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Characterization of one Novel Flavone and four New Source Compounds from the Bark of *Millettia ovalifolia* and In-Vitro Inhibition of Carbonic Anhydrase-II by the Novel Flavonoid

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Abstract: The phytochemical examination of the extract of bark of *Millettia ovalifolia* yielded chemical constituents, which included one novel flavonoid 7-(4-methoxyphenyl)-9H-furo [2, 3-f] chromen-9-one and four new source compounds characterized as 3,7-Dihydroxy-2-phenyl-4H-chromen-4-one, (E)-Ethyl-13-(3,4-dimethoxyphenyl)acrylate, (E)-Methyl-3-(3,4-dimethoxyphenyl)acrylate and N-Ethylacetamide. These compounds were characterized by using advance modern spectroscopic analytical techniques such as UV, IR, 1D, 2D NMR and mass spectrometry. The novel flavonoid (1) displayed significant inhibition of cytosolic form of bovine carbonic anhydrase-II with IC_{50} value of 17.86 ± 0.07 µM. This flavonoid may be used as a new pharmacophore to treat cystic fibrosis, glaucoma, epilepsy, leaukomia and other disorders such as neurology etc.

Keywords: *Millettia ovalifolia* bark; phytochemicals; spectral analysis; novel flavonoid; bovine carbonic anhydrase inhibition.

1. Introduction

The carbonic anhydrases are zinc-containing metallo-proteins, extensively found in almost all life kingdoms [1]. Almost 16 carbonic anhydrase isoforms are recognized in mammals that vary in their sub cellular localizations as well as in catalytic activity [2,3]. These enzymes are proficient catalysts for reversible hydration of carbon dioxide to bicarbonate and thus involved in vital physiological processes like breathing, pH and CO_2 homeostasis, secretion of electrolytes, calcification, bone resorption, bio-synthetic reactions (e.g. lipogenesis, ureagenesis, and gluconeogenesis), tumorigenicity and many other physiological processes [2,4]. The most protruding class of CA inhibitors is aromatic/heterocyclic sulfonamides which have been studied for the development of antitumor, antiglaucoma, antiobesity and anticonvulsant drugs [5,6]. There is a continuous need for novel CA inhibitors belonging to diverse classes of compounds. Such natural compounds then may lead to impending pharmacological applications.

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The genus *Millettia* belongs to family Leguminosae (Papilionaceae); consists of about 150 species distributed in the tropical and sub-tropical regions of the world. Only two species are present in Pakistan i.e. *M. extensa* and *M. ovalifolia* [7]. The genus *Millettia* is well known for its medicinal importance and variety of pharmacological functions. The major class of compounds of this genus is flavonoids. The plant *M. conraui* is well known for its insecticidal, molluscicidal and pesticidal activities [8], while *M. pachycarpa* showed significant inhibition of murine retroviral reverse transcriptase and human DNA polymerases [9]. Compounds; rotenone and 3*α*-hydroxyrotenone isolated from *M. pervilleana* have shown inhibition of TPA-induced ornithine decarboxylase at the level of its m RNA expression and recommended as promising cancer chemo-preventive agents. The prenylated isoflavanone, pervilleanone from the same plants also showed anticancer activity [10]. The flavonoids; chalcones isolated from *M. ovalifolia* showed anti-malarial activity [12], while this plant also contains hypotensive agents [11]. Isoflavonoids, griffonianone and maximaisoflavone charcterized from *M. griffoniana* showed significant cytotoxicity [13]. *M. dura* contains 6α,12α-didehydro-6-oxodeguelin has good insecticidal activity [14]. *M. griffoniana* contains griffonianone C which showed a potent estrogenic activity [13]. The compound Millepurone was reported from *M. atropurpurea* has shown promising antitumor activity [15]. Another flavonoid Osajin isolated from *M. auriculata* showed anti-oxidant activity [16] while *M. duchusnei* contains rotenones used as potent insecticides. In some parts of Africa especially in Cameroon the plants of genus *Millettia* are used by different communities as a potent inhibitor of intestinal parasites in children as well as colic besides oral treatment for boils [17]. *M. ovalifolia* is locally known as shewa (in Pashto), villayati shisham (in Urdu), and rose wood (in English) and this plant widely used in by the communities for different medicinal purposes [7, 8]. Keeping in view the medicinal importance of the genus *Millettia*, the plant *Millettia ovalifolia* was investigated to find biologically active phytochemicals. This research includes the isolation of secondary metabolite and evaluation of a novel flavonoid as inhibitor of carbonic anhydrase-II. This enzyme play crucial role in the treatment of cystic fibrosis, glaucoma, epilepsy, leaukomia and other disorders such as neurology etc.

2. Materials and Methods

2.1. Carbonic anhydrase activity

CA-II activity was conducted at 400 nm via the hydrolysis of 4-Nitrophenyl acetate, a colorless compound transformed into yellow colored compound 4-Nitrophenol. In this assay purified bovine erythrocyte CA-II was liquefied in de-ionized water and 4-Nitrophenyl acetate was used as a substrate. The overall reaction volume of 200 µL contained 140 µL HEPES TRIS buffer, 20 µL freshly prepared bovine erythrocyte CA-II (0.1mg/mL), 20 µL test compound is incubated for 15 minutes and the absorbance is restrained by accumulation 20 µL of substrate at 0.7 mM. The reaction mixture was kept at 25^{o}C for 30 mints [21, 22]. All the chemicals were procured from sigma Aldrich. The spectrophotometer, spectra max 384 from molecular devices (USA) was used for the determination of *in-vitro* inhibition of CA-II. The IC_{50} values were deliberated by using EZ- fit enzyme kinetics software USA.

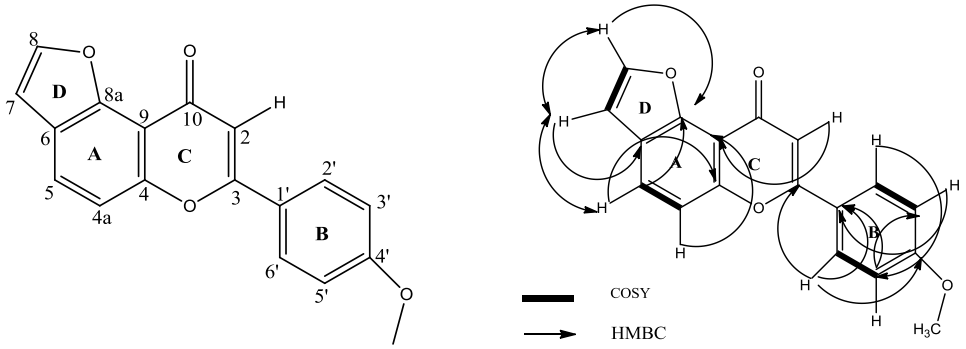
2.2. Plant material

The bark of *M. ovalifolia* was collected during the month of June, 2008 from Pakistan Forest Institute, Peshawar. The plant was identified and authenticated by Dr. Samin Jan, Associate Professor, Department of Botany, Islamia College University, Peshawar, Pakistan. The voucher specimen (SJ-33) was deposited in the herbarium of Botany Department of the University.

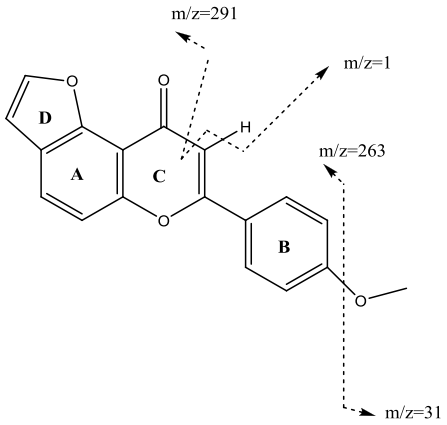
2.3. Extraction and isolation

The stem bark was shade dried and powdered (70 kg) were soaked in 5% aqueous methanol for one week (x3). The combined brownish extract was concentrated under reduce pressure in a vacuum rotary evaporator and brownish residue F1 (5 kg) was obtained, which was suspended in water and partitioned with *n*-hexane to get *n*-hexane fraction FX (1.2 kg). The *n*-hexane insoluble portion was acidified with HCl (pH 2) and subjected to further fractionation with ethyl acetate (x3). This afforded ethyl acetate fraction FX1 (1.0 kg),

while remaining insoluble fraction was basified with ammonia (pH 8) and fractionated with chloroform. Two fractions, chloroform fraction FX2 (1.6 kg) and insoluble fraction FX4 (0.8 kg) were thus obtained. These fractions were studied for antifungal, antibacterial, phytotoxic and insecticidal activity. The chloroform fraction (500 g) was subjected to column chromatography using silica gel with *n*-hexane-chloroform increasing polarity to obtain several fractions, which were combined on the basis of TLC profiles and this yielded six sub fractions (1-6). Fractions 3 (14.3 g), 4 (25 g) and 5 (19.7 g) were combined on the basis of TLC profiles and rechromatographed using silica gel and *n*-hexane-chloroform in increasing polarity to furnish 56 fractions. Fractions 20-40 were combined based on the basis of similarities of TLC profiles and purified by Prep TLC to afford six compounds including one novel 7-(4-methoxyphenyl)-9H-furo [2,3-f] chromen-9-one and five new source compounds.



1a **1b**



1c

Figure 1. Structure **(1a),** selected key COSY ($^{1}H-^{1}H$) and HMBC (H→C) interactions **(1b),** and mass fragmentations **(1c)** of compound **1.**

3. Results and Discussion

3.1. Structure elucidation

Compound 1, a new flavone was isolated as yellow powder. The ESI-MS and CI showed the molecular ion [M]+ peak at m/z 293 (292) and 291 (292) corresponding to the molecular formula $C_{18}H_{12}O_4$. The HRMS spectrum recorded on 123 Jeol JMS HX 110 mass spectrometer indicated m/z 293 (calcd. 292 for $C_{18}H_{12}O_4$).The IR absorption bands at 1641 cm^{-1} indicated the presence of carbonyl group. The UV spectrum showed maximum absorption at 247 nm indicating the presence of conjugated system. The

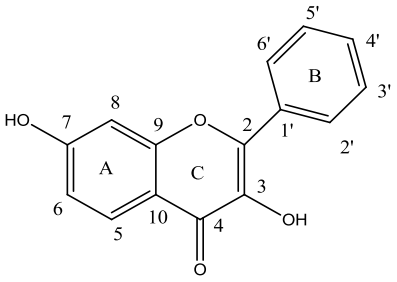
spectral data (Table-1) of **1** indicated the presence of a singlet at δH 6.92, (H-2), and 4'-methoxylated pattern for ring B, where ortho-ortho/meta-meta coupled doublets at δH 7.45, (d, J = 9) and δH 6.98, (d, J = 9) of ring B protons signals resonated. The resonance signals appeared as a doublet at δ_H 7.05, (d, *J* = 10) and δ_H 8.05, (d, *J* = 10) were assigned to H-4a and H-5 of ring A. The peaks appeared at δ_H 6.83, (d, *J* = 10) and δ_H 7.47, (d, *J* = 10) were accredited to the protons resonance signals due H-7 and H-8 of ring D. The singlet at δ_H 3.61 was due to the methoxy protons present at C-4′ of ring B. The stereochemistry of proton of ring D and the presence of methoxy group at C-4′ of ring B was confirmed by NOE difference experiment and HMBC (Figure-1and 2) correlation. The ^{13}C NMR (BB and DEPT) spectra (Table-2) of **1** revealed signals for eighteen carbons. Eight of them were resonated at δc 160.1, 158.1, 113.2, 164.2, 107.1, 178.9, 126.2 and 165.1 were assigned to C-3, C-4, C-6, C-8a, C-9, C-10, C-1'and C-4' respectively, while seven carbon atoms appeared at δ_C103.5, 104.1, 125.1, 147.3, 105.1, 130.2 and 116.2 were assigned to quaternary carbons at C-2, C-4a, C-5, C-7, C-8, C-2′, C-6′,C-3 and C-5′ respectively and one methoxy carbon exhibited δc 55.2. The HMBC correlations (Figure-1 and 2) of H-2 (δ_H 6.92) to C-9 confirmed the connectivity assigned in ring C, While the H-4a (δ_H 7.05) showed correlation with C-9 and H-5 (δ_H 8.05) showed HMBC connection with C-7, while H-8 (δ_H 6.75), H-2′(δ_H 7.0) and H-6′ (δ_H 7.0) showed HMBC correlation with C-4, C-8a respectively. Similarly H-7 (δ_H 6.83) and H-8 (δ_H 7.47) showed HMBC connectivity with C-6 and C-8a of ring D. The H-6′ (δ_H 7.45), H-5′ (δ_H 8.05), H-3′ (δ_H 6.98) and H-2′ (δ_H 7.45) displayed HMBC correlations with (C-3, C-1′), (C-3′, C-1′), (C-5) and (C-5) respectively. The methoxy protons (δ_H 3.61) are connected with both C-3′ and C-5′. The combined ^{1}H-^{1}H COSY (Figure-1 and 2) correlation of **1** can be demonstrated as H-2'/H-3; /H-6'/H-5'; H-4a/H-5 and H-7/H-8 respectively. The NOE spectrum showed the relative configuration at C-8, C-7 and C-5. The NOE experiment revealed β configuration of H-8 (7.47, d,) and H-7 (6.83, d) with respect to ring A by cross peak in the NOE spectrum. The structure was further authenticated by different mass fragments (Figure-1 and 2) observed in the mass spectrum at *m/z* 292 [M^+], 291 [M^+-H] and 263 [M^+-OCH_3]. All the information derived from the spectral data of **1** was characterized as 7-(4-methoxyphenyl)-9H-furo [2, 3-f] chromen-9-one.

Compound **2** was obtained as yellow amorphous powder and the EI-MS showed molecular ion $[M]^+$ peak appeared at *m/z* 254 corresponding to molecular formula $C_{15}H_{10}O_4$. The IR spectrum displayed absorption bands at 3227 (OH) and 1628 cm^{-1} (C = O) groups and the UV spectrum showed maximum absorptions at 266 nm and 211 nm indicating the presence of conjugated system. The spectral data (Table-1) showed the presence of a hydroxyl group at C-7 in ring A while ring B was unsubstituted. All the protons of ring B appeared as a multiplets at δ_H 7.57, while the protons of ring A resonated as two dublets at δ_H 6.65 (d, *J* = 9 Hz, H-8), δ_H 6.14 (d, *J* = 7.8, H-5) along with a multiplet at δ_H 7.54. The spectral data were in accordance with those reported in literature for 3,7-dihydroxy-2-phenyl-4H-chromen-4-one [18].

Compound **3** was isolated as colorless sticky liquid and the EI-MS showed the characteristic molecular ion $[M]^+$ peak observed at *m/z* 236 corresponding to molecular formula $C_{13}H_{16}O_4$. The IR spectrum displayed absorption bands at 2918(C-H), 1673 (C=O) and 1608 (aromatic C= C) and the UV spectrum showed maximum absorption at 313 nm. The ^{1}H NMR spectrum (Table-1) showed aromatic signals at δ_H 7.01 (1H, d, *J* = 2 Hz, H-2), 6.82 (1H, d, *J* = 8 Hz, H-5) and 7.05 (1H, dd, *J* = 8, 2 Hz, H-6) along with a signal at δ_H 3.81 (6H, s) for the two methoxy groups indicated a tri-substituted benzene ring. The signals at δ_H 7.57 (1H, d, *J* = 16 Hz) and 6.27 (1H, d, *J* = 16 Hz) attached to carbons at δ_C 144.4 and 115.9 along with 167.1 for an ester carbonyl group confirmed the presence of a 3,4-dimethoxy-*trans*-cinnamate moiety in the molecule. The signals displayed at δ_H 4.21 (q, *J* = 7 Hz) and δ_H 1.28 (t, *J* = 7 Hz) were ascribed to the methylene (H-10) and methyl (H-11) of ethoxy group at C-9**.** The spectroscopic data analysis leads to establish the following structure of this compound. The ^{13}C NMR (BB and DEPT) spectrum (Table-2) confirmed the presence of a dimethoxycinnamate derivative with resonances attributed to a carbonyl group at δc 167.1 (C-9) and two deshielded oxygen bearing quaternary carbon. The assignments given above deduced the structure of **3** as (*E*)-ethyl 3-(3,4-dimethoxyphenyl) acrylate [19].

Compound **4** was obtained as colorless sticky liquid and the EI-MS showed molecular ion $[M]^+$ peak at *m/z* 222 corresponding to molecular formula $C_{12}H_{13}O_4$. The IR spectrum displayed absorption bands at 2910 (C-H), 1685(C=O) and 1611(aromatic C=C) and the UV spectrum showed maximum absorption at 278 nm. The ^{1}H-NMR spectral data (Table-1) of **4** displayed aromatic signals at δ_H 7.03 (1H, d, *J* = 2 Hz, H-2), 6.84 (1H, d, *J* = 8 Hz, H-5) and 7.07 (1H, dd, *J* = 8, 2 Hz, H-6) for the tri-substituted benzene ring. A six proton singlet resonated in ^{1}H-NMR spectrum at δ_H 3.88 for the two methoxy groups substituted on the benzene ring at C-3 and C-4. The signals at δ_H 7.59 (1H, d, *J* = 16 Hz, H-7) and 6.28 (1H, d, *J* = 16 Hz, H-8) attached to carbons at δ_C 144.6 and 115.3 along with 167.4 for ester carbonyl group confirmed the

presence of a 3,4-dimethoxy cinnamate moiety having trans configuration in the molecule. A singlet resonated at δ_H 1.31 (3H) was assigned to the methoxy group at C-9 [19]. The spectroscopic data analysis leads to establish the following structure of this compound. The structure of **4** was deduced as (*E*)-Methyl 3-(3, 4-dimethoxyphenyl) acrylate.



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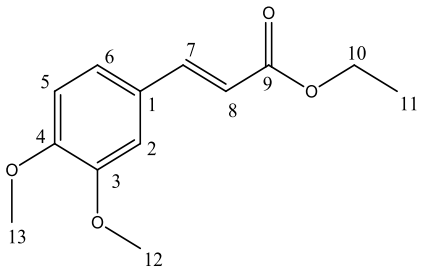

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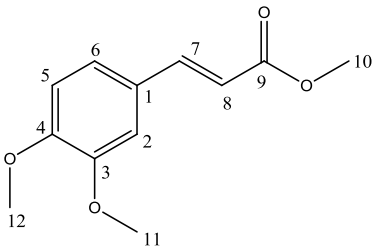

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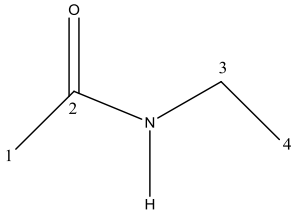

5

Compound **5** was isolated as yellowish amorphous powder and the EI-MS showed molecular ion $[M]^+$ peak at *m/z* 87.1 corresponding to molecular formula C_4H_9NO. The IR absorption displayed ketone group at 1696 and amide group at 3300 cm^{-1}. The ^1H NMR data showed signals for two methyls one methine and amide protons. The spectrum (Table-1) of **5** showed quartet appeared at δ_H3.01 (q, J = 7Hz, H-3) and a singlet displayed at δ_H1.89 (s, H-1) for terminal methyl. One triplet observed at δ_H1.28 (t, J = 7.2 Hz, H-4) ascribed to another terminal methyl group. The spectroscopic data analysis clues to establish the following structure of this compound. The ^{13}C NMR (BB and DEPT) spectrum of **5** showed total of four carbon atoms; include two methyl, one methine and one carbonyl carbon. The carbonyl C-2 was identified from its characteristic signal displayed at δc179.9, while C-3 was resonated at δc 43.3. The two methyl C-1 and C-4were displayed at δc23.9 and δc11.5 respectively. These assignments given above were compaired with the reported value for N-ethylacetamide [20]. The structures of all these compounds were established by advance spectral techniques. The data obtained revealed that this plant is very important from medicinal point of view. The current study discussed above aims to reveal the hidden chemical potential of this plant, which in turns help to explore its medicinal importance. The phytochemical study will help community to control various diseases and develop a method how to isolate and use phytochemicals for their benefits.

Table 1. ^1H NMR data for compounds **1-5** (at 400 and 500 MHz in MeOD and $CDCl_3$, δ in ppm, J in Hz).

Positions (H)	**1**	**2**	**3**	**4**	**5**
1	-	-	-	-	1.89 (s)
2	6.92 (s)	-	7.01 (d, *J* = 2)	7.03 (d, *J* = 2)	-
3	-	-	-	-	3.01 (q, *J* = 7)
4	-	-	-	-	1.28 (t, *J* = 7.2)
4a	7.05 (d, *J* = 10)	-	-	-	-
NH	-	-	-	-	(s)
5	8.05 (d, *J* = 10)	6.14 (d, *J* = 7.8)	6.82 (d, *J* = 8)	6.84 (d, *J* = 8)	-
6	-	7.54 (m)	7.05 (dd, *J* = 8, 2)	7.07 (dd, *J* = 8, 2)	-
7	8.05 (d, *J* = 10)	-	7.57 (d, *J* = 16)	7.59 (d, *J* = 16)	-
8	7.47 (d, *J* = 10)	6.65 (d, *J* = 9)	6.27(d, *J* =16)	6.28 (d, *J* =16)	-
8a	-	-	-	-	-
9	-	-	-	-	-
10	-	-	4.21 (q, *J* = 7)	1.31 (t, *J* = 7)	-
11	-	-	1.28 (t, *J* = 7)	3.88 (s)	-
12	-	-	-	3.88 (s)	-
1′	-	-	-	-	-
2′	7.45 (2H, d, *J* = 9)	7.57 (m)	-	-	-
3′	6.98 (2H, d, *J* = 9)	7.57(m)	-	-	-
4′		7.57(m)	-	-	-
OCH_3	3.61 (s)				
5′	6.98 (2H, d, *J* = 9)	7.57(m)	-	-	-
6′	7.45 (2H, d, *J* = 9)	7.57(m)	-	-	-

Table 2. ^{13}C NMR data for compounds **1-5** (at 100 and 1250 MHz in MeOD and $CDCl_3$, δC in ppm).

Positions (C)	**1**	**2**	**3**	**4**	**5**
1	-	-	127.4(C)	127.4(C)	23.9(CH_3)
2	103.5(CH)	136.3(C)	109.7(CH)	109.7(CH)	179.9(C)
3	160.1(C)	113.1(C)	149.2(C)	149.2(C)	43.3(CH_2)
4	158.1(C)	171.3(C)	151.1(C)	151.1(C)	11.5(CH_3)
4a	104.1(CH	79.5(C)	-	-	-
5	125.1(CH)	108.3(CH)	111.5(CH)	111.5(CH)	-
6	113.2(C)	129.9(CH)	122.5(CH)	122.5(CH)	-
7	147.3(CH)	163.6(C)	144.4(CH)	144.6(CH)	-
8	105.1(CH)	134.6(CH)	115.9(CH)	115.3(CH)	-
8a	164.2(C)	163.3(C)	-	-	-
9	107.1(C)	-	167.1(C)	167.4(C)	-
10	178.9(C)	-	60.2(CH_2)	14.3(CH_3)	-
11	-	-	14.2(CH_3)	55.9(CH_3)	-
12	-	-	-	55.9(CH_3)	-
1′	126.2(C)	79.5(C)	-	-	-
2′	130.2(CH)	130.0(CH)	-	-	-
3′	116.2(CH)	130.0(CH)	-	-	-
4′	165.1(C)	130.0(CH)	-	-	-
5′	116.2(CH)	130.0(CH)	-	-	-
6′	130.2(CH)	128.9(CH)	-	-	-
OCH_3	55.2(CH_3)	-	-	-	-

3.2. Carbonic anhydrase-II inhibition

The novel flavonoid (**1**) isolated from *Millettia ovalifolia* bark was evaluated for the carbonic anhydrase activity. The assay was performed using purified bovine erythrocyte and the results were expressed as IC_{50} value. Zonisamide was used as a positive control for this activity. The results found are

summarized in Table-3, which showed that (**1**) exhibited substantial inhibition of cytosolic form of bovine carbonic anhydrase-II with IC_{50} value of 17.86 ± 0.09 μM against standard inhibitor (zonisamide). Literature studies showed that some compounds like Benz imidazole-based zinc complexes were used as structural carbonic anhydrase models. Furthermore, benzimidazole and indapamide-like benzene sulfonamides have been reported as Rho kinase and carbonic anhydrases I, II, VII, and VIII, respectively [23-25].The compound (**1**) compound may be employed an innovative pharmacophore for the treatment of cystic fibrosis, leaukomia, glaucoma, epilepsy and other be neurological disorders.

Table 3. *In vitro* CA-II inhibition result of compound **1**.

S. No	Compound	IC_{50}[a] µM ± SEM[b]
1	**1**	17.86 ± 0.09
2	Zonisamide (standard)	1.86 ± 0.03

a- Minimum inhibitory concentration
b- Standard error mean

Acknowledgments

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Supporting Information

Supporting Information accompanies this paper on http://www.acgpubs.org/RNP

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