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Original article

Quercetin Mitigates Acetamiprid-Induced Memory Impairment and Neuronal Damage in Rats

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Abstract

Background and objectives: Acetamiprid, a widely used neonicotinoid pesticide in agriculture, acts as a stimulant on nicotinic acetylcholine receptors, potentially causing neurotoxicity. Quercetin, a neuroprotective flavonoid found in fruits and vegetables, shows promise in mitigating neurological disorders. This study investigated quercetin's protective potential against acetamiprid-induced memory impairment. **Methods**: Male rats were divided into four groups: control, acetamiprid (40 mg/kg), quercetin (20 mg/kg), and a combination of acetamiprid and quercetin, administered orally for 28 days. Cognitive performance was assessed using the Morris water maze test; oxidative stress markers in the hippocampus were evaluated, along with histological analysis. **Results:** Rats exposed to 40 mg/kg acetamiprid exhibited significant memory impairment. Notably, co-treatment with quercetin reversed this effect. Acetamiprid induced oxidative stress, as indicated by increased lipid peroxidation, reduced thiol content, and decreased catalase (CAT) enzyme activity. Simultaneous quercetin and acetamiprid administration effectively mitigated these oxidative stress markers. Histological analysis demonstrated quercetin's ability to prevent acetamiprid-induced hippocampal neuronal damage. **Conclusion**: Quercetin shows promise in ameliorating acetamiprid-induced memory deficits and neuronal damage, making it a potentially valuable nutraceutical, especially for individuals exposed to pesticides like agricultural workers.

Keywords: memory; neonicotinoids; oxidative stress; quercetin

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Introduction

Neonicotinoids, with a systemic mechanism of action, constitute the sole significant category of insecticides created within the past thirty years. These compounds are readily absorbed and act rapidly on piercing-sucking pests such as aphids, leafhoppers, and whiteflies. Because of their similarity in structure to nicotine, they act as activators of the nicotinic acetylcholine receptors (nAChRs) with a greater affinity and specificity for insect's nAChRs compared to those in mammals [1,2]. While acute toxicity effects primarily stem from their actions on nAChRs in the brain , chronic exposure is associated with multiple organ toxicity, including hepatotoxicity, nephrotoxicity, neurotoxicity (leading to developmental and cognitive disorders), as well as changes in hematologic parameters [3]. These compounds are absorbed and distributed to various organs, such as the spleen, heart, bones, lungs, adrenal gland, peripheral and central

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nervous systems, liver, and kidneys [4]. Animal studies further indicate neurobehavioral alterations, including learning and memory impairment, as consequences of exposure to neonicotinoids [5].

Acetamiprid, a neonicotinoid pesticide, is extensively employed on a global scale to manage sap-feeding insects on various crops, including vegetables and fruits. When orally administered to rats, acetamiprid is observed to distribute widely throughout their tissues. The highest concentrations of acetamiprid are typically detected in the adrenal gland, liver, and kidney, while lower concentrations, including its metabolites, are present in the brain [1]. In the brain, acetamiprid significantly accumulates in the midbrain compared to other brain regions [6]. Frequent exposures to acetamiprid have been found to alter brain function related to learning and memory [7]. One of the proposed mechanisms by which neonicotinoids disturb the brain function is oxidative stress. Previous studies have shown that imidacloprid as another major member of neonicotinoids induces the generation of reactive oxygen species (ROS), and alters the activity of antioxidant enzymes in the rat brain [3,8]. As with other neonicotinoids, acetamiprid is considered an oxidative agent that can trigger oxidative stress in the brain [9].

Flavonoids are a significant category of polyphenolic compounds known for their wide range of clinical and biochemical functions [10,11]. Quercetin belongs to the flavanol subclass and commonly exists in fruits and vegetables. It possesses a diverse range of biological properties, including anti-inflammatory, memory-enhancing, antioxidant, and anti-aging effects [12]. Quercetin has beneficial effects on neurodegenerative disease [13]. The neuroprotective effect of quercetin on chemical induced-neurotoxicity such as pesticides has been studied [14,15]. The suggested mechanisms underlying the antioxidant activity of quercetin include both the sequestering of free radicals and the enhancement of cellular antioxidant defense pathways. Moreover, quercetin has been shown to inhibit xanthine oxidase and nitric oxide synthetase, as well as acting as an iron and calcium chelator in biological tissues [12,16]. At the molecular level, quercetin upregulates the antioxidant signaling pathways [16,17].

The increasing use of acetamiprid in agriculture has raised concerns about potential neurological effects on humans, arising from the potential high exposure levels linked to its widespread application. Given the neuroprotective and diverse biological activity of quercetin, this study aimed to evaluate the protective effects of quercetin against sub-acute acetamiprid-induced neurotoxicity in rats.

Material and Methods

Ethical considerations

All experimental procedures adhered to approved guidelines established by the ethics committee of Tarbiat Modares University, with the approval ID: IR.MODARES.REC.1397.114.

Chemicals

Quercetin, thiobarbituric acid (TBA), trichloroacetic acid (TCA), and malondialdehyde bis dimethyl acetal, were obtained from Sigma-Aldrich (USA). Acetamiprid was provided by Golsam Gorgan Chemical Company (Iran). Methanol and acetonitrile of HPLC grade were obtained from Amertate Shimico (Iran).

Animals

The study comprised 24 adult male Wistar rats, each weighing approximately 180±20 g, obtained from the Faculty of Medical Sciences, Tarbiat Modares University. These rats were housed in sanitary plastic cages placed in a room maintained at a controlled temperature of 25±1°C, following a 12-hour light/dark cycle. They had unrestricted access to a standard laboratory diet and water.

Treatment

Twenty-four rats were randomly divided into four groups, six rats in each group as follows: control group (without treatment); quercetin group: rats received 20 mg/kg of quercetin orally; acetamiprid group: the rats received 40 mg/kg of acetamiprid orally; acetamiprid quercetin group: the rats were orally administered 40 mg/kg of acetamiprid, followed by 20 mg/kg of quercetin via oral gavage needle, with a 30-minute interval between the two substances. This regimen was repeated daily for 28 consecutive days. Both acetamiprid and quercetin were dissolved in 10 % dimethyl sulfoxide (DMSO). Our previous studies indicated that DMSO up to 20% had no

effects on the behavioral performance of rats in the Morris water maze test or the oxidative stress biomarkers [18,19]. . The specific doses of 20 mg/kg for quercetin and 40 mg/kg for acetamiprid were selected based on previous research findings [18,19].

Morris water maze test

The Morris water maze (MWM) test was employed to evaluate spatial learning and memory performance. The water maze is commonly used to evaluate how different substances affect memory processes, such as acquisition, consolidation, and retrieval. Its widespread use is due to its high validity. The Morris water maze (MWM) is a frequently employed tool for assessing visuospatial learning, effectively identifying cognitive effects from brain lesions and drugs treating cognitive impairments. Notably, performance in the MWM task doesn't require special motivators like food or water deprivation. The MWM is a robust and reliable test strongly linked to hippocampal synaptic plasticity [20]. The test was conducted in a circular pool measuring 1.5 m diameter and 60 cm height. The pool was divided into four equal quadrants and filled with water to a depth of 30 cm. Within the southwest quadrant of the pool, a hidden platform measuring 10×10 cm was positioned 2 cm below the water surface. During each training trial, the rats were placed in the water in a randomly selected quadrant facing the tank wall. The training trial lasted for 90 s, and if a rat was unable to reach the platform within the allocated time, it was gently positioned on the platform and allowed to stay there for 20 s. Subsequently, the animals were lifted out of the water and dried with a clean cloth. The time interval between trials was set at 60 s. Throughout the training trials, all activities were recorded using a video camera placed above the tank. The captured videos were subsequently analyzed to obtain two learning measures: "escape latency" refers to the duration it took for the rats to locate the concealed platform, while "traveled distance" signifies the distance the rats covered in their efforts to find the hidden platform. By analyzing the recorded videos, these measures were determined, enabling an assessment of the rats' spatial learning and memory performance during the Morris water maze test.

After four days of training trials, a probe test was conducted without the platform. During this test, the rats were initially positioned in the northeast quadrant, which was opposite to the original platform location. They were then given 60 s to swim freely. The period that the rats spent in the southwest quadrant, where the platform was originally situated during their training, was recorded as a measure of their spatial memory performance [18,20,21].

Serum collection and tissue preparation

The blood samples were obtained via a heart puncture procedure for measurement of acetamiprid in the serum. Subsequently, the hippocampal tissue was extracted for additional analysis. The hippocampal tissue was first homogenized in a phosphate buffer with a pH of 7.4. This homogenate was then subjected to centrifugation at 3000 × g for 5 min at 4 ºC. The resulting supernatant was employed to measure the quantity of lipid peroxidation, total thiol content, and catalase activity.

Lipid peroxidation measurement

The lipid peroxidation was measured by quantification of malondialdehyde (MDA) as a byproduct of lipid peroxidation [19].To perform this procedure, 200 µL of a thiobarbituric acid (TBA) reagent (3.75% TCA and 0.0925% TBA) was combined with 100 µL of brain homogenate or standard solution. This mixture was then incubated at 90 ºC for 60 min. Following the incubation, the mixture was allowed to cool, and subsequently, 150 µL of N-butanol and 150 µL of NaCl (5 M) were added. The resulting mixture was then subjected to centrifugation at 1000 g for 10 min. The absorbance was measured at 540 nm using a plate reader. (BIOTEK ELX808, Agilent Technologies, United States). The standard calibration curve was established using various concentrations of MDA standard solution. The amount of MDA in the samples was then quantified with reference to the standard calibration curve. Subsequently, the amount of MDA in each sample was expressed as a percentage of the control.

Total thiol content

The measurement of total thiol content was conducted using the Ellman method [22]. This method relies on the reaction between thiols and

DTNB (5, 5'-dithiobis (2-nitrobenzoic acid)), a reagent, leading to the production of a yellow ion called 5-thio-2-nitrobenzoic acid (TNB), which has an absorbance at 405 nm. In brief, 20 µL of brain homogenate was mixed with 180 µL of a reaction buffer containing phosphate buffer (0.3 M, pH: 8), DTNB (0.1 mM), and sodium citrate (0.01%). After thorough mixing, the absorbance of the resulting solution was recorded at 405 nm using a plate reader (BioTek,USA).

Catalase enzyme activity measurement

The catalase enzyme activity was determined by measuring the rate of H_2O_2 decomposition by enzyme [23]. In brief, a 10 mM H_2O_2 solution in phosphate buffer (50 mM, pH 7.4) was prepared. Subsequently, 200 µL of brain homogenate was mixed with 2.8 µL of the H_2O_2 solution, and the rate of reaction was continuously monitored at 240 nm over 3 min. The results were reported as U/mg protein of tissue.

Histological examinations

For histological evaluation, two brain tissues from each group were preserved in a 10% formalin solution and then embedded in paraffin. Subsequently, the brain samples were sectioned into slices of 5 µm thickness, stained with hematoxylin and eosin (H&E), and examined under a light microscope to assess any changes in the hippocampal tissue histological structure.

Quantification of acetamiprid in hippocampus tissue and serum

The extraction of acetamiprid from both serum and hippocampal tissues was conducted using acetone. The hippocampal tissue was homogenized with chilled acetone at a concentration of 10% w/v. For the extraction of the serum sample, one volume of serum and two volumes of acetone were mixed and vigorously shaken on a vortex for 10 min. Subsequently, the mixtures underwent centrifugation at 10,000 g for 15 min. The resulting supernatants were collected, and the pellets were subjected to a second extraction with an equivalent volume of acetone. The combined supernatants were then evaporated to dryness. The resulting pellet was dissolved in 1 mL of acetonitrile, filtered, and subsequently injected into the high-performance liquid chromatography (HPLC) system for analysis [6].

HPLC analysis was conducted utilizing an Agilent HPLC system (Agilent 1200 series, USA). The column employed in this analysis was a C_{18} column (Nucleodur) with dimensions of 25 cm in length, 4.6 mm in diameter, and a particle size of 5 µm. The mobile phase used was a mixture of acetonitrile and water in a ratio of 40/60 (v/v), with a flow rate set at 1 mL/min. The column oven temperature was maintained at 40 °C, and each injection had a volume of 20 µL. The standard calibration curve was constructed by injecting various concentrations of acetamiprid standard solution (0.5-5 µg/mL). Subsequently, the amount of acetamiprid in the samples was quantified using this established standard calibration curve.

Statistical analysis

All bars represent the mean ± SEM. The analysis of behavioral data was carried out using the repeated measured two-way ANOVA test, followed by Tukey's multiple comparison test. The analysis of other data sets was conducted using a one-way ANOVA, followed by Tukey's multiple comparison test. GraphPad Prism 8 software was employed for these statistical analyses. Results with p-values less than 0.05 were considered statistically significant.

Results and Discussion

Morris Water Maze Test was used to assess the spatial learning and memory functions of rats. During the training days, two learning measures were recorded: escape latency and traveled distance. A reduction in these measures during training indicates successful learning. The results for escape latency and traveled distance can be found in Figures 1A and Figure 1B, respectively. Data analysis using repeated measures two-way ANOVA revealed a significant effect of time ($p<0.001$), treatment ($p<0.001$), and the interaction between time and treatment factors ($p<0.001$). Further paired analysis of groups by Tukey's multiple comparison tests indicated significant differences between the acetamiprid group and the control group, as well as between the acetamiprid group and the acetamiprid +quercetin group, specifically on the first and second days of training, in terms of escape latency and traveled distance measures. The speed parameter is depicted in Figure 1C, and no significant differences were observed between the groups or during the training days. In the

probe test, time spent in the target quadrant and opposite quadrant has been compared. The results demonstrated a significant difference in all groups, except for the acetamiprid group. These results are presented in Figure 2.

The concentrations of acetamiprid in the serum and hippocampal tissue of the studied groups are presented in Table 1. The levels of acetamiprid were found to be the same in both the serum and hippocampal tissue of the acetamiprid group and the acetamiprid + quercetin group. Acetamiprid was not detected in the control and quercetin-treated groups.

The MDA level was significantly higher in the acetamiprid group compared to the control group ($p<0.001$). However, in the acetamiprid + quercetin group, the MDA content exhibited a significant decrease when compared to the acetamiprid group ($p<0.001$, Figure 3). There was no significant difference between quercetin treated and control groups.

As depicted in Figure 4, exposure to acetamiprid resulted in a significant oxidation of the thiol groups in the acetamiprid group compared to the control group ($p<0.001$). However, the administration of quercetin to the acetamiprid + quercetin group significantly prevented thiol group oxidation in comparison to the acetamiprid group ($p<0.001$). There was no significant difference between quercetin treated and control groups.

The results demonstrated a significant decrease in catalase activity in the acetamiprid group compared to the control group ($p<0.001$). Conversely, the enzyme activity increased in the acetamiprid + quercetin group when compared to the acetamiprid group ($p<0.001$, Figure 5). There was no significant difference between quercetin and control groups.

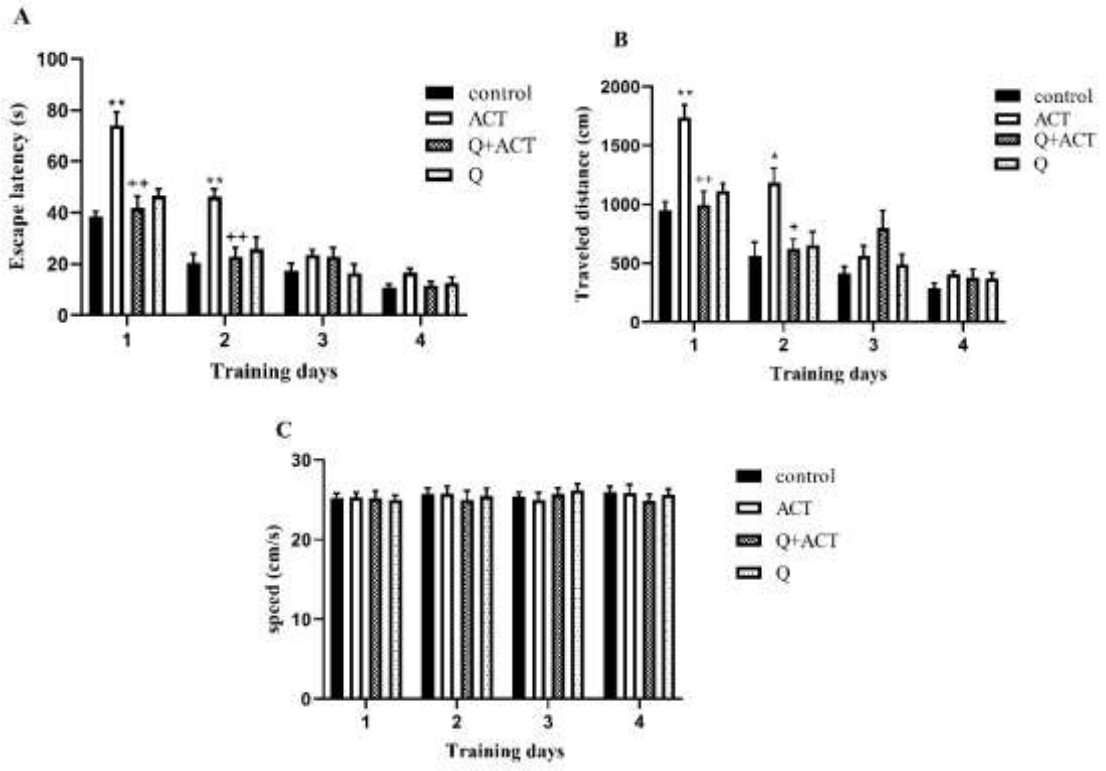

Figure 1. Performance of animals in the Morris water maze task; A: escape latency; B: traveled distance; C: speed; ACT: acetamiprid; Q: quercetin; *$p<0.05$, **$p<0.01$ versus the control group; +$p<0.05$, ++$p<0.01$ versus ACT group; the bars represent the mean ± SEM (n=6).

Table 1. Concentration of acetamiprid in serum and hippocampus

	Control	Q20 mg/kg	ACT-Q	ACT40 mg/kg
Serum (µg/mL)	Not detected	Not detected	13.22± 2.71	12.85± 3.25
Hippocampus (µg/g)	Not detected	Not detected	8.17± 0.63	7.27± 0.96

Data represented as Mean±SD (n=6); ACT: acetamiprid Q: quercetin.

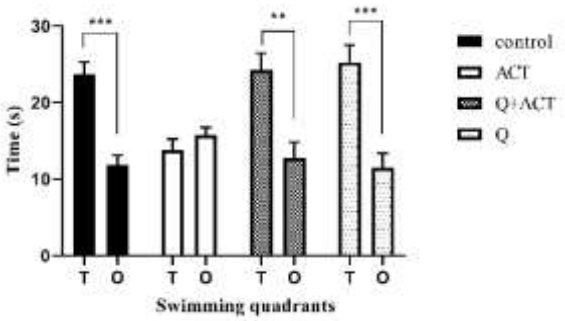

Figure 2. Comparison of time spent in the target and opposite quadrants in each group; ACT: acetamiprid. Q: quercetin; T: target quadrant; O: opposite quadrant; **p<0.01, *** p <0.001 versus the opposite quadrant; the bars represent the mean ± SEM (n=6)

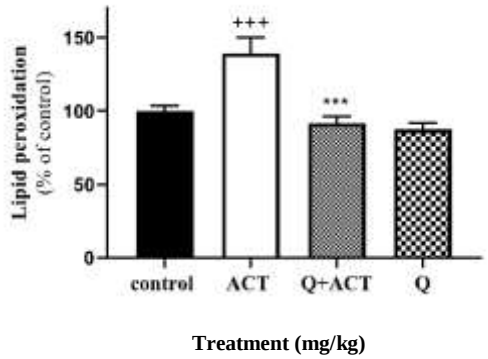

Figure 3. Malondialdehyde (MDA) level in the hippocampus samples; ACT: acetamiprid; Q: quercetin; +++p<0.001 vs. control; ***p<0.001 vs. ACT; the bars represent mean ± SEM (n=6)

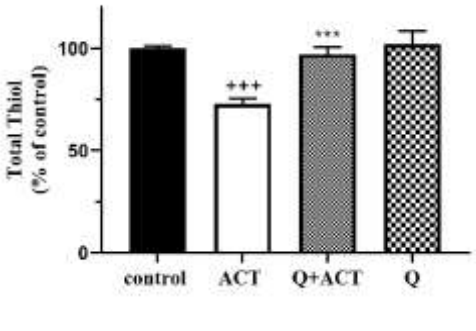

Figure 4. Total thiol concentration in the hippocampus samples; ACT: acetamiprid; Q: quercetin; +++p<0.001 vs. control, ***p<0.001 vs. ACT; the bars represent mean ± SEM (n=6)

Histological evaluation is presented in Figure 6. In the normal control group (A), neurons and neural tissue appear intact with a normal morphology. In the acetamiprid group (B), there is a disorder in the dentate gyrus architecture, along with dilated capillaries and an increase in glial cells. However, in the Acetamiprid + quercetin group (C), the structure is nearly normal. Normal architected of hippocampal tissue is observed in the quercetin group (D)

In the present study, the neurotoxic effects of acetamiprid in the male rat hippocampus and the protective effect of quercetin have been explored. To this end, the effects of sub-acute exposure to acetamiprid on the spatial learning and memory function of rats were explored, and the role of oxidative stress as a possible mechanism of toxicity in the animal hippocampus tissues was examined. Additionally, the ameliorative role of quercetin was assessed.

The results of the Morris Water Maze test showed that the acetamiprid group exhibited a significant increase in escape latency and travel distance on the first and second days of training, as compared to the control group. This finding suggests that acetamiprid disrupted the acquisition phase of learning, and the acetamiprid-treated animals required more trials to learn the task.

Furthermore, the results of the probe test revealed that the acetamiprid-treated group spent approximately equal time in both the target and opposite quadrants, while the animals in the control group spent more time in the target quadrant. This observation suggests that acetamiprid may impair the consolidation and recall of memory. In addition, the results of the MWM test indicated a significant difference in learning measures during the acquisition phase and the probe test between the Acetamiprid + Quercetin group and the Acetamiprid group. These findings suggest that the co-administration of quercetin and acetamiprid leads to an improvement in acetamiprid-induced memory impairment.

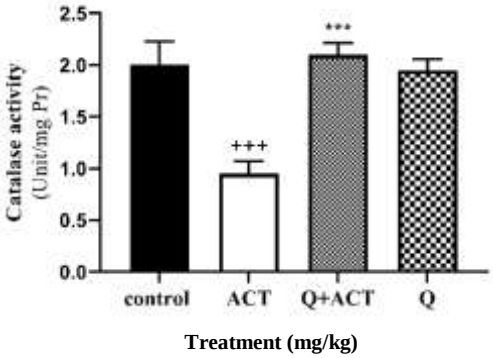

Figure 5. Catalase activity in the hippocampus samples. ACT: Acetamiprid; Q: Quercetin. The bars represent mean ± SEM (n=6). +++p<0.001 vs. control, ***p<0.001 vs. ACT

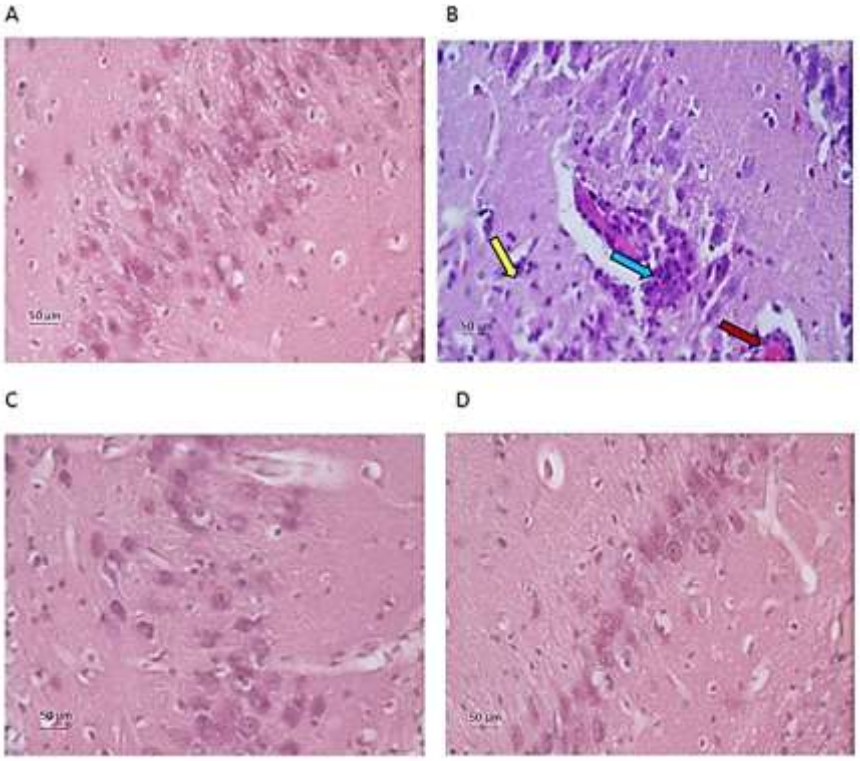

Figure 6. Effects of quercetin on acetamiprid-induced histological damages in the hippocampus of the rats; hippocampus sections were stained using the hematoxylin–eosin method (400X). The control (A); ACT: acetamiprid (B); Q: quercetin (C); Q-ACT (D). The red arrow shows the dilated capillaries, the blue arrow indicates the increase in glial cells, and the yellow arrow indicates the disturbance in normal architecture.

Previous studies have shown the neuroprotective and memory-enhancing effects of quercetin [24,25]. Various mechanisms have been suggested to underlie quercetin's neuroprotective actions, including its antioxidant activity, anti-inflammatory effects, supporting mitochondrial function, and modulation of signaling pathways involved in cell survival [26,27].

The results of our study revealed an increase in lipid peroxidation and a decrease in total thiol and catalase activity in the hippocampus tissue of animals treated with acetamiprid, indicating the induction of oxidative stress following acetamiprid treatment. Furthermore, histological assessment demonstrated structural damage and an elevation in glial cell presence in the hippocampus tissue, particularly in the dentate gyrus area. These findings suggest that oxidative stress inflicted neural cell damage in the hippocampus tissue, thereby playing a pivotal role in acetamiprid-induced learning deficits. The induction of oxidative stress by acetamiprid has been documented in previous studies. For instance, one study demonstrated that treatment of rats with acetamiprid 40 mg/kg for 21 consecutive days induced oxidative stress in the cerebellum, as evidenced by a significant increase in lipid peroxidation and a reduction in antioxidant enzyme activity [28]. Similarly, Gasmi et al. reported that a dose of 3.14 mg/kg of acetamiprid over 90 days induced oxidative stress in the brains of rats [9]. It has also been reported that acetamiprid disrupts mitochondrial function, leading to an elevation in reactive oxygen species (ROS) production [9,29].

As indicated in the results section, the administration of quercetin effectively counteracted the acetamiprid-induced oxidative stress in the hippocampus tissue. Quercetin, a naturally occurring flavonoid known for its antioxidant properties, acts as an antioxidant through both direct and indirect mechanisms. It directly scavenges free radicals and, indirectly, enhances cellular antioxidant defense pathways. Notably, quercetin has a significant role in activating the Nrf2 (nuclear factor [erythroid-derived 2]-like 2) -ARE (antioxidant response element) pathway, which is a critical regulator of

the cellular system to counteract oxidative stress. The Nrf2 pathway modulates the expression of glutathione S-transferases, heme oxygenase-1, glutathione peroxidase, superoxide dismutase, and catalase, thereby providing neuroprotection against oxidative damage [30]. Quercetin, through the upregulation of the paraoxonase 2 pathway, could potentially mitigate chlorpyrifos-induced oxidative stress [19]. Furthermore, studies have demonstrated that quercetin can mitigate oxidative stress in the liver [31], kidneys [32] and immune system [33] which is produced following acetamiprid exposure.

There is documented evidence indicating that oxidative stress induced following acetamiprid exposure is linked to damage to the mitochondria, specifically involving the downregulation of the ND1, ND2, COX1, and COX4 subunits of mitochondrial complexes. These subunits play critical roles in the mitochondrial electron transport chain (ETC), responsible for the majority of cellular ATP production. The downregulation of these subunits disrupts normal mitochondrial function, leading to an imbalance between ROS production and antioxidant defense mechanisms. This imbalance results in oxidative stress, contributing to various pathological conditions [29]. Numerous studies have highlighted quercetin's mitoprotective effects. Quercetin has been revealed to regulate mitochondrial activity by influencing mitochondrial formation, membrane potential, electron transfer chain, ATP production, and intrinsic apoptosis pathways [34,35]. As a result, it is suggested that quercetin alleviates acetamiprid-induced oxidative stress by mitigating mitochondrial damage caused by acetamiprid. However, further investigation is warranted to validate this hypothesis.

The results of the histological assessment revealed disturbances in dentate gyrus architecture, capillary dilation, and an increase in glial cell presence in the acetamiprid group (40 mg/kg). Given that the dentate gyrus is implicated in processes such as learning, memory, and emotional regulation, damage to this region can profoundly impact cognitive and behavioral outcomes [36]. Notably, all alterations observed in the dentate gyrus sub region were mitigated through co-administration of quercetin. Therefore, it is suggested that quercetin, through its antioxidant mechanisms, protects against hippocampal tissue damage and ameliorates memory deficits stemming from acetamiprid treatment.

The analysis of acetamiprid levels in both serum and hippocampus tissue revealed similar amounts of acetamiprid in rats that were administered acetamiprid alone and those that received it in combination with quercetin. This observation suggests that quercetin does not influence the absorption and distribution of acetamiprid and that its protective effects primarily occur at the cellular level.

Conclusion

Our study demonstrated that oral administration of acetamiprid (40 mg/kg) results in its distribution to the hippocampus, inducing oxidative stress that damages hippocampus tissue, consequently impairing learning and memory. Additionally, quercetin, administered at a dose of 20 mg/kg, exhibited significant potential in ameliorating histopathological alterations, oxidative damage, and learning deficits induced by acetamiprid. This beneficial effect is attributed to quercetin's capacity to enhance cellular antioxidant defenses and potentially protect mitochondria. Therefore, it is recommended that further clinical studies evaluate the health benefits of consumption of quercetin-rich foods or quercetin as a nutraceutical agent in individuals directly or indirectly exposed to acetamiprid, such as agricultural workers or those residing near agricultural areas.

Acknowledgments

None

Author contributions

Maliheh Soodi designed and supervised the project, analyzed the data, interpreted the results, and revised the final manuscript; Mohsen Shamsi performed the behavioral experiment, collected samples, conducted HPLC analysis, and prepared the draft of the manuscript; Ameneh Omidi directed the histological experiment and evaluated the results; Alireza Ghazanfari performed the biochemical experiments.

Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the accuracy and integrity of the paper content.

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Abbreviations

nAChRs: nicotinic acetylcholine receptors; MWM: Morris water maze; H&E: Hematoxylin and eosin; ANOVA: one-way analysis of variance; DMSO: dimethyl sulfoxide; HPLC: High Performance Liquid Chromatography; DTNB: 5, 5'-dithiobis (2-nitrobenzoic acid; TNB: 5-thio-2-nitrobenzoic acid); ETC: electron transport chain; Nrf2: nuclear factor [erythroid-derived 2]-like 2); ARE: antioxidant response element; TBA: thiobarbituric acid; TCA: trichloroacetic acid; ROS: reactive oxygen species; MDA: malondialdehyde