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Anti-acetylcholinesterase, Anti-inflammatory and Anti-oxidant Activities of *Mentha longifolia* for Treating Alzheimer Disease

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ABSTRACT

Alzheimer's disease is one of the most common types of dementia in the aging population. We aimed to evaluate anti-acetylcholinesterase, anti-inflammatory and antioxidant activities of M. longifolia extract for treating the disease. alcoholic extract and essential oil of M. longifolia were prepared and tested for their antioxidant activity in in-vitro assays as well as anti Alzheimer effect of extract and essential oil at 10% LD_{50} was evaluated relevant to their anti-inflammatory effect, acetylcholinesterase inhibitory activity and antioxidant activity in aluminium chloride model. In in-vitro study, M. longifolia showed potent antioxidant effects as radical scavenger and inhibitor for lipid peroxidation. In in-vivo study, the essential oil significantly decreased production of hydrogen peroxide, nitric oxide and malondialdehyde in brain tissue as compared to AL-intoxicated group. Mint extract and oil increased glutathione concentration, superoxide dismutase and catalase activities as well as total antioxidant capacity in brain tissue. Treating intoxicated rats with extract or oil decreased the production of proinflammatory cytokines in sera and brain tissue. Extract and oil decreased (IL-1β) ,IL-6 and (TNF-α) in intoxicated rats, also inhibited production of to be nearly at the same level of -ve control group. Both extract and oil showed the same effect on IL-1β in brain tissue but had low effect on TNF-α while they elevated inhibition percentage of IL-6 concentration in brain tissue with concomitant inhibition of cholinergic biomarker as inhibiting acetylcholinesterase, respectively and cholinesterase . M. longifolia extract and essential oil may have anti Alzheimer effect through their effect as antioxidant agents, anti-inflammatory agents and as acetylcholinesterase inhibitors.

Keyword: Acetylcholinesterase inhibitors, Alzheimer, anti-inflammatory, antioxidant, *Menthalongifolia*

INTRODUCTION

The age related disease, Alzheimer's (AD), is common type of dementia in elderly populations. The gradual onset and decline of cognitive function is characterized as Alzheimer[1]. The degeneration of neurological function is due to the reduction in levels of the neurotransmitter acetylcholine, in the brains of the elderly people resulting Alzheimer [2]. Drugs used for treating Alzheimer have various management pathways. Enhancement of acetylcholine levels in the brain is one of the mean targets for disease treatment to ameliorate the cognitive ability [3]. Acetylcholinesterase inhibitors (AChEIs) increased the acetylcholine, cholinergic neurotransmission, level within synaptic region [4].The selective inhibitors that are free from side effects or limited considerably targeted from researchers to be available for sufficient modulation in acetylcholine levels to induce full therapeutic response. The synaptic medications used in AD treatment cause gastrointestinal disturbances [4].Natural products contribute with high compounds number in this concern through various mechanisms like oxidative stress inhibition that reduce the presence of reactive oxygen species resulting inhibition of bio-molecule's oxidation leads to cellular damage and it plays a key pathogenic role in the aging process [5] which consequently suppress aging and neurodegenerative diseases [6]. Many studies noted the presence of common signs of inflammation that confirm the inflammation theory. These studies hypothesized the presence of various inflammatory molecules including pro-

inflammatory cytokines, complement factors and acute-phase proteins in AD brain [7]. Microglia and astrocytes secreted pro-inflammatory substances, macrophage-colony stimulating factor, interleukin-1, interleukin-6, and tumor necrosis factor- α as well as prostaglandins with free radicals [8, 9, 10, 11, 12]. In AD disease, senile plaques are surrounded by high number of macrophages resident of central nervous system which commonly associated physiological features for AD while the another feature is neurofibrillary tangles. Accumulation of plaque through years leads to induce the inflammatory cascade. The primary component of senile plaques, β-amyloid (Aβ), capable to activate microglia and astrocytes as well as it is targeted for advanced alteration which is able to magnify inflammatory pathways [13].Amplification of inflammation caused neurodegenerative disorder results dementia observed in AD patient.

In an effort to discover new sources which can potentially be used in the treatment of AD, *M. longifolia* extract and essential oil were evaluated for their neurodegenerative activity relevant to their acetylcholine esterase inhibitory effect, antioxidant capacity and immuno-inflammatory parameters in brain tissue of mint treated animals.

The study was approved by the ethics committee of the National Research Centre and all subjects gave their informed consent prior to entering this study.

MATERIALS AND METHODS

1. Plant extract preparation

M. longifolia used in this study was originated from north Sinai and propagated in sandy soil in Pelpies district where it is known locally as Habaq. The plant was identified as *Mentha longifolia (L.) huds synonyms Menthaspicata L. var. longifolia*,[14]. The fresh whole plant was cut into small pieces and allowed to dry at room temperature (30 ± 2°C) for ten days then it was ground to powder in a ball mill. The powder (500 g) was exhaustively extracted with ethyl alcohol (70%) by maceration and then concentrated under reduced pressure using rotary evaporator at 40ºC to a small volume to be free from alcohol then the residue was lyophilized. The essential oil of mint dry plant was prepared by hydro-distillation for three hours. The crude ethanolic extract and essential oil were supplied in the *in-vitro* antioxidant activity tests and anti-Alzheimer experiment.

2. In-Vitro antioxidant activity

All chemicals were purchased from Sigma-aldrich, Germany

a. DPPH radical-scavenging activity

The antioxidant activity was determined using DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging model [15]. Three concentrations of tested essential oils (25, 50 and 100 µg/mL) prepared in methanol having a final DPPH radical concentration of 0.1mM. The mixture was shaken vigorously (2500 rpm) for 1min then left to stand for 60 min in the dark. Scavenging capacity was measured spectrophotometrically at 517 nm. Ascorbic acid was used as a positive control. Inhibition (%) was plotted against the sample concentration in the reaction system. The percentage inhibition of the DPPH radical calculated according to the following formula:

% Inhibition = [(A control −A sample)/ A control] X 100

b. Total Antioxidant capacity

Total antioxidant activity was measured according to the method described by [16]. 0.2ml of peroxidase (4.4units/ml), 0.2ml of H_2O_2 (50µM), 0.2ml of ABTS (2,2-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid, diammonium salt, 100 µM) and 1.0ml distilled water were mixed, and kept in the dark for 1h to form a bluish-green complex. After adding 1.0ml of extract, the absorbance at 734nm was measured. The total antioxidant activity was calculated as follows:

Total antioxidant activity (%) = [1-(A sample/ A control)] x 100

c. Inhibition of lipid peroxidation

The ability of crude extract and essential oil to inhibit lipid peroxidation was determined using thiobarbituric acid-reactive substance (TBARS) method [17]. The reaction mixture was composed of 0.5ml extract or essential oil, 0.9 ml phosphate buffer (50mM. pH 7.4), 0.5ml of 0.01mM $FeSO_4$+ 0.1mM ascorbic acid, and 0.1 ml of vehicle or test compounds. The reaction mixtures were incubated at 37^o C for 30min and the reaction was stopped on ice by adding 0.5 ml of 30% (w/v) trichloroacetic acid to the mixture. After centrifugation at 3000rpm for 15min, the supernatant was incubated with 0.5ml of 0.8% thiobarbituric acid at 100^{o}C for 15min. The absorption of TBARS was measured at 532nm.

3. Acute toxicity study:

The acute toxicity test for the *M. longifolia* alcoholic extract (70%) was carried out to evaluate any possible toxicity. Male albino mice obtained from animal house of National Research Centre (n = 8) were divided into six groups then, administered different doses of mint crude extract in intraperitoneal route by increasing or decreasing the dose, according to the response of animal [18]. The dosing patron was 500, 1000, 1500, 2000, 2500, 3000mg/kg body weight. The essential oil of mint was also observed for its acute toxicity using the following doses; 100, 200, 400, 800 and 1000mg/kg b.wt. as intraperitoneal administration, while the control group received only the normal saline. All groups were observed for any gross effect or mortality during 48h. Death of half of examined animals was observed at 1700mg/kg b.wt. for extract while the LD_{50} for essential oil was 440mg/kg b.wt.

4. Anti-alzheimer experiment

Animals and experimental design

Adult male saparguedawely rats from National Research Centre weighing between 150-180g were maintained under normal laboratory conditions and kept in standard polypropylene cages at room temperature of 25-30°C, 60 to 65% relative humidity and provided with standard diet and water *ad libitum*. This study was approved by Medical Research Ethics Committee, National Research Centre, Egypt. Six groups each of fifteen rats were used and treated as follow:

Group I: received the vehicle (saline solution) intraperitoneally and it was served as control group for 105 days.
Group II: received $ALCl_3$ (4.2mg/kg b.wt./45days), serves as ve^+ control group.
Group III: received dimethyl sulphoxide (DMSO) at concentration of 5% in saline which used as a solvent for extract and oil, served as ve^+ control group.
Group IV: received crude aqueous alcoholic extract (400mg/kg b.wt./60days) intraperitoneally and served as ve^+ control group.
Group V: received essential oil (0.15mg/kg b.wt./60days), served as ve^+ control group.
Group VI: received $ALCl_3$ (4.2mg/kg b.wt./45days) then treated with mint crude extract (400mg/kg b.wt./60days).

Group VII: received $ALCl_3$ (4.2mg/kg b.wt./45days) then treated with mint essential oil (0.15mg/kg b.wt./60days).
At the end of the experiment, rats fasted overnight, were subjected to anesthesia by diethyl ether then, sacrificed. The whole brain of each rat was rapidly dissected and washed with isotonic saline and dried on filter paper. Each brain was sagitally divided into two portions. The first portion of each brain was weighed and homogenized to give 10% (w/v) homogenate in ice cold medium containing 50mM Tris-HCl and 300mM sucrose at pH:7.4 [19]. The homogenate was centrifuged at 4ºC. The supernatant (10%) was stored at -80ºC and were used in biochemical analyses including oxidative stress biomarker (nitric oxide concentration, hydrogen peroxide concentration, glutathione conc. and malondialdehyde concentration), antioxidant status (total antioxidant capacity, superoxide dismutase activity and catalase activity). The reduced glutathione level in the brain tissue was determined according to the method of Griffith (1980) [20]. Brain superoxide dismutase (SOD) activity was estimated by the method of Kakkar*et al.* (1984)[21]. Catalase (CAT) activity was measured by following decomposition of H_20_2 according to the method of Berr's and Sizer [22]. The TBARS level in brain tissue, an index of malondialdehyde (MDA) production was determined by the method of Ohkawa *et al.* (1979) [23]. Cholinergic markers (choline esterase and acetylcholine esterase activities) were determined in brain tissue (kits were purchased from Quimica Clinica Aplicada S.A.), neuronal inflammation markers (Interleukin-1β, interleukin-4 and tumor necrosis factor –α). Also brain total protein concentration was measured for calculation of enzyme specific activity [24]. Blood samples were obtained from the experimental and control rats by puncturing retro-orbital plexus. Sera was collected after centrifugation at 4000rpm for 5min/4°C then, used for determination of the neuronal inflammation marker cytokine IL-1β and IL-6 levels using enzyme linked immune adsorbent assay kits in accordance with the manufacturer's recommendations (Quantikine,USA) while tumoral necrosis factor-α(Hycult biotech, Netherelands). The assessment was done by ELISA reader (Dynatech laboratories MRW micro plate reader, 2CXB2445), the sensitivity of assay was20 pg/ml. Oxidative stress biomarker and antioxidant status were determined by colorimetric method using kits purchased from Biodiagnostic Co., Cairo, Egypt.

Statistical Analysis

Conventional statistical methods were used to calculate means and standard deviations of three simultaneous assays carried out with the different methods. Analysis of variance (ANOVA) was applied to data to determine differences ($p< 0.05$). ANOVA one way was applied followed by post hoc for multiple comparisons to obtain least significant difference and standard deviation.

RESULTS

1. ***In-Vitro* antioxidant activity of *Mentha longifolia***

Radical scavenging activity (DPPH)

A significant decrease ($p<0.01$) in the concentration of DPPH was observed due to the scavenging ability of *M. longifolia* extract, essential oil and standards (Table, 1). Radical scavenging effects of mint extract or essential oil were significantly increased with increasing concentration from 25µg/ml to 400µg/ml while the other concentrations, 50, 100 and 200µg/ml, showed insignificant increments between them.

M. longifolia essential oil showed potent effect as radical scavenger against DPPH radicals and it was better than alcoholic extract (IC_{50} were 0.48 and 0.52µg/ml for essential oil and crude extract, respectively). Mint crude extract at 400µg/ ml reached nearly the same level of radical inhibition percentage (98.47%) of ascorbic acid at 100µg/ml and BHT at 200µg/ml. No significant differences were observed between 25 and 50µg/ml also 100 and 200µg/ml for crude extract and essential oil, respectively.

Total antioxidant capacity

The ABTS/H_2O_2 discoloration method is reported to represent the total antioxidant activity of plant extracts. The total antioxidant activity (TAA) of crude mint extract and essential oil at different concentrations is presented in Table (2). The data show that *M. longifolia* extract and essential oil have potent antioxidant effect as measured by ABTS/H_2O_2 assay, IC_{50} were 1.32 and 1.92µg/ml for extract and oil respectively. TAA was significantly increased in a dose dependent manner to reach their maximum activities at maximum concentration (82.81 and 61.15%, respectively). Regarding the essential oil effect, increasing the concentration from 25 to 50µg/ml and from 200 to 400µg/ml didn't show any significant difference in TAA%.

Effect of mint extract and essential oil on Lipid peroxidation

The TBARS assay is sensitive, requires small sample amounts and provides reproducible results. This method is preferable for obtaining useful data in an environment similar to the real-life situation and allows testing of both lipophilic and hydrophilic substances. The inhibition of lipid peroxidation is expressed in terms of concentration which produces a 50% inhibition of lipid peroxidation (IC_{50}). Crude extract or essential oil significantly inhibited TBARS peroxidation at all concentrations. (IC_{50}= 2.32 and 1.49µg/ml). *M. logifolia* essential oil showed potent effect as lipid peroxidation agent more than crude alcoholic extract (Table, 3). Inhibition of lipid peroxidation increased significantly and gradually with increasing concentration of extract or essential oil to reach the maximum inhibition percentage (92 and 96.7%, respectively) at 400µg/ml. The essential oil at 400µg/ml inhibited peroxidation by the same percentage of BHT and it was more effective than ascorbic acid.

2. Anti-alzheimer activity of *Mentha longifolia* relevant to its antioxidant and anti-inflammatory activity in intoxicated rats

Table (4) shows that *M. longifolia* extract and essential oil significantly reduced all oxidative stress parameters ($p<0.05$) as compared to vehicle control group. Brain Hydrogen peroxide, Nitric oxide and Malondialdehyde concentrations were elevated by aluminum chloride treatment as compared to vehicle group.

Mint extract inhibited hydrogen peroxide and nitric oxide production in brain tissue of rats treated with aluminum chloride as compared to Al-intoxicated group followed by significant reduction in lipid peroxidation determined as malondialdehyde. Mint essential oil significantly reduced all oxidative stress parameters by 51.32, 39.77 and 58% for H_2O_2, NO and MDA, respectively. The essential oil was more efficient than crude extract in reduction of oxidative stress in brain tissue.

Reduced glutathione concentration was significantly magnified by extract and essential oil (4.22 and 4.99 µmol/g tissue, respectively) as compared to vehicle group. Also SOD activity was induced when Al-intoxicated rats were treated with extract or oil as compared to Al-intoxicated control group (79.39 and 83.03% for extract and oil, respectively). Catalase activity was elevated by extract and essential oil treatments; it was induced by 204% and 161% for extract and oil, respectively as compared to Al-intoxicated group. These increments in reduced glutathione, SOD and CAT were accompanied with increasing total antioxidant capacity in brain tissue. TAC was increased by 51.16% and 66.79% for extract and oil, respectively, Table (5).

Inflammation in brain tissue by aluminum chloride and reduction of inflammation were recorded by determination of pro-inflammatory cytokines; interleukin-1β, IL-6 and tumor necrosis factor-α in brain tissue and serum by ELISA technique. IL-1β in rats sera was highly elevated by aluminum treatment (761.24pg/ml) while IL-1β concentration of treated Al-intoxicated group remained in the same level of vehicle control group (195 and 164pg/ml for Al-intoxicated group treated with extract and oil, respectively). Data presented in Table (6) showed significant

reduction in IL-6 concentration in sera of intoxicated rats treated with mint crude extract and essential oil, the inhibition percentages were 41.91% and 53.69% for extract and oil, respectively. Tumor necrosis factor-α was also decreased in intoxicated rats treated with mint crude extract or essential oil and they showed nearly the same level of inhibition (83.9 and 85.5% for extract and oil, respectively).

Considering the effect of mint crude extract and essential oil on pro-inflammatory cytokines in brain tissue, data presented in Table (7) showed the same trend of data for their effect on pro-inflammatory cytokines concentrations in sera of intoxicated rats. All determined pro-inflammatory cytokines were significantly decreased in all treated groups. The maximum reduction was recorded for IL-1β. It was reduced by 78.84% and 82.85% in brain tissue of Al-intoxicated animals treated with crude extract or essential oil, respectively. The same trend of data was observed in case of IL-6, it was decreased by 60.97% and 64.77% for extract and oil. Minimum reduction levels were recorded with TNF-α which inhibited by 31.41 and 40.33% for extract and oil, respectively. The essential oil is more efficient than crude extract in inhibiting production of pro-inflammatory cytokines in tissue and sera.

Cholinergic biomarkers were determined in brain tissue as an indicator for loss of cholinergic cells which is accepted as strategy for Alzheimer treatment. Rats treated by mint extract or essential oil as ve^+ control groups have the lowest level of AChE (565.21 and 463.33U/mg protein, respectively), Table (8).

Aluminum chloride highly elevated acetylcholiesterase; increment percentage was 50.42% as compared to vehicle group. On the other hand, it was inhibited by 36.60% when animal treated with crude extract while it was inhibited by 47.52% when rats treated with essential oil. The above mentioned inhibition in AChE activity showed significant increments in Acetylcholine concentration in brain tissue. Acetylcholine was increased by 71.17% in brain tissue of Al-intoxicated rats treated with crude extract while it was elevated by 90.93% when animal treated with essential oil. At the same trend of data, choline- esterase was significantly inhibited by 58.79% when Al-intoxicated animals were treated with mint crude extract. Also essential oil treatment highly reduced cholinesterase activity by inhibition percentage of 62.63% as compared to Al-intoxicated rats.

DISCUSSION

The oxidation of cellular oxidizable substrates can be prevented or delayed by antioxidants from natural sources as edible plants which is a way to keep human health. These antioxidant substances exert their effects through scavenging reactive oxygen species that prevent the reactive oxygen species generation and detoxifying proteins [25].

M. longifolia extract and essential oil showed potent antioxidant activity through their effects as radical scavengers and as inhibitor for lipid peroxidation in *in-vitro* model. This promising effect may exert preferable effects on prevention from generation of ROS in in-vivo model. There is a link between development of neuroinflammation and risk factors involved many interaction complexes that contributes to oxidative stress, neuroinflammation and vascular compromise. By means, when the inflammatory cascade was initiated the neuroinflammatory process becomes highly activated to make further cellular damage to loss their function which is accompanied with neurofibrillary tangles and amyloid plaques formation, Alzheimer's hallmarks [26]. Treating Al-intoxicated rats with aqueous alcoholic extract or essential oil of *M. longifolia* significantly increases the antioxidant parameters in brain tissue. They elevate glutathione concentration, Superoxide dismutase activity, catalase activity and total antioxidant capacity which reflected on significant reduction in oxidative stress in brain tissue to be nearly the same level of vehicle control group.

It was reported that Alzheimer risk factors including environmental, biological and genetic factors correlated with increasing level of inflammatory markers which can induce advanced cognitive impairment. On the other hand, the central nervous system (CNS) considered as immune privileged organ also it was documented that the development of various CNS diseases, e.g. Alzheimer’s disease, is linked to neuroinflammation. Inflammatory process development involve a widely spread range of molecular interactions which represent important CNS changes outcomes. These changes contribute in CNS regulation and function impairment that increased level of inflammatory marker [27]. Mint extract and essential oil significantly reduced the production of all pro-inflammatory cytokines in brain tissue and sera of Al-intoxicated rats as compared to Al-intoxicated rats control group. These promising anti-inflammatory and antioxidant activities of mint crude extract and essential oil was accompanied with significant reduction in acetylcholine concentration in brain tissue resulted from significant inhibition in acetylcholinesterase activity. Cholinesterase activity was inhibited by administration of mint crude extract but essential oil showed promising effect as cholinesterase inhibitor. These results support the idea of anti-alzheimer activity of *Mentha longifolia* essential oil through its role as cholinesterase inhibitor; the cholinesterase inhibitors could alter the release of amyloid precursor protein from superfused cortical slices of rats [28].

CONCLUSION

The findings of this work showed that mint crude extract and essential oil may have a neuroprotective effect by activation of normal amyloid precursor process. In most determined parameters essential oil of *M. longifolia* found to be more effective than crude extract. These mentioned plausible effects of mint support the idea that *M. longifolia* aqueous alcoholic extract and essential oil may have anti-Alzheimer activity through the anti-inflammatory and antioxidant pathway.

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