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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 ± 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 μg/ml (AChE), 34.61 μg/ml (BuChE) and 6.51 μg/ml (AChE), 9.07 μg/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

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 acetylcholineaterase inhibitors (AChEI) such as donopesil, 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 *Erythroxylun 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 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 butyxylcholinesterase (EC 3.1.1.8) which were products of Fluka Co., Germany. Acetylcholine iodide (ATCHI), buthrylcholine chloride (BuCHCl), 5,5-dithio-bis-nitrobenzene acid (DTNB), and physiostigmine (eserine) salicylate were from Sigma Co., UK. Reagents for buffer include disodium hydrogen orthophosphate dihydrate ($Na_2HPO_4{\cdot}2H_2O$) and sodium dihydrogen phosphate ($NaH_2PO_4{\cdot}12H_2O$), both of which were of analytical grade. Also used were silica gel for column (ASTM), and pre-coated TLC plates, $G_{60}PF_{254}$ (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 form 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 eserin ((−) physiostigmine) 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 $a = \Delta A$/min of control; $b = \Delta A$/min of test sample; ΔA = 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, dicholoromethane 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.