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SHORT COMMUNICATION

Conferin, potent antioxidant and anti-inflammatory isoflavone from *Caragana conferta* Benth

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Abstract

Conferin (**1**), a new isoflavone, has been isolated from the ethyl acetate soluble fraction of *Caragana conferta* Benth. along with seven known compounds, namely biochanin A (**2**), *p*-hydroxybenzoic acid (**3**), 3,5-dimethoxybenzoic acid (**4**), ursolic acid (**5**), erythrodiol (**6**), pinoresinol (**7**), and syringresinol (**8**), reported for the first time from this species. The structure of the new isoflavone was deduced on the basis of spectroscopic studies. Compounds **1** and **2** were investigated for biological activities and showed significant anti-inflammatory activity in carrageenan induced paw edema of rats. Evaluation of antioxidant activity by the radical scavenging method indicated that compound **1** is a potent antioxidant while **2** is moderately active. It was also shown that the reducing capability of compound **2** was remarkably increased in a concentration dependent manner as compared to **1**. Compound **1** showed moderate inhibitory activity against the enzyme lipoxygenase, while **2** showed weak activity.

Keywords: Caragana conferta*; papilionaceae; isoflavone; antioxidant; anti-inflammatory*

Introduction

The family Papilionaceae comprises 55 genera and well over 7000 species. The genus *Caragana* has over 80 species, of which 10 have so far been identified in Pakistan[1]. *Caragana conferta* is a shrub which grows in Asia, Africa, and south east Europe. In Pakistan it is mainly found in the Gilgit and Kashmir valleys at an altitude of 7000–12,000 feet above sea level[1]. Plants of the genus *Caragana* are used as folk medicine in China and Korea for the treatment of neuralgia, rheumatism, arthritis, and hypertension[2]. A literature survey revealed that few compounds have so far been reported from this species[3,4]. A methanolic 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 soluble sub-fraction. Further pharmacological screening of this fraction revealed potent antioxidant activity. In this article we report the isolation of a new isoflavone (**1**), named conferin, along with several known compounds: biochanin A (**2**)[5], *p*-hydroxybenzoic acid (**3**)[6], 3,5-dimethoxybenzoic acid (**4**)[6], ursolic acid (**5**)[7], erythrodiol (**6**)[8], pinoresinol (**7**)[9], and syringresinol (**8**)[10]. This study was undertaken to investigate the antioxidant potentials using different tests including 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging activity and reducing power. *In vivo* anti-inflammatory activity was determined by observing the carrageen induced paw edema of rats for compounds **1** and **2**, while the lipoxygenase inhibitory assay was also performed.

Materials and methods

Plant material

The whole plant of *Caragana conferta* Benth. was collected from Gilgit (Pakistan) and identified by Dr. Rubina Ashraf of the National Agriculture Research Center, Islamabad, Pakistan. A voucher specimen has been deposited in the

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herbarium of the Department of Botany, University of Karachi (voucher no. 319).

General experimental procedures

^{1}H- and ^{13}C-nuclear magnetic resonance (NMR), heteronuclear multiple quantum coherence (HMQC), and heteronuclear multiple bond coherence (HMBC) spectra were recorded on Bruker spectrometers operating at 400 MHz for ^{1}H-NMR and 100 MHz for ^{13}C-NMR, respectively. The chemical shift values are reported in units of ppm (δ) and the coupling constants (*J*) are in Hz. Electron impact mass spectra (EIMS) and high resolution EIMS (HREIMS) were recorded on JMS-HX-110 and JMS-DA-5000 mass spectrometers. Ultraviolet (UV) spectra were recorded on a Hitachi UV-3200 spectrophotometer. Infrared (IR) spectra were recorded on a 460 Shimadzu spectrometer. Aluminum sheets precoated with silica gel 60 F_{254} (20 × 20 cm, 0.2 mm thick; E. Merck) were used for thin layer chromatography (TLC) and silica gel (230–400 mesh) was used for column chromatography. Visualization of the TLC plates was carried out under UV light at 254 and 366 nm and by spraying with ceric sulfate reagent solution (with heating).

Extraction and isolation

Shade dried whole plant material (15 kg) was extracted with MeOH (3 × 60 L) at room temperature. The combined methanolic extract was evaporated under reduced pressure to obtain a thick gummy mass (700 g). It was suspended in water and successively extracted with *n*-hexane, ethyl acetate, and *n*-butanol. The ethyl acetate soluble fraction (150 g) was subjected to column chromatography, eluting with $CHCl_3$, $CHCl_3$–MeOH, and MeOH in increasing order of polarity to obtain five fractions (A–E). Fraction A obtained from $CHCl_3$–MeOH (9.9:0.1) was further purified by column chromatography, eluting with $CHCl_3$–MeOH (9.7:0.3) to afford compounds **3** (16 mg) and **4** (15 mg). Fraction B obtained from $CHCl_3$–MeOH (9.8:0.2) was further purified by column chromatography, eluting with $CHCl_3$–MeOH (9.5:0.5) to afford compounds **5** (22 mg) and **6** (25 mg). Fraction C obtained from $CHCl_3$–MeOH (9.5:0.5) was a mixture of two components, which were separated by column chromatography using the solvent system $CHCl_3$–MeOH (9.2:0.8) to afford compounds **2** (30 mg) and **1** (25 mg) from the top and the tail fractions, respectively (Figure 1). Fraction D obtained from $CHCl_3$–MeOH (9.3:0.7) was a mixture of two components, which were separated by column chromatography using the solvent system $CHCl_3$–MeOH (9.0:1.0) to afford compounds **7** (18 mg) and **8** (15 mg) from the top and the tail fractions, respectively.

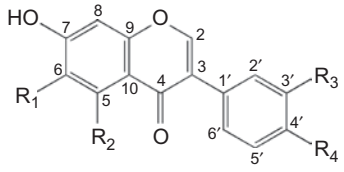

Figure 1. Compounds **1** and **2**.

Compounds **2–8** were identified through comparison of their physical and spectral data with those reported in the literature[5–10].

*Conferin (**1**)* White solid (25 mg), M.p. 218–219°C. UV (MeOH) λ_{max} (log ε): 258 (2.45), 288 (2.75) nm. IR (KBr) cm^{-1}: 3450, 1665 and 1580. EIMS *m/z* (rel. int.): 300 (100), 285 (27), 257 (28), 229 (36), 154 (20), 148 (15), 133 (26). HREIMS: *m/z* 300.0625, calcd. for $C_{16}H_{12}O_6$ 300.0633. ^{1}H-NMR (400 MHz, pyridine-d_5) δ: 3.80 (3H, s, OMe-3′), 7.09 (1H, d, *J* = 8.4 Hz, H-5′), 7.25 (1H, s, H-8), 7.38 (1H, dd, *J* = 8.4, 2.0 Hz, H-6′), 7.89 (1H, d, *J* = 2.0 Hz, H-2′), 8.17 (1H, s, H-2), 8.23 (1H, s, H-5). ^{13}C-NMR (100 MHz, pyridine-d_5) δ: 56.5 (OMe), 152.4 (C-2), 124.1 (C-3), 175.5 (C-4), 109.4 (C-5), 146.5 (C-6), 154.3 (C-7), 103.7 (C-8), 152.1 (C-9), 118.1 (C-10), 125.8 (C-1′), 118.5 (C-2′), 149.2 (C-3′), 147.5 (C-4′), 112.8 (C-5′), 121.0 (C-6′).

Antioxidant assay

The free radical scavenging activity was measured using 1,1-diphenyl-2-picryl-hydrazyl (DPPH)[11]. A solution of DPPH 0.3 mM was prepared in ethanol. Five microliters of each sample of a different concentration (62.5–500 μg) were mixed with 95 μL of DPPH solution in ethanol. The mixture was dispersed in 96-well plates and incubated at 37°C for 30 min. The absorbance at 515 nm was measured by microtiter plate reader (SpectraMax Plus 384; Molecular Devices, Sunnyvale, CA, USA) and percent radical scavenging activity was determined in comparison with the methanol treated control. Butylated hydroxyanisole (BHA) was used as standard.

$$\text{DPPH scavenging effect}(\%) = A_c - \frac{A_s}{A_c} \times 100$$

where Ac is the absorbance of the control (dimethylsulfoxide (DMSO) treated) and As is the absorbance of the sample

Determination of total reducing ability

Total reducing capability of these compounds was estimated according to the method of Oyaizu[12] with some modification. Different concentrations of samples (5–100 μg/mL) in methanol were mixed with phosphate buffer (250 μL, 0.2 M, pH 6.6). Then 250 μL potassium ferricyanide ($K_3[Fe(CN)_6]$) (1%) was added. The mixture was incubated at 50°C for 20 min. After incubation, 250 μL trichloroacetic acid (10%) was added to the mixture which was centrifuged for 10 min; the upper layer of the solution (250 μL) was separated in another set of test tubes and mixed with an equal volume of deionized water (250 μL). Then 50 μL of ferric chloride ($FeCl_3$) (0.1%) was added and the absorbance was measured at 700 nm using a spectrophotometer (Specord 200; Analytik Jena, Germany). A higher absorbance of the reaction mixture indicated a higher reducing power.

Lipoxygenase inhibition assay

All chemicals required for the assay were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Lipoxygenase inhibiting activity was measured by modifying the spectrophotometric method developed by Tappel[13]. A mixture of test compound (10 μL; 1 mM) in MeOH, type-1B lipoxygenase (EC 1.13.11.12; from soyabean) (20 μL; 70 units) in 0.1 M aqueous phosphate buffer (pH 8.0) in a total volume of 160 μL was incubated for 10 min at 25°C. Then the reaction was initiated by the addition of a solution of linoleic acid as substrate (10 μL; 20 μM), resulting in the formation of (9*Z*,11*E*,13*S*)-13-hydroperoxyoctadeca-9,11-dienoate. The change in UV absorbance at 234 nm was followed over a period of 6 min. All reactions were performed in triplicate, and analyzed with a 96-well plate reader (SpectraMax Plus 384; Molecular Devices). The IC_{50} values were calculated using EZ-Fit enzyme-kinetics software (Perrella Scientific, Inc., Amherst, MA, USA).

Anti-inflammatory assay

Inflammation is a defensive reaction to injury[14]. The symptoms of inflammation (redness, edema, heat, pain, and disturbed tissue function) are the result of a complex pathophysiological process[15]. Conventional non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin, ibuprofen, diclofenac, and naproxen are widely used for the treatment of pain and inflammation. The prolonged use of these drugs is associated with a substantial toxicity profile with side effects mainly affecting the gastrointestinal tract, heart, and kidney[16]. In inflammation, the role of both cyclooxygenase (COX) and lipoxygenase (LOX) is apparent. Therefore, the search for anti-inflammatory agents that retain the therapeutic efficacy of classical NSAIDs and have greater safety via equally blocking both the COX and the 5-LOX metabolic pathways is justified[17]. Traditionally used plants could provide useful information in this regard.

In vivo anti-inflammatory assay

The anti-inflammatory potential of compounds **1** and **2** was evaluated in rats using the following chemicals: carrageenan and DMSO obtained from Sigma and diclofenac sodium from Novartis Pharmaceuticals. Wistar rats (120–180 g) of either sex were obtained from the animal house of the Agha Khan University of Karachi. Animals were kept under standard environmental conditions with free access to food and water. Rats were divided into four groups (six in each group). Group 1 served as control and received 0.9% saline while group 2 received diclofenac sodium orally. Group 3 received compound **1** in a concentration 30 mg/kg and group 4 received 30 mg/kg of compound **2**. Acute edema was induced in the right hind paw of rats by injecting 0.1 mL of freshly prepared carrageenan 1% solution subcutaneously into the plantar region. Drugs were given 1 h before carrageenan challenge. Paw volume was measured using a plethysmometer at 0 and 4 h after carrageenan injection. The edema volume and percent inhibition in edema were calculated using the method of Christopher[18].

Results and discussion

The ethyl acetate soluble fraction of the methanolic extract of the whole plant of *Caragana conferta* was subjected to a series of column chromatographic techniques to obtain compounds **1–8**, and their structures were established by UV, IR, MS, and NMR techniques.

Conferin (**1**) was isolated as white amorphous solid which gave violet coloration with $FeCl_3$. The molecular formula $C_{16}H_{12}O_6$ was established by HREIMS showing a $[M+H]^+$ peak at *m/z* 300.0625 (calcd. for $C_{16}H_{12}O_6$ 300.0633) having eleven degrees of unsaturation. The broadband and distortionless enhancement by polarization transfer (DEPT) ^{13}C-NMR spectra of **1** showed 16 carbon signals, including one methyl, six methane, and nine quaternary carbons. The signal at δ 175.5 was assigned to the conjugated carbonyl, while the conjugated olefinic carbon resonated at δ 152.4[4]. The methoxyl carbon was observed at δ 56.5, while oxygenated aromatic carbons gave signals at δ 154.3, 149.2, 147.5, and 146.5. In the ^{1}H-NMR spectrum a singlet of conjugated olefinic proton was observed at δ 8.17. Thus, **1** was deduced to be an isoflavone. In HREIMS the retro-Diels–Alder fragments were observed at *m/z* 154 and 148, revealing the presence of two hydroxyl groups in ring A and one methoxyl and one hydroxyl group in ring B. Ring A showed two singlets in the ^{1}H-NMR spectrum at δ 7.25 and δ 8.23, revealing the presence of hydroxyl groups at C-6 and C-7[19].

The protons of the 1,3,4-trisubstituted ring B were observed at δ 7.89 (1H, d, *J* = 2.0 Hz, H-2′), δ 7.09 (1H, d, *J* = 8.4 Hz, H-5′), and δ 7.38 (1H, dd, *J* = 8.4, 2.0 Hz, H-6′). The signal of the methoxyl group appeared at δ 3.80 (3H, s). The position of the methoxyl group was ascertained through HMBC, showing ^3J correlation of methoxyl protons with C-3′ (δ 149.2). It was supported by the observation of a strong NOESY (nuclear Overhauser effect spectroscopy) correlation between the methoxyl protons and H-2′ (Figure 2). All these data were in complete agreement to the assigned structure of conferin (**1**) as 6,7,4′-trihydroxy-3′-methoxy isoflavone.

Compounds **1** and **2** were evaluated for their antioxidant activity as shown in Table 1. Compound **1** reflected potent antioxidant ability while **2** was moderately active when compared with the standard BHA. This shows that the

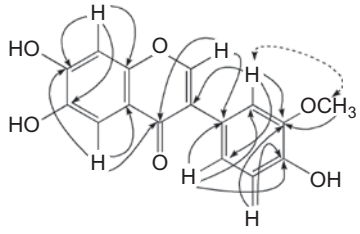

Figure 2. HMBC & NOESY correlations for compound **1**.

number and position of hydroxyl groups are apparently the controlling factors for antioxidant activity. Thus, compound **1** having a greater number of phenolic functionalities including two of them in *ortho* positions induces greater antioxidant activity than **2**.

The reducing power of a compound may serve as a significant indicator of its potential antioxidant activity[20]. The reductive capabilities of compounds are assessed by the extent of conversion of the Fe^{3+}/ferricyanide complex to the Fe^{2+}/ferrous form. The reducing power of compound **1** was observed at different concentrations (50, 100, 150, 200 μg/mL) and results were compared with BHA as shown in Figure 3. Compound **1** showed 65, 70, 75, and 77% reducing capability while the percentage reducing capability of compound **2** was 42, 49, 83, and 102% at concentrations 50, 100, 150, and 200 μg/mL, respectively. This means that compound **1** exhibits powerful reducing ability as compared to compound **2** at the lowest concentration. It is apparent that the phenolic groups at C-6 and C-7, which are adjacent to each other, are responsible for the higher reducing ability of compound **1**. Surprisingly, the reducing power of compound **2** rapidly increased with an increase in concentration, as shown in Figure 3. It is suggested that due to the increase in concentration, some intramolecular changes may occur which cause the free hydroxyl group to become unavailable to react. It is also possible that compound **1** is more reactive toward itself than the reagent at a higher concentration while compound **2** is more reactive toward the reagent.

Table 1. IC_{50} (μM) values of compounds **1** and **2** in antioxidant assay and against lipoxygenase.

Compound	DPPH scavenging activity, IC_{50}[a] (μM)	Lipoxygenase inhibition activity, IC_{50}[a] (μM)
1	19.1 ± 0.11	55.2 ± 0.22
2	99.5 ± 0.15	100 ± 0.12
BHA[b]	44.3 ± 0.09	
Baicalein[c]		22.6 ± 0.08

[a]Value ± SEM (standard mean error of three assays).
[b]Standard DPPH scavenging activity.
[c]Standard inhibitor of lipoxygenase.

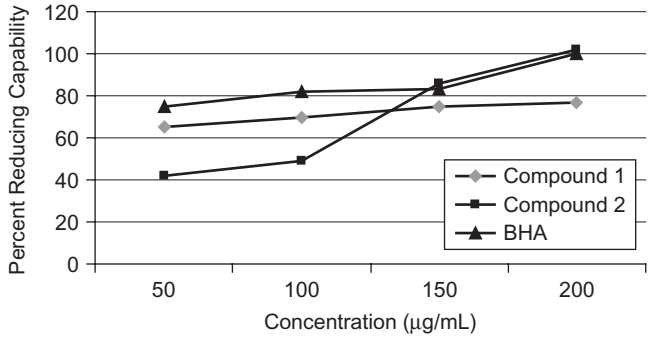

Figure 3. Reducing ability of BHA and compounds **1** and **2** at various concentrations.

Table 2. Anti-inflammatory potential of compounds **1** and **2** in carrageenan-induced paw edema of rats.

Sr no.	Treatment (100 mg/kg)	Initial paw volume[a], Vo	Final volume[a], Vf (2–3 h)	Edema volume[a], Vf – Vo = Vc	Percent inhibition
1	Cage 1, control	0.86 ± 0.08	0.86 ± 0.09	0	—
2	Diclofenac sodium	0.83 ± 0.09	1.04 ± 0.15	0.21 ± 0.23	58
3	**1**	0.93 ± 0.07	1.13 ± 0.05	0.24 ± 0.15	52
4	**2**	0.92 ± 0.13	1.19 ± 0.05	0.27 ± 0.21	54

[a]Measured in mL.

Determination of the lipoxygenase inhibition activity as shown in Table 1 indicates that compound **1** is a moderate lipoxygenase inhibitor with an IC_{50} value of 55.2 μM while compound **2** has an IC_{50} value of 100 μM when compared with baicalein (22.6 μM). It has been found that lipoxygenase inhibitors play an important role in the treatment of a variety of disorders such as bronchial asthma and inflammation[21], and also have a profound influence on the development of several cancers[22]; therefore, the search for clinically efficacious inhibitors is in demand in the global market. Lipoxygenases are therefore potential targets for rational drug design and the discovery of mechanism based inhibitors for the treatment of bronchial asthma, inflammation, and autoimmune diseases.

The anti-inflammatory activity of compounds **1** and **2** was determined in carrageenan induced paw edema of rats, and both were found to be significant when compared with group 2 which received diclofenac sodium (positive control) (Table 2).

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***Declaration of interest*:** The authors report no conflict of interest. The authors are responsible for the content and writing of the paper.

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