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In-vitro Evaluation of Acetylcholinesterase and Butyrylcholinesterase Inhibitory Activities of Antlion Larvae Extracts

Muinat M. Adeyanju[1], Balqees O. Shodiya[1], Olufemi E. Abayomi[1], Adewale B. Awofeso[1], Ayodele O. Akinlade[1], Oluwatayo S. Adebisi[1], Basil O. Ugwu[1], Joseph S. Ashidi[2], Efere M. Obuotor[3]

[1]Department of Biochemistry, Faculty of Basic Medical Sciences, Olabisi Onabanjo University, Sagamu, Ogun State, Nigeria
[2]Department of Plant Sciences, Faculty of Sciences, Olabisi Onabanjo University, Ago-Iwoye Ogun State, Nigeria
[3]Department of Biochemistry, Faculty of Sciences, Obafemi Awolowo University, Ile-Ife, Osun State, Nigeria

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ABSTRACT

Antlion is used in traditional medicine by natives of the southern part of Nigeria particularly the Yorubas for memory enhancement. The progress made so far in the use of this organism as a memory booster lead to investigating the acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) (the key enzymes in the pathogenesis of Alzheimer's disease) inhibitory activities of methanolic and phosphate buffered saline (PBS) extracts. The activities of these enzymes were investigated using Ellman's method. The kinetics of the inhibition patterns was also studied using eserine as the standard inhibitor. The concentration of the extract required for 50% inhibition (IC_{50}) of the AChE was 49.00 ± 1.20 and 271.40 ± 0.10 µg/mL, for the PBS and methanolic extracts respectively, compared to Eserine with IC_{50} of 2.25 x 10^{-2} ± 0.15 x 10^{-2} µg/mL. Similarly, the IC_{50} for the BuChE was 66.30 ± 0.40 and 216.70 ± 1.10 µg/mL respectively, for the methanol and PBS extracts, compared to Eserine with IC_{50} of 1.10 ± 0.30 µg/mL. The pattern of inhibition of the BuChE in the presence of the extracts was non-competitive, while AChE exhibited non-competitive and competitive inhibitions for the methanolic and PBS extracts respectively. It is therefore evident that extracts of the antlion larvae contained cholinesterase inhibitors which might be binding to AChE and BuChE; with the PBS extract inhibitory activity towards AChE being more potent than the methanolic extract. Suggesting a beneficial effect in cognitive deficit and related dementia associated with Alzheimer's disease.

Key words: *Antlion extracts; Acetylcholinesterase inhibitor; Butyrylcholinesterase inhibitor; Alzheimer's disease*

INTRODUCTION

Alzheimer's disease (AD) is the most common cause of dementia (Blennow et al. 2006), which is characterized clinically by progressive decline in cognitive and functional ability and the emergence of behavioural and psychological symptoms. Nowadays, AD prevalence among people over 60 years old is estimated in 40.2 per 1,000, while its incidence proportion is 34.1 per 1,000 (Fiest et al. 2016). These values mean that over 45 million people are suffering from AD symptoms worldwide, whereas this scenario is expected to double every 20 years until 2050 (Scheltens et al. 2016). Cholinergic dysfunction has been closely associated with the early cognitive decline in AD patients (Craig et al. 2011). In fact, early in the 70's, it was observed that cholinergic neurons were prematurely lost in AD process, which resulted in the postulation of the Alzheimer's Cholinergic Hypothesis (Bartus et al. 1982). This hypothesis was further corroborated by observations that cholinergic neurons in basal forebrain were severely damaged during AD progression (Bartus 2000). The treatment of patients with AD has been an

Correspondence: Muinat M. Adeyanju, PhD, Department of Biochemistry, Olabisi Onabanjo University, Sagamu, Nigeria. mmadeyanju@oouagoiwoye.edu.ng, +2348067191759

intensive research topic in the past decades. Current treatments mainly target cholinergic deficiency. The rationale for the use of cholinesterase inhibitors is based on their ability to boost acetylcholine level in synapses in tracts supporting cognitive function (Schneider 2000; Anand and Singh 2013; Andrieu et al. 2015). The brain of mammals contains two major forms of cholinesterases: acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) (Giacobini 2004). Acetylcholine (ACh) is one of the most important neurotransmitters of the central nervous system associated with memory and cognition (Perry et al. 1978). In the human brain, BuChE is found in neurons and glial cells as well as in neuritic plaques and tangles in AD patients. While AChE activity decreases progressively in the brain of AD patients, BuChE activity shows some increase (Giacobini 2004). It was postulated that blocking the enzyme cholinesterase induced hydrolysis of ACh and subsequent increase in ACh concentration in central synapses and enhancement of cholinergic functions provides the symptomatic improvement to AD patient (Ellis 2005; Ezoulin et al. 2005; Ferreira 2006).

Table 1: Mean % inhibition of BuChE and AChE of antlion larvae extracts

Extract Concentration (µg/mL)	MEAN % Inhibition ± SEM of BuChE		MEAN % Inhibition ± SEM of AChE	
	Methanolic Extract	PBS Extract	PBS Extract	Methanolic Extract
250.00	64.43±1.13	50.44±4.90	67.02±1.99	48.35±2.10
125.00	56.44±0.17	48.45±1.00	58.11±3.99	46.34±0.16
625.00	49.30±0.83	44.36±2.32	56.65±3.90	42.66±0.45
312.50	46.55±4.10	42.73±2.49	43.06±1.74	39.54±1.26

Cholinesterase inhibitors were developed to improve the effectiveness of ACh by inhibiting its breakdown and increasing the level in the brain or by strengthening the way nerve cells respond to it. Increased concentrations of ACh in the brain leads to increased communication between nerve cells and may temporarily improve or stabilize the symptoms of AD. These drugs appear to work best in the early and moderate stages of AD (Pope et al. 2005; Van Marum 2008). It has been further suggested that dual inhibition of AChE and BuChE enzymes should be one of the objectives in the treatment of cognitive dysfunction associated with AD (Giacobini 2004; Okello et al. 2004). Studies confirmed the role of BuChE within the nervous system, especially as it coregulates the level of ACh in the brain in cases of neurodegenerative disorders as AD (Darvesh et al. 2003). Therefore, BuChE has an important role in the development and progression of AD, which has been ascribed to its peptidase activity besides the esterase activity (Chattonet and Masson 1986). It cleaves the amyloid precursor protein which is found in abundance in normal brain to β-amyloid protein in AD. Selective BuChE inhibitors, for example N1-phenethyl-norcymserine and rivastigmine also prevent the formation of new β-amyloids plaques (Vuorelaa et al. 2004). Based on the changes of cholinesterase activity in the brain of AD patients, a rational indication of selective BuChE inhibitor (or of mixed double function inhibitors) is the treatment of advanced cases of the disease (Giacobini 2004).

Table 2: Mean % inhibition of BuChE and AChE of eserine

Concentration of Eserine (µg/mL)	Mean % Inhibition ± SEM of BuChE	Mean % Inhibition ± SEM of AChE
0.13	93.34±3.01	76.46±1.02
0.06	96.48± 0.01	68.56 ± 2.32
0.03	88.10± 2.82	55.57 ± 1.22
0.02	86.26± 1.70	49.46±1.31
0.01	79.07± 1.48	38.66±1.76

Meanwhile, about fifty percent of the drugs introduced into the market during the last twenty years are derived directly or indirectly from small biogenic molecules (Vuorelaa et al. 2004). Natural products are now seen to play a major role as active substances and model molecules for the discovery and validation of drug targets (Vuorelaa et al. 2004). Hence, nature can be considered as an important source of new chemical entities for the treatment of various diseases, including AD. Moreover, the drugs currently available in the market for the treatment of various learning and memory disorders are associated with several side effects indicating the need for new medications from alternative system of medicine (Pattewar et al. 2011).

Antlion is an insect found inhabiting the soil, and its larvae called 'Kuluso' in the western part of Nigeria is used by traditional medicine practitioners in the treatment of memory loss referred to as "Ogun-Isoye" though undocumented. The use of antlion has also been reported by Nakatani et al. (2004) in Chinese traditional medicine for the cure of convulsion. These findings instigated this preliminary investigation in the evaluation of cholinesterase inhibitory activities of the antlion larvae which could possibly be the key to the design of a drug in the treatment of cognitive dysfunction, an important characteristic of AD and other related neurodegenerative diseases.

MATERIALS AND METHODS

The live antlion larvae were collected with some earth from the premises of Olabisi Onabanjo University, Remo Campus, Ogun State, Nigeria, and identified at the Department of Zoology as *Nosa tigris*.

Chemicals and Equipment
All chemicals used in this study were of analytical grade and include Ellman's reagent (5,5-dithiol-(2-nitrobenzoic acid; DTNB) D8130, Eserine (physostigmine) and acetylthiocholine iodide (Sigma-Aldrich Co., USA). Equipment used includes visible spectrophotometer, Outrao microplate reader (Model SM 600), dry bath incubator (Model MK 2000-2) and tree electronic precision balance (Model HRB 203).

Methodology
Preparation of the Extracts
The live antlion larvae (2.50 g) were washed in cold saline solution and homogenized in four volumes of 80% methanol for methanol extract and 1× phosphate buffered saline (PBS), pH 7.4 for PBS extract. The homogenates were centrifuged at 4,000 rpm for 5 min and the supernatants obtained were diluted appropriately to give extract concentration of 2.0 mg/mL The methanolic extract was diluted with 0.05M Tris buffer, pH 8.0 while the PBS extract was diluted using 0.10 M phosphate buffer, pH 7.4. The supernatants were stored at -4 ℃ for further analyses while the pellets were discarded.

Cholinesterase Inhibitory Assay
The acetylcholinesterase and butyrylcholinesterase inhibitory activities of the extracts were determined spectrophotometrically by the modified microplate - assay of Ellman et al. (1961).
Assay Protocol: The reaction mixture in the wells of microtitre plate consisted of 20 µL of the different concentrations of the extracts (2.0 to 0.03125 mg/mL), 240 µL of appropriate buffer (for PBS extract: 0.10 M phosphate buffer, pH 7.4; for methanol extract: 0.05M Tris buffer, pH 8.0) and then 20 µL of the BuChE/AChE followed by incubation of the reaction mixture at 37 ℃ for 30 min. To the reaction mixture was added 20 µL of 5,5-dithiobis (2-nitrobenzoic acid) and 20 µL of the substrate; butyrylthiocholine chloride for BuChE and acetylthiocholine iodide for AChE assay. They were read immediately at a wavelength of 412 nm every 30 s for a period of 4 min on a microplate reader. The control (blank) was prepared without the inhibitor solution. Assay protocol was repeated using a standard inhibitor (eserine) at varied concentrations from 0.13-0.01 µg/mL for AChE and 0.50-0.01 µg/mL for BuChE activities. The absorbance obtained at different time interval was analysed using Microsoft Excel Package and the slope determined were recorded for the control, extracts and standard inhibitor. The percentage inhibitions at different concentrations of extract/standard were determined from the equation:

$$\% \text{ INH} = \frac{\text{a} - \text{b}}{a} \times 100$$

where a = ∆A/min of control, b=∆A/min of extract at particular concentration. ∆A = change in absorbance. The IC_{50} was determined by plotting percentage inhibition against concentration of the extract.

Determination of Kinetic Parameters, K_m and V_{max}
The protocol for kinetic study of butyrylcholinesterase Inhibitor (BUCHEI) and acetylcholinesterase Inhibitor (ACHEI) activities followed the same protocol according to the method of Ellman's et al. (1961), while substrate concentration was varied from 625 to 3,125 µg. The kinetic constants K_m and V_{max} of the extracts and standard inhibitor were determined from the transformation of the Michaelis-Menten plots (Michaelis and Menten 1913). The pattern of inhibition was also studied from the plots. Data are means of two independent experiments, each performed in duplicate.

Statistical Analysis
Statistical significance was determined by Student's t-test and differences were considered significant when $p<0.05$. Statistical tests, kinetic parameters, percentages of inhibition and inhibitory potencies were estimated using Prism V 4.0 (GraphPad Software Inc.).

RESULTS

Percentage Inhibition and IC_{50} Values
The mean percentage inhibition of the different concentrations of the methanol and PBS extracts for BuChE and AChE is presented in Table 1 with the PBS extract exhibiting highest activity of 67.02±1.99 % towards AChE. Similarly, the activity of the methanol extract was also high towards BuChE, with a value of 64.43±1.13% at 250 µg/mL of the extract. We also noted that as the concentrations of the extract decreased the activity also decreased showing moderately high activities towards the AChE and BuChE. The mean percentage inhibition of the standard inhibitor (eserine) is shown in Table 2 giving highest activity values of 96.48±0.01 % at 0.06 µg/mL and 76.46±1.02 % at 0.13 µg/mL of the eserine on BuChE and AChE respectively.
The results of the AChE inhibitory assay revealed that the PBS and methanolic extracts of the antlion larvae had mean IC_{50} value of 49.00 ±1.20 and 271.40±0.10 µg/mL respectively as shown in Table 3.

Table 3: Mean IC_{50} ± SEM (µg/mL) of the antlion larvae extracts and eserine on the activities of BUCHE and AChE

	MEAN OF IC_{50} ± SEM (µg/mL)	
	BuChE	**AChE**
PBS Extract	216.70±1.10	49.00±1.20
Methanolic Extract	66.30±0.40	271.40±0.10
Eserine	1.10±0.30	$2.25 \times 10^{-2} \pm 0.15 \times 10^{-2}$

For the BuChE inhibitory assay, we obtained mean IC50 value of 66.30±0.40 and 216.70±1.10 µg/mL for the methanolic and PBS extracts respectively, while eserine gave mean IC_{50} value of 1.10±0.30 and 2.25 x 10^{-2} ± 0.15 x 10^{-2} µg/mL for BuChE and AChE respectively as also shown in Table 3.

Kinetic Parameters

The plots of the AChE and BuChE activities against the substrate concentration in the absence of the extract and at increasing concentrations of the extract gave hyperbolic curves (not s-hown). The extracts obeyed Michealis-Menten kinetics and the Km and V_{max} values obtained from the Lineweaver-Burk's plots are shown in Figure 1 - 4. The mode of inhibition towards BuChE showed non-competitive type for methanol and PBS extracts (Fig. 1 and 2). The Km values of the PBS and methanolic extracts were approximately 556.61 and 860.52 µg/mL respectively, while the estimated V_{max} value of the methanolic extract decreased from 10.00 µmol/min (in the absence of the extract) to 8.91 x10^{-4} µmol/min (increased extract concentration); the PBS extract on the other hand followed the same pattern of the methanolic extract with the Vmax value decreasing from 7.15 µmol/min (in the absence of the extract) to 6.45 × 10^{-4} µmol/min (at increased extract concentrations) as presented in Table 4. Again, the mode of inhibition of the AChE was depicted as competitive type for PBS extract, while for the methanolic extract, we observed non-competitive inhibition. In this case, the K_m value was found to increase from 2000.00±0.06 to 2857.14±0.60 µg/ml and V_{max} remained almost constant at 10.00 x10^{-4} µmol/min, while the K_m value in the presence of the methanolic extract remained constant at approximately 1000.75 µg/ml and V_{max} decreased from 10.00 µmol/min (in the absence of the extract) to 6.67 x10^{-4} µmol/min (at increasing concentrations of the extract) as shown in Table 5 .Thus, reversible modes of inhibition were observed in both type of extracts.

Table 4: K_m and V_{max} values of BuChE at increasing concentration of antlion larvae extracts

	0 µg/mL of Extract	10 µg/mL of Extract	20 µg/mL Extract	40 µg/mL Extract
PBS K_m (µg/mL)	2000.00±0.06	2500.00±0.05	2631.58±0.60	2857.14±0.60
PBS V_{max} × 10^{-4} (µmol/min)	10.00±0.12	10.00±0.22	10.00±0.24	11.00±1.20
Methanolic K_m (µg/mL)	1000.60±0.02	1000.20±0.03	1000.10±0.02	1002.08±0.05
Methanolic V_{max} × 10^{-4} (µmol/m in)	10.00±0.13	7.69±0.11	6.89±0.10	6.67±0.10

DISCUSSION

The current standard of care for mild to moderate AD includes treatment with acetylcholine esterase inhibitors, such as donepezil or rivastigmine, to improve cognitive function. Acetylcholinesterase (AChE) predominates in the healthy brain, with butyrylcholinesterase (BuChE) considered to play a minor role in regulating brain acetylcholine (ACh) levels. Both enzymes therefore represent legitimate therapeutic targets for ameliorating the cholinergic deficit considered to be responsible for the declines in cognitive, behavioural and global functioning characteristic of AD. According to a study both AChE and BuChE may have roles in the aetiology and progression of AD beyond regulation of synaptic Ach levels (Greig et al. 2002). BuChE activity progressively increases as the severity of dementia advances, while AChE activity declines. Therefore, inhibition of BuChE may provide additional benefits (Lane et al. 2006).

The findings of this study demonstrated that aside the eserine which is the standard inhibitor with highest potency, the PBS extract showed an increased inhibitory potency for AChE compared to the methanolic extract; in other words, the PBS extract inhibitory activity was higher than the methanolic extract counterpart. Again, the methanolic extract inhibitory activity against BuChE was more potent than the PBS extract.

The early discovery of a marked cholinergic

Table 5: K_m and V_{max} values of AChE at increasing concentration of antlion larvae extracts

	0 µg/ml of Extract	10 µg/mL of Extract	20 µg/mL Extract	40 µg/mL of Extract
Methanolic K_m (µg/mL)	847.46±0.25	862.07±0.10	862.97±0.15	869.57±7.35
Methanolic V_{max} × 10^{-4} (µmol/min)	10.11±0.01	9.67±0.13	9.28±0.16	8.91±0.18
PBS K_m(µg/mL)	565.00±5.15	555.56±0.25	555.86±0.27	550.00±0.35
PBS V_{max} × 10^{-4} (µmol/min)	7.15±0.11	6.90±0.10	6.67±0.12	6.45±0.13

deficit in the brains of patients with AD led to the study of therapeutically augmenting cholinergic activity (Whitehouse et al. 1982). Plant materials have been a major source of natural therapeutic remedies and are used to treat various infectious diseases in many developing countries (Beverly and Sudarsanam 2011; Dike et al. 2012). A variety of plants has been reported to show AChE inhibitory activity and so may be relevant to the treatment of neurodegenerative disorders such as AD (Mukherjee et al. 2007). The major alkaloids with recognized anti-AChE activity are the classical galantamine and huperzine A, which have been elegantly reviewed (Gulcan et al. 2015; Qian and Ke 2014). Other plants with a variety of activities and compounds include Salvia spp. (Lamiaceae) which have been used for centuries for its beneficial effects on memory disorders (Hamidpour et al. 2014). Likewise, leaves of *Fumaria capreolata*, *Fumaria densiflora*, *Rosmarinus officinalis*, *Conyza bonariensis* and *Majorana syriaca* were used to enhance cognitive and other related diseases with their potencies reported in decreasing order of IC_{50} values of 0.035, 0.62, 2.40, 2.90 and 2.90 mg/mL respectively (Ali Shtayeh et al. 2014). These plants were identified to effectively inhibit AChE enzyme which is considered to be related to the mechanism of memory dysfunction. But, for the first time we reported the use of antlion larvae with lower IC50 values of 49.00±1.20 µg/mL and 66.30±0.40 mg/mL for the PBS and methanolic extracts respectively. This showed higher cholinesterase inhibitory activity than some reported plant extracts, a positive finding and promising alternative for rectifying cholinergic deficits in AD.

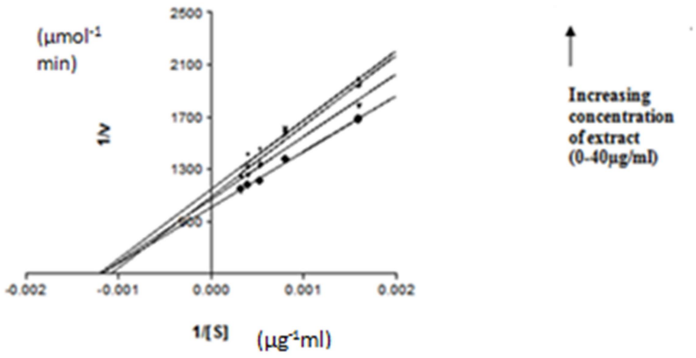

Fig. 1: Lineweaver-Burk's plot of BuChE activity against substrate concentration in the presence of antlion larvae (methanolic extract)

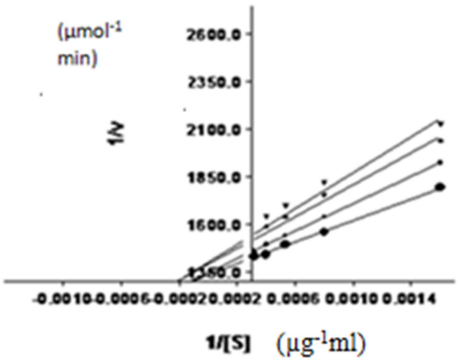

Fig. 2: Lineweaver-Burk's plot of BuChE activity against substrate concentration in the presence of antlion larvae (PBS) extract

As the world population ages, there is a need therefore to investigate other sources asides plant which can be screened to manage neurodegenerative diseases. As the need for novel and effective treatments increases, researchers have turned towards traditional medicine as a resource. Modern evidence based research examining traditional and complementary remedies for AD has generated promising results within the last decade (Cooper and Ma, 2017). This study is therefore novel as we evaluated the cholinesterase inhibitory activities of the larvae of antlion, an animal based extract employed by traditional medicine practitioners to boost memory loss.

Both extracts from the antlion larvae were noted to possess the cholinesterase inhibitory activities though differing activities were reported and this is because different bioactive components of the antlion were extracted using methanol and phosphate buffered saline. Polar compounds in the sample were extracted with methanol; with the BuChEI activity is higher in the methanol extract than in the PBS extract the compound is could be thought of to be polar in nature. The PBS extract on the other hand was employed to disaggregate the tissues and dissociate cells because it maintains pH and osmotic balance as well as provide cells with water and essential inorganic ions. PBS is generally utilized to maintain cells for the short term in a viable condition while the cells are manipulated outside of their regular growth environment. It is noticeable that the use of extracting solvents of distinct polarities, which suggests that the their active compounds might pertain to a wide range of secondary metabolites. Cholinesterase inhibitory activities were confirmed in both extracts though less potent than eserine but since the crude extracts were used it could possibly become more potent if purified.

Years of research have shown that inhibitors are useful for mechanistic studies—they reveal how enzymes interact with their substrates, what role inhibitors play in enzyme regulation, and, based on structure-activity relationships, how to develop drugs that inhibit aberrant biochemical reactions (Mohan et al. 2014).The non-competitive mode of inhibition of the extract on BuChE is an indication that the inhibition of the enzyme by extract is not dependent on substrate concentration, since the inhibitor will only bind on other sites of the enzyme, leading to a conformational change in the active site of the enzyme, thus reducing the affinity of the enzyme for its substrate. But, as seen in the ACHEI we reported a competitive mode of inhibition of the PBS extract just like galanthamine which reversibly and competitively inhibits AChE (Thomsen and Kewitz 1990). Reversible AChEIs play an important role in pharmacological manipulation of the enzyme activity. Medications currently approved by regulatory agencies such as the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA) to treat the cognitive manifestations of AD and improve life quality of the patients are: donepezil, rivastigmine and galantamine as reversible AChE inhibitors, and memantine as an N-methyl-D-aspartic acid (NMDA) receptor antagonist (Wang and Du 2009; Zhou et al. 2009; Lee et al. 2007). Quite a number of the AChEIs currently employed in the treatment of AD are reversible, this include galantamine, tacrine and huperzine A (Colovic et al. 2013) as observed in the antlion larvae extracts. Reversible AChE inhibitors have become the increasingly central role for the treatment of AD. Also, natural molecules and plant secondary metabolites are found to be superior in their inhibiting potential compared to synthetic molecules (Pinho et al. 2013). Another important property of the extracts is the reversible mode of the inhibition observed from the plots since competitive and non-competitive inhibitions were forms of reversible inhibition. The Km values obtained were high except for the PBS Km of the BuChE which was lower indicating more affinity for the substrate and the non-competitive mode of inhibition of the extract on BuChE is an indication that the inhibition of the enzyme by extract is not dependent on substrate concentration, since the inhibitor will only bind on other sites of the enzyme, leading to a conformational change in the active site of the enzyme, thus, reducing the affinity of the enzyme for its substrate..

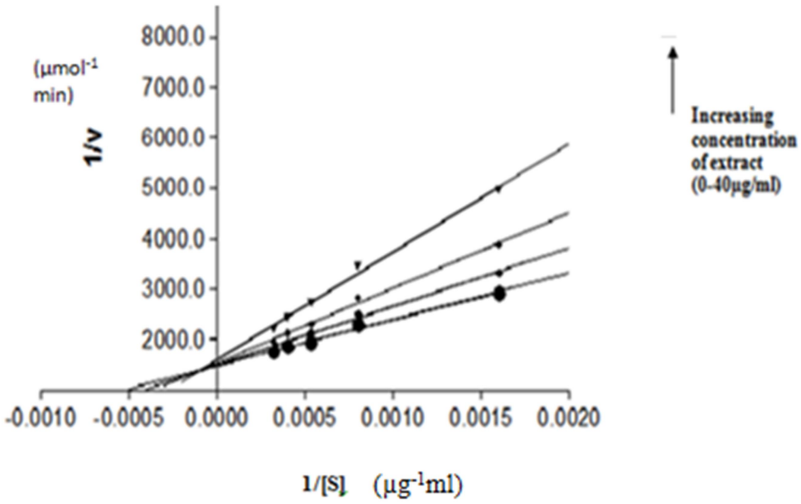

Fig. 3: Lineweaver-Burk's plot of AChE activity against substrate concentration in the presence of antlion larvae (PBS) extract

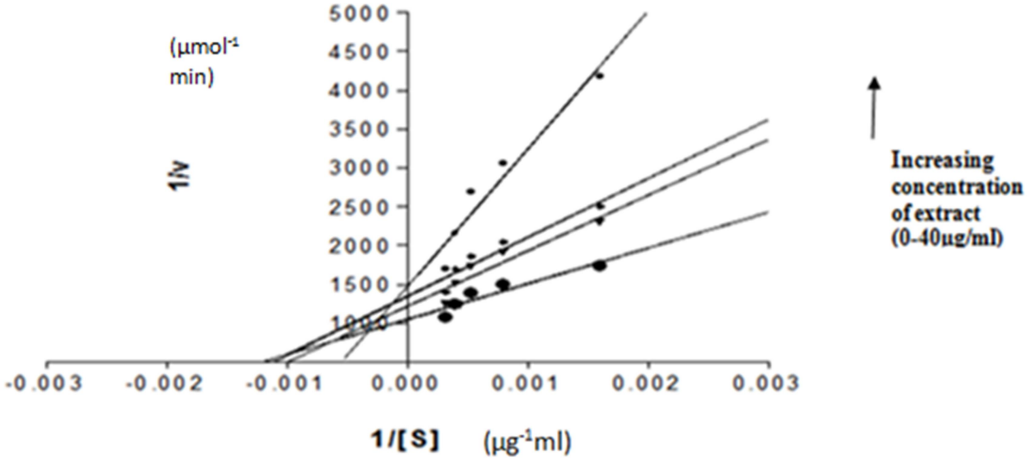

Fig. 4: Line weaver- Burk's plot of AChE activity against substrate concentration in the presence of antlion larvae (methanol) extract.

Conclusion

In conclusion, we established cholinesterase inhibitory activities of the crude antlion larvae extracts with the PBS extract being the most potent for the AChE which also binds competitively and thus could possibly be the

key to the treatment of AD and other forms of dementia if further purified. Further studies wherein identification of active compound(s) and the development of neuroactive compound for pharmacotherapy of AD and other related psychopathologies are therefore recommended.

Conflict of Interest
None declared

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