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ANTICHOLINESTERASE EFFECTS OF *ANNONA MURICATA* LEAF EXTRACT ON ALUMINUM LACTATE-INDUCED ALZHEIMER'S-LIKE DISEASE IN ALBINO RATS

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ABSTRACT: *Alzheimer's disease (AD) is a neurodegenerative disease characterised by β-amyloid plaques and neurofibrillary tangles in the hippocampus, leading to brain cells' death with a concomitant decline in memory and thinking. Cholinesterase inhibitors and N-Methyl-D-Aapartate are the approved classes of drugs for AD treatment. Annona muricata, an Annonacea family, shows various potentials in ethnotraditional medicine e.g. anti-inflammatory potential. This study aimed to determine the anticholinesterase effects of Annona muricata on aluminum lactate-induced Alzheimer's-like disease in rats, compare its effects with that of Neostigmine; and determine the potential of acetylcholinesterase and butyrylcholinesterase in AD diagnosis. Thirty rats were used and grouped into five groups of 6 each: group-I (normal control, administered with distilled water only), group-II (negative control, only induced with the toxicant), group-III (standard control, treated with 2mg/Kg-Neostigmine + toxicant induction), and groups-IV and V (were treated with 250mg/Kg and 500mg/Kg of A. muricata respectively + toxicant induction). The treatment lasted for 28days and the toxicant accompanied it after the third week, for the last 7days. The biochemical analysis was carried out and revealed significant ($p<0.05$) alteration induced by the toxicant in the levels of acetylcholinesterase and butyrylcholinesterase. Treatments with Neostigmine and A. muricata significantly ($p<0.05$) countered these effects at varying capacity and dose dependence; with A. muricata (at 500mg/Kg) having more potency than the standard drug. Conclusively, A. muricata exhibits dose-dependent anticholinesterase potential in the management of AD more than Neostigmine; acetylcholnesterase and butyrylcholinesterase are good candidates for AD diagnosis and management, and aluminum lactate holds promise in inducing AD.*

KEYWORDS: Alzheimer's disease, anticholinesterase, *Annona muricata*, cholinesterase inhibitors and Neostigmine.

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INTRODUCTION

Alzheimer's disease, a public health issue, was named after a German psychiatric, Alois Alzheimer, who first identified the neuropathological changes associated with clinical presentation of progressive dementia and described its syndrome in 1906 and published it in the subsequent year (Fernandez et al., 2020; Breijyeh & Karaman, 2020). Alzheimer's disease is a neurodegenerative disorder resulted majorly from oxidative stress elevation in the brain which leads to extracellular formation of β-amyloid plaques and intracellular deposits of neurofibrillary tangles in the hippocampus, cerebral cortex. It is characterized by a decline in thinking skills and independence in personal daily activities (Breijyeh & Karaman, 2020), difficulty with language, concentration, and orientation (Calderaro et al., 2022), speech disturbances, dyspraxia, partial paralysis, tremors, and marked decline in memory and learning (Ramesh, Jayalakshmi & Edwin, 2016), progressive cognitive impairment, affecting the areas of memory, praxis, awareness, speech and executive function (Apostolova, 2016; Abramo et al., 2020). Many studies revealed that high oxidative stress, which is caused by an imbalance between the antioxidant and prooxidant systems (Ide et al., 2016), plays a significant role in Alzheimer's disease progression (Butterfield, Perluigi & Sultana, 2006; Butterfield, Swomley & Sultana, 2013; Ide et al., 2016; Al-okbi et al., 2017; Breijyeh & Karaman, 2020). Alzheimer's disease, the main and commonest cause of dementia, accounts for 60–80% of all dementia cases in which its prevalence is forecast to reach 115.4million in 2050 (Prince et al., 2013; Ide et al., 2016). As the cost AD care increases with the rise of number of patients, and in order to develop effective therapeutics and prevention approaches, it is fundamental to diversify affordable risk factors and biomarkers of Alzheimer's disease (Bateman et al., 2011; Ide et al., 2016).

Acetylcholine (ACh-synthesised by choline acetyltransferase) is a neurotransmitter that is responsible for memory, learning, sensory information attention, and other critical functions in the brain (Breijyeh & Karaman, 2020). In age related diseases such as Alzheimer's disease and Parkinson disease, there is increase in secretion of cholinesterase enzyme (an enzyme that hydrolyses acetylcholine) which leads to alteration in cholinergic system (acetylcholinesterase-AChE, butyrylcholinesterase-BuChE, choline transferase, nicotinic and muscarinic Ach receptors, and vesicular Ach transporter) as a result of decrease in ACh. The loss of cholinergic synaptic connections is considered one hallmark of AD symptoms. However, this alteration is mitigated by the action of cholinesterase inhibitors (ChEIs-including naturally derived, synthetic and hybrid analogues) as they improve ACh levels in synaptic cleft by inhibiting the activities of ChE, making ChEIs the best choice for effective treatment of AD (Lahiri, et al., 2005; Eldufani & Blaise, 2019; Breijyeh & Karaman, 2020). AChE and butyrylcholinesterase (BuChE) are the two types of cholinesterases (Dhahri et al., 2022). Because of the fact that, plasma is easily accessible for clinical use in monitoring disease progression and management, plasma AChE as a marker for AD was assessed by many researchers (Riba-llena et al., 2010). Hence, could be considered valuable in our present research. It was detected by Riba-Illena et al., (2010), a selective increase of AChE activity in plasma from Alzheimer's disease (AD) patients compared to age and gender-matched controls. Riba-llena et al., (2010) stated that a major problem is posed due to excess amounts of butyrylcholinesterase (BuChE).

Neostigmine (anticholinesterase) is an AChI which improves cholinergic action by facilitating impulse transmission through neuromuscular junctions. Probably, due to inability of neostigmine to cross blood-brain barriers (BBBs) subarachnoidly, its longer action lacks

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neurotoxicity side effects. It could be administered through different routes to offer varying levels of achievement (Eldufani & Blaise, 2019).

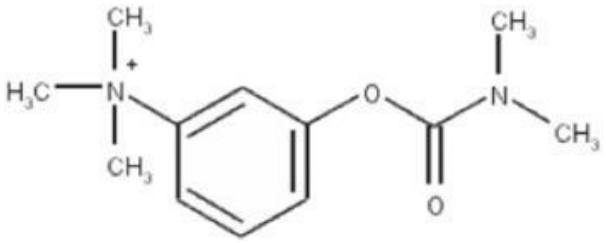

Fig. 1.1: Structural formula of Neostigmine (Eldufani & Blaise, 2019)

Aluminum (Al) is the third-most abundant element (after oxygen and silicon) on Earth and second in production and consumption after steel (Wesdock & Arnold 2014; Qin et al., 2019). It was reported that aluminum intake is associated with neurodegenerative diseases such as Alzheimer's disease with learning and memory impairment (Qin et al., 2019; Shaw, 2018; Colomina, & Peris-Sampedro, 2017; Kandimalla et al., 2016). Aluminum is commonly used in industries like food and drinks, pharmaceutical, cosmetic and other industries, thus, gets into the human systemic circulation and accumulates in the cortex and cerebellum areas to interact with proteins, leading to accumulation of tau protein and Aβ protein in the brain which are the common symbols of Alzheimer's disease (Maya et al., 2016; Al-okbi et al., 2017; Colomina & Peris-Sampedro, 2017; Breijyeh & Karaman, 2020).

Medicinal plants and their extracts are used by humans for the treatment of diseases long time ago. This is seen in various roles they play therapeutically such as antimalarial, antihypertensive, antitussive, and analgesic medicines (Nsofor et al., 2023). Hence, considered vital to community health and to human at large (Dike et al., 2023). *Annona muricata* tree belongs to the Annonaceae family with its fruit popularly known in Nigeria as sour-sop in English (Onuoha et al., 2023), 'tuwon biri' in Hausa, 'shawanshop' in Igbo and 'ebo' in Yoruba (Kelechi et al., 2016)**.** While in South and North America, it is known as graviola or guanabana (Onuoha et al., 2023). It has over 2,300 species and over 130 genera. The plant is widely spread in tropical and subtropical regions like Southeast Asia, South America, Nigeria and the African rainforests. It contains a variety of pharmacologically active substances such as anti-diabetic, anti-parasitic, anti-helminthic, antipyretic and anti-inflammatory properties (Onuoha et al., 2023; Ezerioha et al., 2024). In a study conducted by Uno et al. (2017) revealed that *Annona muricata* tea has the propensity to mitigating oxidative stress caused by caffeine (which is dose dependent) in albino rats as mammalian models. The antioxidant capacity of *A. muricata* was reported to be responsible by the phenolic compounds in it, such as quercetin and gallic acid. Many studies have reported that at least 100 human diseases, including hypertension, diabetes, renal insufficiency, Parkinson's disease and Alzheimer's disease, are related to cellular oxidative damage (Coria-Tellez et al., 2018; Balderrama-carmona et al., 2020). The effect of *A. muricata* leaf extract on neurotoxicity was found to be due to the presence of these bioactive compounds in it (Patel & Patel, 2016)**:** anonaine, isolaureline, xylopine (Fofana et al., 2011), Quercetin 3-O-α-rhamnosyl- (1→6)-β-sophoroside, gallic acid, epicatechin, quercetin 3-O-rutinosid, quercetin 3-O-neohispredoside, chlorogenic acid, argentine (1-

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N,Ndimethylethanyl4,6-dimethoxy-3,8-dihydroxyphenanthrene), kaempferol 3-O-rutinoside, quercetin 3-O-glucoside, quercetin, kaempferol (Nawwar et al., 2012), anonaine, annonamine, (S)-norcorydine, (R)-4′-O-methylcoclaurine, (R)-O,O-dimethylcoclaurine (Matsushige, et al., 2012[b]), annoionol A, annoionol B, annoionol C, annoionoside, vomifoliol, roseoside, turpinionoside, citroside A, blumenol C, (+)-epiloliolide, loliolide, (1S,2S,4R)-trans-2-hydroxy-1,8- cineole β-D-glucopyranoside, (Z)-3-hexenyl β-glucopyranoside, rutin, kaempferol 3-O-rutinoside, kaempferol 3-O-robinobioside, kaempferol 3-O-β-D-(2′′-O-βglucopyranosyl, 6′′-O-αL'Rhamnopyranosyl) glucopyranoside (Matsushige, et al., 2012 a).

MATERIALS AND METHODS

Plants Collection and Extract Preparation

Fresh leaves of *Annona muricata* were collected from trees in Nsukka, Enugu state, Nigeria. The fruits and leaves were identified, authenticated and given voucher number (18098) by Dr. Hyginus C. Ogbuehi of the Department of Crop Science and Biotechnology, Imo State University, Owerri, Nigeria. The plant materials were washed, air dried (at room temperature) to constant weight and ground into powder. Maceration extraction technique was used which involves soaking (in methanol for 48 hours), filtration (using Whatman filter paper) and evaporation (using waterbath). 400g of the pulverised *Annona muricata* was extracted with 1750ml of methanol. All chemicals and reagents were obtained from certified suppliers and were of analytical grade.

Preparation of Toxicant and Standard Drug

1.80g of sodium chloride (NaCl) was dissolved in 240ml of distilled water to form saline solution. 0.64g of aluminum lactate was dissolved in 240ml of saline solution. 1g of NaCl was dissolved in 100ml of distilled water to form a water soluble saline solution and 2ml of neostigmine injection was dissolved in 20ml of the saline solution.

Experimental Animals

Thirty five (35) male albino rats (120-140g) were obtained from the animal house of Abia State University, Uturu. The animals were acclimatized for 14 days at the animal house of Biochemistry Department, FUTO and fed with standard pelletised feed, and water was provided to the animals ad libitum for the 14 days. Thirty (30) of the animals were randomly selected and grouped into 5 groups of 6 rats each. With the exception of group I, all the groups were treated for 28days through oral intubation and were accompanied by induction of 7.5mg/Kg aluminum lactate (the toxicant) via intra-peritoneal injection from the 21st day to the 28th day (1 week). They were labelled: group I (normal control which was administered only distilled water), group II (negative control which was only induced with the toxicant without treatment), group III (standard control: it was treated with 2mg/Kg Neostigmine- the standard drug), and groups IV and V (were treated with 250mg/Kg and 500mg/Kg of *A. muricata* respectively). During the experiment; food intake and body weight of rats were followed twice weekly. After the experiment, total food intake, body weight gained and food efficiency ratio (body weight gained/total food intake) were calculated.

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Table 1: Summary of Animal Grouping and Administration of the Treatments

Treatment	No of rats	Total duration	Toxicant (Aluminum lactate + saline)		Treatment (Normal drug-Neostigmine)		Treatment (Extract-*A. muricata*)	
Group			Dose	Duration	Dose	Duration	Dose	**Duration**
Group I (Normal Control)	6 rats	28 days	Nil	Nil	Nil	Nil	Nil	Nil
Group II (Negative Control)	6 rats	28 days	7.5mg/Kg	7 days	Nil	Nil	Nil	Nil
Group III (Standard Control)	6 rats	28 days	7.5mg/Kg	7 days	2mg/Kg	28 days	Nil	Nil
Group IV (Low Dose)	6 rats	28 days	7.5mg/Kg	7 days	Nil	Nil	250mg/Kg	28 days
Group V (High Dose)	6 rats	28 days	7.5mg/Kg	7 days	Nil	Nil	500mg/Kg	28 days

SAMPLE COLLECTION

At the end of the experiment, after an overnight fast, rats were anesthetized and blood samples were withdrawn through ocular puncture (eye vein orbital) into EDTA and plain sample bottles for laboratory analysis. Blood samples was mixed with heparin as anticoagulant. It was followed by centrifugation at 3000 rpm for 15min to separate plasma. Standard methods were used to determine AChE and BuChE.

MEASUREMENT OF ACETYLCHOLINESTERASE ACTIVITY

Acetylcholinesterase (AChE) activity was assessed by a modified spectrophotometry method of Ellman et al. (1961). The AChE activity was determined in a reaction mixture containing 200L of sample in 0.1M phosphate buffer (pH 8.0), 100L of 3.3mM solution of 5,5-dithio-bis(2-nitrobenzoic) acid (DTNB), in the same buffer solution containing 6mM $NaHCO_3$ and 500L of phosphate buffer (pH 8.0). After incubation for 20min at $25^{O}C$, 100uL of 0.05mM acetylthiocholine iodide solution was added and AChE activity was determined as change in absorbance at 412nm for 3min at $25^{o}C$.

MEASUREMENT OF BUTYRYLCHOLINESTERASE ACTIVITY

The catalytic activity of BuChE was measured spectrophotometrically as described by Ellman et al., (1961), using butyrylthiocholine (BuTCh) (0.9mmol/L) and acetylthiocholine (ATCh) (0.6mmol/L) as substrates. The reaction mixture for the hydrolysis in the sample contained 1mL of a mixture of 3mL, 0.1mol phosphate buffer and 100µL of 0.38mmol/L 5.5-dithio-bis(2-nitrobenzoic acid) (DTNB), 100µL of BTCh or 100µL of ATCh, and 50µL of sample. The increase in absorbance at 412nm and at $25^{o}C$ was monitored for 3min. A blank sample containing the incubation mixture without the sample was run simultaneously to correct for the

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spontaneous substrate breakdown. Enzyme activities were expressed as μmol/L of substrate hydrolysed per min per mL of sample.

Statistical Analysis

The data were analysed by a one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test using the Statistical Package for Social Sciences (SPSS) version 21. p values less than 0.05 ($p<00.5$) was considered as statistically significant.

RESULTS AND DISCUSSION

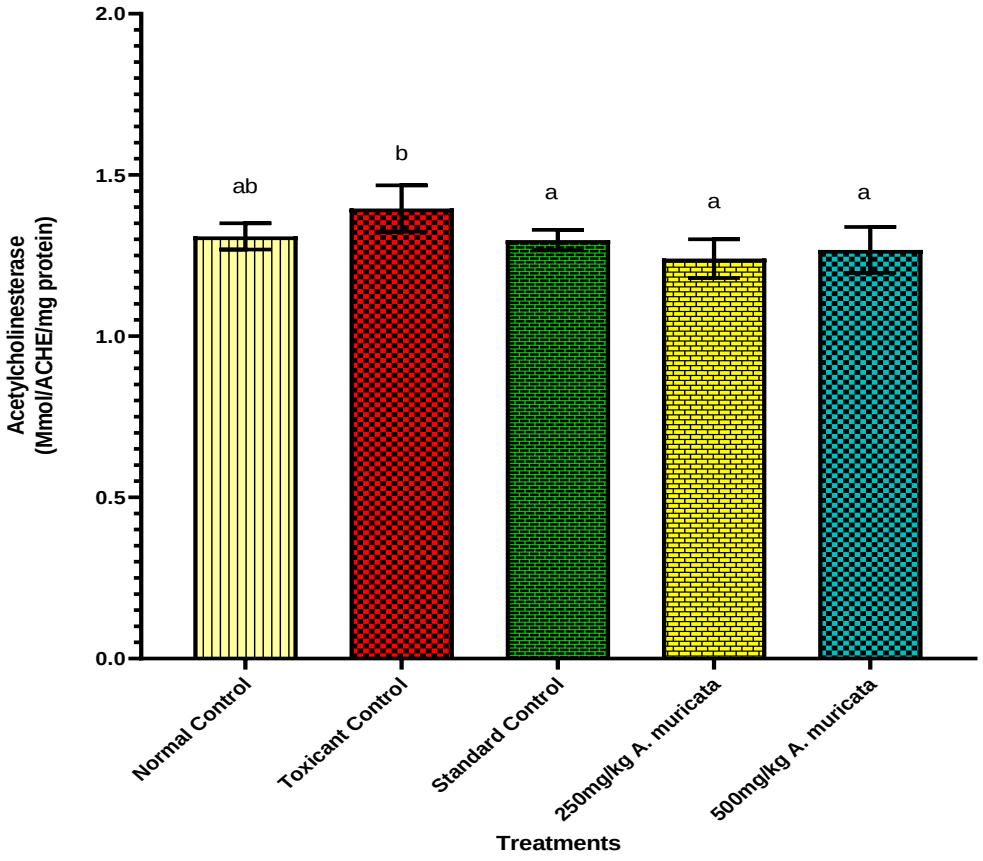

Fig. 2.1 Effects of *A. muricata* Leaf Extracts on AChE in Alzheimer's Disease in Rats Induced by Aluminium Lactate
Bars Represent Mean ± Standard Deviation
Bars bearing different letters are statistically significant ($p<0.05$)

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AChE is an enzyme that involves in the breakdown of the neurotransmitter, acetylcholine. Because of the fact that, plasma is easily accessible for clinical use in monitoring disease progression and management, plasma AChE as a marker for AD was assessed by many researchers (Riba-llena et al., 2010). It was detected by Riba-Illena et al., (2010) a selective increase of AChE activity in plasma from Alzheimer's disease (AD) patients compared to age and gender-matched controls. In age related diseases such as Alzheimer's disease and Parkinson disease, there is increase in secretion of cholinesterase enzyme which leads to alteration in cholinergic system (AChE and BuChE) as a result of decrease in ACh. The loss of cholinergic synaptic connections is considered one hallmark of AD symptoms. However, this alteration is mitigated by the action of cholinesterase inhibitors (ChEIs-including naturally derived, synthetic and hybrid analogues) as they improve ACh levels in synaptic cleft by inhibiting the activities of ChE, making ChEIs the best choice for effective treatment of AD (Lahiri, et al., 2005; Eldufani & Blaise, 2019; Breijyeh & Karaman, 2020). Elevated level of acetylcholinesterase (AChE) in this study (fig. 1.1) was observed in the negative control group (when compared with the normal control and the standard control groups) which was induced by the toxicant (aluminum lactate) alone. This could be due to accumulation of the aluminum lactate in the cortex and cerebellum areas to interact with proteins leading to accumulation of tau protein and Aβ protein in the brain which are the hallmarks of Alzheimer's disease (Maya et al., 2016; Al-okbi et al., 2017; Colomina & Peris-Sampedro, 2017; Breijyeh & Karaman, 2020). This might also be due to phosphorylation of the highly phosphorylated proteins such as tau proteins in the hippocampal and cerebellum, which is indicative of Alzheimer's disease (Colomina & Peris-Sampedro, 2017). The level was significantly ($p<0.05$) reduced when treated with the standard drug and the leaf extracts of *A. muricata* at different doses (250mg/Kg and 500mg/Kg). This could be as a result of inhibition of the enzyme by AChE inhibitors (AChEi), from both the standard drug and the leaf extract, that enhances acetylcholine availability which boosts synaptic transmission and memory function (Dhahri et al., 2022). Acetylcholinesterase inhibitors have been used to combat AD (Dhahri et al., 2022). Natural AChEi became multifunctional therapeutic strategy for preventing the occurrence of AD and its progression. This due to their additional pharmacological properties, mainly antioxidant properties (Ayaz, et al., 2017; Sahoo, et al., 2018). Several studies have isolated and identified natural molecules with potential AChEi activity that have shown positive outcomes as novel anti-AD drugs (Huang, Su, & Li, 2013). The antioxidant capacity of *A. muricata* was reported to be responsible by the phenolic compounds in it, such as quercetin and gallic acid. Many studies have reported that at least 100 human diseases, including hypertension, diabetes, renal insufficiency, Parkinson's disease and Alzheimer's disease, are related to cellular oxidative damage (Coria-Tellez et al., 2018; Balderrama-carmona et al., 2020). The effect of *A. muricata* leaf extract on neurotoxicity was found to be due to the presence of bioactive compounds in it (Patel & Patel, 2016). Comparatively, it was observed that the level of the enzyme in the standard control group (group III) was significantly ($p<0.05$) higher than those in groups treated with the leaf extracts (groups IV and V). This attribute, may be as a result of their additional pharmacological properties, mainly antioxidant properties (Ayaz, et al., 2017; Sahoo, et al., 2018). As well, group V (high dose-500mg/Kg) was found significantly (<0.05) higher than group IV (low dose-250mg/Kg). This indicated that the low dose is more effective than the high dose. The efficacy of AChE inhibitors (both the neostigmine and the *A. muricata*) in AD treatment stresses the significance of AChE in AD diagnosis and management. This study strongly suggests that AChE may serve as a valuable biomarker for the diagnosis and prognosis of AD, as well as a therapeutic target for intervention.

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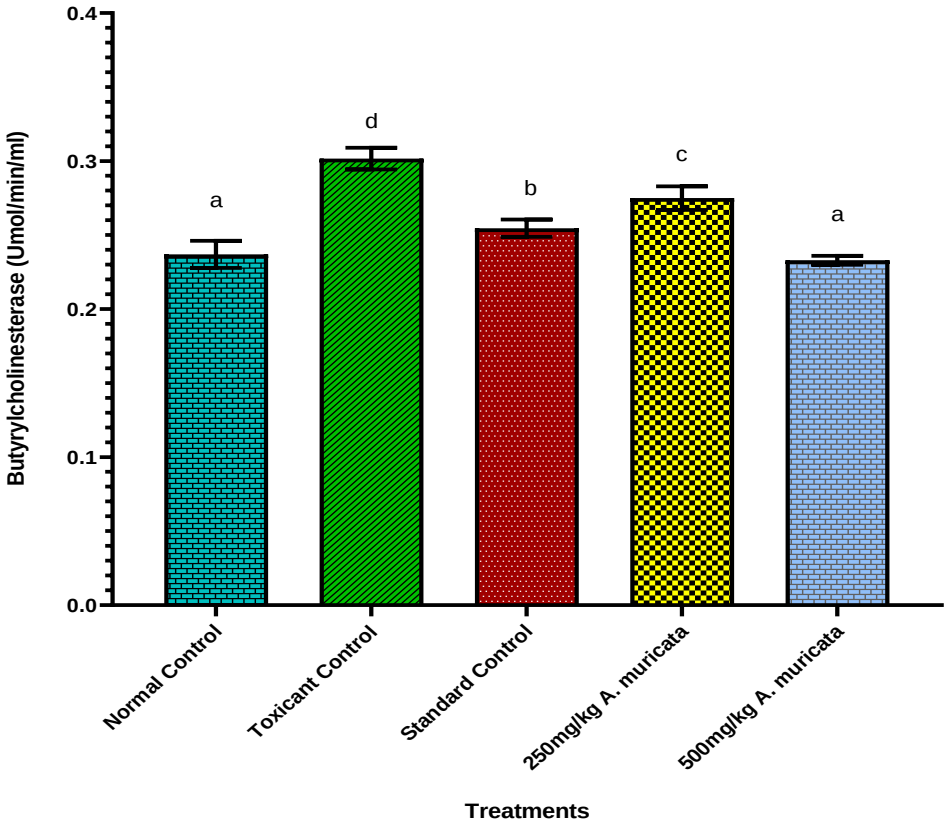

Fig. 2.2 Effects of *A. muricata* Leaf Extracts on BuChE in Alzheimer's Disease in Rats Induced by Aluminium Lactate
Bars Represent Mean ± Standard Deviation
Bars bearing different letters are statistically significant ($p<0.05$)

Physiologically, hydrolysis of excess Ach is the major role of BuChE as it was reported by Giacobini et al. (2002). In support of the above, they reported that in the presence of an AChE specific inhibitor in human brain, butyrylcholine, the surrogate of Ach is hydrolysed by BuChE. In this study (fig. 2.2), the level of BuChE in the negative control group was found to be significantly ($p<0.05$) higher (induced by the toxicant) than those of the normal control and the standard control groups which is observed in AD, as it was reported by Rao et al. (2007) that elevations in plasma and tissue concentrations of butyrylcholinesterase and acetylcholinesterase were revealed in Alzheimer's disease. In the normal brain, BuChE is mostly found in glial cells and has only 10% of AChE activity while in the AD brain, BuChE activity is increased to 40–90% and ACh activity is simultaneously decreased, suggesting that BuChE action may indicate a moderate to severe dementia (Khoury, Rajamanickam, & Grossberg, 2018). The effect of the toxicant was mitigated when treated with the standard drug and the leaf extracts of *A. muricata* at different doses (250mg/Kg and 500mg/Kg). This may be attributed to their cholinesterase inhibitory effect due to the phytochemicals loaded in them that have anti-inflammatory and antioxidant potentials as was reported: many studies have reported that not less than 100 human diseases, including hypertension, diabetes, renal insufficiency, Parkinson's disease and Alzheimer's disease, are

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related to cellular oxidative damage (Coria-Tellez et al., 2018; Balderrama-carmona et al., 2020). The antioxidant capacity of *A. muricata* was reported to be responsible by the phenolic compounds in it, such as quercetin and gallic acid. The effect of *A. muricata* leaf extract on neurotoxicity was found to be due to the presence of bioactive compounds in it (Patel & Patel, 2016). It was observed that the level of BuChE in group V (treated with 500mg/Kg *A. muricata*) was significantly ($p<0.05$) lower than the standard control group. It was reported by Giacobini et al. (2002) that the level of extracellular ACh in cortex is increased with the decreased activity of BuChE by its selective inhibitor. This implied that, *A. muricata* has more BuChE inhibitory effect than Neostigmine, hence, more potent. Unlike the case in AChE, in BuChE, *A. muricata* at high dose was discovered to be more potent than at low dose. This is due to the fact that, the action of the leaf extract at low dose, inhibited AChE significantly ($p<0.005$) more than at the high dose making the level of the BuChE at low dose to rise above its level at high dose. This is in line with the available literatures such as: BuChE is known to localise at the glial cells that proliferate as the cholinergic function decreases with Alzheimer's disease progression which results to loss of brain AChE activity. This phenomenon makes BuChE activity to increase to the extent that, the ratio of BuChE to AChE increases histrionically from 0.6 to 11, reported by Giacobini (2002). Rao et al. (2007), alteration in the levels of butyrylcholinesterase assumes significance in Alzheimer's disease in circumstances whereby there is a deficit or absence of acetylcholinesterase, that is, when acetylcholinesterase is lost up to 85% in definite regions of brain, while levels of butyrylcholinesterase rise with disease progression. These imply that, in the presence of AChEI in AD, decrease in the level of AChE might lead to increase in the level of BuChE.

In anyway, these results indicated that the effect of *A. muricata* is dose dependent just as it was observed by Giacobini et al. (2002) that, the inhibition of plasma BuChE depends on dose. This implies that, *A. muricata* is a strong BuChE inhibitor. Its efficacy and that of standard drug in this research demonstrated the relevance of AChE and BuChE as reliable biomarkers in the diagnosis and management of Alzheimer's disease. Though, *A. muricata* displayed more cholinesterase inhibitory effect than the standard drug, yet both of them demonstrated strong inhibitory potential capable of managing Alzheimer's disease.

Conclusively, *A. muricata* exhibits dose-dependent anticholinesterase potential in the management of AD. It is more than Neostigmine; acetylcholinesterase and butyrylcholinesterase are good candidates for AD diagnosis and management, and aluminum lactate holds promise in inducing AD.

FUTURE RESEARCH

Research should be carried out with the same doses but in varying duration.

Research should also be carried out with higher doses on the same method and the results should be compared with this.

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