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Evaluation of *Origanum Vulgare* L. ssp. Viridis Leaves Extract Effect on Discrimination Learning and LTP Induction in the CA1 Region of the Rat Hippocampus

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Abstract

Objective(s)

The objective of this study was to determine the effect of aqueous extract of *Origanum vulgare* L. ssp. Viridis (ORG) on discrimination learning and long term potentiation (LTP) in CA1 region of the rat hippocampus.

Materials and Methods

A group of adult male Wistar rats weighing 275±25 g received aqueous extract of ORG (150, 300, 450 mg/kg/day) by intraperitoneal injection for one week, and the other group received saline (n= 6). A wooden T-maze was used to evaluate the discrimination learning. In electrophysiological experiments, the effect of ORG leaves extract on induction and maintenance of long term potentiation (LTP) in CA1 hippocampus area was determined. LTP was evaluated in CA1 region after high-frequency stimulation (200 Hz) of the Schaffer collaterals. Also, serum antioxidant levels were analyzed in the two groups (n= 4).

Results

Statistical analysis showed significant decreases in the number of total (significantly at the dose of 300 and 450 mg/kg) and wrong (significantly at the dose of 300 mg/kg) entrance into opposite box of T-maze procedure in ORG-treated animals ($P< 0.05$). In electrophysiological study, the rats which had received ORG (150, 300, and 450 mg/kg) showed an increase in both population spike amplitude (59.7±14.1%, 85±14.7% and 49.3±8.7% respectively, compared to 39±9.2% increase in saline group) and maintenance of LTP in hippocampus CA1 after high frequency stimulation in Schaffer collateral pathway. In serum antioxidant assay, level of antioxidants in ORG groups (300 and 450 mg/kg) remarkably increased in comparison to saline group ($P< 0.05$ and $P< 0.001$, in turn).

Conclusion

Our results suggest that Origanum aqueous extract can improve the learning criteria in rats.

Keywords: Antioxidant assay, Long term potentiation, Medicinal plant, *Origanum vulgare*, Spatial learning, T-maze

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Introduction

Medicinal plants have long been recognized as effective treatments in some human diseases. Nowadays investigations of the therapeutic effects of plants, is the subject of many researches (1-3). *Origanum vulgare* L. ssp. Viridis (ORG) is an aromatic plant with a wide distribution throughout Asia and specially in Iran (4). It is used to cure respiratory diseases (5), hypoglycemic disease (6) and leukemia (1). The major aqueous constituents of ORG (oregano) are rosmarinic acid, eriocitrin, luteolin-7-o-glucoside, apigenin-7-o-glucoside (7), origanol A and B (8) and ursolic acid (9, 10). Rosmarinic acid and origanol A and B, constituents of the most components of aqueous extract of ORG, have anti-oxidative activities (7, 8). So, aqueous extract of ORG has antioxidant activity because of some of its components like flavonoids, (8, 11, 12). Phenols can pass the brain blood barrier and cause dose- dependent anti-oxidative effects (13).

Antioxidants , such as vitamin E and deprenyl, prevent the oxidative damage and delay memory deficits in animal models (14, 15). Phenols, rosmarinic acid and ursalic acid protect memory impairments observed in Alzheimer's disease (10, 16, 17). Rosmarinic acid, the most abundant component of aqueous extract of ORG (7), exhibits a strong scavengeing activity for ONOO− and other free radicals (18). ONOO− has a broaden biological damage in the brains of individuals suffering from Alzheimer's disease (19). Beta-amyloid (Aβ), the major constituent of the senile plaques, is the most important reason of increased ONOO− in the brain of these patients (20). Free radicals cause phospholipid peroxidation, oxidative DNA damage and protein denaturation (21). By increasing H_2O_2, these free radicals reduce synaptic plasticity and inhibit long term potentiation (LTP). LTP is an important cellular mechanism for learning and memory in hippocampus (22-24). In this study, we have therefore, assessed the anti-oxidative effects of aqueous extract of *Origanum vulgare* on discriminate learning and plasticity of neurons in the CA1 area of the rat hippocampus.

Materials and Methods

Animals
A total of sixty four adult male Wistar rats, weighing 275±25 g were used in this study. They were housed in specific cages in 12 hr light 12 hr dark cycle. Room temperature was controlled at 23±2 °C. Animals were maintained with free access to commercial food and drinking water. All methods were in accordance with rules for caring and using laboratory animals in Kerman Neuroscience Research Center (EC/KNRC/86-31).

Drugs and groups
Ainmals were randomly divided into separate groups for three studies: behavioral, electrophysiological and antioxidant assay experiments. In every study, rats were divided into 4 groups as follow:

1) Saline group with intraperitoneal (i.p.) injection of normal saline (1 ml/kg) for 7 days.

2) Three groups with i.p. injection of aqueous extract of ORG (150, 300 and 450 mg/kg) (1 ml/kg) for 7 days.

Every day all injections were carried out 30 minutes prior to the experiment.

T-maze apparatus
To evaluate discrimination learning, a wooden T-maze apparatus with 15 cm high walls with start and goal boxes (15×20×20 cm) was used. Its arm length was 108 cm ended into two L-shaped arms. The lengths of the first and second parts of each arm were 39 cm and 28 cm consecutively. The second part was led to the goal box. The arms were separated from the start box and the L-shaped arms by the guillotine doors. Five days prior to the experiments as well as during experiments, rats were fed with pellets weighing 4-12 g to maintain optimal weight (85% of primary weight). The method used for the T-maze procedure has already been described by Annett *et al* (25). On days 1 to 3 preliminary training took place. On the first day food pellet (4 mg) was left in the stem and arms and goal boxes with all the guillotine doors were opened. Each rat was placed individually in the start box. They had 5 min for exploring the maze and eating food. On days 2 and 3, each rat was placed in the start box and after 10 sec they were allowed to find pellets in either of the goal boxes in about 20 sec. On days 4 and 5, rats had access to pellets on every trial. Before allowing them to choose one of the goal boxes rats were kept in the start box for

10 sec. In the goal boxes, animals had 20 sec. Each day eleven consecutive trials were carried out. The discrimination learning was done on days 6, 7 and 8.

We put food pellets in one arm of T-maze and due to their hunger status, the animals had to go after and look for the plate in the designated goal box, and if the animal did this for 5 consecutive rounds without any lapse between them, so learning had been occurred. For 3 days (days: 6-8), the whole operation mentioned above, had to be successfully repeated for the opposite arm too. If once again the animal followed the order in expected manner, it was concluded that the reversal learning had taken place (as a standard criteria for learning). At the end, based on aforementioned protocol, the numbers of total and wrong entrance trials into opposite box were recorded (25).

Electrophysiological recordings

Animals were anesthetized with an intraperitoneal injection of urethane (1.2 g/kg) and positioned in a sterotaxic frame. The animals' body temperature was maintained at 37 $^{\circ}$C by a servo-controlled heating pad. The stimulation electrode was placed in the Schaffer collateral (3 mm posterior to bregma, 1.5 mm lateral to midline) (26).

A current of 0.2 ms, 0.5-1.5 mA, was delivered at 0.1 Hz through an insulated stainless steel twisted bipolar electrode (0.125 mm diameter, WPI). Recordings were made with a tungsten microelectrode (1-3 MΩ) placed in the stratum radiatum of the CA1 region of the hippocampus (5 mm posterior to bragma, 3-4 laterals to midline) (26). Final positions of the stimulating and recording electrodes were then determined by maximizing the amplitude of the population spikes (PS). Evoked responses were amplified (gain 100-1000$\times$) and filtered at 0.1-3.0 KHz (WPI, Dam 80) and recorded in the personal computer for off-line analysis. Test stimulation was set so population spikes were 50-60% of maximum. After stable baseline recording for at least 30 min, high frequency stimulation (HFS) consisted of 10 trains of stimulus with 20 pulses at 200 Hz with 2 sec interval between each train was used for inducing LTP (27). Then the protocol was followed by resumption of low frequency stimulation continuing for 60 min.

Extraction procedure

Leaves of origanum were collected in spring in Yazd. They were dried in shade and powdered. Powder was refluxed with distilled warm water (below 50 °C) by 1/100 ratio for 24 hr. The extract was filtered with Whatman No.2 filter paper. The mixture was concentrated under reduced pressure at 40 °C by a rota evaporator. This method is used for qualitative chemical studies of terpenoids, flavonoids, alkaloid, etc (28).

Antioxidant assay

Serum antioxidant levels were assayed in each group of saline or ORG (150, 300, 450 mg/kg) (n= 4). The antioxidant was determined using the commercial antioxidant assay kit (Sigma, USA; CS0790) according to the manufacturer instructions (29). Rats were anesthetized by CO_2 inhalation for 30 min after the last injection, and then they were decapitated. Serum bloods were removed by centrifuging at 2500 rpm for 10 min. Samples were freezed immediately and stored at -20 $^{\circ}$C. Trolox, a water-soluble vitamin E analog, serves as a standard or control antioxidant. Trolox standards have been prepared for a standard curve (Figure 1). ABTS substrate working solution has been prepared by adding 25 ml of 3% hydrogen peroxide solution (Sigma, USA; 323381) to 10 ml of ABTS substrate solution. Samples and standards were located in a 96-well plate. In wells for the trolox standard curve, 10 ml of a trolox standard and 20 ml of myoglobin working solution were added. In wells for the test samples, 10 ml of test sample and 20 ml of myoglobin working solution were added. Then, 150 ml of ABTS substrate working solution was added to each well and they were incubated for 5 min at room temperature. A total of 100 ml of stop solution (Sigma, USA; S3446) was added to each well. Finally, the endpoint absorbance was determined at 405 nm using a plate reader. Standard curves of serum antioxidant levels were determined by comparing the absorbance of known standard concentrations.

Statistical analysis

In behavioral study, t-test and one way analysis of variance (ANOVA) followed by low statistical differentiation (LSD) were used for comparing saline with aqueous extract injected groups. In electrophysiological study, data were recorded at 30 sec intervals, and for each animal PS amplitude was averaged over a 30 min baseline- recording period. PS amplitudes were normalized relative to the average baseline response and expressed as percentile change from baseline. For each group the mean (±SEM) percentile change at 60 min post tetanization was analyzed using two-way repeated-measure analysis of variance ANOVA followed by least significant difference (LSD) in SPSS soft ware (version: 15.5). All of the data were represented as mean (±SEM) and the *P*-value less than 0.05 was considered to be significantly different.

Results
The effects of Origanum vulgare L. ssp. viridis leaves extract on antioxidant assay

Total concentrations of antioxidants in ORG groups (300 and 450 mg/kg) were significantly higher than those in saline group (*P*< 0.05 and *P*< 0.001, respectively) (Figure1B).

The effects of Origanum vulgare L. ssp. Viridis leaves extract on discrimination learning

One-way analysis of variance showed that ORG at 300 and 450 mg/kg doses decreases the number of total entrance trials into opposite box of T-maze to achieve the standard criteria of learning (Figure 2A) (*P*< 0.001). In ORG-treated rats, dose of 300 mg/kg decreased the mean (±SEM) of error times to attain the standard criteria of learning comparing with saline group (Figure 2B) (*P*< 0.05).

The effects of Origanum vulgare L. ssp. viridis leaves extract on Electrophysiological parameters

Figure 3 illustrates the mean (±SEM) of population spike (PS) amplitudes as percentile change from 30 min prior to baseline, and 60 min following HFS. Our results showed that PS amplitudes significantly increased in all groups at 10 min post HFS compared to baseline. At this time, we observed a 39±9.2%
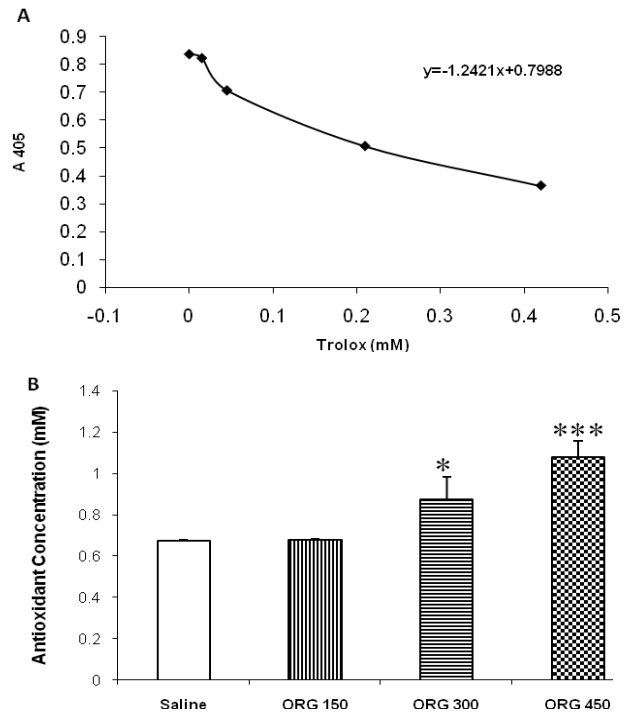
Figure1. (A) Trolox standard curve, (B) Mean (±SEM) of antioxidant concentration levels (mM) of serum in treatment groups, saline and ORG extract (150, 300, 450 mg/kg). *P< 0.05, ***P< 0.001, ORG: Origanum, n= 4.

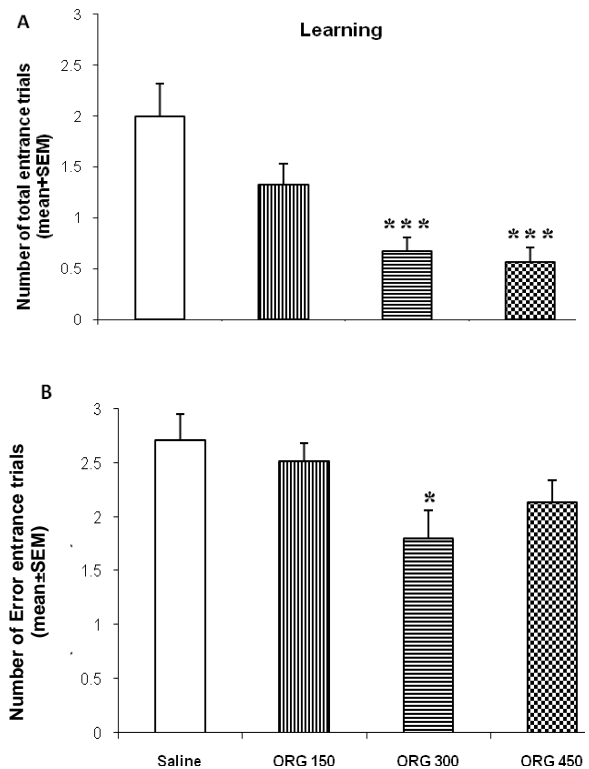Figure 2. Mean±SEM number of total (A) and wrong (B) entrance trials into opposite box of T-maze as an index for discriminatory learning. *P< 0.05, ***P< 0.001 ORG groups (150, 300, 450 mg/kg) compared to saline group, ORG: Origanum, n= 6.
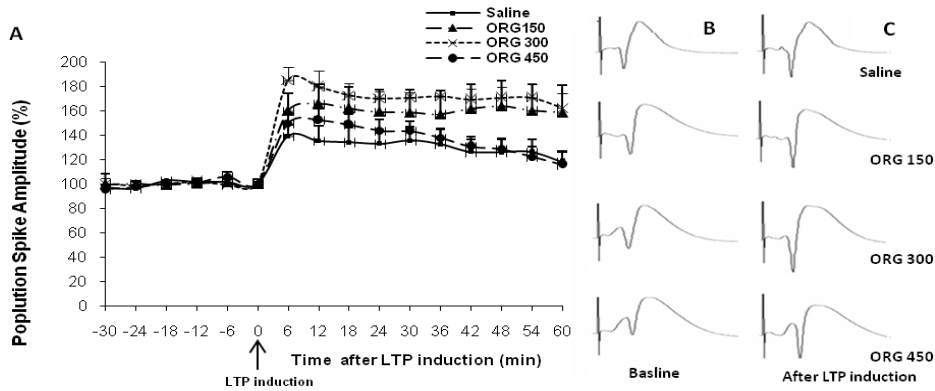
Figure 3. (A) Mean (±SEM) of population spike amplitude as percent change from baseline for CA1 area of hippocampal of anesthetized rats treated with saline and ORG (150, 300, 450 mg/kg). A 30 min baseline period was followed by tetanization and recording continued for 60 min. At 60 min post tetanization, there was a significant difference between ORG 300 mg/kg and saline group during all the times. Representative traces of population spike amplitude recorded from baseline (B) and after induction LTP (C) in CA1 area of hippocampus of anesthetized rats treated with saline and ORG (150, 300, 450 mg/kg), ORG: Origanum, n= 6.

increase in the saline group, while in the ORG-treated groups (150, 300, and 450 mg/kg), the increases were 59.7±14.1%, 85±14.7% and 49.3±8.7%, respectively. In the ORG-treated groups, there were numerically greater LTP than the saline group. At 10 min after tetanic stimulation the PS amplitude was significantly different in hippocampal CA1 area of the ORG- treated groups compared to the saline group ($P< 0.05$). In addition, mean (±SEM) PS amplitude for the ORG-treated groups at 60 min post tetanus differed from the saline group. Administration of ORG, induced a significant increase in LTP maintenance in the CA1 area at 60 min post tetanic stimulation compared to the saline group ($P< 0.05$).

Discussion

This study showed that administration of ORG extract once a day for one week improves discrimination learning in T-maze, induction and maintenance of LTP in CA1 region of hippocampus in rats. We evaluated total antioxidant concentration levels (mM) of serum in the saline group and ORG groups. Serum samples of the ORG groups (300 and 450 mg/kg) had significantly higher concentrations of total antioxidant. The major aqueous constituents of Oregano are demonstrated to be rosmarinic acid, eriocitrin, luteolin-7-O-glucoside and apigenin-7-O-glucoside (7). Anti-oxidative effects of origanol A and B in the aqueous extract are

comparative with those of rosmarinic acid (8). In addition to aromatic phenols, ursolic acid is an important antioxidant being extracted by super critical CO_2 techniques from aqueous extract (9, 10). Ursolic acid is a three pantacyclic acid which exists in many therapeutic plants (9, 30). So, the use of *Origanum Vulgare* L. ssp. Viridis leaves extract can result in an increase in total antioxidant concentration levels (mM) in blood serum.

Ursalic acid and rosmarinic acid which are the major components of aqueous extract of ORG, have anti oxidative activities. In cell culture studies, rosmarinic acid and ursalic acid protect against the reactive oxygen species induced by Aβ (7, 10, 17). In an *in vitro* study, it has been shown that amyloid beta protein increases free radical production and lipid peroxidation in nerve cells, leading to apoptosis and cell death. Pretreatment with ursalic acid and vitamin E inhibited the Abeta-induced neurotoxic effect (10). Further, rosmarinic acid not only inhibits the formation of beta-amyloid fibrils, but also destabilizes preformed beta-amyloid fibrils *in vitro* (31). Free radicals reduce synaptic plasticity and inhibit long term potentiation (LTP). LTP is an important model of synaptic plasticity and is thought to be associated with memory consolidation and it is inducible in the hippocampus by a high frequency stimulation (32). The molecular signaling pathway

responsible for LTP begins with the activation of synaptic *N*-Methyl D-Aspartate (NMDA) receptors (33). Prolonged depolarization relieves a voltage-dependent Mg^{2+} block from the channel and allows it to be conducted. Calcium influx through these channels is associated with the activation of a number of kinases, ultimately leading to an increase in the postsynaptic response through an increase in the number of AMPA receptors at the synapse or an increase in channel conductance (34). Hippocampsal lesions impair spatial learning in the water maze (35). Drugs that antagonize NMDA-receptor activity block LTP and impair water-maze learning (36). This has led to the hypothesis that NMDA receptors, through their involvement in synaptic plasticity, may be necessary for spatial (37) and other forms of learning (38, 39). Our results illustrated that tetanic stimulation produces a significant increase in the population of spikes and LTP maintenance (60 min after LTP induction) following ORG treatment. In addition, origanum improved discrimination learning in T-maze. The mechanism of learning improvment in ORG-treared animals has not been known yet, one possibility may be due to the origanum antioxidative properties. Antioxidants in the central nervous system (CNS) improve neurodegenerative disorders (40). Reactive oxygen species (ROS) can react with unsaturated fatty acids and produce lipid peroxides in brain (11, 41). Increased production of ROS and oxidative stress are demonstrated by increased lipid peroxidation. Antioxidants cause a reduction in lipid peroxidation which has a great role in membrane rigidity and synaptic plasticity (42). In addition, many studies reported that free radicals diminish synaptic plasticity and long term potentiation in hippocampus (23, 24). Some reports showed that Oregano extracts can decrease lipid peroxidation in the neurons (2, 8, 43). Unfortunately we didn't measure the activities of major antioxidant enzymes in brain. But there are many reports showing that the components of aqueous of origanum extracts such as ursolic acid and rosmarinic acid exert a potent antioxidant activity through scavenging free radicals (7, 44). In addition, while rats were trained in a cross maze, acetylcholine was released simultaneously in the hippocampus and striatum (45). It has been shown that the ursolic acid is a potent acetylcholinesterase inhibitor in Alzheimer's disease (30). So, antioxidant activity and acetylcholinesterase property of origanum may be involved in the improvement of the discrimination learning and the LTP induction and its maintenance of CA1 area of hippocampus.

Conclusion

The present study clearly demonstrates that *Origanum vulgare* L. ssp. viridis leaves extract increases the discrimination learning and improve the LTP induction and its maintenance of CA1 area of hippocampus in treated rats. These results suggest that origanum consumption may be useful to improve learning disorders.

Acknowledgment

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