Enzymes Inhibitory Constituents from *Buddleja crispa*

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Steroidal galactoside **1** and aryl esters **2** and **3** have been isolated from *Buddleja crispa*, along with ginipin **4**, gardiol **5**, 1-heptacosanol **6**, and methyl benzoate **7**, isolated for the first time from this species. The structures of all of the compounds were determined by spectroscopic techniques and chemical studies. The steroidal galactoside **1** is an inhibitor of lipoxygenase. Compounds **1 – 3** displayed inhibitory activity against butyrylcholinesterse, while compounds **2** and **3** further showed inhibition against acetylcholinesterase.

Key words: Buddleja crispa, Buddlejaceae, Steroid Galactoside, Aryl Esters, Enzyme Inhibition

Introduction

The genus Buddleja (family Buddlejaceae) is found in the temperate regions of America, Asia and South Africa. It is a genus of about 100 species represented in Pakistan by four species [1]. The flowers, leaves and roots of various species of Buddleja are used in traditional medicine in several parts of the world [2]. Several known pharmacological activities and folkoric uses are attributed to the genus. For example *B. asiata*, which is indigenous to China, India, and Java, is known for its uses as an abortifacient and for treatment of skin diseases [3]. Flowers and buds of *B. asiata* produces a yellow coloured essential oil which is active against several pathogenic fungi [4]. In central and southern region of Chile the aqueous extract of *B. globase* is used for stomach ulcers, wounds and burns [5]. The medicinal importance of the genus prompted us to carry out phytochemical studies on one of its species namely *B. crispa* which is a densely tomentose shrub. The wood of *B. crispa* is fairly hard and is used for fuel, found in southern part of Pakistan. No phytochemical investigation has so far been carried out on this species. A literature survey of the genus *Buddleja* revealed the isolation of various natural products, including sterols [3, 5], aryl esters [5], triterpenoid glycosides [6], phenylethanoids [7, 8], flavonoids [9], phenolic fatty acid esters [10], diterpenes [11] and sesquiterpenes [12, 13]. Herein we report the isolation and structure elucidation of (22*R*)-stigmasta-7,9(11)-dien-22 α-ol-3 β-*O*-β-D-galactopyranoside **1**, nonyl benzoate **2** and hexyl *p*-hydroxy-cinnamate **3** along with ginipin **4** [14], gardiol **5** [15], 1-heptacosanol **6** [16] and methyl benzoate **7**, reported for the first time from this species.

Acetylcholinesterase (AChE; EC 3.1.1.7) and butyrylcholinesterase (BChE; EC 3.1.1.8) are serine hydrolases that share about 55% of amino acid sequence identity, and have similar, yet distinct catalytic properties. The different specificity for substrates, irreversible inhibitors and reversible ligands is dictated by the difference in amino acid residues of the active sites of AChE and BChE [17].

AChE is a key component of cholinergic brain synapses and neuromuscular junctions. The major biological role of the enzyme is the termination of impulse transmission by rapid hydrolysis of the cationic neurotransmitter acetylcholine [18]. According to the cholinergic hypothesis, the memory impairment in the patients with senile dementia of *Alzheimer's* type results from a deficiency in cholinergic function in the brain [19]. Hence the most promising therapeutic strategy for activating central cholinergic functions has been the use of cholinomimetic agents. The aim of AChE inhibitors is to boost the endogenous levels of acetylcholine in the brains of Alzheimer's disease patients and thereby, to boost cholinergic neurotransmission. It has also been found that butyryl-

cholinesterase inhibition may also be an effective tool for the treatment of AD and related dementias [20]. BChE (E.C 3.1.1.8) is produced in the liver and enriched in the circulation. In addition, it is also present in adipose tissue, intestine, smooth muscle cells, white matter of the brain and many other tissues [21]. The exact physiological function of BChE is still elusive. It acts as a scavenger for anticholinesterase compounds.

Lipoxygenases (EC 1.13.11.12) constitute a family of non-haem iron containing dioxygenases that are widely distributed in animals and plants. In mammalian cells these are key enzymes in the biosynthesis of variety of bioregulatory compounds such as hydroxyeicosatetraenoic acids (HETEs), leukotrienes, lipoxins and hepoxylines [22]. It has been found that these lipoxygenase products play a role in a variety of disorders such as bronchial asthma, inflammation [23] and tumor angiogenesis [24]. Lipoxygenases are therefore potential target for the rational drug design and discovery of mechanism-based inhibitors for the treatment of bronchial asthma, inflammation, cancer and autoimmune diseases.

Results and Discussion

The methanolic extract of shade-dried whole plant material (30 kg) of *B. crispa* was evaporated *in vacuo*, the residue suspended in H_2O, and successively partitioned with *n*-hexane, EtOAc, and BuOH. Silica gel column chromatography of the EtOAc extract afforded compounds **1,4,5** while the *n*-hexane extract provided compounds **2**, **3**, **6** and **7**.

Compound **1** was obtained as colorless crystalline solid which gave characteristic colour reactions of sterols. It showed an $[M+H]^+$ peak in the HRFABMS at *m/z* 591.4243 corresponding to molecular formula $C_{35}H_{59}O_7$ (calcd. for $C_{35}H_{59}O_7$, 591.4245). The UV spectrum showed absorptions at λ_{max} 235, 242 and 250 which are characteristic of $\Delta^{7,9(11)}$-sterols [25]. The IR spectrum showed bands due to hydroxyl groups (3490 cm^{-1}) and double bond (1650, 890 cm^{-1}). The EIMS showed peaks at *m/z* 572 $[M\text{-}H_2O]^+$, 428 [M-hexose]$^+$ and 410 $[M\text{-hexose-}H_2O]^+$. The peak at *m/z* 271 represented the loss of both the sugar and side chain from the molecular ion peak.

The ^{1}H NMR spectrum displayed signals for six methyl protons at $\delta_H = 0.81$ (d, $J = 6.9$, 3H, H-26), 0.87 (s, 3H, H-18), 0.89 (t, $J = 7.2$, 3H, H-29), 0.91 (d, $J = 6.9$, 3H, H-27), 0.93 (d, $J = 7.0$, 3H, H-21), 1.02 (s, 3H, H-19). A pair of doublets at $\delta_H = 5.85$ ($J = 5.4$ Hz, 1H, H-7) and 5.56 ($J = 6.1$ Hz, 1H, H-11) were assigned to the olefinic protons. The position of the double bonds were further confirmed by COSY experiments, which established the *J* connectivities between H_2-6 and H-7 and between H_2-12 and H-11. A pair of doublet at $\delta_H = 0.81$ ($J = 6.9$ Hz, H-26) and $\delta_H = 0.91$ ($J = 6.9$ Hz, H-27) and a multiplet at $\delta_H = 1.81$ (H-25) confirmed the presence of an isopropyl group. The position of the ethyl group at C-24 was confirmed by the presence of 3H triplet at $\delta_H = 0.89$ in the ^{1}H NMR and also confirmed by the mass fragmentation which showed a fragment at *m/z* 85 due to the loss of C_6H_{13}. In addition the ^{1}H NMR also showed signals for the β-linked sugar moiety which appeared at $\delta_H = 4.65$ (d, $J = 7.8$ Hz, H-1'), 3.27–3.45 (m, H-2', 3', 5'), 3.31 (t, $J = 1.6$, H-4'), 3.63 (dd, $J = 11.9$, 5.5) and 3.84 (dd, $J = 11.9$, 2.3, H-6'). In COSY spectrum the more downfield oxymethine proton at $\delta_H = 3.66$ showed cross peaks with four other protons and it could consequently be assigned to the more usual C-3 position; its downfield shift being due to glycoside formation. Its larger coupling constant allowed us to assign α and axial configuration to H-3. The comparatively upfield oxymethine proton at $\delta_H = 3.28$ showed connectivity to three other protons including H-20 ($\delta_H = 1.73$), thereby confirming the presence of hydroxyl moiety at C-22.

The ^{13}C NMR spectrum (BB and DPET) showed 35 carbon signals, in which there are 6 methyl, 10 methylene, 15 methine and 4 quaternary carbons. The low-field region of ^{13}C NMR spectrum showed four signals at $\delta_C = 145.7$ (C-9), 133.2 (C-8), 123.1 (C-7), 119.4 (C-11) which could be assigned to olefinic carbons. One of the oxymethine resonated at $\delta_C = 72.5$. The downfield shift of C-3 ($\delta_C = 79.3$) confirmed the linkage of the sugar moiety at this position. The ^{13}C NMR showed signals for the sugar moiety at $\delta_C =$ 100.6 (C-1'), 73.2 (C-2'), 76.6 (C-3'), 70.2 (C-4'), 76.7 (C-5') and 61.2 (C-6'). The sugar could be identified as galactose by comparing its ^{1}H and ^{13}C chemical shifts with those reported in literature and further confirmed through acid hydrolysis of **1** which provided various products, among which the glycone could be separated and identified as galactose by comparing the retention time of its trimethylsilyl (TMS) ether with that of the standard in gas chromatography (GC). The sign of optical rotation allowed us to assign D-configuration to the galactose moiety.

The stereochemistry at C-22 could be established by comparing ^{13}C chemical shifts with literature. In 22-*R*

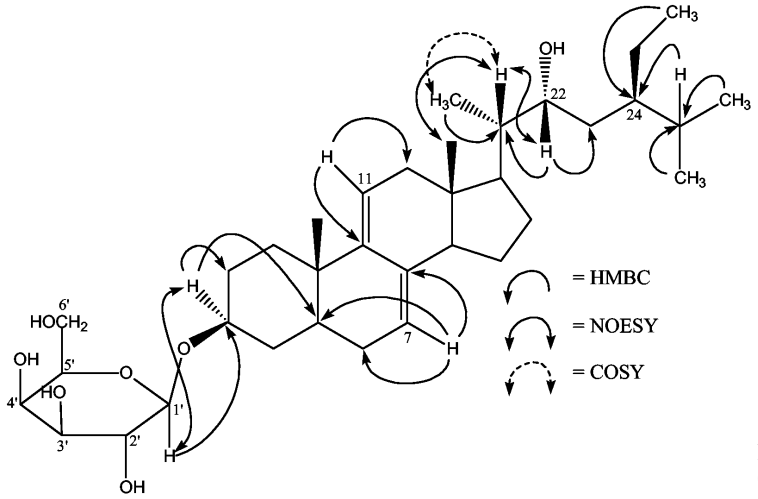

Fig. 1. Important HMBC, NOESY and COESY interaction of **1**.

hydroxysterols the signal of C-22 was observed comparatively upfield and that of C-20 downfield relative to the corresponding *S*-epimer [26, 27]. In **1** the chemical shifts of both C-20 ($\delta_C = 42.8$) and C-22 ($\delta_C = 72.5$) corresponded to those of 22-*R* epimer. The β configuration of both H-20 and H-22 could further be authenticated by NOESY spectrum in which the protons at C-18 showed correlations with both H-20 and H-22. The configuration at C-24 could also be assigned '*R*' based on similar chemical shift difference between 24*R*- and 24*S*-epimers [28]. Thus structure of **1** could be assigned as (22*R*)-stigmasta-7,9(11)-dien-22α-ol-3 β-*O*-β-D-galactopyranoside. The HMBC correlations were in complete agreement of the assigned structure (Fig. 1).

Compound **2** was isolated as an oil. The molecular formula $C_{16}H_{24}O_2$ was determined by HREIMS and ^{13}C NMR spectra. The IR spectrum showed absorption bands at 1720, 1610 and 1460 cm^{-1} suggesting the presence of ester and aromatic functionalities. The ^{1}H NMR spectrum showed the presence of monosubstututed benzene [multiplets at $\delta_H = 7.36$ (2H), 7.47 (1H) and 7.97 (2H), respectively] and a terminal methyl group at $\delta_H = 0.82$ (3H, t, $J = 7.0$ Hz). The signal of oxymethylene protons was observed at $\delta_H = 4.26$ while another methylene group was observed at $\delta_H = 1.50$. The unresolved multiplate at $\delta_H = 1.22$ integrating for twelve protons was due to 6 further methylene groups of long chain. The ^{13}C NMR spectrum (BB & DEPT) showed the signals at $\delta_C = 166.7$ which could be assigned to the ester carbonyl. The signal at $\delta_C = 65.1$ was assigned to the oxygenated methylene while the resonance at $\delta_C = 132.7$, 130.5, 129.5 and 128.3 were assigned to the aromatic ring. The signals between $\delta_C = 32.6$ and $\delta_C = 22.6$ (all methylenes in DEPT) were indicative of a long chain hydrocarbon.

The mass spectrum showed fragment at *m/z* 122, 105 and 77. On the basis of these evidences **2** could be identified as nonyl benzoate. Alkaline hydrolysis of **2** provided benzoic acid and nonanol, respectively, providing conclusive evidence for the assigned structure. This compound is reported as a new natural product following its earlier synthesis by Breusch *et al.* [29].

Compound **3** was also obtained as an oil. The HREIMS showed an $[M]^+$ peak at *m/z* 248.1405 corresponding the molecular formula $C_{15}H_{20}O_3$. It gave greenish-brown color with $FeCl_3$ and the IR spectrum showed absorption bands at 3470 (free OH), 1700 (C=O), 1670 (C=C), 1610, 1530, 1460 (aromatic), 1365 (methyl) 1260, 1160 (C–O) and 720 cm^{-1} [-$(CH_2)_X$-]. The UV spectrum was typical of cinnamic acid ester [30] showing λ_{max} at 235 and 325, respectively.

The ^{1}H NMR spectrum showed characteristic *p*-hydroxycinnamoyl moiety [aromatic protons showing AA', BB' pattern with $\delta_H = 7.39$ (2H, d, $J = 8.4$ Hz) and δ_H 6.87 (2H, d, $J = 8.4$ Hz); trans olefinic protons at $\delta_H = 7.64$ (1H, d, $J = 16$ Hz) and $\delta_H = 6.29$ (1H, d, $J = 16$ Hz)]. In addition it showed a three protons triplet for the terminal methyl group at $\delta_H = 0.87$ (6.34 Hz) and OCH_2CH_2R grouping [$\delta_H = 4.19$, 2H, t, $J = 6.69$ Hz and $\delta_H = 1.65$, 2H, m)]. The unresolved multiplet at $\delta_H = 1.29$ integrating for 6 protons showed the presence of 3 further methylene groups. The mass spectrum showed peaks at *m/z* 164, 147 and 119. The

Table 1. *In vitro* quantitative inhibition of AChE, BChE and LOX enzymes by compounds **1**–**3**.

Compounds	$IC_{50} \pm$ SEM[a] [μM]		
	AChE	BChE	LOX
1	–	46.7 ± 0.2	6.1 ± 0.5
2	53.5 ± 1.2	73.2 ± 1.2	–
3	32.2 ± 0.5	22.5 ± 0.6	–
Galanthamine[b]	0.5 ± 0.01	8.5 ± 0.001	–
Baicalein[c]	–	–	22.7 ± 0.05

[a] Standard mean error of five determinations; [b] positive control used in AChE and BChE inhibiting assays; [c] positive control used in LOX inhibiting assay.

compound **3** could, therefore, be identified as hexyl ester of *p*-hydroxy cinnamic acid. The structure was corroborated by ^{13}C NMR spectrum which included peaks at $\delta_C = 168.6$ for the ester carbonyl and further peaks at $\delta_C = 145.2$ and $\delta_C = 114.8$ for the olefinic carbons. The alkaline hydrolysis of **3** provided *p*-hydroxy cinnamic acid and hexanol, respectively.

Compound **1** showed moderate inhibitory potential against LOX with IC_{50} value (6.1 ± 0.5) μM (Table 1) and weak inhibitory activity against BChE (46.7 ± 0.2) μM whereas the standard inhibitor of LOX (baicalein) and BChE (galanthamine) have IC_{50} values of (22.5 ± 0.2) μM and (8.5 ± 0.01) μM, respectively. Compounds **2** and **3** displayed weak inhibitory activity against AChE with IC_{50} values of (53.5 ± 1.2) μM and (32.2 ± 0.5) μM, and also against BChE with IC_{50} values of (73.2 ± 1.2) μM and (22.5 ± 0.6) μM, respectively. The positive control (galanthamine) used in the assays showed IC_{50} values of (0.5 ± 0.001) μM and (8.5 ± 0.01) μM against AChE and BChE, respectively.

Experimental Section

General: Optical rotations were taken on a JASCO DIP-360 digital polarimeter. IR spectral data were measured on a JASCO 302-A spectrophotometer in $CHCl_3$. UV spectra were obtained on a Hitachi UV-3200 spectrophotometer. NMR spectra were run on a Bruker instrument. Chemical shifts δ are shown in ppm relative to TMS as internal standard and coupling constant *J* are described in Hz. EI-, FAB-, and HREI-MS were recorded on a JEOL JMS-HX-110 and JMS-DA-500 mass spectrometers, *m/z* (relative. int). Silica gel 60 – 200 mesh and 200 – 440 mesh (Merck) was used for column chromatography, respectively. Silica gel plates (Si 60 F_{254}, Merck) were used for TLC.

Plant material: The whole plant material was collected in March 2003 from Baluchistan and identified as *Buddleja crispa* Benth. by Prof. Rasool Bakhsh Tareen, Department of Botany, University of Baluchistan, Pakistan. A voucher specimen (BBU-101) is deposited in the herbarium of the Department of Botany, University of Baluchistan, Quetta, Pakistan.

Extraction and isolation: The shade-dried plant (30 kg) were chopped and extracted thrice with MeOH (60 l) at rt for 96 h. The methanolic extract was evaporated *in vacuo* to give a dark greenish residue (800 g), which was partitioned between *n*-hexane and water. The water fraction was further extracted with EtOAc and *n*-BuOH. The EtOAc fraction (40 g) was subjected to column chromatography eluting with *n*-hexane-EtOAc in increasing order of polarity to give eight fractions. The fraction obtained from *n*-hexane-EtOAc (3:7) was rechromatographed over flash silica using *n*-hexane-EtOAc (6:4-2:8) as solvent systems to give two successive fractions. The first fraction was further purified by column chromatography on silica gel using *n*-hexane-EtOAc (4:6) as eluent to afford compound (**1**) (16 mg). The fraction obtained from *n*-hexane-EtOAc (6:4) was further purified by column chromatography over silica gel using *n*-hexane-EtOAc (8:2-5:5) as eluents to afford compound (**4**) (11 mg) and (**5**) (15 mg), respectively. The *n*-hexane fraction (30 g) was subjected to column chromatography eluting with *n*-hexane and *n*-hexane-$CHCl_3$ in increasing order of polarity to give five fractions. The fraction obtained from *n*-hexane-$CHCl_3$ (8:2) was rechromatographed over flash silica using *n*-hexane- $CHCl_3$ (9:1-6:4) as solvent systems to give three successive fractions. The second fraction was a mixture of two compounds and afforded compounds **2** (40 mg) and **3** (35 mg) by silica gel column chromatography using *n*-hexane, *n*-hexane-$CHCl_3$ (9:1, 8:2) as eluents. The first fraction which was also a mixture of two compounds, was purified through column chromatogrphay over silica gel using *n*-hexane-$CHCl_3$ (9:1) as eluent to afford compounds **6** (18 mg) and **7** (15 mg), respectively.

Acid hydrolysis of compound **1**

A solution of **1** (8 mg) in MeOH (5 ml) containing 1 N HCl (4 ml) was refluxed for 4 h, concentrated under reduced pressure, and diluted with H_2O (8 ml). It was extracted with EtOAc and the residue recovered from the organic phase was found to be an inseperatable mixture of products. The aqueous phase was concentrated and D-galactose was identified by the sign of its optical rotation ($[\alpha]_D^{20} + 80.0^\circ$, $c = 0.02$, H_2O). It was also confirmed based on comparison of the retention time of its TMS ether (α-anomer 3.8 min, β-anomer 5.2 min) with a standard.

Alkaline hydrolysis of **2** *and* **3***:* The ester **2** (20 mg) in benzene (25 ml) was refluxed with 5% methanolic potassium hydroxide (15 ml) for 4 h, concentrated *in vacuo*, diluted with water (30 ml) and extracted with ether. The organic layer on removal of solvent afforded a liquid (Fp -5°, Bp 214°), characterized as nonanol (M^+ peak at *m/z* 144, superimposable IR). The aqueous layer was acidified with dil. hydrochloric acid and extracted with dichloromethane. The residue re-

covered from the organic phase crystallized from H_2O, m. p. 123 °C: It was characterized as benzoic acid (m.m.p., IR and Co-TLC).

Corresponding hydrolysis of **3** provided hexanol (characterized through B. P, IR and co-TLC) and p-hydroxy cinnamic acid, colourless crystals from H_2O, m. p. 212 °C (characterized through m.m.p., Co-TLC, IR).

Compound **1**: – Colourless crystalline. – M. p. 258–260 °C. – $[\alpha]_D$ −29.9 ($c = 0.02$, MeOH). – UV λ_{max} (MeOH) 235, 240, 250. – IR (KBr) $\nu = 3490$ (OH group), 1650, 890 cm^{-1} (double bonds). – Positive HRFABMS *m/z* 591.4243 $[M+H]^+$ (calcd. 591.4245 for $C_{35}H_{59}O_7$). – HREIMS *m/z* 572, 428, 410, 271. – ^{1}H NMR (90 MHz, CD_3OD) $\delta = 0.81$ (d, $J = 6.9$ Hz, 3H, H-26), 0.87 (s, 3H, H-18), 0.89 (t, $J = 7.2$ Hz, 3H, H-29), 0.91 (d, $J = 6.9$, 3H, H-27), 0.93 (d, $J = 7.0$, 3H, H-21), 1.02 (s, 3H, H-19), 1.39 (m, 1H, H-4β), 1.59 (m, 1H, H-2β), 1.88 (m, 1H, H-4α), 1.96 (m, 1H, H-2α), 3.28 (m, 1H, H-22), 3.66 (m, 1H, H-3), 5.56 (d, $J = 6.1$ Hz, 1H, H-11), 5.85 (d, $J = 5.4$ Hz, 1H, H-7), 4.65 (d, $J = 7.8$ Hz, H-1'), 3.27–3.45 (m, H-2',3',5'), 3.31 (t, $J = 1.6$ Hz, H-4'), 3.63 dd ($J = 11.9$, 5.5 Hz) and 3.84 dd ($J = 11.9$, 2.3) (H-6'). – ^{13}C NMR (100 MHz, CD_3OD) $\delta = 37.7$ (C-1), 30.5 (C-2), 79.3 (C-3), 35.5 (C-4), 40.6 (C-5), 31.0 (C-6), 123.1 (C-7), 133.2 (C-8), 145.7 (C-9), 37.0 (C-10), 119.4 (C-11), 44.9 (C-12), 42.6 (C-13), 57.9 (C-14), 25.8 (C-15), 27.9 (C-16), 52.7 (C-17), 13.6 (C-18), 19.6 (C-19), 42.8 (C-20), 12.6 (C-21), 72.5 (C-22), 30.0 (C-23), 41.5 (C-24), 28.9 (C-25), 17.5 (C-26), 20.6 (C-27), 23.6 (C-28), 11.9 (C-29), 100.6 (C-1'), 73.2 (C-2'), 76.6 (C-3'), 70.2 (C-4'), 76.7 (C-5'), 61.2 (C-6').

Compound **2**. – Oil. – IR (KBr) $\nu = 1720$, 1610, 1460. – UV (MeOH) $\lambda_{max} = 227$, 282. – HREIMS *m/z* 248, 122, 105, 77. – ^{1}H NMR (400 MHz, $CDCl_3$) $\delta = 0.82$ (3H, t, $J = 7.0$ Hz, Me), 1.22 [12H, br m, $(CH_2)_6$], 1.50 (2H, m, OCH_2CH_2R), 4.26 (2H, m, OCH_2CH_2R), 7.47 (IH, m), 7.97 2H, m), 7.36 (2H,m). – ^{13}C NMR (100 MHz, $CDCl_3$) $\delta = 166.7$ (C-1'), 132.7 (C-4), 130.5 (C-1), 129.5 (C-2, 6), 128.3 (C-3,5), 65.1 (C-1"), 32.62 (C-2"), 31.8 (C-7"), 25.7 (C-4"), 29.6 (C-5"), 29.4 (C-6"), 26.8 (C-3"), 22.6 (C-8"), 14.2 (C-9").

Compound **3**. – Oil. – IR (KBr) $\nu = 3470$, 1700, 1670, 1610, 1530, 1460, 1365, 1280, 1160, 720 cm^{-1}. – UV (MeOH) $\lambda_{max} = 235$, 325. – HREIMS *m/z* 248, 164, 147, 119. – ^{1}H NMR (400 MHz, $CDCl_3$) $\delta = 0.87$ (3H, t, $J = 6.34$ Hz, Me), 1.29 [6H, br m, $(CH_2)_3$], 1.65 (2H, m, OCH_2CH_2R), 4.19 (2H, t, $J = 6.69$ Hz, OCH_2CH_2R), 6.29 (1H,d, $J = 16.0$ Hz, =CH), 6.87 (2H,d, $J = 8.4$ Hz H-3 and H-5), 7.39 (2H,d, $J = 8.4$ Hz H-2 and H-6), 7.64 (1H,d, $J = 16$ Hz CH=). – ^{13}C NMR (100 MHz, $CDCl_3$) $\delta = 168.7$ (C-3'), 158.7 (C-4), 145.2 (C-1'), 130.0 (C-2 and C-6), 126.6 (C-1), 116.1 (C-3 and C-5), 114.8 (C-2'), 65.1 (C-1"), 31.4 (C-2"), 28.6 (C-3"), 25.6 (C-4"), 22.5 (C-5"), 14.0 (C-6").

In vitro cholinesterase inhibition assay

Electric-eel AChE (EC 3.1.1.7), horse-serum BChE (EC 3.1.1.8), acetylthiocholine iodide, butyrylthiocholine chloride, 5,5'-dithiobis [2-nitrobenzoic acid] (DTNB) and galanthamine were purchased from Sigma (St. Louis, MO, USA). All other chemicals were of analytical grade. AChE and BChE inhibiting activities were measured by the spectrophotometric method developed by Ellman *et al.* [31]. Assay conditions and protocol was the same as described previously [32].

In vitro lipoxygenase inhibition assay

Lipoxygenase inhibiting activity was measured by slightly modifying the spectrometric method developed by *A. L. Tappel* [33]. Lipoxygenase (1.13.11.12) type I-B and linoleic acid were purchased from Sigma (St. Louis, MO, USA). All other chemicals were of analytical grade. The assay conditions and protocol was the same as described previously [34].

Determination of IC_{50} values

The concentrations of the test compounds that inhibited the hydrolysis of substrates (acetylthiocholine, butyrylthiocholine and linoleic acid) by 50% (IC_{50}) were determined by monitoring the effect of various concentrations of these compounds in the assays on the inhibition values. The IC_{50} values were then calculated using the EZ-Fit Enzyme Kinetics program (Perrella Scientific Inc., Amherst, USA).

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