of this spectroscopic data, the structure of compound **1** was deduced as 1,3-*O*-diferuloylsucrose. Interestingly 5'''-methoxy derivative of compound **1** was reported earlier.¹⁶

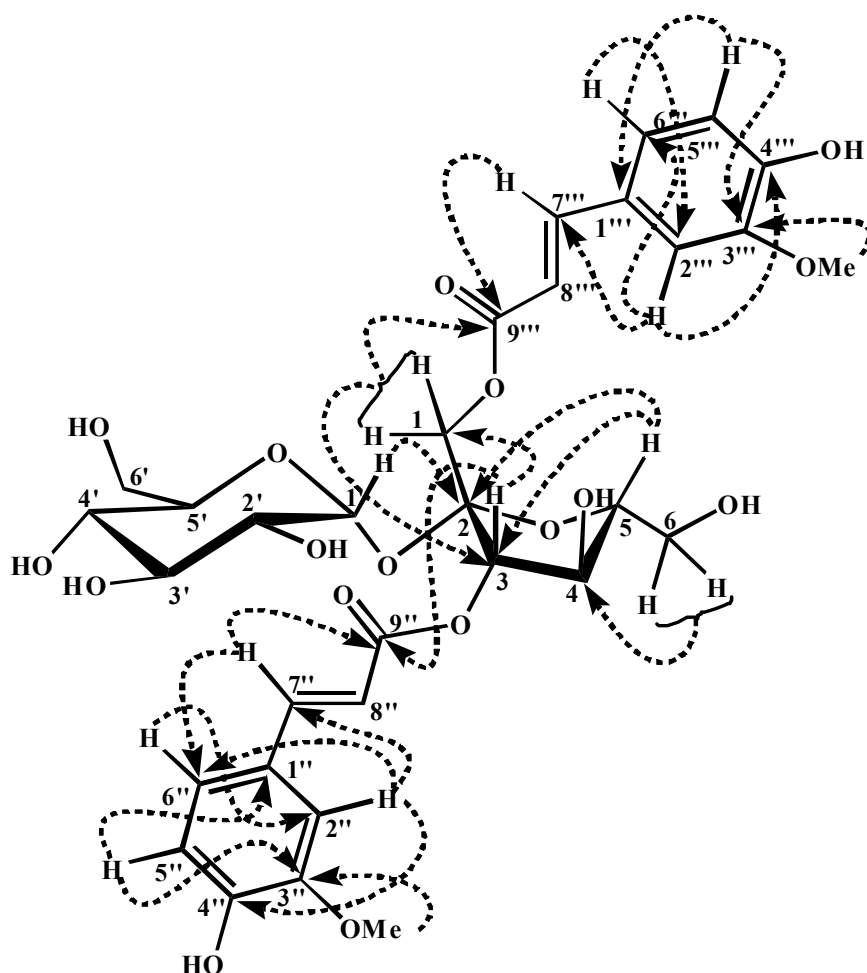


Figure 1. Key HMBC interactions in Compound **1**.

Table 1. ^1H - and ^{13}C -NMR chemical shift data for compound **1** in CD_3OD .

Carbon no.	^{13}C -NMR	^1H -NMR
	δ_{C}	δ_{H} (J in Hz)
1a	66.3	4.38 (d, $J_{1a,1b} = 11.7$)
1b	-	4.26 (d, $J_{1b,1a} = 11.7$)
2	103.3	-
3	79.5	5.58 (d, $J = 8.5$)
4	73.1	4.43 (t, $J = 8.5$)
5	83.9	3.92 (overlap)
6a	62.9	3.83 (overlap)
6b	-	3.79 (overlap)
1'	93.7	5.49 (d, $J_{1',2'} = 3.7$)
2'	72.9	3.43 (dd, $J_{2',3'} = 9.6$, $J_{2',1'} = 3.7$)
3'	74.9	3.64 (t, $J_{3',4'/3',2'} = 9.6$)
4'	71.9	3.41 (overlap)
5'	74.5	3.94 (m)
6'a	62.4	3.88 (overlap)
6'b	-	3.83 (overlap)
1''	127.6	-
2''	111.6	7.14 (brs)
3''	149.4	-
4''	150.7	-
5''	116.4	6.73 (d, $J_{5'',6''} = 8.5$)
6''	124.3	7.05 (dd, $J_{6'',5''} = 8.1$, $J_{6'',2''} = 1.2$)
7''	147.4	7.62 (d, $J_{7'',8''} = 15.9$)
8''	114.8	6.46 (d, $J_{8'',7''} = 15.9$)
9''	168.4	-
1'''	127.6	-
2'''	112.1	7.17 (brs)
3'''	149.4	-
4'''	150.8	-
5'''	116.5	6.79 (d, $J_{5''',6'''} = 8.5$)
6'''	124.4	7.08 (dd, $J_{6''',5'''} = 8.1$, $J_{6''',2'''} = 1.2$)
7'''	148.0	7.72 (d, $J_{7''',8'''} = 15.9$)
8'''	115.0	6.38 (d, $J_{8''',7'''} = 15.9$)
9'''	168.5	-
3''-OCH ₃	56.5	3.86 (s)
3'''-OCH ₃	56.4	3.82 (s)

The fractions eluted by pet. ether - dichloromethane (0.5 : 99.5) from the ethanol soluble part of *I. unguicularis* yielded **2** (12.5 mg) as colorless crystals. Strong IR absorptions at 3430 (OH), 2915 (CH), 1650 (C=O), and 1585 (C=C) cm^{-1} , along with UV bands at 297, 258 and 210 nm, indicated the presence of a benzopyrane skeleton.^{17,18}

The molecular formula of compound **2** was inferred from the HREI-MS which exhibited an M^+ as the base peak at m/z 208.0380 ($C_{10}H_8O_5$, calcd. 208.0371) with nine degrees of unsaturation. A peak at m/z 193 indicated the loss of a methyl group from C-6 position of M^+ ,¹⁹ while another peak at m/z 165 was due to the loss of a carbonyl group from the M^+-CH_3 .²⁰

The 1H -NMR spectrum of compound **2** showed signals for two olefinic and one aromatic protons (Table 2). A singlet at δ_H 6.25 corresponded to the aromatic H-8. Two olefinic protons, resonating at δ_H 7.63 and 5.99 ($J_{2,3/3,2} = 5.9$ Hz), were assigned to the *ortho* coupled H-2 and H-3. A 3H singlet at δ_H 3.70 was due to the *O*-methyl protons, substituted at the C-6 of the aromatic ring.¹⁸ The broad-band (BB) decoupled ^{13}C -NMR spectrum of compound **2** showed resonances for ten carbons, including one methyl, three methine and six quaternary carbons (Table 2). Two olefinic carbons, resonating at δ_C 155.8 and 110.1, were assigned to C-2 and C-3, respectively.²¹ The methoxy carbon signal resonated at δ_C 60.3. The most downfield signal in the ^{13}C -NMR spectrum at δ_C 182.2 was assigned to the C-4 ketonic carbonyl carbon which indicated the presence of a hydroxyl group at the C-5 position.²² Other carbon signals appeared at δ_C 152.4 (C-5), 131.2 (C-6), 156.8 (C-7), 94.2 (C-8), 153.5 (C-9), and 106.1 (C-10).

Table 2. 1H - and ^{13}C -NMR chemical shift data for compound **2** in CD_3OD .

Carbon no.	^{13}C -NMR	1H -NMR
	δ_C	δ_H (J in Hz)
2	155.8	7.63 (d, $J_{2,3} = 5.9$)
3	110.1	5.99 (d, $J_{3,2} = 5.9$)
4	182.2	-
5	152.4	-
6	131.2	-
7	156.8	-
8	94.2	6.25 (s)
9	153.5	-
10	106.1	-
OCH ₃	60.3	3.70 (s)

The NMR assignments were made on the basis of HMQC, HMBC and COSY-45° techniques. In the COSY-45° spectrum, the olefinic protons [H-2 and H-3] showed couplings with each other. Direct one-bond $^1H/^{13}C$ interactions were deduced by the HMQC spectrum. The olefinic and aromatic protons

i.e., H-2 (δ_{H} 7.63), H-3 (δ_{H} 5.99) and H-8 (δ_{H} 6.25), showed connectivities with C-2 (δ_{C} 155.8), C-3 (δ_{C} 110.1), and C-8 (δ_{C} 94.2), respectively. The HMBC spectrum showed long-range interactions of H-2 (δ_{H} 7.63) with C-3 (δ_{C} 110.1), C-4 (δ_{C} 182.2) and C-9 (δ_{C} 153.5). The other HMBC interactions were of H-3 (δ_{H} 5.99) with C-2 (δ_{C} 155.8), C-4 (δ_{C} 182.2) and C-10 (δ_{C} 106.1), and of H-8 (δ_{H} 6.25) with C-6 (δ_{C} 131.2), C-7 (δ_{C} 156.8), C-9 (δ_{C} 153.5), and C-10 (δ_{C} 106.1). The *O*-methyl protons (δ_{H} 3.70), were coupled with the aromatic C-6 (δ_{C} 131.2) (Figure 2). Finally, the structure was unambiguously established by X-ray diffraction analysis (Figure 3). The data indicated that compound **2** is a new natural product with the structure corresponding to 5,7-dihydroxy-6-methoxychromone. This compound was previously reported as a synthetic product.²³

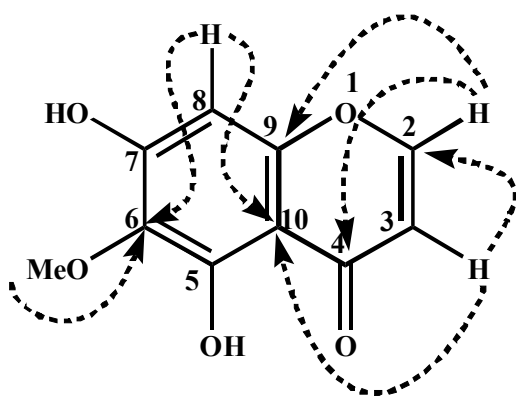


Figure 2. Key HMBC interactions in compound **2**.

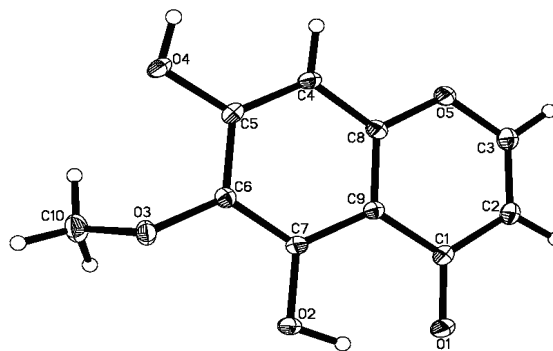


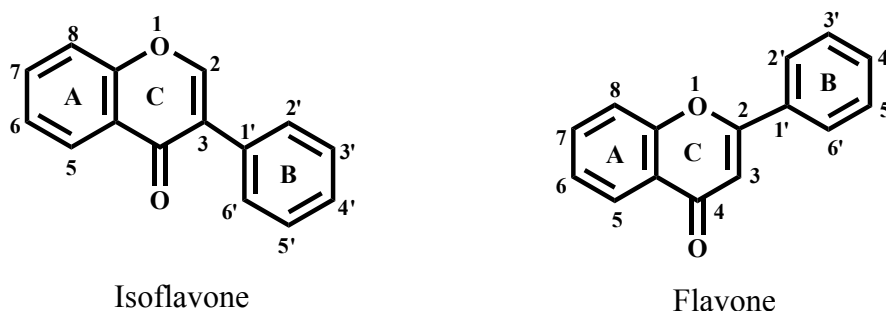
Figure 3. Computer-generated ORTEP diagram of the final X-ray model of **2**.

Compound **1** (1,3-*O*-diferuloylsucrose) is a new compound while the compound **2** (5,7-dihydroxy-6-methoxychromone) has not been reported so far from any natural source.²³ In addition to this, several known compounds have also been isolated, and identified as, irilone (**3**), 4',5,7-trihydroxy-6-methoxyflavanone (**4**), tectorigenin (**5**), kaempferol (**6**), 4',5,7-trihydroxy-3',8-dimethoxyflavanone (**7**), 8-methoxyeriodictyol (**8**), hispidulin (**9**), and mangiferin (**10**).²⁴⁻³⁰ This is the first report of the isolation of compounds **3-10** from *I. unguicularis*.

The compounds of *I. unguicularis* exhibited a dose-dependent anti-radical activity by reducing the stable DPPH radicals to the yellow colored diphenylpicrylhydrazine derivative. Mangiferin (**10**), kaempferol (**6**), 8-methoxyeriodictyol (**8**) and 1,3-*O*-diferuloylsucrose (**1**) were found to be active in this assay. Compound **10** showed potent anti-radical activity with IC_{50} value $22.45 \pm 0.35 \mu\text{M}$, which is lesser than both the standards used during the studies and exhibited greater antiradical potential than standards (BHT, IC_{50} $44.2 \pm 0.20 \mu\text{M}$ and nPG IC_{50} $30.0 \pm 0.27 \mu\text{M}$). Compound **6** also exhibited higher antiradical

potential with IC_{50} value $40.26 \pm 1.04 \mu\text{M}$, which is still lesser than one of the standard used (BHT. IC_{50} $44.2 \pm 0.20 \mu\text{M}$). Compounds **1** and **8** also exhibited a good anti-radical activity in this assay.

Structure-Activity Relationship:



The flavonoids have basic skeleton of diphenylpropanes with different oxidation of the central pyran ring. The analyzed compounds belonged to different sub classes of flavonoids, such as flavones, flavanones and isoflavones.

The difference in antiradical activity of these compounds seems to be due to the structural differences in hydroxylation and methoxylation on the basic skeleton. The presence of *ortho*-dihydroxyl groups was potentially found to be responsible of the higher antiradical activity of these molecules. The tested compounds showed a variety of radical scavenging activities, some of the compounds showed a very strong activity, while others were completely inactive. On the basis of preliminary analysis, it can be concluded that the radical scavenging activities of flavonoids is based on the number of phenolic groups present in the molecules.

Presence of the 3-OH in the C-ring, and the 2,3-double bond, in conjugation with 4-oxo function (carbonyl group) in the C-ring, as in compound **6**, seems to be responsible of the higher activity of this compound. Absence of this 3-OH in the C-ring, as in compounds **9** and **4**, resulted in a loss of activity.

Presence of *ortho*-dihydroxyl groups (catechol structure) in the B-ring, as in compound **8**, is potentially responsible of its higher activity, replacement of the -OH with -OCH₃ at C-3' position in ring B, results in a loss of activity many folds, as in compound **7**. Table 3 presents the antioxidant properties of these compounds in DPPH radical scavenging assay.

Table 3. Antioxidant (Radical Scavenging) activity of compounds **1-10**.

Compounds	IC ₅₀ (μM ± SEM)
1,3- <i>O</i> -Diferuloylsucrose (1)	110.21 ± 2.57
5,7-Dihydroxy-6-methoxychromone (2)	-
Irilone (3)	-
4',5,7-Trihydroxy-6-methoxyflavanone (4)	>500 ± 0.42
Tectorigenin (5)	>500 ± 1.72
Kaempferol (6)	40.26 ± 1.04
4',5,7-Trihydroxy-3',8-dimethoxyflavanone (7)	<500 ± 1.23
8-Methoxyeriodictyol (8)	56.84 ± 0.02
Hispidulin (9)	>500
Mangiferin (10)	22.45 ± 0.35
Standard BHT	44.2 ± 0.20
Standard nPG	30.0 ± 0.27

“-” Inactive

BHT = Butylated Hydroxytoluene (Standard)

nPG = n-Propyl Gallate (Standard)

SEM = Standard Error of Mean of Three Experiments.

RSA = Radical Scavenging Activity

EXPERIMENTAL

General Experimental Procedures. Ultraviolet (UV) spectra were recorded in methanol on Hitachi UV-3200 spectrophotometer and UV absorption (λ_{\max}) values are given in nm. Infrared (IR) spectra were measured in CHCl₃ or as KBr discs on Shimadzu FTIR-8900/Bruker Vector 22 spectrophotometer and presented in cm⁻¹. The ¹H-NMR spectra were measured at 300 MHz and 600 MHz while the ¹³C-NMR spectra were measured at 75 MHz and 150 MHz in deuterated solvents (CDCl₃ or in CD₃OD) on Bruker AVANCE spectrometers. Chemical shifts are measured in ppm (δ) relative to SiMe₄ as internal standard, and the coupling constants (*J*) are given in Hz. CDCl₃ (δ 7.25) and CD₃OD (δ 3.33 and 4.80) were used as solvents and as internal standards.

Mass spectra (EI-MS) was measured in an electron impact mode on Varian MAT 312 double focusing spectrometer or on a JMS-600 H (Jeol, Japan) spectrometers and ions are given in *m/z* (%). High-resolution electron impact mass measurements (HREI-MS) were performed on Jeol HX 110 and MAT 95 XP mass spectrometers Thermo Finnigan. Chromatography was carried out on silica gel (E. Merck, type 60, 70-230 mesh), Sephadex LH-20 (Pharmacia, Uppsala, Sweden), precoated silica gel GF-254 preparative TLC plates (20 x 20, 0.5 mm thick, E. Merck) and by recycling preparative HPLC (Japan Analytical Industry Co., Ltd.). H-80 C₁₈ (ODS) column was used for HPLC separation. Purity of

the samples was checked on precoated TLC plates (silica gel) and by HPLC on Shimadzu LC-6A instrument by using RP-18 column. Melting points were determined on a Gallenkemp apparatus and are uncorrected. X-Ray data was collected on a Bruker Smart Apex I, CCD 4-K area detector diffractometer.

Plant Material. The rhizomes of *Iris unguicularis* Poir. were collected from Antakya, Turkey, in June 2004 and air dried (1 kg). A voucher specimen (GUE 8964) was deposited in the Herbarium of Faculty of Pharmacy, Gazi University, Ankara, Turkey. The plant was identified by Prof. Dr. Filiz Ayanoglu.

Extraction and Isolation. The dried rhizomes (1 kg) were chopped, and soaked in EtOH (10 L) for two weeks at room temperature, and the resulting extract was concentrated to a gum (250.3 gm). The ethanolic extract (250.3 gm) was dissolved in distilled water (3 L) and defatted with pet. ether (5 x 3 L), to obtain 10.4 gm of pet. ether extract. The defatted aqueous extract was further fractionated with CH₂Cl₂ (5 x 3 L), EtOAc (5 x 3 L) and BuOH (5 x 3 L). On evaporation of the organic solvents, 42.3, 45.5 and 12.4 gm of extracts were obtained, respectively. Five compounds **2**, and **3-6** were isolated from the CH₂Cl₂ extract, while five compounds **1**, and **7-10** were obtained from EtOAc extract by using repeated column chromatography (silica gel) and preparative recycling HPLC (ODS column).

The CH₂Cl₂ extract was chromatographed on a column (silica gel) by using pet. ether - dichloromethane - methanol in increasing order of polarity to obtain four major fractions (Fr-1 to Fr-4). These fractions were further subjected to repeated column chromatography on silica gel by using various mixtures of pet. ether - dichloromethane - methanol to afford compounds **2** (12.5 mg), **3** (6.2 mg), **4** (10.1 mg), **5** (9.8 mg), and **6** (12.4 mg).

The EtOAc extract was subjected to column chromatography (silica gel) and eluted with increasing polarities of pet. ether - dichloromethane - methanol mixtures to obtain five major fractions (Fr-1 to Fr-5). These fractions were subjected to further column chromatography and eluted with mixtures of pet. ether - CH₂Cl₂ - MeOH to obtain **1** (10.2 mg), **7** (15.2 mg), **8** (12.4 mg), and **9** (20.1 mg). Compounds **1** and **10** were purified from fraction Fr-5. The fraction Fr-5 were subjected to recycling HPLC by using water-MeOH (1 : 1) as solvent system on H-80 ODS column with 3 mL/min flow rate (*Rt* 28 and 26 min) to obtain compounds **1** (10.2 mg) and **10** (54.2 mg).

The known compounds **3-10** were characterized through comparison of their physical and spectral data with those reported in the literature.²⁴⁻³⁰ These known compounds were isolated for the first time from this plant species.

1,3-O-Diferuloylsucrose (1): IR (KBr) ν_{\max} cm⁻¹: 3401 (OH), 1696 (conjugated ester C=O), 1598 (C=C). UV λ_{\max} (MeOH) (log ϵ) nm: 325, 235, 218, 202, 197. UV λ_{\min} (MeOH) (log ϵ) nm: 263, 228, 210. FAB-MS (-ve) *m/z*: 693 (20) [M-H]⁻, 517 (18), 337 (30). HRFAB-MS (-ve) *m/z*: 693.2019 [M-H]⁻ (calcd.

for C₃₂H₃₇O₁₇: 693.2030), EI-MS (rel. int., %) *m/z*: 370 (10), 338 (21), 194 (79), 177 (100), 150 (54), 135 (36), 77 (40), ¹H-NMR and ¹³C-NMR: Table 1. HMBC: See Figure 1.

5,7-Dihydroxy-6-methoxychromone (2): Mp 77-79 °C. IR (KBr) ν_{\max} cm⁻¹: 3430 (OH), 2915 (CH), 1650 (C=O) and 1585 (C=C). UV λ_{\max} (MeOH) (log ϵ) nm: 297, 258 and 210. UV λ_{\min} (MeOH) (log ϵ) nm: 276, 243. EI-MS *m/z*: 208 (M⁺, 100), 193 (M⁺-CH₃, 40), 190 (34), 178 (8), 165 (M⁺-C=O, 25). HREI-MS *m/z*: 208.0380 (calcd. for C₁₀H₈O₅: 208.0371), ¹H-NMR and ¹³C-NMR: Table 2. HMBC: See Figure 2.

Single-crystal X-ray diffraction data of 5,7-dihydroxy-6-methoxychromone (2): A block-shaped colorless crystal of compound **2** with dimension 0.31 x 0.17 x 0.15 mm was selected for X-ray diffraction studies. C₁₀H₈O₅: Mr 208.16; monoclinic; *a* = 7.5164 (3), *b* = 16.3891 (7), *c* = 7.1054 (3) Å; $\alpha = \gamma = 90^\circ$, $\beta = 91.0910 (10)^\circ$; *V* = 875.13 (6) Å³, space group = P21/c, *Z* = 4, *D*_{calc.} = 1.580 g/cm³, *F*(000) = 432.0, Mo-K α (λ 0.71073 Å). Intensity data of compound **2** was collected on a Bruker Smart Apex I, CCD 4-K area detector diffractometer, attached with a KRYO-FLEX low temperature device. Data reductions were performed by using SAINT program.³¹ The structure was solved by direct methods³² and refined by full-matrix least squares on *F*² using the SHELXTL-PC package. The intensity data within the θ range 2.49-28.30 were collected at 173 (2) K. A total of 11,780 reflections were recorded, of which 2,168 reflections were judged on the basis of *I* > 2 *s* (1). The final *R* and *R*_w values were 0.0383 and 0.1146, respectively. Figure 3 was plotted with the aid of ORTEP.³³ Anisotropic thermal displacement parameters were allowed for hydrogen and non-hydrogen atoms. The *U*_{iso} values were constrained to be 1.5 *U*_{eq} of the carrier atom for methyl H atoms, and 1.2 *U*_{eq} for the remaining H atoms. In the absence of significant anomalous dispersion effects, Friedel pairs were merged before the final refinement.

Crystallographic data for compound **2** has been deposited at the Cambridge Crystallographic Data Center (CCDC-655285) 12 Union Road, Cambridge, CB2 1EZ, UK via Internet at http://www.ccdc.cam.ac.uk/data_request/cif.

DPPH Free Radical Scavenging Assay. For quantitative determination of electron donating ability of any compound, stable radicals have the advantage and their concentrations can be readily and directly measurable.³⁴ Free radical scavenging potential of these compounds were determined by measuring the changes in absorbance of DPPH (1, 1-Diphenyl-2-picrylhydrazyl radical) spectrophotometrically, as described by S. K. Lee.³⁵

1,1-Diphenyl-2-picrylhydrazyl radical (DPPH) is a stable free radical in EtOH. In solution form, it has a deep violet color which absorbs strongly at 515 nm. As the free radical is converted into a neutral

molecule after accepting an electron, the absorption vanishes into a decolorized hydrazine form. The antioxidant activity obtained from DPPH method is comparable to other methods reported, so far.

During this study, test samples were solubilized in DMSO and allowed to react with 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical in EtOH for 30 min at 37 °C. The DPPH concentration was kept 300 µM. After incubation, decrease in absorption was measured at 515 nm by using a multiplate reader (Spectra MAX-340, Molecular Devices, CA, USA). Percent radical scavenging activity of samples was determined by using the following formula, in comparison to a DMSO treated control group.

$$\% \text{ RSA} = 100 - \{(\text{Optical Density test compound} / \text{Optical density control}) \times 100\}$$

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