Original Research Article

Novel cholinesterase inhibitory effect of α-spinasterol isolated from the leaves of *Acacia auriculiformis* A. CUNN Ex. Benth (Fabaceae)

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Abstract

Purpose: To investigate the in vitro anticholinesterase, α-glucosidase and antioxidant activities of α-spinasterol isolated from Acacia auriculiformis leaves.
Methods: The powdered leaves of Acacia auriculiformis were extracted with 70 % ethanol and the dried hydroalcoholic extract was suspended in water and partitioned with ethyl acetate and n-butanol to give their soluble fractions. The in vitro inhibitory activities of α-spinasterol were determined against cholinesterase and, α-glucosidase enzymes, and free radical scavenging potentials using (1,1-diphenyl-2-picrylhydarzyl (DPPH) and 2,2-azino-bis (3-Ethylbenzothiazoline-6-sulphonic acid (ABTS) antioxidant assays.
Results: The compound, α-spinasterol, exhibited moderate anticholinesterase activity (IC_{50} value of 44.19±2.59 µg/mL which was significantly different at ($p < 0.05$) when compared to the standard galanthamine (IC_{50} value of 1.73 ± 1.10 µg/mL). It also displayed a good α-glucosidase inhibitory activity with IC_{50} value of 8.65 ± 1.71µg/mL which was not significantly different when compared to the standard, acarbose with IC_{50} value of 2.79±0.81 µg/mL. This compound, however, exhibited weak free radical scavenging activities at 26.93 ± 0.00 and 35.16 ±0.26 % inhibition of DPPH+ and ABTS+ radicals as compared to ascorbic acid and Trolox (73.88 ± 0.04 and 99.82 ± 0.00%) respectively.
Conclusion: The results show that α-spinasterol isolated from Acacia auriculiformis exerts potent inhibitory effect against cholinesterase enzyme which might serve as a lead in the search for drugs against Alzheimer disease and diabetes mellitus.

Keywords: Acacia auriculiformis, α-Spinasterol, Galanthamine, Acarbose, Trolox, Ascorbic acid

INTRODUCTION

Terrestrial plants are still crucial sources of medicines particularly in the less developed nations of the world medicines especially in the developing parts of the world, where about 80%

of the population primarily derive their health needs. depends on natural products for their health needs [1, 2]. Secondary metabolites present in natural extracts purified from plants and microorganisms are known to possess multiple bioactivities and are considered inexpensive [3].This has necessitated intensive studies for the unfolding of novel chemical compound suitable for drug development through purification and chemical characterization of active constituent [4].

Acetylcholine (ACh) is the most abundant neurotransmitter responsible for cholinergic transmission which is hydrolysed in the synaptic cleft by the enzyme acetylcholinesterase. The enzyme acetylcholinesterase (AChE) is involved in the hydrolysis of Ach [5]. Alzheimers Disease (AD) is a neurodegenerative disorde a condition most prevalent amongst which affects the elderly population. It has been reported that the treatment of AD is focused generally on improving cognitive functions based on the cholinergic hypothesis [6]. Thus, AChE inhibitors are the most developed class of drug proposed for the treatment of AD [7].

Acacia belongs to the family Fabaceae, which consists of about 1,400 species. Most of the species of this family are known to contain phytoconstituents such as Flavonoids, gums and tannins [8]. They are mostly found in tropical countries such as Benin and Nigeria. *Acacia auriculiformis* commonly referred to as Black Wattle, is an important medicinal plant and a widely distributed member of the family Fabaceae. The decoction of the leaves is used by the Ibibio of Niger Delta region to treat malaria. Nigeria uses. The aboringes of Australia were known to use the infusion of the bark of this plant to treat inflammation. An infusion of the bark of this plant is used to treat inflammation among the aborigines of Australia [9].

The antimutagenic and chemoprotective effects of *Acacia auriculiformis* as well as the antioxidant activity of the ethyl acetate and acetone fractions have been reported [10,11]. A new triterpenoid trisaccharide and three new triterpenoids have been isolated from this plant [12,13]. The antimicrobial effect of acaciaside a and b two saponins isolated from this plant was reported by Mandal *et al* [14]. The larvicidal activity of the fruit extract of *Acacia auriculiformis* as well as the anticholinesterase inhibitory activity of the hydroalcoholic extract of the leaves of this plant have been reported [15,16].

In this present work, the effects of α-spinasterol isolated obtained by chromatographic separation of the ethyl acetate soluble fraction of the hydroalcoholic extract of the leaves of *Acacia auriculiformis against* acetyl cholinesterase and α-glucosidase enzymes in addition to the antioxidant properties using DPPH and ABTS was investigated is reported and antioxidant of the isolated compound.

EXPERIMENTAL

Equipment/reagents

Flash column chromatography was carried out on Silica Gel G (200-400 Mesh (40-63μm, Silicycle); analytical and preparative thin layer chromatography were performed on Pre-coated silica gel G TLC plates (Aluminium backed, 0.2mm), and silica gel G (glass backed, 0.5mm). Nuclear magnetic Resonance (NMR) spectra analysis was carried on Bruker Avance 400MHz spectrophotometer, while mass spectrometry was carried out on Shimadzu LCMS – 8040 Triple Quadrupole Mass Spectrophotometer using ESI (electrospray ionization) mode.

Potassium persulfate, 1,1-diphenyl-2-picryl-hydrazyl (DPPH), methanol, glucose, (Bovine serum albumin (BSA), sodium phosphate buffer, ascorbic acid, ethanol, Acarbose 2,20-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), para-nitrophenyl-alpha-glucopyranoside, were purchased from Sigma-Aldrich chemicals, USA,and S.D. Fine Chemicals Limited India.

Plant collection and extraction

The fresh leaves of *Acacia auriculiformis* were collected in July, 2018 from Basawa, Zaria in Kaduna State, Nigeria. A vouchered specimen (1292) was deposited after authentication by Umar S Gallah of Biology Department, Kaduna State University, Nigeria where a voucher specimen (no. 1292) was deposited in the herbarium. The air-dried pulverized leaves (408 g) was extracted to exhaustively at room temperature using 5L of 70% ethanol and removal of the solvent was achieved using rotatory evaporator. This process afforded a dark green mass referred to as hydroalcholic extract that weighed 36.8 g. A portion of this exttract 30 grams was suspended in 200 mL of water and filtered. The filtrate was partitioned exhaustively with a total of 1.5L each of ethyl acetate and n-butanol respectively. Subsequently, the removal of the solvents afforded 5.7 g and 11.7 g of their

respective soluble fractions- ethyl acetate and n-butanol soluble fractions.

Isolation of α-spinasterol

A portion of the ethyl acetate-soluble fraction (5.0 g) was packed in a column (50 x 3 cm) with silica gel and eluted gradiently with using n-hexane (100%) and –n-hexane: ethyl acetate mixtures to 5% methanol in ethyl acetate and finally 5 % methanol in ethyl acetate. Fifty mL aliquots were collected and the separation was checked on progress of elution was monitored on TLC using three solvent systems namely, n-hexane and ethyl acetate mixtures in the ratio 9:1, 5:1, 3:1 and ethyl acetate: chloroform: methanol: water (15:8:4:1). The fraction eluted with 20 % ethyl acetate in n-hexane revealed three spots. This was subjected to repeated preparative Thin Layer Chromatography LC which afforded the steroid α-spiansterol led to the isolation of α-spinasterol. The chemical structure of the isolated compound was deduced using a combination of spectroscopic techniques.

Anticholinesterase assay

The cholinesterase inhibition assay was determined using the modified Ellman's assay method [17] which is one of the standard methods used to determine the *in vitro* acetylcholinesterase (AChE) activity. Each 96-well plate consists of 5µL of the supernatant brain homegentate (acetylcholinesterase source), 5µL of samples of varying concentrations ranging from 5-100 µg/mL diluted in 30mM buffer solution, pH 7.0 and this was incubated over ice. sodium phosphate buffer, pH 7.0 and this was incubated over ice. Galanthamine was used as the standard anti-AChE. Another component of the reaction mixtures includes 95 µL of DTNB, which was added after the incubation. The mixture was thoroughly mixed with pipette and the reaction mixture was initiated by the addition of the substrate (95 µL of acetylcholine iodide) at a final concentration of 0.1mM. The kinetic profile of AChE activity was evaluated at 412 nm at 30s interval for 300s using a multimode reader (Perkin Elmer). The blank contains only the buffer with the substrate without the enzyme and inhibitor. The AChE activity of the compound was first calculated followed by the percentage inhibition using Eqs 1 and 2:

AChE activity (µM min^{-1}) = $\Delta A \times V_T \times$ 1000/E × V$_E$ ……… (1)

where ΔA = change in absorbance per minute; V_T = total volume of reaction mixture (0.2); E =

molar extinction coefficient at 412 nm (1.36 × 10^4); V$_E$ = volume of enzyme (0.05).

% Inhibition = {(A$_c$ – A$_S$)]/A$_C$}100 …....... (2)

Where A$_C$ = AChE activity of control; A$_S$ = AChE activity of sample

Anti-diabetic assay

This was carried out using α-glucosidase inhibition assay kit as described by [18]. The reaction mixture was made up of 20 µl of the compound (α-spinasterol), 100 µl of phosphate buffer solution (PBS) and 50 µl of α glucosidase (enzyme). This was incubated for 5 minutes at room temperature followed by the addition of the substrate (5 mM, p-nitrophenyl-α-glucopyrano-side, prepared in 100 mM phosphate buffer, pH 6.8). Readings were taken at 405 nm. The compound was replaced with the diluent (DMSO) and acarbose (reference standard) for the blank and control wells respectively. The whole mixture was incubated for 10 minutes and the inhibition of the α-glucosidase enzyme was at 405nm using UV-Visible spectrophotometer. measured spectrophotometrically at 405 nm (BioTek synergy, BioTek Instruments Inc, Winooski, VT, USA). Percentage inhibition of α-glucosidase of enzyme inhibition was obtained using equation 3:

% Inhibition = (Absorbance of control - Absorbance of compound)/ (Absorbance of control) X 100

H (%) = {(Abc – A$_{bs}$)}/Abc}100 …………(3)

where Abc= absorbance of control; A$_{bs}$ = absorbance of sample

All readings were taken in triplicates for each concentration and IC$_{50}$ of the compound was calculated applying a suitable regression analysis.

Determination of Antioxidant activity using 2, 2$^/$-diphenyl-1- picrylhydrazyl) free radical scavenging assay (DPPH)

The decolorisation of DPPH radical was determined by reported standard method of Tiwari et al [19]. The reaction mixture containing 25 µL of various dilutions of α spinasterol concentrations (ranging from 10.00 to 0.156 µg/mL),100 µL of Tris HCl buffer (0.1 M, pH 7.4) and 125 µL of 0.5 mM DPPH solution in methanol were thoroughly mixed and incubated in the dark for 15 minutes. The scavenging effect of DPPH was indicated by measuring the change

in colour from deep violet to yellow.The absorbance was recorded at 517 nm using a UV-visible spectrophotometer and ascorbic acid was used as the standard. Scavenging of DPPH radical by α-spinasterol was calculated as inhibition (H) as shown in Eq 4.

$$H\ (\%) = \{(Ab_c - Ab_s)] / Abc\} \times 100$$

Where Ab_c = absorbance of DPPH control with solvent; Ab_s = absorbance of sample

Ascorbic acid was used as standard and data obtained were expressed as mean ± SEM.

Determination of antioxidant activity using 2,2′-Azino-bis(3-Ethylbenzothiazoline-6-sulfonic acid) Assay (ABTS)

The ABTS radical scavenging activity was determined according to a modified method [19]. The stock solution of ABTS was prepared by the addition of 1mL of 6.89 mM of potassium persulphate in phosphate buffer solution, (PBS) pH 8.0. The mixture was stored in dark for 16h to produce the ABTS cation. The scavenging activity of ABTS$^+$ was obtained by the addition of 10µL of α-spinasterol to 190µL of ABTS$^+$ solution in a 96-well plate. Trolox was used as the standard and the absorbance was recorded at 734nm. The percentage scavenging radical of ABTS$^+$ by α-spinasterol was calculated by applying Eq 5.

$$H\ (\%) = [(Ab_c - Ab_s)/Ab_c\}100 \ .... \ (5)$$

where Ab_c = absorbance of control; Ab_s = absorbance of reaction mixture

All tests were carried out in triplicate and the data obtained expressed as mean ± SEM.

Trolox was used as standard and IC_{50} was calculated by regression analysis.

Statistical analysis

All tests were carried out in triplicates and values expressed as means ± SEM. The data obtained were subjected to one way analysis of variance (ANOVA). and the significant difference was determined by the use of Duncan's multiple test was used to calculate the significant difference (P < 0.05), using Graph pad prism, version 6.0.

RESULTS

Spectral characteristics

Compound I was isolated as an amorphous solid which weighed (8 mg).

ESI-MS, m/z: 413 (M+H)$^+$, ^{1}H-NMR (CDCl$_3$),δ (ppm), 5.15 (d,d,1H,23-CH), 5.17 (d,1H, 7-CH), 5.02 (d,d,1H,22-CH),3.56-3.64 (1H,m, 3-OH),1.04 (d,3H,21-CH$_3$),0.86 (d,3H,26 or 27-CH$_3$),0.84 (d,3H,26 or 27-CH$_3$),0.81 (t,3H,29-CH$_3$),0.80 (s,3H,19-CH$_3$) and 0.54 (s,3H,18-CH$_3$)
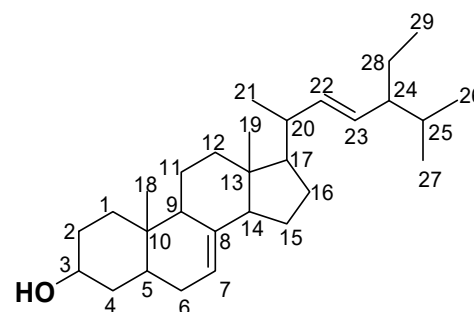

Figure 1: Structure of α-spinasterol compound I

Table 1: Cholinesterase and α-Glucosidase inhibition activities of α-Spinasterol

Compound	Cholinesterase activity IC$_{50}$ (µg/mL)	α-Glucosidase activity IC$_{50}$ (µg/mL)
Spinasterol	44.19 ± 2.59[b]	8.65 ± 1.71[a]
Galanthamine	1.73 ± 1.10[a]	NA
Acarbose	NA	2.79 ± 0.81[a]

*NA: not applicable. Values are expressed as mean ± SEM (n=3); Means with different letters are significantly different at $p < 0.05$
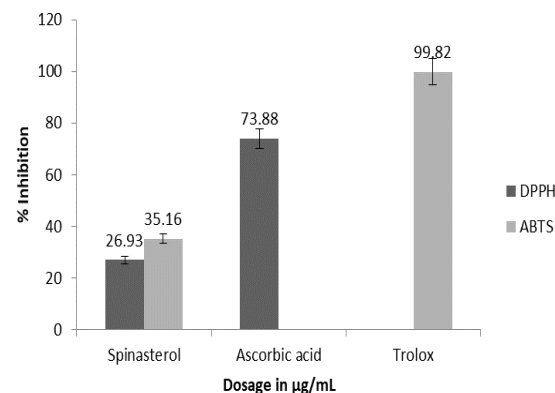

Figure 2: Free radical scavenging activity of spinasterol using DPPH and ABTS

DISCUSSION

The mass spectrum of compound I was isolated as a white solid. The mass spectrum gave an (M + 1) peak at m/z 413 which translates to depict a molecular weight of m/z 412 that depict a molecular formula $C_{29}H_{48}O$ which suggests a steroid. The proton NMR spectra showed the revealed the presence of three vinylic signals at δ = 5.03, 5.06 and 5.17 ppm suggestive of the presence of at least two double bonds in the structure. Six methyl signals were discernible at δ = 0.54, 0.80, 0.81, 0.84, 0.86 and 1.04 ppm respectively, while a multiplet signal at δ = 3.56-3.64 ppm is was due to the hydroxyl methine to the 3-OH proton at C-3 position of the steroidal nucleus. Compound I was identified as α-spinasterol by comparison of its spectral analysis (NMR and MS) with literature data [20]. We have previously isolated spinasterol from *Acacia ataxacantha* stem bark [23]. The presence of this compound in the leaves of *Acacia auriculiformis* is has been reported here for the first time. The steroid α-spinasterol isolated from the leaves of *Acacia auriculiformis* was evaluated for its anticholinesterase, α-glucosidase inhibitory and antioxidant activities in vitro. The enzyme acetyl cholinesterase is the enzyme responsible for the hydrolysis breakdown of acetylcholine in the body and the inhibition of this enzyme is seen as a therapeutic target for the management of neurodegenerative diseases such as Alzheimer and also in the management of glaucoma. The result of the cholinesterase inhibition assay revealed a moderate activity of α-spinasterol with an IC_{50} value of 44.19 ± 2.59 μg/mL which was significantly different (p<0.05) when compared with the value for gallanthamine with an IC_{50} of 1.73 ± 1.10 μg/mL (Table 1). There has been no previous report on the effect of α-spinasterol on the cholinesterase inhibitory activity. However, the hydroalcholic extract of the leaves of *Acacia auriculiformis* has been previously investigated for cholinesterase inhibitory activity [16]. Plant sterols and terpenoids have been reported to exhibit anticholinesterase activity [21]. It is likely that the presence of this compound in *Acacia auriculiformis* might be responsible for the observed activity.

The compound α-spinasterol showed good *in vitro* α-glucosidase inhibitory activity with IC_{50} value of 8.65 ± 1.71 μg/mL, which was not significantly different when compared to the standard, acarbose, with IC_{50} value of 2.79 ± 0.81 μg/mL (Table 1). Alpha-glucosidase inhibitors are classed of as drugs that is are considered among to be one of the effective strategies in the treatment of diabetes mellitus. The compound α-spinasterol has been previously reported to exhibit α-glucosidase inhibitory activity *in vitro*; however, this is the first report with respect to this activity in Acacia species. This compound has also been reported to demonstrate a therapeutic potential of modulating the development and progression of diabetic nephropathy [22].

The in vitro antioxidant activity of α-spinasterol was also investigated using the ABTS and DPPH and ABTS radical scavenging assays. The result revealed that α-spinasterol exhibited a weak radical scavenging activity with percentage inhibitions of 26.93 ± 0.00 and 35.16 ± 0.26 % for DPPH and ABTS assays respectively (thus, it was not possible to calculate the IC_{50} by linear regression). The standard antioxidant, ascorbic acid and trolox showed very high scavenging activities at 73.88 ± 0.04 % and 99.82 ± 0.01% respectively (Figure 1). However, α-spinasterol has been reported to display a high scavenging activity in a dose-dependent manner [23]. It has also been reported to exhibit an increase in the resistance of hippocampal cells to oxidative injury [24]. Generally, it has been established that radical scavenging effects of natural compounds alleviates the symptoms, and assist in the therapeutic management of illnesses such as diabetes mellitus and Alzheimer's disease [25].

CONCLUSION

These results indicate that for the first time that α-spinasterol isolated from *Acacia auriculiformis* leaves exerts potent inhibitory effect against cholinesterase which corroborates the earlier reported cholinesterase inhibitory effect of the ethanol extract of *Acacia auriculiformis*. This plant, this might serve as a lead component in the design and development of an appropriate of chemical entity as drug for the therapeutic management of neurodegenerative diseases such as Alzheimers and diabetes mellitus.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. AAA conceived and designed the study/experiments, A.U and BAL analysed and interpreted the spectra, TE and BO carried out the biological activity and the NMR experiment. All authors have read and approved the manuscript for publication.

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