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Evaluation of the pharmacological effect of Methanol extract of *Cassia tora* and *Moringa oleifera* leaves on Alzheimer's disease *via* inhibition of Acetylcholinesterase

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Alzheimer's disease, Acetylcholine, Acetylcholinesterase, *Cassia tora*, *Moringa oleifera* and Enzyme Kinetics.

Abstract

Alzheimer's disease (AD) is a common neurodegenerative disease of the central nervous system, characterized by low levels neurotransmitter acetylcholine (ACh) in the brain. Acetylcholinesterase (AChE), the predominant cholinesterase in the brain, hydrolyzes ACh to choline and acetate, thereby terminating the effect of this neurotransmitter at cholinergic synapses. Treatment of this disease relies on the enhancement of cholinergic function by stimulation of cholinergic receptors and prolonging the availability of acetylcholine concentrations in the synaptic cleft and enhanced cholinergic transmission by use of agents which improves the levels of acetylcholine. Inhibition of acetylcholinesterase (AChE) and enzymes which breakdown acetylcholine, are considered as a promising strategy for the treatment of AD. The aim of this study is to evaluate *in vitro* effect of methanol extract of *Cassia tora*(CTE)and *Moringa oleifera* (MOET)leaves on extract on Acetylcholinesterase (AChE). The i*n vitro* assay for inhibitory effect of CTE and MOET on AChEactivities was performed according to Ellman's standard protocols by reacting AChE and substrates at different concentration (20- 100 µg/ml) of the extract in relation to galanthamine while the enzyme kinetics (mode of Inhibition) assay for AChE was determined using the Lineweaver-Burk plot. The results revealed that CTE and MOET had a lower IC_{50} (68.5µg/mL and 72.5µg/ mL) than galanthamine 80.2µg/mL, hence the extracts are potent inhibitors than galanthamine. The enzyme kinetics assay, the Lineweaver-Burke plot showed that both extract inhibits AChE in a competitive manner. It can be suggested from this study that CTE and MOET are potent inhibitors for AChE and maybe useful in the treatment of Alzheimer's disease.

1. Introduction

Alzheimer's disease is a progressive neurodegenerative brain disorder that is slow in onset but leads to dementia, unusual behavior, personality change and ultimately death (Jewart *et al*., 2005). The occurrence has been found to rise exponentially with age, ranging from 3.0% in patient's aged 65-74 years to as much as 47.2% in those aged 85 years (Wernicke and Reischies, 1994). Has estimated by World Health organization (WHO) 35.6 million people are currently living with dementia worldwide which will further increase to 65.7 million by 2013 and 115.4 million by 2050 (Wimo and Prince, 2010).

Studies have revealed the involvement of neurotransmitter acetylcholine in Alzheimer's disease resulting into disproportionate deficiency of acetylcholine. It has been documented that markers for acetylcholine esterase, acetylcholine transferase and cholinergic neurons, are responsible for acetylcholine synthesis and its degradation decreases in the hippocampus area and cortex of the brain involved in cognition and memory (Francis *et al*., 1999). The study has demonstrated that the resultant decreased in acetylcholine dependant neurotransmission, is associated with the functional deficit of AD (Wright *et al*., 1993).

The use of cholinesterase inhibitors in the treatment of patient with Alzheimer disease has been found to be better successful strategy (Nordberg and Svensson, 1998; Weinstock, 1999). The tacrine, the acridine derivatives, was the first drug approved by food and drug administration (FDA), USA for general clinical use in Alzheimer's disease. The new choline esterase inhibitors approved by FDA, USA to treat Alzheimer's are Donepezil (Aricept), Rivastigmine (Exelon), Galanthamine (Reminyl) (Bullock, 2001; Bullock, 2002).

The above mentioned drugs are used to treat mild to moderately severe Alzheimer's. The currently available drugs for the treatment of Alzheimer's disease do not alter the condition and progression of the disease. They also produced adverse effects in the patients, not suitable for a prolong time. To alter the current conditions related to present dosage form, need to search alternative effective therapy, which will alter the present condition also retard the progression of the disease by preventing the formation or clearing of plaques.

C*assia tora* Linn. Is well known medicinal plant commonly found in India and other tropical countries. The use of medicinal plants as raw materials in the production of new drugs is increasing day by day because of their potentials in combating the problem of drug resistance in micro-organisms (Choudhary *et al*., 2011). Various ethnobotanical properties of this plant have been reported in the Indian traditional system of medicine as a laxative, antiseptic, antioxidant activity, antiperiodic and useful in treatment of ringworm, bronchitis, leprosy, hepatic disorder, cardiac diseases, liver tonic, hemorrhoids, and ophthalmic, skin diseases (Huang, 1993).

Moringa oleifera belongs to the family of Moringaceae, a fast growing drought- resistant tree, native to sub Himalayan tracts of Northern India but also distributed worldwide in the tropics and sub tropics (Fuglie, 1999). In Nigeria, though the Moringa tree is widespread throughout the states, it is usually found around farms and compounds as fence in Northern part of the country. In Nigeria, it is cultivated for its use as an alternative green vegetable source for human consumption and other medicinal uses. Ethanolic extract of the leaves hasbeen shown to possess antifungal activity against a number of dermatophytes (Chaung *et al*., 2007) while, the methanolic extract was shown to have a CNS depressant activity (Pal *et al*., 1996) while the aqueous extract has also demonstrated anti-fertility activity (Prakash, 1998). *Moringa oleifera*is used to treat stomach ailments (ease stomach pain, ulcer and aiding digestion), poor vision, joint pain, diabetes, anemia and hypertension (Abe and Ohtani, 2013; Popoola and Obembe, 2013),malaria, typhoid fever, parasitic diseases, arthritis, swellings, cuts, diseases of the skin, genito-urinary ailments, hypertension and diabetes (Leone *et al.*, 2015), toothache, as anthelmintic and antiparalytic inflammations, muscle diseases, hysteria, tumours and enlargement of the spleen (Anwar *et al.*, 2007; Yabesh *et al.*, 2014).

Several drugs have been developed to eradicate or reduce the pandemic but are however, these drugs are known to have limitations for clinical use due to their short-half-lives and/or unfavourable side-effects (Sung *et al*., 2002). The combined therapy of extract of *Cassia tora* and *Moringa oleifera extract* may serve as comparatively cheap and potential sources for the development of therapeutic agents for Alzheimer and dementia related diseases. Herbal medicine offers several options to modify the progress and symptoms of AD. There has been a new trend in the preparation

and marketing of drugs based on medicinal plants, and their scientific and commercial significance appears to be gathering momentum in health-relevant areas. The aim of the research is to investigate the possible inhibitory effect of *Cassia tora* and *Moringa oleifera* leaves on acetylcholinestarase in order to point out the role of these plants as potential sources for the development of therapeutic agents for Alzheimer's disease (AD).

2. 0 Materials and Methods

2.1Plant material

A whole fresh plant of *Cassia tora* and *Moringa oleifera* leaves were collected fresh from Lokongoma Kogi State, in October, 2018. The plants were identified at the Herbarium Unit of the Department of Biological Sciences, Kogi State Polytechnic Lokoja; Kogi State, Nigeria. The leaves were air-dried at room temperature for two weeks and pulverized to coarse powder in an electric hammer mill.

2.2 Chemicals and drugs

Acetylcholinestarase (EC 3.1.1.7), acetylthiocholine, (Sigma-Aldrich, UK), galanthamine (Glucobay® Bayer), Methanol (BDH, England), Acetylcholine, tris- HCl buffer (pH 8.0), Ellam reagent (Sigma-Aldrich, UK). Other solvents and reagents used were purchased from the country representative of Sigma Chemical, St. Loius USA and were of analytical grade.

2.3 Preparation of extract

The powdered plant material was extracted with methanol (98% v/v) by cold maceration with occasional shaking for 48 h. The mixture was filtered using Whatman filter paper (No 1) to obtain the filtrate. The filtrates were pooled, concentrated and evaporated to dryness on a hot water bath at 45^{O}C for 48h. The extract will henceforth be referred to as CTE and MOET, then stored in a refrigerator until required for use..

2.4 Enzyme inhibition protocol

2.4.1 Acetylcholinesterase *In vitro* inhibition assay

AChE activity was investigated spectrophotometerically according to the method of Ellman's*et al*. (1961) with slight modification.

Acetylthiocholinethe substrate is hydrolysed by the enzyme resulting in the product thiocholine which reacts with Ellman's reagent (DTNB) to produce 5-thio-2- nitrobenzoate and 2-nitrobenzoate-5-mercaptothiocholine and which can be detected at 412 nm. In a test tube 1000µL of 50 mM Tris–HCl buffer pH 8.0 and 250 µL of plant extracts at the concentrations of 20 – 100 µg/ mL,10 µL 6.67 UmL^{-1}AChE and 20 µL of 10 mM of DTNB (5,5'-dithio-bis[2- nitrobenzoic acid]) in buffer were added. Galanthaminethe Positive control were prepared in serial concentration as same as test extract by dissolving in 50 mM Tris–HCl buffer pH 8.0. The mixture was incubated at 37^{o}C for 15 min. Then,10 µL of acetylthiocholine iodide (200 mM) in buffer were added to the mixture and absorbance was measured at 412 nm every 10 sec for 3 mins, a blank with buffer was used. The enzyme inhibition (%) was calculated from the rate of absorbance change with time (V= Abs/Δt).

$$\text{Inhibition (\%)} = \frac{100 - \text{Change of sample absorbance}}{\text{Change of blank absorbance}} \times 100$$

The experiment was done in triplicate and test extract concentrations that inhibit the hydrolysis of the substrate (acetylcholine) by 50% (IC_{50}) were determined using linear regression analysis between the inhibition percentages versus the extract concentration.

2.4.2 Mode of Acetylcholinesterase Inhibition

The mode of inhibition of acetylcholinesterase by the extracts will be determined using the extract with the lowest $IC_{50.}$ 50 µL of the (5mg/mL) extract was pre-incubated with 100µL of acetylcholinestarase solution (0.5 mg/mL) of or 15 min at 35°C in one set of tubes. In another set of tubes, acetylcholinestarase was pre-incubated with 50µL of Tris–HCl buffer (pH 8.0). 50 µL of 10mM DTNB at increasing concentrations (0.5 - 2.0 mg/ml) was added to both sets of reaction mixtures to start the reaction. Then,10 µL of acetylthiocholine iodide (200 mM) in buffer were added to the mixture and absorbance was measured at 412 nm. A double reciprocal (Lineweaver-Burk) plot (1/v versus 1/[S]) where V is reaction velocity and [S] is substrate concentration will be plotted to determine the mode of inhibition.

2.5 Statistical Analysis

All data were expressed as Mean ± SD and statistical differences between means were determined by one-way ANOVA followed by Duncan *post –hoc* test for multiple comparison tests using SPSS. Values were considered significant at $p \leq 0.05$.

3.0 Results

3.1 Percentage Inhibitory Effect of Various Concentration of Methanol Extract *C. tora* (CTE) and *M. oleifera* Leaves (MOET) on the Activity of Acetylcholinesterase Enzyme.

A concentration-dependent inhibitory effect on the Acetylcholinesterase enzyme was observed various concentration of the extracts used. The Median Inhibitory Concentration (IC_{50}) of the leaves extract of *C.tora*and *M. oleifera on* acetylcholinesterase was estimated to be 68.5µg/ ml, and 72.5µg/ml (Table 1).

Table 1: Percentage Inhibitory Effect of Various Concentration of Methanol Extract *C. tora*(CTE) and *M. oleifera* Leaves (MOET) on the Activity of Acetylcholinesterase Enzyme.

Plant Extract	Concentration (µg/ml)	% Inhibition	IC_{50}	Plant Extract	Concentration (µg/ml)	% Inhibition	IC_{50}
	20	18.70±1.66			20	25.06±2.11	
	40	30.28±0.61			40	32.63±0.45	
CTE	60	41.86±1.12	**68.5µg/mL**	**MOET**	60	42.67±0.11	**72.5µg/mL**
	80	54.04±1.25			80	56.75±0.56	
	100	57.92±0.23			100	70.05±0.13	

Data represented as mean ± S.D. Data was analyzed by one- way ANOVA followed by Duncan's *post – hoc* test for multiple comparisons, (n=3).

3.2: Percentage Inhibitory Effect of Mixed Various Concentration of Methanol Extract of *C. tora* leaves (CTE), *M. oleifera* Leaves (MOET) and Galanthamine (Standard) on the Activity of Acetylcholinesterase Enzyme.

A concentration-dependent inhibitory effect on the acetylcholinesterase enzyme was observed with mixed various concentration of the extract and galanthamine (standard drug) used. The Median Inhibitory Concentration (IC_{50}) of the mixed leaves extract of *C. tora* (CTE) and *M. oleifera* (MOET) on acetylcholinsetase was estimated to be 69.1µg/ ml, while that of the standard; Galanthamine was 80.2µg/ml (Table 2).

Table 2: Percentage Inhibitory Effect of Mixed Various Concentration of Methanol Extract *C. tora* leaves (CTE), *M. oleifera* Leaves (MOET) and Galanthamine on the Activity of Acetylcholinesterase Enzyme.

Plant Extract	Concentration (µg/ml)	% Inhibition	IC_{50}	Standard drug	Concentration (µg/ml)	% Inhibition	IC_{50}
	20	27.26±1.36			20	18.08±1.08	
CTE and	40	34.83±1.61			40	24.57±2.67	
MOET	60	38.71±0.11	**69.1µg/mL**	**Galanthamine**	60	39.62±0.43	**80.2µg/mL**
	80	57.59±0.29			80	50.54±0.67	
	100	76.38±0.43			100	68.02±2.44	

Data represented as mean ± S.D. Data was analyzed by one- way ANOVA followed by Duncan's *post – hoc* test for multiple comparisons, (n=3).

3.3. Lineweaver-Burk Plots for the Investigation of Mode of Inhibition of Acetylcholinesterase Activity by Methanol Extract of *Cassia tora* Seeds (CTE) and Galanthamine (Standard drug)

The mode of inhibition of the enzyme was generated using Lineweaver-Burke plot and the result showed that methanol extract of *C. tora* inhibited acetylcholinesterase in a competitive manner with increasing K_m of 6.992 mM to 37.174 mM and V_{max} of 33.7 × 10^{-3}mM/min to 15.5 × 10^{-3}mM/min while the standard drug galanthamine also inhibits acetylcholinesterase in a competitive manner with increasing K_m of 15.702 mM to 37.174 mM and V_{max} of 33.7 × 10^{-3}mM/min to 8.0 × 10^{-3}mM/min (Figure 1).

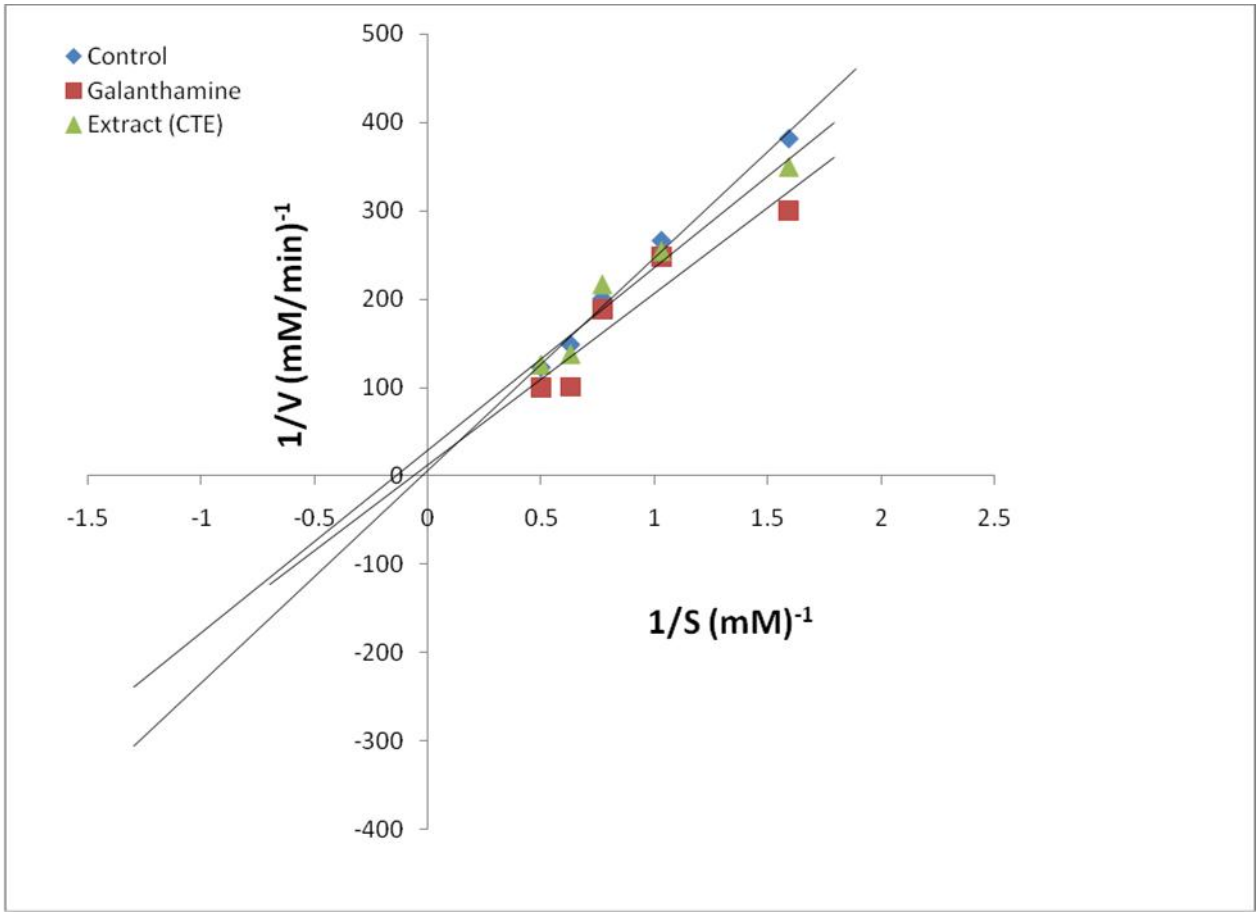

Figure 1: Lineweaver-Burk Plots for the Investigation of Mode of Inhibition of Acetylcholinesterase Activity by Methanol Extract of *Cassia tora* Seeds(CTE) and Galanthamine (Standard Drug).

3.4 Lineweaver-Burk Plots for the Investigation of Mode of Inhibition of Acetylcholinesterase Activity by Methanol Extract of *Moringa oleifera* Seeds (MOET) and Galanthamine (Standard drug)

The mode of inhibition of the enzyme was generated using Lineweaver-Burke plot and the result showed that methanol extract of *M. oleifera* inhibited acetylcholinesterase in a competitive manner with increasing K_m of 2.930 mM to 37.174 mM and V_{max} of 33.7 × 10^{-3}mM/min to 15.5 × 10^{-3}mM/min while the standard drug galanthamine also inhibits acetylcholinesterase in a competitive manner with increasing K_m of 15.702 mM to 37.174 mM and V_{max} of 33.7 × 10^{-3}mM/min to 18.0 × 10^{-3}mM/min (Figure 2).

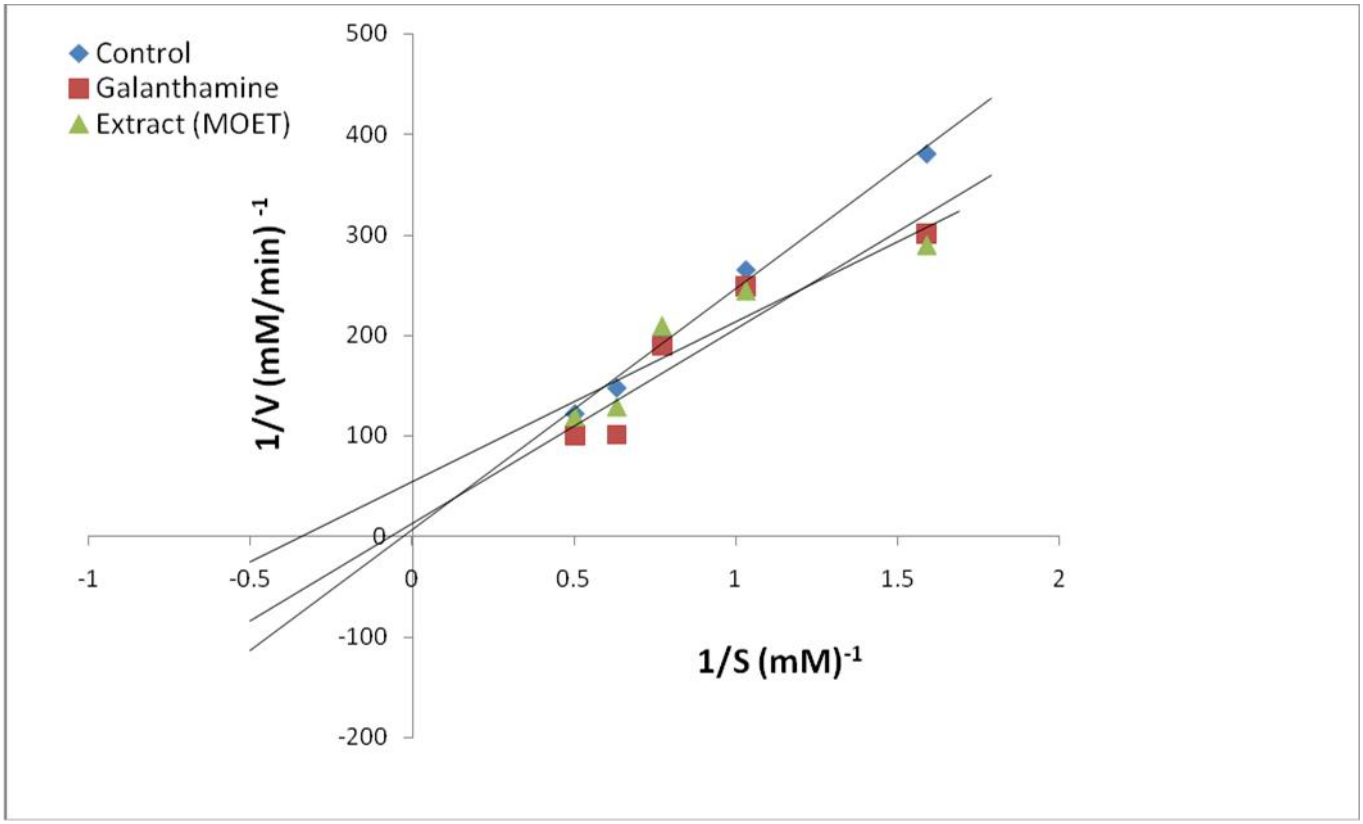

Figure 2: Lineweaver-Burk Plots for the Investigation of Mode of Inhibition of Acetylcholinesterase Activity by Methanol Extract of *Moringa oleifera* Seeds (MOET) and Galanthamine (Standard Drug)

4.0 Discussion

Alzheimer's disease (AD) is a progressive neurodegenerative brain disorder, the most common form of dementia, characterized by memory loss and other intellectual abilities serious enough to interfere with daily life (Thompson *et al*., 2012). The disease is commonly identified with loss of cholinergic neurons in the brain and the reduced level of acetylcholine (Ach)(Lane *et al*.,2006). The main therapeutic target in the AD treatment strategies is the inhibition of brain AChE(Lane *et al*.,2006; Giacobini, 2004). Various physiological processes related to AD, damage or destroy cells that produce and use acetylcholine, thereby reducing the amount available to deliver messages to other cells. Cholinesterase inhibitors, maintain acetylcholine level by decreasing its breakdown rate. Therefore, they boost cholinergic neurotransmission in forebrain regions and compensate for the loss of functioning brain cells. No drug has an indication for delaying or halting the progression of the disease (Stahl, 2000). Medications currently approved by regulatory agencies to treat the cognitive manifestations of AD and improve life quality of the patients are: donepezil, rivastigmine and galanthamine as reversible AChE inhibitors, and memantine as an NMDA receptor antagonist (Birks, 2006; Hyde *et al*., 2013; Bond *et al*., 2012). Tacrine was the first of the AChE inhibitors approved for the AD treatment in 1993, but its use has been abandoned because of a high incidence of side effects including hepatotoxicity (Birks *et al*., 2009; Watkins *et al*., 1994).

Consequently, some people often resort to medicinal plants and foods with medicinal activity but with fewer or no adverse effects while some co-use herbs with conventional anti-cholinergic agents for better AChE control. Herbal extracts have been used directly or indirectly, for the preparation of many modern medicines. Many herbal plant extracts have been reported for their anti-cholinergic properties and acetylcholinesterase inhibitory activity but to date no such report was found for *C. tora* and *M. oleifera* seeds. This study investigated the effect of methanol extract of *C. tora and M. oleifera* seeds on alzheimer's disease via inhibition of acetylcholinesterase enzyme activity.

In the *in vitro* study, galanthamine was used as the positive standard drug; it inhibited the acetylcholinesterase activity with an IC_{50} value 80.2µg/ml while the IC_{50} value of the extract *C. tora* was found to be 68.5µg/ ml and *M. oleifera* 72.5µg/ ml. The high percentage inhibition implies that the methanol extract of *C. tora*and *M. oleifera* leaves are very potentacetylcholinesterase inhibitor in comparison with galanthamine indicating a potential role as an anticholinergic agent from natural source in order to prevent some of the side effects produced by synthetic drugs. The reason is thatany plant with strong inhibitor foracetylcholinesterase could serve as effective therapy for several neurological disorders like AD with minimal side effects. This is also in agreement with the report of Kim *et al.* (1999) found that a methanol extract of the tuber of Corydalis ternata showed significant inhibition of AChE.

Further study was carried on the mode of inhibition of acetylcholinesterase by the methanol extract of *C. tora* and *M. oleifera*. The extract of *C. tora* and *M. oleifaera* both inhibits AChE in a competitive manner. This suggests that the inhibitor binds exclusively to the free enzyme yielding an enzyme-inhibitor complex. The binding of the inhibitor to the free enzyme decrease the apparent affinity of the enzyme for the substrate (K_m value appears to increase; relatively constant V_{max}). This implies that, the active component in the extract is structurally similar to the substrate, the extract binds reversibly to the active site of the enzyme and occupies it in a mutually exclusive manner that causes conformational changes in the enzyme that reduce the affinity of the enzyme for its substrate (Mayur*et al*., 2010). The manner of competitive inhibition of AChE exhibited by galanthamine indicates that the active component is structurally similar to the substrate. This implies that the galanthamine compete for the active site of the enzyme and occupies it in an exclusive manner with the substrate (Kazeem *et al*., 2013).

The inhibitory effects of methanol extract of *C. tora*and *M. oleifera* on AChE activities respectively may be attributed to the presence of phytochemicals such as alkaloids, terpenoids, sterols, flavonoids and phenolic compounds, etc (Houghton *et al.,* 2008; Mukherjee *et al.,* 2007; Orhan*et al.,* 2009). Until 2006 only a few diterpenoids demonstrated to inhibit AChE (Houghton *et al.,* 2008). However, further recent research has reported a larger number of compounds belonging to this group with the ability to exert either moderate or strong AChE inhibitory activity. In addition, a new cassanediterpene named niloticane was isolated from the ethyl acetate bark extract of *Acacia nilotica* subsp. *kraussiana* (Fabaceae), a plant used in African traditional medicine (Eldeen *et al.,* 2010). Decrease in acetylcholinesterase activity by *C. tora* and *M. oleifera* indicates an increase in the basal level of acetylcholine.

5.0 Conclusion

In conclusion, findings from this study have demonstrated the anticholinergic potential of *Cassia tora*and *M. oleifera via* its *in vitro* inhibitory action on acetylcholinesterase and significantly reduce the rate of breakdown of acetylcholine. Therefore, *Cassia tora and Moringa oleifera* leaves extract could be potentially used as natural therapy for the enhancement of cholinergic system in the brain and treatment of Alzheimer's disease with minimal side effects.

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