Enhancement of memory function by antioxidant potential of *Nigella sativa* L. oil in restrained rats

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Abstract: Stress is a vulnerable state to cellular homeostasis which leads to oxidative damage via free radical generation. The acute stress induces alteration in antioxidant enzyme activities to an extent which produce oxidative stress and causes certain pathological conditions. The use of *Nigella sativa* L. oil (NSO) in folk medicine has increased throughout the world for the prevention or treatment of various ailments because of potent antioxidant properties. In the present study, potential therapeutic effects of NSO on memory in both unrestrained and 2h restrained rats were observed. Short-term memory (STM) and long-term memory (LTM) were assessed by elevated plus maze (EPM) and Morris water maze (MWM) respectively. The present study also demonstrated the effect of NSO on lipid peroxidation (LPO) and activities of antioxidant enzymes (superoxide dismutase, catalase and glutathione peroxidase) along with the activity of acetyl cholinesterase (AChE). The results obtained from the present study showed that 2h restraint stress significantly enhanced both short-term memory ($p<0.01$) and long-term memory ($p<0.05$) in rats. Pretreatment with NSO at a dose of 0.2ml/kg/day also significantly improved STM ($p<0.05$) in restrained rats and LTM ($p<0.01$) in unrestrained rats. This study also showed significantly decreased ($p<0.01$) LPO and significantly increased ($p<0.01$) endogenous antioxidant enzymes activity in NSO treated restrained rats. Similarly significant decreased ($p<0.01$) AChE activity was also observed in NSO treated unrestrained and 2h restrained rats. Therefore, current findings suggested that repeated administration of NSO may exert memory enhancing effects against restrained stress and it can be used for therapeutic purpose because of having fewer side effects.

Keywords: Oxidative stress, *Nigella sativa* L. oil, short term memory, long term memory, acetylcholinesterase, restraint stress.

INTRODUCTION

Experience to a diverse stress situation persuades alteration in behaviors through the modulation of physiological, biochemical, hormonal and neurochemical functions (Joels and Baram, 2009). Stress and memory functions are linked with each other. It has been observed that type and extent of the stressors have substantial impact on cognitive functions (Sandi *et al.,* 2007). Stress can either improve or impair and even have no effect on learning and memory processes (Park *et al.,* 2008). Exposure to acute stress often improves memory tasks through the stimulation of sympathetic nervous system as a part of flight or fight response (McEwen and Sapolsky, 1995). Acute stress generally increases brain ability to encode new memories whereas; chronic or prolonged stress causes alteration in the neuronal structures which may dampen memory formation (Jackson *et al.,* 2006). Stressful events may become a key modulator to encode new memory under the influence of stress hormones and neurotransmitters (Groeneweg *et al.,* 2011). Herbal products have been extensively used from ancient times for the treatment of various ailments largely due to their less side effects and adequate efficacy (Cheema *et al.,* 2016). One of the herbal plants of great medicinal importance is *Nigella sativa* L. having rich historical and religious background. It belongs to the family Ranunculaceae and generally known as black cumin, black seed or kalonji (Gillani *et al.,* 2004). The seeds of *Nigella sativa* contain fixed oils which are rich in unsaturated and essential fatty acids including arachidonic acid, palmitic acid, oleic acid and linolenic acid (Ali and Blunden, 2003). It is also a chief source of omega 3 and omega 6 polyunsaturated fatty acids which are found to suppress many diseases. *Nigella sativa* seeds also contain proteins, carbohydrates, alkaloids, coumarins and minerals (Cheikh-Rouhou *et al.,* 2007). Essential oils isolated from *Nigella sativa* seeds have different types of flavonoids and active compounds such as thymoquinone, thymohydroquinone, dithymoquinone, carvacrol and thymol (Randhawa and Alghamdi, 2011).These active compounds are mainly responsible for therapeutic activity of *Nigella sativa* against various diseases. The essential

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oils isolated from the seeds have number of pharmacological activities ranging from antioxidant (Burits and Bucar, 2000), anti-inflammatory and analgesic (Al-Ghamdi, 2001; Ghannadi *et al.,* 2005), cardiovascular, antimicrobial, antiasthamatic and anticonvulsant. Studies reported that *Nigella sativa* oil (NSO) possess potent antidepressant, anxiolytic and memory enhancing properties due to its bioactive component thymoquinone (Al- Naggar *et al.,* 2003). Thymoquinone possess potent anti-carcinogenic and anti-mutagenic properties (Khader *et al.,* 2010). Thymoquinone has the ability to suppress the tumor necrosis factor induced NF-Kappa B activation by various carcinogenic and inflammatory stimuli (Albensi and Mattson, 2000; Sethi *et al.,* 2008).The antioxidant properties of NSO and thymoquinone increase the activity of endogenous enzymes (Ebru *et al.,* 2008) that may cure different kinds of cancer (Khader *et al.,* 2010) through minimizing the toxic effect of anticancerous drugs by an up regulation of antioxidant mechanisms (Alenzi *et al.,* 2010). The generation of oxidative stress is the normal cellular mechanism in living organisms. The body is endowed with endogenous antioxidant enzymes which readily convert reactive oxygen species (ROS) to less reactive compounds (Uttara, Bayani, *et al.,* 2009). Stress can cause alteration in physiological and biochemical reactions as well as enhance production of free radicals causing changes in structure of DNA, lipids and protein; activation of several stresses induced transcriptional factors and may produce pro-inflammatory and inflammatory cytokines (Birben *et al.,* 2012). The malondialdehyde (MDA) is a biomarker of lipid per oxidation which may inactivate several cellular proteins by forming cross linkage that can cause changes in biochemical properties of biomolecules and may lead to different pathological conditions (Ayala *et al.,* 2014). Increased level of LPO followed by decrease level of SOD and CAT activities indicate the generation of ROS (Matos *et al.,* 2007). The enzyme SOD catalyzes the diminution of oxygen anion (O^2-) to hydrogen peroxide (H_2O_2). This H_2O_2 is lethal to cells and hence further converted to water (H_2O) by the mutual scavenging activities of CAT and GPx (Ray and Hussain, 2002). The antioxidant activity of NSO significantly restore the activity of SOD, CAT and GPx and decrease the generation of LPO to the normal level (Ahmad *et al.,* 2013). Studies reported that antioxidant activities of NSO could be involved in acetylcholinesterase (AChE) activity inhibition, thus retaining the effects of acetylcholine in the encoding of new memories (Hasselmo, 2006). Therefore, NSO is an imperative source of antioxidant herb which significantly increases the brain tissue level of antioxidant enzymes (Burits and Bucar, 2000).

Despite a number of acceptable beneficial effects and investigated antioxidant properties (Pitozzi *et al.,* 2010), only few studies have been done on the effects of *Nigella sativa* on memory functions (Sahak *et al.,* 2013; Bin sayeed *et al.,* 2013), however, no work has been done with respect to restraint stress related changes in memory function in rats. Therefore, in this study stress related memory functions and activities of endogenous enzymes following the administration of NSO for three weeks were measured.

MATERIALS AND METHODS

Experimental protocol

Male Albino Wistar rats (150-200g) were purchased from ICCBS, animal house, Karachi, Pakistan (voucher number: 6700/2016). To avoid the effect of social interaction, animals were housed individually with ad libitum access to cubes of standard rodent diet and tap water under a 12:12 h light/dark cycle (lights on at 7: 00 am) at controlled room temperature (22±2°C). Twenty four rats were divided into control and *Nigella sativa* oil treated groups. *Nigella sativa* oil was purchased from local super market. All other chemicals were purchased from Sigma-Aldrich Company (voucher number: 0763/2016). *Nigella Sativa* oil at a dose of 0.2 ml/kg/day, (Mehmet *et al*., 2005) was given orally to the oil treated groups for three weeks, whereas, controls were treated with tap water for the same period of time. After three weeks, control and test groups were equally divided as unrestrained (n=6) and restrained (n=6) rats. The rats of restraint group were given 2h/day for two days restraint stress in specifically designed ventilated closed plastic tubes that allow constrained lateral movement (Emad *et al.,* 2017). The rats of unrestraint group were kept in their cages throughout the experiment. Both restrained and unrestrained rats were then subjected to elevated plus maze test (EPM) and Morris water maze (MWM) to monitor short term and long term memory. Rats were then decapitated using the guillotine and their plasma and brain samples were collected. Their brains were detached from the skull within 30s after decapitation and stored at -70^{o}C until investigated for biochemical estimations. All experiments were carried out between 10:00 am and 5:00 pm in a balanced design to avoid influence of order and time. The experiment was performed after the formal approval from Institutional Review Board (IRB) with reference No.1196 (2010).The guidelines of National Institute of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985) were strictly followed. All efforts were made to reduce animal suffering and to minimize the number of animals used in the experiments.

Behavioral assessment

Elevated plus maze test (EPM)

The short term memory was assessed by EPM in rats (Sharma and Kulkarni, 1992). The EPM apparatus was same as used previously in our laboratory (Emad *et al.,* 2017).

Morris water maze test (MWM)

The MWM is a traditional cognitive testing apparatus, described by Morris, 1981. The MWM apparatus was same as used earlier in our laboratory (Emad *et al.,* 2017).

Biochemical estimations

Estimation of lipid peroxidation (LPO) was performed as described by Chow and Tappel (Chow and Tappel, 1972). LPO was expressed as µmol of MDA/g of brain tissue. The SOD was estimated by the method (Singh *et al.,* 2002), based on the reduction of NBT to water insoluble blue formazan. Units of SOD activity were expressed as the amount of enzyme required to inhibit the reduction of NBT by 50%. The specific activity was expressed in terms of U/g of brain tissue. CAT was estimated using a previously reported method (Sinha, 1972). CAT activity was expressed as consumption of H_2O_2 µmol/min/g of brain. GPx activity was measured by the procedure of Flohe and Gunzler (Flohe and Gunzler, 1984). Activity of GPx was expressed as µmol /min/g of brain. Activity of AChE in homogenate was determined according to the method of Ellman *et al* (Ellman *et al.,* 1961). The activity of AChE was expressed as µmol/g/min of brain tissue.

STATISTICAL ANALYSIS

Results are expressed as mean±S.D. Analyses were performed by two-way analysis of variance (ANOVA) of SPSS version 20.0 to determine the effects of the various factors involved. Post hoc analysis was done by Tukey's test and p>0.05 was considered non-significant.

RESULTS

The present study observed the effect of NSO on short-term and long-term memory in unrestrained and 2h restrained male rats. In addition, the effect of NSO on LPO, antioxidant enzymes (SOD, CAT and GPx) activities along with the AChE activity was also monitored.

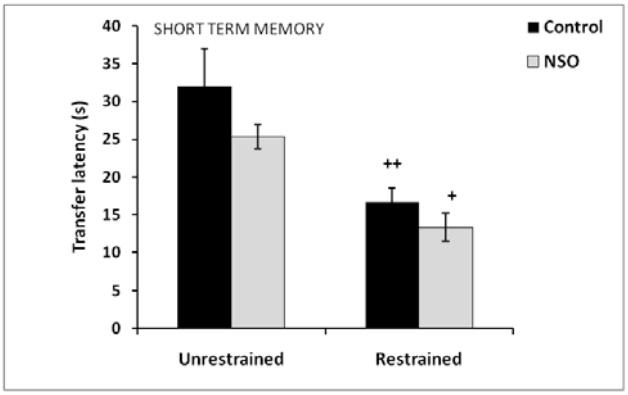

Fig. 1: Effect of NSO on elevated plus maze test in 2h restrained rats. Values are mean±SD (n=6). Significant differences by Tukey's test ++p<0.01 and +p<0.05 from their respective unrestrained controls.

Elevated plus maze test

Fig. 1 shows the effect of NSO on the EPM test in unrestrained and 2h restrained male rats. Data analysis revealed significant effect of stress [F(1,20)=20.947, p<0.01], non significant effect of NSO [F(1,20)=2.804, p>0.05] and interaction among stress x NSO [F(1,20)=0.312, p>0.05]. Tukey's test demonstrated that 2h restraint stress significantly decreased (p<0.01) time to enter closed arm. Repeated administration of NSO also significantly decreased (p<0.05) time to enter closed arm in restrained rats compared to respective unrestrained rats.

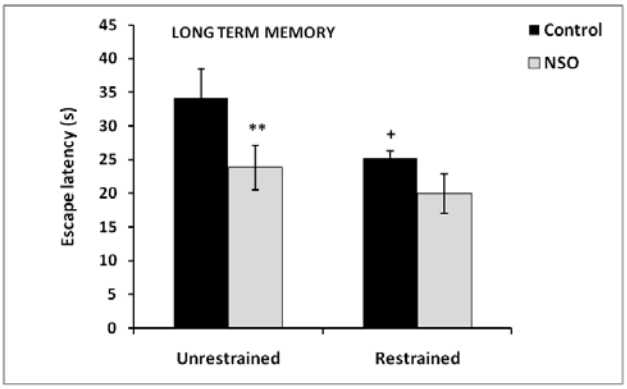

Fig. 2: Effect of NSO on Morris water maze test in 2h restrained rats. Values are mean±SD (n=6). Significant differences by Tukey's test **p<0.01 from their respective controls, +p<0.05 from their respective unrestrained controls.

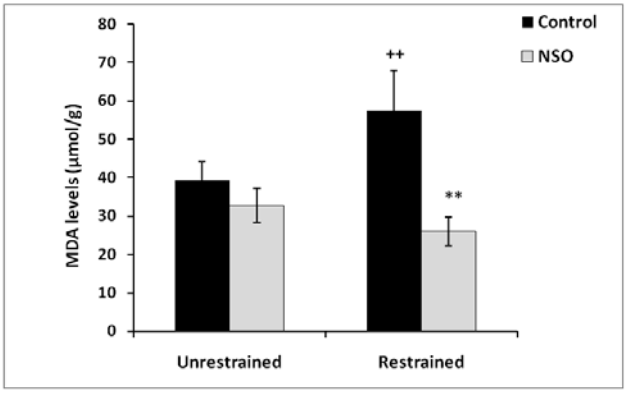

Fig. 3: Effect of NSO on lipid peroxidation in 2h restrained rats. Values are mean±SD (n=6). Significant differences by Tukey's test **p<0.01 from their respective controls, ++p<0.01 from their respective unrestrained controls.

Morris water maze test

Fig. 2 shows the effect of NSO on the MWM test in unrestrained and 2h restrained male rats. Data analysis revealed significant effect of stress [F(1,20)=10.457, p<0.01], NSO [F(1,20)=15.254, P<0.01] and non significant interaction among stress x NSO [F(1,20)=, 1.695p>0.05]. Tukey's test demonstrated that 2h restraint stress significantly decreased (p<0.05) time to reach hidden platform. Repeated administration of NSO

significantly decreased (p<0.01) time to reach hidden platform in unrestrained rats.

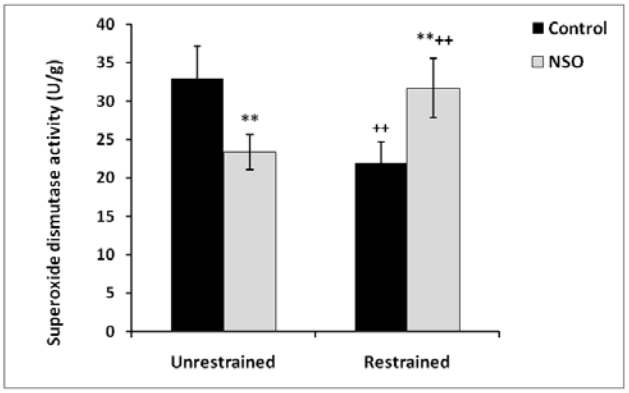

Fig. 4: Effect of NSO on superoxide dismutase activity in 2h restrained rats. Values are mean±SD (n=6). Significant differences by Tukey's test ***p*<0.01 from their respective controls, ++*p*<0.01 from their respective unrestrained controls.

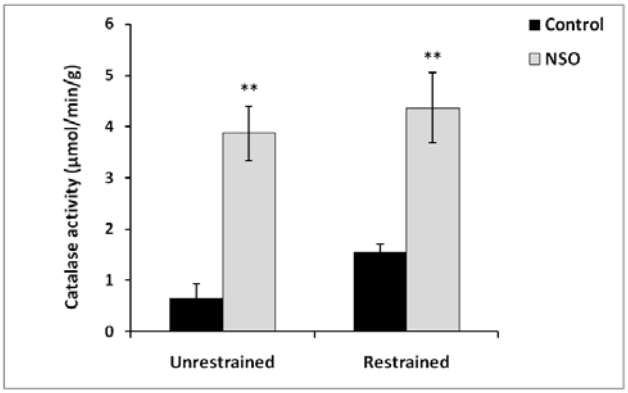

Fig. 5: Effect of NSO on catalase activity in 2h restrained rats. Values are mean±SD (n=6). Significant differences by Tukey's test ***p*<0.01 from their respective controls.

Lipid peroxidation (LPO)

Fig. 3 shows the effect of NSO on lipid peroxidation measured in terms of MDA levels in unrestrained and 2h restrained male rats. Data analysis showed non significant effect of stress [F(1,20)=3.770, p>0.05], significant effect of NSO [F(1,20)=42.126, p<0.01] and interaction among stress x NSO [F(1,20)=18.365, p<0.01]. Tukey's test demonstrated that 2h restraint stress significantly increased (p<0.01) LPO. Repeated administration of NSO significantly decreased (p<0.01) LPO in restrained rats.

Antioxidant enzymes activities (SOD, CAT, GPx)

Fig. 4 shows the effect of NSO on SOD activity in unrestrained and 2h restrained male rats. Data analysis revealed non significant effect of stress [F(1,20)=0.684, p>0.05], NSO [(1,20)=0.003, p>0.05], and significant interaction among stress x NSO [F(1,20)=35.015, p<0.01]. Tukey's test demonstrated that 2h restraint stress significantly decreased (p<0.01) SOD activity. However, significantly increased (p<0.01) SOD activity was observed in NSO treated restrained rats. Fig. 5 shows effect of NSO on CAT activity in unrestrained and 2h restrained male rats. Data analysis revealed significant effect of stress [F(1,20)=5.138, p<0.05], NSO [F(1,20)=95.499, p<0.01], and non significant interaction among stress x NSO [F(1,20)=0.432, p>0.05]. Tukey's test demonstrated significantly increased (p<0.01) CAT activity in NSO treated unrestrained and restrained rats. Fig. 6 shows the effect of NSO on GPx activity in unrestrained and 2h restrained male rats. Data analysis revealed significant effect of stress [F(1,20)=51.838, p<0.01], NSO [F(1,20)=308.026, p<0.01] and non significant interaction among stress x NSO [F(1,20)=2.33 p>0.05]. Tukey's test demonstrated that 2h restraint stress significantly increased (p<0.01) GPx activity. Similarly significantly increased (p<0.01) GPx activity was observed in NSO treated restrained and unrestrained rats as compared to respective control.

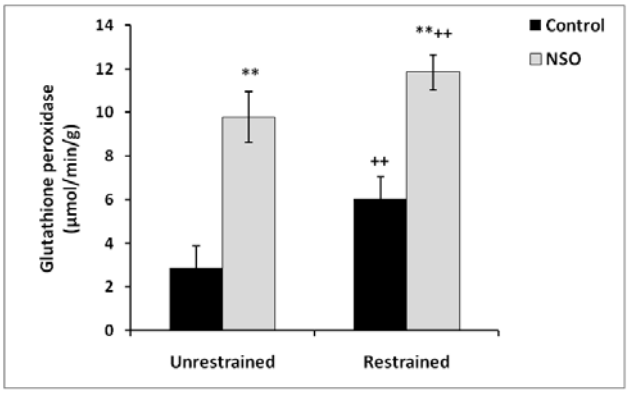

Fig. 6: Effect of NSO on glutathione peroxidase activity in 2h restrained rats. Values are mean±SD (n=6). Significant differences by Tukey's test ***p*<0.01 from their respective controls, ++*p*<0.01 from their respective unrestrained controls.

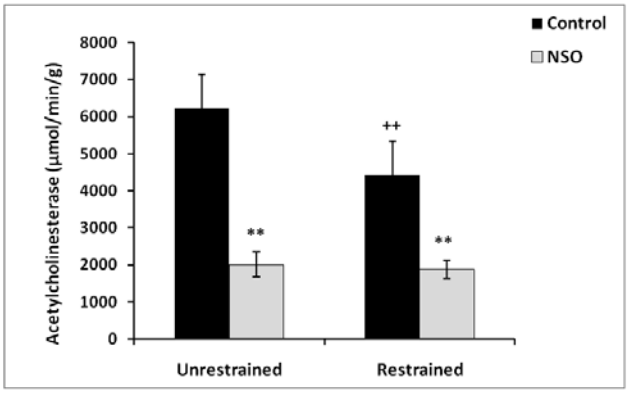

Fig. 7: Effect of NSO on acetylcholinesterase activity in 2h restrained rats. Values are mean±SD (n=6). Significant differences by Tukey's test ***p*<0.01 from their respective controls, ++*p*<0.01 from their respective unrestrained controls.

Assessment of NSO on AChE activity

The effect of NSO on enzyme inhibition of AChE in unrestrained and 2h restrained rats as shown in Fig. 7. Data analysis revealed significant effect of stress [F (1, 20) =10.853, $p<0.01$], NSO [F (1, 20) =133.505, $p<0.01$] and significant interaction among stress x NSO [F (1, 20) =8.066, $p<0.05$]. Tukey's test demonstrated that 2h restraint stress significantly decreased AChE activity. Similarly, repeated administration of NSO significantly decreased AChE activity in unrestrained and restrained rats.

DISCUSSION

The medicinal plants have great attention all over the world for the management of various diseases. *Nigella sativa* is an excellent medicinal plant used to treat acute as well as chronic illness (Darakhshan *et al.,* 2015). The current study demonstrated that exposure to 2h restraint stress improved STM in rats. The improvement in memory in stress could be due to the instantaneous release of glucocorticoids via hypothalamic pituitary adrenal axis (HPA-axis) (Mc Ewen, 2007) as well as increased glutamatergic transmission in rat brain. In present study, repeated administration of NSO has shown to cause further improvement in STM in restrained rats as it is observed by decreased transfer latency in EPM test. Studies reported that adaptogenic properties of *Nigella sativa* extract protect from oxidative stress and enhance memory functions by normalizing HPA axis activity (Sahak *et al.,* 2013). Pharmacological effects of NSO showed that increased level of brain serotonin and dopamine is involved in the enhancement of memory functions as reported earlier (Perveen *et al.,* 2008). The present study also demonstrated improvement in LTM following 2h restraint stress as escape latency in MWM test is decreased in rats. It is reported that acute stress increase activation of mineralocorticoid receptors which enhances long term potentiation (LTP) and memory function (Conrad *et al.,* 1999). The antioxidant effects of NSO have shown significant effect on learning and memory. *Nigella sativa* seeds contain flavonoids, which are able to induce learning and memory processes by increasing synaptic plasticity and long term potentiation (Rendeiro *et al.,* 2009). *Nigella sativa* oil has potential scavenging activities, control free radicals production and serves as a neuroprotective herb by improving memory function in rats (Malik and Haleem, 2012). Brain is highly sensitive to free radical production as having high lipid content, high oxygen consumption and relative rarity of antioxidant enzymes (Cui *et al.,* 2004; Samarghandian *et al.,* 2017). Acute restraint stress cause an imbalance in an antioxidant enzyme system and cause oxidative damage (Atif *et al.,* 2008) that may induced cellular injury through lipid peroxidation largely in the cell membrane. The integrity of membrane changed by lipid peroxidation also leads to tissue damage (Sahin and Gumuslu, 2007).

In present study 2h restraint stress produced oxidation as observed by increased LPO activity. Increased MDA levels as one of the biomarker of brain lipid peroxidation was observed in restrained rats which show that 2h restraint stress disrupted the integrity of brain tissues. It is reported earlier that repeated administration of NSO is beneficial to cope from stress situation (Perveen *et al.,* 2014). Findings of present study demonstrated that repeated administration of NSO normalized MDA levels in restrained rats. NSO decreased the level of LPO due to the presence of bioactive components and essential fatty acids which could replace the polyunsaturated fatty acids components of cellular membrane that have been attacked by oxygen free radicals (Youdim *et al.,* 2000: Carrillo and Feller, 2005). In the current study it has been observed that 2h restraint stress decreased SOD activity in restrained rats. Stress induced inhibition of SOD activity may be due to oxidative modification of polypeptide chains of enzyme (Davydov and Shvets, 2003). Superoxide anion scavenging activity by superoxide dismutase enzyme is considered as the first line of defense against oxidative stress. SOD protect against free radical generation by removing superoxide radicals which damage biological membrane and biological structure (Matos, 2007).

Repeated administration of NSO was found to scavenge superoxide radicals and converted them into less toxic H_2O_2 molecule as compared to superoxide anions (Mansour *et al.,* 2002). Results of present study showed that NSO may prevent from oxidative damage and potentiate the activities of endogenous antioxidant enzyme system as reported earlier (Safhi, 2016). Studies reported that NSO may directly act as an antioxidant by blocking ROS generation and by inhibiting programmed cell death pathway or can directly decrease ROS levels (Becker *et al.,* 2002; Schubert *et al.,* 2006). It is also reported that the active component thymoquinone is responsible for antioxidant activity of NSO which has ability to decrease LPO by inhibiting eicosanoids production as well as increasing the level of endogenous antioxidant enzymes (Houghton *et al.,* 1995; Solati *et al.,* 2014). The results of present study suggest that repeated administration of NSO restored brain levels of endogenous antioxidant enzymes due to its active components which may cross the BBB (Ersahin *et, al* 2011; Huong *et al.,* 1999; Perveen *et al.,* 2013) and protects CNS from damaging effects of acute stress .

Acetylcholine (ACh) is an important neurotransmitter which is involved in learning and memory processes and it is observed that decreased release of acetylcholine impairs memory (Hasselmo, 2006). The acute restraint stress exposure in rodents produce a rapid but transient activation of central cholinergic system and release newly synthesized ACh (Finkelestein, 1985). Acute stress may cause activation of cholinergic system via activation of

corticotrophin-releasing factor (CRF) and modulate memory functions (Steckler and Holsboer, 1999). In the present study, degradation of ACh is inhibited by decreased AChE enzyme activity in restrained rats and this could be the reason of improved memory performance as observed in present study. The beneficial effects of NSO on memory is most likely due to its antioxidant properties which may counteract with memory impairments and neurodegeneration and this enhancement in memory could be due to anticholinesterase properties of NSO (Yassin, 2005). *Nigella sativa* L. oil has bioactive compounds such as thymoquinone which attribute to the learning and memory (Salehi *et al.,* 2012). Thymoquinone and thymohydroquinone usually present in the form of glycosidically bound aglycones which can easily cross the BBB and showed neuroprotective effects (Jukic *et al.,* 2007). On the other hand, thymol and carvacrol also activated amyloid β (Aβ) in dementia and induced cognitive improvement (Azizi *et al.,* 2012). Previous study has also been reported that AChE inhibitory activity of NSO is due to its active component thymoquinone, thymohydroquinone, thymol and carvacrol (Toktam *et al.,* 2011). Studies demonstrated that NSO and its active components inhibit the activity of enzyme AChE thus retaining the effect of ACh in encoding the new memories (Hasselmo, 2006). It is also reported that NSO enhance the effect of donepezil, an inhibitor of AChE (El-Marasy *et al.,* 2012). The memory enhancing effects in rats in present study reflects the cholinergic modulation and oxidative stress alleviation by *Nigella Sativa* L. oil. However, additional studies are required to understand the role of active components of *Nigella Sativa* in memory regulation.

CONCLUSION

In conclusion present study suggested that enhancement in memory functions following repeated administration of NSO in restrained rats is due to its antioxidant potential and the inhibitory effect on acetylcholinesterase activity in rat brain.

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