Conferols A and B, New Anti-inflammatory 4-Hydroxyisoflavones from *Caragana conferta*

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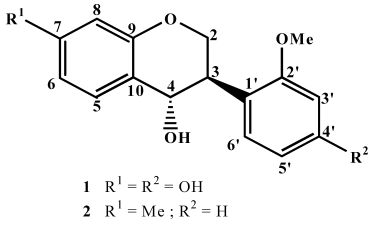
Conferols A (1) and B (2), the new 4-hydroxyisoflavones, have been isolated from the dichloromethane sub-fraction of the methanolic extract of *Caragana conferta* along with 3′,5′-dihydroxy, 7,4′-dimethoxyisoflavone (3), *E*-cinnamic acid (4), tetracosyl 3,4-dihydroxy-*E*-cinnamate (5), docosyl 3,4-dihydroxy-*E*-cinnamate (6), β-sitosterol (7), β-sitostrerol 3-*O*-β-D-glucopyranoside (8), stigmasterol (9) and lupeol (10), respectively, reported for the first time from this species. The structures of the new compounds were elucidated through spectroscopic techniques including MS and 2D-NMR. The compounds 1—3 showed significant anti-inflammatory activity in the respiratory burst assay.

Key words *Caragana conferta*; 4-hydroxyisoflavone; anti-inflammatory activity

The genus *Caragana* belongs to the family Papilionaceae, which comprises 55 genera, and over 7000 species. The genus *Caragana* has over 80 species out of which 10 species have so far been identified in Pakistan.[1] *Caragana conferta* is a shrub which grows in Asia, Africa and southeast Europe. In Pakistan it is mainly found in Gilgit and Kashmir valley at an altitude of 7000—12000 feet above the sea level.[1] The plants of genus *Caragana* are used as folk medicine in China and Korea for the treatment of neuralgia, inflammation, rheumatism, arthritis, and hypertension.[2] Previously an isoflavone has been reported from *C. conferta*. A methanolic extract of this plant showed strong toxicity in brine shrimp lethality test and on subsequent fractionation, the major toxicity was observed in dichloromethane sub-fraction. Further pharmacological screening of the latter fraction revealed potent anti-inflammatory activity. This prompted us to carry out bioassay directed isolation studies on dichloromethane sub-fraction of this plant. As a result we herein report the isolation and structure elucidation of two new 4-hydroxyisoflavones named as conferols A (**1**) and B (**2**). In addition, eight compounds namely 3′,5′-dihydroxy, 7,4′-dimethoxyisoflavone (**3**),[3] *E*-cinnamic acid (**4**),[4] tetracosyl 3,4-dihydroxy-*E*-cinnamate (**5**),[5] docosyl 3,4-dihydroxy-*E*-cinnamate (**6**),[6] β-sitosterol (**7**),[7] β-sitostrerol 3-*O*-β-D-glucopyranoside (**8**),[8] stigmastrerol (**9**)[9,10] and lupeol (**10**),[11] and are also isolated for the first time from this species. The compounds **1**—**3** showed significant anti-inflammatory activity in respiratory burst assay.

Result and Discussion

Conferol A (**1**) was obtained as a yellow gum, $[\alpha]_D^{25}$ −107.6° (chloroform). It gave positive $FeCl_3$ color reaction for a phenol. The high resolution (HR)-EI-MS showed the molecular ion peak at *m*/*z* 288.1164 corresponding to the molecular formula $C_{16}H_{16}O_5$ (Calcd for $C_{16}H_{16}O_5$, 288.1154). The IR spectrum showed absorption bands at 3400 cm^{-1} (OH) and 1640, 1550 cm^{-1} (aromatic). The UV spectrum showed maxima at 298 and 210 nm. The broad band (BB) and distortionless enhancement by polarization transfer (DEPT) ^{13}C-NMR spectra showed sixteen signals comprising of one methyl, one methylene, eight methine and six quarternary carbons. In EI-MS the retero Diels–Alder fragments at *m*/*z* 179 and 148 confirmed the presence of one hydroxyl group in ring A and methoxyl functionality in ring B. The ^{1}H-NMR spectrum showed a pair of one doublet of doublets at δ 3.60 (*J*=11.0, 10.8 Hz), δ 4.20 (*J*=5.0, 10.8 Hz), a doublet of double doublets at δ 3.53 (*J*=5.0, 10.8, 6.5 Hz) and a doublet at δ 5.46 (*J*=6.5 Hz). These signals were assignable to H-2 protons, H-3 and H-4 protons respectively of a 4-hydroxyisoflavone skeleton. The corresponding carbons were identified by heteronuclear multiple quantum coherence (HMQC) as a methylene carbon at δ 66.0 and two methine carbons at δ 39.5 and 78.5 respectively. In the ^{1}H-NMR spectrum further signals were observed showing the presence of one methoxyl group (δ 3.56, singlet), 7-substituted ring A [δ 7.38 (1H, d, *J*=8.41 Hz, H-5), δ 6.53 (1H, dd, *J*=8.4, 2.1 Hz, H-6) and δ 6.40 (1H, d, *J*=2.1 Hz, H-8)] and 2′,4′-disubstituted ring B [δ 7.12 (1H, d, *J*=8.7 Hz, H-6′), δ 6.42 (1H, dd, *J*=8.7, 2.4 Hz, H-5′) and δ 6.44, (1H, d, *J*=2.4 Hz, H-3′)]. The hydroxyl groups were assigned to C-7 and C-4′ while point of attachement of the methoxyl group was confirmed at C-2′ on the basis of heteronuclear multiple bond connectivity (HMBC) correlations illustrated in Table 1 and



1 $R^1 = R^2 = OH$
2 $R^1 = Me$; $R^2 = H$

Fig. 1. Structures of Compounds **1** and **2**

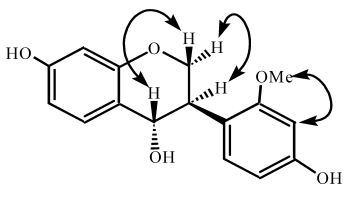


Fig. 2. Important NOESY Correlations in Compounds

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Table 1. ^{1}H- and ^{13}C-NMR Data of the Conferols A (**1**) and B (**2**)

Position	**1**		**2**	
	δ_C	δ_H (mult., *J*, Hz)	δ_C	δ_H (mult., *J*, Hz)
2	66.0	3.60 (dd, 11.0, 10.8) 4.20 (dd, 5.0, 10.8)	66.0	3.60 (dd, 11.0, 10.8) 4.20 (dd, 5.0, 10.8)
3	39.5	3.53 (ddd, 5.0, 10.8, 6.5)	39.5	3.53 (ddd, 5.0, 10.8, 6.5)
4	78.5	5.46 (d, 6.5)	78.5	5.46 (d, 6.5)
5	132.0	7.38 (d, 8.41)	132.0	7.41 (d, 8.3)
6	109.7	6.53 (dd, 8.4, 2.1)	109.0	7.35 (dd, 8.3, 2.3)
7	156.7		135.0	
8	103.9	6.40 (d, 2.1)	96.0	6.40 (d, 2.3)
9	159.9		156.6	
10	135.0		103.0	
1′	119.0		119.0	
2′	161.2		161.0	
3′	99.9	6.44 (d, 2.4)	103.0	7.10 (dd, 8.7, 3.3)
4′	160.7		124.7	6.42 (dd, 8.3, 2.2)
5′	106.7	6.42 (dd, 8.7, 2.4)	127.7	6.42 (dd, 8.3, 3.3)
6′	125.0	7.12 (d, 8.7)	106.0	7.01 (dd, 8.4, 2.2)
2′-OCH_3	55.5	3.56 (s)	55.5	3.56 (s)
7-CH_3			29.0	2.61 (s)

Assignments were confirmed by COSY, HMQC and HMBC experiments. All spectra were recorded in $CDCl_3$ at 400 MHz., δ in ppm, *J* in Hz.

further confirmed through nuclear Overhauser effect spectroscopy (NOESY) correlation between the methoxyl protons and H-3′.

The larger magnitude of coupling constants between H-2β and H-3 inferred α and pseudoaxial orientation of H-3. Moreover, the larger coupling constant between H-3 and H-4 showed their *trans* relationship indicating β and pseudoaxial orientation of H-4. The absolute stereochemistry at C-3 and C-4 was not only confirmed by strong NOESY correlations between H-4 and H-2β and between H-3 and H-2α and also by NMR chemical shifts of C-2, C-3 and C-4 and their respective protons which showed complete agreement with those of bolusanthol A.[12] Thus structure of conferol A (**1**) was assigned as (3*R*,4*R*)-3-(4-hydroxy-2-methoxyphenyl)-3,4-dihydro-2*H*-chromene-4,7-diol.

Conferol B (**2**) was obtained as a brownish gum, $[\alpha]_D^{25}$ −57.03° (chloroform). The IR and UV spectra were very similar to those of **1**. The HR-EI-MS showed M^+ peak at *m*/*z* 270.1286 which corresponded to molecular formula $C_{17}H_{18}O_3$ (Calcd for $C_{17}H_{18}O_3$, 270.1256). The BB and DEPT ^{13}C-NMR spectra showed seventeen carbons signals comprising two methyl, one methylene, nine methine and five quartenary carbons. The ^{13}C-NMR spectra were very similar to those of **1**, except the upfield shift of C-4′ and C-7. The ^{1}H-NMR spectrum also showed similar features except the presence of methyl group as singlet at δ 1.28. Moreover, the chemical shifts of various protons of ring A and B also showed variation from the ^{1}H-NMR spectrum of **1**. It now showed a 2′ monosubstituted ring B [δ 6.42 (1H, dd, *J*=8.3, 3.3 Hz, H-5′), δ 7.10 (1H, dd, *J*=8.7, 3.3 Hz, H-3′), δ 7.01 (1H, dd, *J*=8.4, 2.2 Hz, H-6′) and δ 6.42 (1H, dd, *J*=8.3, 2.2 Hz, H-4′)]. The signals of 7-substitued ring A were observed at δ 7.41 (1H, dd, *J*=8.3 Hz, H-5), δ 7.35 (1-H, dd, *J*=8.3, 2.3 Hz, H-6) and δ 6.40 (1H, d, *J*=2.3 Hz, H-8). The positions of methyl and methoxyl groups were confirmed through HMBC correlations and also by NOESY correlation of both the methyl and methoxyl protons with H-6, H-8 and H-3′, respectively (Table 1).

Table 2. *In Vitro* Respiratory Burst Assay IC_{50} (μM) Values of Compounds **1**—**3** and Positive Controls at 500 μM Concentration

Compound	% Inhibition	IC_{50} (μg/ml)±S.E.M.
1	78.147	673.580±1.023
2	89.256	389.312±3.256
3	82.54	463.146±6.901
Indomethacin	81.36	271.21±2.192

a=positive control. S.E.M.=standard error mean.

Similarity in the coupling constants between H-2β and H-3 as well as H-3 and H-4 with those of **1** allowed us to assign similar absolute configurations at C-3, C-4 which was subsequently confirmed by strong NOESY interactions between H-2α and H-3α as well as H-2β and H-4β. The structure of conferol B (**2**) was therefore assigned as (3*R*,4*R*)-3-(2-methoxyphenyl)-7-methyl-3,4-dihydro-2*H*-chromen-4-ol.

Inflammation occurs as a defensive response, which induces physiological adaptations to limit tissue damage and removes the pathogenic infections.[13] Reactive oxygen species (ROS) are formed subsequent to the assembly and activation of the phagocyte-specific enzyme, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. This process is initiated by the production of superoxide anion (O_2^-), during a respiratory burst of non-mitochondrial oxygen uptake by an NADPH oxidase system.[14] This study used the water soluble tetrazolium salt (WST-1) to measure superoxide production by neutrophils activated by opsonized zymosan, which induces phagocytic activation of neutrophils. This technique is more sensitive and reliable than other available techniques. The aim of this study was to examine the anti-inflammatory activity of the isolated compounds with the help of an *in vitro* assay and explore their potential as non-steroidal anti-inflammatory agents. As a result the compounds **1**—**3** showed significant anti-inflammatory activity and thus have the potential to serve as lead compounds in drug design and discovery. The other isolated compounds did not show any inflammatory activity.

Experimental

The optical rotations were measured on a JASCO DIP-370 digital polarimeter. ^{1}H- 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 ^{1}H- and 100 MHz for ^{13}C-NMR) in $CDCl_3$ with TMS as internal standard. Chemical shifts δ are shown in ppm relative to TMS. The UV spectra were obtained on a Hitachi UV-3200 spectrophotometer. IR spectra measured on a JASCO 302-A spectrometer in $CHCl_3$. The thin layer chromatography (TLC) were carried out on pre-coated silica gel 60 F_{254} plates (E. Merck, 0.25 and 0.50 mm thickness respectively), and visualized under UV light (254 nm) and by spraying with ceric sulphate reagent. Silica gel 230—400 mesh (E. Merck, Darmstadt, Germany) was used for column chromatography. The HR-EI-MS were recorded on a JEOL JMS-HX-110 mass spectrometer.

Heparinized fresh venous blood was drawn from healthy volunteers of a local blood bank and neutrophils were isolated by the reported method.[15] During the biological testing, absorbance was measured on a SpectraMax 340 microplate reader (Molecular Devices). WST-1 (Dojindo Laboratories, Kumamoto, Japan), zymosan A (Sigma Chemicals, St. Louis, MO, U.S.A.) was used.

Plant Material Whole plant of *Caragana conferta* Benth was collected from Gilgit valley (Pakistan) and identified by a Senior Scientist of National Agriculture Research Center (NARC), Islamabad, Pakistan. A voucher specimen has been 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 EtOH (3×30 l) at room temperature. The combined extract was evaporated to yield the residue (500 g), which was divided into dichloromethane (CH_2Cl_2) (80 g), *n*-hexane (30 g), *n*-butanol (270 g) and water (100 g) soluble sub-fractions. The CH_2Cl_2 fraction was subjected to column chromatography (CC) over silica gel eluting with mixtures of *n*-hexane and CH_2Cl_2 in increasing order of polarity to obtain three major fractions A, B and C. The fraction A obtained from *n*-hexane/CH_2Cl_2 (3 : 7) was again chromatographed over Si gel using *n*-hexne/CH_2Cl_2 (9 : 1) as eluent to afford two successive fractions A_A and A_B. CC of the sub fraction A_A gave the compound **1** (15 mg) through elution with *n*-hexne/CH_2Cl_2 (9.5 : 0.5) while elution with *n*-hexane/CH_2Cl_2 (9 : 1) provided compound **2** (13 mg). The fraction A_B was subjected to CC eluting with *n*-hexane/CH_2Cl_2 (2.7 : 7.3) collecting 200 ml fractions to afford 5 sub-fractions that were further purified by Si gel CC eluting with *n*-hexane/CH_2Cl_2 (9.1 : 0.9, 8.9 : 1.1, 8.7 : 1.3, 8.3 : 1.7, 7.9 : 2.1) respectively to afford *E*-cinnamic (**4**) acid, tetracosyl 3,4-dihydroxy-*E*-cinnamate (**5**), docosyl 3,4-dihydroxy-*E*-cinnamate (**6**), β-sitosterol (**7**) and β-sitostrerol 3-*O*-β-D-glucopyranoside (**8**). The fraction B obtained from *n*-hexane/CH_2Cl_2 (5 : 5) was again chromatographed over Si gel using *n*-hexane/CH_2Cl_2 (9 : 1) as eluent to afford two successive fractions B_A and B_B. CC of the sub fraction B_B and elution with *n*-hexane/CH_2Cl_2 (7 : 3) afforded stigmasterol (**9**) (13 mg) and lupeol (**10**) (11 mg) from the top and the tail fractions, respectively. The fraction C obtained from *n*-hexane/CH_2Cl_2 (4 : 6) yielded 3′,5′-dihydroxy, 7,4′-dimethoxy-isoflavone (**3**),[4] (7 mg). All the known compounds were identified by comparision of their physical and spectral data with those reported in literature.

Conferol A (**1**): Yellow gum, $[\alpha]_D^{25}$ −107.6° (*c*=0.3, $CHCl_3$); UV λ_{max} ($CHCl_3$) nm: 298, 210; IR (KBr) cm^{-1}: 3400, 1640, 1550; ^{1}H-NMR ($CDCl_3$, 400 MHz) and ^{13}C-NMR ($CDCl_3$, 100 MHz): see Table 1; HR-EI-MS *m/z* 288.1164, $C_{16}H_{16}O_5$, (Calcd for $C_{16}H_{16}O_5$, 288.1154).

Conferol B (**2**): Brownish gum, $[\alpha]_D^{25}$ −57.03° (*c*=0.3, $CHCl_3$); ^{1}H-NMR ($CDCl_3$, 400 MHz) and ^{13}C-NMR ($CDCl_3$, 100 MHz): see Table 1; HR-EI-MS *m/z* 270.1286, $C_{17}H_{18}O_3$, (Calcd for $C_{17}H_{18}O_3$, 270.1256).

***In Vitro* Anti-inflammatory Assay** Anti-inflammatory activity of the test compounds was determined by using a modified assay of Tan and Berridge.[14] This *in vitro* assay was based on the reduction of highly water-soluble tetrazolium salt (WST-1) in the presence of activated neutrophils. Antiinflammatory activity was determined in a total volume of 200 μl MHS (pH 7.4) containing 1.0×10^4 neutrophils/ml, 250 μM WST-1 and various concentrations of test compounds. The control contained buffer, neutrophils and WST-1. All compounds were equilibrated at 37 °C and the reaction was initiated by adding opsonized zymosan A (15 mg/ml), which was prepared by mixing with human pooled serum, followed by centrifugation at 3000 rpm and the pellet was resuspended in PBS buffer. Absorbance was measured at 450 nm using a Spectra MAX 340-microplate reader (Molecular Devices). Indomethacin was used as positive controls which are widely used as non-steroidal anti-inflammatory drugs (NSAIDs) for the treatment of several inflammatory diseases. IC_{50} values were calculated by comparison with the dimethyl sulfoxide (DMSO) as the blank and expressed as the % inhibition of superoxide anions produced. The percent inhibitory activity by the samples was determined against a DMSO blank and calculated using the following formula:

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

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

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