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Dual inhibitors of urease and carbonic anhydrase-II from *Iris* species

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Abstract: Twenty seven (1–27) known natural organic compounds were isolated for first time from two species of *Iris*, i.e. *loczyi* and *Iris unguicularis*. The structures of these compounds were deduced from the spectral data of NMR, IR, and mass spectrogram. These were evaluated against urease and carbonic anhydrase inhibition studies. For carbonic anhydrase-II inhibition studies, these compounds were evaluated by biochemical mechanism based *in vitro* bio-assay. Some compounds showed significant inhibition against CA-II enzyme. Comparatively, compound (12) showed IC_{50} value of $17.60 \pm 0.08 \mu M$ against urease enzyme, while compound (3) was found to be most active against carbonic anhydrase-II, having an IC_{50} value of $66.27 \pm 0.89 \mu M$. Izalpinin (3), 5,7-dihydroxy-2',6-dimethoxyisoflavone (9), 4',5,7-trihydroxy-6-methoxyflavanone (16), 4',5,7-trihydroxy-3',8-dimethoxyflavanone (20), 8-methoxyeriodictyol (21), and mangiferin (26) were found to be dual inhibitors of both the enzymes. The most active compounds were docked using Autodock Vina and i-GEMDOCK softwares. The docking and *in-vitro* results are in agreement which showed secondary interactions with the enzymes. The compounds can serve as therapeutic agents to treat urease and carbonic anhydrase associated disorders.

Keywords: benzene derivatives; carbonic anhydrase inhibition; Eurasia 2018; flavonoids; flavonoid glycosides; *Iris loczyi* Kan; *Iris unguicularis* Poir; steroids; terpenoids; urease inhibition; xanthon glycosides.

Introduction

Iris (Irideaceae) is a genus of bulbous herbs, widely spread in the north temperate regions of the world. They have the ability to grow in a variety of soils, but highly fragrant varieties grow in dry areas. A lot of species of *Iris* have medicinal importance. These are widely used for the treatment of various diseases, and

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are distributed throughout the world [1]. Fresh rhizomes can be freed from acidity by drying. Minor sores and pimples can be easily treated with the roots of *Iris* species. Their roots are also beneficial for the removal of nauseous obstructions [2]. Indigenous people usually used these roots for pimples and sores. The enzyme urease catalyzes the hydrolysis reaction in which urea reversibly converts into carbon dioxide and ammonia, and *vice versa* [3].

Urease is widely distributed in a variety of bacteria, fungi, and plants, and plays an important role in the circulation of nitrogen in nature [4]. Inhibition of urease activity has been extensively studied because of its potential role in disease conditions such as *Helicobacter pylori*-induced peptic ulcer, urinary lithiasis, pyelonephritis, and hepatic coma, and in other infections caused by *Proteus mirabilis* and *Yersinia enterocolitica*. Urease supports the colony formation of *Helicobacter pylori* by increasing the pH of the stomach, and therefore, plays an important role in the pathogenesis of gastritis and peptic ulcers, as well as cancer [5, 6]. Urease inhibitors are also mixed with fertilizers for controlling rapid urea degradation by soil bacteria [7, 8].

Carbonic anhydrase (EC 4.2.1.1., CA-II) catalyzes a simple physiological inter-conversion between carbon dioxide and the bicarbonate ion [9]. It is involved in certain physiological processes related to respiration and transport of CO₂/bicarbonate between metabolizing tissues and lungs. CA also plays an important role in pH and CO₂ homeostasis, electrolyte secretion, biosynthetic reactions such as gluconeogenesis, lipogenesis and ureogenesis, bone resorption, calcification, tumorigenicity, and many other physiological or pathological processes [10]. Cancer cells require a high flux of bicarbonate in the metabolic pathway, hence the expression of CA's is increased in many tumors where they provide the tumors a growth advantage over normal tissues. It is also reported that the expression of CA-I and CA-II correlates with the aggressiveness of colorectal cancer and synchronous distant metastasis. Carbonic anhydrase-II has been involved in glaucoma, epilepsy, leukemia, and cystic fibrosis [11, 12]. Due to the role of this enzyme in a number of diseases, its inhibition is considered to be therapeutically important. Several polyphenolic compounds have been reported as carbonic anhydrase-II inhibitors. A number of these inhibitors were isolated from various medicinal plants [13, 14].

Urease and carbonic anhydrase (CA) are key enzymes in the chemical reactions of living organisms, and have been found to be associated with calcification in a number of microorganisms, and invertebrates. Furthermore, *Bacillus* species have been known to produce both urease and CA in the process of biocalcification [15]. Urease increases the pH of the stomach, and thus plays an important role in the pathogenesis of gastritis, peptic ulcers and cancer. On the other hand carbonic anhydrase plays a key role in acid-base balance, CO₂ and ion transport. Any change in the carbonic anhydrase activity may cause disturbances in these processes, leading to peptic ulcers and ulcerated cancers [16].

The aim of the current study was to discover dual inhibitors of urease and carbonic anhydrase-II, as an approach to identify leads against the above cited diseases. For this purpose, compounds **1–27**, isolated from two *Iris* species, were evaluated through biochemical mechanism-based assays (Schemes 1 and 2).

Results and discussion

The present work on the ethanolic extracts of two plants, *Iris loczyi*, and *I. unguicularis*, have resulted in the isolation and characterization of twenty seven known compounds (**1–27**). Compounds **1–13** were isolated from *Iris loczyi*, and **14–27** from *I. unguicularis* were isolated. The structures of these compounds were identified on the basis of spectroscopic data as arborinone (**1**), alpinone (**2**), izalpinin (**3**), β -sitosterol (**4**), irisone A (**5**), betavulgarin (**6**), irisone B (**7**), irilin D (**8**), 5,7-dihydroxy-2',6-dimethoxyisoflavone (**9**), irilin B (**10**), tenuifone (**11**), irisoid A (**12**), ethyl- β -D-glucopyranoside (**13**), eupatorin (**14**), 5,7-dihydroxy-6-methoxychromone (**15**), 4',5,7-trihydroxy-6-methoxyflavanone (**16**), tectorigenin (**17**), kaempferol (**18**), apigenin (**19**), 4',5,7-trihydroxy-3',8-dimethoxyflavanone (**20**), 8-methoxyeriodictyol (**21**), (*E*)-methyl-4-hydroxy-3-methoxycinnamate (**22**), hispidulin (**23**), eupafolin (**24**), tectorigenin-4'-glucoside (**25**), mangiferin (**26**), and 1,3-*O*-diferuloylsucrose (**27**). These known compounds have been isolated earlier from *Iris loczyi* and *Iris unguicularis* except **8**, **14**, **19**,

Scheme 1: Compounds 1–13 isolated from *Iris loczyi*.

22, 27, and 25, which were obtained for the first time from these two species. The structures of known compounds 1–27 were characterized through comparison of their physical and spectral data with those reported in the literature [17].

Compounds 1–27 belong to seven different classes, (i) flavanoids, (ii) cinnamic acid derivatives, (iii) terpenoids, (iv) steroids, (v) glycosides, (vi) chromin, and (vii) xanthone glycoside. These include 20 flavonoid derivatives 2, 3, 5–12, 14, 16–21, and 23–25. Compounds 22 and 25 are cinnamic acids derivatives. Compounds 1 and 4 belong to terpenoids and steroids classes, respectively, while compounds 13 and 26 are glycoside and xanthone glycoside, respectively. Compound 15 belongs to the chromin class. These compounds were evaluated for their inhibitory activities against the urease and carbonic anhydrase-II enzymes. All assays were performed according to standard protocol.

Scheme 2: Compounds **14–27** isolated from *Iris unguicularis*.

Out of 20 flavonoids, **11** were found to be active against the enzyme urease, where thiourea was used as an standard. Among them, compound **12** (Irisoid A) showed an excellent inhibition of urease with IC_{50} value $17.60 \pm 0.08 \mu M$, better than the standard thiourea ($IC_{50} = 21.0 \pm 0.11 \mu M$). Compounds **8**, **9**, **20**, **21**, **23**, and **24** showed IC_{50} values in the range of 40.83 ± 0.16 – $95.53 \pm 0.17 \mu M$. The other flavonoids **2**, **10**, **16**, **17**, and **25** were only moderately active, with IC_{50} values in the range of 132.33 ± 0.38 – $367.86 \pm 2.78 \mu M$. The remaining flavonoids (**2**, **5**, **6**, **7**, **11**, **14**, **15**, **18**, and **19**) showed less than 50 % inhibition.

Table 1: Urease inhibitory activities of compounds 1–27.

Compound	IC ₅₀ $\mu\text{M} \pm \text{SEM}^a$	Compound	IC ₅₀ $\mu\text{M} \pm \text{SEM}^a$
Arborinone (1)	NA	5,7-Dihydroxy-6-methoxychromone (15)	NA
Alpinone (2)	NA	4',5,7-Trihydroxy-6-methoxyflavanone (16)	367.86 $\pm$ 2.78
Izalpinin (3)	358.23 $\pm$ 1.79	Tectorigenin (17)	170.60 $\pm$ 0.92
β -Sitosterol (4)	NA	Kaempferol (18)	NA
Irisone A (5)	NA	Apigenin (19)	NA
Betavulgarin (6)	NA	4',5,7-Trihydroxy-3',8-dimethoxyflavanone (20)	58.46 $\pm$ 0.28
Irisone B (7)	NA	8-Methoxyeriodictyol (21)	70.96 $\pm$ 0.72
Irilin D (8)	69.43 $\pm$ 0.14	(E)-Methyl-4-hydroxy-3-methoxycinnamate (22)	NA
5,7-Dihydroxy-2',6-dimethoxyisoflavone (9)	95.53 $\pm$ 0.17	Hispidulin (23)	76.20 $\pm$ 0.47
Irilin B (10)	138.70 $\pm$ 0.98	Eupafolin (24)	40.83 $\pm$ 0.16
Tenuifone (11)	NA	Tectorigenin-4'-glucoside (25)	189.16 $\pm$ 1.57
Irisoid A (12)	17.60 $\pm$ 0.08	Mangiferin (26)	152.20 $\pm$ 0.20
Ethyl- β -D-glucopyranoside (13)	NA	1,3-O-Diferuloylsucrose (27)	322.76 $\pm$ 1.03
Eupatorin (14)	NA	Thiourea	21 $\pm$ 0.011

^aSEM is the standard error of the mean. ^bThe standard inhibitors of the enzyme. NA, Not active.

Out of the two cinnamic acids derivatives (22 and 27), compound 27 (1, 3-*O*-diferuloylsucrose) showed more than 50 % inhibition (IC₅₀ = 322.76 $\pm$ 1.03 μM) against urease. The terpenoid 1, steroid 2, glycoside 13, and chromin 15 did not show any significant inhibition. The xanthone glycoside 26 was found to have an IC₅₀ value of 152.20 $\pm$ 0.20 μM . The results of the assay are presented in Table 1.

Structure-activity relationship of compounds 1–27 against urease

Compound 12 exhibited excellent inhibition potential against urease (IC₅₀ = 17.60 $\pm$ 0.08 μM) in the aforementioned *Iris* compounds. It is a peltagynoid with three hydroxyl groups. These hydroxyl groups may chelate with the Ni ions at the active site of urease. The second most potent compound, hispidulin (23), showed an IC₅₀ value 40.83 $\pm$ 0.16 μM . It is also a member of the peltagynoid class with three hydroxyl substituents. The higher activity of compound 12 as compared to compound 23 may be due to the presence of an extra ring which contains an oxygen atom. These observations indicate that the C-5 and C-7 hydroxyl groups on ring A are mainly responsible for the activity whereas the presence of methoxy group apparently decreases the inhibition potential (e.g. compound 20, IC₅₀ = 58.46 $\pm$ 0.28 μM).

Compounds 8 and 10 are isoflavones with the difference in the presence of hydroxyl group at C-2' in compound 10, while in compound 8, OH groups are present at C-3' and C-4' of ring B. The enhanced activity of 8 (IC₅₀ = 69.43 $\pm$ 0.14 μM) as compared to compound 10 (IC₅₀ = 138.70 $\pm$ 0.98 μM) may be due to this extra hydroxyl group. The structures of compounds 24 and 23 are distinctly similar. Both have hydroxyl group at *para* position, but their enzyme inhibition potential was different due to the presence of a *meta* OH in compound 24, which apparently enhances the activity (IC₅₀ = 40.83 $\pm$ 0.16 μM). This extra OH group on compound 24 may increase the chelating capacity of this compound. Cinnamic acid derivatives, 1, 3-*O*-diferuloylsucrose (27) showed a modest activity with an IC₅₀ value more than 100 μM . Mangiferin (26), a xanthone, was also found to be moderately active with an IC₅₀ of more than 100 μM .

The data presented here indicates that flavonoids have the potential to inhibit urease. The number and position of hydroxyl groups at various positions of the flavonoid skeleton apparently influence the activity. Previously we have also reported a number of new flavonoids as urease inhibitors [18].

Docking studies

The 3D structures of receptor urease (jack bean) and carbonic anhydrase II (CA-II from bovine) were retrieved from the protein data bank (PDB) with accession codes of 4GY7 and 1V9E respectively. The receptor structures

were refined and energy minimization was done through Swiss Pdb viewer v4.1.0. The 2D structures of the compounds were drawn in Chem sketch software [19–21]. The 2D structures were saved in mol format, then hydrogen addition, and energy refinement were performed through Avogadro's software. Finally, the 3D structures of the ligands were saved in PDB format. In this study, the docking were carried out through two docking programs (Autodock Vina and i-GEMDOCK) [22, 23]. Autodock Vina is a suitable software for molecular docking studies [24]. This software can be operated through Autodock tools or PyRex tools [25]. The Autodock Vina was connected with PyRex virtual screening tool. The method optimization of the software was carried out by redocking of the cocrystallized ligand, isolated from the active site of urease (4GY7). For the CA II enzyme, the docking method validation such as self-docking could not be carried because this receptor did not contain any cocrystallized ligand. The docking studies were carried out for the urease receptor first and then for the CA-II receptor. The macromolecules of urease and CA-II were uploaded in the PyRex tool which automatically removed the solvent molecules followed by hydrogen addition, and gasteiger charges calculations. The compounds pdb files were uploaded in PyRex tool linked with Autodock Vina, the receptor and compounds pdb files were converted into pdbqt format. The Grid center was positioned on the active site of receptor urease, and CAII.

The docking simulation was also carried out through i-GEMDOCKv2.1 software. This software was also brought into use for docking of the two receptors (urease and CA-II). The method of the software was also validated by redocking of the cocrystallized ligand from urease (4GY7). The procedure was set up at 70 generations per compound and the population size of 200 random individuals. The top docking conformations were carried out for twice. The software is implemented with genetic evolutionary algorithm and empirical scoring function. The active site of both the receptors were recognized at a distance of 12 Å. The software estimated the empirical scoring function as: $\text{Fitness} = \text{vdW} + \text{Hbond} + \text{Elec}$. The VDW, Hbond, and Elec stand for vander Waal energy, hydrogen bonding energy, and electro statistic energy, respectively.

The analysis of docking simulation was carried out through Discovery studio visualizer version 4.0, PyMOL version 1.7.2, and LIGPLOT+ version v.1.4.5 softwares [26–28].

Molecular docking studies were performed to understand the binding modes of the isolated compounds with best *in vitro* results, to explain the active site interactions of urease (Jack bean) and CA II (Bovine). The docking studies were carried out by using i-GEMDOCK, and Autodock vina softwares. The docking procedures of softwares were validated by redocking of the co-crystallized ligand (ligand of Urease (4GY7), but CAII did not containing any co-crystallized ligand). Generally, the hydrogen bonding and hydrophobic interactions are necessary for inhibition activities of enzymes. The docking studies revealed that all the compounds (**10**, **12**, **15**, **23** and **26** with urease) (**4**, **7**, **11**, **21** and **29**) with CAII can forms hydrogen bonding, and hydrophobic interactions with both receptors.

The docking score (Table 2) of the best compounds with urease showed good docking results than the standard thiourea. The predicted docked compounds possess are shown in Fig. 1. The docking studies predict that compound **10**, and **12** have good docking score than other compounds including standard. The interaction analysis also validates the docking result. Compound **10** (Fig. 2) forms two hydrogen bond interactions with Glu417, Meth636 with a distance of 3.11 Å and 3.20 Å, respectively. The two hydrogen bond interactions were observed from the Gln634 with a distance of 3.13 Å and 3.03 Å. Hydrophobic interactions were also

Table 2: Docking results of urease with compounds **10**, **12**, **20**, **21**, **23**, and standard thiourea.

Compound	Autodock Vina (kcal/mol) B. affinity	i-GEMDOCK (kcal/mol)			
		Total energy	VDW	HBond	Elec
10	−7.0	−105	−67	−38	0
12	−6.7	−103	−82	−21	0
20	−6.3	−91	−64	−27	0
21	−6.4	−95	−66	−29	0
23	−6.0	−89	−66	−23	0
Standard thiourea	−3.4	−44	−24	−20	0

Fig. 1: Predicted docked poses of urease with compounds (green color) **10**, **12**, **20**, **21**, **23**, co-crystallized ligand (red color), and standard thiourea (cyan color).

observed from the residues Gln413, Leu414, Meth587, Gly637, Arg638, and Val639. These interactions are responsible for the best docking result with -7.0 kcal/mol (Autodock vina score) and -105 kcal/mol (i-GEM-DOCK) of the compound **10** against the urease enzyme.

Compound **12** (Fig. 2) also forms three hydrogen bond interactions with Meth636, Gln634, and Val639 with a distance of 3.14 Å, 3.10 Å, and 3.07 Å, respectively. The hydrophobic contacts were also observed from the residues Meth587, Gly637, Arg638, and Gly640. These contacts are responsible for the good predicted interaction energies of compound **12** with urease receptor.

The docking studies of CA II were also carried out by using the two docking softwares. The docking studies (Table 3) predict that all compounds show their best docking results similar to the standard (acetazolamide), but compound **11** shows better results. The predicted docked poses of the compounds are indicated in the Fig. 3.

Compound **11** shows the docking score of -7.1 kcal/mol (Autodock Vina score), and -104 kcal/mol (i-GEM-DOCK score) against the CA II receptor. The interaction analysis indicates (Fig. 4) that compound **11** forms two hydrogen bond interactions with His63, and Asn66 with a distance of 3.11 Å, and 2.71 Å, respectively. The Gln91 residue forms two hydrogen bonds with a distance of 2.80 , and 3.07 Å. The other two hydrogen bond interactions were observed from Thr198 and Pro199 with the same distance of 2.70 Å. There are three residues which are involved in hydrophobic contacts with compound **11** are the His93, Phe129 and Pro200. These interactions are responsible for the good predicted interaction energies of compound **11** against the CA II receptor.

Compound **26** also showed good docking score against CA-II. The interaction analysis indicates that compound **26** forms two hydrogen bond interaction with Asn66, and Asp71, with a distance of 3.25 Å, and 3.02 Å, respectively. The other three hydrogen bonds were observed from Gln91 with a distance of 2.82 Å, 2.99 Å, and 2.27 Å.

Structure-activity relationship of compounds 1–27 against carbonic anhydrase-II

Twenty compounds were evaluated for their carbonic anhydrase-II inhibitory activity (Table 4). The two most active compounds, belonging to flavanoids class, izalpinin (**3**) ($IC_{50} = 66.27 \pm 0.89$ μM), and 8-methoxyeriodictyol (**21**) ($IC_{50} = 102.61 \pm 0.57$ μM), possess $-OH$ groups, particularly at C-5 of ring B. Along with this, izalpinin

Fig. 2: The 2D and 3D binding interactions of compounds (10, 12, 20, 21, 23) against urease receptor.

(3) also possesses an –OH group at C-3 of ring C, which apparently plays a key role in enhancing its activity. The flavonoids (5, 9, 16, 18, and 20) showed IC_{50} values in the range 116.39–401.39 μ M. Terpene 1, and xanthone glycoside 26 also showed IC_{50} values of 198.39 ± 3.44 , and 168.70 ± 10.6 μ M, respectively.

Table 3: Docking result of CA II with compounds **3**, **9**, **20**, **21**, **26**, and standard acetazolamide.

Compound	Autodock Vina (kcal/mol) B. affinity	i-GEMDOCK (kcal/mol)			
		Total energy	VDW	HBond	Elec
3	−6.6	−92	−73	−19	0
9	−6.1	−84	−66	−18	0
20	−7.1	−104	−81	−23	0
21	−6.7	−95	−72	−23	0
26	−6.8	−99	−75	−24	0
Standard acetazolamide	−6.1	−73	−47	−26	0

Fig. 3: The predicted docked poses of CAII with compounds (**3**, **9**, **20**, **21**, **26**) and standard acetazolamide.

The dual inhibitors have more efficiency for their target, as compare to their individual inhibitors. The dual inhibition of type-I and type-II 5 α -reductase leads to improved efficacy in the treatment of male pattern hair loss (MPHL). Dual inhibitors have potential role in cancer therapy by inhibiting human ubiquitin-specific proteases 7 (USP7) and 47 (USP47). The dual inhibition of COX-2 and 5-LOX have also anti-carcinogenic effect in the colonic tumorigenesis promoted by cigarette smoke. *Helicobacter pylori* (*H. pylori*) successfully resides in the human stomach in highly acidic conditions, causing a variety of gastroduodenal lesions, including gastric ulcer, gastric cancer and MALT lymphoma. For acid acclimation of *H. pylori*, two types of enzymes, urease and carbonic anhydrase (CA), play a central role. They cooperatively function to maintain neutral pH in the bacterial cytoplasm and periplasm. The dual inhibition of urease and carbonic anhydrase are thus valuable therapy for the above mention diseases.

This study identifies the natural inhibitors of urease and carbonic anhydrase-II. Data shows that these compounds have more selectivity towards urease, than carbonic anhydrase-II. Both enzymes are involved

Table 4: Carbonic anhydrase-II inhibitory activity of compounds 1–27.

Compound	IC ₅₀ $\mu\text{M} \pm \text{SEM}^a$	Compound	IC ₅₀ $\mu\text{M} \pm \text{SEM}^a$
Arborinone (1)	198.39 $\pm$ 3.44	5,7-Dihydroxy-6-methoxychromone (15)	NA
Alpinone (2)	NA	4',5,7-Trihydroxy-6-methoxyflavanone (16)	207.56 $\pm$ 3.08
Izalpinin (3)	66.27 $\pm$ 0.89	Tectorigenin (17)	NA
β -Sitosterol (4)	NA	Kaempferol (18)	401.39 $\pm$ 0.56
Irisone A (5)	194.68 $\pm$ 1.68	Apigenin (19)	
Betavulgarin (6)	NA	4',5,7-Trihydroxy-3',8-dimethoxyflavanone (20)	152.17 $\pm$ 3.68
Irisone B (7)	NA	8-Methoxyeriodictyol (21)	102.61 $\pm$ 0.57
Irilin D (8)	NA	(E)-Methyl-4-hydroxy-3-methoxycinnamate (22)	NA
5,7-Dihydroxy-2',6-dimethoxyisoflavone (9)	116.39 $\pm$ 1.06	Hispidulin (23)	NA
Irilin B (10)	NA	Eupafolin (24)	NA
Tenuifone (11)	NA	Tectorigenin-4'-glucoside (25)	NA
Irisoid A (12)	NA	Mangiferin (26)	168.70 $\pm$ 10.6
Ethyl- β -D-glucopyranoside (13)		1,3-O-Diferuloylsucrose (27)	NA
Eupatorin (14)	NA	Acetazolamide ^b	0.12 $\pm$ 0.03

^aSEM is the standard error of the mean. ^bThe standard inhibitors of the enzyme. NA, Not active.

in the pathogenesis of different diseases and their inhibition has therapeutic importance. Dual inhibitors of these enzymes can serve as lead molecules for further research.

Experimental

The ultraviolet (UV) spectra were recorded in methanol on Hitachi UV-3200 spectrophotometer. Optical rotations were measured on a JASCO DIP-360 polarimeter. Infrared (IR) spectra were recorded on Shimadzu FTIR-8900/Bruker Vector 22 spectrophotometer. NMR Spectra were recorded on Bruker AVANCE spectrometers at 300 and 400 MHz by using deuterated solvents (CDCl_3 , $\text{C}_5\text{D}_5\text{N}$, or CD_3OD). Mass spectra (EI-MS) were measured in an electron impact mode on Varian MAT 312 double focusing spectrometer or on JMS-600 H (Jeol, Japan) spectrometer. Fast atom bombardment mass spectra (FAB-MS) were recorded on Joel HX 110 machine. Column chromatography was performed on silica gel (E. Merck, type 60, 70–230 mesh), and pre-coated silica gel GF-254 preparative TLC plates, (20 $\times$ 20, 0.5 mm thick, E. Merck). TLC was performed on precoated silica gel plates and ceric sulfate was used as a spray reagent. Melting points were determined on a Gallenkemp apparatus, and were uncorrected.

Plant material

The whole plants of *Iris loczyi* Kan. (Irideaceae) (30 kg) (air dried) was collected from Ziarat Valley, Quetta, Pakistan, in March 2002, and identified by one of us (Prof. Dr. Rasool Bakhsh Tareen), Department of Botany, University of Balochistan, Quetta, where a voucher specimen (no. 1852) was also deposited in the herbarium.

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 of compounds from *Iris loczyi*

The dried whole plant material of *Iris loczyi* Kan. (30 kg) (air dried) was chopped and soaked in 90% EtOH + H_2O (120 L) for 15 days at 25 $^\circ\text{C}$, and after evaporation of the solvent a crude extract (1.8 kg) was

obtained. This crude extract was subjected to vacuum liquid chromatography (VLC) on silica gel, and eluted with pure petroleum ether (pet. ether), pet. Ether-CH₂Cl₂, CH₂Cl₂, CH₂Cl₂-MeOH, and finally with pure MeOH as the mobile phase. Each (1.5 L) solvent was passed through VLC column, and various fractions were collected (1 L each). On evaporation of organic solvents, six major fractions, Fr-1 to Fr-6, were obtained. The fractions were subjected to various chromatographic techniques to obtain compounds **1** (35 mg), **2** (300.4 mg), **3** (35.1 mg), **4** (15.0 mg), **5** (14.8 mg), **6** (32.3 mg), **7** (18.1 mg), **8** (15.1 mg), **9** (24.0 mg), **10** (25.7 mg), **11** (9.3 mg), **12** (45.4 mg), and **13** (45.0 mg).

Extraction and isolation of compounds from *Iris unguicularis*

The dried rhizomes of *Iris unguicularis* Poir. (1 kg) were chopped, and extracted with EtOH (10 L) at room temperature for 2 weeks, and the resulting extract was concentrated to a gum (250.3 g). This gum was dissolved in distilled water (3 L) and defatted with pet. ether (5 × 3 L) to obtain 10.4 g of pet. ether extract. The defatted aqueous extract was further fractionated with CH₂Cl₂ (5 × 3 L), EtOAc (5 × 3 L), and BuOH (5 × 3 L). On evaporation of solvents, 42.3, 45.5, and 12.4 g of extracts were obtained, respectively. Six compounds **14** (15.8 mg), **15** (12.5 mg), **16** (10.1 mg), **17** (9.8 mg), **18** (12.4 mg), and **19** (6.6 mg) were isolated from the CH₂Cl₂ extract, while eight compounds **20** (15.2 mg), **21** (12.4 mg), **22** (6.4 mg), **23** (20.1 mg), **24** (6.7 mg), **25** (7.1 mg), **26** (54.2 mg), and **27** (10.2 mg) were obtained from the EtOAc extract by using repeated column chromatography (silica gel) and preparative recycling HPLC (ODS column).

Urease assay and inhibition

Reaction mixture comprising 25 µL of enzyme (*Canavalia ensiformis* urease) solution and 55 µL of buffers containing 100 mM urea were incubated with 5 µL of test compounds (0.5 mM concentration) at 30 °C for 15 min in 96-well plates. Urease activity was determined by measuring ammonia production by using the indophenol method, as described by Weatherburn. Briefly, 45 µL each phenol reagent (1% w/v phenol and 0.005% w/v sodium nitroprusside) and 70 µL of alkali reagent (0.5% w/v NaOH and 0.1% active chloride NaOCl) were added to each well. The increasing absorbance at 630 nm was measured after 50 min by using a microplate reader (Spectra Max, Molecular Devices, CA, USA). All reactions were performed in triplicate in a final volume of 200 µL. The results (change in absorbance per min) were processed by using soft Max Pro software (Molecular Devices, CA, USA). The entire assays were performed at pH 6.8. Percentage inhibitions were calculated from the formula $100 - (\text{OD}_{\text{testwell}} / \text{OD}_{\text{control}}) \times 100$. Thiourea was used as the standard inhibitor of urease.¹³

Carbonic anhydrase assay and inhibition

The experiment was run with the buffer containing HEPES-*Tris* solution at a total concentration of 20 mM and pH 7.2–7.9. For this, 140 µL of the HEPES-*Tris* solution was mixed with 20 µL of freshly prepared aqueous solution of purified bovine erythrocyte CA-II (0.1–0.2 mg/2000 µL of deionized water for 96-well), Fluka MP Bio-medicals. The test compound was dissolved in 10% DMSO, out of this 20 µL was added in a reaction mixture, followed by the addition of 4-NPA at concentration of 0.8 mM diluted in ethanol. The reaction was initiated by addition of 4-NPA after 15 min incubation of test compound. The compounds were tested in triplicate. In this assay, the reaction was performed by using 96-well plates. To initiate the reaction, the plate was placed in a microplate reader and the amount of reaction product formed was monitored at 1 min interval for 30 min at 400 nm. The reaction temperature was kept between 25 and 28 °C.

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