Original Research Article

Acetylcholinesterase Inhibitory Activities of Aqueous and Ethanolic Leaf Extracts of *Vernonia amygdalina* and Seed Extract of *Myristica fragrans*

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Abstract

Anticholinesterases are drugs that inhibit acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE). Clinical uses of anticholinesterases include: (a) treatment of myasthenia gravis, (b) reversal of the action of non-depolarizing neuromuscular-blocking drugs used during surgical operations, (c) treatment of glaucoma and management of Alzheimer's disease (AD). Studies have reported several medicinal properties of *V. amygdalina and M. fragrans*. However the anticholinesterse inhibitory actions (beneficial in neurodegenerative disorders) of these herbs have not been studied, hence the need for the present study. The leaves of *V. amygdalina* and seeds of *M. fragrans* were extracted in water and ethanol using cold extraction. The cholinesterase inhibitory assay of the extracts was done in five test tubes: the first three contained the extracts, the fourth test tube contained neostigmine, while the fifth contained blank (control). All the test tubes (control and tests) were incubated tor 20 mins at room temperature after addition of acetylcholinesterse and acetylthiocholine. Thereafter, Ellman's spectrophotometric method was used to determine the absorbance at 412 nm wavelength. The phytochemical screening revealed the presence of flavonoids, phenolics and glycosides. Aqueous and ethanolic extracts of the leaves of *V. amygdalina* had mean change in absorbance of 0.032 ± 0.00 and 0.023 ± 0.01 (64 and 74% AChE inhibition respectively), while aqueous seed extract of *M. fragrans* had 0.015 ± 0.01 (83% AChE inhibition). There were statistically significant differences ($p = 0.01$) in the mean change in absorbance per minute in the various test groups. Extracts of leaves of *V. amygdalina* and seeds of *M. fragrans* showed significant in vitro acetylcholinesterase inhibitory activities at concentrations used.

Keywords: Acetylcholinesterase, anticholinesterase, Alzheimer's disease, extracts, inhibition, in vitro, Vernonia amygdalina, Myristica fragrans.

INTRODUCTION

Anticholinesterases are drugs that inhibit acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE). They are divided into three groups according to the nature of their interaction with the active sites of the enzymes which determine their duration of action: (i) Short-acting anticholinesterases (e.g. edrophonium) used in the diagnosis of myasthenia gravis. (ii) Medium-duration anticholinesterases (e.g. neostigmine, pyridostigmine, physostigmine, rivastigmine) used, among other indications, for the treatment of myasthenia gravis and Alzheimer's disease. (iii) Irreversible anticholinesterases (e.g. parathion, echothiophate, soman, sarin) which are variously used as pesticides, insecticides, and nerve gases in warfare [1]. Other clinical uses of anticholinesterases include: (a) treatment of myasthenia gravis, (b) reversal of the action of non-depolarizing neuromuscular-blocking drugs used during surgical operations,(c) treatment of glaucoma (echothiophate eye drops [1].

Alzheimer's disease (AD) is a neurodegenerative disorder with dementia that does not have an antecedent cause, such as stroke, brain tumor or alcohol. Its prevalence rises sharply with age from about 5% at 65 to 90% or more at 95[1]. In 2010, approximately 35 million people worldwide were suffering from AD and this number is believed to reach 65.7 million by 2030 [2]. Hendrie *et al.*, reported that the age-adjusted prevalence rate of Alzheimer's disease in Yorubas in Ibadan, Western Nigeria was 1.41% compared to 3.69% for Yorubas in Indianapolis, USA [3]. These findings show significant differences in the prevalence of Alzheimer's disease in two different communities with similar ethnic origins, thus highlighting the possible effect of environment in the pathogenesis of the disease. A large review involving the Sub-Saharan Africa found that the prevalence of

Alzheimer disease varied between 0.7% and 5.6% in population-based studies and from 1% to 47.8% in hospital-based studies and major risk factors were advanced age and female gender [4].

A promising approach to treating Alzheimer's disease patient is to enhance the level of cholinergic neurotransmitters in the brain by cholinesterase inhibitors [5]. Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) are the two major cholinesterase enzymes and they play an important role in decreasing choline levels in the body [6]. Finding natural anticholinesterases appears promising and has generated a lot of interest [2].

In Nigeria, especially the Southeast geopolitical zone, it is expected that some plants or vegetables that are part of our diet contain useful medicinal substances. Here, in folklore medicine, *V. amygdalina* (bitter leaf) was used in the treatment of memory loss [7]. However, this claim has not been subjected to any scientific verification. Bitter leaf (*Vernonia amygdalina*) which belongs to the Asteraceae family is a small shrub that grows in tropical regions. In Nigeria it is known by various ethnic names such as onugbu (Igbo), etidot (Ibibio), oriwo (Edo), ewuro (Yoruba), chusar-doki (Hausa).

Several studies have reported the medicinal properties of *V. amygdalina* which include antibacterial, antiplasmodial, antimalarial, and analgesic, amoebicidal [1], and wound healing properties [8-11]. However, there are no reported studies on its effect on Alzheimer's disease. Elevation of acetylcholine levels in brain through the use of AChE inhibitors has been considered the most effective treatment method for Alzheimer's disease. However, some of the existing drugs used for this ailment have either lost their efficacy or developed serious side effects which can lead to patient's non-compliance. For instance, tacrin, the first drug approved for treating AD has to be given four times daily and produces cholinergic side effects such as nausea, and abdominal cramps as well as hepatotoxicity in some patients. Therefore, the search for alternative therapeutic approach using medicinal plants and herbs becomes imperative.

This study, therefore, was designed to determine the inhibitory effect of aqueous and ethanolic extracts of this plant on acetylcholinesterase, an enzyme that catalyses the breakdown of acetylcholine. This appears plausible since loss of cholinergic neurons and therefore cholinergic hypoactivity are the pathological and biochemical hallmarks of Alzheimer's disease.

The general objective of the study was to investigate the acetylcholinesterase inhibitory activities of aqueous, and ethanolic leaf extracts of *V. amygdalina and M. fragrans*. The specific objectives included the following:

- To determine the phytochemical constituents of *V. amygdalina* and *M. fragrans.*
- To determine the percentage inhibition of AChE by ethanolic leaf extracts of *V. amygdalina* and that of the seed of *M. fragrans.*
- To compare the percentage inhibition of AChE by aqueous leaf extract of *V. amygdalina* and that of ethanolic leaf extract of *V. amygdalina.*
- To compare the percentage inhibition of AChE by the extracts (*V. amygdalina* and *M. fragrans*) and the positive control (Neostigmine).

Null hypothesis in this study stated that aqueous and ethanol extracts of the leaf of *V. amygdalina* and seed of *M. fragrans* do not inhibit acetylcholinesterase activity in vitro. The alternative hypothesis stated that aqueous and ethanol extracts of the leaf of *V. amygdalina* and the seed of *M. fragrans* inhibit acetylcholinesterase activity in vitro. The null hypothesis would be tested at a significant level (p-value) of 0.05. It would be rejected if the p-value is ≤ 0.05 and accepted if p-value >0.05. At p-value ≤ 0.05, the null hypothesis would be rejected, implying that aqueous and ethanol extracts of the leaf of *V. amygdalina* and seed of *M. fragrans* inhibit acetylcholinesterase activity in vitro.

MATERIALS AND METHODS

The leaves of *V. amygdalina* and the seeds of *M. fragrans* were extracted in water and ethanol using both cold and hot extraction methods [12]. Quantitative determination of the presence of phytochemicals was done using the method of Trease and Evans (2012). Percentage (%) yield of the extracts was calculated by the formula, Yield=Weight of extract/Weight of sample × 100/1. Ellman's method was used for the determination of AChE inhibitory activity [13]. The change in absorbance was measured at 412nm wavelength using Spectrophotometer 20 D (Techmel & Techmel U.S.A) [14].

Acetylcholinesterase inhibitory assay

Ellman's method was used for determination of AChE inhibitory activity.[13] Acetylthiocholine was used as a substrate and hydrolysis of acetylthiocholine was determined by monitoring the formation of the yellow 5-thio-2-nitrobenzoate anion as a result of the reaction with 5, 5-dithio-bis-2 –nitrobenzoic acid with thiocholine. The reaction mixture was set as follows:

1) Control group which contained the following:
   a) 200 µL AChE, 0.45 U/ml solution (Elabscience, USA).
   b) 100 ml of 5, 5-dithio-bis-2-nitro benzoic acid (DTNB) solution (Sigma, India).
   c) 500 µL phosphate Buffer pH 8.0 (BDH, UK).

2) The test groups contained a, b, c above plus the extracts (20 mg, 40 mg, 60 mg, 80 mg, 100 mg).

3. Positive control which contain a, b, c above plus neostigmine100 μL, 2.5 mg/ml solution (Vital Healthcare, India)

All the test tubes (control & tests) were incubated for 20mins at room temperature. 100 μL acetylthiocholine iodide, 0.05 mM solution (Elabscience, USA) was added as the substrate and AChE activity was determined with a spectrophotometer (Techmel & Techmel U.S.A)

The change in absorbance at the wavelength of 412nm was then measured for a period of 3 minutes at room temperature. All assays were carried out in triplicates. The percentage inhibition was calculated as follows: a-b/a ×100/1

Where a= change in absorbance per min of control (ʌA/min), b= Change in absorbance per minute of test sample [14].

Statistical Analysis
The results of triplicate assays were presented. Percentage AChE inhibitions were calculated with a scientific calculator using the formula given above.

The concentrations, change in absorbance per minutes, and the mean ± SEM were calculated. Also, the graphs and bar chart were plotted on excel and exported to Microsoft word. Test of statistical significance for the mean change in absorbance per minute in the various test groups was done using ANOVA followed by post hoc Boferonni and the

results taken as statistically significant if the p-value was < 0.05. Statistical analysis was done using SPSS Version 21.

RESULTS AND DISCUSSION
The qualitative and quantitative phytochemical constituents of the extracts are shown in Table-1. *V. amygdalina* extract contained higher concentrations of alkaloids, tannins and saponins than other extracts while aqueous extracts of *M.fragrans* contained higher concentrations of steroid and glycosides than the *V. amygdalina* extracts as shown inTable-1.

The results showed that aqueous and ethanolic leaf extracts of *V. amygdalina* had mean change in absorbance of 0.032 ±0.00 and 0.023 ±0.01 (64 and 74% cholinesterase inhibition respectively) while aqueous seed extract of *M. fragrans* had 0.015 ±0.01 (83% inhibition) as shown in Table II. The ethanolic seed extract of *M. fragrans* was turbid and therefore could not be analyzed spectrophotometrically.

Percentage inhibition of acetylcholinesterase by aqueous and ethanolic extracts of *V amygdalina* was highest at concentrations of 20 mg/ml and 80 mg/ml as shown in Table-2, Figures 1 and 2. Also, the percentage inhibition of acetylcholinesterase by aqueous extract of M. fragrans was highest at 20 mg/ml and 100 mg/ml as shown in Figure-3. The percentage inhibition of acetylcholinesterase by aqueous extract of M. fragrans was higher than extracts of V. amygdalina and the positive control (Neostigmine) as shown in Figure-4.

Table-1: Quantitative Phytochemical Analysis of extracts of the leaf of *V. amygdalina* and seed of *M. fragrans*

Phytochemical Constituent	EEV (mg/100 g)	AEV(mg/100 g)	AEM(mg/100 g)
Alkaloids	1.27±0.01	0.97±0.020	0.06±0.00
Tannins	4.08±0.03	2.67±0.03	0.77±0.01
Saponins	1.97±0.02	1.81±0.01	1.38±0.03
Flavonoids	1.62±0.01	0.68±0.01	2.81±0.06
Phenolics	1.04±0.01	0.91±0.03	0.08±0.00
Steroids	0.07±0.01	0.00±0.00	0.44±0.02
Glycosides	1.01±0.03	0.82±0.01	1.62±0.03

EEV= Ethanol extract of the leaf of *V. amygdalina.*   AEV=Aqueous extract of the leaf of *V. amygdalina*.   AEM= Aqueous extract of the seed of *M. fragrans.*

Table-2: Change in absorbance per minute and percentage acetylchoinestersae inhibition of the extracts of *V. amygdalina* and *M. fragrans* at different concentrations compared with that of neostigmine

Conc. (mg/ml)	Aqueous leaf extract of *Vernonia amygdalina*		Ethanol leaf extract of *Vernonia amygdalina*		Aqueous seed extract of *Myristica fragrans*		Neostigmine (positive control)	
	Change in absorbance	% inhibition	Change in absorbance	% inhibition	Change in absorbance	% inhibition	Change in absorbance	% inhibition
100	0.032±0.001	64	0.023±0.001	74	0.015±0.008	83	--	--
80	0.018±0.000	80	0.005±0.000	94	0.024±0.000	73	--	--
60	0.031±0.000	65	0.019±0.000	79	0.024±0.000	73	--	--
40	0.032±0.001	64	0.020±0.000	76	0.026±0.000	71	--	--
20	0.008±0.000	91	0.004±0.000	96	0.001±0.000	99	--	--
2.5mg/ml	--	--	--	--	--		0.027	70%
Blank	0.089±0.000		0.089±0.000		0.089±0.000		0.089±0.000	

There were statistically significant differences in the mean change in absorbance per minute in the various test groups using ANOVA followed by Post Hoc Bonferonni as shown in Table-3.

Table-3: Statistically significant differences in mean changes in absorbance per minute in various test and control groups using ANOVA followed by Post Hoc Bonferroni

Concentration(mg/ml)	AEV	EEV	AEM
100	0.032±0.001	0.023±0.001	0.015±0.008
60	0.018±0.000	0.005±0.000	0.024±0.000
40	0.032±0.001	0.020±0.000	0.026±0.000
20	0.008±0.000	0.004±0.000	0.001±0.000
Blank	0.089±0.000	0.089±0.000	0.089±0.000
F- test	2856.96	82.71	98.73
P- value	<0.001*	<0.001*	<0.001*
A vs B	<0.001*	0.05*	0.9
A vs C	1.00	1.00	0.9
A vs D	1.00	1.00	0.4
A vs E	<0.001*	0.033*	0.11
A vs F	<0.001*	<0.001*	<0.001*
B vs C	<0.001*	0.22	1.00
B vs D	<0.001*	0.15	1.00
B vs E	<0.001*	1.00	0.003*
B vs F	<0.001*	<0.001*	<0.001*
C vs D	1.00	1.00	1.00
C vs E	<0.001*	0.15	0.003*
C vs F	<0.001*	< 0.001*	<0.001*
D vs E	<0.001*	0.103	0.001*
D vs F	<0.001*	<0.001*	<0.001*
E vs F	<0.001*	<0.001*	<0.001*

The multiple comparison test (Bonferroni) was done using change in absorbance per minute for the extracts AEV, EEV, and AEM (AEV, aqueous extract of the leaf of *V. amygdalina*; EEV, ethanol extract of the leaf of *V. amygdalina*; AEM, aqueous extract of the seed of *M. fragrans)* at different concentrations: 100mg/ml (A), 80mg/ml (B), 60mg/ml(C), 40mg/ml (D), 20mg/ml (E), and blank (F). *Significant p-values.
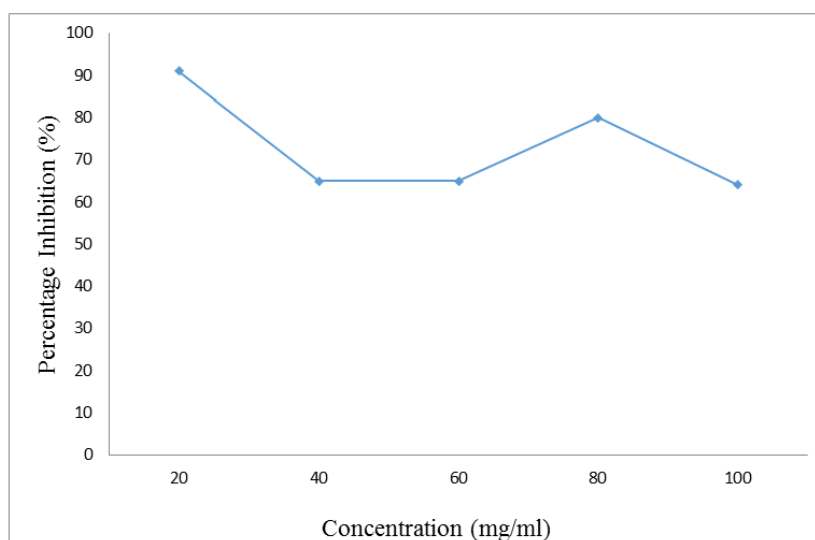

Fig-1: Graph showing percentage inhibition versus concentration of aqueous leaf extract of *V. amygdalina*
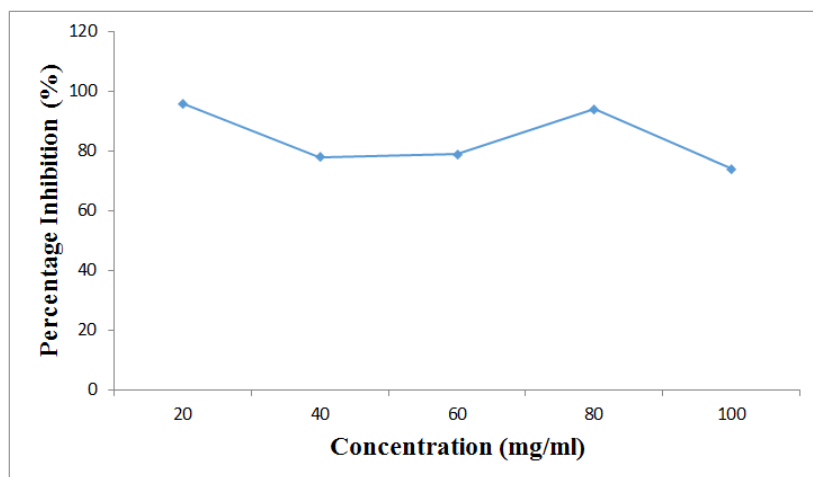
Fig-2: Graph showing percentage inhibition versus concentration of ethanolic extract of leaves of *V. amygdalina*
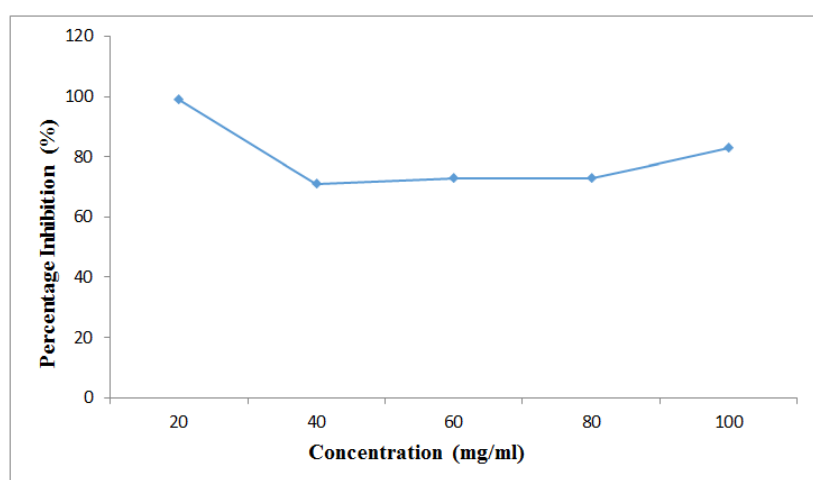

Fig-3: Graph showing percentage inhibition versus concentration of aqueous extract of seeds of *M. fragrans*
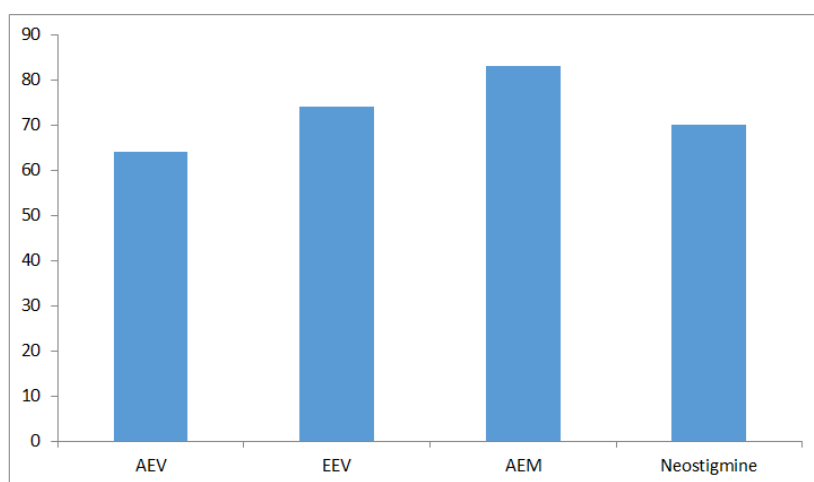

Fig-4: Bar chart showing percentage inhibition of the extract at 100 mg/ml and neostigmine (AEV, Aqueous extract of leaves of *V. amygdalina;* EEV, Ethanolic extract of leaves of *V. amygdalina;* AEM, Aqueous extract of seeds of *M. fragrans)*

The two medicinal plants used in this study demonstrated significant cholinesterase inhibitory activities and the anticholinesterase activities were comparable to that of positive control (neostigmine). However, it must be pointed out that the 70% AChE inhibition of neostigmine may not be optimal. This may be attributed to the concentration of neostigmine (2.5 mg/ml) used in this study which may be suboptimal.

It is also pertinent to observe that the acetylcholinesterase inhibition by the extracts in this study was not dose-dependent. The lowest (20 mg/ml) and the highest (100 mg/ml) doses study produced highest inhibition of AChE compared to other doses used in this. It is possible that there are thresholds for significant inhibitory activity. What could be responsible for the thresholds could be the subject of another study.

Extracts of *V. amygdalina* and nutmeg have been shown to have antioxidant activities [15-17]. The phytochemical screening done on these two medicinal plants also revealed the presence of flavonoids, phenolics, and glycosides. These phytochemical constituents of *V. amygdalina* were in agreemen with the findings of Igile *et al.* and those of Udensi *et al.,* [18, 19]. Likewise the phytochemical constituents of *M. fragrans* agreed with the findings of previous studies [20, 21].

Ethanol extract of leaf of *V. amygdalina* had more constituents than aqueous extract. Thus ethanol extracted more constituents than water. This could be due to the fact that ethanol is both organic and polar solvent. The higher activity of ethanol leaf extract of *V. amygdalina* than the aqueous extract shows that the active components of the plants dissolved more in ethanol than water. Similar conclusions were drawn by different researchers [22].

The cholinesterase inhibitiory activities of *V.amygdalina* and *M. fragrans* may be attributed to some of these phytochemical constituents. In addition to cholinesterase inhibitory action, these constituents could be beneficial in management of AD. For example, some of them are anti-oxidants. Indeed, extracts of leaf of *V. amygdalina* and seed of *M. fragrans* have been shown to have antioxidant activities [15-17].

Oxidative stress is an important process in the pathophysiology of AD and indeed, most neurodegenerative disorders. The brain has high energy needs which are almost entirely met by mitochondrial oxidative phosphorylation, generating adenosine triphosphate (ATP) at the same time as reducing molecular 0_2 to H_2O. Oxidative stress is the result of excessive production of highly reactive oxygen species (ROS).Oxidative stress is both a cause and consequence of inflammation which is a general feature of neurodegenerative disease and is thought to contribute to neuronal damage [1].

Phytochemical analysis done for the extracts of *V. amygalina* and *M. fragrans* showed that both medicinal plants contain significant amounts of flavonoids which are known to be good antioxidants. Luteolin (a flavonoid found in *V. amygdalina*) has been reported to be a strong antioxidant [23]. In fact, Igile *et al.,* confirmed that luteolin is more potent an antioxidant than the synthetic antioxidant butylated hydroxytoluene (BHT) [18]. Thus the antioxidant activities of these two medicinal plants through their flavonoid content may help reduce oxidative stress that is implicated in neurodegenerative disorder as exemplified by AD. Thus the antioxidant activities of these two medicinal plants through their flavonoid content may help in AD.

CONCLUSION

This present study has demonstrated that both extracts of *V. amygdalina* and *M. fragrans* showed significant acetylcholinesterase inhibitory activities in vitro comparable to that of neostigmine. The significance tests produced p values ≤ 0.05, therefore, the null hypothesis was rejected, implying that aqueous and ethanol extracts of the leaf of *V. amygdalina* and seed of *M. fragrans* inhibit acetylcholinesterase activity in vitro.

By extrapolation, this implies that if administered to man, the extracts may help suppress breakdown of acetylcholine by acetylcholinesterase. This may lead to build up of acetylcholine in the brain and subsequently enhance cholinergic transmission thereby overcoming one of the major biochemical deficits in Alzheimer's disease.

It is recommended that further studies on these extracts and acetylcholinesterase inhibition will involve the use of animal models.

REFERENCES

1. Rang, H. P., Ritter, J. M., Flower, R. J., & Henderson, G. (2016). Rang and Dale's Pharmacology, 8th ed. Edinburgh: Churchill Livingstone.
2. Jazayeri, S. B., Amanlou, A., Ghanadian, N., Pasalar, P., & Amanlou, M. (2014). A preliminary investigation of anticholinesterase activity of some Iranian medicinal plants commonly used in traditional medicine. *DARU Journal of Pharmaceutical Sciences*, *22*(1), 17.
3. Hendrie, H., Osuntokun, B. O., Hall, K., Ogunniyi, A. O., Hui, S., Unverzagt, F., & Burdine, V. (1995). Prevalence of Alzheimer's disease and dementia in two communities: Nigerian Africans and African Americans. *American Journal of Psychiatry*, *152*(10), 1485-1492.
4. Schelterns, P., & Feldman, H. (2003). Treatment of Alzheimer's disease; current status and new perspectives. *The Lancet Neurology*, *2*(9), 539-547.
5. Lekoubou, A., Echouffo-Tcheugui, J. B., & Kengne, A. P. (2014). Epidemiology of neurodegenerative diseases in sub-Saharan Africa: a systematic review. *BMC Public Health*, *14*(1), 653.

6. Odubanjo, V. O., Oboh, G., & Ibukun, E. O. (2013). Antioxidant and anticholinesterase activities of aqueous extract of Uraria picta (Jacq.) DC. *African Journal of Pharmacy and Pharmacology*, *7*(41), 2768-2773.

7. Nwauzoma, A. B., & Dappa, M. S. (2013). Ethnobotanical Studies of Port Harcourt Metropolis, Nigeria. *ISRN Botany*, *2013*.

8. Erasto, P., Grierson, D. S., & Afolayan, A. J. (2006). Bioactive sesquiterpene lactones from the leaves of Vernonia amygdalina. *Journal of ethnopharmacology*, *106*(1), 117-120.

9. Njan, A. A., Adzu, B., Agaba, A. G., Byarugaba, D., Díaz-Llera, S., & Bangsberg, D. R. (2008). The analgesic and antiplasmodial activities and toxicology of Vernonia amygdalina. *Journal of medicinal food*, *11*(3), 574-581.

10. Moundipa, P. F., Flore, K. G. M., Bilong, C. F. B., & Bruchhau, I. (2005). In vitro amoebicidal activity of some medicinal plants of the Bamun region (Cameroon).

11. Giday, M., Asfaw, Z., Elmqvist, T., & Woldu, Z. (2003). An ethnobotanical study of medicinal plants used by the Zay people in Ethiopia. *Journal of ethnopharmacology*, *85*(1), 43-52.

12. Nwobu, R. A. U., Uzochukwu, I. C., & Okoye, E. L. (2010). Phytochemical analysis and antimicrobial activity of Hyptis suaveolens. *Medicinal plants: phytochemistry, pharmacology and therapeutics, Volume 1*, 390-396.

13. Ellman, G. L., Courtney, K. D., Andres Jr, V., & Featherstone, R. M. (1961). A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochemical pharmacology*, *7*(2), 88-95.

14. Taiwo, O. E., Efere, M. O., Joseph, M. A., & Saburi, A. A. (2013). Acetyl and buteryl cholinesterase inhibitory effect of Peltophorum pterocarpum (DC) Backer ex K. Heyne (family Leguminosae). *Journal of Pharmacognosy and Phytotherapy*, *5*(5), 77-82.

15. Ijeh, I. I., & Ejike, C. E. (2011). Current perspectives on the medicinal potentials of Vernonia amygdalina Del. *Journal of Medicinal Plants Research*, *5*(7), 1051-1061.

16. Yadav, A. S., & Bhatnagar, D. (2007). Modulatory effect of spice extracts on iron-induced lipid peroxidation in rat liver. *Biofactors*, *29*(2, 3), 147-157.

17. Sharma, M., & Kumar, M. (2007). Radioprotection of Swiss albino mice by Myristica fragrans houtt. *Journal of radiation research*, *48*(2), 135-141.

18. Igile, G. O., Oleszek, W., Jurzysta, M., Burda, S., Fafunso, M., & Fasanmade, A. A. (1994). Flavonoids from Vernonia amygdalina and their antioxidant activities. *Journal of Agricultural and Food Chemistry*, *42*(11), 2445-2448.

19. Udensi, E. A., Ijeh, I. I., & Ogbonna, U. (2002). Effect of traditional processing on the phytochemical and nutrient composition of some local Nigerian leafy vegetables. *Journal of Science and Technology*, *8*, 37-40.

20. Adewole, E., Ajiboye, B. O., Idris, O. O., Ojo, O. A., Onikan, A., Ogunmodede, O. T., & Adewumi, D. F. (2013). Phytochemical, antimicrobial and Gc-Ms of African Nutmeg. *International Journal of Pharmaceutical Science Invention,* 2(51): 25-32.

21. Gayathri, R., & Anuradha, V. (2015). Phytochemical screening and total phenolic content of aqueous and acetone extracts of seed, butter, mace of nutmeg (Myristica Fragrans Houtt). *Int J Pharm Sci Rev Res*, *33*, 236-9.

22. Iheukwumere, I., Uba, B. O., & Ubajekwu, C. C. (2012). Antibacterial activity of *Annona muricate* and *Persea americana* leaves extracts against ampicillin-resistant *Staphylococcus aureus. Journal of Science, Engineering and Technology,* 19**:** 10786-10798.

23. Torel, J., Cillard, J., & Cillard, P. (1986). Antioxidant activity of flavonoids and reactivity with peroxy radical. *Phytochemistry*, *25*(2), 383-385.