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RESEARCH ARTICLE

Protective Effect of *Nigella Sativa* Oil on Hippocampus in Acrylamide-Induced Toxicity in Rats

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ABSTRACT

The aim of this study was to evaluate the possible protective effects of *Nigella sativa* oil against acylamide-induced toxicity in the hippocampus of rats. For this purpose, Wistar rats were divided into four groups; control (C), acrylamide (ACR), *Nigella sativa* (NS) and acrylamide+*Nigella sativa* (ANS). At the end of the experiment, the rats were sacrificed by cervical dislocation, their brains were removed and their weights were measured. Samples were fixed in 10% formaldehyde, embedded in paraffin, and stained with Kluver Barrera, May-Grünwald-Giemsa, and argyrophilic nucleolar organizer regions (AgNOR). After staining, cell counts were performed in the cornu ammonis 1 (CA1), cornu ammonis 2 (CA2), and cornu ammonis 3 (CA3) and dentate gyrus areas of the hippocampus and AgNOR parameters were evaluated. In the acrylamide-administered group, rats showed a significant decrease in body weight and brain weight ($P<0.05$), as well as fatigue and gait abnormalities were observed. Besides, acrylamide caused a significant decrease ($P<0.05$) in the number of neurons in the hippocampus. Contrary to this, *Nigella sativa* oil supplementation resulted in a significant increase in the number of neurons in the hippocampus ($P<0.05$), and improved gait abnormalities without affecting body weight and brain weight. It was determined that the relative AgNOR area significantly decreased ($P<0.05$) in the CA1 and dentate gyrus regions in the ACR group, and significantly increased ($P<0.05$) in the ANS group compared to the ACR group. In the study, it was determined that acrylamide has a neurotoxic effect and *Nigella sativa* oil can affect the reduction of the resulting neurotoxic effect.

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INTRODUCTION

The hippocampus is responsible for the formation of memory in particular for shaping short-term memory (Aminu *et al*., 2019). Also, the hippocampus is one of the regions where neurogenesis occurs (Park *et al*., 2010). The brain is more susceptible to oxidative stress. Oxidative stress directly causes tissue damage and increases inflammatory and proapoptotic cascades. Also, increased reactive oxygen species residues can cause cognitive dysfunction (Zhao *et al*., 2022). Environmental toxins can cause oxidative stress. Acrylamide, an environmental toxin, is also a source of oxidative stress.

Acrylamide is used in laboratories, paper making, petrochemistry, water treatment, textile manufacturing, personal care and grooming products. In addition, acrylamide occurs naturally when foods rich in carbohydrates are cooked at high temperatures. Exposure to acrylamide causes toxic effects such as genotoxicity, neurotoxicity, and carcinogenicity (Rifai and Saleh, 2020; Hatipoğlu *et al*., 2023). Neurological symptoms such as tremors, weakness, numbness, tingling in the limbs, or ataxia are observed in animals exposed to acrylamide (Kopańska *et al*., 2022). Additionally; it has been reported that degeneration occurs in peripheral nerves and nerve endings in some brain regions (cerebral cortex, hypothalamus, and hippocampus) related to memory, learning, and cognitive functions after exposure to acrylamide (Rifai and Saleh, 2020). The possible toxic effect of acrylamide may be caused by oxidative stress. Various studies, aimed to reverse the negative effects of acrylamide by using antioxidant substances. *Nigella sativa* is a medical plant that is being used as an herbal medicine in the treatment of various diseases because it has many pharmacological effects such as neuroprotection, antioxidant, and antiapoptotic properties (Mehri *et al*., 2014). The aims of the study were to investigate the potential neurotoxic effects of acrylamide on the hippocampus and to determine the effects of Nigella sativa oil, specifically whether it has positive or negative

effects. Additionally, the study aimed to explore whether administering *Nigella sativa* oil alongside acrylamide could prevent any potential negative effects of acrylamide on the hippocampus.

MATERIALS AND METHODS

Ethical approval: This study was approved by the Animal Experiments Ethics Committee of Selçuk University Experimental Medicine Application and Research Center with reference number 2020-53 dated 30.11.2020.

Experimental design: Eight-wk-old male Wistar rats (*n*=32) were divided into four groups (n=8), the control (C), acrylamide (ACR), *Nigella sativa* (NS), and acrylamide +*Nigella sativa* (ANS) group. All rats were fed a standard rat diet and water *ad-libitum*. During the experiment (15 days), rats in the ACR group were given 40 mg/kg acrylamide (Sigma, Germany) by gavage. The rats in the *NS group* were given 1 ml/kg of *Nigella sativa* oil (Botalife, Türkiye) via gavage. For the rats in the ANS group, both acrylamide and *Nigella sativa* oil were given via gavage.

Body weight analysis: The weights of the rats were measured at the beginning of the experiment, on the 5th day, on the 10th day, and on the last day of the experiment.

Histomorphometric studies: At the end of the experiment, the rats were sacrificed by cervical dislocation, their brains were removed and their weights were measured. The brain tissues were fixed in 10% formaldehyde, processed under routine histological procedures, and embedded in paraffin. Sections of 6 µm thickness were taken from all paraffin blocks. Paraffin sections were stained with Kluver Barrera, May-Grünwald-Giemsa, and AgNOR.

For Kluver Barrera staining, paraffin sections were deparaffinized and brought to water then stained in Luxol fast blue (Sigma, Germany) solution overnight at 37ºC. The sections were differentiated using 0.05% lithium carbonate (Merck, Germany) for 20 sec then stained with 0.1% cresyl violet (Merck, Germany) for 10 min at room temperature. Stained sections were dehydrated in increasing serial ethanol, cleared in xylene, and mounted with entellan (Culling *et al*., 1985).

For May-Grünwald-Giemsa staining, paraffin sections were deparaffinized and brought to water. Sections were stained in May-Grünwald solution (Merck, Germany) for 20 min and then washed in distilled water. After that sections were stained in Giemsa solution (one droplet Giemsa in one ml distilled water) (Merck, Germany) for 5 min. Stained sections were dehydrated in increasing serial ethanol, cleared in xylene, and mounted with entellan (Culling *et al*., 1985).

For AgNOR staining, paraffin sections were deparaffinized and brought to water. Sections were stained in AgNOR solution for 30 min at 37ºC. AgNOR solution: one volume of 1% gelatine (Sigma, USA) in 1% formic acid (Merck, Germany) solution and two volume of 50% silver nitrate solution were mixed. Stained sections were washed in distilled water, dehydrated in increasing serial ethanol, cleared in xylene, and mounted with entellan (Sur *et al*., 2011).

Cell counting and image analysis: Cell counts were performed in the CA1, CA2, and CA3 areas of the hippocampus at 100X magnification in each section with Kluver Barrera staining from three different regions in each area. After the May-Grünwald-Giemsa staining procedure, cell counts were performed from the dentate gyrus area of the hippocampus at 100X magnification in three different regions. After AgNOR staining, 25 cells were evaluated in the CA1, CA2, CA3 and dentate gyrus areas of the hippocampus in each slide. Nuclear area, AgNOR number, and AgNOR area measurements were performed in each cell and the relative (%) AgNOR area was determined. The slides were examined with a Leica DM2500 (Switzerland) model light microscope with a Leica DFC-320 camera attachment. Measurements were performed using the LAS (Leica Application Suite, Switzerland) image analysis program and the images of the required regions were recorded.

Statistical Analysis: Statistical analysis of the obtained data was performed using analysis of variance (ANOVA) and then Duncan's post hoc analysis to determine the differences between groups. Statistical analyzes were performed using IBM SPSS v22.0 package program.

RESULTS

Body weight analysis and gait abnormalities: Considering the body weight measurements of the rats every five days throughout the experiment. The rat body weights decreased in the ACR group, and this decrease was statistically significant ($P<0.05$). It was observed that the body weights of the rats in the NS group increased similarly to the C group. It was determined that the body weight of the rats in the ANS group decreased, and this decrease was statistically significant when compared to the C group ($P<0.05$). The data obtained regarding the body weights are presented in Table 1.

While it was observed that there was no problem in the C group and NS groups in the rats whose general health status was monitored throughout the experiment, it was observed that the rats in the ANS group were sluggish and were showing difficulty in walking. In the rats in the ACR group, walking difficulties, weakness, and hindleg paralysis were observed.

Histomorphometry of brain: Brain weight decreased in the ACR group compared to the C group. This decrease was statistically significant ($P<0.05$). Brain weight decreased in the ACR group compared to the C group. These decreases were found to be statistically significant ($P<0.05$). An increase in rat brain weight was observed in the NS group compared to the control group, but this increase was not statistically significant ($P>0.05$). The data obtained regarding the brain weights are presented in the Table 2.

Cell count analysis: Regarding the presence of neurons, it was determined that the darkly stained neurons were quite frequent in CA1, CA2, CA3, and dentate gyrus regions of the hippocampus in the ACR group whereas the same neurons were rarely observed in the ANS group.

Table 1: Body weight (g) measurements of rats.

BW/Days	Groups			
	C	ACR	NS	ANS
Day 1st	364,00±7,48	361,00±7,37	349,00±9,92	358,66±8,51
Day 5th	380,33±8,28^{a}	325,33±8,35^{b}	358,66±10,91^{ac}	333,66±10,71^{bc}
Day 10th	401,33±8,55^{a}	303,00±13,41^{b}	376,00±9,98^{a}	295,66±12,10^{b}
Day 15th	414,33±8,05^{a}	241,00±12,94^{b}	392,66±10,04^{a}	246,66±11,43^{b}

The body weights (g) of rats are presented as Mean±SEM. Different superscripts ^{a,b,c} represent the statistical difference in the mean value with P<0.05.

Table 2: Brain weights (g) measurements of rats.

	Groups			
Brain Weight	C	ACR	NS	ANS
	2,87±0,03^{a}	2,60±0,06^{b}	2,91±0,03^{a}	2,58±0,18^{b}

The brain weights (g) of rats are presented as Mean±SEM. Different superscripts ^{a,b} represent the statistical difference in the mean value with P<0.05.

Table 3: AgNOR parameters in neurons in CA1, CA2, CA3, and dentate gyrus regions of the hippocampus.

		Groups			
		C	ACR	NS	ANS
CA1	AgNOR Number	2,53±0,10^{a}	1,94 ±0,08^{b}	2,54±0,11^{a}	2,52±0,11^{a}
	AgNOR Area (µm2)	5,33±0,13^{a}	3,71±0,09^{b}	5,38±0,23^{a}	4,93±0,21^{a}
	Nucleus Area (µm2)	64,30±1,70	63,72±1,46	64,53±1,45	64,26±1,29
	Relative (%) AgNOR Area	9,11±0,54^{a}	6,46±0,26^{b}	9,10±0,47^{a}	8,57±0,52^{a}
CA2	AgNOR Number	2,75±0,11^{a}	2,18±0,11^{b}	2,62±0,12^{a}	2,10±0,10^{b}
	AgNOR Area (µm2)	5,48±0,15^{a}	3,76±0,13^{b}	5,52±0,17^{a}	5,14±0,16^{a}
	Nucleus Area (µm2)	69,81±1,63	67,15±2,27	70,18±1,29	69,44±1,30
	Relative (%) AgNOR Area	8,25±0,24	7,53±0,58	8,48±0,38	7,90±0,36
CA3	AgNOR Number	3,00±0,11^{a}	2,28±0,08^{b}	2,46±0,12^{b}	2,09±0,10^{b}
	AgNOR Area (µm2)	7,86±0,32^{ab}	7,46±0,24^{b}	7,65±0,12^{ab}	8,25±0,23^{a}
	Nucleus Area (µm2)	78,84±2,56	76,05±1,88	79,26±2,92	76,66±1,06
	Relative (%) AgNOR Area	12,32±0,79	10,72±0,44	12,32±0,72	11,05±0,35
Dentate gyrus	AgNOR Number	2,32±0,09^{a}	1,75±0,07^{b}	2,34±0,10^{a}	2,03±0,10^{c}
	AgNOR Area (µm2)	4,20±0,15^{a}	2,89±0,07^{b}	4,37±0,18^{a}	4,06±0,21^{a}
	Nucleus Area (µm2)	35,65±0,89	35,51±0,81	35,95±0,58	35,56±0,72
	Relative (%) AgNOR Area	12,50±0,48^{a}	8,76±0,29^{b}	12,57±0,57^{a}	12,16±0,72^{a}

AgNOR: argyrophilic nucleolar organizer region. Relative AgNOR Area: Ratio of AgNOR area to nucleus area (%). AgNOR parameters are presented as Mean±SEM. Different superscripts ^{a,b} represent the statistical difference in the mean value with P<0.05.

The C group indicated a similar morphology and trend. Cell counts were performed in the CA1, CA2, CA3, and dentate gyrus regions of the hippocampus. Cell count decreased in all regions in the ACR group.

These decreases were found to be statistically significant (P<0.05). It was seen that the NS group was similar to the C group in all regions and there was no statistical difference (P>0.05). It was determined that the cell numbers in the ANS group expressed a significant increase in all regions compared to the ACR group (P<0.05). It was determined that the cell numbers in the ANS group were similar to the C group and there was no statistical difference (P>0.05) (Fig. 1 and 2).

The data obtained regarding the AgNOR parameters is presented in Table 3. It has been observed that acrylamide causes a statistically significant decrease in the relative AgNOR area in the neurons of the CA1 and dentate gyrus regions (P<0.05) (Fig. 3).

DISCUSSION

Gait abnormality is considered a classic morphological feature of acrylamide-induced neurotoxicity and represents a relatively sensitive measure of the onset and progression of neurological changes (Zhao *et al*., 2022). It has been shown that treatment of high doses of acrylamide (50 mg/kg) results in hindlimb paralysis and walking difficulties, which are typical features of acrylamide-mediated neurotoxicity (Park *et al*., 2010, Zhao *et al*., 2022). It was observed that acrylamide treatment caused walking difficulty, weakness, and hind leg paralysis in rats.

With acrylamide treatment, average body weight can change. Lai *et al*. (2017) reported that acrylamide decreased significantly the body weight. Another study showed that acrylamide negatively affected the growth of mice, and the average body weight of mice decreased with medium and high-dose acrylamide treatment (Zhao *et al*., 2022). Asiaei *et al*. (2017) reported that *Nigella sativa* did not affect body weight. It was reported that the BW, which decreases with oxidative stress, didn't change by treatment of thymoquinone (Sheikh and Mohamadin, 2012). Consistent with the literature data in this study, 40 mg/kg acrylamide treatment caused a significant reduction in the body weight of rats. It was observed that *Nigella sativa* oil did not cause any change in rat body weight.

It was reported that the treatment of 30 mg/kg acrylamide caused a significant decrease in brain weight in rats (Sharma *et al*., 2020). It was reported that the brain weights of rats were increased by 1 ml/kg *Nigella sativa* oil treatment for 15 days (Aminu *et al*., 2019). Similarly, it has been shown that 1 ml/kg of Nigella sativa oil treatment increases relative brain weight (Folarin *et al*., 2020). It was reported that the brain weights, which decrease with oxidative stress, were increased by the treatment of thymoquinone (Sheikh and Mohamadin, 2012). In this study, it was determined that the brain weight decreased statistically significantly after acrylamide treatment, similar to the literature data. It was observed that *Nigella sativa* oil caused an increase in brain weight, but this increase was not statistically significant. Unlike the literature data, the fact that Nigella sativa oil did not cause a significant increase in brain weight was evaluated depending on the dose used.

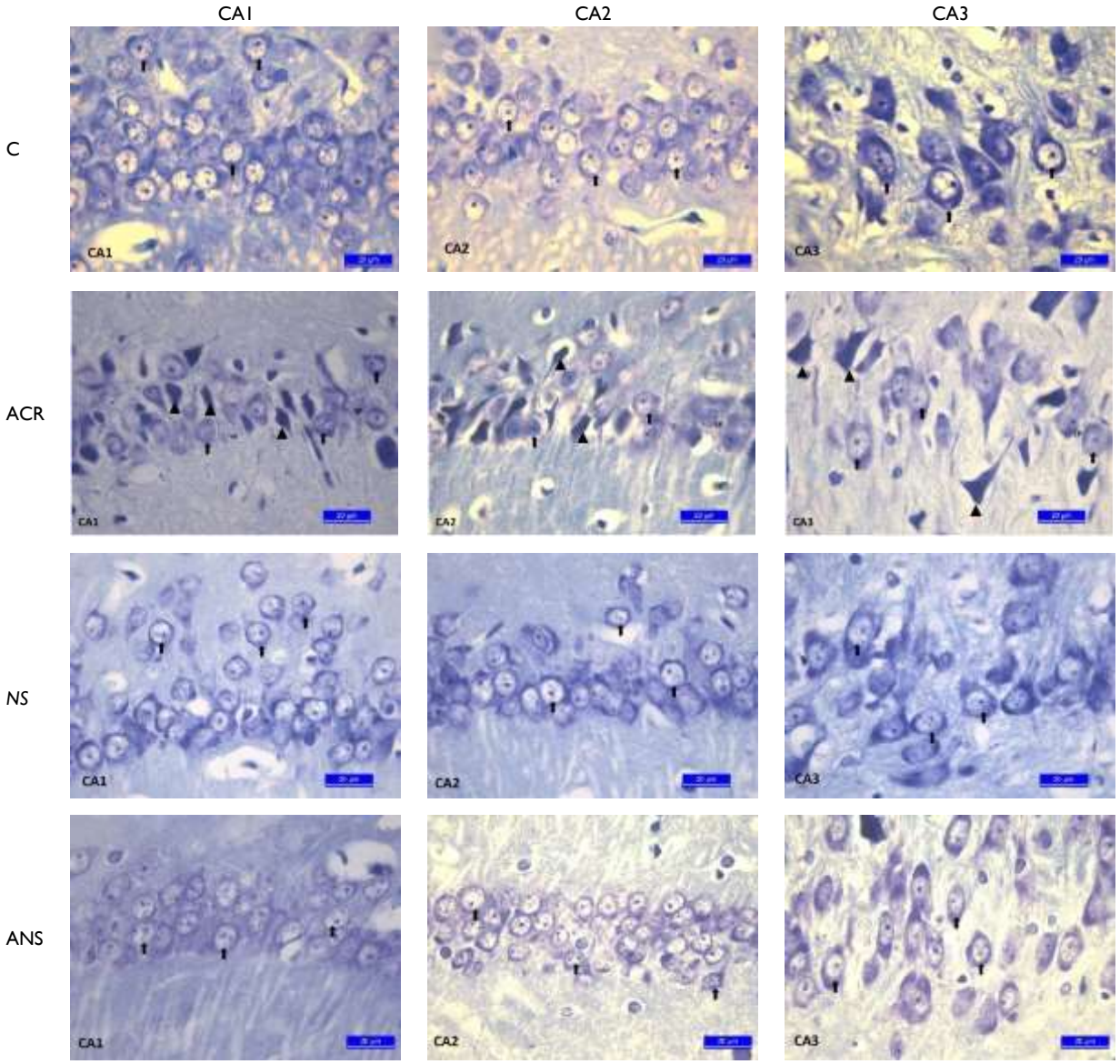

Fig. 1: Protective effect of *Nigella sativa* oil against acrylamide-mediated darkly stained neurons in CA1, CA2, and CA3 regions of the hippocampus in rats. Arrows show healthy neurons. Arrowheads show darkly stained neurons. Kluver barrera staining. Bar: 20 µm.

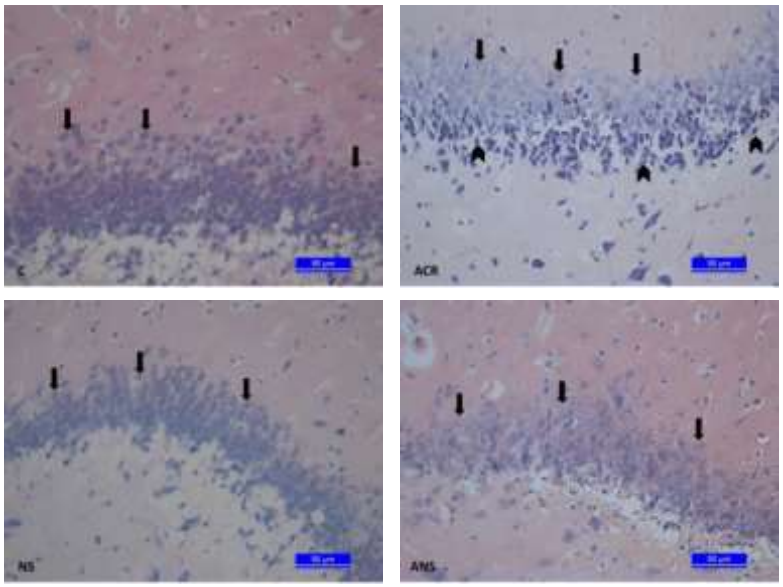

Fig. 2: Protective effect of *Nigella sativa* oil against acrylamide-mediated darkly stained neurons in the dentate gyrus region of the hippocampus in rats. Arrows show healthy neurons. Arrowheads show darkly stained neurons. May-Grünwald-Giemsa staining. Bar: 50 µm.

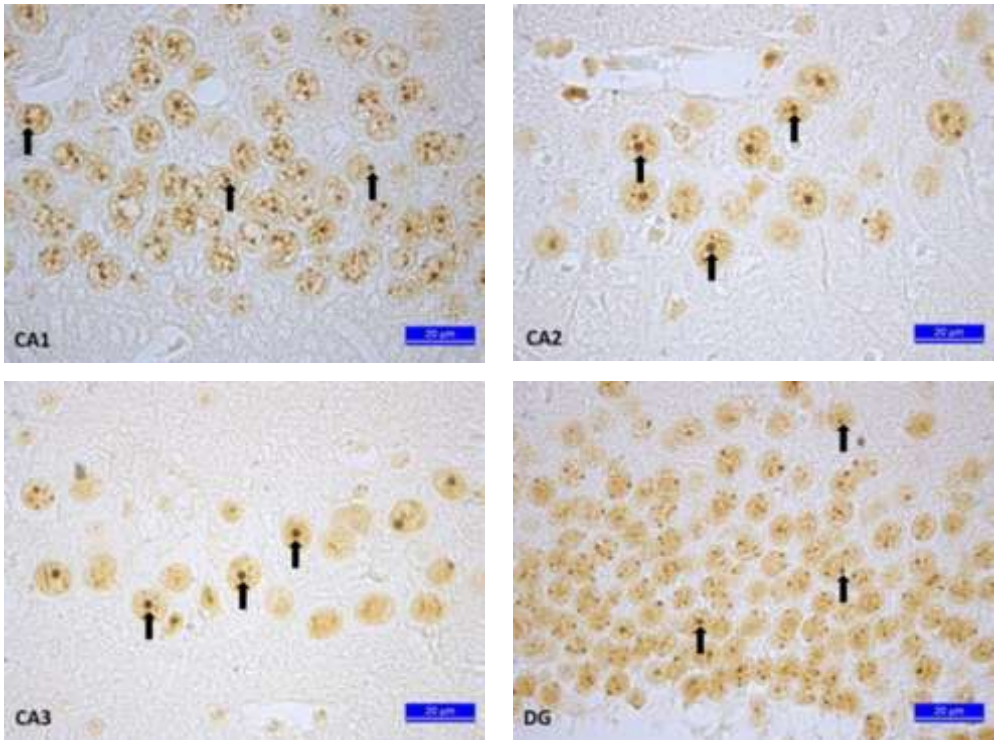

Fig. 3: AgNOR regions in the nucleus of neurons in CA1, CA2, CA3, and dentate gyrus region of Hippocampus. AgNOR regions are seen as black dots in the nucleus of neurons. Arrows show AgNOR regions in the nucleus of neurons. CA: Cornu ammonis. DG: dentate gyrus. AgNOR staining. Bar: 20 µm.

A study by Farouk *et al*. (2021) reported that after 25 mg/kg acrylamide treatment for 21 days, darkly stained shrunken neuron structures with pycnotic nuclei in the hippocampus were observed, unlike the control group. Nassar *et al*. (2022) observed a decrease in pyramidal cell layer thickness and the presence of degenerated neuron structures in the hippocampus after treatment of 50 mg/kg acrylamide in rats for eight wk. An increase in free radicals may cause this tissue damage from acrylamide treatment. Studies were shown that *Nigella sativa* and thymoquinone reverse neuronal damage against neurodegenerative changes and have a protective effect (Al-Majed *et al*., 2006; Gülşen *et al*., 2016; Nassar *et al*., 2022). In this study, in accordance with the literature data, while degeneration was observed in neurons in the hippocampus in the acrylamide group, it was observed that *Nigella sativa* reversed this negative effect.

One of the neurotoxic effects caused by acrylamide is neuronal loss. In a study, it was shown that neuron loss occurs after oral acrylamide treatment in mice (Zhao *et al*., 2022). Guo *et al*. (2020) observed severe neuronal losses in the hippocampus when they treated 40 mg/kg of acrylamide for four weeks. These neuronal losses are also observed in Alzheimer's disease. Poorgholam *et al*. (2018) reported a neuronal loss in CA1 regions in a rat model of Alzheimer's disease. It has been reported that *Nigella sativa* causes morphological improvement in neurodegeneration in the hippocampus (Kanter, 2008). In another study, it was revealed that *Nigella sativa* reduces the neurodegenerative effect and increases the number of neurons (Hobbenaghi *et al*., 2014). It has been reported that Nigella sativa and thymoquinone increase neuronal density, reduce apoptosis and restore neuronal integrity and functions by preventing inflammatory processes (Gülşen *et al*., 2016). Al-Majed *et al*. (2006) reported that ischemia causes dead neuronal cells in the hippocampus and thymoquinone reduces the number of dead cells and causes an increase in the number of intact neurons. In another study by Poorgholam *et al*. (2018), it was revealed that thymoquinone increase surviving pyramidal cells in the hippocampus in a rat model of Alzheimer's disease. In this study, the number of neurons in the CA1, CA2, and CA3 areas of the hippocampus in the ACR group was statistically significantly decreased compared to the C group, similar to the literature data. The number of neurons in the NS group and ANS group was similar to the C group, and *Nigella sativa* oil reduced the neurotoxic effect caused by acrylamide and increased the number of neurons.

While it was believed that nerve cells were produced only in the prenatal period, it is now known that neurogenesis continues in two different regions of the brain. The formation of new neurons throughout life in adult mammals generally occurs in the subventricular region and in the dentate gyrus region. While neurogenesis may decrease with aging, it can be increased through physical exercise, environmental and metabolic stimuli (Park *et al*., 2010). Park *et al*. (2010) reported that there were fewer newly produced cells in the acrylamide-treated group compared to the control group. Since, acrylamide inhibits the proliferation of neurons in the dentate gyrus, it was reported to impair adult hippocampal neurogenesis (Park *et al*., 2010). In a study examining the toxic effect of acrylamide on weaning rats, Lai *et al*. (2017) reported that the layer's thickness and the number of cells in the dentate gyrus decreased after acrylamide treatment. In another study, Lee *et al*. (2018) similarly showed that acrylamide affects neurogenesis, and that high-dose (50 mg/kg) acrylamide treatment for two weeks reduces the number of cells in the dentate gyrus in mice. It

was reported that *Nigella sativa*, which is used for treatment against the neurotoxic effect of acrylamide, can inhibit cell death stimulation in neurons by increasing the antioxidant defense system against neurotoxicity (AbdulAzeez *et al*., 2020). In another study, it was reported that *Nigella sativa* increased neurogenesis in the dentate gyrus (Brito *et al*., 2015). Beker *et al*. (2018) reported that thymoquinone increases neurogenesis in the healthy hippocampus. In this study, it was observed that the number of neurons in the dentate gyrus decreased in rats treated with acrylamide, similar to the literature data. It was noted that there was no statistical difference in the number of cells in the NS group, which was similar to the C group. The number of neurons in the ANS group increased statistically significantly compared to the ACR group.

Subunits of ribosomes are synthesized in the nucleolus region of the Nucleus. The gene regions where ribosomal RNA is synthesized are located in the nucleolus region and form the dark regions. These gene regions on the DNA where ribosomal RNA is synthesized and forms the nucleolus are called nucleolus organizer regions (NOR). Since these regions are argyrophilic, they can be easily stained with silver dyes, appearing as black dots (AgNOR) in the cell nucleus. The size, number, and distribution of AgNORs are considered an indicator of the transcriptional activity of the cell and are suggested to reflect nucleolar activity (Sur *et al.*, 2011). Although, AgNORs have long been used as an indicator of proliferative activity. As a result of the emergence of immunohistochemical markers such as proliferating cell nuclear antigen (PCNA) and Ki-67 the use of AgNORs as proliferative markers has declined. Contrary to immunohistochemical markers, AgNORs can be preferred because they are less affected by fixation, low cost, and ease of use (Sorkun *et al*., 2009). In a study, it has been reported that the number and areas of AgNOR are less in all areas of the hippocampus in the case of ischemia (Mennel and Müller 1994). Ogawa *et al*. (2012) reported a decrease in the number of PCNA-positive cells in the dentate gyrus of acrylamide. Similarly, in another study, the number of Ki-67 positive cells was significantly reduced in high-dose acrylamide treatment compared to the control group (Yu *et al*., 2019). Studies evaluating Ki-67 positive cell counts have reported that *Nigella sativa* oil improves cell proliferation (Aminu *et al*., 2019). AgNOR parameters were evaluated to determine proliferation in this study. The relative AgNOR area was statistically decreased in CA1 and dentate gyrus regions in the ACR group. It was determined that the relative AgNOR area increased in the ANS group compared to the ACR group and it was similar to the C group. It was observed that the obtained acrylamide reduces cell proliferation, and *Nigella sativa* oil improves this situation.

Conclusions: The results suggest that *Nigella sativa* oil may have a protective effect on the hippocampus against acrylamide-induced damage. This implies that incorporating Nigella sativa oil into the diet or using it as a supplement might help reduce the detrimental effects of acrylamide on the hippocampus. However, it is important to note that further research is needed to determine the specific mechanisms by which *Nigella sativa* oil exerts its neuroprotective effects.

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Authors contribution: IU and HHD designed this project. IU performed laboratory studies and experiments in this study. HHD analyzed the data. All authors interpreted the data critically revised the manuscript for important intellectual contents and approved the final version.

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