Aervfuranoside: A New Chlorinated Dibenzofuran Glycoside and other Metabolites from the Flowers of *Aerva javanica*

[1,2] Sara Musaddiq, [1] Muhammad Saqib Saeed, [1] Naheed Riaz, [3] Mamona Nazir
[3] Tasneem Khatoon and [1] Abdul Jabbar
[4] Tayaba Ismail, [1] Muhammad Ashraf, [2] Samia Khakwani, [1] Abida Ashraf and [1] Muhammad Saleem*
[1]*Department of Chemistry, Baghdad-ul-Jadeed Campus, The Islamia University of Bahawalpur, Bahawalpur 63100, Pakistan.*
[2]*Department of Chemistry, Kutchehry Campus, The Women University Multan, Multan 60000, Pakistan.*
[3]*Department of Chemistry, Government Sadiq College Women University, Bahawalpur, Bahawalpur 63100, Pakistan.*
drsaleem_kr@yahoo.com; m.saleem@iub.edu.pk*

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Summary: Aervfuranoside (**1**), a new chlorinated dibenzofuran glycoside together with ten (**2-11**) known compounds were isolated from ethyl acetate soluble fraction of methanolic extract of the flowers of *Aerva javanica*. The structures of isolates were elucidated by the combination of 1D (^{1}H and ^{13}C-NMR), 2D (HMQC, HMBC and COSY) NMR spectroscopy and mass spectrometry (FABMS, HRFABMS) or in comparison with the literature data in case of known metabolites. Among these isolates, only compounds **1-3** were evaluated for DPPH free radical scavenging potential and enzyme inhibitory activity against acetylcholinesterase (AChE), butyrylcholinesterase (BChE), lipoxygenase (LOX) and metallo-*β*-lactamase (IMP-1). The test compound **2** exhibited moderate inhibition of the enzyme lipoxygenase with the IC_{50} value of 53.5 µM.

Key words: *Aerva javanica*, Secondary Metabolites, Characterization, Enzyme Inhibition

Introduction

Aerva javanica is a member of the botanical garden belonging to Cholistan Desert. Ethnobotanical studies revealed that it is used against bladder and kidney stones [1]. The roots of this plant are chewed for teeth cleaning and the paste of roots is applied to remove acne from the face, whereas, the seeds are used to ease headache [2]. The folk use of *A. javanica* prompted us to investigate this plant for its bioactive secondary metabolites. Previously, we have isolated acylated flavonoids and ecdysteroids from this species [3,4]. In the present study, the chromatographic purification of the methanolic extract of the flowers of *A. javanica* resulted in the isolation of a new chlorinated dibenzofuran glycoside; aervfuranoside (**1**), along with kaempferol-3-*O*-*β*-D-(6-*E*-*p*-coumaroyl)-glucoside (**2**) [5], allantoin (**3**) [6], mannitol (**4**) [7], indole acetic acid (**5**) [8], (2*S*,3*S*,4*R*,8*Z*)-2-[(2*R*)-2-hydroxypentacosanoylamino]-8-octadecene-1,3,4-triol-1-*O*-*β*-D-glucopyranoside (**6**) [9], (2*S,*3*S,*4*R,*14*E*)-2-[(2*R*)-2-hydroxyoctadecanoyl] amino}tetraeicos-14-ene-1,3,4-triol-1-*O*-*β*-D-glucopyranoside (**7**) [10], *β*-sitosterol (**8**) [11], *β*-sitosterol 3-*O*-*β*-D-glucopyranoside (**9**) [11], oleanolic acid (**10**) [12] and lupeol (**11**) [13] (Fig. 1).

Experimental

General experimental procedures

Column chromatography was carried out using silica gel of 70-230 and 230-400 mesh size. Aluminum sheets pre-coated with silica gel 60 F_{254} (20 × 20 cm, 0.2 mm thick; E. Merck) were used for TLC to check the purity of the compounds and were visualized under UV light (254 and 366 nm) and or by spraying of ceric sulfate followed by heating. The UV spectra were measured on a Hitachi UV-3200 spectrometer (λ_{max} in nm). The IR spectra were recorded on Shimadzu IR-460 spectrophotometer (υ in cm^{-1}). FABMS and HRFABMS data were measured on Jeol JMS-HX 110 spectrometer with data system. The ^{1}H-NMR spectra were scanned on Bruker AMX-400 MHz instruments using TMS as an internal reference. The chemical shift values are reported in ppm (*δ*) units and the scalar coupling constants (*J*) are in Hz. The ^{13}C-NMR spectra were recorded at 100 MHz on the same instrument. The 2D NMR data was also measured on the same instrument operating at a frequency of 400 MHz.

Plant material

The flowers of *Aerva javanica* Burm were collected from Cholistan Desert (District Bahawalpur, Pakistan) in September 2010 and were identified by Dr. Muhammad Arshad (late), Plant Taxonomist, Cholistan Institute for Desert Studies (CIDS), The Islamia University of Bahawalpur, Pakistan, where a voucher specimen (AJ/CIDS-10-102) is deposited.

*To whom all correspondence should be addressed.

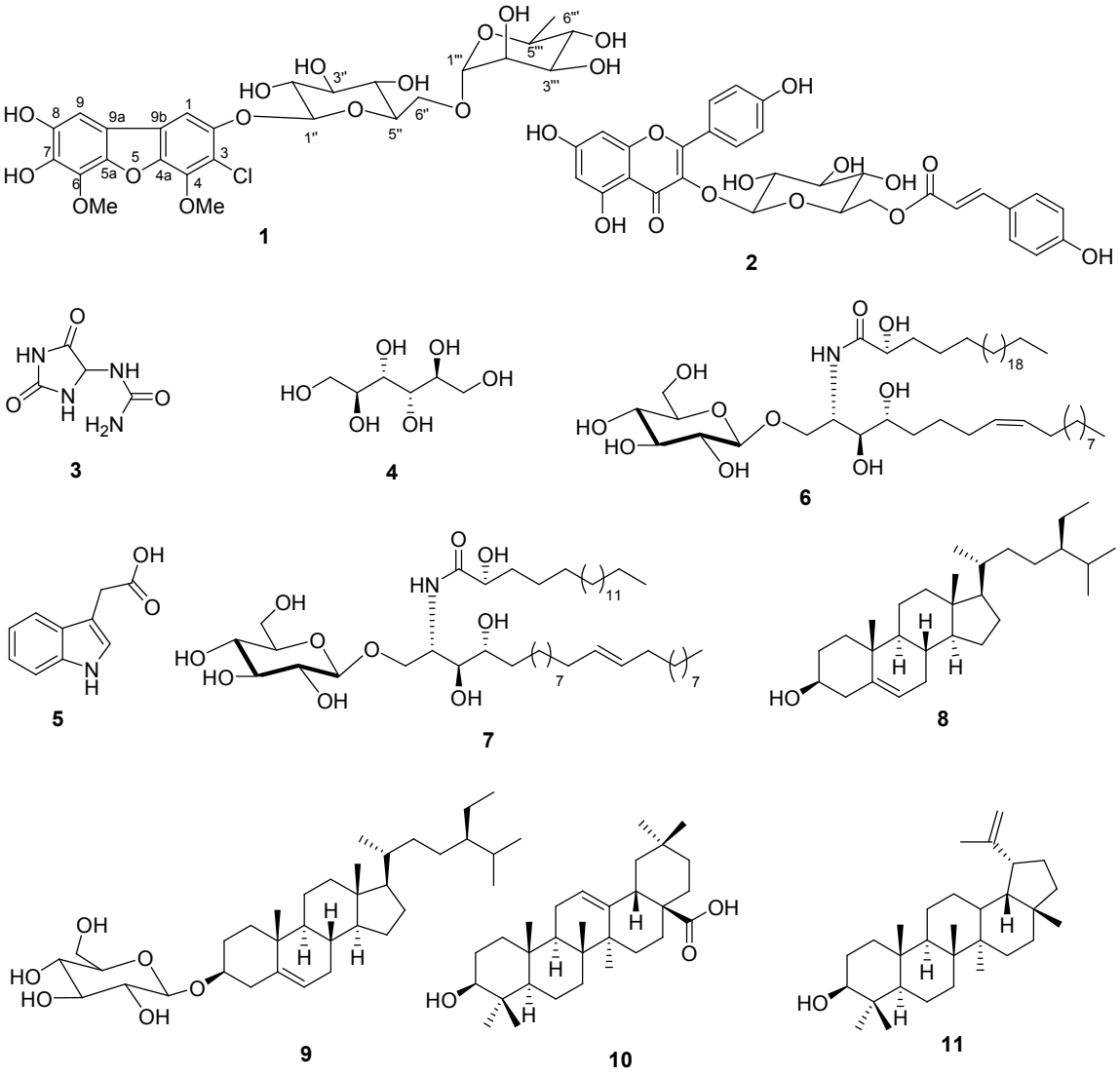

Fig. 1: Structures of the compounds isolated from *Aerva javanica.*

Extraction and Isolation

The shade dried flowers of *A. javanica* (4 kg) were extracted thrice with MeOH (15 L). The combined MeOH extract was concentrated under vacuum, and the residue (250 g) was divided in to *n*-hexane (70 g), chloroform (50 g), ethyl acetate (55 g), *n*-butanol (25 g) and water-soluble (50 g) fractions. The ethyl acetate soluble fraction (55 g) was subjected to column chromatography over silica gel eluting with *n*-hexane, *n*-hexane-$CHCl_3$, $CHCl_3$ and $CHCl_3$-CH_3OH in increasing order of polarity to get ten fractions (A-J). The Fraction H (15 g) obtained with $CHCl_3$:CH_3OH (9.5:0.5) was subjected to silica gel column chromatography eluted with a gradient of *n*-hexane:EtOAc to get six sub fractions (H_1-H_6). The sub fraction H_6, on repeated column silica gel chromatography using an isocratic of $CHCl_3$:CH_3OH (9.8:0.2) yielded **3** (60 mg), **4** (55 mg) and a semi-pure fraction, which was cleaned by passing through sephadex LH-20 eluting with methanol to get compound **1** (25 mg). The sub-fraction H_5 eluted with *n*-hexane:EtOAc (4:6) was further purified over silica gel column using a gradient of *n*-hexane:EtOAc, to get compound **2** (30 mg). The fraction G (2 g) obtained from the column with pure chloroform was further separated on silica gel column with the same mobile phase into two sub-fractions G_1 and G_2. The sub-fraction G_1 yielded compound **6** (32 mg) and **7** (35 mg) on re-silica gel column chromatography with isocratic of $CHCl_3$:CH_3OH (9.9:0.1), whereas, sub-fraction G_2 yielded compound **8** (110 mg) with a mobile phase system of $CHCl_3$:CH_3OH (9.8:0.2). The fraction D (3

g) of the main column, which was eluted with *n*-hexane:$CHCl_3$ (3:7), on repeated silica gel column chromatography yielded compound **9** (170 mg), **10** (90 mg) and **11** (22 mg) when eluted with *n*-hexane:$CHCl_3$ (5:5, 6:4 and 7:3 respectively). The other main fraction A (4.5 g) obtained with *n*-hexane:$CHCl_3$ (8:2) was again subjected to silica gel column chromatography using gradient of *n*-hexane:$CHCl_3$ to get three sub-fractions (A_1-A_3). The sub-fraction A_2 was further purified on silica gel column eluted with *n*-hexane:$CHCl_3$ (8.0:2.0) to get compound **5** (17 mg). Other fractions did not show prominent components, therefore, were not processed.

Aervfuranoside (**1**)

Pale yellow gum (25 mg); $[\alpha]_D^{25}$ - 24.7 (*c* 0.1, MeOH); IR (KBr) υ_{max} (cm^{-1}): 3410, 1595, 1560, 1525, 1030, 755; UV (MeOH) λ_{max} (log ε) nm: 215 (4.49), 239 (4.49), 264 sh (4.38), 298 sh (4.29), 308 (4.31); ^{1}H and ^{13}C-NMR: see Table-1; HRFABMS: *m/z* 619.1426 $[M+H]^+$ (calcd. for $C_{26}H_{32}ClO_{15}$, 619.1430).

Table-1: ^{1}H and ^{13}C-NMR data of **1** (DMSO-d_6, 400 and 100 MHz).

Position	δ_H (*J* in Hz)	δ_C
1	7.79, s	112.3
2	–	151.2
3	–	114.4
4	–	141.9
4a	–	158.3
5a	–	158.4
6	–	140.9
7	–	140.3
8	–	141.7
9	7.51, s	111.7
9a	–	112.8*
9b	–	111.9*
4-OCH_3	4.06, s	61.6
6 -OCH3	4.03, s	60.8
1″	5.12, d (7.5)	101.5
2″	3.34, m	76.3
3″	3.36, m	73.2
4″	3.08, dt (5, 9.5)	71.9
5″	3.57, m	75.5
6″	3.83, dd (7, 11.5) 3.42, br d (10)	66.0
1‴	4.48, br s	100.5
2‴	3.59, m	70.1
3‴	3.15, m	69.7
4‴	3.38, m	70.6
5‴	3.30, m	68.1
6‴	1.01, d (6.0)	17.7
2″ -OH	5.21, br s	–
3″ -OH	5.50, br s	–
4″ -OH	4.59, d (5)	–
2‴-OH	4.49, d (4)	–
3‴-OH	5.20, br s	–
4‴-OH	4.34, d (6)	

*Values interchangeable

Acid hydrolysis of **1**

A solution of compound **1** (10 mg) in MeOH (5 ml) containing 1 N HCl (4 ml) was refluxed for 4h, concentrated under reduced pressure, and diluted with H_2O (10 ml). It was extracted with EtOAc and the residue recovered from the organic phase was found to be dibenzofruran while the aqueous phase was concentrated and purified by preparative TLC using the solvent system (EtOAc-MeOH-H_2O-HOAc; 4:2:2:2) and identified as D- glucose and L-rhamnose by comparative TLC of the hydrolyzed sugars with the authentic samples of glucose and rhamnose and their optical rotation values $[\alpha]_D^{20}$ + 50.5˚and $[\alpha]_D^{23}$ +7.7˚. These sugars were also confirmed by the retention time of their TMS ethers (D-glucose *α*-anomer 4.1 min, *β*-anomer 7.8 min and L-rhamnose 8.6 min) with the standards.

DPPH Free Radical Scavenging Assay

The stable 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) was used for the determination of antioxidant activity [14]. Equal volumes (5 µL) of different concentrations of samples dissolved in DMSO were added to 90 µL of 100 µM methanolic DPPH in a total volume of 100 µL in 96-well plates. The contents were mixed and incubated at 37°C for 30 min. The absorbance was measured at 517 nm, whereas, quercetin was used as standard antioxidant. The experiments were performed in triplicates and the activity was determined by the following formula:

%age scavenging activity = 100 - (Abs of test compound/Abs of control) X 100

Acetylcholinesterase Inhibitory Assay

The AChE inhibition activity was performed according to the reported method [15] with slight modifications. 100 µL of the reaction mixture contained 60 µL Na_2HPO_4 buffer with concentration of 50 mM and pH 7.7, 10 µL test compound (0.5 mM well-1) was added, followed by the addition of 10 µL (0.005 unit $well^{-1}$) of the enzyme. The contents were mixed and pre-read at 405 nm. Then contents were pre-incubated for 10 min at 37ºC. The reaction was initiated by the addition of 10 µL of 0.5 mM $well^{-1}$ substrate (acetylthiocholine iodide), followed by the addition of 10 µL DTNB (0.5 mM well-1). After 15 min of incubation at 37ºC, absorbance was measured at 405 nm using 96-well plate reader. All experiments were carried out with their respective controls in triplicate. Eserine (0.5 mM $well^{-1}$) was used as a positive control.

Butyrylcholinesterase Inhibitory Assay

The BChE inhibition activity was performed according to the method described in literature [15] with slight modification. Total volume of the reaction

mixture was 100 µL containing 60 µL, Na_2HPO_4 buffer, 50 mM and pH 7.7. 10.0 µL of the test compound 0.5 mM well^{-1} was added followed by the addition of 10 µL (0.5 unit well^{-1}) BChE (Sigma Inc.). The contents were mixed and pre-read at 405 nm and then pre-incubated for 10 min at 37ºC. The reaction was initiated by the addition of 10 µL of 0.5 mM well^{-1} substrate (butyrylthiocholine chloride), followed by the addition of 10 µL DTNB, 0.5 mM well^{-1}. After 15 min of incubation at 37ºC, absorbance was measured at 405 nm using 96-well plate reader Synergy HT, Biotek, USA. All experiments were carried out with their respective controls in triplicate. Eserine (0.5 mM well-1) was used as positive control.

Lipoxygenase Inhibitory Assay

The lipoxygenase activity was assayed according to the method of Tappel et al. [16] with slight modifications. A total volume of 200 ml assay mixture contained 160 ml sodium phosphate buffer (100 mM, pH 8.0), 10 ml of test compound, and 20 ml purified lipoxygenase (Sigma Chemicals, Seelze, Germany). The contents were pre-incubated for 10 min at 258°C. The reaction was initiated by the addition of 10 ml linoleic acid (substrate solution). The change in absorbance was observed after 6 min at 234 nm. All reactions were performed in triplicates in 96-well microplate reader (Synergy HT, Biotek, Winooski, Vermont, USA). Baicalein (0.5 mM well^{-1}) was used as a positive control.

Metallo-β-lactamase (IMP-1) Inhibitory Assay

Inhibition assays for IMP-1 were performed in 96 well 400 µL UV multi-titre plates using a UV multi-plate spectrophotometer. The hydrolysis rate of benzyl penicillin (penicillin G sodium salt) was measured at λ = 235 nm [17]. The initial screening assay was performed in duplicate, with benzyl penicillin as the substrate and HEPESX (50 mM HEPES, 0.1M NaCl, 100 mM $ZnCl_2$, pH 7.0) as the buffer at 25°C. The final concentration of the enzyme and benzyl penicillin was 10 nM and 500 µM respectively. Bovine serum albumin (final concentration of 20 µg/ml) was added to the enzyme for stability. For each well, 196 µL substrate and 4 µL inhibitor were followed by the addition of 200 µL of the enzyme and the rate of hydrolysis of substrate was recorded for 5 min.

The percent inhibitions for all assays were calculated by the help of following equation.

$$\text{Inhibition (\%)} = \frac{\text{Control} - \text{Test}}{\text{Control}} \times 100$$

where

Control = Total enzyme activity without inhibitor
Test = Activity in the presence of test compound

Results and Discussion

Compound **1** was isolated as pale yellow gummy material, which absorbed in the IR region at 3410, 1595, 1560 and 1525 cm^{-1} due to hydroxyl and aromatic systems, respectively. The absorption band at 1030 cm^{-1} could be attested for a methoxyl moiety, whereas, an intense band at 755 cm^{-1} indicated the presence of chloride atom in **1**. The positive FABMS exhibited a *pseudo*-molecular ion peak at *m/z* 619 $[M+H]^+$ with an M+2 peak at *m/z* 621. The molecular formula $C_{26}H_{31}ClO_{15}$ with 11 double bond equivalence (DBE) could be established through positive HRFABMS. The UV spectrum of **1** showed absorption maxima at 215, 239, 264, 298 and 308 nm suggesting the presence of oxygen-substituted aromatic system.

Aromatic region of the ^{1}H-NMR spectrum of **1** showed two singlets at δ 7.79 and 7.51 due to aromatic moieties, whereas, two methyl singlets resonated at δ 4.06 and 4.03 were attested for two methoxyl units. The presence of two sugar moieties in **1** were evidenced due to the resonance of two anomeric protons at δ 5.12 (1H, d, J = 7.5 Hz) and 4.48 (1H, br s), with other sugar protons displaying their positions between δ 3.08-3.83. Besides, the same spectrum displayed a doublet methyl at δ 1.01 (J = 6.0 Hz). The analysis of coupling constants of the anomeric protons revealed the sugars to be β- and α-hexoses, respectively, in addition the resonance of doublet methyl indicated that the α-hexose could be rhamnose.

The ^{13}C-NMR spectrum (BB and DEPT) of **1** (Table-1) showed 26 signals for three methyl, one methylene, twelve methine and ten quaternary carbons. The total twelve sp^2 carbon atoms (δ 158.4, 158.3, 151.2, 141.9, 141.7, 140.9, 140.3, 114.4, 112.8, 112.3, 111.9 and 111.7) in ^{13}C-NMR spectrum were attested for two benzene rings that accommodated 08 DBE, whereas 02 DBE could be attributed to two sugar units. The remaining 01 DBE could only be accommodated for another ring system, which was supposed to be present in the form of a furan moiety between two benzene rings. The above discussion led to the identification of **1** as a chloro derivative of dibenzofuran glycoside.

Table-2: Biological activities of compounds **1-3**.

Compound*	AChE (%)	AChE (IC_{50}) μM	BChE (%)	BChE (IC_{50}) μM	LOX (%)	LOX (IC_{50}) μM	IMP-1 (%)	IMP-1 (Kic) μM	DPPH(%)	DPPH (IC_{50}) μM
1	68.56 ± 0.32	198.6 ± 0.11	53.13 ± 0.18	<400	62.88 ± 0.18	235.61 ± 0.5	5.0 ± 0.77	Nil	18.64 ± 0.18	<900
2	76.42 ± 0.17	165.91 ± 0.17	41.96 ± 0.52	<700	63.61 ± 0.04	53.51 ± 0.05	3.0 ± 0.68	Nil	15.22 ± 0.18	Nil
3	NP	-	NP	-	NP	-	17 ± 1.6	Nil	6.8 ± 0.89	Nil
Eserine	91.29±1.17	0.04±0.0001	82.82±1.09	0.85±0.001	-	-	-	-	-	-
Baicalein	-	-	-	-	93.79±1.2	22.4±1.3	-	-	-	-
Quercetin	-	-	-	-	-	-	-	--	93.21±0.97	16.96±0.14
L-Captopril	-	-	-	-	-	-	-	12 ± 2.4**	-	-

*All compounds were prepared in methanol with a concentration of 0.5mM.
All the measurements were done in triplicate, and statistical analysis was performed by Microsoft Excel 2003. Results are presented as mean ± *sem*.
NP= not performed

The two sugar moieties were finally identified as D-glucose and L-rhamnose due to acidic hydrolysis of compound **1** followed by comparative TLC of the hydrolyzed sugars with the authentic samples of glucose and rhamnose and their optical rotation values $[\alpha]_D^{20}$ + 50.5˚(*c*, 10 in H_2O) [18] and $[\alpha]_D^{23}$ +7.7˚ (*c*, 0.2 in H_2O) [19]. The substitutions on dibenzofuran nucleus were fixed through HMBC spectral information (Fig. 2) in which the anomeric proton (δ 5.12) of glucose showed long range interaction with the quaternary carbon at δ 151.2 (C-2) of benzene ring. H-1 (δ 7.79) exhibited HMBC correlation with the carbons at δ 151.2 (C-2), 114.4 (C-3) 158.3 (C-4a) and 112.8 (C-9a), whereas, H-9 (δ 7.51) correlated with the carbons at δ 112.8 (C-9a), 111.9 (C-9b), 140.3 (C-7) and 158.4 (C-5a). The two methoxyl groups (δ 4.03 and 4.06) were fixed at C-4 and C-6 due to their HMBC correlation with the carbons at δ 141.9 and 140.3, respectively. The HMBC interaction of anomeric proton of rhamnose (δ 4.48) with the methylene carbon (δ 66.0) of glucose confirmed its attachment at C-6 of the glucose. The above inferences allowed the placement of chloride atom at C-3. All the assignments were finalized due to COSY, HSQC and HMBC experiments and comparison with literature data of related compounds [20, 21] which finally led to the structure as **1**, which is a new natural product and is named as aervfuranoside.

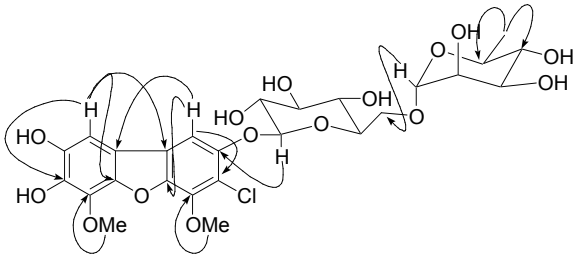

Fig. 2: Important HMBC Correlations observed in the spectrum of **1.**

Biological Studies

Due to limitation in laboratory facilities, only compounds **1-3** were evaluated for DPPH free radical scavenging potential and enzyme inhibitory activity against acetylcholinesterase (AChE), butyrylcholinesterase (BChE), lipoxygenase (LOX) and metallo-β-lactamases (IMP-1) (Table-2). At a concentration of 0.5 mM, **1** and **2** showed weak enzyme inhibitory activity against AChE, whereas, compound **3** was inactive against BChE and IMP-1, however, the test compound **2** was found a potential inhibitor of the enzyme lipoxygenase with an IC_{50} value of 53.5 µM.

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