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RESEARCH ARTICLE

Nigella sativa Oil Ameliorates Chronic Ethanol Induced Anxiety and Impaired Spatial Memory by Modulating Noradrenaline Levels

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ABSTRACT

Nigella sativa, one of the medicinal plants, is gaining global recognition. The plant has been proven to reduce mental illness and improve behavior. For instance, *Nigella sativa* reduces anxiety, improves spatial memory and increases motor activities. Ethanol is a commonly available and widely used addictive substance. Chronic ethanol consumption negatively affects cognition, exerts anxiogenic effects. Ethanol administration may induce behavioral deficits by modulating noradrenaline level. Here we hypothesized that *Nigella sativa* can ameliorate some of the negative health effects of ethanol consumption. To test this hypothesis, we investigated the effect of *Nigella sativa* oil on spatial learning and memory (Morris water maze test), anxiety (Elevated plus maze test) and noradrenaline levels in prefrontal cortex and hippocampus by HPLC-EC of male Wistar rats drinking water or ethanol. Results showed reduced anxiety, improved memory and increased prefrontal and hippocampal noradrenalin levels in ethanolic rats that were treated with *Nigella sativa* oil. *Nigella sativa* oil treated water drinking rats showed reduced noradrenaline levels in hippocampus. In conclusion, *Nigella sativa* oil supplementation can be beneficial to improve memory, reduce the anxiogenic effects of chronic ethanol consumption.

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INTRODUCTION

The use of plants and herbs for disease prevention and treatment of various disease is gaining attention because of the safety and inexpensiveness. Among herbal medicinal plants, *Nigella sativa* is gaining global recognition (Juma and Hayfaa, 2011).

Nigella sativa, a medicinal plant, belongs to the family Ranunculaceae, commonly known as black cumin, nutmeg or fennel flower (Qidwai *et al*., 2009). It is native to North Africa, Southern Europe, and Asia Minor, whereas is also widely cultivated in Pakistan and India. There are reports that *Nigella sativa* possess many therapeutics properties. For example, in vitro, in vivo and preclinical studies have proven that *Nigella sativa* has anti-inflammatory (Ikhsan *et al*., 2018), cytoprotective, neuroprotective (Azali Sahak *et al*., 2016), antitumor (Agbaria *et al*., 2015), antimicrobial and immunopotentiation and antioxidative effects (Bordoni *et al*., 2019). In addition, *Nigella sativa* is useful in the treatment of phenotypes that may have gone away in mental illnesses. For instance, *Nigella sativa* reduces anxiety-like behavior (Ajao *et al*., 2016) and enhances spatial memory in rats (Farimah *et al*., 2016). *Nigella sativa* has also been reported to Increase motor activity (Ajao *et al*., 2016).

According to World Health Organization (WHO) reports, more than 2 billion people are using addictive substances. Furthermore, addiction is placed within the top 10 of most burdensome disorders. It costs the society 179 billion Euros annually. Alcohol/ethanol is among most commonly used beverages in many parts of the world, despite of the risks of adverse effects on health. Each year, 3 million people die worldwide due to harmful effects of alcohol use, and this death ratio is equal to 5.3% of all deaths (WHO, 2018). Ethanol has a major influence on brain functioning, leading to behavioral and cognitive

impairments. There are reports that chronic ethanol consumption is associated with impaired memory. Ethanol use, and anxiety are often connected. In most individuals, ethanol use increases anxiety, as has also been observed in preclinical studies (Homes *et al*., 2012). Ethanol administration may interact with psychological stress to affect noradrenaline signaling. Continuous ethanol consumption decreases brain noradrenaline and reduces the activity of the enzyme dopamine beta-hydroxylase responsible for noradrenaline synthesis in rat hippocampus. Such changes in noradrenaline levels may mediate the ethanol-induced increases in anxiety (Devoto *et al*., 2015).

Given the studies reviewed above, we hypothesized that *Nigella sativa* oil can prevent the adverse effects of ethanol on anxiety and spatial learning and memory. To test this hypothesis, we determined the effects of *Nigella sativa* oil on anxiety, memory and noradrenaline levels in prefrontal cortex and hippocampus of water and ethanol drinking rats with and without *Nigella sativa* oil treatment.

MATERIALS AND METHODS

Animals: Wistar male rats, weighing about 150 g were supplied by the Animal Research Facility, Dr. Panjwani Center for Molecular Medicine and Drug Research. The animals were housed individually under controlled temperature (22±2˚C), humidity (55±10%) and in 12 hours light and 12 hours dark cycle. Animals were acclimatized for 7 days before the experiment started and had free access to water and standard chow during the entire study period. The study protocol design was based on the international guidelines and rules for care and use of animals in research included in the National Institute of Health Guidelines for Care and Use of Laboratory Animals. This study and the experiments were approved by the Institutional Animal Care and Use committee, International Center for chemical and Biological Sciences, University of Karachi.

Chemicals and drugs: Mature seeds of *Nigella sativa* were purchased from a local herbal store of Sukkur. Seeds were of similar growing season. *Nigella sativa* oil was obtained by seed pressing procedure described by the Al-okbi *et al*., 2013. Absolute ethanol was purchased from Merck. It was diluted with tap water to make 5 and 10% (*v/v*) ethanol. Noradrenaline (NA) and sodium octyl sulfate (SOS) were procured from Sigma (St. Louis, Mo, USA).

Experimental protocol: Male Wistar rats were divided into 4 groups (n=6) (i) Water + Water (WW), (ii) Water + *Nigella sativa* oil (WNSO), (iii) Ethanol + Water (EW) and (iv) Ethanol + *Nigella sativa* oil (ENSO). For 26 days, the animals in water drinking group were supplied with tap water and the animals in ethanol drinking group were exposed to 10% ethanol. Water and ethanol solution in bottles were added and changed when needed. From 17th day NSO treatment of group 2 and 4 was started. The Water drinking + *Nigella sativa* oil and ethanol drinking + *Nigella sativa* oil groups were administered with *Nigella sativa* oil at dose of 0.5 ml/kg by oral gavage for 10 days (day 17 to 26) and water drinking + water and ethanol drinking + water groups received same dose of tap water by same route from 10:00 to 10:30 h. Behavioral tests were performed at 11:00 h onwards. The elevated plus maze was performed on day 25. Morris water maze test were performed on 26. On day 26, animals were decapitated, and brains were immediately dissected. The samples were stored at -80˚C. All the experiments were conducted during standard light and dark cycle and testing was done from 9am to 3 pm.

Elevated plus maze: The Elevated plus maze (EPM) test was used to find out the anxiolytic effects of *Nigella sativa* oil in water and ethanol treated rats as previously described (Cheema *et al*., 2016). The EPM consisted of 4 arms (16 x 5 cm), two open arms and two closed arms placed above (60 cm) the ground. Animals were placed in the center of the maze facing the open arm and left in it for 5 minutes. The number of entries and time spent in an open arm were recorded.

Morris water maze test: Spatial memory was evaluated by an apparatus named Water Maze. Apparatus was consisted of a circular pool of (90cm diameter, 37 cm height) and a platform of 10x10cm. Water maze test was performed as described previously (Cheema *et al*., 2016). Pool was filled with opaque water (30cm depth) and platform was submerged (2cm) in water dedicated to fixed direction. The maze was given 4 directions or quadrants named North (N), South (S), East (E) and West (W). Animals underwent trial session in which they were trained to escape hidden platform from three directions (except the platform quadrant) followed by acquisition after 2 hours of last trial to evaluate short term memory (STM). Retention and long-term memory (LTM) were checked on next day. Escape Latency time was recorded to assess the acquisition and retention results.

Neurochemistry: Noradrenaline levels in prefrontal cortex and hippocampus were measured by high performance liquid chromatography with electrochemical detector (HPLC-EC). Briefly, the prefrontal cortex and hippocampus tissues were homogenized in 5 volume of extraction medium (0.1% sodium metabisulphite with 0.4M perchloric acid, 0.01% cystein and 0.01% EDTA). The homogenate was centrifuged for 15 minutes (4°C) at the speed of 12000 rpm. The supernatant was collected and was spinned down again at same speed and temperature for 5 minutes. 20µl of the supernatant and standard was filtered and auto-injected on top of the column to detect the levels by reverse phase Water Alliances e2695 HPLC system with electrochemical detector. Commercially available C18 analytical column was used as stationary phase. The mobile phase (pH: 2.9) was made up of 0.1M phosphate buffer containing 10% methanol, 0.005% EDTA and 0.023% ocetyl sodium sulphate. Separation was attained by mobile phase at the operating pressure of 1500 to 2500psi and operating potential was +0.8- +1.0 V. All the signals received from detector were recorded by computer software (Empower^{TM} 3 software by Water Alliance HPLC system).

Statistical analysis: Statistical analyses were done using the IBM SPSS software version 16. Data are presented as mean ± SD. Behavioral data was analyzed by two-way

ANOVA. Individual comparisons were made by Tukey's Post-hoc Test. A value of P<0.05 was considered significant.

RESULTS

Anxiety-like behavior

Entries in open arms: Data were analyzed by two-way ANVOVA. We found significant effects of ethanol drinking (F=111.141, df=1,20, P<0.01) and interaction effect between ethanol consumption and *Nigella sativa oil* treatment (F=32.527, df=1,20, P<0.01) on entries in the open arms. Post-hoc test analysis for group differences shows that ethanol drinking animals showed significant (P<0.01) less entries into the open arms compared to water drinking animals. The number of entries into the open arms was significantly increased (P<0.01) in ethanol drinking + *Nigella sativa* oil treated rats compared to ethanol drinking + water treated control animals. *Nigella sativa* oil treatment of water drinking animals decreased (P<0.01) the number of open arm entries as compared to water drinking + water treated animals (Fig. 1A). These data indicate that *Nigella sativa* oil ameliorates the anxiety like effects of ethanol drinking.

Time spent in the open arms: We found a significant effect of ethanol drinking (F=100.284, df=1,20, P<0.01) and interaction between ethanol consumption and *Nigella sativa* oil treatment (F=61.184, df=1,20, P<0.01) on time spent in the open arms. Post-hoc analysis for group differences showed that ethanol drinking animals spent a significant lower (P<0.01) amount of time the open arms compared to water drinking animals. *Nigella sativa* oil treatment significantly (P<0.01) enhanced the time spent the open arms in ethanol drinking rats and decreased (P<0.01) time spent in open arms in water drinking animals as compared to water treated control animals. These data indicate that *Nigella sativa* oil treatment decreased anxiety-like behavior in ethanol drinking animals (Fig. 1B).

Spatial learning and memory: Data were analyzed by two-way ANVOVA. We found a significant effect of ethanol consumption (F=209.716, df=1,20, P<0.01), *Nigella sativa oil* treatment (F=125.980, df=1,20, P<0.01) and ethanol consumption x *Nigella sativa oil* treatment (F= 116.623, df=1,20, P<0.01) on latency to reach platform in Morris water maze test. Post-hoc testing done for group differences shows that ethanol drinking animals took more time and showed increased latency time (P<0.01) to reach the platform compared to water drinking. *Nigella sativa* oil treatment of ethanol drinking animals improved the memory and latency time was reduced (P<0.01) compared to ethanol drinking + water treated animals (Fig. 2).

Noradrenaline in prefrontal cortex and Hippocampus:

Noradrenaline in Prefrontal cortex: Two-way ANOVA on noradrenaline levels in the prefrontal cortex revealed significant effects of ethanol consumption (F=131.271, df=1,20, P<0.01), *Nigella sativa* oil treatment (F=424.32, df=1,20, P<0.01) and ethanol consumption x *Nigella sativa* oil treatment (F=26.282, df=1,20, P<0.01). Post-hoc analysis shows that noradrenaline levels were significantly (P<0.01) increased in prefrontal cortex of *Nigella sativa* oil treated water and ethanol drinking rats compared to respective controls. When ethanol drinking + *Nigella sativa* oil and water drinking + *Nigella sativa* oil groups were compared, we found that this increase was more significant (P<0.01) in ethanol drinking than water dinking rats, which is showing the interaction of *Nigella sativa* oil and ethanol drinking (Fig. 3). These data indicated that *Nigella sativa* oil is capable to increase noradrenaline level in the prefrontal cortex.

Noradrenaline in the hippocampus: Data on hippocampal noradrenaline level were analyzed using 2-way ANOVA, revealing significant effects of ethanol consumption (F= 16.645, df=1,20, P<0.01), *Nigella sativa* oil treatment (F=9.107, df=1,20, P<0.01) and a significant ethanol consumption x *Nigella sativa* oil treatment interaction effect (F=33.28, df=1,20, P<0.01) on noradrenaline level in hippocampus.

Post-hoc data analysis revealed a significant (P<0.01) decrease in noradrenaline levels in the hippocampus of water drinking + *Nigella sativa* oil treated rats compared to water drinking + water treated rats. No significant difference in noradrenaline concentration was observed in ethanol drinking + *Nigella sativa* oil treated animals and ethanol drinking + water treated animals. Ethanol drinking + water treated animals displayed significant (P<0.01) lower noradrenaline levels compared to water drinking + water treated animals (Fig. 4). These data indicate that *Nigella sativa* oil can reduce noradrenaline level in the hippocampus of water drinking rats.

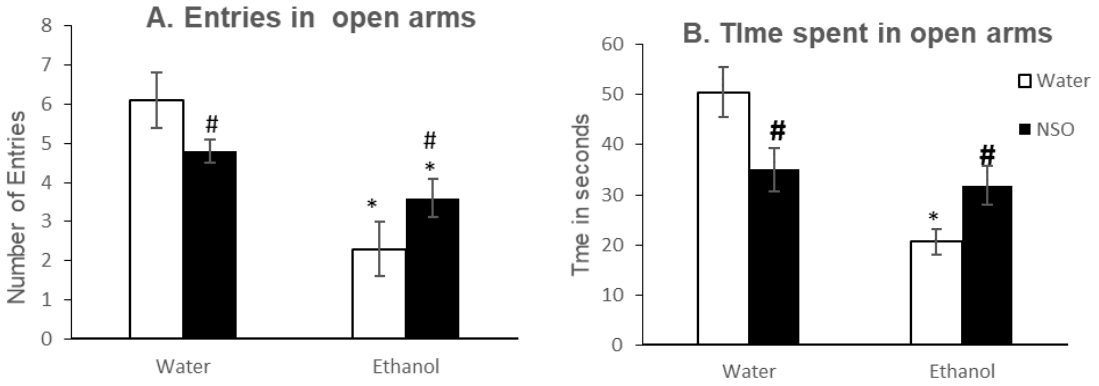

Fig. 1: Effects of *Nigella sativa* oil treatment (NSO) for 10 days of ethanol and water drinking rats on anxiety as measured in the elevated plus maze. Values represent means ± SD. (A) The number of open arm entries. *P<0.05 different from water drinking + *Nigella sativa* oil treated animals, #P<0.01 different from respective water treated controls. (B) Time spent in open arms. # P<0.01 different from water treated controls of ethanol and water drinking animals. *P<0.05 different from water drinking + *Nigella sativa* oil treated animals.

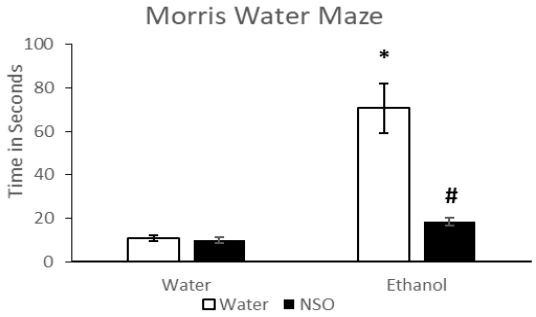

Fig. 2: Effects of *Nigella sativa* oil treatment (NSO) of ethanol and water drinking rats on memory as measured in Morris water maze test. Values represent means ± SD. #P<0.01 different from respective ethanol drinking + water treated control and *P<0.01 from respective water drinking animals.

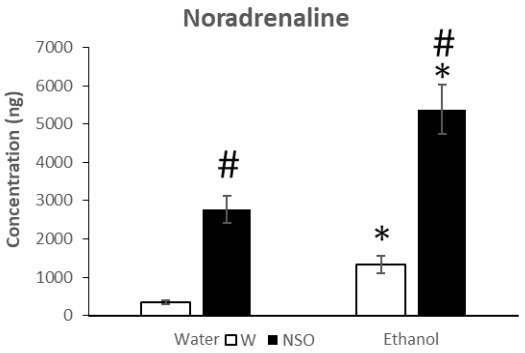

Fig. 3: Effects of ethanol consumption and *Nigella sativa* oil (NSO) 10 days treatment on noradrenaline level in the prefrontal cortex. Values represent means ± SD. #P<0.01 and *P<0.01 different from water drinking + water treated controls; #P<0.01 and *P<0.01 different from water drinking + *Nigella sativa* oil treated animals.

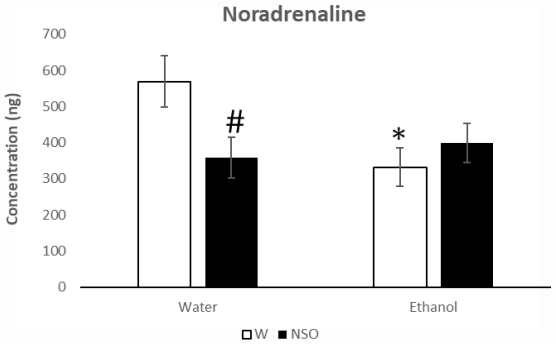

Fig. 4: Effects of ethanol consumption and 10 days of *Nigella sativa* oil (NSO) treatment on noradrenaline levels in the hippocampus. Values represent means ± SD. #P<0.01 and *P<0.01 different from water drinking + water treated controls.

DISCUSSION

This research demonstrated that *Nigella sativa* oil improves spatial memory and reduces anxiety in ethanolic rats. *Nigella sativa* oil increased noradrenaline levels in the prefrontal cortex of water and ethanol consuming rats. However, in water drinking groups *Nigella sativa* oil decreased noradrenaline levels in hippocampus. These findings show that *Nigella sativa* counteracts the anxiogenic effects of chronic ethanol exposure.

Anxiety: Ethanol drinking rats showed anxiety-like behavior in the elevated plus maze compared to water drinking controls. In line with our findings, Holmes and coworkers, (2012) also reported that in most individuals, ethanol use increases anxiety. Ajao and coworkers, (2016) reported that *Nigella sativa* oil reduces anxiety and anxiety-related behavior. Interestingly, *Nigella sativa* oil treatment of ethanol drinking animals reduced anxiety and spent more time in open arms of elevated plus maze compared to ethanol drinking controls. Many studies have demonstrated the effect of *Nigella sativa* oil on anxiety but no study to date showed the effect of *Nigella sativa* oil on anxiety induced by chronic ethanol consumption.

A previous study reported that when rats were treated with *Nigella sativa* oil at a dose of 0.4 ml/kg for 4 weeks, anxiety reduced. This was associated with an increase in activity (Parveen *et al*., 2009). In contrast, smaller doses of *Nigella sativa* oil, in the range of 0.1 and 0.25 ml/kg for 5 weeks, did not affect anxiety in rats (Cheema *et al*., 2016). In our study, the dose 0.5 mg/kg was used, and it was found that oral administration of *Nigella sativa* oil for 10 days produced significant anxiolytic effects and reduced ethanol-induced anxiety. Our results demonstrated that *Nigella sativa* oil can reduce anxiety when given at bit higher dose.

Spatial learning and memory: Ethanol has a major influence on brain functioning, leading to behavioral and cognitive impairments (Samson and Harris, 1992). In our study, ethanol resulted in impaired spatial learning and memory compared to water drinking animals. There are reports that chronic ethanol consumption is associated with impaired memory (Wixted, 2005; Staples and Mandyam, 2016). These studies are in line with our results. When animals were treated with *Nigella sativa* oil, we found that the ethanol drinking + *Nigella sativa* oil treated group showed improved memory and gave quick response in Morris water maze test which is showing the memory reformation. Many studies are in line with our results and showed that *Nigella sativa* oil enhances memory in rats (Farimah *et al.* 2016; Anaeigoudari *et al*., 2018). Our results are showing the protective effects of *Nigella sativa* oil on ethanol prompted impaired spatial memory.

Noradrenaline in prefrontal cortex and hippocampus: Neurotransmitters influence the functioning of neuroanatomic circuits that offer support to cognition, anxiety and fear. Noradrenaline is one of the best-known neurotransmitters that modulate the synaptic functions. In the central nervous system, the primary source of noradrenaline is the locus coeruleus (Sara *et al*., 1994), a structure located in the brain stem that sends noradrenergic projections to many brain regions.

Continuous ethanol exposure resulted in reduced noradrenaline in hypothalamus, midbrain and hippocampus. These results are in favor of our results where ethanol drinking rats showed decreased noradrenaline levels in the hippocampus (Ishida *et al*., 2000; Arivazhagan and Panneerselvam 2002). This reduction in noradrenaline levels may be interpreted as a manifestation of an accelerated noradrenaline turnover or an enhanced release of noradrenaline. Several clinical and scientific research have demonstrated the role of noradrenaline in a range of psychological and physiological processes including memory, learning, anxiety, sleep, adaptation and arousal (Terbeck *et al*., 2016). Continuous ethanol consumption decreases brain noradrenaline and reduces the activity of the enzyme dopamine beta-hydroxylase responsible for noradrenaline

synthesis in rat hippocampus. Such changes in noradrenaline levels may mediate the ethanol-induced increases in anxiety. In contrast to the ethanol-induced decreases in noradrenaline levels in the hippocampus, increases in noradrenaline were found in the prefrontal cortex. Prefrontal cortex possesses more dopamine-β hydroxylase varicosities relative to sensory cortical regions (Arnsten, 2015). This raises the possibility that prefrontal cortex areas might be subjected greater levels of noradrenaline in synaptic transmission, which may explain why the prefrontal cortex is sensitive to anxiety and stress (Park and Moghaddam, 2017). We furthermore found that *Nigella sativa* oil treatment reduced noradrenaline levels in the hippocampus of water drinking animals, and significantly increased noradrenaline levels in the prefrontal cortex.

By looking at the hippocampus data, it shows that ethanol reduced noradrenaline levels in the hippocampus, and that *Nigella sativa* oil tends to antagonize that in the ethanolic rats because there is no further decrease. The hippocampus has major role in integrating and processing spatial information important in formation and retrieval of memory (Hansen, 2017) and locus coeruleus projections to hippocampus has impact on learning (Wagatsuma *et al*., 2018). Noradrenaline can influence the function of hippocampus and locus coeruleus stimulation. Many studies have suggested that noradrenaline increase long-term potentiation (LTP) in hippocampus, specifically in CA1 and dentate gyrus, which is dependent upon βAR and α1 mechanisms (Harley, 2007). High level of noradrenaline enhances excitatory post synaptic potentials, reduce spike on set latency, and enhances the population spike amplitude. These effects promote long-term potentiation induction and are important for memory formation (Harley, 2007).

Park and Moghadda (2017) reported that impaired cognition is a hallmark deficit associated with anxiety. In line with this results anxiety with impaired memory was observed in ethanolic rats. *Nigella sativa* oil treatment ameliorated the ethanol induced anxiety and improved memory by increasing hippocampal and prefrontal noradrenaline. These results showed that higher level of hippocampal noradrenaline decreased anxiety and improved spatial memory by increasing the hippocampal long-term potentiation.

Conclusions: This research demonstrates that *Nigella sativa* oil can prevent the adverse effects of chronic ethanol consumption on spatial learning and memory, anxiety, noradrenaline levels in hippocampus and prefrontal cortex.

Authors contribution: BA: study conception and design, performed all experiments, collection and/or assembly of data, data analysis and interpretation, wrote whole manuscript, final approval of the manuscript; IU: helped in study conception; JH: guided in data analysis, manuscript writing and final approval of manuscript; DJH: experimental design, supervised this research and helped in manuscript writing.

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