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In vitro and *in vivo* Anticholinesterase Activity of the Volatile Oil of the Aerial Parts of *Ocimum basilicum* L. and *O. africanum* Lour. Growing in Egypt

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***Abstract*—**In this study, the *in vitro* anticholinesterase activity of the volatile oils of both *O. basilicum* and *O. africanum* was investigated and both samples showed significant activity. The major constituents of the two oils were isolated using several column chromatographies. Linalool, 1,8-cineol and eugenol were isolated from the volatile oil of *O. basilicum* and camphor was isolated from the volatile oil of *O. africanum.* The anticholinesterase activities of the isolated compounds were also evaluated where 1,8-cineol showed the highest inhibitory activity followed by camphor. To confirm these activities, learning and memory enhancing effects were tested in mice. Memory impairment was induced by scopolamine, a cholinergic muscarinic receptor antagonist. Anti-amnesic effects of both volatile oils and their terpenoids were investigated by the passive avoidance task in mice. We also examined their effects on brain acetylcholinesterase activity. Results showed that scopolamine-induced cognitive dysfunction was significantly attenuated by administration of the volatile oils and their terpenoids, eugenol and camphor, in the passive avoidance task and inhibited brain acetylcholinesterase activity. These results suggest that *O. basilicum* and *O. africanum* volatile oils can be good candidates for further studies on Alzheimer's disease via their acetylcholinesterase inhibitory actions.

***Keywords*—**Acetylcholinesterase, *Ocimum basilicum, Ocimum africanum,* passive avoidance.

I. Introduction

MEMORY is the ability of an individual to record, retain and recall information and to use them to adapt environmental reponses [1]. Alzheimer's disease (AD) is the most common neurodegenerative disease of this century and the most prevalent cause of dementia among the elderly [2]. This irreversible neurological disorder is characterized by memory and cognitive impairment, behavioral deficits and disturbances in daily activities [3]. One of the most remarkable biochemical changes in AD patients is the reduction of acetylcholine levels in the hippocampus and cortex of the brain. That is why the most commonly used symptomatic treatment of mild AD is through improving the cholinergic deficit via the inhibition of acetylcholinesterase (AChE) [4], [5].However, currently used cholinesterase inhibitors are limited and have multiple adverse effects [6], [7]. Therefore, there is a need for developing new compounds with multiple potencies and minimal side effect profiles. Medicinal plants have been primary source of medicines and are rich sources of secondary metabolites and oils that are important in therapeutics. The most important advantage of them is their low price and availability worldwide beside their safety [8].

The genus *Ocimum,* a member of family Lamiaceae comprises about 30 species which are found in tropical and subtropical regions [9]. They are rich in essential oils and have received considerable attention for their potential therapeutic properties. These include hypoglycemic, hypolipidemic [10], antiulcerogenic [11], antimicrobial [12], chemopreventive [13], antimutagenic [14], antioxidant [15] and antihypertensive [16] effects. Sweet basil (*Ocimum basilicum* L.) is an aromatic, herbaceous, autogamous plant that is annual and perennial [17]. This plant is 20-60 cm long, white-purple flowering plant, and is originally native to India and other regions of Asia and also Africa, South America, and the Mediterranean but widely cultivated in many countries [18], [19]. Sweet basil is used in Mediterranean cuisine and foods such as soup, cream cheese for sandwiches and pasta dishes [20], [21].

Wild populations of *Ocimum basilicum* and *Ocimum africanum,* which are grown in Egypt, differ in essential oil composition. Previously, a comprehensive volatile profile of aerial parts of the two *Ocimum* species was performed by GC/MS analysis. In this study, the hypothesis that the volatile oils extracted from the aerial parts of *O. basilicum* L. and *O. Africanum* Lour. can inhibit the activity of the enzyme AChE, was tested. In addition, their ability to enhance memory in scopolamine-induced amnesic mice was investigated.

II. Materials

A. Drugs and Chemicals

Acetylcholinesterase (Electric-eel EC 3.1.1.7), acetylthiocholine iodide, dimethyl sulfoxide (DMSO) and 5,5′-dithiobis(2-nitro) benzoic acid (DTNB), eserine and scopolamine were purchased from Sigma (St. Louis, MO, USA). Buffers and other chemicals were of analytical grade.

B. Animals

Swiss Albino Mice of 20–25 g were used for *in vivo* testing of cholinesterase inhibitory activity of the tested agents. They were housed in plastic cages in groups of four or five per cage under a controlled 12/12-h light–dark cycle, at constant temperature 20±2 ◦C and humidity of 50±5% and allowed free

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access to food and water. On the day of the experiment, animals were brought to the experimental room and permitted to habituate to the environmental conditions for approximately 60 min before the start of the experiment. Handling and experimentation were conducted according to the international ethical guidelines concerning the care and use of laboratory animals. The experimental protocol was approved by Ain Shams University Faculty of Pharmacy Review Committee for the use of Animal Subjects.

C. Animal Treatments

Mice were divided into 13 groups (6 animals each). The first group served as control animals that received DMSO (1 ml/100 g, i.p.) for seven days. One hour after the last dose and 30 min before training sessions, mice were intraperitoneally injected with saline. The second group received DMSO (1 ml/100 g, i.p.) for seven days. One hour after the last dose and 30 min before training sessions mice received scopolamine dissolved in saline (1 mg/kg, i.p.) [22]. Groups 3 to 8 received different doses of *Ocimum basilicum* and *Ocimum africanum* (100, 200, 400 mg/kg i.p.) for seven days. One hour after the last dose and 30 min before training sessions mice received scopolamine dissolved in saline (1 mg/kg, i.p.). Retention (memory) was recorded after 24 h. The volatile oil dose(s) that show(s) effective memory retention was used for calculating the doses of linalool (22%), 1,8-cineol (7%), eugenol (17%) and camphor (50%) to be administered intraperitoneally to groups 9 to 12, respectively, for seven days followed by scopolamine. Eserine, as an established anticholinesterase agent, was dissolved in normal saline and administered (0.2 mg/kg, i.p) to positive control group (group 13) followed by scopolamine. Animals that exhibited antiamnestic effects were then sacrificed. Brains were dissected out and stored at -80°C till AChE activity estimation.

III. Methods

A. In vitro Cholinesterase Inhibition Assay and Determination of IC50

Acetylcholinesterase inhibiting activity was measured according to a slightly modified spectrophotometric method [23]. Test compounds were dissolved in DMSO. Acetylthiocholine iodide was used as substrate to assay acetylcholinesterase. 5,5´-Dithiobis[2-nitrobenzoic-acid] (DTNB) was used for the measurement of cholinesterase activity. 100 mM sodium phosphate buffer (pH 8.0, 140 µL), DTNB (10 µL), test compound solution (20 µL) and acetylcholinesterase (20 µL) were mixed and incubated for 15 minutes (25º C). The reaction was then initiated by the addition of acetylthiocholine (10 µL). The hydrolysis of acetylthiocholine was monitored by the formation of yellow 5-thio-2-nitrobenzoate anion as the result of the reaction of DTNB with thiocholine, released by the enzymatic hydrolysis of acetylthiocholine at a wavelength of 412 nm. The rate of hydrolysis of AChE was measured over 15 min using a 96-well microplate reader. The concentrations of test compounds that inhibited the hydrolysis of substrate (acetylthiocholine) by 50% (IC50) were determined by monitoring the effect of increasing concentrations of these compounds in the assays on the inhibition values. The IC50 values were then calculated using a software program (GraphPad Prism, version 5.01, Inc., 2007, San Diego California USA). The concentration of the compounds which caused 50% inhibition of the AChE activity (IC50) was calculated via nonlinear regression analysis. The anticholinesterase activity of the compounds was compared with that of eserine.

B. Passive Shock Avoidance (Step-Through) Paradigm

A step-through passive avoidance apparatus for mice was used (Ugo Basile, Italy). Each mouse was subjected to two sessions: a training session and a test session. During the training session, each mouse was trained by gently placing it in the light compartment. When it stepped through the dark compartment putting all its paws on the grid floor, the door automatically closed and an electric shock of 1mA was delivered for 1 s. Mice that failed to step through within a cut-off time of 90 s were not used.

Test session: Twenty-four hours after training, each mouse was introduced to the light compartment and the latency to step through to the dark compartment was recorded as a passive avoidance behavior indicating memory acquisition, with an upper cut-off time of 300 s. No electric shock was delivered during this test session [24].

C. Estimation of Whole Brain Acetylcholinesterase Activity

The estimation of whole brain acetylcholinesterase activity is carried out based on Ellman's method with slight modifications. Following the behavioral testing, animals were decapitated and brains were dissected out immediately and placed in ice-cold saline. The tissue was weighed and homogenized in 0.1 M Phosphate buffer pH 8 (10%w/v). Homogenized tissue was centrifuged to 15,375 ×g for 10 min. 0.4 ml aliquot of the supernatant was added to the other reagents and processed as described before.

D. Statistical Analysis

In vitro assay was undertaken in triplicate and the concentration of tested samples required to inhibit 50% of the activity under the assay conditions was determined from dose-response curves and defined as the IC50 value and expressed as mean ± standard deviation.

Other data were expressed as mean ± standard error and analyzed by one-way ANOVA followed by Tukey test was to assess significant differences among the treatment groups. Probability values of less than 0.05 were considered statistically significant.

IV. Results

A. In vitro Cholinesterase Inhibition Assay and Determination of IC50

Volatile oils of both *O. basilicum* and *O. africanum* showed significant *in vitro* inhibitory activity against AChE. The major constituents of the two oils: Linalool, 1,8-cineol, eugenol and camphor showed anticholinesterase activity

where the highest inhibitory activity was for 1,8-cineol isolated from *O. basilicum* followed by camphor isolated from *O. africanum*.

TABLE I
IN VITRO ACETYLCHOLINESTERASE INHIBITORY ACTIVITY

Sample	IC 50 (mg/ml) against AChE
Eserine	0.27 ± 0.15
O. baselicum volatiles	0.22 ± 0.2
O. africanum volatiles	0.175± 0.09
Linalool	23.209 ± 1.9
1,8-Cineol	0.3552 ± 0.02
Eugenol	6.622 ± 0.49
Camphor	3.263 ± 0.27

Results were expressed as mean ± SD (n = 3)

B. Step-Through Latencies of Mice in Passive Avoidance Paradigm

Scopolamine-treated mice showed significant decline in latency time as compared to the control group ($p< 0.001$). Treatment of mice with 400 mg/kg of *O. basilicum* and 200 mg/kg of *O. africanum* significantly delayed latency times in retention trials in comparison to scopolamine-treated mice ($p< 0.01$, $p<0.05$, respectively). Mice treated with eugenol (70 mg/kg) and camphor (100 mg/kg) exhibited significantly delayed latency times ($p<0.05$). These effects were not significantly different from those elicited by eserine. However, treatment with linalool (90 mg/kg) and 1, 8-cineol (30 mg/kg) didn't significantly affect latency (Fig. 1).

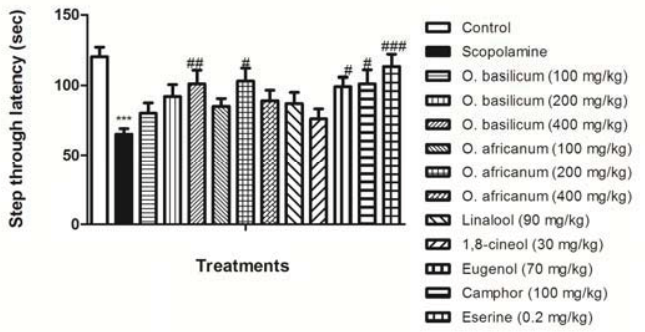

Fig. 1 Effects of *O. basilicum* and *O. africanum* and their terpenoids on scopolamine-induced amnesia of a step-through passive avoidance task in mice (The oils were intraperitoneally administered for 7 days. Scopolamine (1 mg/kg, i.p.) was injected 1 h following treatments of day 7, followed 30 min later by the training session. Step through latency values are presented as means ± S.E.M. (n = 6). #p<0.05, ##p< 0.01 , ###p<0.001compared to scopolamine-treated group, ***p<0.001 compared to control group (one-way ANOVA followed by Tukey test))

C. *Brain AChE Activity*

As shown in Fig. 2, mice treated with scopolamine showed significantly high AChE activity in comparison to control group ($p<0.001$). Treatment of mice with 400 mg/kg of *O. basilicum* and 200 mg/kg of *O. africanum* significantly inhibited AChE activity in comparison to scopolamine-treated mice ($p<0.01$). Mice treated with eugenol (70 mg/kg) and camphor (100 mg/kg) exhibited significantly reduced AChE activity ($p<0.05$, $p<0.01$, respectively). These effects were not significantly different from those elicited by eserine. However, treatment with linalool (90 mg/kg) and 1, 8-cineol (30 mg/kg) did not significantly affect AChE activity.

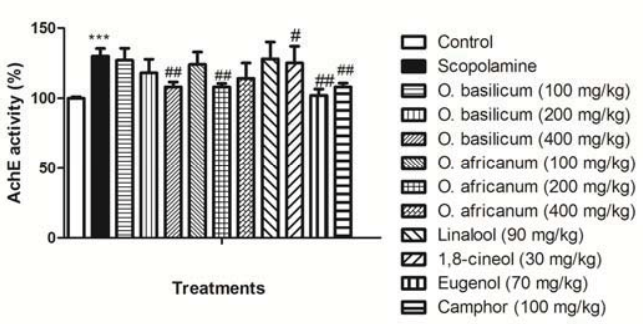

Fig. 2 Inhibitory effects of *O.basilicum* and *O. Africanum* volatile oils and their terpenoids against brain AChE activity in scopolamine-treated mice (Inhibition value of AChE (%) was calculated and compared to that of control group. Data represent the mean ±S.E.M (n=6) and were analyzed by one-way ANOVA followed by Tukey test. #p<0.05, ##p< 0.01 compared to scopolamine-treated group, ***p< 0.001 compared to control group)

V. DISCUSSION

Different strategies are adopted to improve cholinergic transmission in Alzheimer's disease patients, including increase of acetylcholine synthesis, the augmentation of pre-synaptic acetlycholine release, and the stimulation of cholinergic post synaptic muscarinic and nicotinic receptors. Another important strategy towards treatment of Alzheimer's disease is inhibition of acetylcholine synaptic degradation by employing cholinesterase inhibitors. The severity and prevalence of this disease are not yet under control despite the availability of various treatment strategies. Hence, alternative and complementary medicines including herbal extracts containing phytochemicals are being used in the management of Alzheimer's disease [25].

This study showed that the oils of *Ocimum basilicum* and *Ocimum africanum* and their terpenoids exhibited cholinesterase inhibitory activity in *in vitro* conditions.

In the current study, passive avoidance step through model was used to evaluate memory retention of mice. The results show that 400 mg/kg of green *O. basilicum* for seven days as well as 200 mg/kg of *O. africanum* and their eugenol and camphor terpenoids increased memory retention. Our findings are in agreement with earlier studies on other species of *Ocimum*. Reference [26] revealed that IP injection of *O. sanctum* water extract enhanced memory in mice. It has also been reported that *O. sanctum* hydroalcoholic extract enhanced memory in restraint stress induced memory impaired rats [27]. Also, *O. tenuiflorum* ethanol extract increased step-down latency significantly [28]. Further, it is known that ethyl acetate extract of *O. basilicum* administration before global cerebral ischemia prevents cerebral ischemia-induced impairment of short-term memory [29] and this plant is effective in reducing oxidative damage which is attributed to its anti-oxidant properties [30].

The *in vivo* acetylcholinesterase activity has been shown to be increased within and around amnesic brain. Scopolamine induced amnesia leads to increased calcium influx followed by oxidative stress which in turn increases activity of acetylcholinesterase. In this study, the acetylcholinesterase activity in the brain was increased in mice treated with Scopolamine when compared with the normal; in addition, Scopolamine induced increase in acetylcholinesterase was attenuated by the oils of *Ocimum basilicum* and *Ocimum africanum* and their terpenoids, eugenol and camphor. GC-MS analysis also showed that essential oil extracts of *Ocimum basilicum* and *Ocimum africanum* possess considerable amount of different types of terpenoids. Terpenoids are secondary metabolites synthesized by seaweeds and represent a form of essential oils and are classified according to their isoprene unit such as mono-, sesqui-, di-, and triterpene [31]. They possess high therapeutic potentials like anticancer, antioxidant, and anti-inflammatory activities either independently or synergistically [32]. Recent findings reveal that terpenoids have potential neuroprotective effects against ischemic and glutamatergic neurotoxicity, 6-hydroxydopamine toxicity and oxidative stress [31]. Studies on other extracts such as ethanolic extract of *Salvia potentillifolia* show that the essential oils containing mono- and sesquiterpenoids obtained from them exhibit excellent cholinesterase inhibitory activity in *in vitro* condition [33]. Reference [34] demonstrated the anticholinesterase activity of monoterpenes isolated from fungi. These terpenoids, on the other hand, due to their small molecular size and lipophilicity, readily cross the blood-brain barrier and are effective in the treatment of AD [35].

VI. Conclusion

In conclusion, the findings of this study imply memory enhancing property of *Ocimum basilicum* and *Ocimum africanum* in mice, Hence, their volatile oils could be useful in conditions associated with neurodegenerative disorders of Alzheimer's type.

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