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Isolation and Characterizations of Bergenin from *Peltophorum pterocarpum* Leaves and Its Cholinesterase Inhibitory Activities

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Authors' contributions

This work was done through collaboration between the authors. Author TOE performed the experiments, did the literature searches and wrote the first draft of this paper. Author EMO managed the experiments and provided technical assistance. Author JMA supervised the work and contributed to the protocol. Author SAA designed the study and wrote the protocol. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The study identified a cholinesterase inhibitory constituent in *P. pterocarpum*, a medicinal plant used in Nigerian ethno-medicine for the management of memory loss.
Study Design: Activity directed phytochemical investigation of *P. pterocarpum* using *in vitro* anti cholinesterase assay and spectroscopic analysis of isolated compound.
Methodology: Combined chromatographic (VLC and CC) and spectroscopic (NMR and MS) analyses revealed bergenin as the major constituent of the most active ethyl acetate fraction of *P. pterocarpum*.
Results: The extract, fractions and pure isolated bergenin tested for their AChE and BuChE

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inhibitory activities showed significant inhibition of both enzymes with bergenin having an IC_{50} of (13.17 µM) towards AChE and (14.60 µM) towards BuChE.
Conclusion: We conclude that *Peltophorum pterocarpum* may be good in traditional management of memory loss associated with AD and bergenin may be a good drug candidate for the treatment of AD after further investigations in AD models.

Keywords: Peltophorum pterocarpum; bergenin; butyryl cholinesterase; acetyl cholinesterase; Alzheimer's disease.

1. INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disease which causes dementia and affects several million people in both developed and developing countries of the world [1]. With more and more people reaching old age, the prevalence of AD is generally on the increase, with approximately 24 million sufferers in 2001 [2] and an estimated rise to 42.3 million by 2020 and 81.1 million by 2040 [1]. Despite these facts, AD remains incurable till date [3] largely due to its multifactorial nature [1]. Thus, further research into discovering useful medications is imperative.

Memory loss, an abnormal degree of forgetfulness and/or inability to recall past events, is qualitatively different in normal ageing and disease states such as AD. However, acetylcholine is very important for the normal functioning of memory, weather in normal ageing or in AD and the majority of research aimed at getting treatment for memory defects focus on acetylcholine [4].

Quite a number of cholinesterase inhibitors have been developed and they differ in their selectivity for AChE and BuChE as well as in their inhibition mechanism and reversibility. Although the use of cholinesterase inhibitors with dual ability to inhibit both AChE and BuChE has been associated with some side effects, it is still believed this duality could produce better clinical results especially in AD patients [5].

Current medications for AD such as donepezil, rivastigmine and galanthamine are based on inhibition of acetyl cholinesterase [3], thus attenuating cholinergic deficit associated with AD. Galanthamine is an alkaloid obtained from the bulbs and flowers of *Galanthus species* (Amaryllidaceae) and related genera like *Narcissus.* New naturally occurring inhibitors of both acetyl and butyryl cholinesterase are constantly being identified in many plant species around the world.

Medicinal plants are good sources of potential therapeutic agents [6] and the usefulness of plants, plant extracts as well as plant derived pure compounds to treat or manage ailments is a practice that has been for a long time [7]. Furthermore, memory related ailments have been managed with plant remedies for centuries [8] and cholinesterase inhibitory activity of plants used traditionally for managing memory loss have been reported by many researchers [9,10,11].

Peltophorum pterocarpum (DC) Backer ex K. Heyne (Leguminosae) is a plant found useful in Nigerian ethno-medicine as a memory enhancer. It is an upright, semi-evergreen, spreading handsome tree with rounded canopy [12]. The bark is used in fermenting palm wine in Indonesia [13]. It is also used to relieve sprain, bruises, swellings and pain at child birth as well as used as lotion for eye troubles [14].

The antifungal [15], anti-inflammatory and anti-bacterial [14], and the anti-microbial [16] activities of *Peltophorum pterocarpum* have been reported. The cholinesterase inhibitory activity [11] of the methanolic extract has also been reported. The ability of extract of *P. pterocarpum* to modulate cell surface hydrophobicity of enterohemorrhagic *E. coli* with high bacteriostatic and bacteriocidal activities [17] has also been shown. The plant also has significant anti-glycemic activity with the active constituent identified as quercetin-3-O-β-d-galactopyrano-side [18]. The aqueous extract of the plant has also been reported to exhibit potent inhibitory effect against *Epstein—Barr* virus early antigen (EBV-EA) activation induced with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in *Raji* cells and against melanogenesis in *α*-melanocyte-stimulating hormone (*α*-MSH)-stimulated B16 melanoma cells [19].

Several compounds have been isolated from this plant and these include (-) epicatechin and leucocyanidin from the bark [20], bergenin, norbergenin, 3-0-methyl-D-(+) Pinitol,

myomositol, hentriacontanol, kaempferol and quercetin from the flower [21]. Also reported in the plant are campesterol, Stigmasterol, β-Sitosterol, Lupeol and Lupenone [22]. GC-MS of ethyl acetate fraction revealed thirty one compounds with several associated activities [23]. An antifungal amidase designated as peltopterin was also purified from the seed [24]. A cinnamic acid derived bisamide alkaloid (E, E)-terrestribisamide was isolated from the flower and shown to possess anti-oxidant, anti-microbial and cytotoxic activities [25].

In the present study, the cholinesterase (AChE and BuChE) inhibitory activity of bergenin isolated from *P. pterocarpum* was studied.

2. MATERIALS AND METHODS

2.1 Plant Collection and Identification

The leaves of *Peltophorum pterocarpum* were collected on Obafemi Awolowo University campus and authenticated at the Department of Botany where voucher specimen with herbarium number (IFE 3171) was deposited. The leaves were oven dried at 40℃, powdered and stored in an air-tight container till use.

2.2 Extraction and Isolation of Bergenin from *Peltophorum pterocarpum*

The powdered sample (1 kg) was extracted with 80% methanol and concentrated *in vacuo*. The methanolic extract (44.34 g) was successively partitioned between n-hexane, ethyl acetate and water. Vacuum liquid chromatography (VLC) of the ethyl acetate fraction on silica gel with gradient elution from N-hexane through ethyl acetate to methanol yielded five sub-fractions. Fraction A was purified by column chromatography on silica gel 60 with gradient elution from n-hexane in ethyl acetate through 100% ethyl acetate to 100% methanol. This yielded one hundred and twelve (112) subfractions bulked into twenty five (25) (a-y) based on their TLC patterns. Bergenin (29.96 mg) was isolated after repeated crystallizations in methanol of sub-fractions d and e pulled together.

2.3 Cholinesterase Inhibitory Activity

Cholinesterase inhibitory activities, both AChE and BuChE of the extracts, fractions and isolated compound were determined using a 96 well micro-plate reader according to the modified method of Ellman [26,27,28]. The reaction mixture was made up of 2000mL 100mM phosphate buffer pH8.0, 100 mL of test sample stock solution in methanol (final concentration was 42.5 µg/ml), 100 mL of 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 mixed properly and pre-incubated on water bath at 37℃ for 30 minutes. The reaction was then started by the addition of 100 µL of acetyl thiocholine iodide (ATChI) or butyrylthiocholine chloride (BTChCl) at a final concentration of 0.5mM. Methanol was used ss a negative control while eserin ((-) physiostigmine) was used as positive control. Change in absorbance at λmax 412 was then measured every 30 sec for a period of 5 minutes at ambient temperature. All assays were done in triplicate and percentage inhibition calculated as:

$$\frac{a - b}{a} \times 100$$

Where

a = ΔA/min of control
b = ΔA/min of test sample
ΔA = change in absorbance

Active spots were also monitored by TLC bio-autographic assay method [29]. Various samples were spotted on pre-coated (G60 PF 254) TLC aluminum plate after which they were developed in appropriate solvent system. The developed plates were then air dried and sprayed first with 2.55x10-3 units/ml of the cholinesterase enzyme until saturated. The plates were then incubated at 37℃ for at least 20 minutes before spraying with 0.5 mM of the substrate (ATChI or BTChCl) and then DTNB.

2.4 Spectroscopy

Both 1D and 2D NMR spectroscopic analysis were carried out. The ^{1}H and ^{13}C NMR (in both methanol and acetone), COSY, NOESY, and HMBC were recorded using a 600 MHz instrument and results presented in Table 1.

3. RESULTS AND DISCUSSION

In our continuing effort to search for new naturally occurring inhibitors of cholinesterase

enzyme, the methanol extract and ethyl acetate fraction of *Peltophorum pterocarpum* showed significant inhibitory activity of both acetyl and butyryl cholinesterase [26]. Thus, an activity directed phytochemical investigation was carried out to isolate and characterize the cholinesterase inhibitory compound from the plant. The crude methanol extract was partitioned into n-hexane, ethyl acetate and water. The most active ethyl acetate fraction was subjected to VLC on silica gel and this produced five pulled sub-fractions based on their TLC patterns. Thin layer chromatography is used primarily for the rapid qualitative analysis of mixtures of organic compounds. As the mixture of the organic compounds moves over a solid phase, the different components are adsorbed to the solid phase to a different extent and are thus continuously being partitioned between the solid phase and the mobile phase resulting in their separation. These sub-fractions were tested for inhibition of both AChE and BuChE with sub-fraction A being the most active. Final purification of active sub-fraction A was achieved by column chromatography on silica gel with gradient elution from N-hexane in ethyl acetate through 100% ethyl acetate to methanol. Bergenin was isolated from this sub-fraction through repeated crystallizations.

Bergenin was isolated as a brown crystalline compound with molecular formula of $C_{14}H_{16}O_9.H_2O$ corresponding to molecular weight of 328 as detected by mass spectrum. It was identified on the basis of its NMR data which were compared with data already reported in literature [30,31] (Table 1).

The NMR data were recorded both in methanol and acetone (CD_3OD, CD_3COCD_3, 600MHz). Standard pulses were also measured on 2D. These included ^1H-^1H COSY, HSQC, HMBC and NOESY. All data were taken at room temperature. Diagnostic signals on the ^{13}C NMR include a quaternary carbon that has a carbonyl at δ165 indicating a carbonyl group which has HMBC correlation with the aromatic CH. The benzene ring has Cq signals at δ119 and δ117 showing no substitution at these points while the other carbons bear hydroxyl groupings. Also of importance is the methoxy signal at δ60.0 with the proton signal at 3.94 (s). The ^1H and ^{13}C NMR data (Table 1) were very similar to those of bergenin previously isolated [21,30].

The isolated compound (Fig. 1) was tested for both acetyl and butyryl cholinesterase inhibitory activities according to a modified Ellman method. Significant time dependent inhibition of both enzymes was observed. However, inhibition of AChE was better and more prolonged, still being up to 65% after 180 sec when compared with that of BuChE which reduced to about 52% over the same time period. The IC_{50} values, defined as the concentration at which 50% of the enzymes were inhibited were 13.17µM and 14.60 µM against AChE and BuChE respectively.

Table 1. NMR spectra data of isolated compound

Structure numbering		^{13}C	^1H	[30]
1				
2	C	165		164.6
3	C	119		118.2
4	CH	111	7.08 s	109.2
5	C	152		151.1
6	C	142		141.1
7	C	149		148.2
8	C	117		116.1
9	CH	74	4.95 d	73.1
10				
11	CH	83	3.70 m	81.9
12	CH	71	3.49 dd	70.7
13	CH	75	3.80 dd	74.4
14	CH	81	4.90 dd	80.2
15	OCH_3	60	3.94 s	59.8
16	CH_2	62	4.06 dd	61.5
			3.70 m	

Reference [30]

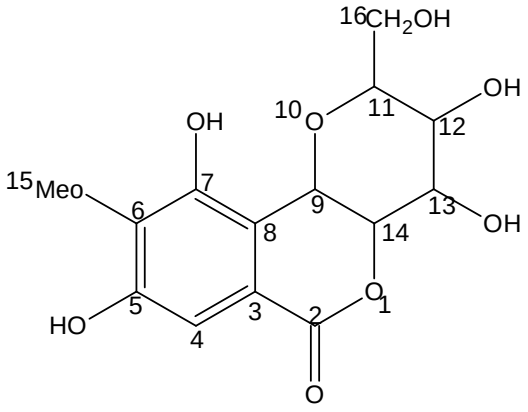

Fig. 1. Bergenin

Bergenin has previously been reported in a number of plant species such as *Peltophorum africanum* [32], *Peltophorum pterocarpum* flowers [21], *Endopleura uchi* [30], and *Bergenia* species [33]. This compound has also been reported to have several activities such as anti-inflammatory [30], anti- microbial [31], neuro-protective effects [34]. The neuro protective effects of bergenin make it of great value in the management of neurodegenerative diseases such as AD.

4. CONCLUSION

The isolation of bergenin from *Peltophorum pterocarpum* leaves as well as its usefulness as a cholinesterase inhibitor is being reported for the first time. The ability of this compound to significantly inhibit both acetyl and butyryl cholinesterase enzymes are highlighted in these findings.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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