INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print); 2222-5234 (Online)
Website: https://www.innspub.net
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Vol. 27, Issue: 6, p. 44-53, 2025

RESEARCH PAPER OPEN ACCESS

Acetylcholinesterase inhibitory and antioxidant activities of whole plant ethanolic extract of *Ophiorrhiza recurvipetala* in aluminium chloride induced *Drosophila melanogaster* model of Alzheimer's disease

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Key words: *Ophiorrhiza recurvipetala*, *Drosophila melanogaster,* Antioxidants, Acetylcholinesterase, Negative geotaxis

DOI: https://dx.doi.org/10.12692/ijb/27.6.44-53 Published: December 08, 2025

ABSTRACT

Alzheimer's disease (AD) is characterized by progressive memory impairment and deficits in cognitive functions. *Drosophila melanogaster*, is used as a model organism owing to the genetic and cost-effective advantages. Aluminium exposure in *Drosophila* affects cognitive function and genes related with AD. Previous study from our lab indicated ethanolic extract of *Ophiorrhiza recurvipetala*. (OREE) explored the potent radical scavenging properties. Therefore, it was aimed to study the survival rate, acetylcholinesterase inhibitory, antioxidant and climbing activities of ethanolic extract of OR in an aluminium chloride induced flies. Flies were divided based on the survival rate into 4 groups, Group I - normal diet for 7 days; Group II - diet with 0.5 ml of $AlCl_3$ in 100 ml of distilled water for 7 days; Group III - diet with $AlCl_3$ as group II + OREE (1.0 %) for 7 days; Group IV - diet with OREE (1.0 %) for 7 days. This study demonstrated the reduced TBARS levels and anticholinesterase activities andenhanced survival rate, antioxidant and climbing activitiesof OREE suggesting its neuroprotective function. Further investigations are needed to evaluate the protective role of OREE on Aβ and tau proteins that are the hallmarks of AD in other animal models.

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INTRODUCTION

Dementia is one among the top 10 worldwide incidences of death (World Health Organization, 2019). Most predominant form of dementia is Alzheimer's disease (AD) which is projected to get tripled by the year 2050, mainly of population aging (Nicholas *et al.*, 2019). Globally, about 32 million were affected by mild, to severe forms and 87 million people by early AD (Gustavsson *et al.*, 2023). AD is the characterized by gradual deficits in cognitive functions, which are caused by the accumulation of neuronal amyloid plaques and neurofibrillary tangles. The epidemiology of AD remains complex and elusive linking various pathological processes like oxidative imbalance, inflammation, loss of mitochondrial function and apoptosis (Chauhan *et al.*, 2024). Current therapeutic ailments can only manage the symptoms without curing AD. But, due to ethical issues and technical limits obstruct the clinical studies. The models of AD ranging from yeast, Caenorhabditis elegans, *Drosophila melanogaster* (*D. melanogaster*) to mammals and human cell culture systems to elucidate the mechanism and therapeutic implications.

Due to the combination of handling, behavioural, structural, genetic and cost-effective advantages, flieshave arisen as a key model organism in the research of neurodegenerative diseases (Lu and Vogel, 2009; Nitta and Sugie, 2022). The mechanistic role of metal ions like aluminium (Al), zinc, lead and cadmium on AD pathology were reported (Chauhan *et al.*, 2024). After exposure with excess Al, several neurodegenerative phenotypes such as diminished locomotion and life span, enhanced olfactory learning irregularities and brain vacuolization were found in Drosophila (Fonte *et al.*, 2002). Moreover, *D. melanogaster* displays age-linked behavioural alterations and pathological processes including oxidative stress, that resembles the features of human NDDs (Prüßing *et al.,* 2013; Tsintzas and Niccoli, 2024). The human genes linked with AD like β-secretase and APP - have homologous counterparts in *Drosophila* (β-secretase-like enzyme and dAPPl) (Fortini *et al.,* 2000). These collective characteristics convert *D. melanogaster* as a vital tool for examining potential therapeutic targets (McGurk *et al.,* 2015).

The plant-based medicines possess numerous secondary metabolites with multiple pharmacological activities were used in treating chronic diseases by their synergistic functions. Thorough knowledge about them helps to develop the novel drugs from medicinal plants (Alum, 2024). The *Ophiorrhiza* genus of Rubiaceae family was found in Asia, Australia and the Pacific Islands (Schanzer, 2005). In Ayurvedha, these plants were used to treat stomach problems including ulcers, snakebite and wounds (Krishnan *et al.,* 2004; Prabha *et al.,* 2018). It is able to reduce pain, inflammation, cancer, and infections caused by bacteria and virus. It is reported to have antioxidant, antitussive and pain-relieving properties (Martins and Nunez, 2015; Sibi *et al.,* 2012). The root extract of the plants is used to treat leprosy, stomach and mensural problems, while having sedative and purgativeproperties (Preethamol and Thoppil, 2022). *O. jacobii* and *O. japonica* revealed antioxidant potential while *O. fasciculata* has exhibited antioxidant and anti-inflammatory activities, due to its enriched bioactive compounds (Preethamol and Thoppil, 2022; Bu *et al.,* 2022, Rashid *et al.,*2023). In India, a new species (*Ophiorrhiza recurvipetala*) is reported in recent study by Bhuyan *et al.* (2021) which closely mimics *Ophiorrhiza ochroleuca* Hook.f., but differs in few morphological characters. The phytochemical analysis of *Ophiorrhiza recurvipetala* (OREE) and *in vitro* antioxidant assays were performed previously in our lab showed the presence of various bioactive components with anti-oxidant and anti-inflammatory properties and also explored the potent radical scavenging properties of OREE (Vidya *et al.,* 2025) suggesting its neuroprotective activity. Therapeutic agents diminishing oxidative stress and/or enhancing acetylcholine levels play a vital role in the management of AD. Therefore, this investigation was aimed to study the acetylcholinesterase inhibitory and antioxidant activities of ethanolic extract of OR in an aluminium chloride induced *D. melanogaster* model of AD, which will be the first attempt.

MATERIALS AND METHODS

Chemicals

Aluminium chloride, phenyl methosulphate (PMS), nitroblue tetrazolium (NBT), NADH, glacial acetic acid, n-butanol, tricholoroacetic acid, thiobarbituric acid, HCl and nicotinamide adenine dinucleotide were obtained from Himedia Pvt. Ltd., Mumbai.

Drosophila melanogaster stock and culture

Wild-type (WT) of *D. melanogaster* were obtained from Centre for Cellular and Molecular Biology (CCMB), Hyderabad, Telangana, India. Flies were cultured on standard cornmeal fly medium and retained at 20-25 °C with a 12h Light-Dark (12:12) cycle in the *Drosophila* culture incubator (Rays Scientifics Instruments, Chennai, India) (Kani and Subramanian, 2021).

Dose dependent/Survival study

5 groups of flies were separately maintained as Group I – normal diet; Group II - diet with 0.5 ml of $AlCl_3$ in 100 ml dH_2O (Saliu *et al.*, 2024); Group III - diet with $AlCl_3$ as group II+ OREE (0.1 %); Group IV - diet with $AlCl_3$ as group II + OREE (1.0 %); Group V - diet with OREE (1.0 %). After 15 days, flies were observed for viability on day-to-day basis and it was quantified (Abolaji *et al.,* 2017) by determining the number of alive flies found.

Experimental design

Flies (male and female) were separately maintained as 4 groups. Normal dietwas given to the Group I for 7 days; Group II - diet with $AlCl_3$ for 7 days (Saliu *et al.*, 2024); Group III - diet with $AlCl_3$ as group II + OREE (1.0 %) for 7 days; Group IV - diet with OREE (1.0 %) for 7 days.

Negative geotaxis: Response to gravity

The flies (n =30) were exposed to diethyl ether and transferred to a glass tube (12 cm X 1.5 cm) which is plugged with cotton at top. After five minutes, flies were allowedto settle. Following a minute, moving and resting flies (percentage) were counted separately. This assay was repeated thrice and mean ± SEM was calculated.

Estimation of thiobarbituric acid reactive substances (TBARS)

The whole-bodyhomogenate was mixed with TBA-TCA-HCl reagent and placedin boiling H_2O. Then, tubes were cooled and centrifuged (Niehaus and Samuelsson, 1968). The supernatant was used to observe OD at 535 nm.

Assay of superoxide dismutase (SOD)

To 0.5 ml of sample, same volume of water, 1.5 ml and 2.5 ml of chloroform and ethanol were added, mixed and centrifuged in a cooling centrifuge. To the supernatant, NBT, PMS, sodium pyrophosphate bufferand NADH was added and maintained in room temperatureand to stop the reaction, it was mixed with glacial acetic acid. Then n-butanol was added, centrifuged after 10 minutes, precipitate was removed, and OD was read at 510 nm (Kakkar *et al.*, 1984).

Assay of catalase

Hydrogen peroxide and phosphate bufferwere added to sample followed bydichromate-acetic acid at various time intervalsfor stopping the reaction and were placed in hot water bath, cooled and OD was observed at 620 nm (Sinha, 1972).

Assay of glutathione-s-transferase (GST)

To thesample, distilled water, phosphate buffer, CDNB (37 °C; 5 min) and glutathione was added and optical density was observedat 340 nm (Habig *et al.,* 1974).

Assay of acetylcholine esterase

Estimation of acetylcholinesterase activity was done by Ellman *et al.* (1959) method. To tissue homogenate, potassium phosphate buffer, DTNB andacetylthiocholine were added. The OD was measured at 412 nm.

Statistical analysis

Using the Statistical Package for Social Sciences (SPSS) version 12.0, one-way analysis of variance and Duncan's Multiple Range Test (DMRT) were used for statistical analysis. Results were expressed as mean±

SEM for four experiments of flies. p values < 0.05 were considered significant.

RESULTS

Survival rate of flies exposed to $AlCl_3$ and OREE

The flies exposed to 40mMol concentration of $AlCl_3$ had significantly decreased percentage survival rate within 15 days as compared to the control flies. Flies co-exposed with $AlCl_3$ and OREE showing the reduced mortality and restored survival rate as compared to $AlCl_3$ alone exposed group. As compared to low dose of OREE (0.1 %), high dose of OREE (1.0 %) showed enhancedviability in $AlCl_3$ exposed flies. So, the high concentration of OREE is considered as the effective dose for further experiments. The OREE (1.0 %) alone exposed flies revealed no significant alterations in survival rate than control group (Fig. 1).

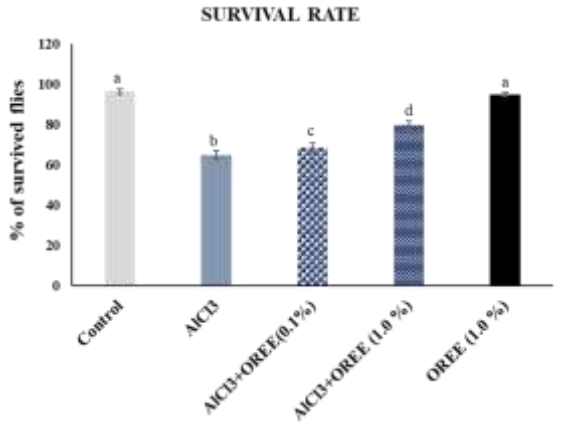

Fig. 1. Effect of AlCl3 and OREE on the percentage survival rate of flies

Data are presented as mean±SEM. Experiments were repeated for four times. Values with different superscript alphabets differ significantly, p< 0.05.

Negative geotaxis of flies exposed to $AlCl_3$ and OREE

The protective role of the OREE on the climbingability of control and $AlCl_3$ exposed fliesexhibited that $AlCl_3$ exposure reduced the activity of flies (diminished negative geotaxis) as compared to control flies. OREE enhanced the climbing activity (enhanced negative geotaxis) in $AlCl_3$ assaulted flies as compared to $AlCl_3$ alone exposed flies. The OREE alone exposed flies revealed no substantial changes in climbing activity than control group (Fig. 2).

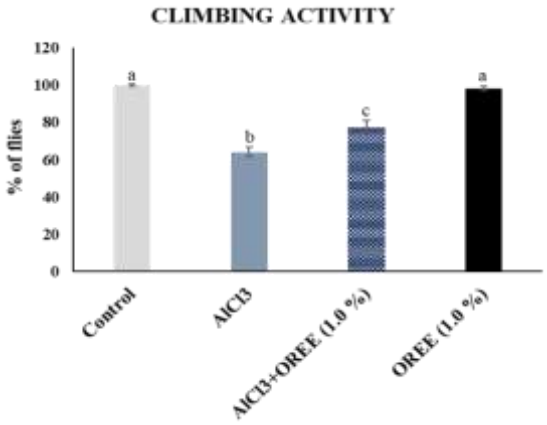

Fig. 2. Effect of AlCl3 and OREE on the climbing rate of flies

Data are presented as mean± SEM. Experiments were repeated for four times. Values with different superscript alphabets differ significantly, p< 0.05.

TBARS concentration inflies treated with $AlCl_3$ and OREE

In flies exposed to $AlCl_3$, a notable increase in TBARS levels was noted. However, the flies co-exposed to $AlCl_3$ and OREE exhibited a lowered TBARS level as compared to $AlCl_3$ alone treated flies. The flies treated with OREE alone showed no discernible change in TBARS levels as when compared with the control group (Fig. 3A).

Enzymatic antioxidant activities of flies treated with $AlCl_3$ and OREE

A significant reduction in the activities of as SOD (Fig. 3B), catalase (Fig. 3C) and GST (Fig. 3D) were found in $AlCl_3$ alone exposed flies as compared to the control flies. However, there was a significant enhancement in the activities of those enzymatic antioxidants in OREE (1.0 %) and $AlCl_3$ cotreated flies as compared to $AlCl_3$ alone exposed flies. The OREE (1.0 %) alone treated flies revealed no significant alterations in the activities of these enzymes than control group.

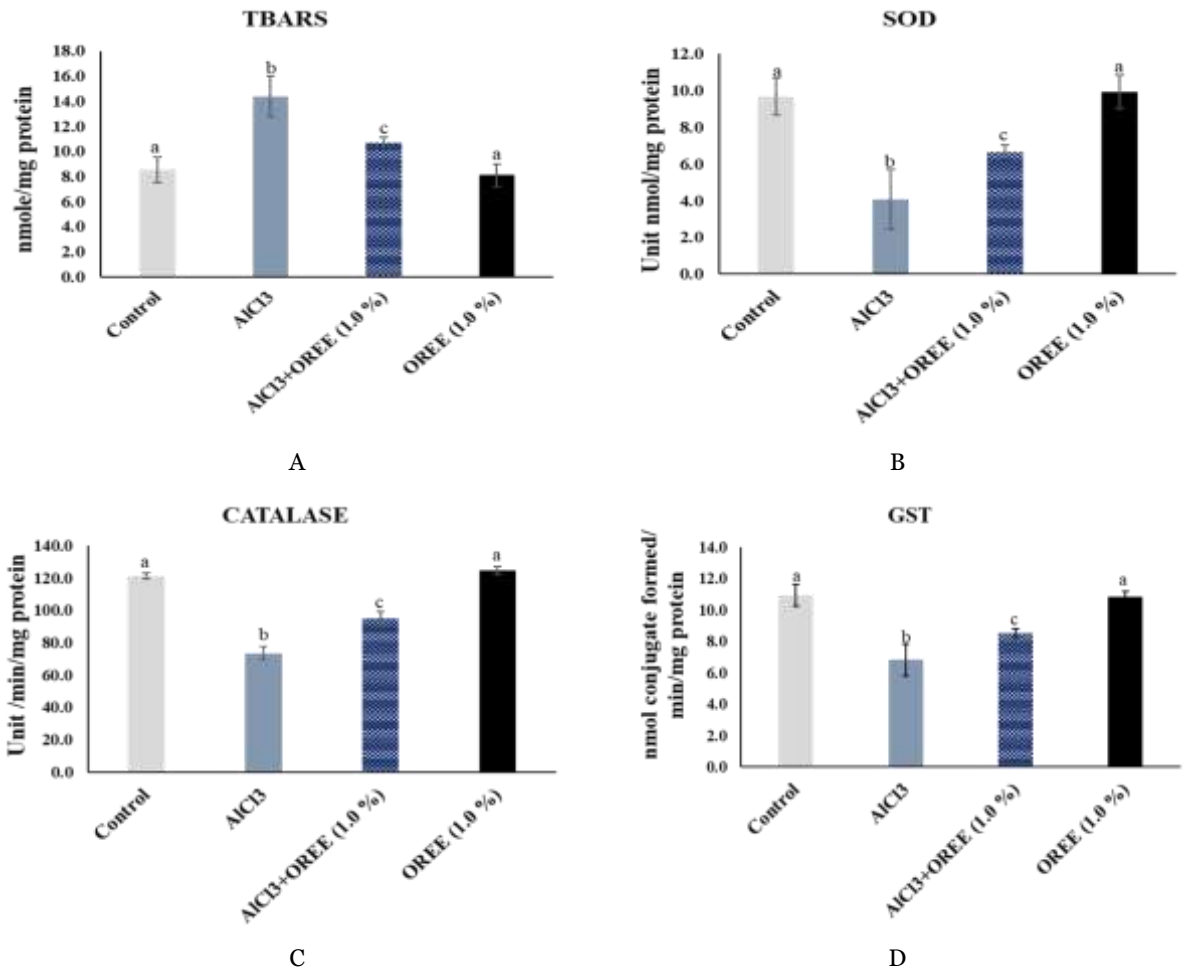

Fig. 3. Effect of $AlCl_3$ and OREE on the levels of TBARS (A), activities of SOD (B), catalase (C) and GST (D) in flies
Data are presented as mean±SEM. Experiments were repeated for four times. Values with different superscript alphabets differ significantly, $p< 0.05$.

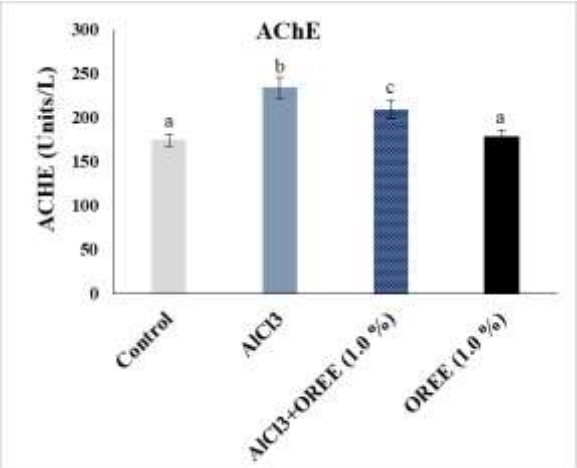

Fig. 4. Effect of $AlCl_3$ and OREE on the activity of AChE in flies
Data are presented as mean±SEM. Experiments were repeated for four times. Values with different superscript alphabets differ significantly, $p< 0.05$.

AchE activity in $AlCl_3$ and OREE exposed flies

The $AlCl_3$-exposedexhibited elevated activity of AChE when compared with normal flies. However, it was found that flies fed with $AlCl_3$ and OREE exhibited a discernible diminutionin AChE activity as compared to $AlCl_3$ alone-exposed flies (Fig. 4).

DISCUSSION

Acetylcholine is a chemical messenger that is reported to involve in episodic and spatial memories, learning, attention, motivation and arousal behaviours in brain. Acetylcholinesterase (AChE) hydrolyses the acetylcholine and an elevated AChE enzyme levels is the main biomarker of AD (Mesulam *et al.,* 2002). Al affected over two hundred reactions in biological system and it causes contrary effects in mammalian CNS (Kawahara and Kato-Negishi, 2011).

Al activated AChE by its allosteric activity and also by its direct binding ability to the enzyme in the β-anionic site (Patočka and Bajgar, 1987). Our observed results marked the increased AChE activity of Al exposed flies, as like prior results (Ogunsuyi *et al.,* 2021; Oboh *et al.,* 2021; Adedayo *et al.,* 2022).

Enhancement of acetylcholine by increasing AChE inhibition was the most important strategy for AD treatment. Hyperforin and galantamine, the plant-based drugs modulated the release of acetylcholine in the CNS (Kumar *et al.,* 2017). Several plants, fungus and marine organisms containing alkaloids (piperidine, quinolizidine, indole, isoquinoline, and steroids) and non-alkaloidal (flavonoids, terpenoids and other phenolic compounds) with potent AChE inhibition activities were reported (Houghton *et al.,* 2006; Williams *et al.,* 2011; Murray *et al.,* 2013). The obtained results indicated the existence of several phytochemical compounds (Vidya *et al.,* 2025) in the OREE may responsible for its AChEi activity, which may use to manage AD.

In negative geotaxis assay, the lowered climbing activity or impairment in locomotion in $AlCl_3$ exposed flies demonstrated the occurrence of neurodegeneration, a main indicator of AD (Oyetayo *et al.,*2020). The obtained results are in concordant with published reports representing $AlCl_3$ mediated locomotor deficits (Oboh *et al.*, 2021), which could result from compromised cholinergic transmission because acetylcholine is essential for the regulation of movement (Day *et al.,* 1991). Enhanced activity of AChE can reduce the levels of acetylcholine and impair the locomotion (Halmenschelager and da Rocha, 2019). Dietary exposure of OREE can able to enhanced the climbing activity and attenuated locomotion deficits of $AlCl_3$ exposed flies which may due to its anti-cholinesterase activity.

The results demonstrated that an enhanced formation of TBARS, an index of lipid peroxidation processes, in Al-exposed flies, while the OREE attenuating the level of TBARS formed. As shown by Exley, 2004 and Zatta *et al.* (2002) Al is reported to have a potent prooxidant activity and metal-based oxidative processes. Moreover, Al combines with superoxide ions and forms complex, a strong oxidant than superoxide anion alone. Further, Al induces hydrogen peroxide and hydroxyl radicals production leading to severe oxidative imbalance (Exley, 2004).

Antioxidants are the low concentrated molecules that impede or interrupt the oxidation of a substrates, thereby counteracting free radical mediated impairment of macromolecules. SOD is called as primary defence enzyme which is reported to convert more toxic superoxide anion into oxygen molecule or less toxic H_2O_2. Uncontrolled superoxide anion production may result in cellular damage (Hayyan *et al.,* 2016). Catalase is a ubiquitous enzyme present in all aerobic organisms, which involved in the breakdown of H_2O_2 into water and molecular oxygen (Pan *et al.,* 2024). It is also a crucial enzyme that protect the cell from damage induced by free radicals. Instead, GST is a key enzyme mainly involving in detoxification of xenobiotics. It catalyses the reactions between reduced glutathione and toxic substance (Lushchak, 2012). The obtained result indicated that there was a reduction in SOD, catalase and GST activities of $AlCl_3$ treated flies, which can be linked with the increase in formation of ROS, a normal process found during the oxidative stress conditions. An increased SOD, catalase and GST actions of $AlCl_3$ and OREE co-treated flies, indicated the antioxidant potential of OREE.

The flies exposed to the diet containing $AlCl_3$ exhibited reduction in the viability of flies due to the toxic effects of $AlCl_3$ which agrees with the previous reports (Burger and Promislow, 2004; Kijak *et al.,* 2014). Impairment in cholinergic neurons, enhanced oxidative stress of $AlCl_3$ exposed flies may be accountable for the reduced survival rate (Oboh *et al.,* 2021). But, OREE exposure along with $AlCl_3$, attenuated $AlCl_3$-mediated death could be due to the existence of antioxidants in the extract.

CONCLUSION

This study demonstrated the antioxidant and anticholinesterase activities of OREE, which attributed to therapeutic action against $AlCl_3$ mediated toxicity and specifically AD. In future, further investigations are needed to reveal the protective action of OREE on Aβ and tau proteins that are the hallmarks of AD in animal models will lead to its utilisation in the management of AD.

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