

Original Article

Cholinesterase inhibitory activity and structure elucidation of a new phytol derivative and a new cinnamic acid ester from *Pycnanthus angolensis*



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ABSTRACT

The leaves of *Pycnanthus angolensis* (Welw.) Warb., Myristicaceae, are used as memory enhancer and anti-ageing in Nigerian ethnomedicine. This study aimed at evaluating the cholinesterase inhibitory property as well as isolates the bioactive compounds from the plant. The acetylcholinesterase and butyrylcholinesterase inhibitory potentials of extracts, fractions, and isolated compounds were evaluated by colorimetric and TLC bioautographic assay techniques. The extract inhibited both enzymes with activity increasing with purification, ethyl acetate fraction being most active fraction at $65.66 \pm 1.06\%$ and $49.38 \pm 1.66\%$ against acetylcholinesterase and butyrylcholinesterase, respectively while the supernatant had $77.44 \pm 1.18\%$ inhibition against acetylcholinesterase. Two new bioactive compounds, (2*E*, 18*E*)-3,7,11,15,18-pentamethylhenicosa-2,18-dien-1-ol (named eluptol) and [12-(4-hydroxy-3-methyl-oxocyclopenta-1,3-dien-1yl)-11-methyl-dodecyl](*E*)-3-(3,4-dimethylphenyl)prop-2-enoate (named omifoate A) were isolated from the plant with IC_{50} of 22.26 $\mu\text{g/ml}$ (AChE), 34.61 $\mu\text{g/ml}$ (BuChE) and 6.51 $\mu\text{g/ml}$ (AChE), 9.07 $\mu\text{g/ml}$ (BuChE) respectively. The results showed that the plant has cholinesterase inhibitory activity which might be responsible for its memory enhancing action, thus justifying its inclusion in traditional memory enhancing preparations

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Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder affecting several people and is yet incurable (Prinz et al., 2013). This makes an urgent need for the development of highly effective medications and therapies very imperative, even though the multifactorial nature of the disease, involving several unbalanced network of receptors and enzymes has made both diagnosis and treatment very difficult (Ballard et al., 2011).

Nevertheless, current management strategies for AD are based on *N*-methyl-D-aspartate receptor (NMDA) antagonist memantine and acetylcholinesterase inhibitors (AChEI) such as donepezil, rivastigmine and galanthamine (Prinz et al., 2013). Although memantine can slow down the rate of neurodegeneration in AD, it does not provide a cure for the disease (Massoud and Gauthier, 2010). Cholinesterase inhibitors on the other hand improve

cholinergic activity in the brain of AD patient and still remain good treatment option.

It is a known fact that the use of alternative medicine is on the increase all over the world with the most increase involving the use of herbal medicine, folk medicine, homoeopathy and massage (Eisenberg et al., 1998; Ernst, 2000). There may be several reasons for this increase but three basic theories: patient dissatisfaction with conventional treatment as a result of ineffectiveness, adverse effects and cost, patients need for more personal control over their health and better compatibility with patients' values, spiritual/religious belief and world view, have been proposed to provide some explanations (Astin, 1998). It has also been suggested that with time, this continuing demand for alternative therapies will have great effect on health care delivery (Kessler et al., 2001). Little wonder why great research efforts are being concentrated in this area with particular emphasis on herbal medicine and medicinal plants.

Medicinal plants have also been good sources of clinical drugs in general for many years (Li and Vederas, 2009; Silverman and Holladay, 2014). Many drugs in clinical practice today are either

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directly from medicinal plants or have their basic template from compounds derived from plants (Lazarus, 2008) and plants have also contributed significantly in providing drugs for the treatment of CNS disorders. These include tropane alkaloids from *Erythroxylum coca*, opium alkaloids from *Papava somniferum*, and the cholinesterase inhibitor physostigmine from *Physostigma venenosum* (Burger, 2003) as well as galantamine from *Narcissus* species (Berkov et al., 2009). Several other cholinesterase inhibitors in particular have been isolated from medicinal plants (Mukherjee et al., 2007; Ahmed et al., 2013).

Pycnanthus angolensis (Welw.) Warb., Myristicaceae, commonly called African nutmeg, is an evergreen tree about 25–35 m high and 60–100 cm in diameter (Orwa et al., 2009). The use of different parts of the plant in folklore is well documented (Achel et al., 2012). The leaf juice has been used for oral thrush in children (Abbiw, 1990) while a decoction of the leaves has been found useful in the ulcer, wound healing, and haemorrhoids (Agyare et al., 2009). The stem has also been reported useful in jaundice, coated tongue and tuberculosis (Fort et al., 2000; Tsaassi et al., 2010; Ashidi et al., 2010). Several bioactive compounds, some of which are potential drug leads have been isolated from the plant. The cytotoxic effect of flavonoids isolated from the plant has been reported (Mansoor et al., 2011). Analgesic and anti-inflammatory fatty acids have also been reported in the plant (Brill et al., 2004). Other reported activities include antioxidant (Oladimeji and Akpan, 2015), anti-helminthic (Onocha and Otunla, 2010), antimalarial (Ancolio et al., 2002) and cholesterol lowering (Leonard, 2004), antinociceptive and antiulcer (Sofidiya and Awolesi, 2015).

We have also previously reported the cholinesterase, both acetyl and butyryl, inhibitory activity of crude extracts of this plant (Elufioye et al., 2010). In this study, we isolated and characterized the cholinesterase inhibitory constituent from the plant.

Materials and methods

Chemicals

The chemicals used include electric eel acetylcholinesterase (EC 3.1.1.7, type VI-s) and Horse butyrylcholinesterase (EC 3.1.1.8) which were products of Fluka Co., Germany. Acetylcholine iodide (ATChI), butyrylcholine chloride (BuChCl), 5,5-dithio-bis-nitrobenzene acid (DTNB), and physostigmine (eserine) salicylate were from Sigma Co., UK. Reagents for buffer include disodium hydrogen orthophosphate dihydrate ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$) and sodium dihydrogen phosphate ($\text{NaH}_2\text{PO}_4 \cdot 12\text{H}_2\text{O}$), both of which were of analytical grade. Also used were silica gel for column (ASTM), and pre-coated TLC plates, G₆₀PF₂₅₄ (Merck).

Plant material collection and authentication

The plant *Pycnanthus angolensis* (Welw.) Warb., Myristicaceae, was identified by Mr. Oladele of the Department of Pharmacognosy, Faculty of Pharmacy, and authenticated by Dr. H. Illoh of the Botany Department, Obafemi Awolowo University, Ile-Ife with herbarium number IFE 13039. The leaves were collected from Road 7, O.A.U Campus in August 2005.

Preparation of extract and fractions

The powdered leaves were macerated with 80% methanol for 72 h and extract was concentrated *in vacuo* to dryness at 40 °C. The methanolic extract was partitioned into hexane, ethylacetate and water. Both the extract and the various fractions were screened for their AChE and BuChE inhibitory activity.

Ethyl acetate extraction and precipitation studies

Powdered leaves of *P. angolensis* were bulk extracted with 100% ethylacetate and the extract concentrated. Lipid constituent were precipitated out by gradual addition of methanol. The precipitates were filtered and weighed. Both supernatants and precipitates were then tested for cholinesterase inhibitory activity.

Phytochemical and cholinesterase analysis

The TLC of both the precipitates and the supernatant were carried out using chloroform:hexane (7:3) as solvent system. Some developed plates were sprayed with different phytochemical screening reagents like vanillin/sulphuric acid, antimony trichloride, Dragendorff's reagent and anisaldehyde spray reagents. The other developed plates were subjected to TLC bioautographic enzyme assay.

Cholinesterase inhibitory assay

The cholinesterase (both AChE and BuChE) inhibitory activities of the crude extract, fractions, precipitate, supernatant and isolated compounds were carried out using a 96 well micro-plate reader according to the modified method of Ellman (Ellman et al., 1961; Houghton et al., 2004; Elufioye et al., 2013).

The reaction mixture contained 2000 ml 100 mM phosphate buffer at pH 8.0, 100 ml of test sample stock solution in methanol at a final concentration of 42.5 µg/ml, 100 ml of the enzyme, either acetylcholinesterase (AChE) or butyrylcholinesterase (BuChE) at a final concentration of 0.003 µ/ml and 0.001 µ/ml respectively. 100 µl of di-thio-nitrobenzoate (DTNB) (0.3 mM) dissolved in 100 M phosphate buffer pH 7.0 containing 120 mM sodium bicarbonate. The assay mixture was pre-incubated on water bath at 37 °C for 30 min after proper mixing. The reaction was started by adding of 100 µl of acetyl thiocholine iodide (ATChI) or butyrylthiocholine chloride (BTChCl) at a final concentration of 0.5 mM. Methanol and eserine ((-)-physostigmine) were used as negative and positive controls respectively. Change in absorbance at λ_{max} 412 was measured every 30 s over a period of 5 min at ambient temperature. All assays were carried out in triplicate and the percentage inhibition calculated as:

$$\text{Percentage inhibition} = \frac{a - b}{a} \times 100$$

where $a = \Delta A/\text{min}$ of control; $b = \Delta A/\text{min}$ of test sample; $\Delta A =$ change in absorbance.

Active spots were also monitored by TLC bio-autographic assay method according to Rhee et al. (2001). The samples were spotted on pre-coated (G60 PF 254) TLC aluminium plate and developed in appropriate solvent system. The developed plates were then air dried and sprayed with 2.55×10^{-3} units/ml of the cholinesterase enzyme until saturated. The plates were then incubated at 37 °C for at least 20 min before spraying with 0.5 mM of the substrate (ATChI or BTChCl) and then DTNB. White spots on a yellow background indicate positive result.

Isolation of bioactive components

The supernatant (120.36 g) was subjected to Vacuum Liquid Chromatography (VLC) on silica gel using hexane, dichloromethane and methanol mixtures as the solvent system. A total of 53 fractions were collected and bulked into six based on their TLC profile. The bulked fractions were subjected to TLC autobiographic assay and fractions showing activity were further purified by repeated VLC and PTLC leading to the isolation of the compounds.

Spectroscopic analysis

Spectroscopic analysis, both 1D and 2D NMR were carried out. Structure elucidation was done based on ^1H and ^{13}C NMR, COSY, HMQC, and HMBC spectra data.

Results and discussion

The precipitate and supernatant were spotted on pre-coated silica gel plates and subjected to preliminary phytochemical screening by spraying with vanillin/ H_2SO_4 , Dragendoff reagent, antimony trichloride, and anisaldehyde spray reagent using the same solvent system (hexane:chloroform (3:7)). The TLC plate after spraying with vanillin/ H_2SO_4 showed that supernatant gave better colour reaction to the spraying reagent. The spots gave different colours to the reagent and this could be indicative of the nature of the constituents in the plants. Concentrated sulphuric acid is used in the general detection of organic compounds (Harborne, 1973). It is also useful in the detection of steroids, terpenes, lipids and essential oils (Pothier, 2000). Positive detection is indicated by a number of colours, blue for linalol, red or violet for thymol, yellow–brown for eugenol, etc. (Pothier, 2000). The above plate which showed different colours with vanillin/ H_2SO_4 indicates the presence of organic compounds such as terpenes, steroids or essential oils.

The TLC plate sprayed with Dragendoff reagent indicated the presence of alkaloid in the precipitates of *P. angolensis*, and *C. jagus* with positive reaction is indicated for alkaloids by orange–brown zones against a yellow back ground (Pothier, 2000). The presence of alkaloid may be true because alkaloids have been found to have AChE inhibitory activity (Houghton et al., 2004). Both eserine from *Physostigma venenosum* and galanthamine from *Crinum* are alkaloids which have been implicated as AChE inhibitors.

Antimony trichloride is used in the detection of cardiac glycosides and saponins (Pothier, 2000). Positive result is usually indicated by coloured zones for terpenoids and flavonoids. The plates showed some coloured (yellow) zones which could be terpenoids or flavonoids in the precipitate and supernatant of *P. angolensis*.

Spraying with anisaldehyde was used for the detection of terpenoids (usually purple, blue or red) and some other compounds e.g. lignands, sugar and flavonoids (Pothier, 2000). The plate showed the presence of terpenoidal compounds in both supernatant and precipitate.

Several compounds belonging to various classes have been previously reported in *P. angolensis*. These include flavonoids (Mansoor et al., 2011), fatty acids (Brill et al., 2004), terpenoid quinones (Wabo et al., 2007), lignands (Abrantes et al., 2008; Eric et al., 2010) and steroids (Connolly, 2006) thus supporting the validity of the phytochemical screening.

Comparing the phytochemical analyses using the various spray reagents and the TLC AChE and BuChE inhibitory activities, it could be observed that supernatants gave more active spots. These spots could be steroids, terpenoids, or terpenes.

Both the quantitative and qualitative cholinesterase inhibitory assays of the precipitate and supernatant showed that the activity was higher in the supernatant when compared with the precipitate (Table 1). It was also observed from the plate that BuChE appeared not to show as many active zones as AChE. Most of these compounds are likely to be terpenoidal in nature because of their purple colour in vanillin/ H_2SO_4 . Thus, the supernatant was subjected to further phytochemical analysis.

TLC bioautographic AChE assay of fractions from the VLC as well as parallel spraying with vanillin/ H_2SO_4 revealed the active spots. The active sub-fractions were subjected to repeated VLC separately followed by PTLC to isolate compounds **1** and **2**.

Table 1

Anticholinesterase activity of extract and fractions of *Pycnanthus angolensis*.

<i>Pycnanthus angolensis</i>	% Inhibition (AChE)	% Inhibition (BuChE)
Methanol extract	43.96 ± 3.04	43.59 ± 1.77
Hexane fraction	23.94 ± 2.24	11.49 ± 1.97
Ethyl acetate fraction	65.66 ± 1.06	49.38 ± 1.66
Aqueous fraction	48.80 ± 1.15	42.17 ± 1.44
Precipitate	72.60 ± 3.34	ND
Supernatant	77.44 ± 1.18	ND
Eserin	92.63 ± 3.66	89.30 ± 2.11

ND, not determined.

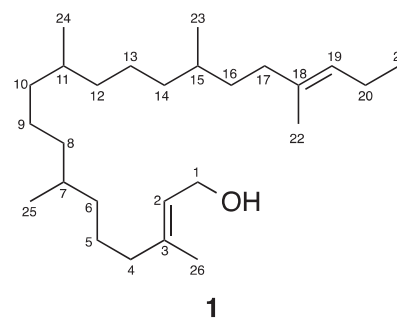
Spectroscopic analysis of the isolated compounds was done to confirm their structures.

Compound **1** was observed as oil with brownish yellow colour. The ^1H NMR spectrum, (CDCl_3 , 300 Hz) showed signals at δ 6.4(s), δ 5.4 (t), δ 4.1(d), δ 2.0(d), δ 1.4(m), δ 0.85(m), δ 0.87(m), δ 0.9(m), δ 1.70(s) and δ 1.60(s). The ^{13}C NMR (CDCl_3 , 300 Hz) data are: δ 59.63 (C-1), 123.30 (C-2), 140.50 (C-3), 40.08 (C-4), 26.93 (C-5), 37.51 (C-6), 33.90 (C-7), 37.64 (C-8), 25.35 (C-9), 39.95 (C-10), 33.01 (C-11), 39.58 (C-12), 25.00 (C-13), 37.50 (C-14), 28.19 (C-15), 29.91 (C-16), 36.88 (C-17), 135.50 (C-18), 123.48 (C-19), 24.68 (C-20), 16.23 (C-21), 16.38 (C-22), 19.96 (C-23), 22.83 (C-24), 22.92 (C-25), 19.93 (C-26).

The signal at 5.4(t) represents the olefinic protons assigned to the protons on C-2 and C-19. The signal at δ 4.1(d) represents an alcohol proton and is assigned to the proton residing on C-1. The multiplet signals at δ 1.40 to δ 1.35 represent the methylene protons on C-7, C-11 and C-15. The multiplets at δ 1.30 to δ 1.00 were assigned to protons on C-6, C-8, C-9, C-10, C-12, C-13, C-14, C-16, and C-17. The signal at δ 1.60(s) was assigned to the methyl protons on C-22 and C-26 while the signal at δ 1.70 was assigned to the OH group. The remaining signals at δ 0.85(m), δ 0.87(m) and δ 0.9(m) were assigned to the methyl protons on C-21, C-23, C-24 and C-25.

The ^{13}C spectrum showed that there were 6 CH_3 , 13 CH_2 , 5CH and 2C. Thus, compound **1** appears to be a C-26 carbon compound. Diagnostic are the oxygenated terminal methylene carbon resonating at δ 59.39 (C-1), the methine carbons resonating at δ 123.39 and δ 123.48 (C-2 and C-19), respectively, and the quaternary carbons C-3 and C-19 resonating at δ 140.50 and δ 135.50 respectively. The tertiary methyl groups (C-22 and C-26) on C-3 and C-18 resonated at δ 16.38 and δ 19.93, respectively; the secondary methyl groups (C-23, C-24 and C-25) resonated at δ 19.96, δ 22.83 and δ 22.92 while the terminal methyl group (C-21) resonated at δ 16.23.

On analyzing the spectra of compound **1**, it appears to be an extension of phytol by additional double bond and methyl groups. While phytol is C-20 (Arigoni et al., 1999), compound **1** is a C-26 compound with additional CH_3 at C-22, CH_2 at C-16, C-17 and C-20, CH at C-19 and Cq at C-18. This compound (2*E*, 18*E*)-3,7,11,15,18-pentamethylhenicosa-2,18-dien-1-ol, named eluoptol, appears new and it is also being reported for cholinesterase inhibitory activity for the first time.

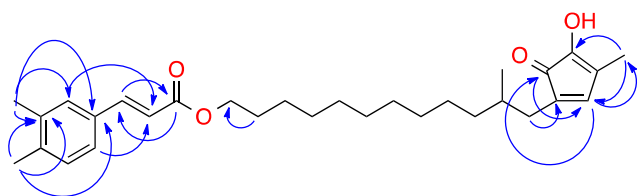


Spectra data for compound **2** are as follows:

^1H NMR (CDCl_3 , 300 Hz): δ 6.6(s), δ 6.4(s), δ 6.0(t), δ 5.1(m), δ 4.2(t), δ 3.1(d), δ 2.6(dd), δ 2.2(m), δ 2.0(m), δ 1.6(m), and δ 1.2(m). ^{13}C NMR (CDCl_3 , 300 Hz) δ 132.30 (C-1), 124.73 (C-2), 132.42 (C-3), 134.77 (C-4), 139.94 (C-5), 123.74 (C-6), 145.69 (C-7), 118.32 (C-8), 173.52 (C-9), 68.33 (C-10), 28.11 (C-11), 26.56 (C-12), 28.38 (C-13), 29.29 (C-14), 34.76 (C-15), 29.91 (C-16), 29.58 (C-17), 27.76 (C-18), 39.26 (C-19), 25.85 (C-20), 39.79 (C-21), 146.10 (C-22), 133.33 (C-23), 130.91 (C-24), 148.68 (C-25), 188.18 (C-26), 16.11 (C-27), 17.88 (C-28), 16.15 (C-29), 16.28 (C-30).

The ^{13}C spectrum of compound **2** showed that there were 4CH_3 , 11CH_2 , 7CH and 8C . Thus, compound **2** appears to be a C-30 compound. Particular are the carbonyl carbons C-9 and C-26 which resonated at δ_c 173.52 and δ_c 188.18, respectively. Also diagnostic is the oxygenated methylene carbon at C-10 which acts as a bridge between the two aromatic ring systems and resonated at δ_c 68.33. Diagnostic also is the methine carbons C-7 and C-8 resonating at δ_c 145.69 and δ_c 118.32, respectively which are in HMBC correlating with the carbonyl at C-9. The quaternary carbons C-25 (δ_c 148.68) was differentiated from that at C-24 (δ_c 130.91) due to the hydroxyl group on C-25 which made it absorb at a higher value. The tertiary methyl groups C-27, C-29 and C-30 resonated at δ_c 16.11, δ_c 16.15 and δ_c 16.28 while the secondary methyl carbon C-28 resonated at δ_c 17.88.

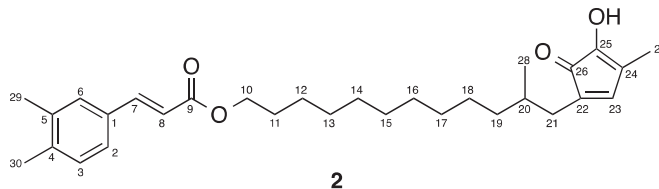
The proton NMR signal at δ 6.6 (s) represents the methylene proton residing on C-23 resonating at δ 133.33 in the HMQC while that at δ 6.4 (s) resides on C-3 at δ 132.42. The triplet at δ 6.0 was shown to reside on the carbon at δ 145.69 which was assigned as C-7. The multiplet at δ 5.1 showed correlation with the carbons at δ 124.73 (C-2), δ 123.74 (C-6), and δ 118.32 (C-8) in the HMQC spectra. The signal at δ 4.2 (t), showed correlation with the diagnostic OCH_2 carbon at δ 68.33 and is assigned to C-10 while the signal at δ 3.1 (d) correlated with the carbon at δ 27.76 assigned to C-18. The multiplet at δ 2.2 to δ 2.0 correlated with carbon signals at δ 26.56, δ 34.76, δ 29.58, and δ 39.79 and were assigned to carbons C-12, C-15, C-17 and C-21 while the multiplet at δ 1.6 to δ 1.2 were assigned to the methyl groups at C-27, C-28, C-29 and C-30. In the HMBC, the diagnostic CH_2 at C-10 showed correlation with the CH_2 signal at δ 28.11 which was assigned to C-11. Also the CH_2 at δ 39.79 (C-21) is coupled to the quaternary carbon at δ 146.10 (C-22) while the carbonyl carbon at δ 188.18 (C-26) is coupled to the carbon resonating at δ 133.33 (C-23). The HMBC spectra also showed that the CH at δ 145.69 (C-7) coupled with the quaternary carbon at δ 173.52 (C-9).



HMBC correlations of compound **2**

When compared with literature (Renmin et al., 2004; Venkateswara et al., 2011), it was observed that compound **2** is a cinnamic acid derivative. There are, however, differences at C-4 and C-5 of the isolated compound and cinnamic acid because of the 4,5-dimethyl substitution on PA-3 which made these carbons absorb at slightly higher δ values (δ 134.77 and δ 139.94), respectively. Derivatives of cinnamic acid in the literature are mostly 2,3-dimethoxy or 2,3-dihydroxy unlike compound **2** which is a 2,3-dimethyl derivative. Also, the group attached to the cinnamic acid through the ester linkage has never been reported

as such in the literature. Thus compound **2**, [12-(4-hydroxy-3-methyl-oxo-cyclopenta-1,3-dien-1yl)-11-methyl-dodecyl] (*E*)-3-(3,4-dimethylphenyl)prop-2-enoate, named omifoate A appears to be new as well and it is being reported as cholinesterase inhibitor for the first time.



In conclusion, extracts of the leaves of *P. angolensis* inhibited cholinesterase enzymes and two new compounds with significant cholinesterase inhibitory activity were isolated from the supernatant of the most active ethyl acetate fraction.

Author's contribution

TOE performed the experiments, did the literature searches, and wrote the manuscript. EMO managed the experimental process and provided technical assistance. Author JMA contributed to the protocol and provided general supervision of the study. Author SAA designed the study and wrote the protocol. All authors have read and approved the submission of the manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bjp.2016.01.010.

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