Identification of bioactive compounds from crab hemolymph to treat Alzheimer's disease

Bakthavachalam Dhivya and Arumugam Sivakumar*
School of Bio sciences and Technology, VIT University, Vellore, Tamil Nadu, INDIA
*siva_kumar.a@vit.ac.in

Abstract

Natural products are now the most challenging sources to use when developing drugs for conditions like diabetes, high blood pressure, heart disease etc. They are crucial for managing human health conditions and developing into a promising drug. Utilizing natural resources like vegetables, spinach, seafood etc.; in our regular diet, could be an advantage over synthesized resources. A healthy lifestyle is maintained by many naturally occurring resources, therefore future generations will benefit from investigating potential chemicals from these sources. In the present study, rats are given $AlCl_3$ orally for 30 days and behavioral evaluations were performed. Bioactive substances from crab hemolymph were coated with liposomes and given orally and intranasally, once the disease condition was induced. Donepezil (10 mg/mL) was used as a standard. After being subjected to behavioral examinations, the rats were subjected to biochemical analyses which included lipid peroxidation assays, superoxide dismutase activity, reactive oxygen species levels and histological examinations for neuronal changes in the tissue.

The radial arm maze test revealed several remarkable variations in rats given $AlCl_3$. Bioactive compounds administered orally and intranasally to rats induced with $AlCl_3$ improved the animals' performance to some extent. Consequently, the proteins helped to restore the rats' cognitive function and they may be a potential treatment for Alzheimer's disease. For further research, the proteins must also be purified and characterized.

Keywords: Alzheimer's disease, Oxidative stress, Protein-based therapy, Radial arm maze, $AlCl_3$ –induced brain damage, Liposome-coated proteins.

Introduction

An impairment of cognitive performance is the main feature of Alzheimer's disease (AD), a neurodegenerative disorder that mostly affects older people worldwide[2,22]. There was a correlation between high BMI, high consumption of sugar-sweetened drinks and high fasting plasma glucose incidence in people, according to the global, regional and national burden of Alzheimer's disease and other dementias. These health conditions were considerably more common in high-income North America, the high-income Asia-Pacific region, western Europe and east Asia[66]. World Health Organization (WHO) research estimates that AD will impact around 78 million individuals. Consequently, the World Health Organization has classified AD as a disease of public health concern[15,18].

AD can be described as a multifaceted illness. Short-term memory loss is one of the common signs of AD. Other symptoms include confusion, aggressive behavior, mood and behavioral changes and long-term memory loss. One of the pathological characteristic of the disease is the abnormal hyperphosphorylation of tau proteins, which causes neurofibrillary tangles and the development of amyloid beta plaques in the neurons[46,61,62]. The pathophysiology of AD is explained by a variety of concepts which include the oxidative stress, cholinergic, amyloid cascade, metal ion and neuroinflammation theories[31,61]. However, the actual underlying cause of the condition remains unknown. AD is linked to a number of risk variables such as age, gender, genetics, down syndrome and inflammation. Furthermore, there is a substantial correlation between AD and exposure to heavy metals[51].

As the most abundant metal on the planet, aluminum (Al) eventually finds its way into the living systems that include food, cooking utensils, deodorants, medications, drinking water and food additives[53]. Environmental acidification which includes the use of chemical fertilizers in agricultural regions and water pollution from the discharge of industrial waste, increases the bioavailability of aluminum. Multiple research studies on both people and animals have shown that algal deposits deteriorate with age and get lodged in the brain. Higher exposure levels or a diminished capacity to eliminate Al from the brain might be the cause of this. Al may be able to get through the blood-brain barrier and settle in the cerebral cortex and hippocampus, two areas of the brain that are necessary for memory and learning, according to a large body of studies[5,49].

Additionally, the brains of rats treated with $AlCl_3$ exhibit neuronal degeneration and inflammatory cell penetration[49]. Al accumulation in the brain has also been linked to degenerative conditions including Parkinson's disease and Alzheimer's disease, according to reports[12].

AD is now being treated with galantamine, aducanumab, donepezil, memantine and other FDA-approved cholinesterase inhibitors. These drugs reduce the cognitive symptoms of mild to moderate illnesses. Additionally, these drugs may cause undesirable side effects like anorexia, vomiting and diarrhea[27].

Several attempts have been made in recent years to use natural substances to treat the illness in its early stages, with a focus on peptide-based research. The study of natural products for the treatment of AD is a significant and promising therapeutic development strategy. These products, which have long been a component of traditional medicine and have demonstrated promise in treating a wide range of disorders including AD, come from a number of sources including plants and marine life[20,42].

Peptides or proteins have been utilized as a promising therapeutic approach in several fields to date. Their high selectivity, efficacy and low toxicity are contributing factors of this. Advanced drug design can address the disadvantages of peptide-based drugs, including their short half-lives *in vivo* and poor brain bioavailability[32,39]. Since proteins are a great source of vitamin supplements, they can be utilized as medicine[38,40]. Certain animal studies have demonstrated that peptides derived from bovine casein (CH-3) can stop cognitive decline in AD mice models by reducing oxidative stress and inflammation and bridging the blood-brain barrier. This supports the benefit of protein as a therapy[4].

Intranasal administration of peptides is a successful method that enables drugs to pass across the blood-brain barrier without causing systemic adverse reactions[23]. Liposome-based drug delivery has attracted focus due to its size and ability to encapsulate hydrophilic and hydrophobic bioactive compounds[30,55,58]. They have been used to enhance the solubility, stability and bioavailability of bioactive components in the food, cosmetic and pharmaceutical sectors to enable targeted administration and controlled release. A number of investigations have suggested liposomal-formulated bioactive compounds including curcumin[57], icariin[56], antidiabetic peptides[35], therapeutic peptides[41] and asenapine maleate (an antipsychotic drug)[37], for the drug's oral delivery.

Since the majority of approved medications or those undergoing advanced stage clinical trials are protein-based biopharmaceuticals[59] and more than half of approved medications are being evaluated for oral delivery, oral distribution of proteins and peptides receives more attention than the other treatments.

At present, tramiprosate, a naturally occurring amino acid derived from seaweed, has progressed to phase III clinical trials and reduces neurotoxicity and fibrillary fiber formation. Additionally, the oral small-molecule medication ALZ-801, the prodrug of tramiprosate, is now being developed. It has shown long-term effectiveness and favorable safety profiles in AD patients, particularly those who have the APOE4 gene, a key genetic risk factor for AD. ALZ-801 is now undergoing phase III clinical trials (NCT04770220)[36].

For several decades, crustaceans have been used as useful model animals in neurology and electrophysiological research. Functional evaluations of individual cells and neural networks are made available by the comparatively basic nervous system and clearly characterized neuronal circuitry. The functions of signaling molecules known as crustacean neuropeptides in a variety of regulatory activities, including as feeding and stress exposure, have been well studied[9]. Furthermore, because peptides and proteins are exposed to depletion, hydrolysis and obstruction by mucus or certain cellular barriers in the gastrointestinal system, oral delivery of these substances has several disadvantages[7]. Oral medications are used to treat neurodegenerative diseases, but they only assist to control the disease's symptoms; they cannot treat the condition completely.

Marine environments, which comprise more than 70% of the planet's surface, are home to around half of all species. As a result, researchers continue to investigate these resources in an effort to develop drugs that can treat human diseases. Marine-based natural products have demonstrated their promise in a variety of biological effects, such as anti-thrombotic, anti-inflammatory, anti-hypertensive, anti-diabetic, heart-protective and neuroprotective action, according to several research. The difficulties are that turning these compounds into pharmaceuticals is more cost-effective and takes a long time to get clinical approval up to ten years. Furthermore, the process of extracting, isolating and characterizing compounds from natural materials is essential to the development of novel medications.

However, there remained a significant research gap in the creation of protein-based medications derived from marine and natural sources that may effectively treat AD. This work aims to fill these gaps by evaluating crude hemolymph protein that is isolated from marine crustacean crabs for the treatment of AD. LCMS analysis was used to characterize the isolated protein. In order to avoid hydrolysis, obstruction and deprivation by mucus or certain cellular barriers in the gastrointestinal system during oral delivery, the isolated protein was then loaded onto liposomes.

This study examined the effectiveness of crude protein by inducing Alzheimer's disease in animal models using $AlCl_3$, followed by oral and intranasal administration of crude protein coated with liposomes. Further, histopathological examinations were done by fixing the tissue in 10% formalin and also by biochemical analysis to measure the level of oxidative stress in the animal. These experimental findings will help in the development of novel protein-based orally administered therapeutics from the marine source for the treatment of AD.

Material and Methods

Animals: Thirty-five Swiss albino Wistar male rats (200-250 g) 12-16 weeks of age were procured from the animal house VIT, Vellore. Rats were maintained at standard laboratory feed and water *ad libitum* and exposure to light 12 h and 12 h dark cycle. Rats were allowed to acclimatize to environmental conditions for a week and then the

experiment was initiated. Dementia was induced in rats by oral administration of $AlCl_3$ 100 mg/kg for 30 days (10 mg/300 g).

Chemicals and Reagents: The materials and chemicals used in this study were of analytical grade as: Aluminum chloride (SDFine chemical, Product code- 37073), donepezil (Standard Drug) (Merck, Product code- D6821), diethyl ether extra pure AR (99.5%) (SRL, Product code- 25049), methanol (SDFCL, cas no: 67-56-1), chloroform (SDFCL cas no: 67-66-3), BSA (Himedia cas no: 9048-46-8), cholesterol (Himedia cas no: 57-88-5), NBT (Himedia cas no: 298-83-9), TCA (SDFCL cas no: 76-03-9), TBA (SRL cas no: 504-17-6), DCFH-DA (SRL cas no: 4091-99-0), formalin (Spectrum chemical cas no: 50-00-0), hemotoxylin (Merck cas no: 517-28-2) and eosin (Merck cas no: 150322-02-4).

Experimental design
Group I: Negative control and administered 0.9% saline orally (n=7).
Group II: Positive control and received $AlCl_3$ (10 mg/300 g/day) orally (n=7).
Group III: Same as group 2, $AlCl_3$ (10 mg/300 g/day) was given orally. After the 30th day, the rats were treated with the standard drug donepezil (10 mg) orally administered (n=7).
Group IV: Received $AlCl_3$ (10 mg/300 g/day) similar to group 2 and was treated with crude peptide (1.137 mg/mL) coated with liposomes, administered orally (0.5 mL) through oral gavage (n=7).
Group V: Received $AlCl_3$ (10 mg/300 g/day) similar to group 2 and was treated with crude peptide (1.137 mg/mL) coated with liposomes administered intranasal (alternative days 40 µL) (n=7).

According to G Power software, using one-way ANOVA analysis, the animals were randomly divided into 5 groups containing 7 animals each (n=7)[11]. The animals were subjected to behavioural assessment before and after administration of $AlCl_3$ to assess the behavioural changes of rats. BACE 1 enzyme analysis was performed after the 30th day. When the last dose was given within 24 h, the animals were sacrificed and the blood, brain tissue (hippocampus) were preserved under proper conditions for further biochemical analysis.

Evaluation of learning and cognitive function by Radial-arm Maze: A radial-arm maze with an 8-arm baited arm was connected with Any maze software version 7.4, used to test the behavioural activity of the rats. Rats were given food via a feeding window and water was provided *ad libitum* daily.

Initially, the animals were given training before inducing the $AlCl_3$. Behavioural testing sessions occurred from Monday to Friday between 9.30 am- 1.00 pm, the rats were given trials for 5 min duration (300 s) in which all 8 arms of the radial maze were provided with feed. Then, rats received a 5-min test trial per day for five days, restricted with 4 arms baited. Every day the 4 arms were accessed for the rats in all the groups.

During the test trial, the rats retrieved all 4 pellets from the baited arms. The experimental trials were performed in triplicates. First entries into non-baited arms were regarded as reference memory errors and entries into arms that had already been visited, were regarded as working memory errors[19].

Collection of samples: Animals were sacrificed by cervical dislocation under mild exposure to diethyl ether for 3 min. The animals were exposed to CO_2 chambers for euthanasia and disposal was carried out by Kenbio Links Pvt. Limited. Blood was collected in a separate tube and later centrifuged at 3000 rpm for 10 min to obtain serum and preserved for further analysis. The animal brains were removed and homogenized in phosphate buffer (pH=7.4). The homogenates were then centrifuged at 8000 rpm for 15 min. The supernatant of homogenates was collected and used for biochemical measures[16].

LCMS/MS Analysis of crude hemolymph: The crude hemolymph from the crab was analysed by SDS-PAGE, further the gel was cut and processed for trypsin digestion. The gel sample was analysed by mixing 10 µL of 0.2% of formic acid and analysed by orbitrap LC-MS/MS (Ultimate 3000 nano UHPLC system, attached to an OrbitrapQ Exactive HF mass spectrometer with Nanospray Flex Ion Source, Thermo Scientific)[9]. C18 column was used with a flow rate of 5 µL/min. Two solvents were used for the elution: Solvent A- Acetonitrile 1% + 0.1% formic acid and Solvent B- 99% Acetonitrile + 0.1% formic acid.

The gradient used for the study was as follows: 2% of B in 0-5 min, 15% of B in 5-80 min, 45% of B in 80-100 min, 95% of B in 100-105 min, 95% of B in 105-110 min, 2% of B in 110-115 min, 2% of B in 115-120 min. The MS/MS method was set up in a data-dependent acquisition mode and the full scan ranging from 350 to 2000 m/z was acquired using single high resolution of 70,000 (at 250 m/z). All the spectra were analysed using peak studio 11 software against the proteome database, which was used to identify the possible proteins from the crude sample.

Preparation of Liposomes: Liposomes were prepared by the thin film hydration technique. 300 µL of extracted phospholipid (from egg yolk) was mixed with 5 mg of cholesterol, 3 mL of methanol, 7 mL of chloroform and 5 µL of crude protein in a sterile round-bottom flask and shaken vigorously (drug to lipid ratio 1:20 w/w).

The solvents were then evaporated to produce a thin film, which was thoroughly vacuum-dried overnight to remove the residual organic solvent. The lipid film was hydrated in 10 mL of phosphate-buffered saline (pH 7.4). The solution was then sonicated at 40 kHz for 45 min to ease the formation of liposomes[13].

Human beta-secretase enzyme -1 assay for rat serum: To perform the human beta-secretase-1 assay, the 100 μL of serum was diluted to 1:25 in 1X assay diluent and incubated for 2.5 h at room temperature with gentle shaking. After incubation, the plates were washed 4 times to remove any liquids completely. Then 1X biotinylated antibody (100 μL) was added to each well and incubated at room temperature for 1 h with gentle shaking. Again, the wells were washed and streptavidin-HRP solution (100 μL) was added to each well and incubated for 45 min at room temperature with gentle shaking. Then, the wells were washed and TMB substrate (100 μL) was added to all wells and kept for 30 min incubation at room temperature in the dark with gentle shaking. The substrate turned blue and 50 μL of stop solution was added and gently mixed. The solution in the well changes from blue to yellow. The absorbance was measured at 450 nm. The concentration of beta site APP cleaving enzyme 1 (BACE1) (ng/mL) was estimated using a standard curve[44]. The rat serum was obtained after every 10 days administration of $AlCl_3$ and treatment with peptides to test the level of BACE-1 enzyme in the blood. In each group, one animal was randomly picked and used for the analysis.

Superoxide Dismutase (SOD) assay: The SOD activities of the supernatant of brain tissue homogenates of the rats were measured with hydroxylamine with nitro-blue tetrazolium (NBT) as a detector. The concentration of SOD was determined based on its ability to inhibit the superoxide-mediated reduction. In this assay, SOD and NBT compete with each other for the O_2^- produced by the visible illumination. Absorbance was measured at 560 nm to determine the NBT reduction. The unit of SOD activity is concluded by the quantity of the enzyme that prevents color production by 50%[29].

Lipid peroxidation (LPO) assay: Lipid peroxidation was carried out to measure the total amount of malondialdehyde (MDA) in the sample. To the supernatant of the brain homogenized tissue sample, the experiments were performed in duplicate and 2 mL of TCA-TBA mixture was added, vortexed and heated to 90°C for 30 min. The mixture was allowed to cool down and was centrifuged at 7000 rpm. The amount of the MDA-TBA mixture from the supernatant was measured at an absorbance of 532 nm using a microplate reader (xMark, BioRad)[47].

Total protein concentration: Bradford assay was performed to measure the total content of protein for all the supernatants of brain tissue homogenates. The concentration of protein in the sample was measured from the standard graph. BSA is used as a standard, in concentrations ranging from 20 to 250 µg/mL. Absorbance was measured at 595 nm using a plate reader and the experiments were performed in duplicate[14].

Reactive oxygen species (ROS) generation: The generation of ROS in the brain tissues was measured using the dye DCFH-DA (permeable dye, 2-7, dichlorofluorescein -diacetate). The dye acts as a bio-indicator of stress on biological organisms which is caused by several external factors. 2 µL of the 100 M DCFH-DA was added to 2 mL of the homogenized brain tissue supernatant and kept for incubation in a dark condition at 37ºC for 30 mins. The excitation and emission wavelength (530 nm and 485 nm) of the dye were analysed using a fluorescence spectrophotometer and the experiments were performed in duplicates[60].

Behavioural analysis: Rat behaviour was analysed using the tracking software ANY-maze version 7.4. In this software, we recorded the average speed (m/s) of the animal, the distance travelled (m), the number of entries in the correct zone and the mean visits in the incorrect zone (s). Behavioural analysis was performed for all the animals in each group. The graph was plotted by calculating the average value of each behavioural assessment. Statistical analysis was calculated using One-way Analysis of Variance (ANOVA) analysis in graph pad prism.

Histopathological examinations: All the animal groups were sacrificed and the hippocampus sections were isolated separately. The tissue samples were washed with cold phosphate-buffered saline, fixed in 10% formalin and embedded in paraffin wax. After sectioning, the paraffin sections were stained with hematoxylin and eosin. The slides were examined using a light microscope and the magnified images of tissues were captured[50].

Statistical analysis: The results were expressed as mean ± standard error of the mean (S.E.M.). The data obtained from various groups was statistically analysed in Graph-pad prism using one-way ANOVA followed by Tukey's multiple range test, $P < 0.05$ was considered to be statistically significant.

Results

Protein identification using LC-MS/MS analysis: The possible protein from the crude hemolymph is summarized in table 1. The results confirm the possibility of sarcoplasmic calcium binding protein, hemocyanin-rich protein, Ig like domain containing protein and several other uncharacterized proteins identified from the crab species *Scylla serrata*[17]. Thus, these findings could be useful for studying more about the proteins from this species and further need to be validated by bioactivity assays.

Expression of β-secretase enzyme -1 in animal serum: The expression of BACE 1 in animal serum was detected among the grouped animals. Elevated enzyme expressions in the hippocampus region denoted the accumulation of lesions resulting in Alzheimer's disease. The enzyme expression was comparatively higher in $AlCl_3$-induced rats than in other groups, as shown in figure 1 ($P < 0.05$). The $AlCl_3$ induced group (Group II) showed 32.92 ± 0.71 ng/mL of enzymes; there is a decrease in the level of β-secretase enzyme expression about 17.33 ± 3.7 ng/mL (group III) when treated with standard drug (Donepezil) whereas the

animal group with oral administration (Group IV) 21.5 ± 1.5 ng/mL and intranasal administration (Group V) 21.02 ± 2.2 ng/mL showed decrease in expression when compared to the $AlCl_3$ group (Figure 1).

Table 1
Possible Proteins list from the crab hemolymph retrieved from Proteome database

Accession	Description	Coverage (%)	Peptides	PSMs	Score sequest
P86909	Sarcoplasmic calcium-binding protein (Fragment) OS=Chionoecetes opilio OX=41210 PE=1 SV=1	100	1	1	2.18
A0A5B7CY39	Fibrinogen C-terminal domain-containing protein OS=Portunus trituberculatus OX=210409 GN=E2C01_007042 PE=4 SV=1	22	1	1	2.99
A0A8J4YDT5	Estradiol 17-beta-dehydrogenase 8 OS=Chionoecetes opilio OX=41210 GN=HSD17B8_7 PE=3 SV=1	22	1	1	2.74
A0A5B7FSN7	Uncharacterized protein OS=Portunus trituberculatus OX=210409 GN=E2C01_043325 PE=4 SV=1	17	1	1	1.9
A0A8J4YKV1	Nucleoporin SEH1-A OS=Chionoecetes opilio OX=41210 GN=seh1l-a PE=3 SV=1	13	1	1	3.12
A0A8J5CFI0	Ig-like domain-containing protein OS=Chionoecetes opilio OX=41210 GN=GWK47_019977 PE=4 SV=1	13	1	1	2.92
A0A5B7KEY3	Hemocyanin C chain OS=Portunus trituberculatus OX=210409 GN=HCYC_0 PE=4 SV=1	7	2	4	11.87
A0A5B7G319	Uncharacterized protein OS=Portunus trituberculatus OX=210409 GN=E2C01_047221 PE=4 SV=1	7	1	1	1.93
A0A0P4WBI5	Ig-like domain-containing protein OS=Scylla olivacea OX=85551 PE=4 SV=1	6	1	1	2.49
A0A0U1ZX76	Hemocyanin subunit 3 OS=Scylla paramamosain OX=85552 PE=2 SV=1	6	5	11	29.79
A0A0P4WHV0	rRNA adenine N(6)-methyltransferase OS=Scylla olivacea OX=85551 PE=3 SV=1	5	1	1	2.49
A0A0P4WKJ1	Chitin-binding type-2 domain-containing protein OS=Scylla olivacea OX=85551 PE=4 SV=1	5	1	1	2.3
A0A8J4YN36	Myb/SANT-like DNA-binding domain-containing protein OS=Chionoecetes opilio OX=41210 GN=GWK47_037436 PE=4 SV=1	4	1	1	2.65
A0A5B7CNU1	Antigen KI-67 OS=Portunus trituberculatus OX=210409 GN=MKI67 PE=4 SV=1	4	1	1	2.67
A0A8J5CR26	Uncharacterized protein OS=Chionoecetes opilio OX=41210 GN=GWK47_053796 PE=4 SV=1	4	1	1	2.32
A0A5B7CYI1	ATP-binding cassette sub-family A member 1 OS=Portunus trituberculatus OX=210409 GN=Abca1_0 PE=4 SV=1	2	1	1	2.61
A0A8J4XQP2	Ankyrin repeat domain-containing protein 13C-B OS=Chionoecetes opilio OX=41210 GN=ankrd13c-b PE=4 SV=1	2	1	1	2.49
A0A5B7EIQ7	Tensin OS=Portunus trituberculatus OX=210409 GN=TNS_1 PE=3 SV=1	2	1	1	3.03
A0A0P4WHI9	Dymeclin OS=Scylla olivacea OX=85551 PE=3 SV=1	1	1	1	2.2
A0A0P4W1V9	Myosin motor domain-containing protein OS=Scylla olivacea OX=85551 PE=3 SV=1	0	1	1	1.95

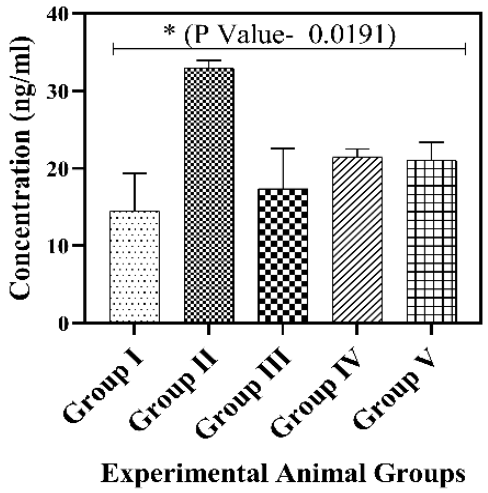


Figure 1: Determination of Beta-secretase enzyme levels in the experimental animal groups. The amount of beta-secretase enzyme present in the animal serum was measured for control, $AlCl_3$ and other groups III, IV and V. The concentration was measured in ng/ml according to the standard curve.

Level of SOD enzyme: SOD levels were decreased in $AlCl_3$-induced rats, but they were dramatically recovered in treatment groups, which implied that the protein treatment restored the SOD levels. Comparatively SOD production was less in the animal group treated with the standard drug and the crude protein through intranasal route of administration (P value 0.0070). The levels of SOD production in the animal's groups were depicted in the figure 2. The enzyme level in control group showed 7.46 ± 0.21 U mL^{-1} (Group I) whereas in treated group, it showed 10.958 U mL^{-1} (Group IV) and 10.5 U mL^{-1} (Group V).

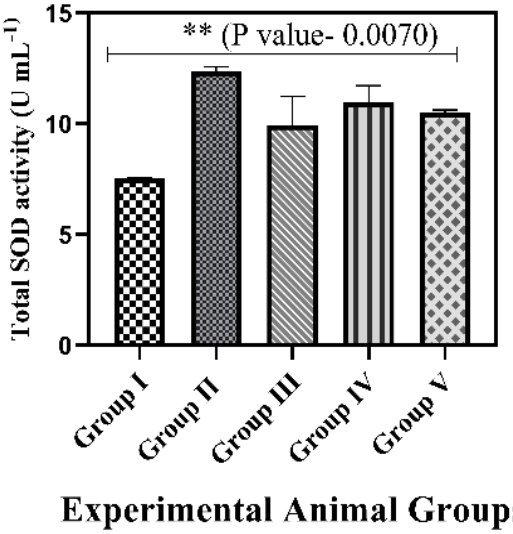


Figure 2: Superoxide activity of $AlCl_3$-induced rat and other experimental groups treated with standard drug (10 mg/mL) and crude protein (1.137 mg/mL).

Level of MDA in the brain tissue: Oxidative stress was measured in terms of the concentration of MDA in the sample. The MDA level in group II was significantly higher ($P < 0.0122$) when compared to the control group, while those treated with a standard drug (Donepezil), significantly decreased in MDA levels when compared with group II ($P < 0.0122$). On the contrary, MDA levels are significantly lower for oral and intranasal administration of protein ($P < 0.0122$) compared with $AlCl_3$-induced rats (Figure 3). The oxidative stress marker (MDA) showed the highest value in the $AlCl_3$ (23.74 ± 2.23 µmol/g (Group II)) after the drug treatment, the level of MDA reduced up to 11.87 ± 1.25 µmol/g (Group V).

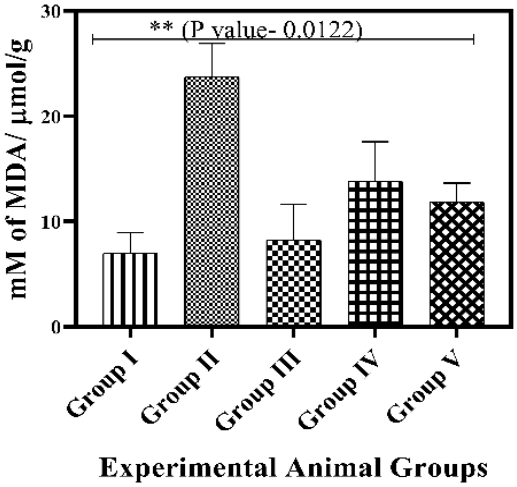


Figure 3: Levels of MDA in tissue homogenates of $AlCl_3$-induced rat and other experimental groups treated with standard drug (10 mg/mL) and crude protein (1.137 mg/mL).

Level of ROS in the brain tissue: ROS generated in tissue homogenate of the animal sample was shown in figure 4. Compared to the control group, $AlCl_3$-induced rats showed significantly higher oxygen species ($P < 0.0001$) whereas the standard drug-treated group showed significantly less than the $AlCl_3$-treated groups ($P < 0.0001$). The oral and intranasal showed significantly less when compared with other groups (Figure 4). The level of ROS in the control group (Group I) showed 81.02 ± 3.65 %. After treatment, it showed up to 88.1071 ± 0.44 % (Group IV).

Analysis of Total Protein Concentration: The total protein concentration of tissue homogenates was determined by the Bradford assay. Compared with the control group, the $AlCl_3$-induced group has a lesser protein concentration and there was no significant value (P value 0.6368). Similarly, the standard group, oral and intranasal administration group had higher protein concentrations (Figure 5). The concentration of the protein in the control group (0.134 ± 0 µg/mL) was higher than the treated group (0.133 ± 0 µg/mL) (Group IV).

Behaviour analysis: The effect of oral and intranasal administration of crude protein on the experimental rats was represented in the figures 6a-6d. The rats memory and behaviour were analysed using ANY-maze software version 7.4.

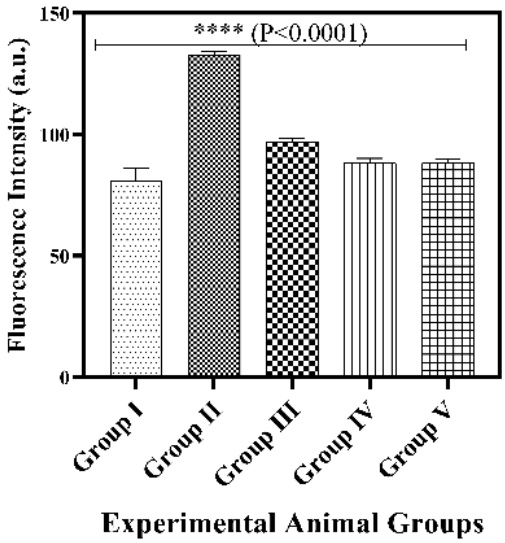


Figure 4: Level of ROS in the tissue homogenates of Group I to Group V.

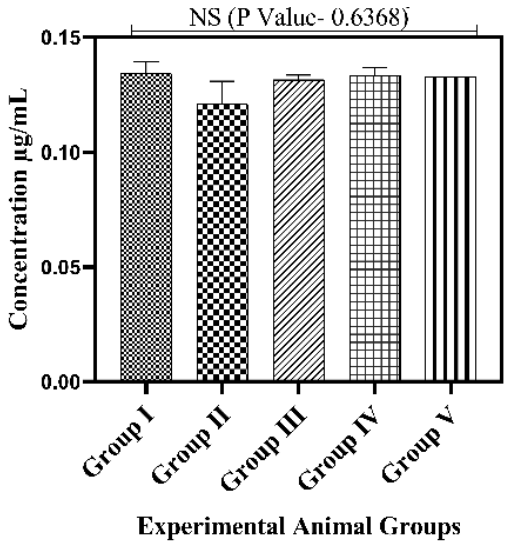
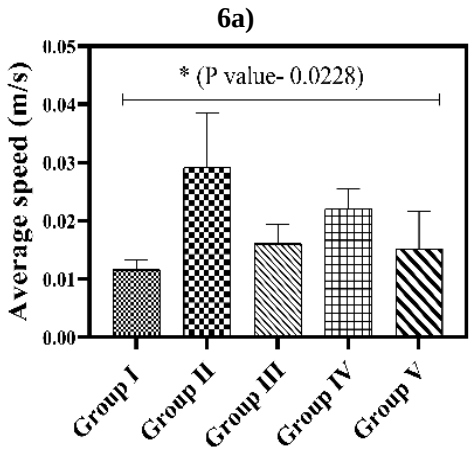
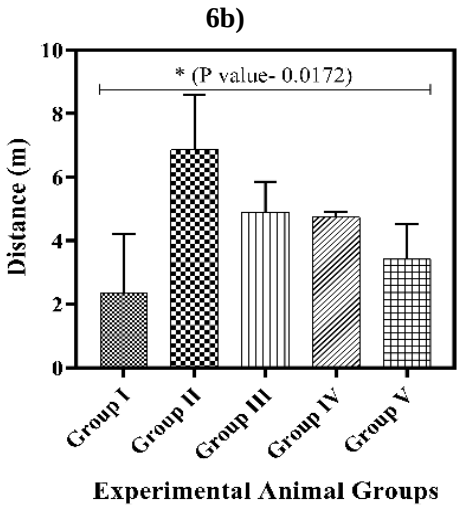
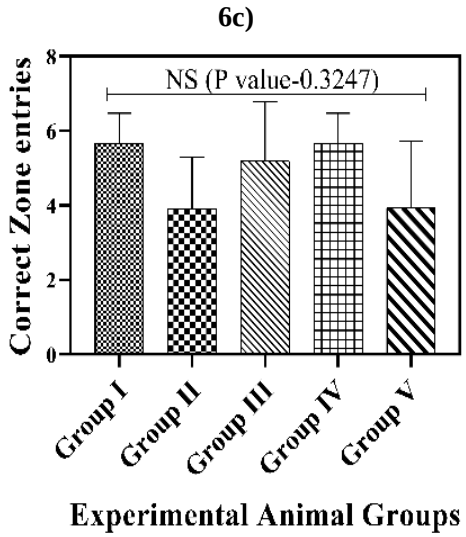


Figure 5: The total protein concentration present in the tissue homogenates was analysed by Bradford assay for all the experimental groups.







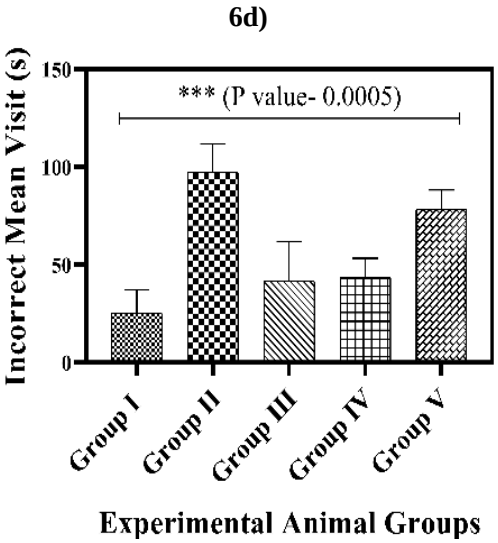


Figure 6: Effect of the crude protein on the $AlCl_3$-induced behavioural assessment in AD rats by Radial arm maze test. Results were depicted as mean ± SD of four discrete experiments. One-way ANOVA analysis was used to examine the data and there was no significant difference. 6a-Average speed in m/s, 6b-Distance travelled by the animal (m), 6c-Number of correct zone entries and 6d- Mean visit in incorrect zone (s)

Each rat was placed in the Radial arm maze and made to explore for 5 min (300 s) to discover the hidden food in the baited arm. The test was performed manually to identify the rat's behaviour. The average speed (m/s) (Figure 6a) travelled by the rats was significantly lesser for the control and treated groups ($P<0.05$) when compared to $AlCl_3$ induced rats. The distance (m) (Figure 6b) travelled by the rats significantly elevated ($P<0.05$) for the $AlCl_3$-induced rats when compared with the rats in the control group. The effect of oral and intranasal administration on the rats showed significantly less in all the values when compared to $AlCl_3$-induced groups.

The correct zone entries (Figure 6c) showed no statistical significance ($P>0.05$) and the $AlCl_3$-induced groups represented lesser average entries compared to other control groups and drug treated groups. The mean visit in the incorrect zone (s) (Figure 6d) showed significantly greater values (P value<0.05) for the treated groups than for $AlCl_3$-induced groups. The results indicate that post-treatment with the crude protein showed memory impairment by the $AlCl_3$ and resulted in improved cognitive memory.

Histopathological examinations of brain tissues: Histopathological examinations of the control group showed an ideal cerebral cortex and hippocampus. On the other hand, the $AlCl_3$- induced AD groups exhibited morphological changes in the hippocampal tissue section. The hippocampus region of the $AlCl_3$-induced group tissue section had necrosis and gliosis when compared to the control group also, the number of neurons were lesser and darkly spotted. Excessive exposure to $AlCl_3$ leads to a reduced number of neurons in the cerebral cortex and different regions of the hippocampus. In contrast, the tissue section of the standard drug (donepezil) showed some similar morphology and neuron arrangement to the control group. The oral and intranasal administration of proteins to the animal groups showed neurons similar to the control group indicating the restoration of neurons in treated group of test animals. (Figures 7a-7e).

Discussion

Generally, all the neurodegenerative disorders including AD are associated with the oxidative stress as common risk factor. The exposure to the heavy metals also causes the oxidative stress that results in the neurodegeneration. Metals like $AlCl_3$ are frequently used in the chemical industries like paints, pesticides, pesticides, pharmaceuticals and mainly in food through water and diet[6]. $AlCl_3$ gets absorbed in the blood-brain barrier and settles chiefly in the hippocampus which is crucial for memory and learning. Accumulation of these heavy metal leads to the aggregation of amyloid beta and neurofibrillary tangles, which results in neurotoxicity[28].

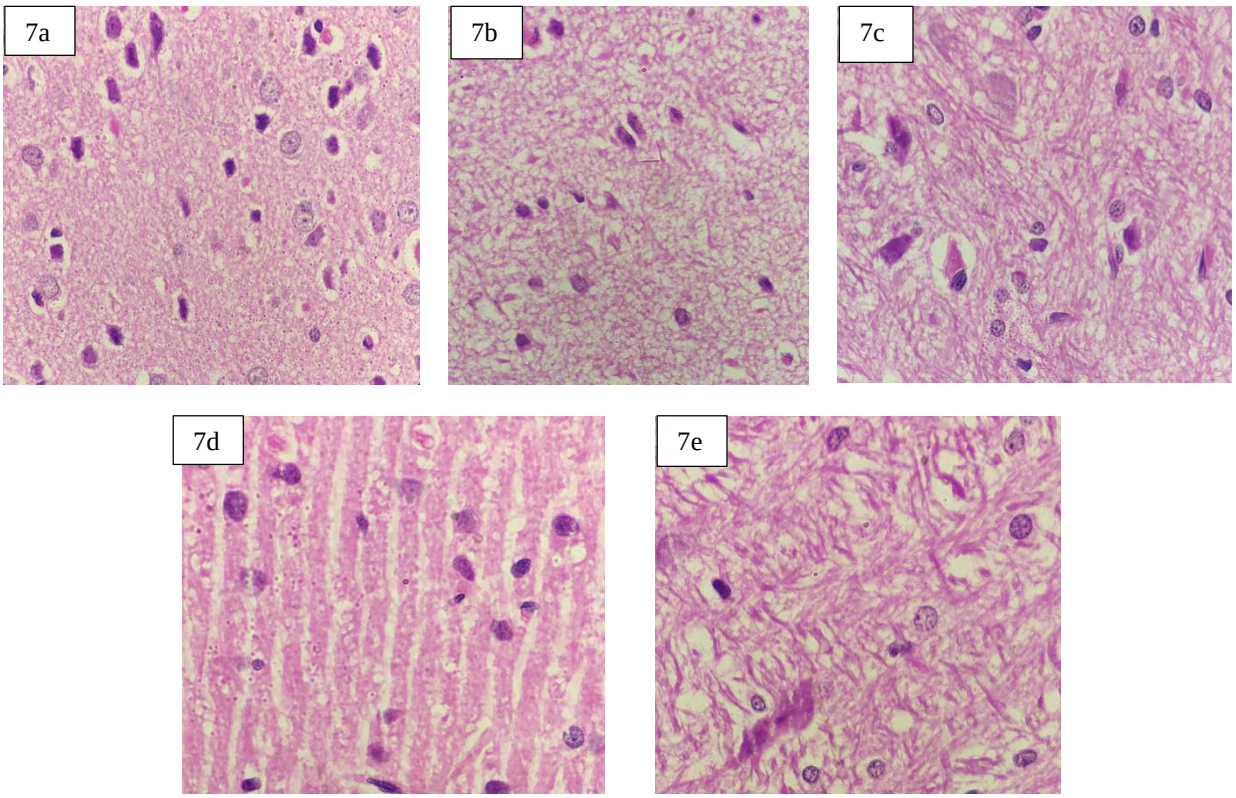


Figure 7: The Histopathological modifications resulted from treating with liposome coated protein at concentration (1.137 mg/mL). The hippocampus region of the control animals showed normal intact cellular arrangements, whereas $AlCl_3$-induced AD animals exhibited cell necrosis, active inflammation and neuronal loss in the brain tissues. Protein treated animals and the standard drug-treated group showed no significant pathology and neuronal cell arrangement was similar to control group. 7a- Control group, 7b- $AlCl_3$-induced AD group, 7c- Standard group, 7d- Oral administration, 7e- Intranasal administration

It is widely recognized that apart from the level of ROS, other free radicals such as nitric oxide, hydrogen peroxide, superoxide anion and hydroxyl radical are crucial for biochemical changes, cell deterioration and biological evolution, as well as the degenerative process of neural cells. ROS can be produced by organisms exogenously by chemical reactions or endogenously by mitochondria. They can control the functions of biomolecules including proteins, lipids and nucleic acids, as well as related biochemical processes, like the oxidation and peroxidation of lipids, particularly the polyunsaturated fatty acids found in abundance in neuronal membranes[45].

When ROS production is higher, the body's natural antioxidant defence system and oxidative stress happen[3], leading to neuronal damage and cognitive impairments via lipid peroxidation, enzyme inactivation and DNA modification[21]. According to literature findings, proteins deliver brain health benefits that focus mainly on their activities, besides neuro-inflammation, aging, damage, oxidative stress, damage to the cholinergic system and A*β*-induced neuronal degeneration. Mitigation of neuronal deficits by neuroprotective peptides leads to the destruction of cognitive impairments in mouse models[8,48].

Peptides appear to influence the brain through the microbiota-gut-brain axis, making it essential to acquire them with suitable properties, like drug therapy for several diseases. So many bioactive peptides exist in natural resources that need to be used by employing proper hydrolysis conditions, which can break down the proteins into small molecules or short peptides, which will be easier for formulating as drug molecules. Peptides are abundantly recognized and isolated from common food protein sources which include animal (whey, fish, egg etc.) and plant sources (rice, peanut, soybean etc). Following consumption of these neuroprotective peptides could either influence the body directly or function as building blocks for physiologically active substances that enhance brain function[52].

Several animal models have been used to estimate the efficiency of peptides and proteins to improve memory by performing behavioral analysis, physiological indicators and brain tissue morphology between control and model animals[25]. Some marine natural products like gracilins derived from marine sponges and fucosterol from brown algae exhibit antioxidant, anti-inflammatory and inhibition of BACE1 enzyme properties. Further clinical studies have to be conducted to bring out the potential of these compounds[24]. Experiments for analysing the behavioural performances of the rats include identification learning like the Morris water maze, radial arm maze, Barnes maze etc.[10,34,64]

To observe the morphological changes in the hippocampus region of the animal brain tissues, several electron microscope techniques have been employed with various dyes to visualize the neurons. Also, a series of biochemical analyses are performed to test the oxidative stress indicators to identify cholinergic system changes and the expression of learning and memory-related genes and proteins[54]. Due to difficulties in extraction and purification process in the natural products, it is difficult to explore many bioactive compounds from the natural sources, also there is a high rate of failure in clinical trials. Peptides or proteins from marine sources could be a promising and challenging drug therapy for many diseases. Since it contains essential amino acids and edible products, it can be used as a therapy.

The protein from the crab species *Scylla serrata* has abundant proteins in it, which have to be characterized in the future to know the potential of the compounds and there are no proteins or peptides characterized for any applications. The route of administration of peptides faces a challenging process in clinical trials. Intranasal and oral administration of peptides by coating them with liposomes could deliver the drug to the targeted area. Liposome-based drug delivery for treating neurodegenerative diseases has been widely used because of its biocompatibility and nontoxic properties[65]. In this study, $AlCl_3$ was administered orally to study its influence on memory and learning processes. Induction of aluminum weakened the animal's capability to learn and memorize the targeted region in the radial arm maze test; this may be due to the defect in the hippocampus region[26].

Extreme oxidative damage due to aluminum results in neuronal injury and disturbs the brain's antioxidant defence system. Thus, the imbalance of the antioxidant defence system and oxidative stress is linked to the impairment of cognitive function. Since the brain ingests the highest amount of oxygen, it ends up in oxidative damage that results in higher lipid peroxidation and deterioration in antioxidant markers[1,43]. An increase in MDA levels is responsible for oxidative damage by damaging the cell membrane. Aluminum administration increased the MDA, ROS levels and decreased the antioxidant enzyme SOD as depicted in figures 2- 4[33]. After induction of AD in rat it was treated with the crude protein extracted from the *Scylla serrata* crab.

Crude hemolymph which was characterized by LCMS/MS analysis contains majorly hemocyanin protein, which is a copper rich protein. Further the crude protein was loaded into liposome and used as a drug to treat the AD in the rat after induction through the oral administration of $AlCl_3$. Binding Cu (II) with Aβ results in dimerization and oligomerization, but actual mechanism is still not clear. N-terminal of Aβ has a high affinity for Cu(II), which delays the fibrillation upon addition of 0.4 equimolar Cu(II). Fibrillation leads to the formation amyloid plaques. Complexes of Cu(II) and Aβ have also been identified in computational chemistry, indicating that Cu(II) affects the compaction of monomers and small oligomers and thus affects the formation of fibril and forms shapeless aggregation for the 1:1 Cu(II) to Aβ complex[63].

In these experimental studies after treatment with the crude hemolymph proteins, there was a decrease in the MDA and ROS levels repairing the antioxidant levels. The human beta-secretase enzyme assay implied that the enzyme concentration was decreased in the treated animals and elevated in the diseased groups, as shown in figure 1. As shown in figures 6a-6d, behavioural studies depict that the $AlCl_3$ administered group had impairment in the memory process, so it reached the targeted area late when compared with the control group. Standard donepezil, oral and nasal administration groups of experimental rats showed some similar changes and attained the targeted area soon by remembering the working memory.

In the animal studies, the crude protein was used for treating the rats. Possibly the copper containing proteins could have helped to decrease the plaques or slowed down the fibril formation which is clearly depicted in histopathological examinations. This concludes that the proteins from the natural source retained the memory of the animals. Administration of proteins showed histopathological alterations in hippocampus tissue regions and showed effects against $AlCl_3$ induced AD as discussed in the results.

Conclusion

The crude proteins from the crab hemolymph coated with liposomes was administered via oral and intranasal routes to the $AlCl_3$-induced Alzheimer rats. $AlCl_3$ impaired cognitive function when treated with the proteins which showed positively anticipated results. Through the behavioural changes in the rats, it could be concluded that crab hemolymph hemocyani rich proteins restored the memory and learning function in the diseased rats and helped balancing the antioxidant and oxidative stress. However, the proteins identified in this study for treating rats have to be further purified.

Further analytical techniques are required to purify and exactly identify the peptide groups that are responsible for the therapeutic activity and know its structural and functional properties which could be a promising therapy to treat AD when compared with existing drugs owing to the speed of recovery and other side effects. Further studies are to be conducted on short peptides isolated from crab hemolymph to explore the protective properties against Alzheimer's disease.

Acknowledgement

The authors would like to thank Sponsored Research and Industrial Consultancy VIT, Vellore for extending their support to this study, Christian Medical College, Vellore for histopathological analysis. Crab sample was authenticated by Dr. A. Gokul, Scientist C, Southern Regional centre, Zoological Survey of India, Chennai. Authentication reference number – F.No: 23-14/2018-Tech/Id&Adv/240.

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(Received 31st August 2025, accepted 01st October 2025)