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Phytochemical and Anti-Glycationactivity of Constituents of *Astragalus bicuspis*

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Abstract: The structure of known compound, 7, 2'- dihydroxy-3', 4'-dimethoxyisoflavan (**1**) was confirmed with the help of X-ray crystallography. Antiglycation activities of isolated compounds **2-4** were determined, where compound cyclosieversigenin-3-*O*-*β*-D-xylopyranoside (**2**) showed a moderate activity (IC_{50} = 57.56 ± 10 µM), when compared with standard drug, rutin (IC_{50} = 41.6 ± 2.3 µM).

Key words: X-Ray crystallography · Anti-glycation activity · HPLC

INTRODUCTION

Astragalus Linn. (Leguminosae), one of the largest genera having about 380 species, is widely distributed in Turkey, India, Australia, Syria, North Africa, North America and in Central, Eastern and Southern Europe and Central Asia. In Pakistan, it is found in Punjab, Hazara, Balochistan and other sub-tropical and temperate regions having altitude 6,000-7,000 ft [1-3]. The various constituents of these plants are used in traditional medicine as antiperspirant, diuretic, tonic [4] and in the treatment of nephritis, diabetes, leukemia and uterine cancer [5, 6]. These plants also promote the discharge of pus and the growth of new tissues [7]. Antitumor, immunodepressant, leishmanicidaland antiviral activities have been reported from the chemical constituents of these plants [8-10].

In continuation of our detailed phytochemical studies on *Astraglusbicuspis*, we isolated compound 7, 2'- dihydroxy-3', 4'-dimethoxyisoflavan (**1**) and screened for antiglycation potentialalong with three other compounds including cyclosieversigenin-3-*O*-*β*-D-xylopyranoside (**2**), bicusposide B (**3**) and bicusposide F (**4**) [11, 12].

MATERIALS and METHODS

General Procedures: IR Spectra were recorded as KBr discs on a JASCO A-302 spectrophotometer. The ^{1}H NMR spectra were recorded on a Bruker AV-300 spectrometer operating at 300 MHz. The chemical shifts were reported in δ (ppm), referenced with respect to the residual solvent signals of MeOH/$CHCl_3$. Coupling constants (*J*) were measured in Hz. Mass spectra were recorded on a Q-STAR XL (Applied Biosystem). Each compound (4μg/mL, dissolved in acetonitrile: 0.1% $HCOOH_{aq}$ (2:1) was directly infused into the mass spectrometer at a flow rate of 3µL/min to acquire full scan and product ion mass spectra. The electrospray voltage at the spraying needle was optimized at 5200 V for positive modes of ionization.

Column chromatography (CC) was performed by using polyamide-6 DF (Riedel-De Haen AG) and silica gel (E. Merck, 160-200 μm mesh) as stationary phases. Preparative recycling HPLC (H-1 and H-2 JAIGEL columns) was also utilized. Purity of samples was checked on pre-coated silica gel TLC (20 x 20 cm, 0.5 mm thick, DC-Alugram 60 UV_{254} of E. Merck). The compounds were spotted on TLC and then spots were observed first under UV light (254 nm) and then stained with cerium (IV) sulfate spray reagent and heated until coloration developed. All reagents used were of analytical grades. HPLC solvents were used in preparative separations.

Collection of Plant Material: The *Astragalus bicuspis* Fisch (whole plant) was collected in July 2003 from Khaltaran-Haramosh regions of Gilgit (Pakistan). Plant was identified by SherWali Khan, a taxonomist at botany department, University of Karachi. The voucher specimen (# 67854) has been deposited at the Herbarium of the Botany Department, University of Karachi, Karachi.

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Extraction and Isolation: The plant material (collected during flowering season) was kept under shade and air dried for three days. The air-dried plant material (2 Kg) was chopped into thick pieces and extracted with 80% aqueous MeOH (3 x 10 L, each soaking was for 1 week). The resulting extract was filtered and concentrated. The crude methanolic extract (AB, 120 g), was subjected to Vacuum Liquid Chromatography (VLC) on silica gel (1000 g, 160-200 μm). Elution was carried out initially with hexane and then with solvents of increasing polarity: 30% $CHCl_3$: hexane (5 L x 3), 50% $CHCl_3$: hexane (5 L x 2), 70% $CHCl_3$: hexane (5 L x 3), $CHCl_3$ (5 L x 3), 30% MeOH/$CHCl_3$ (10 L x 3), 50% MeOH/$CHCl_3$ (10 L x 2) and 100% MeOH (15 L). Seven main fractions (1-7), namely Fr-1 (16 g), Fr-2 (14 g), Fr-3 (12 g), Fr-4 (8.5 g), Fr-5 (40 g), Fr-6 (10 g) and Fr-7 (14 g) were obtained.

Fraction-4 obtained initially from the VLC of methanolic extract (4AB, 8.5 g, eluted with 100% Chloroform) was chromatographed on a silica gel column (diameter 6 mm, height 200 cm) and eluted successively with acetone: hexane (20:80, 30:70, 40:60, 100:0) to afford 16 sub-fractions. Fraction-E (2 g, eluted at 30% acetone: hexane) was subjected to column chromatography, by elution with a mixture of hexane/ethyl acetate in gradient manner, afforded eleven sub-fractions. Sub-fraction-11' (4AB-11, 27 mg), was subjected to recycling preparative High Pressure Liquid Chromatography (Re-HPLC, H-1 and H-2 JAIGEL columns) by using chloroform as a mobile phase, which afforded known compound **1** (9 mg)at a flow rate of 3.5 mL/min.

Fraction-5 (40 g, eluted with 30% MeOH: $CHCl_3$), was subjected to *CC*on silica gel column (diameter 6.0, height 200 cm), by eluting successively with acetone/hexane to afford 7 sub-fractions. Sub-fraction E (5AB-5, 9 g, eluted with 60% acetone: hexane), was subjected to polyamide column chromatography (diameter 8 mm and height 200 cm) with $CHCl_3$/MeOH as eluting solvents in a gradient manner, which yielded 9 small fractions (1'-9'). Fraction-5'(5AB-5A, 4 g, eluted with 2% MeOH: $CHCl_3$), after silica gel column chromatography (height 3 mm and height 60 cm) and further elution with MeOH/$CHCl_3$(1: 10), yielded known compounds **2-4**.

Antiglycation Activity: Bovine Serum Albumin (BSA) was purchased from Research Organics (Cleveland, USA), while other chemicals [glucose anhydrous, trichloroacetic acid, sodium azide, dimethyl sulfoxide, sodium dihydrogen phosphate (NaH_2PO_4), disodium drogen phosphate (Na_2HPO_4), disodium hydrogen phosphate (Na_2HPO_4), potassium chloride, dimethyl sulphoxide and sodium hydroxide] were purchased from Sigma Aldrich. Sodium phosphate buffer (pH 7.4) was prepared by mixing Na_2HPO_4 and NaH_2PO_4 (67Mm) containing sodium azide (3 mM). Phosphate buffer saline (PBS) (pH 10) was prepared by mixing NaCl (137 mM), Na_2PHO_4 (8.1 mM), KCl (2.68 mM) and KH_2PO_4 (1.47 mM). pH was adjusted with NaOH (0.25 mM), while BSA (10 mg/mL) and glucose anhydrous (50 mg/mL) solutions were prepared in sodium phosphate buffer. Samples were prepared in DMSO (1 mg/mL) for pure compounds (1 mM/mL).

McPherson method was employed in this activity [13]. In 96-well plates assays, each well contains 60 µL reaction mixtures [20 µL BSA (10 mg/mL), 20 µL of glucose anhudrous (50 mg/ml) and 20 µL test sample]. Glycated control contains 20 µL BSA, 20 µl glucose and 20 µL sodium phosphate buffer while blank control contains 20 µL BSA and 40 µL sodium phosphate buffer. Reaction mixture was incubated at 37 °C for seven days. After incubation, 6 µL of 100% TCA was added in each well and centrifuged (15,000 rpm) for 4 minutes at 4 °C. After centrifugation, the pellet was re-washed with 60 µL 5% TCA. The supernatant containing glucose, inhibitor and interfering substance was removed and pellet contained AGE-BSA, which was dissolved in 60 µLPBS. Assesment of fluoresence spectrum (ex. 370 nm) and change in fluoresence intensity (ex. 440), based on AGEs, were monitored by using spectrofluoresence RF-1500 (Shimadzu, Japan). Rutin was used as a positive control. The percent inhibition of AGE formation in the test sample versus control was calculated for each inhibitor compound by using the following formula:

% Inhibition= (1 - fluorescence of test sample/ fluorescence of the control group) × 100

(3*r*)-7,2'-Dihydroxy-3',4'-dimethoxy Isoflavan(1): Colorless crystals, $[\alpha]^{25}_{D}$: -8° (*c* 0.1 EtOH), R*f*: 0.53 (acetone: hexane, 2: 3), EI-MS *m/z* : 302 $[M]^+$ (45%), 180 (100%), 167 (52%), 133 (39%), ^{1}H NMR (MeOH, 300 MHz):δ_H 6.16 (1H, d, $J_{8,6}$= 2.4 Hz, H-8), 6.23 (1H, dd, $J_{6,5}$= 8.2 Hz, $J_{6,8}$ = 2.4 Hz, H-6), 6.75 (1H, d, $J_{5,6}$= 8.2 Hz, H-5), 6.63 (1H, d, $J_{6',5'}$= 8.6 Hz, H-6'), 6.27 (1H, d, $J_{5',6'}$ = 8.7 Hz, H-5'), 4.12 (1H, dd, $J_{2\alpha,3}$= 9.9 Hz, $J_{2\alpha,2\beta}$= 1.7 Hz, H-2α), 3.90 (1H, t, $J_{2\beta,3/2\beta,2\alpha}$= 9.9 Hz, H-2$\beta$), 3.36 (1H, m, H-3), 2.76 (2H, dd, $J_{4\beta,3\alpha}$= 10.3 Hz, $J_{4\beta,4\alpha}$= 5.3 Hz, H-4).

RESULTS AND DISCUSSION

Structure Elucidation: Compound **1** was obtainedfrom fraction-5 (8.5 g, eluted with 100% Chloroform), which was chromatographed on silica gel column and eluted successively with acetone: hexane in gradient manner to afford eleven sub-fractions. The sub-fraction-11′ (13 mg)

Table 1.1: Activity of compound 2 in Anti-glycation Assay

Compound	Quantity	%Inhibition	IC_{50} ± SEM (µM)
2	1mM	61.2	57.56 ± 10
Rutin	1mM	85.9	41.6 ± 2.3

Table 1.2: Crystal data of 7, 2'- dihydroxy-3', 4'-dimethoxyisoflavan (**1**)

Empirical formula	$C_{17}H_{18}O_5$
Formula weight	302.31
Temperature	293 (2) K
Wavelength	0.71073 Å
Crystal system	Monoclinic
Space group	P 2 (1)
Unit cell dimensions	a = 8.9147 (19) A, $\alpha = 90°$
	b = 8.7063 (19) A, $\beta = 95.459°$
	c = 9.633 (2) A, $\gamma = 90°$
Volume	744.3 (3), A^3
Z	2
Calculated density	1.349 Mg/m^3
Absorption coefficient	0.099 mm^{-1}
F (000)	320
Crystal size	0.49 x 0.14 x 0.04 mm
Theta data for data collection	2.12 to 25°
Limiting indices	-9 < = h< = 10, -10 < = k< = 10, -11 < = l< = 9
Reflections collected / unique	3941 / 2483 [*R* (int) = 0.0217]
Completeness to theta = 25.00	99.7%
Absorption correction	None
Max. and min. Transmission	0.9960 and 0.9530
Refinement Method	Full-matrix least-square on F^2
Data / restraints / parameters	2483 / 1 / 199
Goodness-of-fit on F^2	1.090
Final R indices [I > 2 sigma (1)]	R_1= 0.0465, wR_2= 0.0891
Rindices (all data)	R_1= 0.0577, wR_2 = 0.0958
Absolute structure parameter	2.1 (13)
Largest diff. Peak and hole	0.167 and -0.122 e. $Å^{-3}$

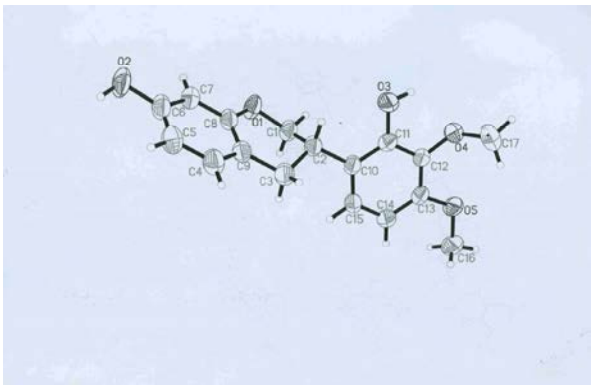

Fig. 1.1: Computer-generated ORTEP diagram of **1**.

was subjected to recycling preparative HPLC (H-1 and H-2 JAIGEL columns) by using chloroform as a mobile phase. This afforded a known compound **1** (9 mg), which was identified as (3*R*)-7,2'-dihydroxy-3',4'-dimethoxy isoflavan through single-crystal X-ray differection technique (Fig 1.1, Table 1.2).

E.I-MS of compound **1** displayed the M^+ at *m/z* 302, corresponding to the formula $C_{17}H_{18}O_5$. The base peak at *m/z* 180 ($C_{10}H_{12}O_3$) and a prominent peak at *m/z* 167 ($C_9H_{11}O_3$), were indicative of an isoflavan skeleton, substituted with OH and two methoxy groups.

The ^{1}H NMR spectrum (300 MHz, $CDCl_3$) of **1** revealed the presence of two methoxy (δ_H 3.68, 6H, s) and five aromatic protons [δ_H 6.16 (1H, d, $J_{8,6}$= 2.4 Hz, H-8), 6.23 (1H, dd, $J_{6,5}$= 8.2 Hz, $J_{6,8}$ = 2.4 Hz, H-6), 6.75 (1H, d, $J_{5,6}$ = 8.2 Hz, H-5), 6.63 (1H, d, $J_{6',5'}$= 8.6 Hz, H-6'), 6.27 (1H, d, $J_{5',6'}$= 8.7 Hz, H-5')]. Two of these aromatic protons were

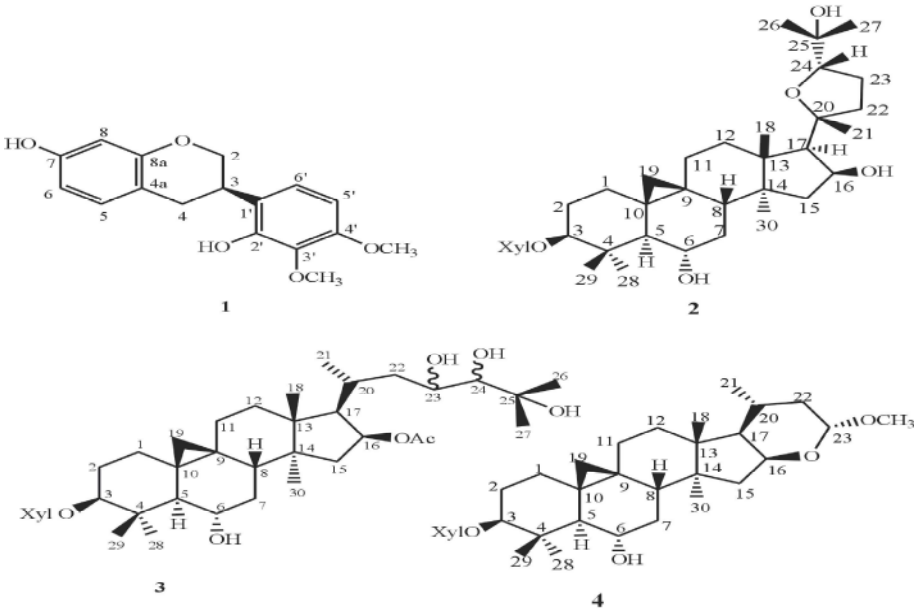

Fig 1.2: Compounds **1-4** isolated from *Astragalus bicuspis*

corresponded to an AB system with *ortho*couplings (8.6 Hz) in a tetrasubstituted benzene ring. Other signals were due to C-2 protons [δ_H4.12 (1H, dd, $J_{2\alpha,3}$= 9.9 Hz, $J_{2\alpha,2\beta}$= 1.7 Hz, H-2α), 3.90 (1H, t, $J_{2\beta,3/2\beta,2\alpha}$= 9.9 Hz, H-2$\beta$)], C-3 protons [$\delta_H$3.36 (1H, m, H-3)] and C-4 protons [δ_H2.76 (2H, dd, $J_{4,3}$ = 10.3 Hz, 5.3 Hz, H-4)], consistent with an isoflavan system. The structure was unambiguously confirmed by single-crystal x-ray data (Fig. 1.1, Table-1.2) as (3*R*)-7, 2'-dihydroxy-3', 4'-dimethoxy isoflavan, which is a known constituent of *Astragalu*s plants [14].

Anti-glycation Activity: The NH_2 groups of aminoacids and other nitrogen-containing compounds form Schiff base adducts with the carbonyl moieties of sugars. These adducts are damaged and non-functioning structures. This interaction is mainly responsible for inhibiting the formation of advanced glycation end product (AGEs) [15]. Glycated proteins are reliable markers of diseases [16]. Increased glycation can alter protein conformation and impair function by altering cross-linking of structural proteins [17]. Drugs have been developed which can open up the posibility of reversing th diabetic complications. [18, 19]. Various natural products have exhibited their anti-glycating role [20, 21]. There is a growing interest in natural products with antiglycation properties. Based on this, we have evaluated isolated cycloartane triterpenoids for their antiglycation potential *in vitro*. For our anti-glycation studies a standard, namely rutin, was used.

The activity mainly depends on the number, as well as the position of hydroxyl substituents. Compounds **2-4** are allmulti hydroxy substituted, but compound **2** showed better activity (IC_{50} = 57.56 ± 10 µM), than the standard rutin (IC_{50} = 41.6 ± 2.3 µM) (Table 1.1). The activity of compound **2** might be due to its capacity to inhibit glycoxidation more than **3** and **4**.

CONCLUSION

Phytochemical study was carried out on *Astragalus bicuspis* Fisch. The structure elucidation as wel as anti-glycation potential of isolated constituents was determined. X-Ray data remained very useful for the confirmation of the structure of 7, 2'- dihydroxy-3', 4'-dimethoxyisoflavan (**1**). Compound cyclosieversigenin-3-*O*-β-D-xylopyranoside (**2**) was found to have moderate activity (IC_{50} = 57.56 ± 10 µM), when compared with standard drug, rutin (IC_{50} = 41.6 ± 2.3 µM).

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