Effect of *Annona squamosa Linn* against aluminium chloride induced Alzheimer's disease in rats

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Background and objective
Alzheimer's disease (AD) is an age-related neurodegenerative disorder characterized by memory deficits. Various studies have been conducted to find therapeutic approaches for AD. However, a suitable treatment alternative is still not available. The present study is aimed to investigate the polyphenolic fraction of *Annona squamosa Linn* (PFAS) against aluminium chloride ($AlCl_3$)-induced AD in rats.

Materials and methods
Wistar rats were divided into group I (normal control), which received distilled water; group II, which received $AlCl_3$ (100 mg/kg, intraperitoneal route); groups III and IV received PFAS (200 and 400 mg/kg, oral, respectively) and inducing agent ($AlCl_3$ 100 mg/kg, oral); and group V received donepezil (1 mg/kg, oral) and inducing agent ($AlCl_3$ 100 mg/kg, oral). The rats were given respective treatment for 28 days, and behavioral parameters were determined on first day, 15th, and 28th day. After 28th day, rats were killed, and antioxidant parameters and brain acetylcholinesterase content were determined, and histopathological studies were done.

Results and conclusion
The PFAS showed dose-dependent protective effect against AD by significant improvement in locomotor activity, motor coordination, spatial memory, and conditioned avoidance response; significant decrease in lipid peroxidation and acetyl cholinesterase; and increase in antioxidants compared with $AlCl_3$-treated rats. PFAS mitigated the $AlCl_3$-induced histological changes by dose dependently. PFAS showed potent neuroprotective effect against $AlCl_3$-induced oxidative stress in rats. Hence, it would be promising compound to treat AD.

Keywords:
Alzheimer's disease, *Annona squamosa Linn*, neuroprotective effect

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Introduction

Alzheimer's disease (AD) is a neurodegenerative disease distressing older adults [1]. It is a progressive disease leading to disturbances of memory and cognitive function. It is estimated that approximately five million people with age 65 years or older and 200 000 people younger than 65 years are affected by AD [2]. Current therapy for AD only has a partial effect and poor management over the disease-causing neurons associated with Alzheimer's [3]. Natural products could be a resource of neuroprotective drugs as they can keep normal cellular communication in the brain and diminish the loss of neuronal functions in pathological conditions [4]. Till date, there are no scientific reports on the neuroprotective effect of leaves extract of *Annona squamosa Linn* against AD. Therefore, the present study was aimed to explore the neuroprotective effect of a polyphenolic fraction of leaves of an extract of *A. squamosa L.* (PFAS) against aluminium chloride ($AlCl_3$)-induced AD in rats.

Materials and methods

Chemicals

1, 1 diphenyl-2-picrylhydrazyl (DPPH), 5, 5-dithio-bis-(2-nitrobenzoic acid), Tris-HCl, trichloroacetic acid, thiobarbituric acid, and $AlCl_3$ were the products of Hi-Media Pvt. Ltd (Mumbai, Maharashtra, India). Gallic acid and quercetin hydrate was the products of TCI Chemicals (Chennai, India). Folin-Ciocalteu phenol reagent was obtained from Loba Chemie Pvt. Ltd (Mumbai, Maharashtra, India). All other chemicals were of analytical grade.

Plant material collection

Leaves of *A. squamosa L.* were collected from Ananthapuramu region, Andhra Pradesh, India. The

specimen was identified and authenticated by Dr. J. Raveendra Reddy, pharmacognosist, Raghavendra Institute of Pharmaceutical Education & Research (RIPER).

Extraction

A weighed quantity (1000 g) of fresh, finely grounded powder was mixed with ethanol+water (water : ethanol, 1 : 3) and subjected to maceration with alternate shaking for 8 days. The extract then obtained was collected by filtration using a muslin cloth. Thus, obtained filtrate was subjected to solvent evaporation to obtain solid extract, which was weighed and stored in a desiccator.

Qualitative phytochemical screening

The plant extract is tested for the screening of phytoconstituents like flavonoids, tannins and phenolic compounds, alkaloids, terpenoids, and saponins [5].

Fractionation

Fractionation of crude extract was done by trituration method. The hydroalcoholic extract (20 g) was added with n-hexane and then shaken for 10 min. After shaking, it was filtered with sinter glass, and then the filtrate is collected. It is repeated three times with n-hexane. The collected filtrate was condensed using a rotary evaporator, and it was called n-hexane fraction. The residue is called n-hexane non-soluble fraction, was then further with ethyl acetate and then shaken for 10 min. After shaking, it was filtered with sinter glass, and then the filtrate is collected. It is repeated three times with ethyl acetate. The collected filtrate was condensed using a rotary evaporator, and it was called ethyl acetate fraction. The rest is called ethyl acetate nonsoluble fraction, which was then added with chloroform and then shaken for 10 min. After shaking, it was filtered with sinter glass, and then the filtrate is collected. It is repeated three times with chloroform. The collected filtrate was condensed using a rotary evaporator, and it was called chloroform fraction. The residue is called chloroform nonsoluble fraction [6].

Estimation of total phenolic content

Overall, 1 ml of extract or standard solution of gallic acid at concentrations of 20, 40, 60, 80, and 100 mg/ml is added to a 25-ml volumetric flask, which already contains 9 ml of distilled deionized water. To the aforementioned solution, 1 ml of Folin-Ciocalteu reagent is added and shaken well. Then, 10 ml of 7% Na_2CO_3 solution was added after 5 min to the aforementioned solution. Then, the solution is diluted by using 25 ml of water, and mixing is done. The solution is incubated for 90 min at room temperature, and absorbance was determined at 750 nm using a ultraviolet (UV)–visible spectrophotometer. The total phenolic content was expressed in units as milligrams of gallic acid equivalents per 100 g dry mass (mg gallic acid equivalents/100 g). All samples were analyzed in triplicate [7].

Estimation of flavonoid content

Overall, 1 ml of extract and standard solutions of quercetin at concentration of 20, 40, 60, 80, and 100 mg/ml was added to a 10-ml volumetric flask already containing 4 ml of distilled deionized water. To the aforementioned solution, 0.3 ml of 5% sodium nitrate was added. Then, 0.3 ml of 10% $AlCl_3$ was added after 5 min, and at 6th min, 2 ml of 1 M NaOH was added, and the total volume was made up to 10 ml with distilled water. The whole solution was mix up, and absorbance was measured at 510 nm in UV–visible spectrophotometer. The data of the total flavonoid content was expressed as milligrams of (+) quercetin equivalents per 100 g dry mass (mg quercetin equivalents/100 g/dm). Analysis of each sample was done in triplicate manner [8].

In vitro antioxidant activity (1, 1-diphenyl-2-picrylhydrazyl)

Aliquots (3 ml) of PFAS and ascorbic acid at different concentrations (25–500 μg/ml) in methanol were added to 1 ml of a 0.04% w/v DPPH solution in triplicate manner. An equal amount of methanol is added to DPPH and used as a control. The mixture was allowed to stand in the dark at 25°C for 30 min, and the absorbance of the mixture was recorded at 517 nm using a double-beam UV/visible spectrophotometer. The IC_{50} was determined from the absorbance versus concentration plot [9].

Experimental animals

Male Wistar rats (180–200 g) were procured from Raghavendra Enterprises (Bangalore, Karnataka, India). They were fed with a standard laboratory diet, water ad libitum, and maintained at a temperature of 21±2°C, relative humidity of 55±5%, with a 12-h light and dark cycle. All the animal experiments were conducted according to the protocols approved by the Institutional Animal Ethical Committee (Protocol No: IAEC/XI/01/RIPER/2018) according to prescribed guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India.

Acute toxicity study

As per OECD guideline 423, acute oral toxicity test of the PFAS was evaluated in mice. Swiss albino mice 15–25 g were randomly divided into four groups, with three animals in each group. The animals were fasted overnight and PFAS was orally administered to different groups of mice at doses of 300, 500, 1000, and 2000 mg/kg body weight, respectively. The animals were observed continuously for further 24 h. Behavior of the animals and any other toxic symptoms were also monitored for 72 h, and they were kept back under observation up to 14 days.

Dose selection

As there were no observable acute toxic effects at dose of 2000 mg/kg, hence PFAS was selected 1/10 of maximum dose as lower dose and 1/5 of maximum dose as higher dose, which are 200 and 400 mg/kg (postoperative) of body weight, respectively.

Treatment schedule

A total of 30 animals were randomly divided into five groups, and each group consisted of six animals and received the treatment for the period of 28 days (Table 1).

All the animals at predetermined dates such as at 1, 15, and 28 days were checked for behavioral parameters. After 28th day, rats were killed, and antioxidant parameters and brain acetylcholinesterase content were determined, along with histopathological studies.

Evaluation of behavioral parameters

Locomotor activity

Actophotometer contains an enclosed area, in which measurement of exploration and voluntary locomotion activity is done. The animals were placed individually in a 30×30 cm black metal chamber with a screen floor and a light-tight lid. Six beams of red light were focused on Photocells and photocell connected to counter. Each beam interruption by the animal movement was registered in the external counter. The light beam breaks were counted for 5 min [10].

Table 1 Treatment schedule for Alzheimer's disease

Groups	Treatment	Dose
I	Normal control	Normal water and normal feed
II	Induced group	$AlCl_3$ (100 mg/kg-oral)
III	PFAS 200 mg/kg (low dose)	$AlCl_3$ (100 mg/kg-PO)+PFAS (200 mg/kg-PO)
IV	PFAS 400 mg/kg (high dose)	$AlCl_3$ (100 mg/kg-PO)+PFAS (400 mg/kg-PO)
V	Donepezil	1 mg/kg-PO

$AlCl_3$, aluminium chloride; PFAS, polyphenolic fraction of *Annona squamosa L*; PO, postoperative.

Motor coordination (Rota rod test)

The Rota rod apparatus consists of a rotating rod and adjusted to a speed of 20 revolutions/min during the test session. The latency to fall in a test session of 180 s was taken as a measure of motor coordination [11].

Pole climb response test in rats

Conditioned avoidance response is to assess the nootropic and neuroprotective effect of medicinal agents by using Pole climb response apparatus. It consists of 2.5-MHz speakers, and a 20-V lamp was attached on the top of the chamber. Before 10 days of starting the experiment, animals are trained by giving buzzing sound 15 s followed by a scrambled shock to the grid floor at a rate of 1.5 mV for 26 s at least 10 trials per day, with an interval of 1 min, for days before the main study in such a way that animals climb the suspended pole to escape from the noxious shock. The response to buzzing sound is called condition response, and response to electrical shock is called as an unconditional response. On the day of testing, test drug was administered before 1 h of recording results [12].

Elevated plus-maze test for spatial memory in rats

The elevated plus-maze analysis is used for measuring long-term memory. It is made up of two identical open (50×10 cm) and closed (50×10×40) arms opposite to each other, and it is elevated 50 cm from the floor. Before experimentation (acquisition period), an individual rat was kept at the distal end of any one of the open arms by facing toward the center of the maze, and the time required for the animal to enter any one of the closed arm (transfer latency time) was noted down. Repeated experimentation reduces the transfer latency time, which indicates the animal's learning and memory retention ability. During experimentation, the transfer latency time of all the animals was noted down by keeping the individual animal in the plus-maze per the period of 5 min (observation time), and if the animal fails to move into closed arm within the observation time, the transfer latency time was considered as 5 min [13].

Estimation of biochemical parameters

The animals were killed by euthanasia, and the brain was isolated and quickly transferred to ice-cold phosphate buffered saline (pH, 7.4). It was maintained free of blood and other tissue fluids and weighed. The brain tissue was nicely chopped with a surgical blade into small slices. Then the pieces were placed in an ice-cold 0.25 M sucrose solution and quickly placed on a filter paper. After that, the pieces were crushed and homogenized in ice-cold

tris-HCl buffer of strength 10 Mm of pH-7.4 to a concentration of 10% w/v. The obtained homogenate was centrifuged at 7000 rpm for 25 min under normal conditions. The clear supernatant fluid was used for the purpose of estimation of different biochemical parameters. Estimation of superoxide dismutase was predictable by the method of Misra and Fridovich (1967). Catalase is estimated (CAT) by Hugo E. Aebi Method (1974). Reduced glutathione was estimated by the method of Maron *et al.* (1979). Lipid peroxidation is estimated by the method of Ohkawa *et al.* (1979) [14].

Histopathology

The spared part of the brain tissue was immediately fixed with 10% formalin. Thereafter, the specimens were embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin.

Statistical analysis

The analysis of data was done by using one-way analysis of variance, followed by Tukey's multiple comparison tests by using Graph pad prism 5.0 software (Graph pad, San Diego, California, USA).

Results and discussion

AD is an age-linked neurodegenerative disease characterized by severe dementia. Symptoms of AD occur over a long period of time, and therefore, treatments should target this silent period of the disease to retard the disease progression toward dementia [15]. This disease is characterized by accumulation of amyloid β plaques, progress of neurofibrillary tangles, inflammation, and neuronal loss in specific regions of the forebrain that gradually destroys memory and thinking skills and ultimately the ability to carry out the simplest tasks [16]. At present, most accepted AD treatment strategy is inhibitors of acetylcholinesterase, including rivastigmine, donepezil, tacrine, galantamine, and memantine. However, there is no drug for AD, and drugs available only calm the disease symptoms. There is no proper treatment leading to cure of AD till now [17]. Enormous efforts are intended toward discovery of various disease-modifying therapies and drugs targeting molecular pathways and blocking progression of AD [18]. Natural polyphenolic phytochemicals have recently gained greater attention as alternative therapeutic agents against AD [19]. They are considered less toxic and more effective than novel synthetic drugs [20]. However, commonly herbal medicines are prepared from the crude materials, which raise questions regarding their mechanism of action and medicinal effects. Recently, research has been focused on specific active components rather than on an entire herb [21]. Therefore, there is a need to identify a number of active constituents and to characterize them according to their therapeutic potentials, focusing on their effects toward neurodegenerative diseases such as AD [22]. The present study is aimed to explore the anti-Alzheimer's activity of PFAS in rats. Numerous reports state that aqueous mixtures of organic solvents are the most suitable for extraction of phenolic compounds from plant sources [23]. The hydroalcoholic extract of *A. squamosa L.* showed the presence of flavonoids, tannins, phenolic compounds, alkaloids, terpenoids, and saponins (Table 2). Recently, a great number of natural medicinal plants have been tested for their therapeutic properties, showing that the raw extracts or isolated pure compounds from them have more effective properties than the whole plant, as an alternative for the treatment of AD. Polyphenolic compounds gain popularity with structural divergent and broad pharmacological activities. They have free –OH group with which form stable phenoxyl radicals by interacting toxic free radical and make them nontoxic chemicals, which further limits neurodegeneration of cholinergic neuron, which are sources of synthesis and release of acetylcholine [2,24]. So determination of total phenolic and flavonoid content helps to analyze the antioxidant potential of fraction [25]. The PFAS has considerable amount of phenolic and flavonoid compounds (Table 3). The PFAS has been shown to have good antioxidant activity by DPPH method (Table 4), and our results were in line with previous studies [26].

Table 2 Phytochemical screening of hydroalcoholic extract of *Annona squamosa*

Serial numbers	Test	Hydroalcoholic extract of *Annona squamosa Linn*
1	Flavonoids	+++
2	Tannins and phenolic compounds	+++
3	Alkaloids	+
4	Terpenoids	+
5	Saponins	+

Table 3 Total phenolic contents and total flavonoid contents of polyphenolic fraction of *Annona squamosa L*

Extracts	TPC (mg of GAE/g)	TFC (mg of QE/g)
PFAS	60.08±3.63	54.67±3.33

Values are expressed as mean±SD (n=3). GAE, gallic acid equivalents; PFAS, polyphenolic fraction of *Annona squamosa L.*; QE, quercetin equivalents.

Aluminum is found in our daily life, such as in drinking water, soil, and tooth pastes. Moreover, it is used to manufacture cooking utensils. Aluminum causes oxidative deterioration of cellular lipids, proteins, and DNA. Lipid peroxidation can cause tissue injury under chronic condition. Therefore, aluminum can be considered as a fundamental factor in AD [27]. In the present study, an AD-mimicking model was induced by injection of $AlCl_3$ (100 mg/kg, oral) daily for 28 days [28]. $AlCl_3$-treated rats showed significant decline in psychological state when tested by actophotometer (locomotor activity) (Table 5), decline in motor coordination when tested by rota rod (Table 6), decline in spatial memory when tested by elevated plus maze and in agreement with the results of Auti and Kulkarni [29] (Table 7), in addition to significant increased levels of brain acetylcholinesterase (AchE) (Table 8). Neurotoxic effect of aluminum significantly increases AchE activity, which is responsible for hydrolysis of acetylcholine. The increase in activity of AchE by aluminum could be owing to the allosteric interaction between cation (Al+3) and anionic sites of AchE enzyme, which lead to alteration in secondary structure of AchE in the brain [30], significant decrease in antioxidants like superoxide dismutase, catalase, and reduced glutathione and significant increase in lipid peroxidation compared with normal control group rats because of the prolonged and extensive exposure of the aluminum toxicity, which is the root cause for the development of the AD that damages the neurons by increasing oxidative stress [31] (Table 9). These results were confirmed by the histopathological examination of the hippocampus of the same rats which revealed the presence of neuronal shrinkage and neuronal degeneration. Treatment of AD rats with PFAS at dose 200 and 400 mg/kg and donepezil at dose of 1 mg/

Table 4 EC50 values (μg/ml) of radical scavenging activity of polyphenolic fraction of *Annona squamosa L.*

Extract	EC 50 values (μg/ml)
PFAS	94.73±1.55

Values are expressed as mean±SD (*n*=3). PFAS, polyphenolic fraction of *Annona squamosa L.*

Table 5 Effect of polyphenolic fraction of *Annona squamosa L.* on locomotor activity

		Locomotor activity (number of counts/5 min)		
Serial numbers	Group	1st day	15th day	28th day
1	Normal group	310±5.79	309±3.376	326±4.361
2	$AlCl_3$ 100 mg/kg	314±4.00^{ns}	228.24±4****	226.02± 5.11****
3	PFAS 200 mg/kg	305.20±2.71^{ns}	252.66±2.57***	250.10±2.57***
4	PFAS 400 mg/kg	306.6±6.34^{ns}	253.88±1.74###	250.50±3.05###
5	Donepezil 1 mg/kg	318.400±4.10^{ns}	316.200±5.34###	323.800±1.91###

$AlCl_3$, aluminium chloride; PFAS, polyphenolic fraction of *Annona squamosa L.* ****P value less than 0.0001 versus normal group, ###P value less than 0.001 versus $AlCl_3$ group.

Table 6 Effect of polyphenolic fraction of *Annona squamosa L* on motor coordination test

		Fall of time (s)		
Serial numbers	Group	1st day	15th day	28th day
1	Normal group	14.520±0.36	18.03±0.34	15.29±0.28
2	$AlCl_3$ 100 mg/kg	14.93±0.340^{ns}	10.98±0.47****	10.40±0.45****
3	PFAS 200 mg/kg	14.73±0.28^{ns}	13.52±0.33$^{##}$	12.560±0.36$^{##}$
4	PFAS 400 mg/kg	16.86±0.58	13.85±0.58$^{##}$	12.984±0.48$^{##}$
5	Donepezil 1 mg/kg	14.670±0.472^{ns}	15.872±0.0.553$^{###}$	16.138±0.200$^{###}$

$AlCl_3$, aluminium chloride; PFAS, polyphenolic fraction of *Annona squamosa L.* ****P value less than 0.0001 versus normal group, ###P value less than 0.001 versus $AlCl_3$, ##P value less than 0.01 versus $AlCl_3$.

Table 7 Effect of polyphenolic fraction of *Annona squamosa L* on elevated plus-maze test

		Transfer latency (s)		
Serial numbers	Group	1st day	15th day	28th day
1	Normal group	35.33±0.35	38.27±1.026	40.070±1.226
2	$AlCl_3$ 100 mg/kg	35.66±0.44^{ns}	48.33±1.609****	49.46±0.90****
3	PFAS 200 mg/kg	35.33±0.35^{ns}	38.27±1.026$^{##}$	44.70±1.36$^{##}$
4	PFAS 400 mg/kg	35.47±0.70^{ns}	43.60±0.439$^{##}$	44.70±0.49$^{##}$
5	Donepezil 1 mg/kg	35.93±0.92^{ns}	37.47±0.928$^{###}$	37.39±0.696$^{###}$

$AlCl_3$, aluminium chloride; PFAS, polyphenolic fraction of *Annona squamosa L.* ****P value less than 0.0001 versus normal group, ###P value less than 0.001, ##P value less than 0.01 versus $AlCl_3$.

Table 8 Effect of polyphenolic fraction of *Annona squamosa L* on acetylcholinesterase activity

Serial numbers	Group	AchE (μmol/min/mg wet tissue)
1	Normal group	15.38±0.58
2	$AlCl_3$ 100 mg/kg	22.35±0.54***
3	PFAS 200 mg/kg	18.25±0.84###
4	PFAS 400 mg/kg	15.63±1.46####
5	Donepezil 1 mg/kg	16.67±0.6363####

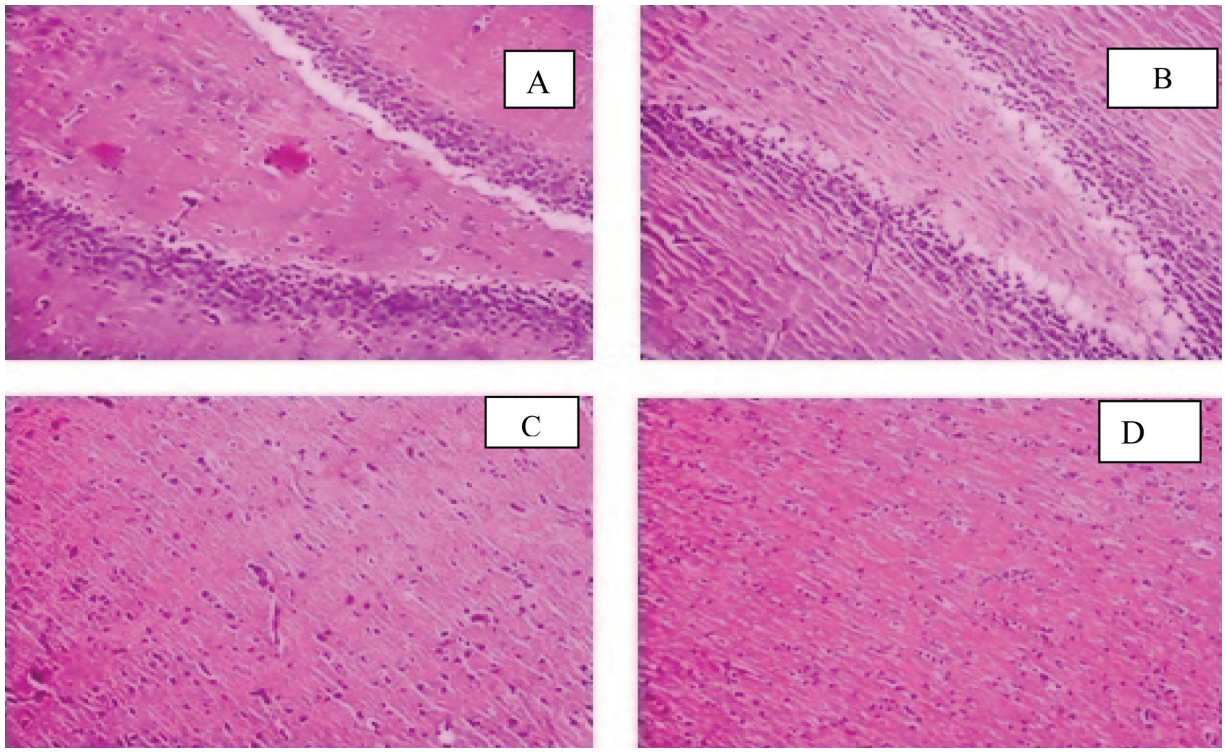
AchE, acetylcholinesterase; $AlCl_3$, aluminium chloride; PFAS, polyphenolic fraction of *Annona squamosa L.* ***P value less than 0.001 versus normal group, ####P value less than 0.0001,###P value less than 0.001 versus $AlCl_3$.

Table 9 Effect of polyphenolic fraction of *Annona squamosa L.* on oxidative parameters in brain tissue homogenate

Serial numbers	Group	SOD (U/mg wet tissue)	CAT (μmole H_2O_2 decomposed/mg wet tissue	GSH (nmol GSH/mg wet tissue)	LPO (nmol MDA/mg wet tissue)
1	Normal group	29.17±0.40	43.14±1.08	46.98±1.80	56.59±1.45
2	$AlCl_3$ 100 mg/kg	19.83±0.33***	27.44±2.85****	31±1.79***	68.98±1.68***
3	PFAS 200 mg/kg	23.54±0.80##	39.15±0.64##	40.84±0.27###	53.6±2.97###
4	PFAS 400 mg/kg	24±0.50##	39.83±0.61##	41.16±0.82####	59.01±0.19####
5	Donepezil 1 mg/kg	29±0.83####	41.85±1.43###	47.67±2.50###	52.62±1.66###

$AlCl_3$, aluminium chloride; CAT, catalase; GSH, glutathione; LPO, lipid peroxidation; PFAS, polyphenolic fraction of *Annona squamosa L*; SOD, superoxide dismutase. ****P value less than 0.0001, ***P value less than 0.001 versus normal group, ####P value less than 0.0001, ###P value less than 0.001, ##P value less than 0.01 versus $AlCl_3$.

Figure 1



Effect of polyphenolic fraction of *Annona squamosa* (PFAS) on aluminium chloride-induced pathological changes in the hippocampus of rats revealed by hematoxylin and eosin staining. (a). Normal group rats showed normal architecture of hippocampus region of brain tissue. (b). Aluminium chloride ($AlCl_3$)-induced rats showed the neuronal shrinkage and neuronal degeneration in hippocampus region of brain tissue. (c). PFAS (200 mg/kg body weight) treated rats showed regenerative changes in brain tissue. (d). PFAS (400 mg/kg body weight) treated rats showed near to normal cyto-architecture of hippocampus region.

kg exhibited significant progress in AD-like status in rats, as evidenced by increase in locomotor activity (Table 5), improvement in motor coordination (Table 6), and improvement in spatial memory, which is also supported by previous data [32] (Table 7); moreover, there are significant decreased

levels of brain AchE, a finding which might be owing to the enzyme inhibition by polyphenolic content in the fraction (Table 8), and significant increased levels of antioxidants like superoxide dismutase, catalase, and reduced glutathione and significant decreased level of lipid peroxidation compared with $AlCl_3$-induced rats, owing to the fact that antioxidative activity of polyphenols is related to their ability to chelate metal ions and acting as ROS scavengers [33] (Table 9). Histopathological findings of PFAS-treated rats at dose 200 mg/kg are regenerative changes in brain tissue, whereas PFAS-treated rats at dose 400 mg/kg are near to normal cyto-architecture of hippocampus region. However, the high dose of PFAS (400 mg/kg) exhibited a better effect than the low dose (200 mg/kg) (Fig. 1).

Conclusion

The PFAS exhibited dose-dependent protective effect against AD by significant improvement in behavioral parameters, restoration of acetylcholine and antioxidants, and mitigation of histological changes caused by $AlCl_3$. PFAS showed potent neuroprotective effects against $AlCl_3$-induced oxidative stress in rats owing to direct scavenging of reactive oxygen species. Hence, it would be a promising compound to treat AD. Further research on minutiae of efficacy studies on human participants is well needed.

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Conflicts of interest

There are no conflicts of interest.

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