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Cognition enhancing activity of essential oil of *Hedychium coronarium* j. Koenig in memory-impaired animal models

Research Article

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Abstract

Alzheimer's disease (AD) is a degenerative neurological condition that causes loss of memory and cognition. Numerous variables, such as age, environment, lifestyle, and genetics, influence the occurrence of such neurodegenerative diseases. *Hedychium coronarium* j. Koenig is being used traditionally for various skin problems. In, this study, we evaluated the effect of the anticholinesterase and antioxidant activity of the essential oil of *Hedychium coronarium* j. Koenig by in vitro as well as in vivo tests in Aluminum chloride ($AlCl_3$) induced zebrafish and mouse models of memory impairment. *Hedychium coronarium* j. Koenig significantly retained the spatial and fear memory in zebrafish and mice. Oral administration of the essential oil of *Hedychium coronarium* j. Koenig has neuroprotective effects in the pathogenesis of Alzheimer's disease by regulating lipid peroxidation and reducing oxidative stress in the brains of Aluminum chloride-treated mice and zebrafish, according to biochemical and histopathological studies. These findings suggest that *Hedychium coronarium* j. Koenig essential oil has anti-amnesic effects via acetylcholine esterase enzyme inhibition and modulation of oxidative stress indicators in neuronal cells. As a result, the current research also suggests that *Hedychium coronarium* j. Koenig may be used as a natural, complementary therapy for cognitive problems linked to Alzheimer's disease (AD).

Keywords: Alzheimer's disease, Cognition, memory, Degeneration, *Hedychium coronarium, A*nticholinesterase, Antioxidants.

Introduction

Brain activity results from the manifestation of behavior, subjective experience, and cognitive function. The negative alterations in this network cause a progressive decline in cognition with aging. Alzheimer's disease (AD), a neurodegenerative ailment linked with severe physical and social impairments, causes motor and psychological disruptions that result in memory deficiencies, concentration difficulties, speech loss, personality changes, and depression. According to the World Health Organisation and the World Bank's global burden of disease study, dementia accounts for approximately 4.1% of all disability-adjusted life years. Alzheimer's disease is the most common cause of learning and memory problems, and the efforts involved in discovering a treatment for Alzheimer's disease have never been at such a high point on an international and national scale.

The clinical lesions of AD include an accumulation of amyloid plaques and neurofibrillary tangles (NFTs), which cause a decrease in acetylcholine (ACh) levels within synapses. In addition to these factors, the pathogenesis of AD is also greatly influenced by oxidative stress, inflammatory responses, metal ion dysregulation, and cell regulatory cycle.

Despite the identification of numerous pathogenic variables associated with Alzheimer's disease, very few medicines have been investigated in recent years. In the treatment of Alzheimer's disease, the acetylcholine esterase enzyme (AChE) inhibitor class of medications such as donepezil, tacrine, and rivastigmine are often utilized. These are the only mainstay treatments for Alzheimer's disease however, they have non-selective action, short half-lives, and severe side effects (1).

Therefore, the search for novel, safer AChEIs is necessary. Numerous phytochemicals have a great deal of potential to develop into the AChEIs of the future (2).

AChE inhibitors have been discovered to be naturally occurring in plants. *Hedychium coronarium*'s leaves and rhizomes are used to produce the volatile oils, which contain a variety of compounds including linalool, limonene, 1,8-cineole, terpinene, α-terpineol, and terpinolene, as well as borneol and other volatile oils. AChE is inhibited by 1,8-cineole; α-terpineol reduces brain plaques and enhances neurogenesis and memory; α- and β-pinene and other compounds lessen oxidative stress and exhibit antioxidant activity (3-5). Therefore, the goal of the current study was designed to evaluate the essential oil of the white ginger lily plant (*Hedychium coronarium*) for an AChE inhibition and antioxidant activity in vitro and for cognition-enhancing and neuroprotective effect in $AlCl_3$-induced zebrafish and mice model of memory impairment.

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Materials and Methods

DPPH (2, 2-diphenyl-1-picrylhydrazyl), Ferric chloride, Ascorbic acid, Magnesium Chloride ($MgCl_2$), Tris buffer, Sodium Chloride (NaCl), EDTA, Trichloroacetic acid (TCA), 2-Thiobarbituric acid (TBA), Sodium nitrite ($NaNO_3$), Potassium chloride (KCl), Concentrated HCl was procured from Research-lab Fine Chem. Industries (Mumbai). Pyrogallol and DTNB were procured from Sigma Aldrich, Pvt Ltd, (Bangalore). All the employed chemicals were analytical grade.

Plant material

The rhizomes and leaves of the *Hedychium coronarium* j. Koenig plants were collected in January and February 2023 from the local market, Sangli, Maharashtra (India). The authentication of plant material was done by Prof. M.D. Wadmare, Department of Botany Smt. Kasturbai Walchand College, Sangli.

Preparation of essential oil

Using a modified Clevenger apparatus fitted to a round-bottom flask with a capacity of 500mL, the essential oils from fresh rhizomes and leaves were steam-distilled for two hours. The resulting samples underwent a five-minute centrifugation. Repeating the extraction process as necessary led to the creation of an adequate amount of oil, which was then kept in a refrigerator for later use.

Phytochemical screening of essential oil of *Hedychium coronarium*

Qualitative tests were performed on the essential oil of *Hedychium coronarium* j. Koenig plant to evaluate for the presence of glycoside, flavonoids, alkaloids, steroids, phenols, tannins, amino acids, proteins, and carbohydrates (6).

Quantitative analysis

GC-MS study: Gas chromatography-mass spectroscopy (GC-MS) was done to examine the compositions of essential oil.

Study of In-vitro activities

In vitro antioxidant study (DPPH scavenging activity)

The procedure outlined by Braca et al. (2001) (7)was used to assess the essential oil from *H. coronarium*'s capacity to scavenge DPPH free radicals.

Test tubes were filled with the extract or standard at various concentrations (25, 50, 100, and 500µg/mL), and 3mL of a methanolic solution of DPPH (0.135mM) was then added. The tube was kept in a dark area at room temperature for 30 minutes. A spectrophotometer was used to test the solution's absorbance at 517 nm in comparison to a blank. In place of HC oil or standard solution, a control reaction was also constructed using a methanolic solution of DPPH and other chemicals.

In-vitro AChE inhibition study

AChE inhibition titrimetric assay: The titrimetric assay was used for in vitro studies of AChE inhibition. Five identically sized and weighed fully developed zebrafish were taken away and anesthetized by being instantly submerged in the ice water (2-4°C), immobilizing them and rendering them unconscious. Rapid chilling induces lethal shock due to disruption of osmoregulatory functions. The experimental procedures for animals were in accordance with the criteria specified in the CPCSEA guidelines for fish. Their brains were dissected, weighed, and homogenized using 0.01M phosphate buffer, pH 7.2 in a mortar and pestle under ice-cold conditions. The homogenization was done slowly to prevent the generation of heat. The resulting homogenized solution was then centrifuged at a speed of 5000 rpm for five minutes. The supernatant collected from the centrifuge tubes was then diluted two-fold with distilled water and used as the enzyme source. 0.1M NaOH, 3.8 ml of 0.01M phosphate buffer, 1 ml of enzyme source, and 0.2 ml of acetylcholine substrate were taken in four test tubes. To the first, second, and third test tubes, the plant extract was added at a concentration of 1, 2, and 3 mg/ml respectively. The fourth tube was maintained as a control without the plant extract. All three tubes were incubated for 30 minutes at 28°C. Once the reaction was complete, the enzyme activity was ceased by incubating tubes in a hot water bath. The products formed were titrated against 0.1 M NaOH solution using phenolphthalein as an indicator. The amount of acetic acid released by the action of acetylcholine esterase was calculated which in turn indicates the enzyme activity (8).

AChE inhibition assay

The spectrophotometric technique developed by Ellman et al. was used to measure the cholinesterase inhibitory activity. The previously published method was used to produce AChE from mouse brain homogenate (5). In brief, brain tissues were collected from mice, homogenized in 50mM Tris-HCl (pH 7.4) containing 1.0M NaCl and 50mM $MgCl_2$, and then centrifuged at 5,000 rpm for 20 minutes at 4°C to yield the crude AChE. Acetylthiocholine iodide (ATCI) was employed as a substrate for AChE. Hydrolysis of ATCI by cholinesterase was monitored spectrophotometrically. 200 µL of enzyme solution was mixed with 500 µL of the test sample and kept for 20 minutes at 37°C. Absorbance was measured at 405 nm continuously for 5 minutes at intervals of 1 minute after adding Ellman's reaction mixture (3.5 mL; 0.5 mM acetylthiocholine Iodide, 1 mM DTNB) in a phosphate buffer (pH 8.0, 50 mM). Saline was used in place of the enzyme to estimate a blank reaction, and saline was used in place of the inhibitor to estimate a control reaction. The reference standard for AChE was donepezil. The proportion of cholinesterase enzyme activity that was inhibited was calculated using the formula below (9):

$$\%\ \text{enzyme inhibition} = \frac{\left(\Delta A_{control} - \Delta A_{sample}\right)}{\Delta A_{control}} \times 100$$

Where $\Delta A_{control}$ depicts the rate of change of absorbance for the control reaction;
ΔA_{sample} represents the change in absorbance per minute for test sample reactions.

In vivo study

In vivo experiments were performed to assess the effects of essential oil obtained from *Hedychium coronarium* j. Koenig employs an $AlCl_3$-induced zebrafish and mouse model of memory impairment.

Experimental animals

Swiss albino mice and Danio rerio of either sex were used in the experiment. Mice were obtained from the animal house of Appasaheb Birnale College of Pharmacy, Sangli. Danio rerio of either sex were purchased from fish aquarium from local market. Form B protocol was created and submitted to the Institutional Animal Ethics Committee (IAEC). IAEC approval was obtained prior to the study of the use of animals. The IAEC gave its approval to the experimental protocol with the number IAEC/ABCP/ 08/022-23.

Housing of animals

The fish were kept for an acclimatisation period before the commencement of study, in an acrylic glass house tank with RO water at a density of 30 fish per 5 litres with continuous aeration and fish food as diet. The mice were kept in a room that was well-ventilated and maintained a humidity level of 65-70% while being fed a regular pellet diet and given mineral water. Animals were kept in a 12-hour natural light-dark cycle with free access to food and water. The Committee for Control and Supervision of Experiments on Animals (CCSEA) provided recommendations on how to care for laboratory animals and conduct procedures involving them were followed.

Treatment schedule

1) Danio rerio (Zebrafish) Model

Acute Toxicity Study: The toxicity study of the essential oil of *Hedychium coronarium* Koen was carried out as per the OECD guideline 203. Using the procedure described in the Guideline, a limit test was performed for 96 hours at 100 mg/L to demonstrate that the LC_{50} is greater than this concentration. Seven fish were used in the limit test, and the same number was used in the control group.

Treatment schedule in zebrafish: The fish were divided into six groups with 5 fish in each group (n = 5). The fish were exposed for 96 hours to the inducer and treatment. We had to check the neuroprotective effect of the drug, hence all the groups were induced by $AlCl_3$, and simultaneously the treatment was given to the fish except the normal group which was used for observation of the effect of other environmental factors. The dosing schedule was done as per table No. 1.

Table No.1 : Groups involved in behavioural study

Group I	**Normal (fed by water)**
Group II	Induced by $AlCl_3$ (50µg/L water in tank)
Group III	Donepezil (1.5mg/kg) + $AlCl_3$ (50µg/L water in tank)
Group IV	HC 5mg/L + $AlCl_3$ (50µg/L water in tank)
Group V	HC 10mg/L + $AlCl_3$ (50µg/L water in tank)
Group VI	HC 15mg/L + $AlCl_3$ (50µg/L water in tank)

Physical parameters in zebrafish

Inhibitory avoidance test

An acrylic glass tank with aquarium dimensions of 18 cm x 9 cm x 7 cm was split into two equal white and dark compartments by a sliding door for the inhibitory avoidance test. In the dark region, an electrode was connected to an electrical stimulator. On practice day, zebrafish were placed in the white container. One minute later, the sliding door opened one centimetre above the tank floor, allowing the fish to enter the dark compartment. After the zebrafish entered the dark chamber, the sliding door was abruptly closed, and a 3mA shock current was administered for two seconds. Zebrafish were evaluated the following day (during the testing session), but without receiving an electrical shock. Both the training and testing sessions checked for the latency time to enter the dark chamber.

Colour-biased appetite conditioning T-maze apparatus

Colour-biased appetite conditioning tank the T-maze test was performed using an acrylic glass T-shaped maze measuring 50 cm by 10 cm by 10 cm for the long arm and 20 cm by 10 cm by 10 cm for the two short arms. Zebrafish were inserted into the long arm's terminal during the training session. The sliding door finally opened a minute later, letting the fish swim across to the short arm. Once the fish entered any of the short arms, another sliding door at the junction was closed. The fish were allowed to swim in the short arms for 4 minutes and were watched. The fish was fed if it swam to the green arm. If the fish swam to the red arm, it was turned to training for another three days. During the testing period, fish entered the test by repeating the training process without food in the green arm and without punishment in the red arm. The total number of entries into the green arm and red arm were evaluated.

Biochemical estimation

On the 4th day of the animal study program, the fishes were anesthetized by immobilizing them by submerging them in ice water (2-4°C) immediately followed by taking the fishes out and removing the excess moisture. The fish's brains were separated and homogenized in the extraction buffer (50 mM Tris-HCl buffer containing 1% Triton X-100) by a homogenizer. The resulting fish brain homogenate (FBH) was centrifuged at 2000 rpm for 30 minutes to obtain the supernatant and stored in the fridge for further experiments.

Estimation of protein

Lowry et al. (1951) estimated the protein content of brain homogenate. The procedure employs a bovine serum albumin calibration curve. The results were calculated as mg of protein present per ml of fish brain homogenate (FBH).

AChE activity

The Ellman et al. method determined the AChE enzyme activity in fish brain homogenate (1961). 200

µL fish brain homogenate (FBH) was added to 2.2mL 50mM Tris-HCl buffer and 200 µL DTNB (15mM) and incubated for 15 minutes at room temperature. Then, immediately following the addition of 400 µL of acetylthiocholine iodide (3.75mM) to the mixture, a change in absorbance per minute at 412 nm was noticed. The specific enzyme activity was expressed as U/mg of protein in FBH.

Brain reduced GSH level

rGSH level was estimated in FBH according to the method of Ellman et al. (1959). 0.25 ml FBH was mixed with 1.25 ml phosphate buffer (0.1 M, pH 7.2) and 1.5 ml distilled water.20µL of DTNB was then added, and the mixture was incubated at room temperature for an additional hour. At 412 nm, the mixture's absorbance was measured. The amount of reduced glutathione was calculated with the Beer–Lambert formula using the extinction coefficient value of 13,600 $M^{-1}cm^{-1}$. The results are expressed as µmol rGSH per mg of protein in FBH.

Brain malonaldehyde (MDA) level

MDA level in FBH was determined by using Esterbauer and Cheeseman (1990) method. 0.25 ml FBH and 2.75 ml trichloroacetic acid (TCA, 10% w/v) were mixed and centrifuged to discard the protein pellets. The resulting supernatant was then mixed with 3 ml TBA (0.67% w/v) and heated at 100 °C for 10 minutes in a water bath. At 532 nm, the solution's absorbance was examined. The amount of MDA was calculated using Beer–Lambert's formula with the extinction coefficient value of 1.56×10^5 $M^{-1}cm^{-1}$. The result is expressed as nmol MDA per mg protein in FBH.

Brain Superoxide dismutase (SOD) activity

SOD activity was determined by the method described earlier, based on the enzyme's ability to inhibit pyrogallol's autoxidation. 50µL FBH was added to 1 ml Tris-EDTA buffer and then the change in absorbance per minute was noted immediately after adding 1 ml pyrogallol at 420nm against Tris-EDTA buffer as blank. On the other hand, for the control reaction, the FBH was substituted with distilled water. SOD activities are expressed as units/mg of protein in FBH. The amount of enzyme necessary to prevent 50% of pyrogallol autoxidation from occurring is referred to as one unit of SOD activity.

Brain Catalase (CAT) activity

CAT activity was assessed using Aebi et al.'s (1974) methodology. 0.1mL FBH was added to the cuvette containing 1.9mL of 50mM phosphate buffer (pH 7.0). The reaction was started by adding 1.0mL of freshly prepared 30mM H_2O_2. The rate of decomposition of H_2O_2 by catalase was measured spectrophotometrically from changes in absorbance at 240 nm. A control reaction was also devised with the FBH replaced with 50 mM phosphate buffer (pH 7.0). The results were calculated using the molar extinction coefficient of 43.6 $M^{-1}cm^{-1}$ for hydrogen. peroxide and expressed as units/mg protein in FBH (9).

2) Mice Model

Acute toxicity study: The acute oral toxicity study was carried out using Swiss albino mice weighing 25–30 g in accordance with OECD recommendations 423.

Principle of acute toxicity study: A follow-up test called the Limit test employs up to five animals. A dosage of 2000 mg/kg was used as a control. A different option is 5000mg/kg. Intentionally biased towards rejecting the limit test for substances with LD50 values near the limit dose, a sequential test plan boosts statistical power and is created to be on the safe side. The likelihood of accurately classifying a drug reduces as the real LD50 gets closer to the limit dose, as is the case with any limit test technique (10).

Behavioural study: The animals were divided into six groups with each group having six animals. (n=6) The animals were subjected to dosing for 28 days. The schedule of dosing of mice is give in table no. 2

Table No. 2: Groups involved in animal study

Group I	**Normal - administered by vehicle for 28 days**
Group II	$AlCl_3$ induced for 28 days (300mg/kg)
Group III	$AlCl_3$ (300mg/kg) + Donepezil will be administered to each mouse for consecutive 28 days
Group IV	$AlCl_3$ (300mg/kg) + HC 200mg/kg will be administered to each mouse for consecutive 28 days
Group V	$AlCl_3$ (300mg/kg) + HC 400mg/kg will be administered to each mouse for consecutive 28 days
Group VI	$AlCl_3$ (300mg/kg) + HC 600mg/kg will be administered to each mouse for consecutive 28 days

All animals except the normal group were induced by $AlCl_3$. Group II served as the control group, group III was induced by $AlCl_3$ and treated with donepezil while Groups IV, V, VI were induced by $AlCl_3$ and treated with the drug of study.

Elevated Plus Maze

Two open arms with dimensions 25 x 5 cm crossed with two enclosed arms having dimensions 25 x 5 x 20 cm made up the raised plus maze. A 5-by-5-cm platform served as the connection point for the arms. The equipment was placed in a room with 25 W of low-intensity lighting and raised to a height of 25 cm. At the open end of the elevated plus maze's arm that faces away from the center, each mouse was positioned separately. Transfer latency (TL) is the measurement of how long it took the mouse to enter the enclosing arm. On day 7, TL (L1) was noted. Mice were released into their individual cages after 2 minutes of maze exploration following the measurement of the TL. On the 7^{th}, 14^{th}, 21^{st}, and 28^{th} days, the transfer latencies were tested as L2, L3, and L4.

Morris Water Maze

In the Morris water maze test, a circular acrylic platform measuring 10 cm by 35 cm was positioned 1 cm below the water's surface and a circular pool of 100

cm by 50 cm was filled with water (to a depth of 44 cm). The pool's edge was evenly distributed in the directions of North (N), East (E), South (S), and West (W), and the temperature was kept at 25 ± 2 °C. The platform is continuously positioned in the centre of the northeast (NE) quadrant of the tank, 1 cm below the water's surface, to maintain the water at room temperature. The mice were permitted to move around the submerged platform during the training sessions. Mice were permitted a maximum of 120 seconds to search the submerged platform, and they were allowed to stay on the platform for roughly 30 seconds. Each trial measured the animals' escape latency (time to reach the platform), and only the first session was used to place the animals on the platform if they were unable to reach it within 120 seconds. Five trials each day were conducted on the animals. Mice underwent a probing trial on the eleventh day following the final training session. The training period was extended for ten days in consecutive days. In this experiment, the platform was removed and the mice were free to swim for 120 seconds while looking for the platform that had been set up in earlier training sessions (11).

Biochemical estimation

On the 38th day of the animal study program, the mice were euthanised by using a CO_2 chamber and the brains were isolated. The mice's brains were separated and homogenised in the extraction buffer (50 mM Tris-HCl buffer containing 1% Triton X-100) by a homogeniser. The resulting mice brain homogenate (MBH) was centrifuged at 2000 rpm for 30 minutes to obtain the supernatant and stored in the fridge for further experiments.

The estimation of protein concentration, brain AChE activity, brain reduced glutathione level, brain malonaldehyde level, brain SOD activity, and brain CAT activity was done by using the same procedure used for the biochemical estimation of the zebrafish model.

Histopathological Study

Haematoxylin and eosin staining were used for the histopathologic examination, which was conducted on 2-3 brain samples from each group and inspected under a microscope.

Results

Phytochemical analysis

Qualitative tests were performed on the essential oil of *Hedychium coronarium* j. Koenig plant to detect the presence of saponin glycoside, steroids, phenols, tannins, amino acids, proteins, and carbohydrates.

GC-MS analysis:

The essential oil compositions of *H. coronarium* leaves and rhizomes are presented in Table No. 3. The oil from rhizomes had 21 components, accounting for 86.1% of the total makeup.

Figure 1: Chromatogram of the essential oil from *Hedychium coronarium* rhizomes and leaves by GC-MS analysis

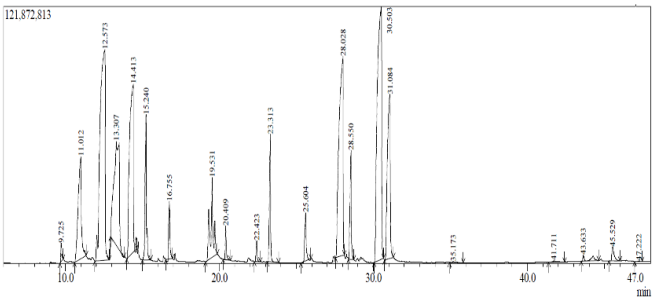


Table 3: Compounds present in the essential oil of *Hedychium coronarium* j. Koenig

Compound	RT	% composition
Benzene, 1-methoxy-4-methyl-	9.725	0.31
Benzyl alcohol	11.012	5.72
Linalyl isobutyrate	13.307	17.49
2-Pyridineethanol, 1-oxide	12.573	9.46
Acetic acid, phenylmethyl ester	14.413	11.02
alpha-Terpineol	15.240	3.37
2-Methylbenzyl alcohol, trifluoroacetate	16.755	1.13
3-Allyl-6-methoxyphenol	19.531	3.52
2-Cyclopenten-1-one, 2-methyl-3-pentyl	20.409	0.54
2,4-Pentadien-1-ol, 3-pentyl-, (2Z)-	22.423	0.36
Butylated Hydroxytoluene	23.313	2.59
2(3H)-Furanone, 5-heptyldihydro	25.604	1.11
Cyclopentanebutanoic acid, 2-oxo-, methyl ester	28.028	13.58
Aromadendrene epoxide	28.550	2.41
Succinic acid, dodec-2-en-1-yl phenethyl ester	43.633	0.39
3-(1,4-Dimethyl-1-vinyl-hex-5-enyl)-1H-indole	45.529	0.55

In vitro antioxidant activity

The antioxidant activity of the *H. coronarium* essential oil was evaluated by the in vitro method of DPPH free radical scavenging activity. The results are shown in the figure. The results of the DPPH radical scavenging assay demonstrated that the essential oil exhibited scavenging activity, which varied with dosage. The IC50 of essential oil of *Hedychium coronarium* was determined to be 167.90268 µg/ml. Furthermore, when compared to the powerful antioxidant ascorbic acid, which had an IC50 value of 21.66 µg/ mL, the activity of *Hedychium coronarium* essential oil proved to be a good antioxidant.

In vitro anti-acetylcholinesterase activity

AChE inhibition assay- titrimetric assay

The experiment, which used acetylcholine as the substrate and AChE from brain homogenate as the enzyme, revealed that plant extracts at 1mg/ml, 2 mg/ml, and 3 mg/ml concentrations reduced enzyme activity. In the titrimetric experiment, the AChE enzyme

was utilized to calculate the amount of acetic acid created from acetylcholine. AChE activity was lowered by 12.90% when the plant extract was used at a concentration of 1 mg/ml. When a 2mg/ml extract was used, the decline was much more extreme, equating to a 25.80% decrease in enzyme activity. When 3mg/ml extract was used, the decline was much more extreme, equating to a 38.7% decrease in enzyme activity. This exemplifies the anti-AChE activity of HC essential oil.

In-vitro AChE inhibition assay

The result in vitro AChE inhibition assay revealed that the HC oil showed AChE inhibition activity with IC50 234.8792 µg/ml indicating that it has moderate AChE inhibitory activity when compared to standard (donepezil) showing IC50 11.0610 µg/ml.

In vivo study

Zebrafish

Acute Toxicity Study

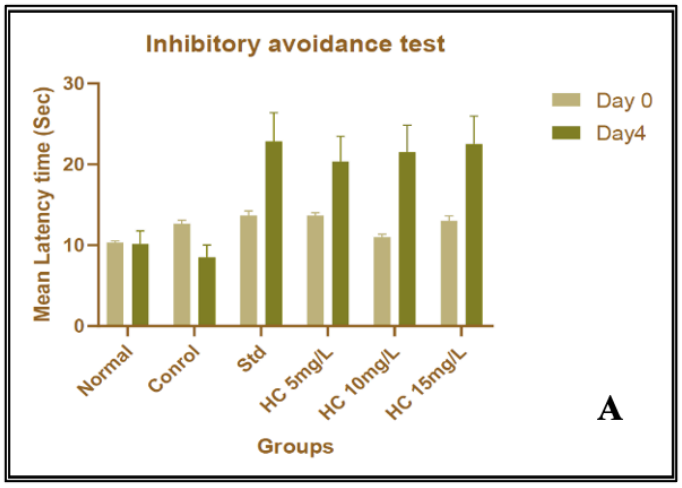
The fish were exposed to the test substances for 96 hours. Changes in behaviour movements such as swimming, pigmentation, and survival were monitored in the treated groups every 24 hours until 96 hours. Mortality rates were recorded at 24, 48, 72, and 96 hours, and the concentration that killed 50% of the fish was calculated. The control group fishes showed no aberrant swimming behaviour, Colour, or survival changes. As a result, the compound's LD50 was determined to be more than 100 mg/L.

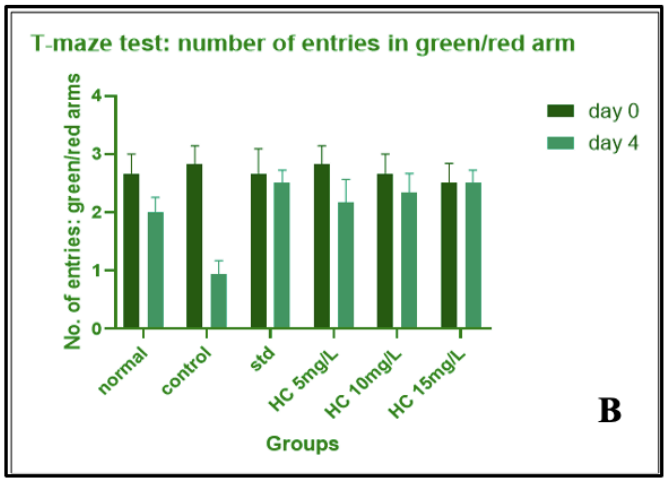
Behavioural Models

Inhibitory avoidance test

In the inhibitory avoidance test, zebrafish given HC oil at doses of 5, 10, and 15mg/ml exhibited significantly shorter latency times of 23.53±604868, 21.53±347631, and 22.53±458543 sec, compared to the $AlCl_3$ group (8.5 ± 1.546322 sec) (Figure 2A). Furthermore, there were no statistically significant changes in HC dosages. These findings revealed that HC was efficacious at all doses against memory impairment caused by $AlCl_3$, particularly fear memory in zebrafish.

Figure 2: A) Effect of $AlCl_3$, Donepezil, and HC oil on the inhibitory avoidance test. Mean latency time in inhibitory avoidance test expressed as mean ± SEM. ****p value <0.0001 compared to the control group. B) Effect of $AlCl_3$, Donepezil, and HC oil on ratio of no. of entries in green and red arm of the Colour-biased appetite conditioning T-maze test. The mean difference of time spent in the green arm and red arm is expressed as mean ± SEM.





Colour-biased appetite conditioning T maze test

The mean difference (green arm/red arm) was computed using the total number of entries in the green arm and red arm tests. For groups with a mean total number of entries greater than one, it was assumed that zebrafish showed more no. of entries in the green arms than the red arms, implying that zebrafish in these groups had strong memory performance. Zebrafish recipients. The HC 5mg/L, HC 10mg/L, and HC 15mg/L treated group showed retention of memory when compared to $AlCl_3$ treated group. The results of HC-treated groups were quite similar to that of the standard (Donepezil) treated group. The results are expressed in graphical format in Figure 2B.

Biochemical estimation

In comparison to the normal group, $AlCl_3$ treatment resulted in considerably increased AChE activity ($p < 0.0001$). $AlCl_3$ and donepezil were administered together, and this significantly decreased AChE activity in zebrafish when compared to the $AlCl_3$ alone group ($p<0.0001$). The activity of AChE in the brain was reduced in zebrafish treated with 5mg/L, 10mg/L, and 15mg/L essential oil of *Hedychium coronarium* when compared to the $AlCl_3$ alone group mice shown in Table 4.

It was found that as compared to the normal group, the administration of $AlCl_3$ fishes significantly (37.49%, $p < 0.0001$) decreased the amount of SOD, an important enzyme involved in the scavenging of superoxide radicals. In comparison to individuals who were just exposed to $AlCl_3$, donepezil, and HC oil (5,

10, and 15 mg/L) administration resulted in a considerable rise in enzyme activity (74.93%, $p < 0.0001$ and 62.38, 74, and 98%, $p < 0.0001$ respectively) (Table 4).

Zebrafish that were only exposed to $AlCl_3$ had a 25% reduction in brain GSH concentration as compared to the control group. In comparison to the $AlCl_3$ control group, donepezil or essential oil treatment (5, 10 or 15 mg/L) enhanced the amount of GSH in the brain (Table 4).

$AlCl_3$ treatment in the current study considerably raised brain MDA levels compared to the control group ($p < 0.0001$). Compared to the $AlCl_3$ control group, donepezil decreased the $AlCl_3$-induced increase of MDA. When compared to the $AlCl_3$-induced group, treatment with HC oil 5, 10, and 15 mg/L significantly ($p < 0.001$) reduced the enhanced MDA content (Table 4).

Table 4: Effect of donepezil or the essential oil of *Hedychium coronarium* on the levels of AChE, MDA, SOD, CAT, and GSH in fish brain homogenates from normal and fish exposed to AlCl3

Sr. no.	Groups	AChE activity (U/mg of protein)	SOD (U/mg protein/Min)	CAT (U/mg protein/ Min)	GSH (µmoles/ Mg of protein)	MDA (mM/mL of brain homogenate)
1	Normal	3.43021 ± 0.0856	45.7142±0.0004	14.575 ± 0.085	0.2009±0.577	0.0142 ± 0.0004
2	Control	13.3485 ± 0.1107	17.142±0.00	1.43 ± 0.008	0.0300±0.0336	0.0245± 0.001
3	Standard	4.0518 ± 0.1097 ****	34.285±0.0001****	15.11 ± 0.087****	0.3369±0.9782	0.013 ± 0.00 ****
4	HC 5mg/L	6.3350 ± 0.1278 ****	28.571±0.00****	9.995 ± 0.064****	0.2682±0.5787	0.015 ± 0.001 ****
5	HC 10mg/L	6.1231 ± 0.1476 ****	34.285±0.00****	10.335 ± 0.063****	0.308±0.815	0.019 ± 0.00***
6	HC 15mg/L	4.264± 0.1124 ****	45.014±0.001****	11.26 ± 0.065****	0.32892±0.954	0.0135 ± 0.0004 ****

Values are expressed in Mean ± SEM and n= 6. ****p<0.0001, ***p<0.1 is considered as criteria for significance when compared with control using one-way ANOVA coupled with Dunnett's multiple comparisons tests

Mice Model

Acute Toxicity Study

Mortality: Mortality was not seen in any group at a dose of 2000 mg/kg body weight.

When mice were given HC oil orally, their body weights changed. The minimal alterations demonstrated that the HC oil had no impact on the animals' normal growth.

A number of wellness factors were used to assess the oil of HC's toxicity. The treatment and control of animals' fur, mucous membranes, eyes, salivation, behavioural patterns, and sleep were all observed as normal. None of the animals displayed any signs of coma, tremors, diarrhoea, or lethargy; instead, they all behaved normally.

An effort has been made in this study to assess how well they can protect neurons, which improves cognition.

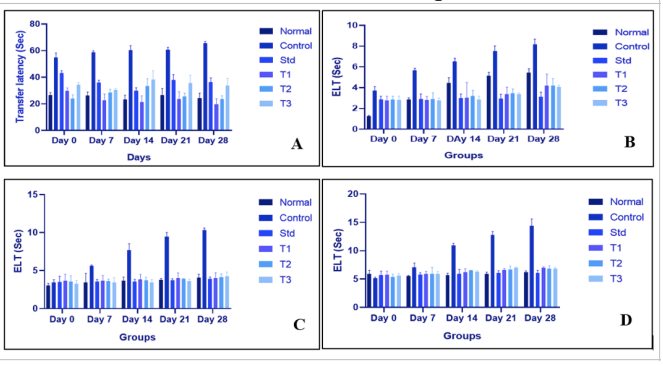
In-vivo studies in mice

Behavioural parameters

Effect of treatment on behaviour on Elevated plus maze apparatus

The effect was measured in the form of transfer latency from the open arm to the closed arm in seconds. The control group treated with $AlCl_3$ showed an increase in transfer latency with an increase in the number of days. $AlCl_3$ had a substantial impact on mice's transfer latency on EPM ($p < 0.001$). Additionally, these $AlCl_3$-induced effects in the animals were greatly diminished by HC. When compared to the normal, standard, and HC groups, the $AlCl_3$-treated mice in the EPM test had a significant ($p < 0.001$) prolongation of the transfer latency in the closed arm. Additionally, the HC groups demonstrated significantly ($p < 0.001$) reduced transfer latency time in the closed arm compared to the $AlCl_3$-treated mice (Figure 3A).

Figure 3: A) Graphical representation of the effect of treatment on transfer latency (TL) of mice on EPM test B) Effect of treatment on escape latency time (ELT) of mice on MWM from the south-east quadrant C) Effect of treatment on escape latency time (ELT) of mice on MWM from the south-west quadrant D) Effect of treatment on escape latency time (ELT) of mice on MWM from the north-east quadrant.



Effect of treatment on Morris water maze test

Mice's capacity to discover the hidden platform in the target quadrant gradually improved as a result of the weekly Morris water maze task testing. The $AlCl_3$-treated animals took longer to locate the platform (longer escape latencies) than the animals in the control group ($p<0.001$), indicating learning and memory impairment. Figures 3B, 3C, and 3D show that groups

treated with HC and donepezil, respectively, both significantly decreased the effects of $AlCl_3$ on escape latency ($p < 0.001$).

Biochemical Estimation

AChE activity and Oxidative biomarkers

In comparison to the control group, $AlCl_3$ treatment resulted in a considerably higher AChE activity (25.71%, $p < 0.0001$). In comparison to mice treated with $AlCl_3$ alone, donepezil and $AlCl_3$ together dramatically reduced AChE activity (84.67%, $p < 0.001$). As shown in Table 5, mice treated with 200, 400, and 600 mg/kg of HC essential oil in combination with $AlCl_3$ showed decreases in AChE activity of 54.14% ($p < 0.0001$), 56.01% ($p<0.0001$) and 80.42% ($p < 0.0001$) compared to animals in the $AlCl_3$ alone group.

When compared to the normal group, $AlCl_3$ considerably (45.08%, $p < 0.05$) increases lipid peroxidation in the brain. In comparison to the $AlCl_3$ control group, treatment with donepezil or HC (400, and 600 mg/ kg) reduced lipid peroxidation in the brain by 80.90%, 80%, and 80% ($p < 0.05$), respectively (Table 7). The GSH level in the brain was lower in the $AlCl_3$-treated group than in the normal group by 17.91% ($p < 0.05$). In comparison to the $AlCl_3$ control group, donepezil (98.5%) or HC 200, 400, and 600mg/ kg considerably raised the lowered GSH level by 92.95%, 94.43%, and 95.27% respectively (Table 5). In comparison to the control group, the $AlCl_3$-treated group's catalase and SOD activity in the brain was significantly reduced (17.94%, $p < 0.0001$ and 47.42%, $p < 0.0001$, respectively) whereas treated groups showed increased the level of CAT and SOD.

Table 5: Antioxidant biomarker levels in different groups of animals

Sr. no.	Groups	AChE activity (U/ mg of protein)	SOD (U/mg protein/Min)	CAT (U/mg protein/Min)	GSH (µmoles/ Mg of protein)	MDA (mM/mL of brain homogenate)
1	Normal	3.430± 0.085	81.171 ± 0.002	16.06 ± 0.106	0.8323 ± 1.688	0.011 ± 0.0001
2	Control	13.348± 0.110	38.493 ± 0.001	2.995 ± 0.017	0.1491 ± 0.320	0.0244 ± 0.00
3	Standard	4.051±0.1097 ****	94.560 ± 0.003 ****	11.47 ± 0.066 ****	0.825 ± 1.699	0.0089 ± 0.00 *
4	HC 200mg/kg (T1)	6.335±0.127886 ****	44.351 ± 0.0015 ****	6.035 ± 0.0457 ****	0.7737 ± 1.244	0.012 ± 0.007 ns
5	HC 400mg/kg (T2)	6.1231±0.1476 ****	77.824 ± 0.0026 ****	10.095 ± 0.058 ****	0.786 ± 1.551	0.0088 ± 0.001*
6	HC 600mg/kg (T3)	4.264± 0.112 ****	95.397 ± 0.003 ****	10.22 ± 0.059 ****	0.7934 ± 1.825	0.0088 ± 0.001*

Values are expressed in Mean ± SEM and n= 6. ****p<0.0001, *p<0.1 is considered as criteria for significance when compared with control using one-way ANOVA coupled with Dunnett's multiple comparisons tests

Figure 4: Graphical representation of effect of treatment on AChE activity of A) Zebrafish and B) Mice. Graphical representation of the Effect of HC essential oil on Antioxidant markers like SOD, CAT, GSH, and MDA in $AlCl_3$-induced neurotoxicity in A) Zebrafish B) mice. Values are expressed in Mean ± SEM and n= 6. ****P<0.0001, *p<0.05 is considered as criteria for significance when compared with control using one-way ANOVA coupled with Dunnett's multiple comparisons tests

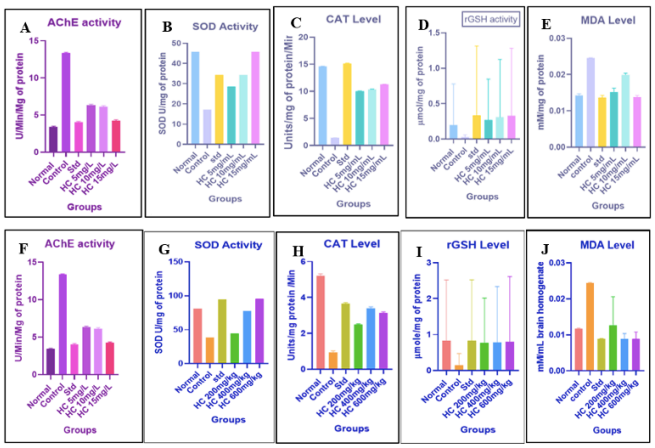
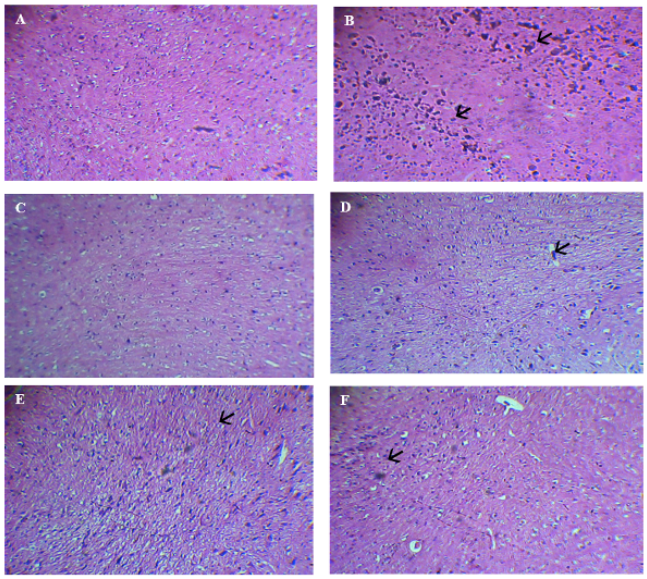


Figure 5: Histopathological observations of mice brain A) brain cells of Normal mice showing healthy glial cells B) brain cells of the Control group. Arrows point neuronal degeneration C) brain cells of Standard group D) brain cells of HC 200mg/kg E) brain cells of HC 400mg/kg F) brain cells of HC 600mg /kg



Histopathological studies

The neuroprotective activity of the essential oil of *Hedychium coronarium* was confirmed by the histopathologic examination of the brain tissue of control and oil-treated animals.

Discussion

Alzheimer's disease (AD) is a neurological condition that affects the elderly and causes a gradual loss of memory and cognition. Numerous etiologic variables in Alzheimer's disease have been identified, with acetylcholine, A-beta, and tau proteins playing critical roles in the pathogenesis (12). According to the cholinergic hypothesis, the steady depletion of acetylcholine in Alzheimer's disease is intimately associated with memory and cognition loss (13). This hypothesis is currently being used to develop new drugs to treat AD. Aβ protein accumulation is considered the central cause in the pathogenesis of AD as it is highly produced in AD, getting aggregated results into neurotoxicity and neurological damage (14). It has also been discovered that oxidative stress is linked to memory and cognition decline. Due to the multifactorial character of Alzheimer's disease, a multitargeted therapy rather than a single-targeted treatment has been proposed and investigated as an AD therapeutic (15).

Many recent efforts have been focused on medicinal herbs, which are used in traditional medicine to treat Alzheimer's disease and similar diseases. Scientists are always looking for plant-derived AChE inhibitors that can be utilized to treat Alzheimer's disease. Our current study attempted this using an essential oil of the plant *Hedychium coronarium* (HC). *Hedychium coronarium* is a perennial erect herb with a wide range of biological activity, including considerable cytotoxic and anti-inflammatory, anti-tumor, anti-allergic, analgesic, and antihelmintic properties (5).

The volatile oil from *Hedychium coronariums*' leaves and rhizomes extracted, which contain a variety of compounds including linalool, limonene, 1,8-cineole, 2,8-diene, terpinene, α-terpineol, and terpinolene, as well as borneol and other volatile oils. AChE is inhibited by 1,8-cineole; α-terpineol reduces brain plaques and enhances neurogenesis and memory; α-and β-pinene and other compounds lessen oxidative stress and exhibit antioxidant activity (3-5).

The essential oil from the *Hedychium coronarium* shows the presence of saponin glycoside, steroids, phenols, tannins, amino acids, proteins, and carbohydrates. The GC-MS of the essential oil was done and various constituents were detected including well-known compounds such as alpha terpineol, benzyl benzoate, linalyl isobutyrate, 1,8-cineole, α-pinene all of which are found in essential oils.

In Vitro Studies

Antioxidant activity

The in-vitro antioxidant activity of *Hedychium coronarium* essential oil was evaluated by using DPPH free radicals scavenging capacity. The results showed that the essential oil has concentration-dependent DPPH radical scavenging action, with efficacy increasing as concentration increases.

The oil then was investigated for in-vitro AChE inhibition assay.

In-vitro titrimetric assay

The results of the in vitro technique utilizing zebrafish brain homogenate. The result showed a significant decrease in AChE activity as the concentration of the test drug increased. This may indicate that the essential oil of Hedychium coronarium shows AChE inhibitory activity.

In vitro AChE inhibition assay

AChE is an enzyme that degrades the neurotransmitter acetylcholine in the neurological system. Since the cholinergic neurotransmitter acetylcholine declines in Alzheimer's disease, inhibitors of cholinesterases have been demonstrated to increase the activity of surviving cholinergic neurons in people with Alzheimer's disease, resulting in improved memory and cognition (17). The result in vitro AChE inhibition assay revealed that the HC oil showed AChE inhibition activity with IC50 234.8792 µg/ml indicating that it has moderate AChE inhibitory activity when compared to standard (donepezil).

Acetylcholine plays an important role in learning and memory (18). Acetylcholinesterase terminates impulse transmission at cholinergic synapses by rapidly hydrolyzing the neurotransmitter ACh into acetate and choline (19). Hence, acetylcholinesterase inhibition is one of the key treatments of AD.

The deterioration in memory and cognitive abilities has also been linked to oxidative stress. Because Alzheimer's disease (AD) is so complicated and diverse, there is a growing interest in creating multi-targeted medications as viable therapy options rather than single-target drugs. This strategy tries to target the many elements involved in the course of Alzheimer's disease and represents a possible therapy option (6).

In Vivo studies

$AlCl_3$-induced memory deficit

Aluminum causes brain poisoning which is recognized as a danger factor for neurological disorders. Furthermore, Al may inhibit antioxidant enzyme activity, altering brain neurochemistry and causing DNA damage in the brain (20). $AlCl_3$ has been shown to increase reactive oxygen species (ROS) and inflammatory mediators, both of which contribute to neurodegeneration. The brains of zebrafish and mice exposed to $AlCl_3$ undergo behavioral, biochemical, and neurochemical alterations (21).

Zebrafish Model

The fear memory is associated with the medial pallium of zebrafish which is similar to the amygdala in the human brain (22). An inhibitory avoidance test was done to check the fear memory of zebrafish. The inhibitory avoidance test examined the latency time to enter a dark compartment to investigate the influence of HC oil on learning and memory. Aluminum inhibits antioxidant enzyme activity, altering brain neurochemistry and causing DNA damage in the brain (20). Thus, the mean difference in latency time between testing and training days of the $AlCl_3$-treated group (control group) was lowered. The Donepezil and HC oil-treated groups at all doses significantly increased the mean difference in latency time compared to the control group. Donepezil was used as a positive control and had a memory-enhancing effect. The HC treatment showed a significant retainment of fear memory than that of the $AlCl_3$ treated group.

Colour-biased appetite conditioning T maze test was done to evaluate the spatial memory in zebrafish. The spatial memory is related to the lateral pallium in zebrafish. The lateral pallium is analogous to the hippocampus in mammals, and is responsible for remembering the learning process (10). The red and green were employed in our zebrafish learning and recognition experiment, with feeding on the green arm to boost learning and memory, a process known as synaesthesia. The T-maze test was used to count the number of entries in the green and red arms as a signal for measuring zebrafish learning and memory (22).

According to this study, a total number of entries in a green and red arm ratio greater than one indicated that zebrafish had strong memory performance. The donepezil group recorded the highest total number of entries ratio. The HC-treated groups showed a similar effect to the standard (donepezil) group suggesting that HC was effective in spatial learning and recognition than the control group due to activity that may inhibit the AChE enzyme.

The AChE activity was determined by biochemical estimation and it revealed that AChE activity was significantly lowered in the standard (Donepezil) and HC-treated group as compared to the control group. This may suggest that the oil showing anti-AChE activity. AChE inhibitory activity can be due to the presence of 1,8 cineole in the oil (3).

The oxidative stress markers were tested to see if HC enhances antioxidant capacity in vivo. Exposure to $AlCl_3$ resulted in a significant decrease in the specific activities of the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), and reduced glutathione (GSH) along with increased level lipid peroxidation (malondialdehyde-MDA). Furthermore, HC therapy (5 mg/L, 10 mg/L, 15 mg/L) significantly reversed the decline in SOD, CAT, and GSH and reduced the level of MDA.

This may be due to the presence of α-terpineol, α- and β-pinene which are reported to have antioxidant activity (4,5).

Mice Model

The neuroprotective effect of HC essential oil on learning and memory impairment was evaluated in an $AlCl_3$-induced mice model of memory impairment.

Since the mice model captures the important features of AD like cholinergic deficit and oxidative stress, we have selected this mode l(23). Aluminium is a neurotoxic metal that can cause neurological disorders. Several investigations have shown that $AlCl_3$ is associated with biochemical and neurological consequences related to cognitive deficits comparable to those seen in Alzheimer's disease (24). The improvement in learning and memory was measured by using the Elevated Plus maze task and the Morris water maze task.

The Elevated Plus Maze apparatus is used to test short-term memory (25). In the EPM task, it was found that the transfer latency from the open arm to the closed arm was recorded. The transfer latency (TL) was decreased in the donepezil (standard) treated group and the HC essential oil-treated group as compared to the control group. This indicated that the HC showed a memory-protective effect.

Morris Water Maze was used to test the nootropic activity, and the results point to an improvement in animal performance when looking for hidden platforms. Hippocampal-dependent spatial learning ability is frequently assessed using the Morris water maze screening task. The Morris water maze test made it easier to compare working memory and reference memory simultaneously. Reduced escape latency over time reflects learning in terms of long-term or reference memory. Long-term memory impairment or weakening was observed in the $AlCl_3$-treated group.

In our findings, the concentration of AChE activity was remarkably increased in control compared to normal, indicating memory dysfunction. Treatment groups like Standard (Donepezil), HC 200mg/kg, HC 400mg/kg, and HC 600mg/kg showed a decrease in acetylcholinesterase enzyme which indicates an enhancement in memory. This suggests that the memory-enhancing effect of the treatment may be due to the inhibition of the AChE enzyme. The results were in accordance with that of previous work (6).

The generation of free radicals, as well as the formation of destructive byproducts of oxidative metabolism, produce oxidative stress, which is known to cause organic damage to the biological system. It is widely accepted that boosting antioxidant levels might delay or reverse free radical damage to neurons. Oxidative stress in the brain produces oxygen free radicals such as hydroxyl radical, superoxide anion, and hydrogen peroxide, which act on polyunsaturated fatty acids (PUFA) in the brain. Many antioxidant enzymes, such as superoxide dismutase (SOD), reduced glutathione (rGSH), and catalase (CAT), help to reduce oxidative stress. These findings align with the results reported in prior research (6).

The histopathological study revealed that the standard and HC-treated group showed a higher number of healthy glial cells and neurons whereas the control group showed degenerated glial cells and neurons.

In the present study, we found that reduction of antioxidant enzymes such as rGSH, SOD, and CAT in the brain of mice models, and an increase in oxidized molecules such as the MDA in the control group. These effects were reversed by treatment with the donepezil and HC, suggesting that the HC is capable of reducing oxidative stress in $AlCl_3$-treated mice. Taken together, our results indicate that the HC contributes to improving the ability of learning and memory through inhibition of AChE and oxidative stress.

Conclusion

The essential oil of *Hedychium coronarium* j. Koenig has been investigated for its possible therapeutic benefits in cognitive deficit models in mice and zebrafish. A comprehensive study that included in-vitro antioxidant assay, in-vitro AChE inhibition assays, behavioral assessments, biochemical analyses, and histopathological examinations revealed that *Hedychium coronarium* treatment has neuroprotective and cognition enhancement benefits in the etiology of Alzheimer's disease (AD). This neuroprotection is accomplished by controlling lipid peroxidation, minimizing oxidative stress, and decreasing neuronal cell death. Also, the cognition-enhancing activity is due the lowered acetylcholinesterase activity.

The broad range of essential oil of *Hedychium coronarium* 's effects, as well as its ability to modulate disease progression, may offer significant promise for the future of Alzheimer's medication therapy. It is worth noting that *Hedychium coronarium* is non-toxic and inexpensive. Nonetheless, its practical utility is dependent on aspects such as its efficacy, specific modes of action, and bioavailability.

As a result, additional research efforts are required to determine the underlying processes responsible for the reported impacts. Furthermore, other experimental models must be used to test these findings and bridge the gap between laboratory discoveries and clinical applicability.

Abbreviations

AD- Alzheimer's disease; NFTs- Neurofibrillary tangles; Ach- Acetylcholine; AChE - Acetylcholine esterase; AChEIs- Acetylcholine esterase inhibitors; HC- *Hedychium coronarium*; DPPH - 2, 2-diphenyl-1-picrylhydrazyl; $MgCl_2$ - Magnesium Chloride; NaCl - Sodium Chloride; EDTA- Ethylenediamine tetraacetic acid; TCA - Trichloroacetic acid; TBA - 2-Thiobarbituric acid; $NaNO_3$ - Sodium nitrite; KCl -Potassium chloride; DTNB- 5.5'-dithiobis-(2-nitrobenzoic acid); $AlCl_3$ - Aluminum chloride; GC-MS - Gas chromatography-mass spectroscopy; IAEC - Institutional Animal Ethics Committee; CCSEA - Committee for Control and Supervision of Experiments on Animals; TL - transfer latency; EPM – Elevated Plus Maze SOD- Superoxide dismutase; rGSH - reduced glutathione; CAT – catalase; MDA – malondialdehyde; PUFA - polyunsaturated fatty acids, TL- Transfer latency; ELT – Escape Latency Time; ANOVA- Analysis of Variance

Conflict of Interest

The authors have no conflicts of interest regarding this investigation**.**

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