Sesquiterpene Lactones from *Cynara cornigera*: Acetyl Cholinesterase Inhibition and *In Silico* Ligand Docking

**Abstract**

Wild artichoke (*Cynara cornigera*), a thistle-like perennial belonging to the Asteraceae family, is native to the Mediterranean region, northwestern Africa, and the Canary Islands. While the pleasant, albeit bitter, taste of the leaves and flowers is attributed to the sesquiterpene lactones cynaropicrin and cynarin, a comprehensive phytochemical investigation still needs to be reported. In this study seven sesquiterpene lactones were isolated from an aqueous methanol plant extract, including a new halogenated metabolite (1), the naturally isolated compound sibthorpine (2), and five metabolites isolated for the first time from *C. cornigera*. Structures were established by spectroscopic methods, including HREIMS, $^1$H, $^{13}$C, DEPT, $^1$H-$^1$H COSY, HMQC, and HMBC-NMR experiments as well as by X-ray analysis. The isolated biactive nutrients were analyzed for their antioxidant and metal chelating activity. Compound 1 exhibited a potent metal chelating activity as well as a high antioxidant capacity. Moreover, select compounds were effective as acetyl cholinesterase inhibitors presenting the possibility for such compounds to be examined for anti-neurodegenerative activity. A computational pharmacophore elucidation and docking study was performed to estimate the pharmacophoric features and binding conformation of isolated compounds in the acetyl cholinesterase active site.

**Introduction**

Wild artichoke (*Cynara cornigera*) belongs to the Asteraceae family and is native to the Mediterranean region; today the plant is widely cultivated around the world. Artichoke extracts have been used in traditional folk medicine for their, hepato-protective, choleretic, diuretic and spasmyloytic effects. Because of these observed biological activities, artichoke is reported in traditional phytopharmaceuticals with a particular focus on its hepatoprotective characteristics [1–4]. Artichoke leaves can also reduce symptoms of irritable bowel syndrome [5], hyperlipoproteinemia [6], and choleretic effects [7]. Artichoke extracts have been reported to inhibit oxidative stress generated by reactive oxygen species in human leukocytes. This biological activity of the extracts originates from polyphenols, such as cynarin, caffeic acid, chlorogenic acid, and luteolin [8]. Due to the close association between oxidative stress, cellular damage and the aging process, biologically active artichoke extracts may provide a phytochemical link between antioxidant activity and degenerative diseases [9]. Treatment of age-related disorders, including an antiatherosclerotic effect by inhibiting the hepatic cholesterol biosynthesis without blocking the hydroxymethyl-glutaryl (HMG)-CoA reductase activity, has already been shown. In addition, oxygen free radicals (OFRs) have been implicated in the pathogenesis of an increasing number of diseases including the most common form of dementia, Alzheimer’s disease (AD) [10]. AD is a progressive age-related disorder, which is characterized by the degeneration of neurological functions due to a reduction of the level of the neurotransmitter acetylcholine with disease progression, resulting in loss of cognitive ability [11]. Acetylcholinesterase (AChE) inhibitors have been shown to function by increasing the acetylcholine level within the synaptic region, thereby restoring deficient cholinergic neurotransmissions [12, 13]. Selective cholinesterase inhibitors that are free of dose-limiting side effects, are currently not available and current compounds cannot always provide a sufficient modulation of the acetylcholine levels to elicit an effective therapeutic response [11].
Therefore, the search for novel AchE inhibitors with high efficacy is necessary. It seems promising to screen sesquiterpene lactones as possible AchE inhibitors for AD therapy [14]. Although Cynara cornigera Lindl. shows a promising antioxidant activity, few phytochemical investigations have been performed [15]. Herein, we report the isolation and chemical characterization of metabolites from C. cornigera as well as their biological activity as antioxidants, free radical scavengers, and acetylcholine esterase inhibitors based on in vitro and in silico analyses.

Results and Discussion

Extensive fractionation and purification of an organic extract of C. cornigera leaves lead to the isolation of seven sesquiterpene lactones (Fig. 1). Compound 1 was obtained as a brown oil. The HR-FAB-MS exhibited a [M + Na]+ ion at m/z 321.0879 (calc. 321.0870), indicating a molecular formula of C_{15}H_{20}O_{5}. 13C NMR (Table 1) and the DEPT spectrum exhibited 15 carbon signals establishing six methylenes, five methines, and four quaternary carbons. The spectrum also revealed the presence of a carbonyl ester at δc 176.9 (C-12), two exomethylene functionalities at δc 111.4/149.7 and 111.2/154.3, one oxygenated methylene at δc 42.9, two oxymethine carbons at δc 73.6 and 83.9, and one oxygenated quaternary carbon at δc 79.9. These spectroscopic data were consistent with those of guaianolide-type sesquiterpene lactones; in fact, 1 was very similar to 6, a compound that has previously been reported by Reis et al. (1992) [16], except for the NMR signals near C-3 raising the possibility that 1 was an epimer of 6. Based on the biogenic precedent, H-7 was assigned to an alpha orientation and the H-6–H-7 associated coupling constant of 9.7 Hz was consistent with a trans configuration. NOE correlations between H-6 and H-5 and H-5 and H-1, and as well as with an up-field methylene hydrogen at δH 1.80 (H-1a) indicated these protons are on the same alpha face. The other proton signal at δH 2.01 (H-3) establishing the hydroxyl group at C-3 in an α-orientation (Figs. 2 and 3). Therefore, 1 was identified as 3α,11a-dihydroxy-13-chloro-1αH,5αH,7αH-guaian-10(14)–en-12,6,8-olide (cornigeraline A), a new chlorinated sesquiterpene lactone.

Compound 2 was obtained as colorless crystals. The HR-FAB-MS exhibited a [M + Na]+ ion at m/z 303.1202 (303.1208), indicating a molecular formula of C_{15}H_{20}O_{5}, which was supported by 1D and 2D NMR. The NMR assignments (Table 1) were determined by an analysis of DQF-COSY, HMQC and HMBC data (Fig. 2). 1H- and 13C NMR spectra were similar to isolated diacetate deriva-

**Table 1** 1H and 13C spectral data (CD3OD, 600 MHz) of compound 1 and 2.

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<td>111.20 (t)</td>
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<td>5.33 br s</td>
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Hegazy M-EF et al. Sesquiterpene Lactones from... Planta Med 2016; 82: 138–146
tives of sibthorpine published by Omar et al. (1984) [17] and almost identical to 1 except for a substitution of a hydroxyl group at the chlorine position. First-time X-ray analysis (CCDC 1059018) confirmed a hydroxyl at C-13 and 2 was positively identified as sibthorpine. In addition to 1 and 2 five known metabolites were isolated, including 3-hydroxy-grosheimin (3) [18], grosheimin (4) [19–22], solstitialin A (5), 13-chlorosolstitialine (6) [16], and cyanaropicrin (7) [16, 23–25].

To probe the scope of antioxidant activity for these individually isolated sesquiterpene lactones, a series of in vitro assays was initially performed. The total antioxidant capacity determined by a diammonium salt (ABTS)-peroxidase system showed that 1 and 3 are potent antioxidants exhibiting greater activity than 4–7 (Fig. S4, Supporting Information). Compound 1 exhibited also the most effective metal chelating activity compared with 2–7 as well as with the standard butylated hydroxyl toluene (BHT; Fig. S5, Supporting Information). Radical scavenging activity against 1,1-diphenyl-2-picryl-hydrazil (DPPH) radicals was observed for all compounds with an even greater activity than the commercially available antioxidant BHT that was employed as a positive control (Fig. S1, Supporting Information). Compounds 2, 4, and 6 showed nearly the same inhibition level (ca. 96%) which was the highest scavenging percentage (Fig. S1, Supporting Information). The ferric to ferrous reduction capability of the isolated metabolites was also greater than that of BHT at all assayed concentrations. Compound 4 and 6 showed a maximum reducing power at 5 µg/mL, while compounds 5 and 7 were weaker (Fig. S2, Supporting Information). Superoxide radicals are generated in a phenazine methosulphate (PMS)–nicotinamide adenine dinucleotide (NADH) system by oxidation of NADH and can be assayed by the reduction of nitroblue tetrazolium (NBT). In this assay, the reference compound BHT produced the lowest effect as scavenger at all concentrations, while the most effective scavenger was 6 reaching 100% scavenging at 20 and 40 µg/mL (Fig. S3, Supporting Information). With all isolated sesquiterpene lactones exhibiting an antioxidant activity employed in a range of standard in vitro protocols, including radical scavenging ABTS-peroxidase and DPPH assays as well as ferric and NBT reduction, a study of the identified artichoke metabolites as inhibitors of the more specific AChE enzyme assay was performed.

AChE inhibitory activity was observed for all isolated compounds at assayed concentrations of 5, 10, 20 and 40 µg/mL. Compound 1 exhibited the highest enzyme inhibition at all assayed concentrations and showed 100% inhibition at 20 µg/mL (Fig. 4, and Table 2). The order of effectiveness for the isolated acetylcholinesterase inhibitors was 1, 5, 3, 2, 7, 4 and 6, with IC50 values of 6.1, 7.2, 8.1, 10, 10, 16 and 18.55 µg/mL, respectively (Fig. 5). To examine possible ligand-structure/enzyme-binding relationships, common structural features were first identified based on pharmacophore modeling, and then in silico docking studies were performed with a high resolution AChE crystal structure. The generated pharmacophore model predicted four structural features that are present in the biologically active metabolites 1–3, 5 and 7. There are two hydrophobic moieties located in the sesquiterpene nucleus, centered close to C-10 and between C-1 and C-3 (Fig. 7A). In addition, two H-bond donors and/or acceptors appear due to the hydroxyl or chloro group associated with C-13 and to a second hydroxyl group associated with C-8 (present only in 3 and 4). Since these four identified pharmacophores are
superimposable on the isolated AChE-inhibitory metabolites except for 4 and 6 (Fig. 7 B–D), they provide a chemical fingerprint for ligand docking with AChE.

Since the commercially available electric eel acetylcholine esterase (EeAChE) employed with the in vitro assays (Fig. 5) only has low-resolution structural data, docking studies were based on a high resolution AChE crystal structure isolated from the Pacific electric ray, Torpedo californica (TcAChE). Both protein sources have a high homology at their active sites [26]. Physostigmine, the positive control used, is known to inhibit AChE through active-site carbamoylation of Ser200. The carbamoylated-enzyme complex is more stable than the choline-enzyme complex. Compounds 1–7 in this study showed a binding to the AChE active site different from that of physostigmine. Compounds 1, 3 and 5, the most potent inhibitors of AChE, showed an almost similar placement in the catalytic site and interaction with amino acids in the anionic sub-site (AS; Fig. 8).

Predicted binding of TcAChE with 1 is via Trp84 with the lactone ring hydroxyl, and Glu199 with the cyclopentane hydroxyl (Fig. 8 A, B). In addition, polar, basic and hydrophobic interactions with Ser200, His440, and Phe330, respectively, were observed. Compound 1 is also estimated to bind to Glu199 and Tyr130 through water molecule. Compound 3 (Fig. 8 C, D) is predicted to bind to the AS Glu199 in a similar fashion to 1. In addition, it is estimated to bind by the hydroxyl group on the seven-member ring to Ser122, Tyr121, Asp72 and Ser81 through the active site conserved water molecules. Moreover, it showed interactions with other active site amino acids residues, such as Trp84, His440 and Gly118. Compound 5 (Fig. 8 E, F) is predicted to bind to Trp84, Glu82 and Tyr442 with the cyclopentane hydroxyl. In addition, 5 shows lactone ring hydroxyl hydrogen bonding with Gly118. In summary, both pharmacophore and docking models predict hydroxyl groups as H-bond donors that bind to AChE active site residues. In addition, hydrophobic ligand portions are predicted to interact with non-polar residues, including Phe330 and Trp84.

Phytochemical investigations of solvent-extracted foliar C. cornigerum afforded seven sesquiterpene lactones that exhibited antioxidant, radical scavenging and metal chelating activity. AChE inhibition with the isolated terpenes observed in vitro served as impetus for examining ligand-enzyme binding in silico. Specifically, 1 exhibited a potent antioxidant and metal chelating activity as well as AChE inhibition.

From previous docking studies, it is well established that the TcAChE active site is located in a deep narrow pocket, referred to as the active site gorge [27]. This catalytic site consists of an amino acid triad of Ser200–His440–Glu327. An AS consists of Trp84.
Tyr334. In fact, with both Tyr130 and Glu199 via water bridging the ligand with ion-Phe330 that is involved in ligand recognition [28, 29]. Finally, a peptide site, the active site contains many aromatic residues such as Tyr130, Glu199, His441, and His444, which are all residues that can participate in inhibitor binding [28]. In fact, Tyr130 and Glu199 via water bridging the ligand with the enzyme. At the A5, the indole side chain of Trp84 makes a cation–π interaction with the quaternary amino groups of acetylcholine as well as AChE inhibitors [28] or lactone ring hydroxyl in 1. Other important amino acid residues are Gly118, Gly119, and Ala201 that form the oxyanion hole of the active site. Compounds such as 5 can bind to Gly118 at this oxyanion hole through the lactone ring hydroxyl hydrogen. In addition to an anionic binding site, the active site contains many aromatic residues such as Phe330 that is involved in ligand recognition [28, 29]. Finally, a peripheral anionic site (PAS) is placed near to the top of the gorge at 14 Å from the active site includes Trp279, Trp70, AP72, Tyr279 and Tyr334 [28].

In many instances sesquiterpene lactones have been reported to possess cytotoxicity [30]. The compounds of this study have not yet been tested for such possible toxic effects, so that the assessment of their potential usefulness as AChE inhibitors leads against neurodegenerative disorders, which remains to be investigated in future studies.

### Materials and Methods

#### General

$^1$H and $^{13}$C NMR spectra were recorded in CDCl$_3$ on a JEOL ECA-600 spectrometer (600 MHz for $^1$H and 150 MHz for $^{13}$C, respectively). All chemical shifts ($\delta$) are given in ppm units with reference to TMS as an internal standard and coupling constants ($J$) are reported in Hz. FAB-MS was performed on a Finnigan LCQ ion trap mass spectrometer and HR-FAB-MS experiments were performed on Fourier transform ion cyclotron mass spectrometer. Ei-MS experiments were performed using a Thermo ISQ Single Quadrupole system. High performance liquid chromatography (HPLC) was performed on variable wavelength UV detector at 254 nm and a semi-preparative reverse-phase column. YM-Pack ODS-A (YMC, Inc., 250 × 4.6 mm i.d.) and (250 × 10 mm i.d.) columns were used for analytical and semipreparative purposes, respectively. Silica gel 60 (230–400 mesh) was used for column chromatography. Pre-coated silica gel plates (Kieselgel 60 F254, 0.25 mm) were used for TLC analyses. Spots were visualized by heating after spraying with 10% H$_2$SO$_4$. 5,5-Dithiobis-2-nitrobenzoic acid (DTNB), Tris-Cl, butylated hydroxy toluene (BHT, purity > 99.0%), PMS, nicotinamide adenine dinucleotide (NADH), Tris–HCl, eserine hemisulfate (purity > 98%, Sigma), 1,1-diphenyl-2-picryl-hydrazil (DPPH•), potassium ferricyanide [K$_3$Fe(CN)$_6$], FeCl$_3$, butylated hydroxyl toluene (BHT, purity > 99.0%), PMS, nicotinamide adenine dinucleotide (NADH), NBT, Horse radish peroxidase, acetylcholinesterase (E.C. No. 3.1.1.7) Type VI-S: from Electric Eel, 2-azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid) diammonium salt (ABTS), FeCl$_2$, hexosulphate salt and acetyltiocholine iodide were purchased from Sigma (Sigma-Aldrich, Germany). All solvents are HPLC grade from Merck.

#### Plant material

Aerial plant parts of the wild artichoke herb C. cornigera were collected and air dried during the flowering period (March 2011) from Marsa-Matroh, Egypt. Plant material was kindly authenticated by Prof. Dr. Ibrahim El-Garf, Botany Department, Faculty of Science, Cairo University, Cairo, Egypt. A voucher specimen (M-30) was deposited in the herbarium of the National Research Centre, Cairo, Egypt.

#### Extraction and isolation

Five hundred grams of dried and powdered plant material were extracted three times with aqueous methanol (80%). Combined extracts were evaporated in vacuo at 45°C to yield circa 100 g of a dark brown residue. The residue was fractionated on a silica gel column (6 × 120 cm) eluting with n-hexane (3 L) followed by a

<table>
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<th>Isolated compounds</th>
<th>Percentage of inhibition</th>
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<tr>
<td>2</td>
<td>26.15 ± 1.64</td>
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<td>38.27 ± 1.84</td>
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<tr>
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<tr>
<td>5</td>
<td>48.64 ± 1.55</td>
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<tr>
<td>6</td>
<td>8.97 ± 1.76</td>
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<tr>
<td>7</td>
<td>28.67 ± 1.45</td>
</tr>
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</table>

Eserine hemisulphate (positive control) it reproduced IC$_{50}$ = 0.092 µM

Determinations were done in triplicates; standard deviation was of three assays. Data were analyzed by one way ANOVA followed by Post Hoc for multiple comparisons. There is no significant difference between values belonging to the same group indicated by a, b, c, d. IC$_{50}$ = concentration required for 50% inhibition.

**Fig. 6** AChE inhibition (%) by compounds 1–7 presented as calculated IC$_{50}$.
gradient of n-hexane-CH₂Cl₂ up to 100%, CH₂Cl₂ and CH₂Cl₂-MeOH up to 15% MeOH (2 L each of the solvent mixture).

The n-hexane:CH₂Cl₂ (1:1) fraction (3.5 g) was subjected to a second silica gel column (3 × 120 cm) eluted with n-hexane:CH₂Cl₂ (6:1) generating two subfractions. Subfraction 1A (0.8 g) was further purified by HPLC eluted with MeOH:H₂O (75:25). The flow rate was set at 2.0 mL/min was at 0–60 min to afford 1 (10 mg, purity > 98% by HPLC), (eluent hexane/EtOAc 1:1, Rf = 0.40) and 2 (15 mg purity > 96% by HPLC), (eluent hexane/EtOAc 1:1, Rf = 0.30). Subfraction 2A (1 g) was also purified by HPLC eluted with MeOH:H₂O (75:25). The flow rate was set at 1.5 mL/min was at 0–60 min to afford 2 (15 mg, purity > 98% by HPLC), (eluent hexane/EtOAc 1:2, Rf = 0.35) and 4 (20 mg, purity > 97% by HPLC), (eluent hexane/EtOAc 1:1, Rf = 0.35). An n-hexane CH₂Cl₂ (1:3) fraction (4.2 g) was subjected to a silica gel fractionation (3 × 120 cm) eluted with n-hexane-CH₂Cl₂-MeOH to give two subfractions. Subfraction 1B (1.2 g) was further purified by HPLC eluted with MeOH-H₂O (70:30). The flow rate was set at 2.0 mL/min was at 0–60 min to afford compound 5 (5 mg, purity > 98% by HPLC), eluted with hexane/EtOAc 1:1, Rf = 0.35; subfraction 2B (1.5 g) was also purified by HPLC eluted with MeOH-H₂O (70:30). The flow rate was set at 1.5 mL/min was at 0–60 min to afford 6 (12 mg, purity > 98% by HPLC), (eluent hexane/EtOAc 1:1, Rf = 0.40). The 100% CH₂Cl₂ fraction was subjected to HPLC eluted with MeOH-H₂O (65:35). The flow rate was set at 2.0 mL/min was at 0–60 min to afford 7 (17 mg, purity > 96% by HPLC), (eluent hexane/EtOAc 1:2, Rf = 0.45).

Corinigeraline (1): Colorless needles +22.0 (c 0.001, MeOH); 1H (CD₂OD, 600 MHz) and 13C (CD₂OD, 150 MHz) NMR, see Table 1; FABMS m/z 321 [M + Na]+, HRFABMS m/z 321.0879 (calcld. for C₁₅H₁₉O₄ClNa: 321.0879); IR (νmax ·cm⁻¹) = 3540 (OH), 1775 (γ-lactone CO), 1635, 905 (C=CH).

Solstitalin (2): Colorless needles +40.0 (c 0.001, MeOH); 1H (CD₂OD, 600 MHz) and 13C (CD₂OD, 150 MHz) NMR, see Table 1; FABMS m/z 303 [M+Na]+, HRFABMS m/z 303.1202 (calcld. for C₁₅H₂₀O₃Na: 303.1208); IR (νmax ·cm⁻¹) = 3540 (OH), 1775 (γ-lactone CO), 1635, 905 (C=CH).

**Single-crystal X-ray crystallography**

X-ray analysis established the complete structure and relative configuration of 2. The crystal data can be summarized as follows: C₁₅H₂₀O₅, formula wt. 280.32; orthorhombic, space group P1 (#1), a = 12.162(2) Å, b = 12.256(2) Å, c = 15.462(3) Å, V = 2235.5(5) Å³, Z = 4, Dcald = 1.593 g/cm³, crystal size 0.350 × 0.130 × 0.070 mm³. All diagrams and calculations were performed using a Rigaku R-AXIS RAPID diffractometer, with graphite monochromated CuKα radiation (λ = 0.71075 Å). The structures were refined by full matrix least squares on FZ using Brucker SHELX-97.22). The final R and Rw were 0.0390 and 0.1048, respectively.

**Acetylcholinesterase inhibition assay**

The enzymatic activity was measured using an adaptation of Ingkaninan et al. [31]. Five hundred µL of DTNB (3 mM), 100 µL of ACh (15 mM), 275 µL of Tris-Cl buffer (50 mM, pH 8) and 100 µL of each compound at different concentrations (5, 10, 20 and 40 µg/mL) were dissolved in ethanol and were added to a 1 mL cuvette, which was used as blank. In the reaction cuvette, 25 µL of buffer were replaced by the same volume of an enzyme solution containing 0.28 µM/l. The reaction was monitored for 5 min at 405 nm and reaction velocities were calculated. Enzyme activity was calculated as a percentage of the velocities compared to that of the assay using buffer instead of inhibitor (compounds). Inhibitory activity was calculated from 100 subtracted by the percentage of enzyme activity. Data presented here are the average of three replicates.

**Antioxidant activity**

**Free radical scavenging activity:** The free radical scavenging activity of the plant extracts was measured with 1,1-diphenyl-2-picryl-hydrazil (DPPH•) [32]. Briefly, DPPH• (0.1 mM) was prepared in methanol and an aliquot (1 mL) was added to each isolated natural product (3 mL) prepared a concentrations of 5, 10, 20 and 40 µg/mL. The mixtures were vigorously shaken and allowed to stand at room temperature for 50 min before recording the absorbance at 517 nm (Jasco V630 spectrophotometer). Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. The DPPH• radical concentration in the reac-
Fig. 8 Docking of the isolated sesquiterpenes to the acetylcholine esterase active site. H-bonds are represented by dashed lines; compounds are colored green. A 2D-ligand interaction of compound 1 with the active site. B 3D docking of compound 1 to active site. C 2D-ligand interaction of compound 3 with the active site. D 3D docking of compound 3 to active site. E 2D-ligand interaction of compound 5 with the active site. F 3D docking of compound 5 to active site. (Color figure available online only.)
DPPH+ scavenging effect (%) = 100 − [(A0 − A1)/A0] × 100

where A0 was the absorbance of the control reaction, and A1 was the absorbance in the presence of the sample [33].

Reduction capability: Plant extract reduction capacities were determined according to Oyaizu et al. [34]. Isolated compounds diluted in MeOH 5, 10, 20 and 40 µg/mL (1 mL) were added to phosphate buffer (2.5 mL, 0.2 M, pH 6.6) containing potassium ferri-cyanide [K₃Fe(CN)₆] (2.5 mL, 1%). The assay mixture was incubated at 50°C for 20 min, then the reaction was stopped by the addition of TCA (10%, 2.5 mL) and the mixture was centrifuged for 10 min at 1000×g (MSE Mistral 2000). The upper layer (ca. 2.5 mL) was diluted with methanol (2.5 mL) containing FeCl₃ (0.5 mL, 0.1%) and the absorbance was measured at 700 nm. BHT was used as a positive control. Higher absorbance of the reaction mixture indicated greater reducing power.

Superoxide anion scavenging activity: Superoxide anion scavenging activity was based on the method described by Gülçin et al. (2003) [35], in which superoxide radicals are generated in a PMS-NADH system by oxidation of NADH and assayed by the reduction of NBT. Superoxide radicals were generated in Tris–HCl buffer (16 mM, pH 8.0, 3 mL), containing NBT (50 µM, 1 mL), NADH (78 µM, 1 mL) and the isolated natural products to be assayed (1 mL) at different concentrations. The reaction was started by adding PMS solution (10 µM, 1 mL). The reaction mixture was incubated at 25°C for 5 min and absorbance readings (560 nm) were performed. Decreasing absorbance indicated increasing superoxide anion scavenging activity. The percentage inhibition of superoxide anion generation was calculated as:

Percent of inhibition = [(A0 − A1)/A0] × 100

where A0 was the absorbance of the control (l-ascorbic acid), and A1 was the absorbance of test metabolite.

Total antioxidant capacity was measured according to the method described by Miller & Rice-Evans [36]. Peroxidase (4.4 units/mL, 0.2 mL), H₂O₂ (50 µM, 0.2 mL), 2,2-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid, diammonium salt [ABTS]) (100 µM, 0.2 mL) were prepared in MeOH (1 mL) then were mixed, and placed in the dark for one hour; isolated compounds (5, 10, 20 and 40 µg/mL, 1 mL) or the positive control BHT were added and absorbance (734 nm) of the bluish-green complex was recorded. Total antioxidant capacity was calculated based on the equation:

Total antioxidant capacity (%) = [1 − (A_{sample}/A_{control})] × 100

where A₀ was the absorbance of control while A₁ was the absorbance in the presence of test sample and standard.

Pharmacophore elucidation for the acetylcholinesterase inhibitors

The pharmacophore model was generated based on the Molecular Operating Environment (MOE) software 10/2008 (Chemical Computing Group). Prior to the pharmacophore elucidation, a database of isolated metabolites with IC₅₀ values was prepared. 3D conformations of the compounds were generated using a conformation import function with a default MMFF94x force field for energy minimization. The activity field was adjusted to respective IC₅₀ values. The features used were Aro/Pir with radius 1.4 Å, Hyd (1.4 Å), Don and Acc (1.0 Å), Cat and Ani (1.0 Å), and O₂ (1.4 Å) (for abbreviations see legend to Fig. 8). The query cluster was adjusted to 1.25 and conformation to As-Is.

Docking study

The crystal structure of TCAChe was selected for structure homology to the assayed EeAChE [26]. The crystal structure of tetracaine bound to acetyl choline esterase active site (1AC) was downloaded from the protein data bank (www.pdb.org). The 3D structures of the tested compounds were generated using the ligx function of the MOE program followed by energy minimization of the generated structures. The protein structure was prepared for docking by 3D protonation, adjusting the temperature to 300 K and pH to 7. The electrostatics functional form was used for calculation; electrostatic interactions are computed using GB/VI (generalized born/volume internal formalism) between two atoms if their separation distance is smaller than the cutoff value of 10 Å. The electrostatic constant of solute and solvent were set to 1 and 80, respectively, the van der Waals functional was set to 800R3. The protein energy was minimized using the MMFF94 force field. The active site was detected using the site finder function of the program. Docking was carried out by setting placement to triangle matcher using affinity dg as rescoring and retainer set to 10. Refinement was set to 1.5 forcefield and rescoring to affinity dg with retainer set to 10. The previous docking parameters were selected as they gave the best re-docking result for the co-crystal ligand with a root mean square deviation (RMSD) of 0.06 Å.

Statistical analysis

The results obtained were presented as a mean of triplicates ± SD while analysis of variance (p ≤ 0.05) was performed by one way ANOVA procedure (SPSS 09.05) followed by Post Hoc analysis for multiple comparisons.

Supporting information

1D and 2D NMR spectroscopic data of compounds 1–7, and experimental details are available as supporting information.

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Conflict of Interest

The authors declare that they have no competing interests.

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