



The Evaluation of Neuroprotective Effect of *Nigella sativa* Linn Seed Using Zebrafish Model

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Naturally occurring materials that can serve as preservatives in foodstuffs are substances found in their composition. *Nigella sativa* Linn, a member of the Ranunculaceae family, is a widely recognized medicinal herb with global usage. For centuries, *N. sativa* L., seeds have been employed to address various ailments and health issues. In behavioral research, the bioactive compound derived from *N. sativa* L., namely Kaempferol 3-(2-galloyl- α -L-arabinopyranoside), has gained significant attention. The escalating prevalence of Alzheimer's disease among elderly individuals worldwide necessitates exploration beyond traditional mammalian and rodent models. This has led to the adoption of zebrafish (*Danio rerio*) as a unique model organism in biomedical research, behavior analysis and human disorders. Experiments involving scopolamine-induced T-maze, escape, place preference and bite tests have strongly suggested that behavior in zebrafish is

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governed by conserved regulatory processes. The results of the behavior tests demonstrated that *N. sativa* L., enhances learning and memory, which was otherwise affected by scopolamine treatment. Notably, a thorough analysis of histoarchitecture revealed no adverse effects of Kaempferol 3-(2-galloyl- α -L-arabinopyranoside), which might be a promising therapeutic option for such a multi-factorial Alzheimer's disease.

Keywords: Zebrafish; Alzheimer's disease; behavioral analysis; histoarchitecture.

1. INTRODUCTION

The prevalence of brain diseases such as Alzheimer's disease (AD), a multidimensional neurodegenerative ailment that is progressing and is the primary cause of 70% of dementia worldwide, is rising globally [1]. In 2030, there will be 78 million AD patients worldwide. The overall quantity is estimated to climb to 139 million (WHO, 2021). Globally, the incidence of neurodegenerative illnesses is rising, necessitating the urgent identification and expansion of pharmaceutical treatment options [2]. Alzheimer's disease stands as the most pervasive neurodegenerative disorder, with limited efficacious remedies. The majority of Alzheimer's disease research on histopathology, molecular mechanisms, and pharmaceutical treatments involves rodent models [3].

1.1 Zebrafish as an Experimental Animal for Determining the Toxicity of Medicinal Plants

Neurodevelopmental toxicological investigations are carried out *in vitro* using zebrafish (*Danio rerio*) as both an infancy and larval model. Zebrafish can be employed in early screening assays to assess the cytotoxicity of materials, frequently in a high-throughput way. the embryotoxicity of zebrafish [4]. Due to the quick turn around time for tests, transparency of embryos, brief life span, high fertility and strong research data comparability, the platform is at the forefront of toxicological research. Zebrafish toxicity research spans from evaluating the toxicity of bioactive substances or unrefined plant extracts to figuring out the best procedure. The majority of the studied extractions, which were employed to measure cytotoxicity and biological properties, were neutral, like ethanol, methanol, and aqueous solutions [5].

The zebrafish (*Danio rerio*) is a unique model animal for biomedical research, including studies of biological processes and human diseases. Zebrafish are sometimes identified as relative to classic rodent models or alternative models, but

the use of fish in addition to classic mammalian models [6]. The role of frontal cortical and hippocampal structures in learning and memory cannot be studied with fish since these are not evident, but other attributes of fish make them valuable models in behavioral neuroscience research. Developmental processes can be continuously visualized in species that have a clear egg sack [7]. Zebrafish, in particular, have been well-used in genetics, neuroscience, pharmacology, and toxicology. The next and ongoing step is to extend the zebrafish model to pursue questions of behavioral neuroscience, an undertaking that requires valid, reliable, and efficient methods of behavioral assessment [8].

Zebrafish (*Danio rerio*) is a unique modeling organism involving biomedical research, including examinations of biological procedures and human illnesses in conventional mammalian and rodent models [9]. Zebrafish are quickly becoming valuable animals for diseased behavior as well as simulating complex brain illnesses. The use of model organisms and compiling a thorough list of behavioral characteristics are crucial tactics in translational neuroscience studies [10]. To study the role of the frontal cortex and hippocampal regions in memory and learning because they are not visible but have other characteristics that make them unique models for behavioral neuroscience research. To make it easier to comprehend the particular brain systems that proliferate, differentiate, migrate, and project, reporter systems may highlight specific brain systems [11].

Nigella sativa Linn. is recognized as one of the best evidence-based therapeutic herbs due to its extraordinary healing qualities. It has been demonstrated to have excellent potential in Islamic literature [12]. It was traditionally recommended to apply this often in Tibb-e-Nabwi (prophetic therapy). There are several traditional systems of medicine in India, such as Siddha, Ayurveda, Unani medicine, or Tibb, that utilize *N. sativa* Linn, known as black kalonji [13]. Black kalonji is a commonly used and popular plant. It can heal the majority of illnesses and protect

against mortality. It is used in cooking as an astringent, stimulant, diuretic, anthelmintic, jaundice, intermittent fever, paralysis and piles, having the ability to treat practically all ailments save for life [14].

However, a lack of thorough knowledge of the causes, pathogenesis and best therapies for Alzheimer's disease is partly a result of the drawbacks of employing rats, such as their higher economic cost, which can have an impact on sample size and eventually statistical power [15]. Expanding our arsenal of animal models is crucial to offering various approaches for research on Alzheimer's disease. The development of intracellular neurofibrillary tangles (NFT) and amyloid beta (A β) plaques are the two main pathologic traits [16].

The anticholinergic substance scopolamine, commonly known as hyoscyne or Devil's Breath, is a tropane alkaloid that can be produced naturally or synthetically. It is used to treat motion sickness, nausea and vomiting that follow surgery. In order to reduce saliva, it is occasionally given before surgery [17]. Effects from injectable use can last up to 8 hours and start to take effect after around 20 minutes. Since its transdermal bioavailability has long been established, it can also be applied topically and as a transdermal patch. The antimuscarinic medication scopolamine acts by obstructing part of acetylcholine's actions on the nervous system [18].

The bioactive component Kaempferol3-(2-galloyl-alpha-L-arabinopyranoside), found in *Nigella sativa* L., seeds, was evaluated in the current study for behavioral and histoarchitecture analysis. The bioactive substance that is responsible for the neuroprotective effect was identified through behavioral analysis. If further research is conducted, it may be possible to formulate this extract into an affordable and potent anti-Alzheimer's medicine.

2. MATERIALS AND METHODS

2.1 *In vivo* Safety Assessment Conducted on Zebrafish

2.1.1 Zebrafish (*Danio rerio*) caring and housing

All experiments were performed on wild-type zebrafish (*Danio rerio*) obtained as adults from a local distributor (Golden Aqua Pets, Tiruchirappalli, Tamil Nadu, India). The aqua pet

store population was used with the intent to increase phenotypical variation, which would increase individual behavioral differences [19]. All fishes were allowed to acclimate for about one month and were initially maintained in a 76 Lr aquarium at 26 $\pm$ 1°C, pH = 7.3–7.5, under a 14:10 light/dark cycle, semi-closed water system with aeration, mechanical and biological filtration. Fishes were fed twice a day with a commercial diet and fed flake food daily by using the Feedwale online automatic feed calculator [20]. All fishes was cared for and managed according to the guidelines provided by the Organization for Economic Co-operation and Development (IAEC Study No. MB-IAEC/2024/01/12). The fish was kept alive through appropriate feeding practices [21].

Calculation of Feed conversion ratio (FCR) =
Weight of the feed fed to the fish / Weight of fish growth

2.2 Training Zebrafish for Behavioral Analysis

The fishes selected for the study was placed in individually labeled compartments for a day before the behavioral experiments, following the approach [22]. To ensure accurate identification of individuals over time, the individual chambers was designed similarly to those implemented. To maintain consistent environmental conditions for each fishes, multiple chambers containing individual fishes was placed inside an additional water bath, which stimulated their sense of smell and sight, as suggested [23]. To minimize the influence of the 14-hour light and 10-hour dark cycles, all trials was conducted between 10:00 a.m. and 2:00 p.m. Throughout the behavioral analyses, all fishes was tracked for three weeks following their confinement to specific compartments [24].

2.3 Scopolamine Treatment for Anesthesia

Scopolamine was prepared at a concentration of 800 mM/L using distilled water. The effects of scopolamine exposure on locomotor activity in wild-type zebrafish was assessed using a laboratory principle [25]. In this study, only larvae at 7 days post-fertilization (dpf) was used, and all relevant parameters was taken into consideration [26]. Before scopolamine exposure, the wild-type zebrafish was acclimatized to the behavioral environment and then exposed to scopolamine (800 mM/L) for 30 minutes, followed by the acquisition of three 10-minute videos capturing

their spontaneous free-swimming behavior [27]. Throughout the video acquisition process, the wild-type zebrafish remained in the presence of scopolamine. Only wild-type zebrafish that exhibited activity under both the control and treatment conditions was included in this investigation [28].

Following the administration of anesthesia, the animals were placed in a 5 L tank (25 x 15 x 15 cm) filled with water and maintained at a temperature of $28 \pm 0.5^\circ\text{C}$. Throughout the 24-hour recovery period, continuous monitoring and interaction with neighboring tanks was ensured. The water system employed mechanical, biological, and aeration filters, with a complete water change performed daily along with aeration. UV-sterilized water was used in each tank of this semi-closed water system [29]. The animal facility followed regular monitoring practices to assess the general health and welfare of the animals. This involved observing indicators such as food consumption, water equilibrium, changes in mucosa color, prolonged erratic movements, swimming behavior, responsiveness to touch, and water agitation. Throughout the experiment, the animals were closely observed at 1, 5, 24, and 48 hours after anesthesia administration [30].

2.4 Experimental Approach for Zebrafish

A total of fifty zebrafish was used in the experiment after being acclimated to the laboratory conditions for three weeks. These fish were randomly assigned to five groups, following the methodology described in [31]. Detailed information regarding the dosage and treatment of scopolamine and the Kaempferol 3-(2"-galloyl-alpha-L-arabinopyranoside) compound is given in Table 1.

2.5 Behavioral Analysis of Zebrafish (*Danio rerio*)

Four behavioral tests were conducted, including the T-maze test, escape test, bite test, and place preference test. To facilitate the transfer, zebrafish were moved between compartments and acclimatization tanks using small containers. Each fish was given a 10-minute adaptation period before each test. Fish were carefully transferred from the acclimatization tank to the designated test area using a small vessel [32]. The following behavioral parameters were recorded in each experiment: total number of bites, horizontal displays, dart throwing, charges, and time spent in contact with the stimuli (Fig. 1).

Table 1. Details regarding dosage and treatment of Scopolamine and Kaempferol 3-(2"-galloyl-alpha-L-arabinopyranoside) compound in zebrafish (*Danio rerio*)

Group	Treatment	Dosage
I	Control	De-chlorinated tap water
II	Scopolamine treatment	800 mM/L
III	Scopolamine + Donepezil	800 mM/L + 2.5 mg/L
IV	Scopolamine + Kaempferol 3-(2"-galloyl-alpha-L-arabinopyranoside)	800 mM/L + 10 mg/L
V	Scopolamine + Kaempferol 3-(2"-galloyl-alpha-L-arabinopyranoside)	800 mM/L + 1 mg/L



Fig. 1. Behavioral analysis apparatus with documentation

2.5.1 T-maze test

The primary component of the apparatus needed to conduct this test on zebrafish is a translucent Plexiglas T-maze. Although the size can be changed, the maze pattern is either cross-shaped (70 cm x 50 cm x 10 cm) or symmetrical (50 cm x 50 cm x 10 cm). The right and left arms of the maze was separated from the start and goal zones by removable, opaque Plexiglas doors [33]. The walls of the maze had colored or patterned sleeves (horizontal vs. vertical) placed along each arm or only in the goal boxes. Performance was considered when choosing colors, indicating an aversion towards red and green. Filtered tap water that has been prepared with a purifier and tank water must be combined and the mixture must be poured into the maze to a height of about 8 to 10 cm [34]. The water within the maze was kept at a temperature of between 25.5 °C and 28.5 °C. Home tanks need continuous filtering and aeration. Experiments were monitored using a timer as well. Stainless steel tweezers was used to distribute food into the proper goal zone and the study contains rewards (Fig. 2).

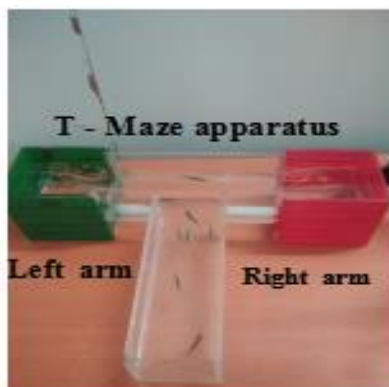


Fig. 2. T- Maze apparatus

To analyze different genotypes or the effects of a chemical, coloration or patterning bias, encompassing learning, extermination, and reversing periods, the T-maze analysis was carried out based on the observations of latencies in achieving the goal zone [35]. Fish have been given five minutes to explore the T-maze during their initial habituation. The fishes underwent two habituation tests for an additional hour, which helped to reduce interaction shock and overall methodological innovation anxiety for three days. However, in the absence of colorful sleeves, a total of three sessions with a total of three tests of exposure to food incentives were

performed. These tests can be conducted on multiple fishes at once [36].

Following the testing, the number of groups was gradually reduced until individual testing was conducted within 3 days. It is possible to change the overall quantity, frequency and variety of trials for each training session [37]. This is possible to alter and conduct through individual training sessions with the total number, frequency, and number of trials. Three trials were run in each session, with a 24-hour gap between each experiment. For pattern discrimination, the target box should be on the left arm for half the fishes and the right arm for the other, with a subsequent reversal. Before allowing the fishes to explore the maze, each one was placed in the start box for two to five minutes with the door closed [38].

As soon as the fishes exited the start box, the door was locked. The maximum time to get to the goal box is ten minutes. If food as a reward, it ought to be given after making the right decision [39]. After each session, the fish was netted 30 seconds after making an assessment and returned to the start box or the home tank. During the appropriate training trials, it is important to note that the experimental fishes consumed information [40].

2.5.2 Escape test

A revolving acrylic apparatus surrounds a circular container with transparent walls that serve as the zebrafish rotation test device. For investigations on zebrafish retinal degeneration, this setup was marked using signals [41]. Usually, the acrylic is denoted with a black segment. To stop the zebrafish from swimming across the midline of the inner chamber, a central support was placed inside the backlight, which is also included under the tool [42].

The device consists of a transparent, stationary, circular container with a diameter of around 12 cm. A revolving acrylic setup covered in white paper surrounds this stationary object. Additionally, the setup has a roughly 5x5 cm black stripe that represents a threatening item [43]. The middle of the inner container features an opaque post with a diameter of about 3cm that stops fishes from swimming across the middle of the container [44]. The intensity of the white light source used to illuminate the setup from above varies depending on the goals of the experiment. The belt connected to a motor turns

the setup at a speed of 10 rpm [45]. Using the support of a mobile device with a camera, the individual's movement was recorded (Fig. 3).



Fig. 3. Escape apparatus

2.5.3 Place preference

With the noticeable change towards the experimental (drug-conditioned) compartment in the adult zebrafish conditional place preference (CPP) behavior, the experimental treatment is assumed to be rewarding [46]. The device comprises a Plexiglas CPP tank with approximate measurements of 54 cm (L), 18 cm (W), and 24 cm (H) [47]. Two opaque separators (one complete and the other perforated) separate the tank into two sections, forming a central path for the individual's entrance (Fig: 4). The CPP tank's fishes activity was recorded [48].



Fig. 4. Place preference apparatus

2.5.4 Bite test

By exposing the beads to various colors, bite test equipment can be utilized to detect the behavior of the biters [49]. Zebrafish exhibit an assortment of reactions in response to this multicolored

display, which promotes the evolution of their eating and biting behaviors. The acrylic apparatus comprises the tank with approximate dimensions of 54 cm (L), 18 cm (W) and 24 cm (H). The connection between the apparatus and the interior of the home tank was made through a 3 cm-wide aperture in the device. The zebrafish can enter and exit the device through this aperture since it is sufficiently wide [50]. Round beads with a diameter of about 2.5 mm were mounted on a transparent vertical rod with a diameter of about 2 mm to begin the experiment. At the other end of the box from the aperture, at the far end, this configuration is fixed [51].

The bead is lowered to the fish's eye level, 1 cm below the water surface, to allow the fishes to circulate freely under the view being observed [52]. The zebra fish's ability to move freely inside the device is useful for examining the part of its brain that is tasked with deciding in reaction to certain responses or stimuli (Fig: 5). Both models' bite test chambers are simply detachable for cleaning and sterilization [53].



Fig. 5. Bite test apparatus

2.6 Histoarchitecture Analysis from the Zebrafish Brain

Following the completion of the behavioral analysis during the experimental period, the total number of zebrafish in each group (consisting of 10 fish) was randomized, resulting in five fishes per group [54]. Subsequently, the brains of these fishes were dissected for further examination. The brain was subjected to histoarchitecture testing to evaluate its cellular structure and organization [55].

3. RESULTS AND DISCUSSION

3.1 Behavioral Analysis of Zebrafish (*Danio rerio*)

3.1.1 Scopolamine treatment

The development of appropriate anesthetics for this species in research has not kept pace with the growth in the use of zebrafish models. The most common anesthetic for fish, scopolamine, can cause aversion, a decrease in heart rate, and a significant mortality rate as a result, especially after prolonged exposure.

3.2 T-Maze Test

The T-maze test in zebrafish relies on either food reward or stimulation with conspecifics. However, a challenge in conducting spatial learning and memory tests in zebrafish is the extended training period, typically lasting eight to ten days. In the present study, the following entries were recorded for the red and green arms of the T-maze test: the total number of entries and the total time spent by groups 1 to V. The recorded values from the green arm for groups I to V were 11.21 ± 3.35 , 13.51 ± 3.98 , 6.28 ± 2.09 , 6.88 ± 2.48 , and 8.76 ± 2.98 , respectively. Each experiment was performed in triplicate (Fig. 6).

3.3 Escape Test

Zebrafish larvae exhibit a remarkable auditory response when exposed to tactile or visual stimuli, and they instinctively avoid dark areas and moving objects by lightly touching their heads or tails. In the present study, the recorded entries for the light and dark compartments of the

escape test included the total number of entries and the total time spent by groups 1 to V. The recorded values from the dark compartments for groups I to V were 9.51 ± 2.97 , 9.06 ± 2.37 , 7.58 ± 2.66 , 4.21 ± 2.07 and 7.68 ± 2.45 , respectively. Each experiment was performed in triplicate (Fig. 7).

3.4 Place Preference

The conditioned place preference (CPP), a form of Pavlovian conditioning, is employed to investigate the rewarding effects associated with drug abuse. Adult zebrafish are trained to associate specific environmental cues with drug intake. In the present study, the recorded entries for the conditioned place preference compartments (CPP) included the total number of entries and the total time spent with and without rewards by groups 1 to V in the place preference test. The recorded values with reward for groups I to V were 11.21 ± 3.34 , 12.58 ± 3.51 , 8.51 ± 2.99 , 6.08 ± 2.25 and 7.36 ± 2.70 , respectively. Each experiment was performed in triplicate (Fig. 8).

3.5 Bite Test

The biting test is a well-established experimental paradigm used to investigate the social and aggressive behavior of adult zebrafish. In the present study, the recorded entries for the conditional chamber of the mirror bite test included the total number of entries and the total time spent with and without rewards by groups 1 to V. The recorded values with reward for groups I to V were 9.91 ± 2.87 , 11.51 ± 3.01 , 8.58 ± 2.56 , 5.08 ± 2.05 , and 7.76 ± 2.40 , respectively. Each experiment was performed in triplicate (Fig. 9).

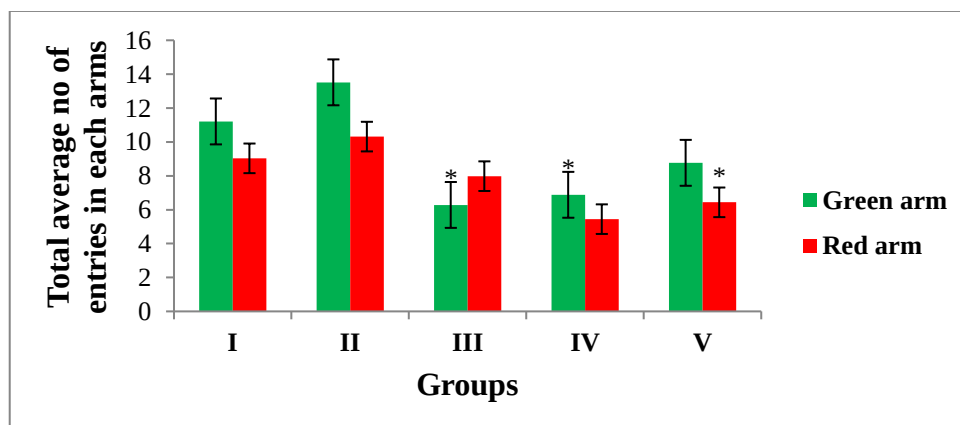


Fig. 6. T-Maze test for behavioral analysis

The results were expressed as Mean ± SD, *p < 0.05 is determined as significant value

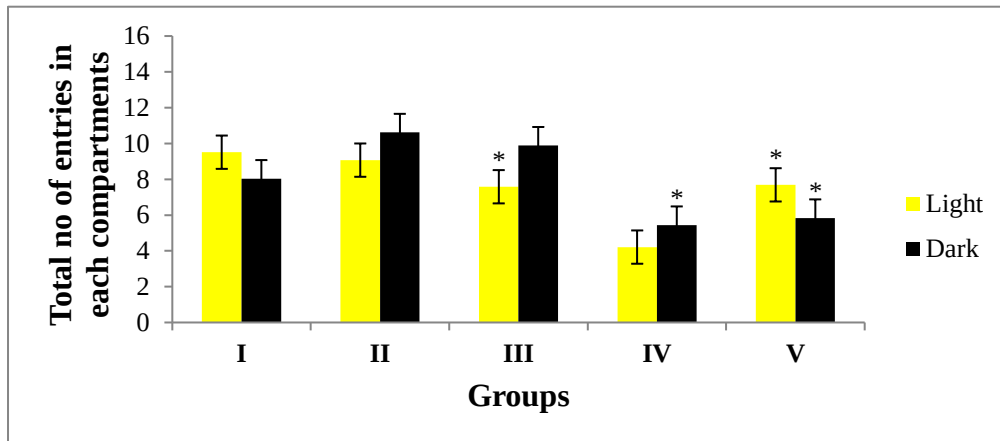


Fig. 7. Escape test for behavioral analysis

The results were expressed as Mean±SD, *p<0.05 is determined as significant value

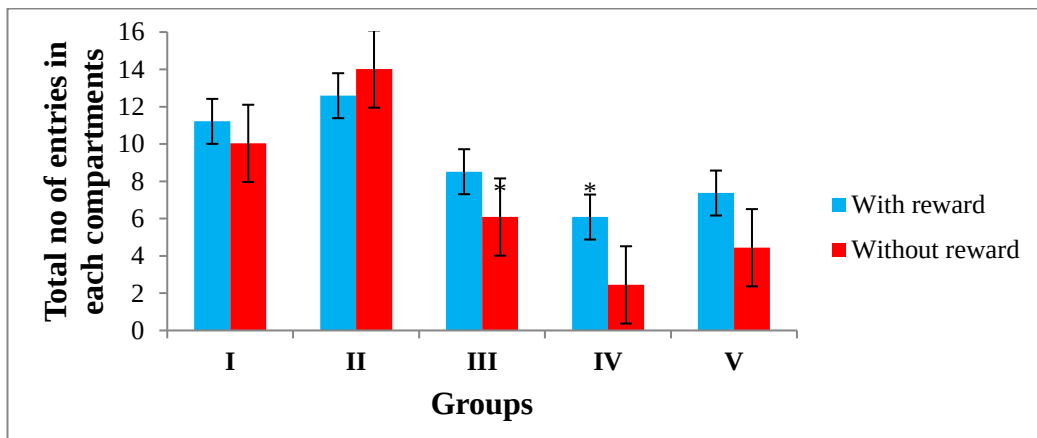


Fig. 8. Place preference for behavioral analysis

The results were expressed as Mean±SD, *p<0.05 is determined as significant value

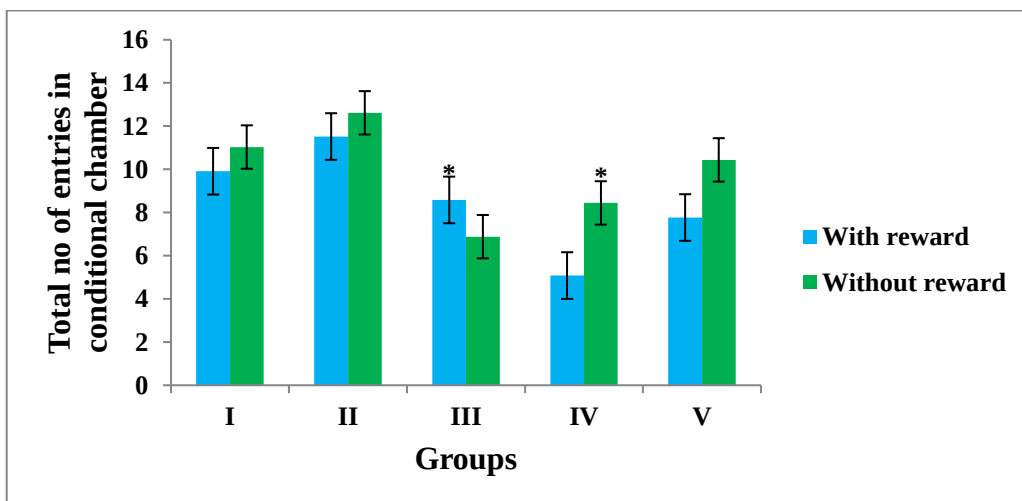


Fig. 9. Bite test for behavioral analysis

The results were expressed as Mean±SD, *p<0.05 is determined as significant value

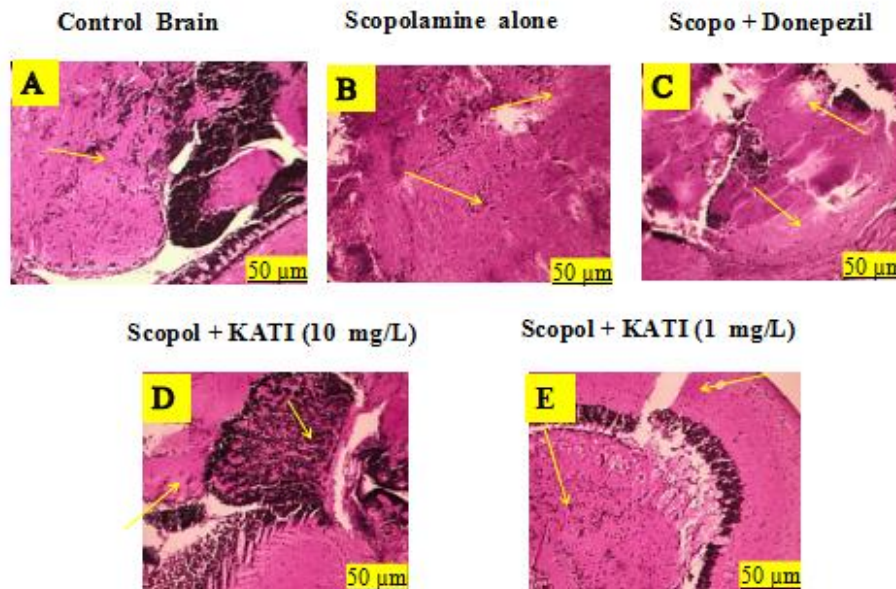


Fig. 10. Histoarchitecture analysis of the effect of *N. Sativa L.*, on *Zebrafish* treated with Control (A), Scopolamine alone (B), Scopolamine with Donepezil (C), Scopolamine with higher concentration (10 mg/L) (D) and Scopolamine with lower concentration (1 mg/L) (E). Original magnification x400; scale bar = 50µm

3.6 Histoarchitecture Analysis from the Brain

The loss of neuronal connections in the brain is considered one of the primary symptoms of Alzheimer's disease. In this study, no abnormalities were found in the experimental neuron or neuropil morphology of the zebrafish brain tissues examined in Group 1. However, in group II, lower basal dendritic spine density and neutrophil infiltration were observed. On the other hand, group III exhibited a higher spine density in the dentate gyrus, granular cells and hyperplastic neurons of the brain. In group IV, neurons in the cerebellum, occasional neurons, congestion and reduction of necrotic neurons and no discernible cerebral lesion were observed. Additionally, in group V, neuronal cells and congestion and reduction of necrotic neurons in the cerebellum (Fig. 10).

4. CONCLUSION

The absence of a definitive treatment or preventive measure for Alzheimer's disease (AD) has spurred the need for continued exploration and innovation, particularly given the growing number of individuals affected by this condition.

The exact etiology of AD is intricate and still not fully comprehended, with multiple factors contributing to neuronal cell death, rendering the quest for a therapeutic approach exceptionally challenging. In this context, it is prudent to consider various factors while seeking a potential treatment, especially in light of the failures in recent clinical trials involving synthetic compounds, often due to toxicity or exacerbation of cognitive decline. The present study underscores the advantageous impact of Kaempferol 3-(2"-galloyl-alpha-L-arabinopyranoside) in enhancing cognitive functions related to learning and memory in zebrafish. The histoarchitecture examinations further substantiate the observed behavioral improvements.

Furthermore, the study investigates the effects of *N.sativa L.*, methanolic extract bioactive component, Kaempferol 3-(2"-galloyl-alpha-L-arabinopyranoside), on zebrafish with scopolamine-induced memory deficits. The results indicate that this extract mitigated memory impairment across various behavioral tests, including the T-maze test, escape test, bite test, and place preference test. These findings hold promise for future research aimed at

developing *Nigella sativa* L., methanolic extract's bioactive component, Kaempferol 3-(2"-galloyl-alpha-L-arabinopyranoside), as a potential health product for alleviating memory impairment or Alzheimer's disease.

The ethnomedicinal value of *N.sativa* L., methanolic extract bioactive component, Kaempferol 3-(2"-galloyl-alpha-L-arabinopyranoside), underscores its potential role in neurological conditions and its favorable effects on the central nervous system. *Nigella sativa* L., methanolic extract bioactive component Kaempferol 3-(2"-galloyl-alpha-L-arabinopyranoside). However, minimal research has been conducted to pinpoint promising pathways for the development of either extracts derived from *N.sativa* L., the methanolic extract bioactive component Kaempferol 3-(2"-galloyl-alpha-L-arabinopyranoside), or the discovery of specific compounds that could be refined and enhanced as foundational elements for AD therapeutics. A more comprehensive exploration of the underlying molecular mechanisms is still warranted. Additionally, determining the pharmacokinetics, pharmacodynamics, bioavailability and optimal route of administration for *Nigella sativa* L., methanolic extract bioactive component Kaempferol 3-(2"-galloyl-alpha-L-arabinopyranoside) extracts may offer further insights into the potential utilization of mint-based products for brain health. Research into the toxicity and the ability of mint extracts to traverse the blood-brain barrier and access neurons in the brain represents an area that merits further investigation.

COMPETING INTERESTS

Authors have declared that they have no known competing financial interests or non-financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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