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Original Article

Correlation of Total Phenolic, Flavonoid and Tannin Content of *Bryophyllum pinnatum* (*Lam.*) (Crassulaceae) Extract with the Antioxidant and Anticholinesterase Activities

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ABSTRACT

Bryophyllum pinnatum is a perennial herb used in traditional medicine against varieties of ailments such as memory disorder. This study quantitatively estimated the total phenolic (TPC), total flavonoid (TFC) and total proautocyanidin (TPA) contents of extract and fractions of *B. pinnatum* and correlated them with its antioxidant and anticholinesterase activities. Methanolic extract of the dried leaves was partitioned into n-hexane, ethyl acetate and aqueous fractions. Total phenolic, flavonoids and proanthocyanidins content were estimated as gallic acid or quercetin equivalents. DPPH and nitric oxide scavenging activity as well as ferric reducing power assays were used to evaluate antioxidant activity, using 2,6-di-tert-butyl-4-methylphenol (DDM) and ascorbic acid as standards. *In vitro* anticholinesterase activity was evaluated by Ellmann's colorimetry assay with phsiostigmine (serine) and donepezil as positive control. The crude methanol extract had the highest phenolic, flavonoid and tannin content. The ethyl acetate fraction had the highest DPPH radical scavenging effect (IC_{50} 0.004 mg/ml) while the aqueous fraction had the highest NO scavenging and ferric reducing effects with values of IC_{50} 0.012 mg/ml and 0.007 mg/ml respectively. The ethyl acetate fraction had the best cholinesterase inhibitory effect (IC_{50} 0.951 µg/ml AChE; 3.546 µg/ml BuChE). DPPH radical scavenging effect correlated strongly with total phenolic, flavonoids and proautocyanidins (r^2 0.896, 0.651 and 0.619 respectively) while ferric reducing power showed weak correlation and NO scavenging as well as AChE inhibition had no correlation. The study shows DPPH radical scavenging could be due to the phenolic content while other class of compounds are responsible for the cholinesterase inhibition.

Key words: *Bryophyllum pinnatum*, Anticholinesterase, Antioxidant, Total Phenolics, Total Flavonoids.

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INTRODUCTION

Phenolics, which are compounds with aromatic rings and hydroxyl groups, are the most abundant, widely distributed secondary metabolites of plants, with about 8,000 structures currently identified.[1] They are generally classified based on the number of hydroxylic groups, chemical composition and substitutes on the carbon skeleton[2] and are involved in defense against pathogens, parasites, predators,[3] ultraviolet radiation[4] as well as contribute to plants' colors.[5] Plant phenolics include phenolic acids, flavonoids, tannins, stilbenes and lignans. Several pharmacological activities have been associated with phenolic compounds. These include anti-ulcer[6], anticancer, antioxidant[7], anti-inflammatory[8], antimicrobial[9] and anticholinesterase[10] among others.

Antioxidants are substances that can prevent or delay damage to cells caused by free radicals, by scavenging them and reduce oxidative stress. Oxidative stress is important in the pathogenesis of several disease conditions such as degenerative diseases, heart disease, cancer and aging.[11-14] Several compounds including those with free sulfhydryl groups (e.g., lipoic acid), those with multiple double bonds and conjugation (e.g., carotenoids), polyphenols (e.g. quercetin), compounds that inhibit reactive oxygen generation (i.e., **NADPHoxidase** inhibitors), and compounds that induce oxidant defenses (e.g., Nrf2 activation-sulforaphane) have been classified as antioxidants.[15] Because of the relative importance of antioxidants, more researches are still ongoing to find new ones especially from natural sources.

Bryophyllum pinnatum is a perennial herb used ethanomedically for the treatment of several ailments such as diarrhea, vomiting, earache, burns, insect bites, smallpox, cough, asthma, palpitations, headache and convulsion.[16,17] The species possesses anthelmintic[18], immunosuppressive[19], hepatoprotective[20], antinociceptive[21], anti-inflammatory[22], antioxidant, antimicrobial[23] among other effects. Several classes of compounds such as alkaloids[24], terpenoids[25], flavonoids[26] have been reported in the plant. This study aims to correlate the phenolic content of *B. pinnatum* with its antioxidant and cholinesterase inhibitory effects.

MATERIALS AND METHODS

Chemicals

Acetylthocholine iodide (Sigma), buterylthiocholine iodide (Sigma-Aldrich), 5,5-dinitro-bis-nitrobenzoic

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acid (Aldrich),2, 2-diphenyl-1-picrylhydrazyl hydrate (Sigma-Aldrich), Folin-Ciocalteu reagent (Fluka Biochemical), vanillin (Fluka Biochemical), potassium ferricyanide (BDH reagents, England), Trichloro acetic acid (Merck chemicals, LTD), sodium nitroprusside (Thomas Baker chemical limited India), Naphthylethylenediamine dichloride (Sigma-Aldrich), All other reagents used in this study were of analytical grade.

Plant material

Fresh leaves of *B. pinnatum* were collected in May 2018 from Ipetu – Ijesa in Osun State Nigeria. The plant was identified and authenticated by Mr. T.K Odewo, Assistant Chief Superintendent Officer, Forestry Research Institute of Nigeria (FRIN) Ibadan, Nigeria. Voucher specimen with FHI number 112041 was deposited at the Department of Pharmacognosy herbarium, University of Ibadan, Nigeria.

Extraction and partitioning

The collected plant was oven dried at 40°C for two weeks using Gallenkamp oven and grinded. Powdered sample (2.03Kg) was macerated in 80% methanol for 72 hours, filtered and concentrated *in vacuo* using rotary evaporator. Methanol extracts (225 g) was partitioned into n-hexane, ethyl acetate and water to obtain the respective fractions.

Total phenolic content

The total phenolic content of the extract and fractions of *B. pinnatum* was determined using the Folin-Ciocalteau's reagent according to previous report.[27] Specifically, 0.5 ml each of the extract (1 mg/ml) was mixed with 2.3 ml of 10% Folin-Ciocalteau's reagent and 2 ml of 15% sodium carbonate was added to the mixture and incubated at 45°C for 15 mins. Absorbance was taken at 765 nm. A standard curve was prepared to estimate the phenolic content, using gallic acid at a concentration range of 0.031 – 1.0 mg/ml. Total phenolic content was expressed as mg gallic acid equivalents (GAE)/g.All experiments were carried out in triplicates with distilled water as blank.

Determination of total flavonoids content

Total flavonoid content was measured using the aluminum chloride colorimetric assay.[28,29] 1 ml of extract (1mg/ml) was made up to 3 ml with methanol in a test tube. This was mixed with 5.6 mls of distilled water, 0.2 mls of 10% aluminum chloride and 0.2 ml of 1M potassium acetate and the whole mixture was left at room temperature for 30 min after which absorbance was measured at 415 nm against the blank. The total flavonoid content was estimated from a calibration curve using different concentrations of quercetin and the result expressed as mg quercetin equivalent per gram dry weight. All experiments were carried out in triplicates.

Proanthocyanidin content

Proanthocyanidin content was estimated according to previously reported procedure.[30,33] In this method, 0.5 ml of the extract (1 mg/mL) was mixed with 3 ml of 4% vanillin in methanol solution and 1.5 ml hydrochloric acid. The mixture was mixed thoroughly and allowed to stand for 15 min at room temperature. The absorbance was measured at 500 nm and the proanthocyanidin content was expressed as mg gallic equivalents (mg GAE) using a calibration curve from gallic acid at different concentrations (1.0, 0.5,0. 25,0.125, 0.063 and 0.031 mg/mL). Experiments were carried out in triplicates.

DPPH radical scavenging

Radical scavenging activity of the extract and fractions was determined using the stable radical 2, 2-diphenyl-1-picrylhydrazyl hydrate (DPPH) according to standard method.[31] Briefly, 1 ml of 0.1 mM of the DPPH was mixed with 1ml of extract/fractions at different concentration (0.031 mg/ml – 1.0 mg/mL). The reaction was vortexed and left in the dark at room temperature for 30 mins. Thereafter, absorbance was taken at 517 nm against a control containing only 1 ml methanol. Ascorbic acid and DDM over a concentration range of 0.125 – 1.0 mg/ml were used as standard drugs. The percentage DPPH radical scavenging activity was calculated as follows:

$$\% \text{ Inhibition of DPPH radical} = [(A_{control} - A_{test})/ A_{control}]*100$$

where $A_{control}$ is the absorbance of the control reaction (containing all reagents except the test compound), A_{test} is the absorbance of the test compound.

Determination of nitric oxide scavenging

Nitric oxide scavenging activity of extract and fractions was determined using Griess IlIosvoyreaction.[32,33] In this protocol, 2 mls of sodium nitroprusside was mixed with 0.5 ml of phosphate buffer pH 7.4 and 0.5 ml of the extract/fractions at varying concentrations (0.0031 – 1.0 mg/ml). The mixture was incubated at 25˚C for 150 mins and absorbance taken at 540 nm (A0). After that, 0.5 ml of the incubated mixture was mixed with 1ml of sulfanilic acid reagent and 1 ml of naphthylethylenediamine dichloride (0.1% w/v) and incubated again at room temperature for 30 mins before taking the absorbance at 540 nm (A1). The same reaction mixture without the test samples but with equivalent amount of methanol served as the control. Ascorbic acid and DDM at various concentrations was used as standard. All experiments were done in triplicates. The percentage inhibition was linearized against the concentrations of each extract and standard antioxidant. The percentage nitrite radical scavenging activity of the extracts and standard were calculated as follows:

$$\% \text{ nitrite radical scavenging activity} = [(A_{control} - A_{test})/ A_{control}]*100$$

where $A_{control}$ is the absorbance of the control reaction (containing all reagents except the test compound), A_{test} is the absorbance of the test compound.

IC_{50}values, which is an inhibitory concentration of each fraction required to reduce 50% of the nitric oxide formation was determined.

Ferric reducing power

Equal volume (0.2 ml) of extract/fractions at various concentration, phosphate buffer and potassium ferricyanide were mixed together and incubated at 50°C for 20 minutes. After cooling, 0.2 ml of 10% trichloro acetic acid (TCA) was added and centrifuged at 4500 rpm for 10 min. 100 µl of the upper layer of the solution was mixed with 20 µl of ferric chloride solution and 100 µl of distilled water. Absorbance was thereafter measured at 700 nm. Control was prepared in similar manner excluding samples. Ascorbic acid, and 2,6-di tert-butyl- 4-metyl phenol (DDM) at various concentrations were used as standard drugs. The assays were carried out in triplicate and percentage reducing power as well as IC_{50} were calculated.[34]

Acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) inhibitory assay

The cholinesterase inhibitory activity of the extract and fractions was determined using the colorimetric method as described by Ellam.[35]The reaction mixture consisted of 240 µl of buffer (50mMTris-HCL, pH 8.0), 20 µl of varying concentrations of the extract/fractions (1, 0.5, 0.25, 0.125, 0.0625, and 0.03125 mg/ml), and 20 µl of the enzyme. This reaction mixture was incubated for 30 mins at 37˚C, after which 20 µl of 10 mM DTNB was added and the reaction started by the addition of 20 µl of the substrate (either 25 mM ATChI or BuTChI). The rate of hydrolysis of the substrate was then determined spectrophotometrically at 412 nm every 30 s for 4 min by measuring the change in absorbance per minute (ΔA/min) due to the formation of the yellow 5-thio-2-

nitrobenzoate anion. Assay with extract/fractions was performed in triplicate at concentrations of 1, 0.5, 0.25, 0.125, 0.0625, and 0.03125 mg/ml. A buffer solution was used as negative control while eserin and donepezil were used as standard drugs. The percentage inhibition was calculated and the extract concentration producing 50% inhibition (IC_{50}) determined. The percentage inhibition (I%) was calculated as follows:

$$I(\%)=[(Vo-Vi)/Vo]*100$$

Where: I(%)=Percentage inhibition

Vi =enzyme activity in the presence of extract.

Vo = enzyme activity in the absence of extract.

Statistical analysis

Statistical analysis was carried out using graph pad prism software. All analyses were performed in triplicate and data are reported as mean ± standard error of the mean of at least three independent experiments. Correlation and regression analysis of antioxidant/anticholinesterase activity (Y) versus the total phenolic/flavonoid/tannin content (X) was carried out using the online Quest Graph™ Linear, Logarithmic, Semi-Log Regression Calculator.[36]

RESULTS AND DISCUSSIONS

Total phenolic, total flavonoid and total tannin content

The total phenolic, total flavonoid and total pro-anthocyanins contents were quantified against either gallic acid or quercetin using standard methods. The crude methanolic had the highest amount of phenolics, flavonoids and tannins (8.30 ± 0.054%, 17.49 ± 0.104%, 17.03 ± 0.102%). This was followed by the aqueous and ethyl acetate fractions respectively (Table 1). Polar solvents appear to have extracted the constituents better than the non-polar solvent.

Phenolics are a major class of secondary metabolites present in medicinal plants. This class of compounds which can be divided into polyphenols and phenolic acids can occur either as mono or polysaccharides and as derivatives like esters or alkyl esters.[1,37,38] Simple phenolic acids and flavonoids are the most common phenolic compounds and they generally occur in plants as insoluble (phenolic acids) or soluble glycosides (flavonoids).[39] Phenolic acids, flavonoids and tannins are the main dietary phenolic compounds.[40] Usually, they also have unsubstituted OH groups, which may cause an increase in their polarity thus accounting for the better solubility in polar solvents.

Data are expressed as Mean ± SEM (n=3). GAE: Gallic Acid Equivalent, QUE: quercetin equivalent, CR: crude methanol extract, AF: Aqueous fraction, EF: Ethyl acetate fraction, HF: Hexane fraction

The antioxidant effect of extracts and fractions of *B. pinnatum* were evaluated using the DPPH radical scavenging, nitric oxide scavenging and ferric reducing power assays. The percentage inhibition for the DPPH scavenging effect is as presented in Figure 1. The ethyl acetate fraction had the highest DPPH radical scavenging activity with an IC_{50} of 0.004 (Table 2).

Data are expressed as Mean ± SEM (n=3). ME: crude methanol extract, HF: Hexane fraction, EF: Ethyl acetate fraction, AF: Aqueous fraction, DDM: 2,6-di-tert-butyl-4-methylphenol .

In the nitric acid scavenging assay, percentage inhibition of which is presented in Figure 2, the aqueous fraction (IC_{50} 0.012), showed the

Table 1: Total phenolics, flavonoids and anthocyanidins of extract and fractions of *Bryophyllum pinnatum*.

Assays	CR	AF	EF	HF
% Yield	11.25	62.57	19.78	4.84
Total phenolics (mg GAE/g)	8.30 ± 0.054	7.07 ± 0.016	7.28 ± 0.136	4.02 ± 0.012
Total flavonoids (mg QUE/g)	17.49 ± 0.104	16.95 ± 0.011	9.02 ± 0.014	3.99 ± 0.122
Total tannins (mg GAE/g)	17.03 ± 0.102	16.99 ± 0.106	9.22 ± 0.107	3.11 ± 0.102

Table 2: IC_{50} values of various antioxidant assays.

	IC_{50} values (mg/ml)					
	ME	HF	EF	AF	Ascorbic acid	DDM
DPPH	0.017	0.161	0.004	0.019	0.015	0.022
Nitric oxide	0.871	0.562	0.158	0.012	0.847	1.400
Ferric reducing	0.059	0.128	0.012	0.007	0.251	0.378

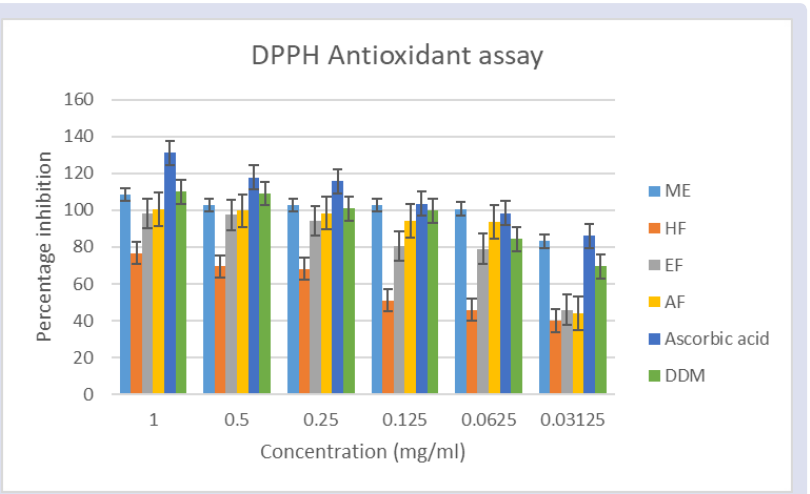

Figure 1: DPPH radical scavenging activity of extract and fractions of *Bryophyllum pinnatum*.

best scavenging effect. Also, in the ferric reducing power, percentage inhibition presented in Figure 3, the aqueous fraction still had the highest activity (IC_{50} 0.007) (Table 2).

Data are expressed as Mean ± SEM (n=3). ME: crude methanol extract, HF: Hexane fraction, EF: Ethyl acetate fraction, AF: Aqueous fraction, DDM: 2,6-di-tert-butyl-4-methylphenol.

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DPPH radical scavenging is one of the most common antioxidant assay method, based on the reduction of the purple $DPPH^{\cdot}$ to 1,1-diphenyl-2-picryl hydrazine. This may be due to its relative convenience, stability and sensitivity.[41] The mechanism involves electron transfer and reduction of a colored oxidant.[42] The extracts and fractions of *B. pinnatum* had good DPPH radical scavenging with the best activity observed in the ethyl acetate fraction followed by the methanol extract and aqueous fraction respectively (Table 2). This is consistent with previous findings on the plant.[43]

Nitric oxide is required for the regulation of several physiological functions under normal conditions.[44] However, excess production of NO can result into tissue damage and has been associated with several disease conditions including inflammation, neurodegeneration and hypertension.[44-46] Thus, different studies are being carried out to discover natural NO inhibitors that may be useful as antioxidant in the management of diseases. In this study, the aqueous fraction exhibited the best NO scavenging effect. This indicates that the compounds responsible for the activity are likely to be polar.

The ferric reducing antioxidant assays is also an electron transfer reaction that measures the ability of the extract/fractions to donate electron to Fe(III).[47] The better the donating power, the better the reduction of Fe^{3+} to the ferrous form, producing different shades of blue color that can be monitored spectrometrically. From our studies, the aqueous (IC_{50} 0.007) fraction had the highest reducing power followed by the ethyl acetate fraction (IC_{50} 0.012) and the crude methanol extract (IC_{50} 0.059) (Table 2). This result suggests polar constituents as responsible for the activity and is comparable to an earlier report.[43]

The antioxidant mechanism of plants is important in reducing lipid peroxidation and thus contributes to the reduction of risks of developing various diseases.[48,49] Studies have shown that diet rich in phenolics contributes to reduced risk of diseases such as cancer, cardiovascular and neurodegenartive diseases that are linked to inflammation and oxidative stress.[50,51]

Inhibitors of cholinesterase still remain a treatment option for neurodegenerative diseases.[52,53] And because of the complexity of these diseases, poly-pharmacology is a prescribed approach of management.[54-56] Thus, enzyme inhibitors with antioxidant potential are considered very promising.[57,58] The crude methanolic extract as well as the various fractions of *B. pinnatum* were also assessed for their

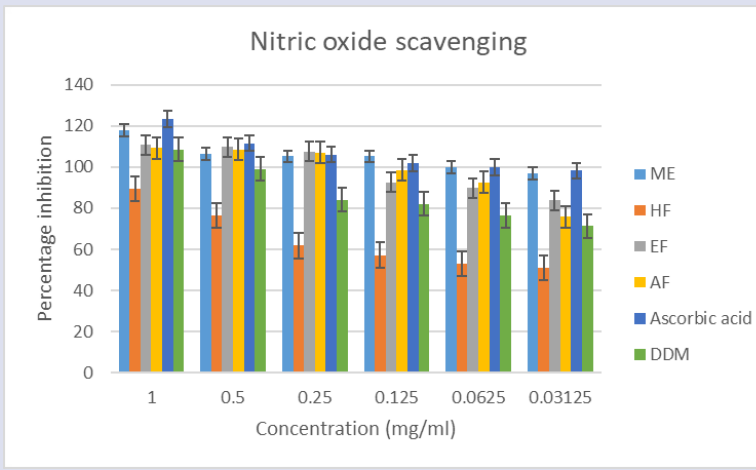

Figure 2: Nitric oxide scavenging activity of extract and fractions of *Bryophyllum pinnatum*.

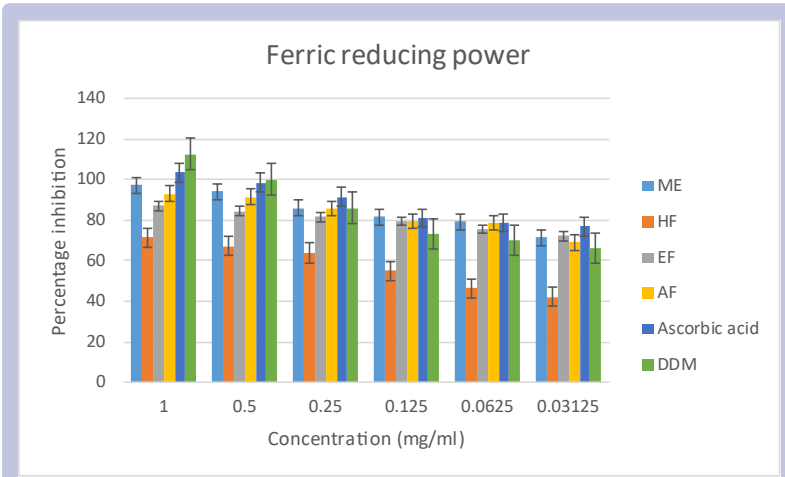

Figure 3: Ferric reducing power of extract and fractions of *Bryophyllum pinnatum*.

ability to inhibit both acetyl and butyryl cholinesterase enzymes. The percentage inhibition of acetyl and butyryl cholinesterase enzymes are as shown in Figures 4 and 5 respectively while the IC_{50} values are reported in Table 3. From the study, the ethyl acetate fraction had the best activity on both enzymes with respective IC_{50} values of 0.951 and 3.546 µg/ml. This result is also found consistent with an earlier report.[43]

Data are expressed as Mean ± SEM (n=3). Hex: hexane fraction; EtOAC: ethyl acetate fraction;

Aqueous: aqueous fraction; Crude: methanol extract.

Data are expressed as Mean ± SEM (n=3). Hex: hexane fraction; EtOAC: ethyl acetate fraction; Aqueous: aqueous fraction; Crude: methanol extract

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The correlation between phytoconstituents of *B. pinnatum* and the observed activities was determined using Quest Graph™ Linear, Logarithmic, Semi-Log Regression Calculator. Several studies have reported strong and positive correlations between antioxidant potential and phenolic contents of medicinal plants.[59-62] Also, the antioxidant effect of phenolics have been attributed to their ability to scavenge free radicals, chelate metal ions or donate hydrogen atoms/electrons.[63,64] Our results suggest strong and positive correlation between the DPPH radical scavenging and total phenolic, flavonoids and proantocyanidins, r^2 0.896, 0.651 and 0.619 respectively (Table 4). Moderate correlation was also observed with the ferric reducing power. However, there was poor correlation with NOscavenging as well as acetylcholinesterase inhibition. Previous studies have demonstrated that phenolic groups are not essential for nitric oxide scavenging effect.[65,66] Also, other classes of compounds such as alkaloids and terpenoids, which have

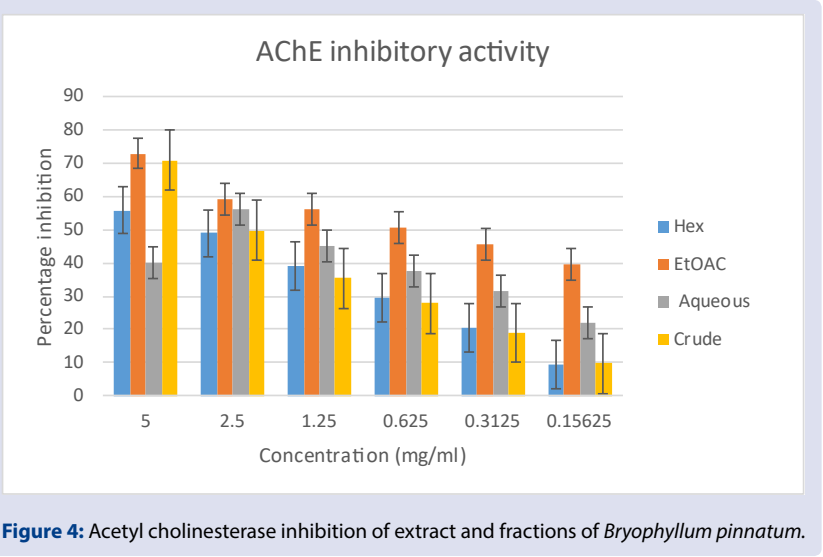

Figure 4: Acetyl cholinesterase inhibition of extract and fractions of *Bryophyllum pinnatum*.

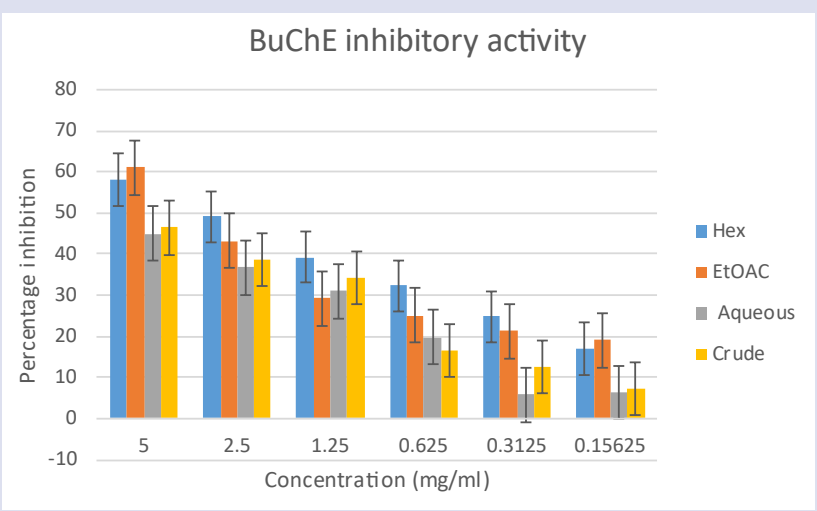

Figure 5: Butyrylcholinesterase cholinesterase inhibition of extract and fractions of *Bryophyllum pinnatum*.

Table 3: IC_{50} values of cholinesterase assays.

	IC_{50} values (µg/ml)					
	Hex	EtOAC	Aqueous	Crude	Donepezil	Eserine
AChE assay	2.203	0.951	1.822	2.403	0.037	0.051
BuChE assay	3.326	3.546	5.115	4.451	0.037	0.051

Table 4: The correlation values.

Assays	r^2 values		
	Total phenolics	Total flavonoids	Proantocyanidin
DPPH scavenging	0.896	0.561	0.619
NO scavenging	2.72×10^{-4}	1.36×10^{-4}	1.78×10^{-3}
Ferric reducing	0.560	0.377	0.442
AChE inhibition	0.028	0.048	0.024
BuChE inhibition	0.345	0.834	0.831

been previously reported in this plant could be responsible for the cholinesterase inhibition observed in the plant.

CONCLUSION

This study confirms the potential of *B.pinnatum* as an antioxidant and anticholinesterase agent with a good prospect for drug development as well as identified phenolics as being responsible for the antioxidant property while suggesting that other class of compounds might be responsible for the anticholinesterase effect.

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CONFLICTS OF INTEREST

Authors declare no conflicts of interest

ABBREVIATIONS

DDM: 2,6-di-tert-butyl-4-methylphenol; AChE: Acetylcholinesterase; BuChE: Butyrylcholinesterase; DTNB: 5,5-dinitro-bis-nitrobenzoic acid; DPPH: 2, 2-diphenyl-1-picrylhydrazyl hydrate; TCA: Trichloro acetic acid; FHI: Forest Herbarium Ibadan; GAE: Gallic acid equivalents; ATChI: Acetyl thiocholine iodide; BuChI: Buteryl thiocholine iodide; What of NO?

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