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PHENOLIC SUBSTANCES OF *Caragana conferta* AND THEIR SUPEROXIDE SCAVENGING ACTIVITY

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*New phenolic constituents **1** and **2** have been isolated from the ethyl acetate soluble fraction of* Caragana conferta *along with 7-hydroxy-4′-methoxyisoflavone (**3**) and 5,6,7-trihydroxy-4′-methoxyflavone (**4**), reported for the first time from this species. Their structures were determined by spectroscopic studies. Compounds **1** and **3** showed a significant superoxide scavenging activity, while **2** was found to be moderately active.*

Keywords: *Caragana conferta*, Papilionaceae, phenolic constitutents, superoxide scavenging activity.

The genus *Caragana* (Papilionaceae) comprises over 80 species, out of which 10 have also been reported from Pakistan [1]. Plants of the genus *Caragana* are used as folk medicine in China and Korea for the treatment of neuralgia, inflammation, rheumatism, arthritis, and hypertension [2]. The ethnopharmacological and chemotaxonomic significance of this genus prompted us to study *Caragana conferta* Benth, which is a shrub growing wildly in Asia, Africa, and southeast Europe. In Pakistan it is mainly found in the Gilgit and Kashmir valleys at an altitude of 7000–12000 feet [1].

The ethanolic extract of this plant showed strong toxicity in the brine shrimp lethality test, and on subsequent fractionation, the major toxicity was observed in the ethyl acetate (EtOAc) soluble subfraction. A bioassay-directed isolation study on the ethyl acetate soluble subfraction has resulted in the isolation of two new phenolic compounds, **1** and **2**, along with the known flavonoids 7-hydroxy-4′-methoxyisoflavone (**3**) and 5,6,7-trihydroxy-4′-methoxyflavone (**4**), reported for the first time from this species. Compounds **1** and **3** showed a significant superoxide scavenging activity, while compound **2** was only moderately active.

Compound **1** was obtained as off-white needles, mp 249–251°C, and showed positive Mg-HCl (reddish) and $FeCl_3$ (greenish-brown) color tests for a phenol. The infrared (IR) spectrum showed absorption bands for hydroxyl OH (3420 cm^{-1}), conjugated ketone C=O (1640 cm^{-1}), and aromatic C=C (1545 cm^{-1}) functionalities. UV absorptions at 228, 255, 292, and 308 nm were characteristic of an isoflavone-type skeleton [3]. A bathochromic shift was observed in the UV spectrum on addition of NaOAc solution, indicating a free phenolic group at C-7 of the isoflavone structure. The high-resolution electron impact mass spectrum (HR-EI-MS) of **1** exhibited the $[M]^+$ peak at *m/z* 284.0689 ($C_{16}H_{12}O_5$; calcd 284.0684) with eleven degrees of unsaturation. This conforms to an isoflavone skeleton with two hydroxyl and one methoxyl groups. The EI-MS showed two diagnostic peaks at *m/z* 148 ($C_9H_8O_2$) and 137 ($C_7H_5O_3$) resulted from retro Diels-Alder cleavage of ring B. Thus, ring A contains one hydroxyl group and ring B contains one hydroxyl and one methoxyl groups. In the ^1H NMR spectrum, the olefinic proton resonated at δ 8.2 along with the signals for trisubstituted ring B at δ 7.01(d, J = 8.4 Hz), 7.30 (dd, J = 8.4 Hz, J = 2.1 Hz), and 7.80 (d, J = 2.1 Hz). The protons of ring A resonated at δ 8.41(dd, J = 9.0 Hz), 7.18 (dd, J = 9.0, J = 2.1 Hz), and 7.07 (d, J = 2.1 Hz). The broad-band decoupled (BB) and DEPT ^{13}C NMR spectra of **1** showed 16 signals, comprising one methyl, seven methine, and eight quaternary carbons (Table 1). A signal at δ 175.6 was assigned to the conjugated carbonyl, while the conjugated olefinic carbons resonated at δ_C 152.7 and 126.3. The methoxyl carbon resonated at δ_C 55.9, while oxygenated aromatic carbons appeared at δ 164.0, 148.0, and 148.7. The relative positions of the substituents were assigned on the basis of HMBC correlations (Fig. 1). On the basis of these evidences, compound **1** was assigned the structure 7,5′-dihydroxy-2′-methoxyisoflavone.

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TABLE 1. ^{1}H and ^{13}C NMR Data (C_5D_5N, CD_3OD) of Compounds **1** and **2** at 400 and 100 MHz, Respectively (δ, ppm, J/Hz). Assignments Were Confirmed by COSY, HMQC, and HMBC Experiments

C atom	**1**		**2**	
	δ_H	δ_C	δ_H	δ_C
2	8.17 (s)	152.7	–	92.4
3	–	126.3	7.71 (dd, J = 8.6, 2.1)	123.9
4	–	175.6	7.54 (t, J = 8.6)	134.0
5	8.41 (d, J = 9.0)	128.2	7.54 (t, J = 8.6)	128.9
6	7.18 (dd, J = 8.4, 2.1)	115.8	7.84 (dd, J = 7.6, 2.1)	125.5
7	–	164.0	–	125.0
8	7.07 (d, J = 2.1)	103.1	–	152.8
9	–	158.5	–	170.6
10	–	118.0	–	–
1′	–	124.9	–	131.5
2′	–	148.0	7.10 (d, J = 8.7)	128.3
3′	7.01 (d, J = 8.4)	112.4	6.71 (d, J = 8.7)	114.9
4′	7.30 (dd, J = 8.4, 2.1)	120.4	–	156.9
5′	–	148.7	6.71 (d, J = 8.7)	114.9
6′	7.80 (d, J = 2.1)	117.8	7.10 (d, J = 8.7)	128.3
1″	–	–	–	131.5
2″	–	–	7.10 (d, J = 8.7)	128.3
3″	–	–	6.71 (d)	114.9
4″	–	–	–	156.9
5″	–	–	6.71 (d, J = 8.7)	114.9
6″	–	–	7.10 (d, J = 8.7)	128.3
2′-OCH_3	3.76 (s)	55.9	–	–

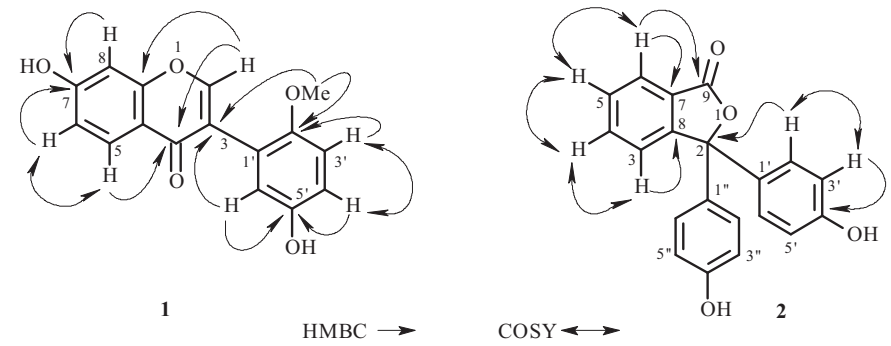

Fig. 1. Key COSY and HMBC correlations in compound **1** and **2**.

Compound **2** was isolated as a white amorphous powder. The HR-EI-MS showed $[M]^+$ at *m/z* 318.3231 corresponding to the formula $C_{20}H_{14}O_4$ (calcd 318.0892). The IR spectrum showed absorptions between 3292–3385 cm^{-1} for hydroxyl groups, while a band at 1735 cm^{-1} was for a carbonyl group together with a shoulder peak at 1716 cm^{-1}, characteristic of a five-membered lactone [4]. This was also inferred from a peak at *m/z* 274 due to the loss of a CO_2 molecule. The ^{1}H NMR spectrum exhibited two doublets (2H each) at δ 7.10 and 6.71 (J = 8.7 Hz), due to two isochronous *para*-substituted aromatic rings. The doublet (4H) at δ 7.10 was assigned to H-2′, H-6′, H-2″, and H-6″, while a doublet (4H) at δ 6.71 was assigned to H-3′, H-5′, H-3″, and H-5″ on the basis of ^{1}H–^{1}H COSY and HMBC spectra (Fig. 1). A set of two-proton triplets at δ 7.54 (J = 8.6 Hz) was attributed to H-4 and H-5, while a double doublet at δ 7.84 (J = 7.6 Hz, J = 2.1 Hz) was ascribed to H-6. Similarly, a double doublet at δ 7.71 (J = 8.6 Hz, J = 2.1 Hz) was assigned to H-3.

TABLE 2. Superoxide Scavenging Acivity of Compounds **1–4** (IC_{50} [μM]) in a Cell-Free System

Compound	IC_{50} ± S. E. M. (μM)	Compound	IC_{50} ± S. E. M. (μM)
1	136.023 ± 3.12	**4**	Inactive
2	321.457 ± 1.25	Propylgallate*	106.03 ± 05.00
3	111.249 ± 2.47		

*Positive control.

The BB and DEPT ^{13}C NMR spectra of compound **2** showed twenty carbon signals comprising eight quaternary and twelve methine carbons. Two peaks for four carbons each at δ 128.3 and δ 114.9 further supported the presence of two *para*-substituted benzene rings. The signal at δ 170.6 was assigned to the lactone carbonyl carbon. On the basis of these evidences, compound **2** was assigned the structure 3,3-bis(4-hydroxyphenyl)isobenzofuran-1-one. This compound is commercially known as phenolphthalein, which is used as an indicator in acid-base titrations. Although various synthetic routes are available for this compound, this is the first report of its isolation from the natural source.

Xanthine oxidase is an enzyme involved in defending the body against external agents during inflammatory responses. It usually produces superoxide by the reduction of molecular oxygen and oxidation of xanthine to uric acid, which is a parallel event. Its activity in neutrophils during inflammation is greater compared to other sites of the body. It is found to play a critical role in diabetes and other metabolic disorders.

It has been reported that a large number of flavonoids exhibit superoxide scavenging activity. Therefore, they can be used for the treatment of human gout and ischemia by decreasing superoxide concentrations in human tissues [5]. The potential of compounds **1–4** as superoxide scavengers were investigated, and the results are shown in Table 2. Compounds **1** and **3** showed a significant superoxide scavenging activity, while **2** was only moderately active. All of these compounds were also tested against xanthine oxidase but showed only a weak inhibitory potential.

EXPERIMENTAL

General Experimental Procedures. Melting points were determined on a Gallenkemp apparatus and are uncorrected. ^1H and ^{13}C NMR spectra and two-dimensional correlation spectroscopy (COSY, NOSEY, HMQC, and HMBC) were recorded on a Bruker AV-400 spectrometer (400 MHz for ^1H and 100 MHz for ^{13}C NMR) in C_5D_5N and CD_3OD with TMS as an internal standard. Chemical shifts (δ) are shown in ppm relative to TMS. UV spectra were obtained on a Hitachi UV-3200 spectrophotometer. IR Spectra were measured on a JASCO 302-A spectrometer in $CHCl_3$. Thin layer chromatography (TLC) was carried out on pre-coated silica gel 60 F254 plates (20 × 20 cm, Merck, Darmstadt, Germany) and visualized under UV light (254 nm) and also by spraying with ceric sulfate reagent. Silica gel 230–400 mesh (Merck, Darmstadt, Germany) was used for column chromatography. The HR-EI-MS were recorded on a JEOL JMS-HX-110 mass spectrometer. During the biological testing, absorbance was measured on a Spectra Max 340 microplate reader (Molecular Devices, Dojindo Laboratories, Kumamoto, Japan).

Plant Material. Whole plant of *Caragana conferta* Benth was collected from Gilgit valley (Pakistan) and identified by a Senior Scientist of the National Agriculture Research Center (NARC), Islamabad, Pakistan. A voucher specimen was deposited in the Herbarium of the Department of Botany, University of Karachi (voucher No. 319).

Extraction and Isolation. The air-dried chopped plant material (22 kg) was extracted with ethanol (3 × 30 L) at room temperature. The combined extract was evaporated to yield the residue (500 g), which was divided into *n*-hexane (30 g), dichloromethane (80 g), ethyl acetate (120 g), *n*-butanol (270 g), and water (100 g) soluble subfractions. The EtOAc fraction was subjected to column chromatography (CC) over silica gel, eluting with mixtures of *n*-hexane and EtOAc in increasing order of polarity to obtain three major fractions A, B, and C. The fraction A obtained from *n*-hexane–EtOAc (3:7) was again chromatographed over Si gel using *n*-hexane–EtOAc (9:1) as eluent to afford two successive fractions A_A and A_B. CC of subfraction A_A gave compound **1** (25 mg) through elution with *n*-hexane–EtOAc (3:7), while elution with *n*-hexane–EtOAc (9:1) provided compound **2** (13 mg). Fraction A_B was subjected to CC, eluting with *n*-hexane–EtOAc (2.7:7.3) and collecting 200 mL fractions to afford two subfractions that were further purified by Si gel CC, eluting with *n*-hexane–EtOAc (9.1:0.9, 8.9:1.1), respectively, to afford compounds **3** and **4**. These were characterized by comparison of physical and spectral data with those reported in literature [6–8].

7,5′-Dihydroxy-2′-methoxyisoflavone (1). Mp 249–251°C. UV (MeOH, λ_{max}, nm): 228, 255, 292, 308. IR (KBr, ν_{max}, cm^{-1}): 3420 (OH), 1640 (conjugated ketone C=O), 1545 (aromatic C=C). ^{1}H and ^{13}C NMR, see Table 1. EI-MS *m/z*: 284 (100) $[M]^+$, 269 (27), 255 (7), 241 (35), 213 (38), 148 (8), 137 (20), and 105 (11). HR-EI-MS *m/z*: 284.0689 ($C_{16}H_{12}O_5$; calcd 284.0684).

3,3-bis(4-Hydroxyphenyl)isobenzofuran-1-one (2). Mp 260–262°C. UV (MeOH, λ_{max}, nm): 203. IR (KBr, ν_{max}, cm^{-1}): 3292–3385 (OH), 1737, 1718 (lactone C=O). ^{1}H and ^{13}C NMR, see Table 1. EI-MS *m/z*: 318 $[M]^+$, 274 (100), 257 (35), 197 (23), 181 (38), 152 (21), 121 (43), and 93 (18). HR-EI-MS *m/z*: 318.3231 ($C_{20}H_{14}O_4$; calcd 318.0892).

***In vitro* Superoxide Scavenging Activity**. Supperoxide scavenging activity was assayed according to the method of Candan [9]. The phosphate buffer (0.1 M, pH 7.5). XO (003 unit/well) and test sample in DMSO were mixed in 96-well microliter plates and preincubated for ten minutes at room temperature; then WST-1 (15 μM) was added. The reaction was initiated by adding 0.1 mM of xanthine, and uric acid formation was measured spectrophotometrically at 295 nm. The reduction of the WST-1 was read at 450 nm using a Spectramax 340 spectrometer, Molecular Devices, CA, USA. The percent inhibitory activity of the samples was determined against a DMSO blank using the following formula:

$$\% \text{ Inhibition} = 100 - \{(\text{OD test compound/OD control}) \times 100\}$$

IC_{50} of samples was determined by using EZ-FIT Windows-based software.

REFERENCES

1. S. I. Ali and M. Qaiser, *Flora of Pakistan*, Department of Botany, University of Karachi, 2001, Ferozsons Printing Press, Karachi, **100**, 98 (1977).
2. S. Kitanaka, T. Ikezawa, and K. Yasukawa, *Chem. Pharm. Bull*., **44**, 565 (1996).
3. K. R. Markham and T. J. Mabry, *The Flavonoids*, Part 1, Academic Press, New York, 1975, p. 50.
4. H. Sugiura, T. Kato, H. Senda, Ko-Ki. Kunimoto, A. Kuwae, and K. Hanai, *Anal. Sci*., **15**, 611 (1999).
5. N. Cotelle, J. L. Bernier, J. P. Henichart, J. P. Catteau, E. Gaydou, and J. C. Wallet, *Free Rad. Biol. Med.,* **13**, 211 (1992).
6. E. Wong, *The Isoflavonoids in the Flavonoids* (J. B. Harborne, T. J. Mabry, H. Mabry, eds.), Chapman and Hall Ltd., London, 1975, p. 743.
7. P. Lebreton, K. R. Markham, W. T. Swift, Oung-Boran, and T. J. Mabry, *Phytochemistry*, **6**, 1675 (1967).
8. J. B. Harborne and B. Valdes, *Phytochemistry*, **10**, 2850 (1971).
9. F. Candan, *J. Enzym. Inhib. Med. Chem*., **18**, 59 (2003).