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Neuroprotective Activity Of Hydroalcoholic Extract Of Fruits Of Punica Granatum Against Aluminium Chloride Induced Alzheimer In Rats.

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ABSTRACT

This study investigated the neuroprotective effects of hydroalcoholic extracts of fruits *Punica granatum* (HAEPG 100 and HAEPG 200 mg/kg) on learning and memory in rats with Alzheimer's disease (AD) induced by aluminium chloride ($AlCl_3$). All Wistar rats in groups II to IV received the appropriate treatment dosages (100/200 mg/kg) 30 minutes prior to the oral administration of aluminium chloride (100 mg/kg), which was administered daily for 42 days. Rats' gross behavioural activity was assessed using an open field arena (OFA) on days 20, 21, and 42 after the beginning of the administration of aluminium chloride. Line crossing, centre square entries, rearing, and grooming were used to score behavioural performance.
Morris Water Maze (MWM) and the Elevated Plus Maze (EPM) were used to assess cognitive performance. On days 19 and 20, MWM-rats were trained to swim to a platform in a circular pool. It was determined how long it took to get to the visual platform (acquisition latency). The first retention latency (1 RL) (21 day) and the second retention latency (2 RL) (42 day) were measured as the amount of time it took to identify the hidden platform after the first day of aluminium chloride administration. On day 20, EPM-rats were trained, and each rat was placed at the end of an open arm. The initial transfer latency, which measures the time taken by the rat to go from the end of the open arm to either of the closed arms, was then recorded (ITL). The rats were examined for retention latency on days 21 and 42, and the results were referred to as first RTL and second RTL, respectively. According to data from OFA, MWM, and EPM, the corresponding treatment groups (100/200 mg/kg, p.o.) significantly defended against Alzheimer's disease caused by aluminium chloride.

Keywords: HAEPG, OFA, MWM, EPM

INTRODUCTION

Cognitive abilities are thought to be rather steady until a person reaches the age of 60, at which point they start to decline, especially between the ages of 60 and 80. There is some evidence to suggest that brain function can begin to decline as early as age 45. Additionally,

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AD and other neurodegenerative diseases for which there is currently no cure are more likely to develop in older people. (**1**)

Amyloid beta (AB) and tau protein buildup in the brain is linked to the neurodegenerative condition Alzheimer's disease (AD), which causes behavioral changes as well as progressively worsening cognitive and functional deficits. (**2**)

The basal forebrain, amygdaloid body, hippocampus, entorhinal cortex, neocortex, and several brain stem nuclei are the areas of the brain that are most susceptible to Alzheimer's disease. (**3**)

The long-standing free radical hypothesis of ageing centres on reactive oxygen species (ROS), which build up with ageing and harm vital cell components such the nucleus, mitochondrial DNA, membranes, and cytoplasmic proteins. The imbalance between the production of free radicals and ROS has been suggested by several authors over a long length of time to play a part in the emergence of the majority of neurodegenerative diseases, including AD. (**4**)

Globally, 66 million individuals are expected to have dementia by 2030, and that number is expected to climb to 115 million by 2050. The most common cause of dementia in elderly people is Alzheimer's disease. Alzheimer's disease can be categorised clinically into early-onset (patients under 65) and late-onset (patients over 65), whereas it is identified pathologically by the presence of plaques of amyloid peptides and intraneuronal tangles of hyperphosphorylated forms of the microtubule associated protein tau (MAPT). (**5**)

For AD, there are currently no efficient or disease-modifying drugs. Amyloid buildup, neuroinflammation, tau accumulation, neuronal degeneration, cognitive decline, and the emergence of behavioural and mental problems are all clinical and molecular events that accompany the course of AD. Clinical experiments that are intended to stop these incidents are being evaluated. Due to recent failures in anti-amyloid studies, research focus has shifted to people with prodromal or preclinical phases and positive diagnostic biomarkers. Since the amyloid theory has been challenged, there were much fewer anti-amyloid phase 3 trials in 2019. Phase 1 and phase 2 trial aims might vary widely, but trends show that neuroprotection and antineuroinflammation are being targeted more frequently in each phase. (**6**)

At the end of the day, AD therapies have a lot of potential. A effectively designed mitochondrial antioxidant offers the fragile, ageing neuron a protective barrier against the oxidative cascade of neurodegeneration if it is given in the right amounts. Importantly, however, once sizable amounts of oxidative damage accumulate within the cell and the secondary pathologies of AD become apparent, any hope of reversing the course of the disease remains beyond the scope of straightforward antioxidant therapy. This is true regardless of how effective such therapies may be for those who have not yet entered the neurodegenerative cascade mentioned above. Because of this, while such a preventative therapy approach is suitable for the young-to-middle-aged population, it is not beneficial to those who exhibit the "oxidative steady state" within damaged cells. Secondary or symptomatic therapies must then be started at that time. (**7**)

Punica granatum L., a member of the Punicaceae family and the Punica L. genus, is an ancient fruit that is indigenous to Central Asia and can be found there as well as in the Mediterranean region, the Middle East, Iran, Turkmenistan, and northern India. (**8**)

Fructose, sucrose, and glucose are all found in good amounts in pomegranate juice. Ascorbic

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acid, citric acid, fumaric acid, and malic acid are only a few of the simple organic acids that are present. Additionally, it has minor concentrations of each amino acid, particularly proline, methionine, and valine. Polyphenols are abundant in both the juice and the peel. The two greatest classes, tannins and flavonoids, suggest the pomegranate's medicinal potential due to its peculiar antioxidant and preservation properties. (**9**)

In order to measure the phenolic components and antioxidant activity of 10 juices made from Iranian-harvested pomegranate arils and entire fruits, Akhavan and colleagues conducted a study in 2015. They demonstrated that juices obtained solely from the arils had significantly lower quantities of phenolics than juices obtained from the entire fruit, which in turn had higher antioxidant activity. (**10**)

Because the hydroxyl groups in pomegranate polyphenols readily give hydrogen to reducing substances, pomegranate polyphenolic compounds go through redox processes. The phenolic compounds' capacity to neutralise free radicals or chelate metal cations accounts for their antioxidant effect. (**11**)

Materials and Methods

Plant Material

Pomegranate (*Punica granatum*) field workers in Kopargaon, Maharashtra, India, harvested ripened fresh pomegranate fruits in December 2016. A 90% (v/v) hydroalcoholic (ethanolic) solution was used to extract the peel after it had been physically separated (3 kg) and macerated at room temperature for five days in a dark space. The hydroalcoholic extracts were filtered, dried at 40 °C, and then stored at -10 ^{0}C in a freezer. The pulps were similarly removed, then the peel extract was combined with it. Rats were given this crude extract to test the extract's neuroprotective effects in rats exposed to aluminium chloride. (**12**)

Animals

Male Albino Wistar rats (180–300 g) were procured from National Institute of Biosciences, Bhor, Pune and maintained at 12/12 h light/dark cycle, 24 ^{0}C temperature and 60 % humidity with food and water *ad libitum*. The experimental protocols comply with the Compendium of CPCSEA, New Delhi, 2018 and were approved by the Animal Ethics Committee of the LSHGCT's Gahlot Institute of Pharmacy (Reg. No: 1485/PO/Re/S/11/CPCSEA, Proposal No. GIP/IAEC/2021/13/1).

Chemicals

Aluminium chloride, Bovine Serum Albumin, Thiobarbituric acid, DTNB (5,5′-dithiobis-(2-nitrobenzoic acid) were purchased from Sigma - Aldrich, Mumbai, India and used in this study. All other chemicals used were of analytical grade.

Dosage Fixation

We conducted a pilot investigation using two different dosages of HAEPG (200 and 300 mg/kg) in an $AlCl_3$-induced experimental model of AD to determine the therapeutic dose. According to behavioural (Morris water maze and Elevated plus maze tests), oral treatment of AlCl3 over 42 days led to memory impairment.

It was found that the treatment doses of 200 and 300 mg/kg reduced Al levels and caused equivalent amounts of neuroprotection. As a result, we have decided to proceed with the trial at the lesser dose of 100 mg/kg rather than the larger dose of 300 mg/kg.

Experimental Design

The solutions of aluminium chloride and all medications being tested for oral administration

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were freshly prepared at the start of each trial, and the total volume consumed is 0.5 ml/100 g body weight. All groups, excluding Group-I, received 100 mg/kg of aluminium chloride orally every day for 42 days. The aluminium chloride was dissolved in sterile water. Animals will be placed into the following groups, each consisting of seven rats:
I (Control) Group: included healthy, normal rats that were administered vehicle P.O. (Saline) at a dose of 0.5 mL per 100 g of body weight each day for 42 days. The appropriate doses of aluminium chloride (100 mg/kg), given to each group from II to IV 30 minutes after to oral administration of treatment doses, each day for 42 days.
Group II (Untreated AD): Vehicle P.O. (Saline) 0.5 mL/100 g body weight.
Group III (HAEPG 100 mg/kg): Oral dose of HAEPG 100 mg/kg.
Group IV (HAEPG 200 mg/kg): Oral dose of HAEPG 200 mg/kg.
On day 43 rats were sacrificed, brains were excised and used for the biochemical estimation.

Biochemical assessment

Measurement of brain Lipid peroxidation (**13-15**)

The Ohkawa et al.-described thiobarbituric acid (TBA) reaction method was used to determine the quantity of lipid peroxides. The malondialdehyde (MDA) generated in peroxidizing lipid systems is supposedly measured by the TBA test. Results, are expressed in nmoles/mg protein.

Estimation of brain Nitrite (**16**)

The spectrophotometric detection of nitrite produced by the spontaneous oxidation of NO under physiological conditions using the Griess diazotization process. The detection limit for this method is 1.0 μM nitrite. Through the catalytic reduction of nitrate to nitrite, the Griess reaction can also be used to examine nitrate. Sulfanilic acid reacts with nitrite in acid solution to quantitatively transform it into a diazonium salt. Following this, the diazonium salt is joined with N-(1-naphthyl) ethylenediamine to create an azo dye that can be quantitated spectrophotometrically using its absorbance at 548 nm.
Results are given as nmoles/ mg protein when reading nitrite concentrations according to the absorbance of experimental samples from the standard plot.

Estimation of brain Reduced glutathione (**17**)

Reduced glutathione is determined by the technique of (Ellaman 1959). When glutathione reacts with DTNB, a yellow chromophore is produced that is detected spectrophotometrically at 412 nm. The standard curve was constructed using known levels of GSH, and the GSH concentrations in the samples were expressed as ug/mg protein.

Estimation of brain Superoxide dismutase (SOD) (**18**)

A initial line of defence against free radical damage is provided by superoxide dismutase, which scavenges the super oxide (O*2). The super oxide anion (O*2) is dismutated by the SOD family of metallo enzymes into molecular oxygen and hydrogen peroxide. The ability of SOD to prevent epinephrine's spontaneous oxidation to adrenochrome was measured.
Units (U) of SOD activity per mg of protein are used to express the results.

Estimation of catalase (CAT) (**19**)

H_2O_2 exhibits a continuous increase in absorption with decreasing wavelength in the U.V. range. The fast conversion of hydrogen peroxide to water is catalysed by catalase. The

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decline in absorbance at 240 nm can be directly related to the oxidation of H_2O_2. The amount of catalase activity is determined by the variation in absorbance per unit.

Estimation of brain Glutathione S-transferase (**20**)
Glutathione S-transferases start the detoxication alkylating agents by catalysing their interaction with glutathione's -SH group. This neutralises the electrophilic sites of the alkylating agents' products and increases their water solubility. The final product, mercapturic acid, is assumed to be produced by further metabolising glutathione conjugates by cleaving the glutamate and glycine residues, then acetylating the resulting free amino group of the cysteinyl residue.
The quantity of enzyme required to catalyse the production of 1 mole of product per minute under the conditions of the particular assay is referred to as a unit of activity.

Estimation of brain acetylcholinesterase (**21**)
George Ellman created the Ellman's method, or the technique for estimating AChE activity, in 1961. Thiocholine is allowed to react with the SH reagent 5, 5-dithio-bis-(2, nitrobenzoic acid) (DTNB), which is reduced to thionitrobenzoic acid, a yellow anion with an absorbance maximum at 412 nm, as a result of the acetylcholine iodide by AChE being broken down. Thionitrobenzoic acid has an extinction coefficient of 1.36 10^4 molar/cm. Utilizing a UV spectrophotometer, the amount of thionitrobenzoic acid is identified and used as a direct indicator of the AChE activity, expressed in nmoles/min/gram.

Estimation of brain total proteins (Modified Biuret, End Point Assay) (**22**)
Alkaline solution's cupric ions and protein peptide bonds combine to generate a coloured chelate, whose absorbance is measured at 578 nm. Sodium-Potassium Tartrate, a component of the Biuret Reagent, aids in preserving the solubility of this complex at an alkaline pH. The sample's total protein concentration, measured in g/dL, directly correlates to the absorbance of the final colour.

Estimation of brain Aluminium (**23**)
The brain's cortex and hippocampus were used to examine the aluminium using the Zumkley wet acid digestion procedure. The tissue was treated with 2.5 ml of a perchloric acid/nitric acid (1:4 by volume) solution before being submerged in a sand bath at 40°C to 50°C for 44 hours until a white ash or residue was produced. Then, 2.5 ml of 10 mM nitric acid was used to dissolve the residues. This sample was put in the sample holder of an atomic absorption spectrophotometer (in liquid form). The total aluminium content was calculated in µg/g of tissue.

Behavioral studies
Open Field Test-(**24**)
This was constructed of white plywood, had walls with height 36 cm , and measured 72 by 72 cm. Rats could be seen within the device since one of the walls was made of Plexiglas, which is translucent. The green lines that had been marked with a marker on the ground could be seen through the transparent Plexiglas floor. The lines divided the floor into sixteen 18 by 18 centimetre squares. A central square (18 cm x 18 cm) was drawn in the middle of the open field. The centre square is employed because some rat strains have high locomotor activity and regularly cross the lines of the test chamber during a test session. The central square also has enough space surrounding it to distinguish it from the nearby neighbourhoods. Rats were

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left in the centre or a corner of the open field for five minutes to investigate the apparatus. Each rat's exploration of the wall's 12 neighbouring outer squares and central square was counted separately. a variety of grooming actions, including licking the fur, washing the face, and scratching. The number of animals that were raising, or standing on their hind limbs with their forelegs periodically leaning against the wall, sniffing, and surveying their surroundings, was counted for five minutes. After the five-minute test, the rats were returned to their cages, and the open field was cleaned with 70% ethyl alcohol and allowed to dry in between tests.
On days 20, 21, and 42 following the oral dose of aluminium chloride, rats were repeatedly submitted to OFA for the measurement of gross behavioural activity.

Assessment of cognitive performance by the Morris water maze task-(**25**)
The ability to learn and retain information was tested using the Morris water maze. Two threads fixed at right angles to one another divided a large circular pool (150 cm in diameter, 45 cm in height, filled to a depth of 30 cm with water at 28 $\pm 1^0$C), which served as the basis of the Morris water maze, into four equal quadrants. The pool was located in a room with plenty of coloured light cues and good lighting. These extraneous cues act as the reference memory and endure throughout the research. A circular platform with a diameter of 4.5 cm was placed in one pool quadrant during the acquisition phase, 1 cm above the water. The same platform was positioned 1 cm below the water's surface for the retention phase. During the evaluation of the two phases, the platform's position was not altered in any quadrant. Each animal underwent four consecutive trials, each separated by five minutes. Each trial's drop point was a different quadrant of the pool, and the animal was gently lowered into the water there. Then, the animal had 120 seconds to find the platform. The animal was then given 20 seconds to remain on the platform. The animal was escorted to the platform and given 20 seconds to stay there if it didn't get there in the allotted 120 seconds.

Maze acquisition phase (training)-
The animals had two days of training in a row. Each rat was placed into the water during the acquisition phase in one of four beginning positions, the order of which was chosen at random. It was determined how long it took to get to the visual platform (acquisition latency).

Maze retention phase (testing for retention of the learned task)-
Animals were tested twice after the acquisition phase; to gauge memory retention, each animal was released at random from one of the pool's sides facing the wall. First retention latency (1st RL) and second retention latency (2nd RL) are terms used to describe the time lag to locate the underwater platform, respectively.
Following oral treatment of aluminium chloride, rats were tested for acquisition latency on days 19 and 20, and first and second retention latency on days 21 and 42.

Elevated plus maze-(**26**)
On day 20, each rat's ITL was comparatively constant and didn't exhibit any appreciable fluctuations. Within 60 seconds, every rat entered the arm that was closed. After training, rats in the Control, Untreated (Aluminum Chloride-induced), and chronic HAEPG treatment groups entered the closed arm quickly, and the retention transfer latencies (first RTL and second RTL) to enter the closed arm on days 21 and 42 were shorter than the ITL on day 20 of each group, respectively.
The retention transfer latencies on days 21 and 42 relative to the ITL on day 20 did not alter in Aluminium chloride-induced rats, indicating a severe memory impairment. In contrast,

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these rats performed poorly throughout the experiment. The retention latencies on days 21 and 42 after the administration of the aluminium chloride were considerably decreased by the chronic administration of HAEPG therapy (each 100 and 200 mg/kg) (P< .05 compared to the Untreated group).

Statistical Analysis

Values are expressed as the mean ± SD. The behavioral assessment data and biochemical estimations were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett test. P< .05 was considered significant.

Results

Effect of HAEPG on lipid peroxidation, nitrite, reduced glutathione, glutathione S-transferase, superoxide dismutase, catalase activity and total protein in whole brains of rats treated with aluminium chloride

In comparison to control group, chronic treatment of aluminium chloride increased MDA and nitrite concentration, depleted reduced GSH, and decreased glutathione S-transferase, superoxide dismutase, catalase, and total protein levels in the whole brain (P< .05). In contrast to untreated rats, chronic HAEPG administration to the rats (100 and 200 mg/kg) significantly decreases oxidative damage (as shown by reductions in MDA, nitrite concentration, and reduced GSH, and increased glutathione S-transferase, superoxide dismutase, and catalase activities). (Table 1)

Groups	MDA (nmol MDA/ min/mg protein)	Nitrite (µmol/mg)	GSH (µg/g)	SOD (unit/ mg)	Catalase (units/ mg protein)	GST (µmol/ ml/min)	Total protein (g/dl)
Control	4.89**** ± 0.15	244.3**** ± 7.8	130.8**** ± 2.02	45.02**** ± 2.07	2.66**** ± 0.04	97.0**** ± 5.21	4.89**** ± 0.15
Untreated	7.99 ± 0.09	625.7 ± 17.7	42.7 ± 0.88	15.13 ± 1.27	1.39 ± 0.01	34.5 ± 0.80	7.99 ± 0.09
HAEPG 100	7.71** ± 0.12	526.2**** ± 7.5	74.2**** ± 1.78	20.91**** ± 0.30	1.70**** ± 0.02	52.1**** ± 1.04	2.40*** ± 0.24
HAEPG 200	6.08**** ± 0.12	461.5**** ± 22.7	90.0**** ± 1.60	30.63**** ± 0.50	1.83**** ± 0.01	73.3**** ± 1.31	2.58**** ± 0.52

Effect HAEPG on lipid peroxidation, nitrite, reduced glutathione, glutathione S-transferase, superoxide dismutase, catalase activity and total protein in whole brains of rats treated with aluminium chloride (Table 1)

Effect of HAEPG on acetylcholinesterase (AChE) activity in aluminium chloride treated rats

When compared to control rats, chronic aluminium chloride administration greatly raised the AChE activity throughout the entire brain. However, when compared to untreated rats,

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chronic HAEPG (100 and 200 mg/kg) given to the rats significantly reduced AChE activity ($P< .05$). (**Fig. 1**).

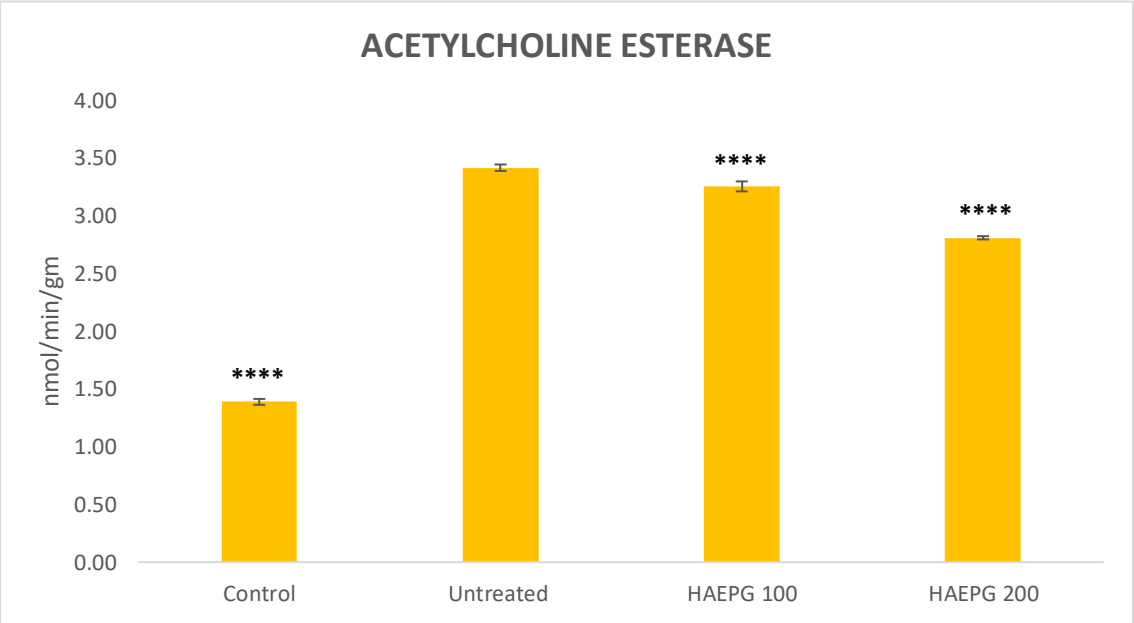

Fig. 1, Effect of HAEPG on acetylcholinesterase (AChE) activity in aluminium chloride treated rats.

Effect of HAEPG on aluminium concentration in aluminium chloride treated rats

Rats receiving an aluminium chloride therapy had considerably more aluminium in their cortex and hippocampus than control group. But compared to untreated rats, chronic HAEPG (each 100 and 200 mg/kg) therapy significantly reduced the level of aluminium in the cortex and hippocampus ($P< .05$). (**Fig. 2**).

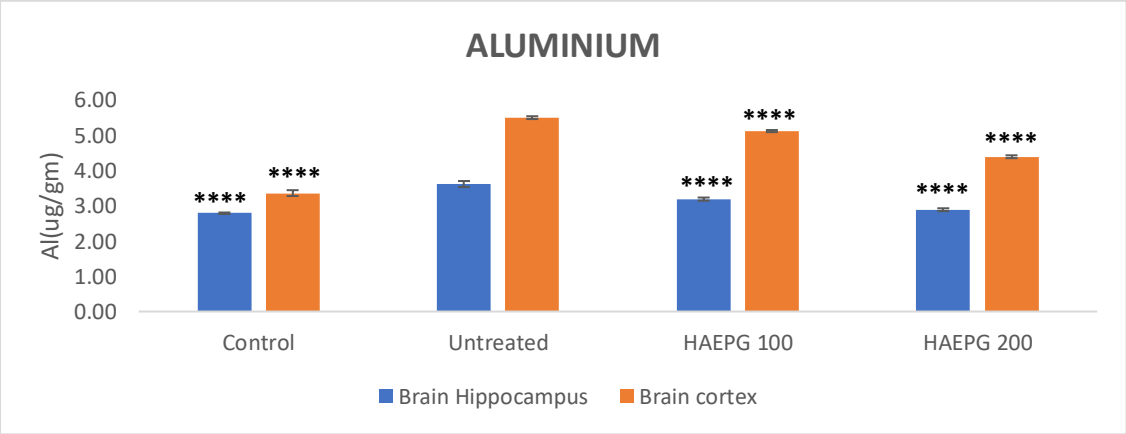

Fig. 2, Effect of HAEPG on aluminium concentration in aluminium chloride treated rats.

Effect of HAEPG on Movement and activities of animals using open field test in aluminium chloride induced rats

Peripheral and central movements, as well as raising and grooming behaviours, were significantly reduced ($P< .05$) in the $AlCl_3$ group. But when AlCl3-treated rats received oral doses of HAEPG (each 100 and 200 mg/kg), their mobility and activity levels significantly increased ($P< .05$) in comparison to the $AlCl_3$ group. (**Fig.3 and 4**)

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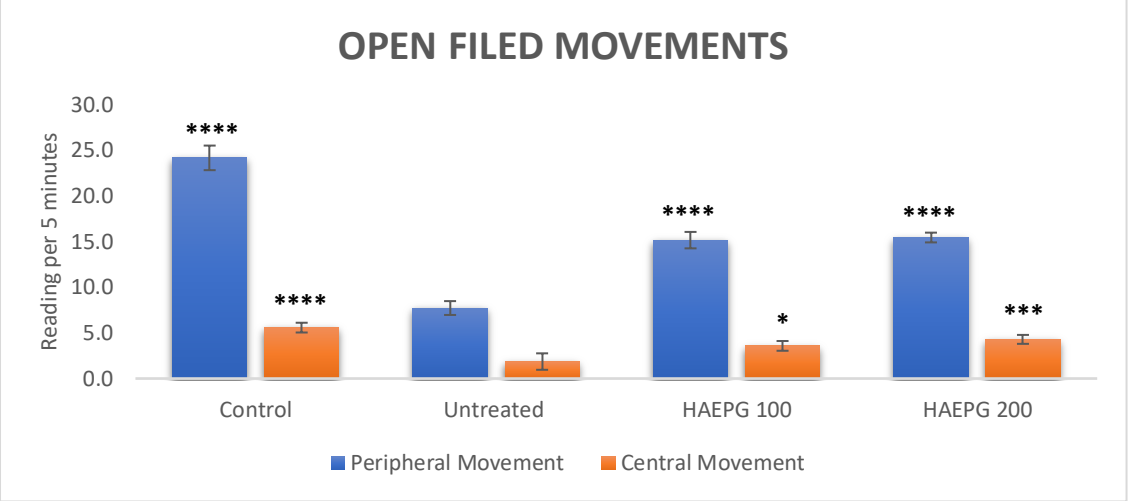

Fig. 3, Effect of HAEPG on Movement of animals using open field test in aluminium chloride induced rats.

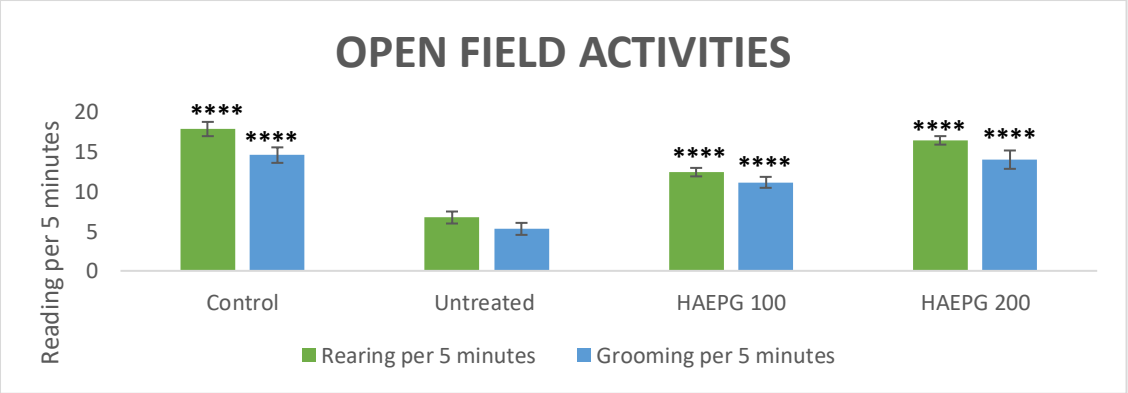

Fig. 4, Effect of HAEPG on activities of animals using open field test in aluminium chloride induced rats.

Effect of HAEPG on memory performance in the Morris water maze task in aluminium chloride induced rats

In comparison to the control group, aluminium chloride-treated rats dramatically increased the acquisition latency to reach the visual platform, showing memory impairments. On days 19 and 20, the aluminium chloride treated group's receiving HAEPG treatment significantly enhanced this memory performance (i.e., decreased average acquisition latency) ($P< .05$). The visual platform was hidden after training. The average acquisition latency (on day 20) and retention latencies (first and second RL on days 21 and 42, respectively) for the aluminium chloride-induced group (Untreated) were then found to be substantially longer than those for the control group to reach the hidden platform. These findings revealed that aluminium chloride significantly impaired cognitive function. Furthermore, compared to untreated rats, chronic HAEPG therapy (each 100 and 200 mg/kg) significantly boosted memory retention for the first and second RL on days 21 and 42, respectively. (**Fig. 5**).

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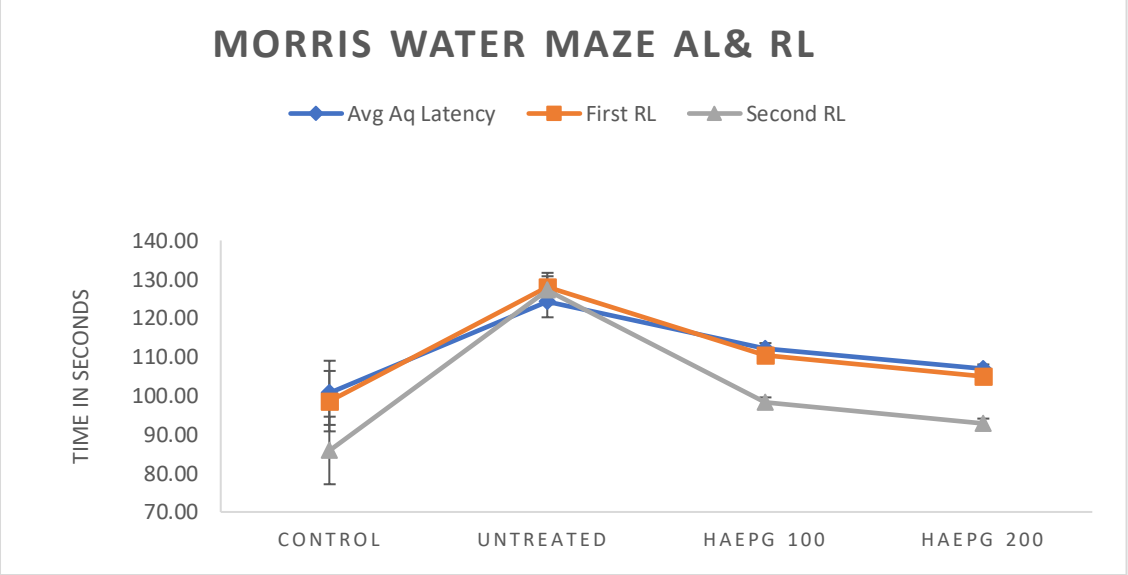

Fig. 5, Effect of HAEPG on memory performance in the Morris water maze task in aluminium chloride induced rats.

Effect of HAEPG on memory performance in the elevated plus maze in aluminium chloride induced rats

On day 20, each rat's ITL was comparatively constant and didn't exhibit any appreciable fluctuations. Within 60 seconds, every rat entered the arm that was closed. After training, rats in the Control, Untreated (Aluminum Chloride-induced), and chronic HAEPG treatment groups entered the closed arm quickly, and the retention transfer latencies (first RTL and second RTL) to enter the closed arm on days 21 and 42 were shorter than the ITL on day 20 of each group, respectively.

The retention transfer latencies on days 21 and 42 relative to the ITL on day 20 did not alter in Aluminium chloride-induced rats, indicating a severe memory impairment. In contrast, these rats performed poorly throughout the experiment. The retention latencies on days 21 and 42 after the administration of the aluminium chloride were considerably decreased by the chronic administration of HAEPG therapy (each 100 and 200 mg/kg) (P< .05 compared to the Untreated group). (**Fig. 6**).

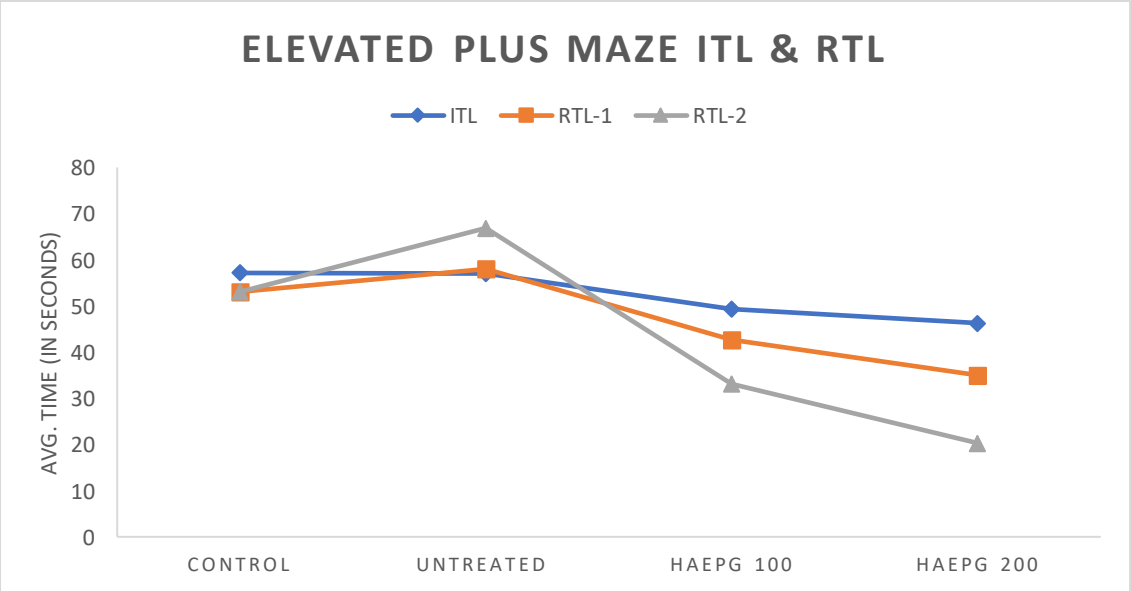

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Fig. 6, Effect of HAEPG on memory performance in the elevated plus maze in aluminium chloride induced rats.

Discussion

As it is an intoxicating pro-oxidant that is known to increase lipid peroxides in the hippocampus, aluminium can cause oxidative stress in the neurons. This was substantial in our investigation, as shown by the significantly higher MDA level in the rats treated with AlCl3. The administration of the HAEPG extract notably returned MDA levels to normal. The extract's beneficial effects on oxidative stress may be attributable to its high concentration of flavonoids and phenolic antioxidants. Rats exposed to aluminium are protected by polyphenols against brain neuroinflammation and cognitive decline. (**27**).

Aluminium is a well-known neurotoxin that has been shown to cause neurodegeneration and symptoms resembling AD. Aluminium causes these consequences through a variety of processes, including as oxidative stress, neuroinflammation, and apoptosis. Our findings show that HAEPG reduces neuroinflammation by reducing oxidative stress which is reflected with increased levels of various antioxidant enzymes in rat models of $AlCl_3$-induced AD. (**28**)

The fundamental mechanism by which Al is damaging to neurons is by increasing cellular oxidative stress. (**29**)

Additionally, HAEPG prevented a decline in brain AChE activity. According to observation, cholinergic neurotransmission is modulated in order to promote memory function.

According to tests using the Morris water maze and the Elevated Plus maze, administration of aluminium chloride caused a progressive decline in spatial memory. This memory loss was prevented due to treatment with HAEPG.

The current study shows that HAEPG therapy enhances behavioural activity, as measured by the OFA test.

CONCLUSIONS

In summary, this study shows that HAEPG therapy protects rat brains from memory loss, cholinergic deficiency, and aluminium loading caused by aluminium chloride. However more investigation is required to determine the exact mechanism of action against Al-induced Alzheimer in rats.

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