In vitro anti-inflammatory and acetylcholinesterase inhibition efficiency of plant extracts from Sinai-Egypt

Abeer Y Ibrahim1, Abdelaaty A Shahat2,3,*, Tarik A Mohamed3, Abdelsamed I Elshamy4, Ali S Alqahtani2, Ibrahim A Saleh5, Emad M Hassan1 & Mohamed-Elamir F Hegazy5

1Medicinal and Aromatic Plants Researches Department, National Research Centre, 33 El Bohouth St. 12622, Dokki, Giza, Egypt
2Pharmacognosy Department, College of Pharmacy, King Saud University, Riyadh 11451, Saudi Arabia
3Phytochemistry Department, National Research Centre, 33 El Bohouth St. 12622, Dokki, Giza, Egypt
4Natural Compounds Chemistry Department, National Research Centre, 33 El Bohouth St., 12622, Dokki, Giza, Egypt
E-mail: *ashahat@ksu.edu.sa; aashahat@hotmail.com

Ten plant extracts were prepared and tested in in vitro assays against COX-2, COX-1 and acetylcholinesterase with evaluation of their antioxidant properties. The tested extracts exhibited varied anti COX-2 effect and they were superior to celecoxibe (inhibition percentage was 42.67% at 50 µg/mL), reference drug. Lavandula coronopifolia and Scrophularia Libanotica extracts were the efficient inhibitors (100% and 91% at 50 µg/mL respectively). Launaeaspinosa and Pulicaria undulata were the powerful AChE inhibitor (IC50 values were 16.69 and 29.06 µg/mL, respectively) followed with L. coronopifolia and S. libanotica extracts (IC50 values were 61.89 and 49.83 µg/mL, respectively) and they were efficient in scavenging superoxide radicals and metal ions, nitric oxide formation inhibition, as well as, lipid peroxide production suppression. L. coronopifolia and S. Libanotica extracts can be introduced as natural cyclooxygenase-2 inhibitors without affecting cyclooxygenase-1 whereas L. spinosa and P. undulata extracts were potent suppressor for AChE with robust antioxidant properties which suggest the possibility of using the four extracts, L. coronopifolia, S. libanotica, L. spinosa and P. undulata as natural agent in treating neurodegenerative disorder.

Keywords: Anti-inflammatory, Acetylcholinesterase, Cyclooxygenase inhibition, Sinai Egyptian wild plants

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Saint Catherine’s Reserve, an ecological and cultural heritage site, is the Sinai Peninsula’s most biologically diverse region. Since ancient Egyptian times, herbal plants have been used as a source of medications which have been confirmed by several studies through isolation and identification of biologically active metabolites as pharmaceutical drugs. Nonsteroidal anti-inflammatory drugs (NSAIDs) are commonly utilized pharmaceutical agents in many diseases. NSAIDs mechanism of action is contributed to their ability to inhibit prostaglandin biosynthesis, the activity possessed by two individual cyclooxygenase isozymes, COX-1 and COX-2. Researchers around the world work for discovery of new potent naturally NSAIDs selective for COX-2, with fewer side effects than traditional NSAIDs. The arachidonic acid (AA) is enzymatically transforms to endoperoxide-containing prostaglandin G2 (PGG2), potent bioactive lipid messengers derived from AA, in the cyclooxygenase reaction. The reduction of a hydroperoxyl to a hydroxyl form in PGG2 and PGH2, respectively, via a

*Corresponding author
selection of Sinai wild plant species based on the unique ecosystem giving rise to great plant diversity due to large variations in landforms, water resources, aridity conditions and temperatures. Herein, we aimed to give a scientific approval and/or new biological finding for some of the wild plants growing in Sinai Peninsula. As a part of our continuous research for natural products discovery from plants growing in Egypt through evaluation of ten plant extracts as anti-inflammatory natural products by evaluating their cyclooxygenases inhibitor activity and testing their effect against acetylcholinesterase which play important role in many neuroinflammatory diseases16-21.

Materials and methods

Plant material and extraction
The air-dried aerial parts of wild plants “Launaea spinosa (SK-219), Teucrium polium (SK-105), Tanacetum sinaicum (SK-120), Pulicaria undulata (SK-103), Chilaidenus montanus (SK-1001), Ballota undulata (SK-113), Scrophularia libanotica (SK-107), Euphorbia sanctae-cathariniae (SK-212), Lavandula coronopifolia (SK-215) and then Stachys aegyptiaca (SK-213)” were collected in June 2014 from South Sinai, Egypt. The identified and voucher specimens have been deposited in the herbarium of Saint Catherine protectorate Egypt. The collection was completed under the agreement of Saint Catherine protectorate for scientific reason through officially permission from the National Research Center. The powdered aerial parts of each plant (100 g) were extracted at room temperature with CH3Cl2-MeOH (1:1). The crude extracts were achieved using rotary evaporator concentrated filtrate solvents extract, resulting in 10 crude extracts containing various compounds polarity.

Chemicals
1,3-diethyl thiobarbituric acid (DETBA), Ammonium thiocyanate and linoleic acid were purchased from E. Merck. Peroxidase, phenazine methosulphate (PMS)-nicotinamide adenine dinucleotide (NADH), Cyclooxygenase enzyme (COX-1 from sheep, EC. 1.14.99.1 or COX-2), 2, 2-azino-bis (3-ethylbenzthiazoline- 6-sulfonic acid) diammonium salt, nitroblue tetrazolum (NBT), Tris-HCl buffer, Greiss reagent, sodium nitroprusside, ferrous chloride, 3-(2-pyridyl)-5,6-bis (4-phenyl-

sulfonic acid)-1,2,4-triazine (ferrozine), nicotinamide adenine dinucleotide (NADH), Ascorbic acid (Vc), butylated hydroxytoluene (BHT), Leuco-2,7-dichlorofluorescien diacetate, hematin, arachidonic acid, Tris-buffer, acetylcholinesterase (E.C. No. 3.1.1.7) Type VI-S: from Electric Eel, serum hemisulfate salt, acetylthiocholine iodide, DTNB (dithiobis nitrobenzoic acid), were purchased from Sigma-Aldrich, USA.

Cyclooxygenases inhibitory activity
The optimum method designated by Larsen et al. (1996)22 is endorsed for the determination of cyclooxygenase inhibitors activity of natural or synthetic materials. The leuco-dichlorofluorescisin (L-DCF) oxidation by the hydroperoxide formed in the cyclooxygenase reaction in the presence of phenol can be used as a sensitive spectrophotometric assay for PGH synthase activity22 (Larsen et al., 1996). The concentrations 25, 50, 100, 200 and 400 µg/mL for fractions were investigated and Celecoxib was used as a reference compound.

Acetylcholinesterase inhibition assay
Ingkaninan et al. (2003)23 method is recommended for determination of acetylcholinesterase enzymatic activity. The reaction mixture contains AChI (15 µM), DTNB (3 µM) and Tris–HCl buffer (50 µM, pH 8) dissolved in ethanol then each sample, plant extracts at 25, 50, 100, 200 and 400 µg/mL, was added and accompanied with blank sample. One ml of reaction mixture and tested extracts at different concentration in three replicates were transferred to cuvette. The enzyme solution (0.28 U mL−1 25 µL) were exchanged by the same volume of the buffer in the reaction cuvette. The absorbance was examined for 5 min at 405 nm for enzymatic activity calculation23 and Eserinehemisulphate was used as a reference drug.

Nitric Oxide radical scavenging activity
Plant extracts at 25, 50, 100, 200 and 400 µg/mL were tested using Greiss reagent24. The reaction mixture (2 mL) including various extract concentrations as well as standard compounds and SNP (10 µM) were incubated at 25°C for 150 min in phosphate buffered saline (PBS) pH 7.4. After incubation, the reaction mixtures for each tested sample (1 mL) were diluted with Greiss reagent (1:1, v/v) and the absorbance was measured at 540 nm.
Antioxidant capacity

The total antioxidant capacity of tested extracts at 25, 50, 100, 200 and 400 µg/mL were determined using Peroxidase-ABTS technique. The mixture was kept in dark for one hour and then one mL of each sample or standard was added followed monitoring of absorbance at 734 nm using the following equation for total capacity calculation:

\[
\text{Total antioxidant activity} \% = \left[1 - \frac{(A_{\text{sample}})}{A_{\text{control}}}\right] \times 100.
\]

Metal chelating activity

The chelating of ferrous ions by extracts and standards was estimated by the method of Dinis et al. Briefly, extracts and standards of various tested concentrations were added to FeCl₂ (2 µM). Ferrrozine was used for reaction initiation (5 µM) and the mixture was shaken vigorously and left standing at room temperature for ten minutes. The absorbance was then measured at 562 nm in a spectrophotometer. The inhibition percentage of ferrozine-Fe²⁺ complex formation was given by the formula:

\[
\text{Inhibition} \% = \left[1 - \frac{(A_{\text{control}} - A_{\text{sample}})}{A_{\text{control}}}\right] \times 100,
\]

where A₀ was the absorbance of the control reaction and A₁ was the absorbance in the presence of the sample of extracts and standards. The control contains FeCl₂ and ferrozine.

Superoxide anion scavenging activity

Measurement of superoxide anion scavenging activity of extracts was based on the method described by Liu et al. Superoxide radicals are generated in phenazine methosulphate (PMS)–nicotinamide adenine dinucleotide (NADH) systems by oxidation of NADH and assayed by the reduction of nitroblue tetrazolium (NBT). L-ascorbic acid and BHT were used as controls. Decrease in absorbance of the reaction mixture indicated increased superoxide anion scavenging activity. The percentage inhibition of superoxide anion generation was calculated using the following formula:

\[
\text{Inhibition} \% = \left[1 - \frac{(A_{\text{control}} - A_{\text{sample}})}{A_{\text{control}}}\right] \times 100,
\]

where A₀ was the absorbance of the control (l-Ascorbic acid), and A₁ was the absorbance of extracts or standards.

Lipid Peroxidation- Ammonium Thiocyanate Method

The antioxidant activity of plant extracts under study was investigated using a modified Gulcin method. A pre-emulsion and samples preparation were performed as previously described by us. The percentage of lipid peroxidation inhibition was calculated according to the following equation:

\[
\text{Inhibition} \% = \left[1 - \frac{(A_{\text{control}} - A_{\text{sample}})}{A_{\text{control}}}\right] \times 100 \text{ (1)},
\]

where A₀ was the absorbance of the control reaction and A₁ was the absorbance in the presence of tested extracts and/or standard compounds.

Statistical analysis

All data are expressed as means of triplicates±S.D. One- way ANOVA was used for analysis of variance for statistical evaluation followed with Tukey’s Multiple Comparison test using SPSS 9 program.

Results and Discussion

The inhibitory effect of plant extracts against COX-2 and COX-1 were evaluated as indicator of their role in inflammatory response in comparing to celecoxib, reference compound. Data recorded indicated the gradual increasing inhibitory effect of tested extracts with increasing concentrations and they exhibited more inhibition percentages at (50, 100 and 200 µg mL⁻¹) than celecoxibe except B. undulata and C. montanus extracts, Fig. (1). L. coronopifolia presented significant inhibition (p≤0.05) and it was powerful in COX-2 inhibition (100% at 50, 100 and 200 µg mL⁻¹) while the minimum mean inhibitory value was observed with B. undulata, extract (74.34% at 200 µg mL⁻¹). S. libanotica extract completely inhibited COX-2 at 100 and 200 µg mL⁻¹ whereas S. aegyptiaca, E. sanctae-catharinae, L. spinosa, T. sinaicum and P. undulata extracts showed the same effect at the highest concentration only.

On the other hand, the plant extracts showed varied effects to COX-1. Generally, they partially inhibited COX-1 with low values comparing to reference drug, celecoxib, at all concentrations in a concentration dependent manner. The minimum inhibitory effect
was recorded for \( T. \text{ polium}, \) \( L. \text{ spinosa} \) and \( T. \text{ sinaicum} \) (15, 14 and 17%, respectively, at 200 µg mL\(^{-1}\)) whereas \( L. \text{ coronopifolia}, \) \( S. \text{ libanotica}, \) \( B. \text{ undulata}, \) \( C. \text{ montanus} \) and \( P. \text{ undulata} \) produced the same effect (24-26% at 200 µg mL\(^{-1}\)), Fig. (2). \( S. \text{ aegyptiaca} \) and \( E. \text{ sanctae catheirinae} \) extracts showed the highest inhibition values (30% at 200 µg mL\(^{-1}\)) which were lower than celecoxibe (61%).

It's evident from cyclooxygenases presented results that the ten tested extracts significantly inhibited COX-2 with low inhibitory effect against COX-1 as compared to the reference drug. \( L. \text{ coronopifolia} \) extract exhibited the highest inhibition percentage (100% at low conc., 50 µg mL\(^{-1}\)) against COX-2 with reduced COX-1 inhibition (20.71%) at the same concentration while celecoxibe reached its maximum inhibitory COX-2 values (89%) at 200 µg/mL with

![Fig. 2 — Inhibitory effect of plant extracts and standard drug against COX-1. Data are expressed as mean of triplicates ± S.D. Significant difference calculated as compared to celecoxibe (p<0.05).](image)

Table 1 — Inhibitory effect of plant extracts and standard compound against Acetylcholinesterase

<table>
<thead>
<tr>
<th>Plant extract and standard</th>
<th>25</th>
<th>50</th>
<th>100</th>
<th>200</th>
<th>IC(\text{S}_0) (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( S. \text{ aegyptiaca} )</td>
<td>18.18±0.12(^{a})</td>
<td>65.52±0.42</td>
<td>77.76±0.91(^{h})</td>
<td>81.12±0.36(^{k})</td>
<td>30.84</td>
</tr>
<tr>
<td>( T. \text{ polium} )</td>
<td>19.26±0.22(^{a})</td>
<td>19.44±0.41</td>
<td>38.44±0.82</td>
<td>61.70±0.39</td>
<td>135.50</td>
</tr>
<tr>
<td>( L. \text{ coronopifolia} )</td>
<td>30±0.34(^{c})</td>
<td>36±0.33</td>
<td>100±0.61</td>
<td>100.00±0.27(^{i})</td>
<td>61.89</td>
</tr>
<tr>
<td>( E. \text{ sanctae catheirinae} )</td>
<td>36.66±0.27(^{b})</td>
<td>42.94±0.27(^{e})</td>
<td>78.29±0.9(^{b})</td>
<td>100.00±0.41(^{j})</td>
<td>79.25</td>
</tr>
<tr>
<td>( S. \text{ libanotica} )</td>
<td>48.22±0.09(^{d})</td>
<td>76.49±0.19(^{f})</td>
<td>78.29±0.72(^{h})</td>
<td>100.00±0.46(^{l})</td>
<td>49.83</td>
</tr>
<tr>
<td>( L. \text{ spinosa} )</td>
<td>78.64±0.31</td>
<td>78±0.36(^{f})</td>
<td>84.43±0.68(^{f})</td>
<td>100.00±0.46(^{j})</td>
<td>16.69</td>
</tr>
<tr>
<td>( T. \text{ sinaicum} )</td>
<td>22.81±0.26</td>
<td>48.78±0.64(^{d})</td>
<td>84.85±0.31(^{i})</td>
<td>100.00±0.71(^{i})</td>
<td>49.73</td>
</tr>
<tr>
<td>( B. \text{ undulata} )</td>
<td>36.42±0.60(^{b})</td>
<td>48.72±0.44(^{d})</td>
<td>79.35±0.42(^{h})</td>
<td>100.00±0.81(^{l})</td>
<td>74.56</td>
</tr>
<tr>
<td>( C. \text{ montanus} )</td>
<td>31.22±0.41(^{c})</td>
<td>42.18±0.38(^{c})</td>
<td>63.72±0.19</td>
<td>83.46±0.61(^{k})</td>
<td>69.76</td>
</tr>
<tr>
<td>( P. \text{ undulata} )</td>
<td>49.26±0.35(^{d})</td>
<td>74.51±0.09(^{f})</td>
<td>93.28±0.32</td>
<td>100.00±0.37(^{j})</td>
<td>29.06</td>
</tr>
<tr>
<td>Eserinehemisulphate</td>
<td>It reproduced IC(\text{S}_0) = 0.03 µg/mL.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data are presented as mean of triplicates ± standard deviation. Data were analyzed by ANOVA one way followed with Post Hoc for multiple comparisons. Groups have the same letter have no significant difference between them. IC\(\text{S}_0\) is a concentration that reproduces 50% inhibition.
The antioxidant compounds participate strongly in the earlier stage of Alzheimer by many means including clearance of toxic materials from cells that can affect biomolecules. Nitric oxide, small toxic bioproduckt in human cells, formation plays an important role in cyclooxygenase-2 activity induction, which activates the inflammatory cascade, therefore, the inflammation related diseases; cancers and neurodegenerative diseases, inhibition of nitric oxide formation is an important factor in cyclooxygenase-2 inhibition in human biological system. The two extracts produced (11.53, 12.63 and 11.44 µg/mL) followed with B. undulata, C. montanus and P. undulata produced the same nitrite formation inhibition level and they were the most effective extracts, IC50 values were (11.53, 12.63 and 11.44 µg/mL, respectively) followed with S. aegyptiaca, E. sanctae-cathariniae and L. spinosa (13.31, 13.53 and 14.87 µg/mL, respectively). However, T. polium, S. libanotica and T. sinaicum represented the languorous effect which is nearly the same standard materials, Table 2.

Tested plant extracts quenched superoxide radicals produced by phenazine in IC50 range from 27 to 51 µg/mL. S. aegyptiaca, S. libanotica and T. sinaicum extracts (IC50 values were 36.95, 36.22 and 35.46 µg/mL, respectively) came after L. spinosa, C. montanus and P. undulata which extensively trapped superoxide radicals in the reaction media, IC50 values were 28.29, 29.56 and 27.43 µg/mL, respectively. Nevertheless, T. polium, L. Coronopifolia, E. sanctae-cathariniae, B. undulate proceeded the lowest effects, IC50 were 43.64, 43.25, 41.71 and 43.26 µg/mL, respectively (Table 2). The presence of excessive metal ions in tissue caused deteriorative effect represented in maximizing oxidative stress that contributes in many diseases. S. aegyptiaca seems to be the potent extract in chelation of metal ion (IC50, 27.31 µg/mL) followed with L. Coronopifolia (IC50, 30.1 µg/mL), T. polium (IC50, 39.54 µg/mL), T. sinaicum (IC50, 42.16 µg/mL) and then B. undulata (IC50, 39.52 µg/mL). The other extracts produced weak chelating effect against ferrous ions, S. libanotica (48.63 µg/mL), E. sanctae-cathariniae and L. spinosa (58 µg/mL) and then C. montanus and P. undulata (61.72 µg/mL), Table 2. Total antioxidant capacity was determined for all extracts at four concentrations. B. undulata and L. spinosa exhibited the same IC50 value (18 µg/mL) followed with E. sanctae-cathariniae, T. sinaicum and S. libanotica (20 µg/mL), S. aegyptiaca and C. montanus (26 µg/mL) and then T. polium and L. coronopifolia (29 µg/mL) while P. undulata possessed the lowest capacity, IC50 value is 30 µg/mL.

Lipid peroxides production in linoleic assay was significantly suppressed by the presence of E. sanctae-cathariniae, IC50 was 9 µg/mL, whereas T. polium, L. coronopifolia and B. undulata came at the second inhibition level, IC50 was 10 µg/mL, with in significant difference with S. aegyptiaca (IC50 was 11 µg/mL). On the other hand, T. sinaicum

<table>
<thead>
<tr>
<th>Test Plant extract</th>
<th>NO scavenging</th>
<th>Superoxide anion scavenging</th>
<th>Chelation of ferrous ions</th>
<th>Total antioxidant capacity</th>
<th>Inhibition of lipid peroxidation %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stachys aegyptiaca</td>
<td>13.31±1.02a</td>
<td>36.95±1.14c</td>
<td>27.31±1.16f</td>
<td>26.12±1.33k</td>
<td>11.99±0.94e</td>
</tr>
<tr>
<td>Teucrium polium</td>
<td>19.07±0.96c</td>
<td>43.64±1.20c</td>
<td>39.54±0.97b</td>
<td>29.73±1.05b</td>
<td>10.31±1.01f</td>
</tr>
<tr>
<td>Lavandula coronopifolia</td>
<td>16.91±0.88c</td>
<td>43.25±0.95c</td>
<td>30.10±1.13d</td>
<td>29.52±1.11c</td>
<td>10.81±0.76c</td>
</tr>
<tr>
<td>Euphorbia sanctae-cathariniae</td>
<td>13.35±1.00c</td>
<td>41.71±1.03c</td>
<td>58.45±1.14f</td>
<td>20.21±0.86f</td>
<td>9.76±0.89</td>
</tr>
<tr>
<td>Scrophularia libanotica</td>
<td>18.02±0.97c</td>
<td>36.22±0.95c</td>
<td>48.63±1.08</td>
<td>20.29±0.94k</td>
<td>13.37±1.00e</td>
</tr>
<tr>
<td>Launaea spinosa</td>
<td>14.87±1.03c</td>
<td>28.29±1.05c</td>
<td>58.61±1.00f</td>
<td>18.66±1.12k</td>
<td>13.66±1.12c</td>
</tr>
<tr>
<td>Tanacetum sinaicum</td>
<td>18.26±1.10c</td>
<td>35.46±1.10h</td>
<td>42.16±0.96f</td>
<td>20.51±1.03c</td>
<td>12.66±1.01c</td>
</tr>
<tr>
<td>Ballota undulata</td>
<td>11.53±0.89c</td>
<td>43.26±1.22c</td>
<td>39.52±0.97b</td>
<td>18.46±0.99f</td>
<td>10.69±0.93c</td>
</tr>
<tr>
<td>Chiladenus montanus</td>
<td>12.63±0.93c</td>
<td>29.56±1.03c</td>
<td>61.25±1.11g</td>
<td>26.12±0.85k</td>
<td>12.56±0.98e</td>
</tr>
<tr>
<td>Pulicaria undulata</td>
<td>11.44±1.04c</td>
<td>27.43±0.99d</td>
<td>61.72±1.07e</td>
<td>30.43±1.01c</td>
<td>13.36±1.03c</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>17.94±0.91c</td>
<td>51.8±0.91i</td>
<td>68.76±0.94</td>
<td>30.48±1.23e</td>
<td>12.34±1.14e</td>
</tr>
<tr>
<td>BHT</td>
<td>17.75±1.03c</td>
<td>43.83±0.87d</td>
<td>58.91±1.10f</td>
<td>28.35±0.98k</td>
<td>13.25±1.02e</td>
</tr>
</tbody>
</table>

Data are expressed as mean of triplicates ± SD The significant difference evaluated p<0.05. There is insignificant difference between groups have the same letter. IC50 values are the concentration that produced 50% effect percentage.
and *C. montanus* depressed 50% of lipid peroxides formation at IC$_{50}$ value at 12 µg/mL. The existence of 50% of lipid peroxides was blocked by extracts of *S. libanotica, L. spinosa* and *P. undulata* at 13 µg/mL.

Focusing on the mentioned data, it can be concluded that *L. coronopifolia, S. libanotica, E. sanctae-catharinae* and *P. undulata* extracts were effective in suppression of cyclooxygenase-2 with weak effects on cyclooxygenase-1, which support their sufficient and safe role as non-steroidal anti-inflammatory agent. The same extract came at the second level after *L. Spinosa* and *P. undulata* as potent anti-acetylcholinesterase agent which can keep the neurotransmitters, acetylcholine, from hydrolysis with valuable effect as ion chelator, scavenger for radicals and preventer for nitric oxide formation. According to the tested activities, these extracts can be recommended for further in-vivo studies for neurodegenerative diseases.

**Conclusion**

The present work introduced *L. coronopifolia* and *S. libanotica* extracts as potent natural cyclooxygenase-2 inhibitors with minor effects on cell maintenance via reduced effect on cyclooxygenase-1. Additionally, their suppressive effect on acetylcholinesterase with plausible antioxidant properties which suggest the possibility of using them as natural agent in treating neurodegenerative disorder. The analgesic and anti-inflammatory activities have also been reported from other *Lavandula* species extracts including polyphenolic fractions and essential oils$^{33-35}$. In addition, previous studies showed that the aqueous alcohol extract of *S. libanotica* showed potent anti-inflammatory activity due to its glycoproteinoids content$^{36,37}$.

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