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Neuroprotective Effect of *Centella asiatica* Leaf Alkaloid Extract on Enzymes Linked with Aluminium-Induced Alzheimer's Disease in *Drosophila melanogaster*

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ABSTRACT

The neurodegenerative disease, Alzheimer's disease (AD) is linked with a depletion of the nerve cells (cholinergic neurons) that utilize Acetylcholine for the transmission of signals in the Central Nervous System (CNS). Inhibition of Acetylcholinesterase (AChE) activity in the brain is the major target in AD's treatment. In this study, the neuroprotective effect of dietary supplementation with *Centella asiatica* leaf alkaloid extract on enzymes linked with Aluminium-induced AD in *Drosophila melanogaster* was investigated. Alkaloid extract was prepared by extraction method and 10 mg/ml of the extract was used for the experiment. Flies were induced with Alzheimer's disease by adding 40 mM of Aluminium chloride ($AlCl_3$) to their diet, after which the flies were treated for seven days using a diet supplemented with alkaloid extract of *Centella asiatica* leaf. The mortality record of the flies was taken daily and their locomotor performance was measured on Day 7. The flies were thereafter homogenized and assayed for acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) activities. Results did not show a significant difference ($P<0.05$) in the rate of survival and locomotor performance of induced flies treated with the extract and induced flies treated with the standard drug, Donepezil, when compared with the control. Also, activities of the enzymes (AChE and BChE) linked with Alzheimer's disease were reduced significantly ($P<0.05$) in the group treated with the extract and in the group treated with Donepezil, when compared to the control. This study therefore suggests that alkaloid extract of *Centella asiatica* leaf have neuroprotective effect on the enzymes linked with aluminium-induced Alzheimer's disease in *D. melanogaster*.

Keywords: Alzheimer's disease, *Drosophila melanogaster, Centella asiatica*, Dietary supplementation, Acetylcholinesterase, Butyrylcholinesterase.

Introduction

Alzheimer's disease (AD) is a progressive and chronic neurodegenerative disease linked to depletion of cholinergic neurons in the brain as well as a decreased level of the neurotransmitter, Acetylcholine (Nigel *et al.*, 2005). AD is the most common type of dementia (Alzheimer's Disease Facts and Figures, 2022), significantly affecting daily living of people, especially those that are 65 years and above, with abnormalities such as cholinergic dysfunction, neuron death, cognitive disorders and memory loss (Yıldırım and Güzeldemirci, 2023). Other symptoms that characterize AD include inability to perform everyday activities, depression, anxiety, behavioral changes, poor judgment, impaired communication and difficulty in walking (Alzheimer's Association,

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2021). More than 50 million of the world's population are estimated to suffer from some form of dementia and by 2050, the figure is expected to rise to about 139 million (GBD Neurology Collaborators, 2016; WHO, 2022).

The major pathological characteristics of brains affected with the disease include neuron loss, neurofibrillary tangles, the presence of senile plaques which is mainly made up of beta-amyloid peptide, neuroinflammation, tau protein phosphorylation, oxidative stress and low levels of the neurotransmitter, acetylcholine in the cortical and hippocampal part of the brain (Gauthier *et al.*, 2021; Bhagat *et al.*, 2021; Bortolami *et al.*, 2021; Carcak-Yilmaz *et al.*, 2017; El-Khatabi *et al.*, 2021; Nguyen *et al.*, 2021). The major target in AD's treatment involves inhibiting Acetylcholinesterase (AChE) activity in the brain (Savelev *et al.*, 2004). However, current available medications for the disease namely Donepezil, Rivastigmine, Galantamine and Memantine (Hyde *et al.*, 2013), are unable to stop the disease progression but can only reduce its symptoms (Nguyen *et al.,* 2021). The side effects of drugs as well as high cost associated with the treatment (Anand and Singh, 2013; Tayeb *et al.*, 2012), has prompted the search for a more holistic approach and less cost with natural source for AD's treatment.

Medicinal plants have been used as natural sources of medicines all around the world (Saranya *et al.,* 2017). *Centella asiatica,* also known as Indian Pennywort and Gotu Kola, is a vital medicinal plant that is utilized globally (Sakshi *et al.*, 2010; Saranya *et al.*, 2017). The medicinal potential of *Centella asiatica* is mainly as a result of secondary metabolites such as alkaloids, saponins, flavonoids, glycosides, reducing compounds, vitamins and minerals, that are present in it (Vinoth *et al.,* 2011; Latif *et al.*, 2019).

Several literatures have reported that *C. asiatica* has neuroprotective effects including anxiolytic, rejuvenant, sedative and intelligence promoting property, on the central nervous system and related ailments (Kumar and Gupta, 2002). Due to its diverse biological components and actions, *C. asiatica* is well known and has been used for centuries for various medical problems such as cognitive dysfunctions (Gohil *et al.,* 2010; Orhan, 2012).

The fruit fly*, Drosophila Melanogaster* is used to study numerous diseases including neurodegenerative diseases such as Alzheimer's, Huntington's, Parkinson's, Spinocerebellar ataxia, oxidative stress and diabetes (Lenz *et al.*, 2013). Though several animal models have been used in order to understand, as well as find new and more effective treatments for neurodegenerative diseases (Giannakou and Crowther, 2011), knowledge of the anatomy, brain and nervous system of *D. Melanogaster* as well as its genetic, behavioral, methodological and economic advantages along with its short life span, make the fruit fly a leading model for research (Prüßing *et al.*, 2013). In addition, the worldwide drift from using animal models to using insect models for experimental studies due to ethical issues such as animal care and preservation of animals has brought about the use of *D. melanogaster* model in this current research.

In spite of the therapeutic ability present in alkaloids for AD's treatment, it may be stated that research associated with the alkaloids of *C. asiatica* seems to be highly overlooked (Abbas *et al.*, 2019). The effect of centella leaf alkaloid extract on the activities of both Acetylcholinesterase (AChE) and Butyrylcholinesterase (BChE) in a fly model of AD is also not fully explored. Hence, this experiment investigated the effect of dietary inclusion of alkaloid extract of *Centella asiatica* leaf on the enzymes (AChE and BChE) linked with Alzheimer's disease, using wild Oregon strain of *Drosophila melanogaster*.

Materials and Methods

Collection of Plant Material

Fresh *Centella asiatica* leaves were collected from New Era Farm, Ile Oluji, Oke-Igbo Local Government Area, Ondo State, Nigeria. Authentication was done in the Department of Crop, Soil and Pest Management, at The Federal University of Technology, Akure,

Nigeria.

Reagents and Materials

All the chemical reagents used for this experiment were of analytical grade, obtained from Sigma Al-drich, Co. (St. Louis, Missouri, USA). The water used was glass distilled.

Preparation of Alkaloid Extract from *Centella asiatica* Leaves

Freshly collected leaves were thoroughly rinsed under running tap water, sun-dried, and ground into a fine powder. Alkaloid extract of the sample was prepared using the method of Harborne (1998), with minor modifications. 50g of the powdered leaves was soaked in 150ml of ethanol and acetic acid solution (9:1 v/v) in an air tight container and placed on an orbital shaker. The mixture was filtered with a muslin cloth and filter paper (Whatman No. 1). The filtrate was allowed to air-dry for five days after which the dried extract was stored in a refrigerator until it was required for use.

Culturing of *Drosophila melanogaster*

The cultivation of *Drosophila melanogaster* was carried out with slight adjustments following the method outlined by Hosamani & Muralidhara, 2009. The wild Oregon strain of *Drosophila melanogaster* was sourced from the Drosophila Laboratory at The Federal University of Technology, Akure (FUTA), Ondo State, Nigeria. These flies were raised and kept in the fly laboratory under standard conditions, specifically at 22±°C with 70-80% relative humidity. The procedure by Farombi *et al.*, 2018, (with minor modifications) was used to formulate the fly diet. The diet consisted of 0.85 L of water, 52 g of cornmeal, 5 g of yeast, 7.9 g of agar agar, 1 g of methyl parabene/ nipagene (preservative) and 1 ml of absolute ethanol. Briefly, the first portion of water (0.7 L) was allowed to boil in a pot while the second portion (0.15 L) was used to dissolve the cornmeal after which the yeast was dissolved in a little amount of hot water. The agar agar was added to the boiling water and allowed to boil for about 10 minutes, while stirring with a turning stick. The dissolved cornmeal was then poured into the boiling water and allowed to cook for another 10 minutes while stirring occasionally after which the dissolved yeast was poured into it. The cornmeal was allowed to cook for 15 more minutes while stirring occasionally before the pot was removed from the heat. After allowing the cornmeal to cool for some minutes, a nipagene-ethanol mixture prepared by dissolving the nipagene in 1 ml of absolute ethanol, was properly added. The prepared cornmeal was distributed in the glass vials into which the flies were introduced.

Experimental Model

The experimental model was conducted using the method of Oboh *et al.*, 2018, with slight modifications. The flies were induced with Alzheimer's disease using 40 mM of $AlCl_3$, according to a prior study on the toxicity of aluminum in *D. melanogaster* by Wu *et al.* (2012).

The flies used for the experimental model were grouped into five (40 flies per replicate; 3 replicates per group) as shown below and the experiment was carried out for 7 days.

Group 1 – (Control) – 1 ml of distilled water with the diet, all to 5 g

Group 2 – 1 ml of 40 mM $AlCl_3$ with the diet, all to 5 g

Group 3 – 1 ml of 40 mM $AlCl_3$ + 1 ml of 10 mg/ml *Centella asiatica* extract with the diet, all to 5 g

Group 4 – 1 ml of 10 mg/ml *Centella asiatica* extract with the diet, all to 5 g

Group 5 – 1 ml of 40 mM $AlCl_3$ + 1 ml of 20 µM Donepezil with the diet, all to 5 g

Lethality Response

Flies were monitored for the occurrence of mortality and the number of dead flies were recorded for each group on a daily basis. The data obtained

were analyzed and thereafter plotted as cumulative mortality and percentage of surviving flies.

Measurement of the Locomotor Performance using Negative Geotaxis Assay

After the 7 days of treatment, the extent of locomotor performance in the flies present in the various groups was assessed using the Negative Geotaxis Assay described by Bland *et al.* (2009) with slight modifications. Surviving flies from each group were immobilized in ice and then transferred into different labeled sterilized tubes (3.5cm diameter and 11 cm length) with the tubes covered, after which the flies were given a recovery period of 10 minutes. The tubes were gently tapped at the bottom and the number of flies that crossed the 6cm mark of the tubes under 6 seconds were recorded. Flies that do not have locomotor dysfunction usually move rapidly to the top while flies having locomotor dysfunction usually move slowly and they stay at the bottom. The obtained results were expressed as the percentage of flies that moved beyond 6cm under 6 seconds. Thereafter, the climbing mean was calculated.

Preparation of Tissue Homogenates

The preparation of the flies' tissue homogenates was done using the method by Farombi *et al.*, 2018, (with minor modifications). The flies that survived from the various treatment groups were anesthetized on ice, weighed and then homogenized in Eppendorf tubes with 0.1 M of phosphate buffer at pH 7.0 (1/10 w/v) using a Teflon homogenizer. The supernatants were prepared by centrifuging the homogenates at 10,000 X g and 4°C for 10 minutes in a Kenxin refrigerated centrifuge Model KX3400C (KENXIN Intl. Co., Hong Kong). The obtained supernatants were separated from the pellets, transferred into labeled Eppendorf tubes and used for the various biochemical assays.

Determination of Total Protein

The total protein present in the fly homogenates was determined using the Coomassie blue method described by Bradford (1976), with the use of 1 mg/ml serum albumin as standard. 10 µL of fly homogenates and 250 µL of Coomasie blue were added to 20 µL of distilled water. The reaction mixture was incubated at room temperature for 30 minutes after which the absorbance was measured at 595 nm in the spectrophotometer. Subsequently, the total protein content was calculated.

Determination of Acetylcholinesterase Activity

The activity of acetylcholinesterase in the tissue homogenate was measured according to a modified version of Ellman's colorimetric method (Perry *et al.,* 2000). A reaction mixture that included 200 µL of fly tissue homogenate in a 0.1 M phosphate buffer at pH 8.0, 100 µL of a 3.3 mM solution of 5,5'-dithio-bis (2-nitrobenzoic) acid (DTNB) in a 0.1 M phosphate buffered solution at pH 7.0 with 6 mM NaHCO6, varying amounts of alkaloid extracts (0–100 µL), and 500 µL of phosphate buffer at pH 8.0, was used to determine the AChE activity. The reaction mixture was incubated for 20 minutes at 25 °C after which the reaction was initiated by the addition of AChE substrate, which was 100 µL of a 0.05 mM solution of acetylthiocholine iodide. The absorbance of the mixture was recorded every 15 seconds over a 5 minutes period at 412 nm using a spectrophotometer. The activity of AChE was subsequently calculated and recorded as µmol AChE/h/mg protein.

Determination of Butyrylcholinesterase Activity

The butyrylcholinesterase activity of the tissue homogenate was determined according to a modified colorimetric method of Ellman (Perry *et al.,* 2000). BChE activity was determined in a reaction mixture containing 200 µL of fly tissue homogenate in 0.1 M phosphate buffer (pH 8.0), 100 µL of 3.3 mM solution of 5,5' -dithio-bis (2- nitrobenzoic) acid (DTNB) in 0.1 M phosphate buffered solution (pH 7.0) containing 6 mM $NaHCO_6$, alkaloid extracts (0–100 µL) and 500 µL of phosphate buffer (pH 8.0). The reaction mixture was incubated for 20 minutes at 25 °C and the reaction was subsequently initiated by adding BChE substrate (100 µL of 0.05 mM solution of butyrylthiocholine iodide). The absorbance was recorded for 5 minutes (15 seconds interval) at 412 nm in a spectrophotometer. Thereafter, BChE activity

was calculated and expressed as µmol BChE/h/mg protein.

Statistical Analysis

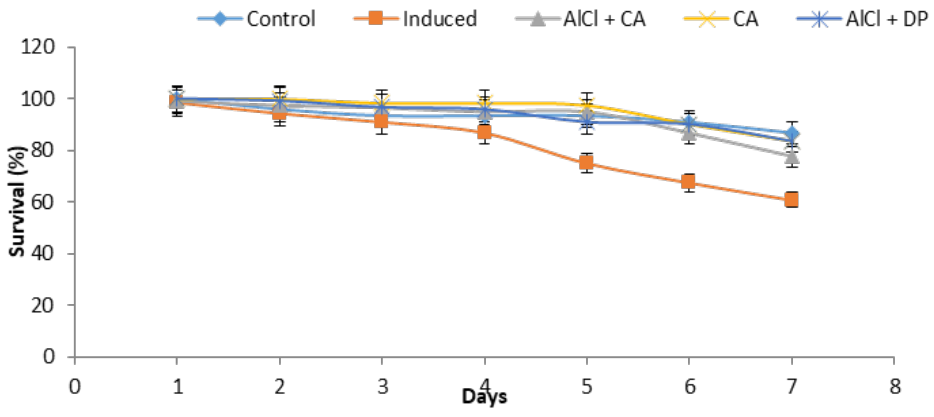
Results were obtained in replicates and expressed as mean ± standard deviation (S.D) values, data for three replicate analysis was subjected to statistical evaluation with the use of the Graph pad PRISM (V.5.0) software. The results were analyzed statistically, using the One-way Analysis of Variance (ANOVA) followed by Tukey's Multiple Comparison Test. The significance difference was accepted at $p \leq 0.05$.

Results and Discussion

In Figure 1, the rate of survival (%) of Alzheimer's disease induced flies and treated flies is shown. The result shows a significant decrease in the survival rate of induced flies (81.9%). When compared with the control, there was no significant decline ($P<0.05$) in the survival rate of induced flies treated with alkaloid extract of *Centella asiatica* leaves (92.5%), induced flies treated with the standard drug (93%), Donepezil and normal flies fed with the extract (93%). This shows that the extract as well as the standard drug were able to increase the rate of survival of flies induced with Alzheimer's disease.

Locomotor performance (%) values of AD induced and treated *Drosophila melanogaster* is shown in Figure 2. In comparison with the control group, climbing behavior of induced flies significantly declined while climbing behavior of normal flies fed with the extract significantly increased. No significant difference ($P<0.05$) was observed in the locomotor performance of induced flies treated with the extract and that of the induced flies treated with the standard drug, Donepezil in comparison with the control. The percentages of flies that went across the 6cm mark under 6 seconds were 80% for flies in the control group; 42.8% for flies induced with aluminium; 78.7% for induced flies fed with diet containing extract, 98.7% for normal flies fed with diet containing extract alone and 76.2% for induced flies fed with diet containing the standard drug, Donepezil.

Figure 3 shows AChE activity after 7 days of treatment for *Drosophila melanogaster* used in the experiment. When compared to the control group ($P<0.05$), a high significant difference was observed in AChE activity of induced flies, as the AChE level greatly increased. The AChE activities of induced flies fed with diet containing extract, normal flies fed with extract and induced flies fed with diet containing Donepezil were



KEY:

Group A = Control

Group B =Aluminium induced flies fed with normal corn meal

Group C = Aluminium induced flies fed with diet containing 10.0 mg/ml of alkaloid extract of Centella asiatica leaves

Group D = Normal flies fed with diet containing 10.0 mg/ml of alkaloid extract of Centella asiatica leaves

Group E = Aluminium induced flies with 20 µM Standard drug, Donepezil included in their diet

Figure 1: Survival rate (%) of *Drosophila melanogaster* used for the experimental model. Values represent mean ± Standard Deviation (n=40).

not different significantly from the AChE activity of flies in the control group (P<0.05).

Presented in Figure 4 is BChE activity of treated and induced flies used in the experiment. There was significant change in BChE activity of induced flies upon comparison with the control group (P<0.05) as BChE activity level greatly increased. The BChE activities of induced flies fed with diet containing extract, normal flies fed with extract and induced flies fed with diet containing the standard drug, Donepezil were not different significantly from the BChE activity of flies in the control group (P<0.05).

This study investigated the neuroprotective effect of *Centella asiatica* leaf alkaloid extract

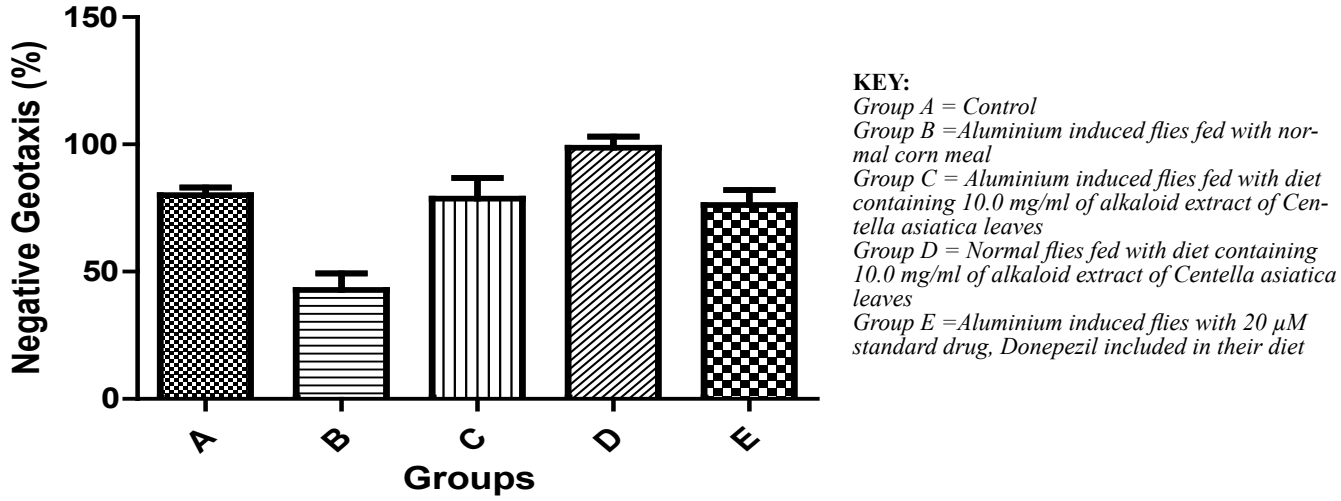


Figure 2: Percentage locomotor performance (Negative Geotaxis) of *Drosophila melanogaster* used for the experimental model. Values represent mean ± Standard Deviation (n=40).

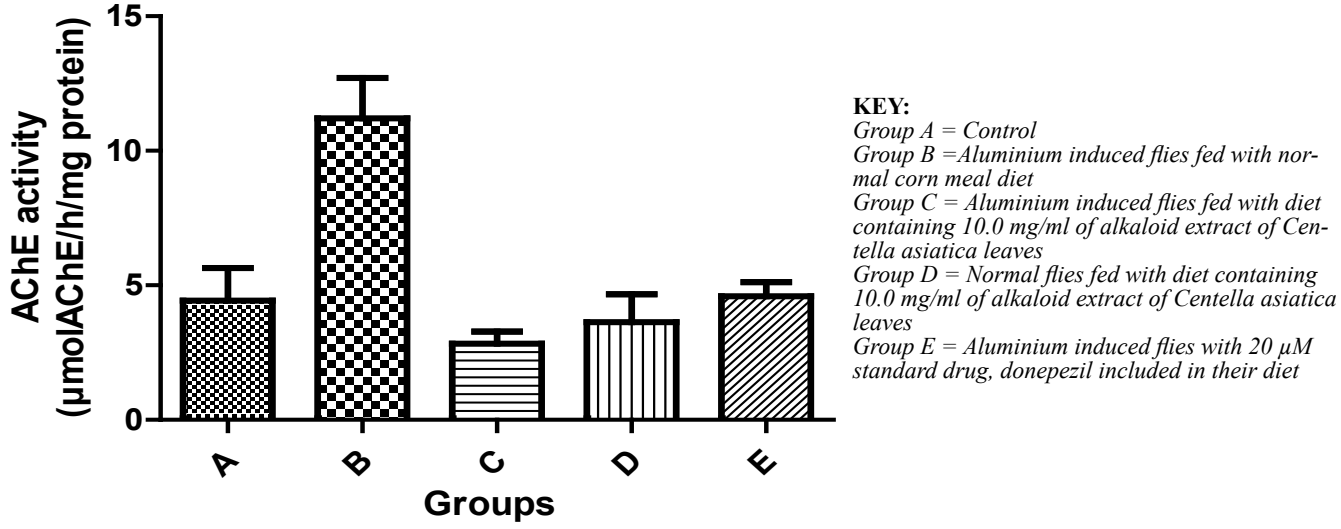


Figure 3: Acetylcholinesterase (AChE) activity in *Drosophila melanogaster* used for the experimental model. Values represent mean ± Standard Deviation (n=40).

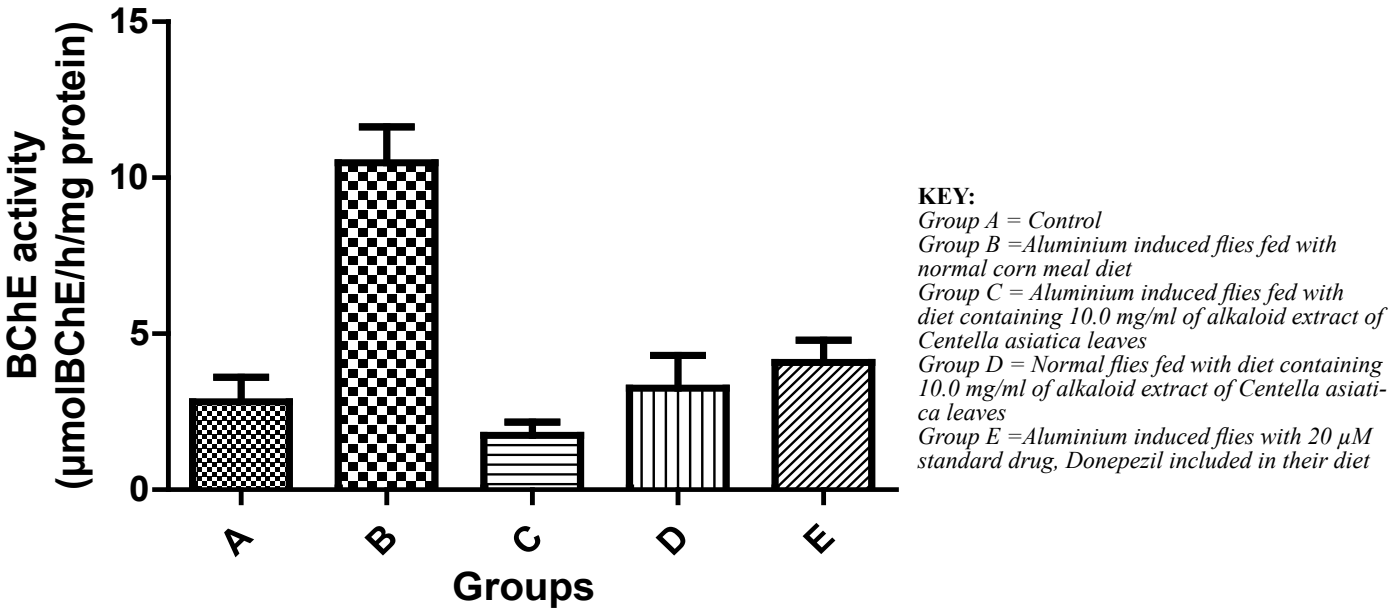


Figure 4: Butyrylcholinesterase (BChE) activity in *Drosophila melanogaster used for the experimental model*. Values represent mean ± Standard Deviation (n=40).

on enzymes linked with Alzheimer's disease (acetylcholinesterase and butyrylcholoinesterase) in *Drosophila melanogaster.* Vital findings of this study are that post-Aluminium treatment of *D. melanogaster* with *C. asiatica* leaf alkaloid extract improved survival rates as well as locomotor performances and decreased activities of the two cholinesterases, acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) in the flies.

Heavy metal toxicity is one of the major causes of Alzheimer's disease (AD). Several heavy metals like aluminium, iron, lead, cadmium, etc. have been constantly associated with AD. Aluminum toxicity is connected to oxidative stress, which has a well-established connection to neurodegeneration. Several research have also shown high amount of aluminum in the brain of people plagued with memory loss (Choudhury *et al.,* 2023). Numerous research that involve a connection between aluminum and AD have been conducted, and they suggested that aluminium is effective in inducing the disease (Mahdi *et al.,* 2019).

Administration of aluminium to the *Drosophila melanogaster* in this study could have been responsible for the loss as well as death of cholinergic neurons, which led to the reduced rate of survival as well as poor locomotor performances reported in the flies. However, treatment of the induced flies with *Centella asiatica* alkaloid leaf extract improved the rate of survival and flies' locomotor performance, confirming the extract's neuroprotective effect. Also, there was no significant difference ($p \leq 0.05$) in the rate of survival and locomotor performance of flies in the group treated with the extract when compared to the control and the group treated with the standard drug, Donepezil. This also proves that alkaloid extract of *Centella asiatica* leaf was not toxic to the flies at the 10 mg/ml used in this study.

Alkaloids are a group of organic compounds which occur naturally, consisting of at least one atom of nitrogen. Qualitative screening of the phytochemicals present in *C. asiatica* leaves showed the presence of alkaloids in the plant (Saranya *et al.*, 2017). The liquid chromatography - mass spectrometry (LC-MS)

analyses of *C. asiatica* extract by Ondeko *et al.*, 2020, also confirm the presence of alkaloids in the plant. Alkaloids exert neuroprotective effects by inhibiting cholinesterase activity and apoptosis, decreasing oxidative stress, ameliorating neuroinflammation and modulating autophagy (Ali *et al.,* 2023). This support the neuroprotective effect of the extract shown in this study.

The pathology of how AD arises is complex. However, the most accepted theory for AD is the cholinergic hypothesis. The neurotransmitter, Acetylcholine (ACh) is important for autonomous nervous system as well as the central nervous system (CNS). ACh plays a key role in learning and memory by making communication available between two nerve cells in the brain. During the process of neurotransmission, Acetylcholine is released from the nerve into the synaptic cleft and it binds to ACh receptors (muscarinic and nicotinic) on the post-synaptic membrane, thereby relaying the signal from the nerve. ACh is the biomarker in AD (Yıldırım and Güzeldemirci, 2023).

Cholinesterases are esterases that break down choline-based esters, most of which function as neurotransmitters. They catalyze the hydrolysis of cholinergic neurotransmitters, for example by breaking down acetylcholine into acetic acid and choline. These reactions are vital to enable a cholinergic neuron go back to its resting state after activation. The two types of cholinesterases that exist are acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) (Dorland, 2014). AChE which is located on the post-synaptic membrane, carries out a major function in regulating acetylcholine when it stops the signal transmission by hydrolyzing ACh to choline and acetate. AChE activity increases in AD brain, thus triggering acetylcholine degradation (Bhagat *et al.*, 2021; Design, 2021). Hence, inhibiting AChE activity reduces the breakdown of ACh. BChE, the isoenzyme of acetylcholinesterase, is a minor element in a healthy brain. The activity of BChE increases as a result of plaques and tangles in the brain of AD patients. Therefore an impairement in cholinergic transmission can also be treated by inhibiting BChE activity (Li *et al.*, 2018; Weinreb *et al.*, 2012).

Based on the cholinergic hypothesis in AD pathology, inhibition of the activities of cholinesterases using cholinesterase inhibitors, which attempt to block acetylcholinesterase and butyrylcholinesterase enzymes, is the most effective approach in AD treatment. This tries to increase and restore ACh levels at cholinergic synapses, thereby making up for cholinergic losses and increasing cholinergic transmission (Alptüzün *et al.,* 2006; Bhagat *et al.,* 2021; João *et al.,* 2021; Miles *et al.,* 2021).

Most research conducted on cholinesterase activity usually focus on assessing and inhibiting the activity of AChE alone. However, this current research focused on assessing and inhibiting the dual effect of both AChE and BChE in Alzheimer's disease. From the results shown in this research, administration of aluminium on the flies increased both AChE and BChE activities. However, treatment with *Centella asiatica* alkaloid leaf extract inhibited and reduced the activities of both AChE and BChE in *D. melanogaster* flies induced with Alzheimer's disease. In addition, no significant difference ($p \leq 0.05$) was recorded in the AChE and BChE activities of flies in the group treated with the extract when compared to the control. Flies treated with the extract also appeared to show better levels of acetylcholinesterase and butyrylcholinesterase activities when compared to the flies treated with the standard drug, Donepezil.

A decrease in AChE and BChE activities in the flies used for this study could have brought about increased ACh levels, as well as restored cholinergic losses and increased cholinergic transmission in the flies, as reported in this study. This corroborates the findings from an earlier research by Rahman *et al.*, 2012, where an in-vitro test for anti-AChE activity of the ethanolic extract of *C. asiatica* confirmed that the plant had a significant level of anti-AChE activity.

Conclusion

This study shows that the alkaloid extract of *C. asiatica* leaf decreased the mortality rate and improved locomotor performance in flies induced with Alzheimer's disease, using aluminium. The extract also inhibited activities of the cholinesterases AChE and BChE, thereby reducing the loss of cholinergic neurons. Therefore, it may be concluded that *C. asiatica* leaf alkaloid extract have neuroprotective effect on the enzymes (acetylcholinesterase and butyrylcholinesterase) linked with aluminium-induced Alzheimer's disease in *D. melanogaster*.

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