Vol.59: e16150354, January-December 2016
http://dx.doi.org/10.1590/1678-4324-2016150354
ISSN 1678-4324 Online Edition

BRAZILIAN ARCHIVES OF
BIOLOGY AND TECHNOLOGY
A N I N T E R N A T I O N A L J O U R N A L

Antioxidant activity of oral administration of Rosmarinus officinalis leaves extract on rat's hippocampus which exposed to 6-Hydroxydopamine

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ABSTRACT

Carnosic acid, a diterpene of Rosemarinus officinalis leaves extract (RE), has potent antioxidant activity in vitro. The dopaminergic connection of substantia nigra pars compacta to the hippocampus might be affected by oxidative stress which caused cognitive impairment observed in the early phase of Parkinson's disease (PD). Adult male Wistar rats were lesioned bilaterally by intra-nigral injection of 6-OHDA, and divided into six groups: four groups that orally given RE containing 40% of carnosic acid, at doses of 25, 50 and 100 mg/kg (treated rats) and distilled water (H2O), once daily for a period of 14 days before and after the injury. There were also two another groups as control rats which injected by normal saline and untreated lesion group. The injured animals were evaluated for their spatial memory performance by Morris Water Maze test. Lesioned rats showed significant increase in escape latency, as compared with control group. Two weeks after injury, tissue samples were collected from the hippocampus. Levels of catalase (CAT), glutathione peroxidase (GPX) and superoxide dismutase (SOD), malondialdehyde (MDA) and reactive oxygen species (ROS) were determined. There were significant increase of SOD, GPX and CAT enzymes activities in RE50 treated group as compared to lesioned rats. We found a significant decrease of ROS in RE50 treated group as compared to Lesioned rats. These findings provide evidence that 50mg/kg of RE decreased oxidative damage of the hippocampus induced by 6-OHDA and serve as potential candidate for the treatment of PD.

Key words*:* Rosmarinus officinalis leaves extract, Antioxidant enzymes, Hippocampus, 6-Hydroxydopamine

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INTRODUCTION

The majority of dopaminergic innervations to the hippocampus (HPC) arise from the ventral tegmental area (VTA) as mesolimbic system, it was demonstrated that dopaminergic afferents from the VTA can stimulate neurogenesis and facilitate long-term synaptic plasticity in the HPC (Bethus et al. 2010; Martig et al . 2011; Ghanbarian et al. 2013). Parkinson's disease (PD) patients show a significant decrease in dopamine level in the hippocampus (Calabresi et al. 2013). Previous MRI studies clearly revealed that decreased hippocampal volume accompanied the cognitive impairment in PD patients (Apostolova et al. 2012).

The most widely mechanism of dopaminergic cell death in PD is a cycle of oxidative stress (Choi et al. 2012; Hwang 2013). Oxidative stress can damage the cell through the oxidization of cellular elements like membrane lipids, proteins and DNA (Halliwell 1992). Accumulating lines of evidence have demonstrated that oxidative stress plays a crucial role on the pathogenesis of PD, and dopamine-rich areas of the brain are particularly vulnerable to oxidative stress, because metabolism of dopamine itself leads to the generation of ROS, including hydrogen peroxide and hydroxyl radicals (Sriraksa et al.2012). The presence of neuromelanin in dopaminergic cells and autoxidation of dopamine responsible for the high basal levels of oxidative stress in substantia nigra (Munoz et al. 2012). Because of the dentate gyrus of the hippocampus receives dopaminergic projections from the ventral tegmental area and substantia nigra, these connections might be affected by oxidative stress (Hritcu et al. 2011; Sriraksa et al.2012). The sensitivity of the mesohippocampal pathway to selective toxins also demonstrated its vulnerability to free radical attack. For example, 6-hydroxydopamine (6-OHDA) destroys dopaminergic neurons through free radical–mediated mechanisms (Hauser and Hastings 2013).

More recently, an increasing amount of evidence has suggested that the mesohippocampal pathway is also critically involved in learning and memory processes, as indicated by the fact that many cognitive impairments, including memory deficits, occur during the early stage of PD even before the development of its classical symptoms (Whittington et al. 2006; Costa et al. 2012).

The non-motor symptoms that include cognitive deficits can be more important than the motor deficits to determine the patients' quality of life and represent an important factor to determine the need for nursing home care (Martig et al. 2011; Hanna-Pladdy et al. 2013). However, studies conducted over the last two decades have shown that cognitive impairments, including spatial working memory deficits, occur even in the early stage of PD when motor symptoms are barely observed (Hirano et al 2003; Varcin et al. 2012). The motor symptoms of PD are more known than cognitive disabilities. Oxidative stress induced by reactive oxygen species (ROS) is common central player in human PD brains and experimental animal models of PD (Varcin et al. 2012). Oxidative stress in brain is an important factor in the neuropathology of PD. It was reported that rats with SNc lesion induced 6-OHDA present deficits in spatial memory tasks without gross motor alterations (Ferro et al. 2005; Bellissimo et al. 2004).

The possible underlying mechanism of neurotoxicity induced by 6-OHDA has been reported to be related to the oxidative stress caused by the production of hydroxyl radicals during autoxidation (Soto-Otero et al. 2000; Sriraksa et al. 2012) and the inhibition of complex I (Glinka and Youdim 1995) resulting in excessive oxidative stress and leading to neuronal death. 6-OHDA is one of the most common neurotoxins used in experiments in order to mimic Parkinsonism in rodents. 6-OHDA acts mainly by generating reactive oxygen species due to its oxidation which can occur spontaneously or can be catalyzed by MAO or iron (Pienaar et al. 2010). Unilateral 6-OHDA SNc-lesioned rats present an almost complete loss of dopaminergic neurons in the SNc, a proportional depletion of striatal dopamine and gross motor disturbances, like turning behavior and these symptoms showed a model of advanced phase of PD characterized by gross motor alterations (Bellissimo et al. 2004). It was reported that, bilaterally instead of unilaterally SNc-lesioned rats was used to avoid motor alterations caused by an asymmetric depletion of striatal dopamine. This model of PD seems to be appropriate for this purpose because, in contrast to unilaterally SNc-lesioned rats, animals with bilateral lesioned do not present gross motor alterations that would otherwise confound the interpretation of poor scores in memory tasks as indicative of cognitive impairment (Ferro et al.

2005). Therefore, in this study the bilaterally 6-OHDA SNc-lesioned rats were used. In these rats, like in PD, cognitive impairment was caused by a marked loss of dopaminergic cells in the SNc and depletion of hippocampal dopamine (Veena et al. 2011). Recently, it was found that 6-OHDA was recognized as a good model for the early stages of PD, especially in terms of emotional and cognitive deficits (Munoz et al. 2012). In the present study, 6-OHDA was employed, because it was easy to be oxidized and of the formation of ROS, thus leading to cell death. 6-OHDA is a selective catecholaminergic neurotoxin that has been widely used to produce PD models in vitro and in vivo because it induces apoptotic activity through oxidative stress (Veena et al. 2011). Our previous reports demonstrated that rats treated with 6-OHDA suffered impairments in memory processes, which are associated with alterations in the brain antioxidant status and lipid peroxidation (Ciobica et al. 2012). Consequently, several reports suggested that many plant extracts have neuroprotective activity against 6-OHDA-induced toxicity through antioxidant and antiapoptotic activities in PD models (Hritcu et al. 2011; Hwang et al. 2011).

Oxidative stress results from an imbalance between the cellular production of ROS and antioxidant mechanisms that remove them, although several antioxidant molecules in the brain such as superoxide dismutase (SOD), glutathione peroxidase and ascorbate can remove the ROS (Brieger et al. 2012). For these reasons, much interest has focused on the antioxidant defenses including supplement with exogenous antioxidant. Antioxidant substances have the role to protect cell from pathogenic oxidation.

Based on the role of oxidative stress in the pathophysiology of PD, the neuroprotective and cognitive enhancing effects of substances possessing antioxidant activity have gained much attention. Therefore, drugs that exhibit multiple properties including free radical scavenging, such as *Rosemarinus officinalis* leaves extract (RE) may serve as potential candidate for the treatment of memory impairment of PD. There are also radical scavengers such as ascorbate (Vitamin C), urate, and glutathione, as tocopherol (Vitamin E), carotenoids, flavonoids and selegiline (Ciccone 1998). Therefore, dietary supplementation with antioxidants could conceivably protect against the molecular effects of lipid peroxidation, free radicals and ROS and delay the progress of many chronic diseases. Results of the relevant studies showed that there are biologically active compounds in rosemary essential oil exhibiting neuroprotective, antioxidant, anti-carcinogenic and cognition-enhancing properties (Kosaka et al. 2010; Zhang et al. 2010).

It is suggested that the neuroprotective effect of RE occurs partly because of decreased oxidative damage. Leaves of *Rosemarinus officinalis* possess a variety of bioactivities, including antioxidant, antitumor, anti-inflamatory and anti-HIV (Halliwell 1992; Ramirez et al. 2006; Altinier et al. 2007). It is composed of a vast number of polyphenolics such as *carnosic acid*, *carnosol*, *rosemarinic acid*, *ursolic acid*. Among these, *carnosic acid* (a phenolic diterpene compound), and *carnosol* are the most potent antioxidant constituents (about 90% of antioxidant activity) (Azad et al. 2011; Sharmila et al. 2012). *Carnosic acid* (CA) is a free radical scavenger, due to its phenolic skeleton (del Bano et al. 2003).

The present study was designed to determine whether dietary supplementation of three concentrations of *Rosmarinus officinalis* leaves extract (25, 50 and 100 mg/kg), containing 40% CA (with high free radical scavenging capacity) could enhance antioxidant status in the hippocampus of 6-OHDA induced Parkinsonian rats. We focused our attention on its effects on the activities of catalase (CAT), glutathione peroxidase (GPX) and superoxide dismutase (SOD) as well as on the levels of malondialdehyde (MDA) and reactive oxygen species (ROS).

MATERIAL AND METHODS

Animals

The experimental protocol was approved by the Research and Ethics Committee of Damghan University. Adult male Wistar rats weighing 200–250g at the start of the experiment were used. The animals were housed in a temperature- and light-controlled room (22°C, a 12-h cycle starting at 08:00 h) and were fed and allowed to drink water ad libitum.

Surgery

All surgical procedures were conducted under aseptic conditions, under ketamine-xylazine (60-3 mg/kg b.w., i.p., SIGMA) anesthesia. Rats were mounted in the tereotaxic apparatus with the nose oriented 11° below horizontal zero plane. Bilateral lesioned of dopaminergic neurons located in SNc

were produced with 6-OHDA-HBr (SIGMA, St. Louis, MO, USA). Six micrograms, of 6-OHDA, dissolved in 2 µl physiological saline containing 0.1% ascorbic acid were administrated through Hamilton syringe at a rate of 0.33µl/min., and the syringe was left in place for 5 min after injection before being slowly removed (lesioned rats). The control rats were injected with saline. The following coordinates were used: 5.0mm posterior to bregma; 2.1 mm right and left of the sagittal suture; 7.7mm ventral to the surface of the cortex (Paxinos 2005).

The rest of lesioned rats were divided into four groups which treated with RE (*carnosic acid* powder CAP25-110401, was purchased from hunan genham biomedical technology ltd,China) containing 40% of *carnosic acid* at doses of 25, 50 and 100 mg/kg (treated rats) and distilled water (H2O rats) respectively, once daily for a period of 14 days before and 14 days after the injury by oral gavage. Two weeks after injury, all rats were sacrificed and tissue samples were collected from the hippocampus. Afterwards all samples were washed in ice-cold phosphate buffer saline. The washed samples were immediately stored at 70º C until biochemical study. Then the frozen tissue samples were homogenized 1:10 in ice-cold 50 mM HCl-Tris buffer (pH 8) and centrifuged at 12000g for 15 min at 4º C. The supernatants were separated and used for biochemical assay.

Behavioral analysis

Behavioral test (Morris Water Maze) for lesion and control groups used to show parkinsonism animal model.

Morris Water Maze

Nine days after surgery, all animals were evaluated for their spatial memory performance by MWM test. The swimming pool used for the test was 190 cm in diameter and 60 cm deep. The escape platform (100 cm^2) was fixed in a permanent position 2 cm under the water surface during the course of the MWM training procedure. The quadrant housing the escape platform was defined as the target zone. Spatial reference cues (arrow, star, circle, and rectangle) around the pool were remained constant during the test. For spatial learning acquisition test, the rats were trained in MWM for 4 consecutive days using 4-trial-per-day. The rats were placed into the pool facing the wall randomly from one of the three starting points located in the three quadrants except the quadrant with the platform. If the animals failed to find the platform by the maximum period of 60 seconds, they would be gently placed on the platform. At the end of each trial, the rats were allowed to rest on the platform for 20 s. A video camera that was mounted directly above the water maze pool linked to a computer recorded the animals' movement. For this purpose, the versatile tracking system of EthoVision (Noldus Information Technology, Wageningen) was employed and the spatial memory was tested by measuring escape latency. Probe trial was performed 1 day after the last training trial, during which the platform was removed from the pool, while all other factors remained unchanged. Rats were allowed to swim for 90s time spent to find out platform (escape latency) each rat were recorded and used to assess the performance of the animal in this memory test (D'Hooge and De Deyn 2001; Da Cunha et al. 2002; Bromley-Brits et al. 2011)

The ROS generation was investigated using the 2',7'-dichlorfluorescein-diacetate (DCFH-DA) (LeBel et al. 1992).

Thiobarbituric acid reactive substances were measured according to Ohkawa et al (Ohkawa et al. 1979). Briefly, to glass tubes were added, in order of appearance: 500µL of sample; 50µL of sodium dodecyl sulfate8.1%; 1500µL of 20% acetic acid in aqueous solution (v/v) pH 3.5; 1500µL of 0.8 % thiobarbituric acid; and 700µL of distilled water. The mixture was vortexed and the reaction was carried out in a boiling water bath for 1 h. The mixture was allowed to cool water for 5 min and was centrifuged 750g for 10 Min. The resulting pink stained TBARS were determined in a spectrophotometer at 532 Nm. TBARS were calculated as nmol/mg protein. A calibration curve was performed using 1,1,3,3-tetramethoxypropane as a standard.

CAT activity was assayed by the method of Aebi (Aebi 1984) using a spectrophotometer. This method is based on the disappearance of H2O2 at 240nm in a reaction medium containing 20 mM H2O2 and 10 mM potassium phosphate buffer pH 7.0. Enzymatic activity was represented according to absorbance changes per minute/mg protein (Mohammadi et al 2014).

GPx activity was measured according to the method of Wendel (Wendel 1981) using tert-butyl hydroperoxide as substrate. NADPH disappearance was monitored at 340nm using a spectrophotometer. The reaction medium contained 2 mM glutathione, 0.15 U/mL

glutathione reductase, 0.4 mM azide, 0.5 mMtert-butyl hydroperoxide and 0.1 mM NADPH. Enzymatic activity was represented according to absorbance changes per minute/mg protein (Mohammadi et al 2014).

SOD activity was assayed by the inhibition of the photochemical reduction of NBT, as described by Giannopolitis and Ries (Giannopolitis and Ries 1977). The reaction medium comprised 0.37 ml 50 mm K-phosphate (pH 7.8), with 0.1mm Na2EDTA, 4 ml regenerating solution and 30 Ml enzyme. The regenerating solution contained 2.2 µM riboflavin, 14.3 mM methionine, and 82.5 µM NBT. Glass test tubes containing the mixtures were placed under a fluorescent lamp. The reaction was initiated by turning the light on and the reduction of NBT was followed by reading the A560 for 10 min. Blanks and controls were run the same way but without illumination and enzyme, respectively. One unit of SOD was defined as the amount of enzyme which produced a 50% inhibition of NBT reduction under the assay conditions (Mohammadi et al 2014). Protein was determined according to lowry method and BSA as standard (Lowry 1951).

Statistical analyses

Data are expressed as means ± S.E.M. and were analyzed statistically by one-way ANOVA, followed by post hoc (LSD) analysis using Tukey test. The results were considered statistically significant P-value < 0.05. All calculations were performed using SPSS 11.0 statistical software.

RESULTS

Behavioral test (Morris Water Maze) for lesion and control groups used to show Parkinsonism animal model. Following 4 days of training in the MWM in our experiments injured group was weak in learning. At the end of training there was a significant increase in escape latency in injured group compared to the control group which indicated an impairment in spatial memory at two weeks after injury (Ferro et al. 2005; Napatr Sriraksa et al.2012; Mojtaba Dolatshahi et al. 2015).

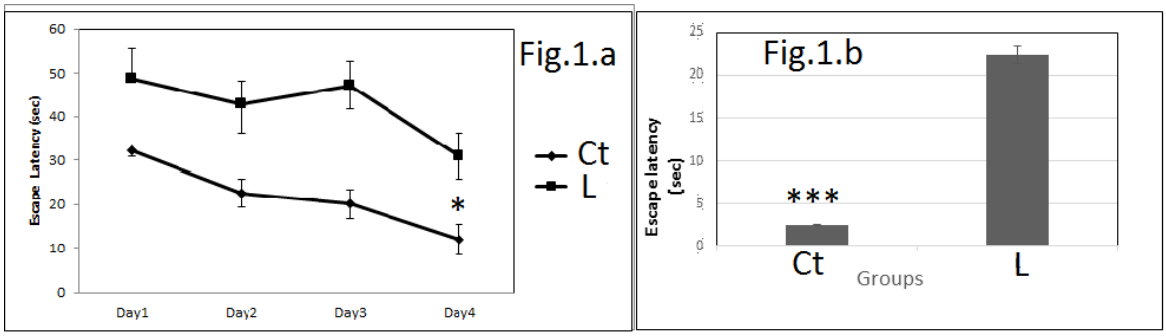

Figure1- a: There was a significant difference between the fourth and the first day of training for the task of finding the hidden platform in terms of escape latency in control group *(P<0.05). No difference was found in escape latency between all days in lesioned group. CT: Control. L: Lesion group. Figure.1. b: There was a significant increased in the escape latency in lesioned group compared to the control group ***(P<0.000).

Determination of Malondialdehyde, reactive oxygen species levels and scavenging enzymes activities (Mohammadi et al. 2014). Biochemical analyses showed of the MDA level. In figure.2, the MDA level was not significantly difference in all doses of RE-treated rats compared to H2O and control groups.

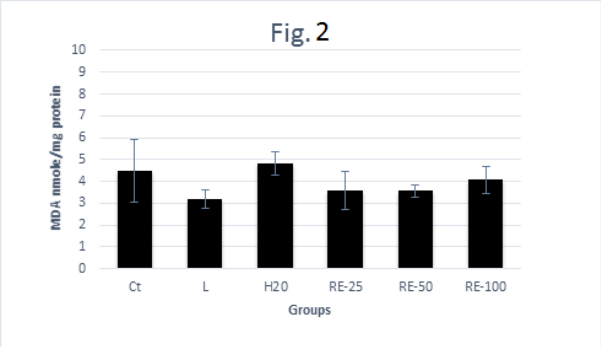

Figure 2- Effect of rosemary leaves extract treatment on MDA level in the rat hippocampus. Data are presented as the mean± SEM, the MDA level was not significantly difference in all doses of RE-treated rats compared to H2O and control groups CT: Control. L: Lesion. H2O: H2O-gavaged rats. RE25: Gavaged rats by RE at a dose of 25 mg/kg-RE50: Gavaged rats gavaged by RE at a dose of 50 mg/kg-RE100: Gavaged rats gavaged by RE at a dose of 100 mg/kg

In Figure 3, ROS level was increased in H2O and lesioned rats as compared to control and RE-treated groups. Administration of RE at a dose of 50 mg/kg to 6-OHDA injured animals by gavage presented a significant decrease of ROS level compared to lesioned and H2O-gavaged animals (P<0/05).

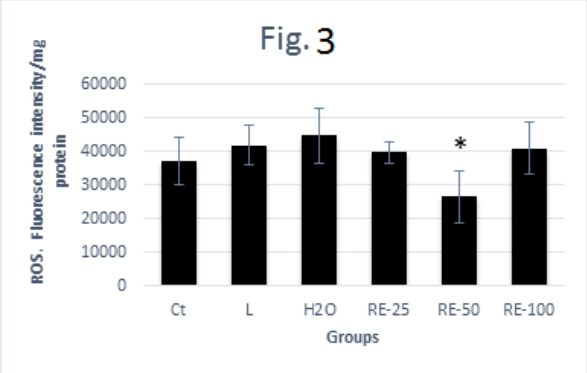

Figure 3 - Effect of rosemary leaves extract treatment on ROS level in the rat hippocampus. Data are presented as the mean ± SEM. ROS level was increased in H2O and lesioned rats as compared to control and RE-treated groups. Oral gavaged rats by RE at a dose of 50 mg/kg presented a significant decrease of ROS level compared to lesioned and H_2O groups. * P<0/05 compare to H_2O and lesioned groups.

In Figure 4, the activity of SOD in lesioned and H2O groups was lower than that of the control and also there was a significant difference between lesion and control groups. Analysis of data showed a significant increase of SOD activity in all doses of rosemary treated animals compared to lesion group. It was shown a significant decrease of SOD activity in the lesion compared to control. Moreover we found a significant increase of SOD activity in RE25 vs. lesion, RE50 treated rats vs. lesion and H2O groups.

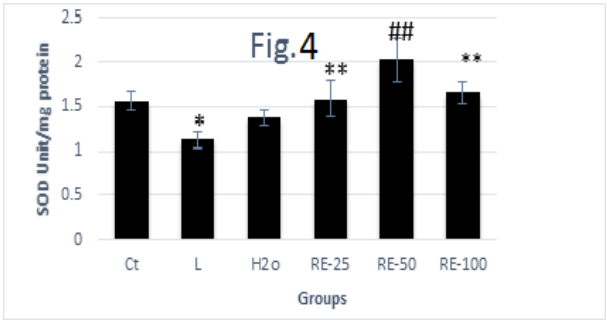

Figure 4 - Effect of rosemary leaves extract treatment on SOD activity in the rat hippocampus. Data are presented as the mean ± SEM; there was a significant decrease of SOD activity of lesioned rats as compared to control. There was a significant increase of SOD activity in all doses of rosemary treated animals compared to lesion group. In addition, there were significant increase of SOD activity in RE25 vs. lesion, RE50 treated rats vs. lesion and H2O groups. * P<0/05 compared to control, ** P<0/05 compared to lesion, ## P<0/001 compared to lesion and H2O groups.

The Glutathione peroxidase activity was increased significantly in all RE-treated groups compared to lesioned and H2O gavaged rats (Fig.5). In addition RE50 and RE100-treated groups showed a significant increase of Glutation activity as compared to control.
We also observed a significant increase of the GPX activity in 6-OHDA-lesioned groups treated with 50 mg/kg of the RE compared to control group.

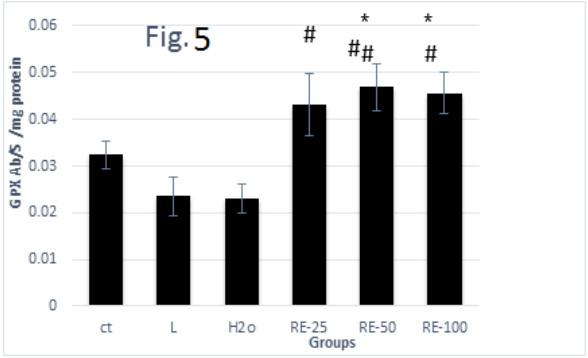

Figure 5- Effect of rosemary leaves extract treatment on GPx activity in the rat hippocampus. Data are presented as the mean ± SEM; The GPX activity was increased significantly in all RE-treated groups as compared to

lesioned and H2O gavaged rats. RE50 and RE100-treated groups showed a significant increase of GPX as compared to control. There was a significant increase of the GPX activity in RE50-treated rats as compared to control group. # P<0/01 compared to lesion and H2O, ## P<0/001 compared to lesion and H2O. *P<0/001 compared to control group.

The CAT activity was increased in all doses of RE-treated groups as compared to lesioned and H2O treated rats. And also, the CAT activity of RE50 and RE50,100-treated animals were increased significantly as compared to lesion and control groups respectively. There was a decreased enzyme activity of lesion and H2O groups as compared to control (Fig.6).

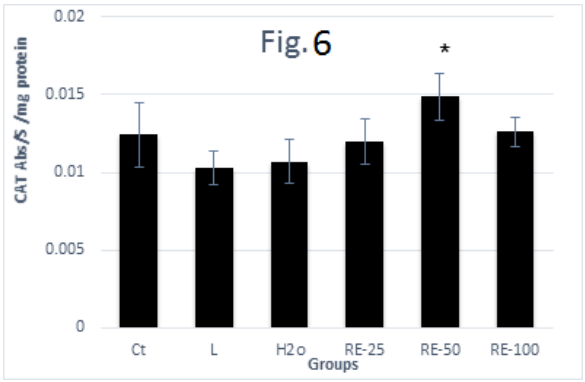

Figure 6- Effect of rosemary leaves extract treatment on CAT activity in the rat hippocampus. Data are presented as the mean ± SEM; the CAT activity was increased in all doses of RE-treated groups as compared to lesion and H2O groups. The CAT activity of RE50 and RE50,100-treated animals was increased significantly as compared to lesion and control groups respectively. There was a decreased activity of CAT in lesion and H2O groups as compared to control. *P<0/05 compared to lesion.

DISCUSSION

There is considerable evidence suggesting that mitochondrial dysfunction and oxidative damages may play a role in the pathogenesis of PD (Hwang 2013; Morris and Berk 2015). Evidence of increase in lipid peroxidtion and oxidation of DNA and proteins has indeed been seen in the substantia nigra of patients affected with PD (Munoz et al. 2012). Decreased levels of antioxidant enzyme activity have been reported in patients with PD (Ciccone et al. 2013). Clearly, therapeutic strategies aimed at limiting free radical production, oxidative stress and damage may slow or stop the progression of neurodegenerative processes of PD. Antioxidants are expected to have a major impact on the treatment of PD. The natural occurrence of antiparkinsonian drugs in plants: anticholinergics in *Datura stramonium*, levodopa in *Mucuna pruriens* and *Vicia faba*, dopamine agonist activity in *Claviceps purpura*, and MAO inhibitor activity in *Banisteria caapi* and *Ginkgo biloba* leaves extract are known (Hritcu et al. 2011; Hwang et al. 2011; Song et al. 2012; Song SS et al. 2012; Li et al. 2013). Green and black tea extracts attenuated neuronal apoptosis induced by 6-OHDA (Chaturvedi et al. 2006; Guo et al. 2007; Bitu Pinto et al. 2015). The extract of the Chinese herb *Tripterygium wilfordii* promotes axonal elongation and protects dopaminergic neurons from the lesion induced by 1-methyl-4-phenylpyridinium ion (Xiong et al. 2013).

In the present study, we prepared a parkinsonian rat model with 6-OHDA by bilateral lesion of the dopaminergic neurons located in the SN. This model represents a good model of the early phase of PD because of its mild motor impairment and cognitive deficits and ideal to see the suitability of neuroprotective agents (Hritcu et al. 2011). Importantly, in recent years, it was indicated that non motor deficits became an important part of patient management (Whittington et al. 2006, Hanna-Pladdy et al. 2013). Additionally, it has been reported that 6-OHDA is an oxidative neurotoxin to injure dopaminergic (DA) neurons in vivo and in vitro. Oxidative stress has been reported to induce cognitive impairment in PD, as indicated by the fact that many cognitive impairments, including memory deficits, occur during the early stage of PD even before the development of its classical symptoms (Tadaiesky et al. 2008). The toxicity of 6-OHDA is attributed to its oxidation by molecular oxygen and/or monoamine oxidase. This leads to the production of intracellular H_2O_2 which can be transformed into highly reactive hydroxyl radicals and produce cell damage (Glinka et al. 1997; Wu et al. 2015). It was determined that treatment with 6-OHDA leads to neuronal death by the production of ROS and by affecting mitochondria (Saito et al. 2007; Weidinger and Kozlov 2015). Dopaminergic neurons, the predominant cells lost in PD are believed to be highly prone to oxidative damage. This is due to the propensity of dopamine to autooxidize and thereby produce an elevated levels

of H_2O_2 which reacts with transition metal iron (Fe^{+3}) and forms the highly reactive and cytotoxic hydroxyl radicals (OH) which are known to damage lipids, proteins and DNA. Iron is frequently associated with neurodegerative process (Halliwell 1992; Hwang et al. 2013; Ayton et al. 2015). The basal ganglia in particular, the globus pallidus and substantia nigra contain high concentration of iron which contributes to free radical production (Halliwell 1992). Accumulation of iron in substantia nigra contributes to the cell death by enhancing lipid peroxidation, as judged by raised levels of both malondialdehyde and lipid hydroperoxides (Hauser et al. 2013; Ward et al. 2015). But the hippocampus is the most vulnerable area to oxidative stress, it was demonstrated that an early event in the course of dopamine depletion following 6-OHDA administration is the generation of oxidative stress in the hippocampus and it caused memory impairment (Hritcu et al. 2011; Sriraksa et al. 2012; Zaltieri et al. 2015). The post-mortem studies from PD patients suggested the involvement of an excess formation of free radicals and the onset of oxidative stress in the disease progression (Ren et al. 2009).

In this study, Although following 4 days of training in the MWM in our experiments, control and lesioned groups learned well as indicated by decrease for finding the hidden platform, but lesioned group was weak in learning. At the end of training there was a significant increase in escape latency in lesioned group compared to the control group. To assess spatial memory retention, a probe trial was performed, during which the platform was removed from the pool. No significant difference in the swimming time tests between two experimental groups emerged, so 6-OHDA-induced bilateral destruction of dopaminergic neurons produced a significant decrease in short-term memory without significantly affecting locomotion. There was a significant difference of escape latency between lesioned and control animals, which indicated an impairment in spatial memory at two weeks after injury (Ferro et al. 2005; Napatr Sriraksa et al.2012; Mojtaba Dolatshahi et al. 2015). The activity of MDA was not significantly decreased in all RE treated rats compared to H_2O and control groups, maybe the treatment period must be shorter or longer than 14 days after neurotoxin injection. MDA level in H_2O-gavaged rats were not increased significantly compared to control group, because of *Rosmarinus officinalis* leaves extract pretreatment in H_2O group and also it would be better to determine MDA level immediately after neurotoxin injection and 14 days treatment after lesion caused MDA level returned to normal level. It was revealed non-significant statistical differences between control and H_2O groups, because of RE pretreatment. *Rosmarinus officinalis* leaves extract with antioxidant effect inhibited high MDA level. Sofia Sanchez (2007) reported that intrastriatal injection of 6-OHDA caused oxidative stress, which increased during the first 2-day post injection and returned to approximate level before neurotoxin administration after 17 days (Sanchez et al. 2007). And also GPx activity of 6-OHDA injured and H2O groups was not decreased significantly compared to control, so it protected cell membrane against lipid peroxidation, as a result we didn't observe an increase of MDA level in injured and H_2O-gavaged rats. It was suggested that, when the treatment starts before injury, it causes the reduction of ROS level and delayed neuronal degeneration.

Even nowadays, medicinal herbs is still very popular for treatment of PD in Asian countries such as China, Japan and Korea (Howes et al. 2003). An extract of rosemary leaf (RE) has strong antioxidant activity due to *carnosic acid* and *carnosol* (Petersen et al. 2003; Perez-Fons et al. 2010). There have been relatively few studies that have characterized antioxidant effect of RE in animal model of PD.

We therefore suggest that *Rosmarinus officinalis* leaves extract has an antioxidant effect as a free radical scavenger. Reducing free-radicals from cigarette smoke by using a filter impregnated by an extract of a rosemary plant in oil was reported (Alexandrov et al. 2006). *Rosmarinic acid* could exert its neuroprotective and anti-oxidative effects against 6-OHDA-induced neurotoxicity in dopaminergic neurons (Ren et al. 2009; Wang et al. 2012; Noguchi et al. 2015).

Our results also demonstrated that RE at dose of 50 mg/kg body weight could decrease oxidative stress by increasing the activities of SOD, CAT and GPx in hippocampus resulting in the decrease of lipid peroxidation level in lesioned region. SOD catalyzes the removal of superoxide radical O2, which would otherwise damage the cell membrane and biological structures. GPx catalyzes the reduction of H_2O_2 to H_2O and O_2. In general, a reduction in the activity of these enzymes is associated with an accumulation of highly reactive free radicals, and can lead to deleterious effects

such as loss of cell membrane integrity and function. We found increased levels of CAT activity in hippocampus tissues in *Rosmarinus officinalis* leaves extract treated rats, and this increase was statistically significant in RE50-treated animals compared to H2O group. This decrease in the enzymatic activity in H_2O-treated animals was increased in hippocampus of all *Rosmarinus officinalis* leaves extract concentrations (25,50,100), but only the 50 mg/kg concentration had beneficial effect. The rats subjected to the 50 mg/kg dose of RE had significantly enhanced SOD, CAT, and GPx activity in the hippocampus ($p < 0.01$, 0.01, and 0.05 resp. compared to H2O group). Administration of RE to 6-OHDA injured animals by gavage showed a significant increase of antioxidants level. Antioxidants level in animals gavaged with H2O instead of RE showed no significant statistical differences in the hippocampus tissue compared to lesion group.

Antioxidant activity in hippocampus was affected by *Rosmarinus officinalis* leaves extract at a concentration of 50 mg/kg. Our results also demonstrated that RE could decrease oxidative stress by increasing the activity of SOD in hippocampus, and increased the activities of CAT and GPx in hippocampus resulting in the decrease of lipid peroxidation level in mentioned area.

Therefore, the neuroprotective effect of *Rosmarinus officinalis* leaves extract might be related to its antioxidant effect and it could be incorporated to the diet as a nutritional supplement, to augment the body's defenses against oxidative stress caused by PD. *Rosmarinic acid* has been reported to protect biomembranes against peroxidative damage (Liu et al. 1992). Additionally, Sharmila (2012) reported that *Rosmarinic acid* had potent anti-cancer, anti-lipid peroxidative and apoptotic effect in skin carcinogenic mice models (Sharmila et al. 2012). Recently studies showed that there are biologically active compounds in rosemary essential oil exhibiting cytotoxic, antioxidant, anti-carcinogenic and cognition enhancing properties (Halliwell 1992). Azad (2010) showed that pretreatment with *carnosic acid* can reduce cellular death in the hippocampus in experimental model of Alzheimer's disease (Azad et al. 2011).

It has been shown in an in vitro study that *carnosic acid* has direct action as an antioxidant (Azad et al. 2011). *Carnosic acid*, like *rosmarinic acid*, has been shown to be neuroprotective in both in vitro models of neuronal death and in vivo models of neurodegenerative disease (Liu et al. 1992). Lee reported the antioxidant and protective effects of RA on H_2O_2 induced neurotoxicity in human dopaminergic neurons (Kelsey et al. 2010).

Generally, to achieve efficacy, a candidate antioxidant must penetrate the blood-brain-barrier (BBB) to attain a critical therapeutic level within the CNS and must be given as early as possible, before the irreversible neuronal loss. It also should fit the precise ROS physiology, for example, the type of ROS involved, the place of generation, and the severity of the damage (Azad et al. 2011; Luca et al. 2015). Thus, antioxidant cocktails or antioxidants combined with other drugs may have more successful synergistic effects. Further well-designed intervention trials, as well as observational investigations based on larger cohorts studied over a longer period of time with several methods for assessing antioxidant exposure, including relation to BBB penetration, are needed to test this hypothesis. RE could penetrate the BBB easily. Supplementation of RE with antioxidant property along with diet will maintain a balanced antioxidant status and may slow down the progression of the disease. As far as we know this is the first work that shows antioxidant effects of *Rosmarinus officinalis* leaves extract in 6-OHDA treated rats as a model of early phase of PD.

In conclusion, the data presented here show the cytoprotective effect of RA against 6-OHDA toxicity in dopaminergic cells, which might provide the pharmacological basis underlying the traditional use of this space to improve memory deficit of PD. Because of the neuroprotective effect of RE might be related to its antioxidant effect, It hopes that these data may be beneficial for future medicinal herbs to prevent the progression of PD.

Furthermore, the present study demonstrates that increase of the antioxidant enzyme activities in 6-OHDA-lesioned groups treated with 50 mg/kg of the RE leaves extract is significantly correlated to a decrease of lipid peroxidation (MDA), in the hippocampus. This could suggest that the increase of the antioxidant defence and decrease of lipid peroxidation could be correlated with the involvement of the RE in neuroprotection against 6-OHDA-induced neuronal oxidative stress generation. However, further clinical trial study is still required.

CONCLUSIONS

This study showed that bilateral injection of 6-OHDA could induce neurodegenerative damage while oral administration of *carnosic acid* could protect hippocampal neurons from oxidative stress. These protective effects may be due to its antioxidant properties. Thus, *carnosic acid* might be used as a nutritional supplement.

ACKNOWLEDGMENTS

This study was supported financially by Damghan University. There is no conflict of interest in this article. The authors express their appreciation to the biological school of Damghan university for supporting this research

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Received: October 08, 2015;
Accepted: February 04, 2015.