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ACETYLCHOLINESTERASE INHIBITORY ACTIVITY OF A CYCLITOL ISOLATED FROM THE LEAVES OF *Bauhinia rufescens*

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ABSTRACT

Bauhinia rufescens is a medicinal plant used in the treatment of mycosis, diarrhea and malaria. Leaves of B. rufescens were extracted with organic solvent in a gradient of petroleum ether, ethyl acetate and methanol. Silica gel column purification of methanol extract afforded the isolation of a cyclitol, identified as 3-O-methyl-D-inositol. The crude extracts and the isolated compound were evaluated for their anticholinesterase inhibitory activity using bioautographic and microplate techniques. The cyclitol demonstrated higher activity in the microplate assay relative to the crude extract with IC_{50} value of 463.77 µM. The Inhibitory activity exhibited by the cyclitol against acetylcholinesterase enzyme indicates its potential as a drug agent for the treatment of Alzheimer's disease.

Keywords: Bauhinia rufescens, cyclitol, cholinesterase

INTRODUCTION

Rational drug discovery from plants started at the beginning of the 19th century, when the German apothecary assistant Friedrich Sertürner succeeded in isolating the analgesic and sleep-inducing agent from opium which he named morphium (morphine) (Atanasov *et al.*, 2015). This triggered the examination of other medicinal herbs, and during the following decades of the 19th century, many bioactive natural products, primarily alkaloids, such as quinine, caffeine, nicotine, codeine, atropine, colchicine, cocaine, capsaicin were isolated from their natural sources (Atanasov *et al.*, 2015).

Research on plants which are used for the treatment of memory dysfunction in folk medicine led to the isolation of acetylcholinesterase inhibitor, conypododiol from methanol extract of *Asparagus adscendens* (Khan *et al.*, 2010). *Bauhinia rufescens* is a shrub in the family Fabaceae with a medicinal application in the treatment of mycosis, diarrhea and malaria etc. The plant is reported to contain p-coumaric acid, ferulic acid, hyperoside from its Leaves (Compaoré *et al.*, 2011). In this work, Leaves of *B. rufescens* were investigated for a potent anti-alzheimer's drug using acetylcholinesterase inhibitory activity.

MATERIALS AND METHODS

UV spectra were measured with a Shimadzu UV 1601PC spectrophotometer, and IR spectra were recorded on a Perkin-Elmer 1600 FTIR spectrophotometer. The ^{1}H- and ^{13}C-NMR spectra were recorded on Bruker Avance 400 MHz spectrometer. Chemical shifts were recorded in parts per million (δ) in deuterated methanol. Mass spectra were obtained from National University Singapore (NUS) Mass Spectrometry Service, Singapore. Vacuum liquid chromatography (VLC) was carried out on silica gel 230-400 mesh (Merck). Column chromatography (CC) was carried out on silica gel 70-230 mesh (Merck). Silica gel 60 F_{254} precoated aluminum plates (0.2 mm, Merck) were used for TLC analysis; detection was performed by spraying with 5% H_2SO_4 in methanol and 1% vanillin in methanol, followed by heating at 120°C for 5 min.

Plant material

The stem bark of *B. rufescens* was collected at Kiru, Kano State, Nigeria in August 2011. A voucher specimen (Acc. 99) was deposited in the herbarium of the Department of Biological Sciences, Bayero University, Kano, Nigeria.

Extraction and isolation

The dried leaves of *B. rufecsens* (400 g) was ground and extracted successively with petroleum ether (3.0 L), ethyl acetate (3.0 L) and methanol (3.0 L) in a soxhlet extractor each for 18 h. The samples were concentrated using rotary evaporator to give sticky brown substances of petroleum ether (8.57 g, 2.14%), ethyl acetate (10.88 g, 2.72%) and methanol (27.55 g, 6.89%).

A portion of the methanol (MeOH) extract from the leaves of *B. rufescens* (BRLM, 12.00 g) was subjected to VLC (180 g silica, column size 7.0 cm x 10.0 cm), eluted with hexane-chloroform-ethyl acetate-methanol to give 32 fractions (BRLM1-BRLM32). Purification of fraction BRLM16 over a repeated silica gel column chromatography led to the isolation of a cyclitol.

Spectral Data of Cyclitol: R_f = 0.54 (EtOAc-MeOH, 9:1); IR (KBr) ν_{max} cm^{-1}: 3423, 2923, 1073; ^{1}H NMR (CD_3OD): δ (ppm) 3.89 (2H, d, *J* = 2.0 Hz, H-1, H-3), 3.75 (1H, dd, *J* = 9.6, 2.0 Hz H-6), 3.71 (1H, dd, *J* = 9.6, 2.0 Hz H-4), 3.61 (3H, s, OCH_3), 3.59 (1H, d, *J* = 9.6 Hz, H-2), 3.25 (1H, t, *J* = 9.6 Hz, H-5); ^{13}C NMR (CD_3OD): δ (ppm) 83.5 (C-5), 72.9 (C-1), 72.3 (C-2), 72.0 (C-3), 71.1 (C-4), 70.6 (C-6), 59.3 (OCH_3); EIMS: *m/z* (%) = 193 $[M - H]^+$ (9), 179 (12), 97 (73), 57 (100).

TLC Bioautographic Assay for Acetylcholinesterase Inhibition

The TLC bioautographic assay for AChE inhibitory activity of the samples on silica gel plate were detected by spraying the substrate, dye and enzyme according to method described by Yang *et al.*, (2012) with slight modification. Sample solution (5 µL) was spotted on a silica gel TLC plate and developed with appropriate solvent system. The plate was dried with a hair dryer. Then AChE (1U/mL in buffer pH 7.8) and acetylthiocholine iodide, ATCI, (1 mM in buffer pH 7.8) were sprayed on to TLC plate subsequently. After each solution was sprayed, TLC plate was blown quickly with cold wind from a hair dryer until no free liquid was found on it. The plate was then incubated at 37°C for 20 min in a humid atmosphere. Finally, 5,5´-dithio-bis-2-nitro-benzoic acid, DTNB, (1 mM in buffer pH 7.8) was sprayed on to the plate. Galanthamine hydrobromide was used as a positive control. White spots on a yellow background showed AChE inhibitory activity. False-positive results due to inhibition of ATCI reaction with DTNB were eliminated. A TLC plate identical to the one in the TLC bioautographic assay was prepared. The developed TLC plate was sprayed with tris–HCl (pH 7.8), ATCI and DTNB in sequence. Appearance of white spots on a yellow background indicated false-positive results.

Microplate assay for Acetylcholinesterase Inhibition

AChE inhibitory activities of the samples were assayed by the spectrophotometric method (Dong *et al.*, 2013; Yang *et al.*, 2012). Acetylthiocholine iodide, 5,5´-dithio-bis-(2-nitrobenzoic) acid (DTNB, Ellman's reagent), AChE were purchased from Sigma Chemical. The reaction mixture (200 µL) containing phosphate buffer (pH 8.0), test sample (100, 10, 1 µM), and AChE (0.22 U/mL), was incubated for 20 min (37°C). The reaction was initiated by the addition of 40 µL of solution containing DTNB (0.01 M) and acetylthiocholine iodide (0.075 M). Galanthamine hydrobromide was used as positive control. All the reactions were performed in triplicate. The percentage inhibition was calculated as follows:

$$Inhibition = \left(\frac{E - S}{E}\right) x100$$

where, E = activity of the enzyme without test sample; and S = activity of enzyme with test sample. IC_{50} values were determined using probit analysis with SPSS 16 software.

Results and Discussion

The methanol extract (12.00 g) was fractionated using VLC with chloroform-ethyl acetate-methanol gradient as eluents to afford 32 fractions. Thin layer chromatographic profile displayed an interesting spots in fraction 16 which was subjected to further purification using column chromatography with n-hexane-chloroform, chloroform-methanol, acetone-methanol, methanol polarity gradient to afford a cyclitol (37.1 mg) as a brown sticky substance.

The IR data showed broad absorption bands at 3423 cm^{-1} for the presence of O-H group, whereas 1073 cm^{-1} was associated with C-O stretching band. The ^{1}H NMR and ^{1}H-^{1}H COSY (**Tables 1 and 2**) indicated the presence of singlet at δ 3.61 integrating for three protons assignable to OCH_3 group. The proton signals at δ 3.89 (d, *J* = 2.0 Hz, H-1, H-3), 3.75 (dd, *J* = 9.6, 2.0 Hz H-6), 3.71 (dd, *J* = 9.6, 2.0 Hz H-4), 3.59 (d, *J* = 9.6 Hz, H-2), 3.25 (t, *J* = 9.6 Hz, H-5) were attributed to the presence of oxymethine protons.

Table 1: ^{1}H (400 MHz) and ^{13}C NMR (100 MHz) Data of the Cyclitol (MeOD-d4)

H/C No.	δH in ppm (multiplicity, *J* in Hz)	δC in ppm (C-type)*
1	3.89 (d, 2.0)	72.9 (CH)
2	3.59 (d, 9.6)	72.3 (CH)
3	3.89 (d, 2.0)	72.0 (CH)
4	3.71 (dd, 9.6, 2.0)	71.1 (CH)
5	3.25 (t, 9.6)	83.5 (CH)
6	3.75 (dd, 9.6, 2.0)	70.6 (CH)
7	3.61 (s)	59.3 (OCH_3)

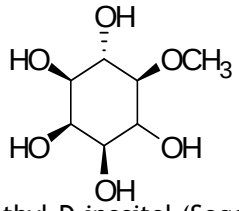
(C-type)* obtained from DEPT experiment

The ^{13}C NMR and DEPT spectra (**Table 1**) showed seven signals ascribed for seven carbon atoms, which were classified into one methoxyl and six oxymethine carbon atoms. The HMQC spectrum (**Table 2**) displayed the correlations of carbon signals at δ 83.5 (C-5), 72.9 (C-1), 72.3 (C-2), 72.0 (C-3), 71.1 (C-4), 70.6 (C-6), 59.3 (OCH_3) with proton resonances at δ 3.25 (H-5), 3.89 (H-1), 3.59 (H-2), 3.89 (H-3), 3.71 (H-4), 3.75 (H-6), 3.61 (OCH_3) respectively.

Table 2: ^{1}H-^{1}H COSY and ^{1}H-^{13}C HMQC NMR Data of the Cyclitol

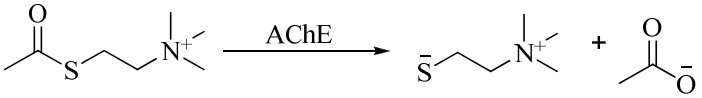
Proton No.	^{1}H-^{1}H COSY	^{1}H-^{13}C HMQC
H-1	-	C-1
H-2	-	C-2
H-3	H-1	C-3
H-4	H-3, H-5	C-4
H-5	H-4, H-6	C-5
H-6	H-5	C-6
H-7	-	C-7

The electron impact mass spectrometry (EIMS) of the isolated compound showed a molecular ion signals at *m/z* 193 for $[M-H]^+$ consistent with molecular formula $C_7H_{14}O_6$. Consequently, the spectral data were consistent with a cyclitol found in *Croton celtidifolius* (Mukherjee and Axt, 1984) which was identified as 3-*O*-methyl-D-inositol (Seqouyitol).



3-*O*-methyl-D-inositol (Seqouyitol)

The plant extracts and the isolated compound (seqouyitol) were investigated for potent anti-acetylcholinesterase activities using Ellman's reagent. The enzyme is involved in the termination of nerve impulse transmission at the cholinergic synapses by rapid hydrolysis of acetylthiocholine (**scheme 1**). Thus inhibition of acetylcholinesterase serves as strategy for the treatment of alzhemer's disease. The disease is a progressive degenerative neurologic disorder resulting in impaired memory and behaviour (Mukherjee *et al.*, 2007).



Scheme 1: Hydrolysis of Acetylcholine

The TLC bioautographic assay for acetylcholinesterase inhibitory activity was carried out on the extracts and isolated compounds of *B. rufescens*. The results were expressed as the lowest concentration at which an inhibitor is detected in microgram (µg) i.e, the detection limit, L_D. Evaluation of the detection limit of the extracts exhibited inhibitions against acetylcholinesterase activity in the range of 30.0 µg to 40.0 µg. While the sequoyitol had the lowest activity with L_D = 97.0 µg, however, positive control galanthamine hydrobromide was found to be more potent with L_D=18.4 µg (**Table3).**

Table 3: TLC Bioautographic Assay of the Crude Extracts and Isolated Compound

Samples*	Molecular weight (g/mol)	Conc.[a]	L_D (µg)
Petroleum ether extract	-	8	40.0
Ethyl acetate extract	-	8	40.0
Methanol extract	-	6	30.0
Seqouyitol	194	100	97.0
Galantamine hydrobromide	368	10	18.4

a: concentration in mg/mL (crude extracts) and mM (isolated compound)
*Volume spotted = 5 µL.

Crude extracts and isolated compound from *B. rufescens* were also investigated for their inhibitory activity on the hydrolysis of acetylcholine by acetylcholinesterase using Ellman's method (Dong *et al.*, 2013). The results were expressed as the inhibitory concentrations at 50% (IC_{50}) of the test samples (Table 4).

Table 4: Acetylcholinesterase Inhibitory Activity

Samples	[a]IC_{50}
Petroleum ether extract	>1000
Ethyl acetate extract	>1000
Methanol extract	>1000
Seqouyitol	463.77
Galantamine hydrobromide	2.92

a: IC_{50} in µg/mL (crude extracts) and µM (isolated compound)

All the extracts were weakly active with IC_{50} value >1000 µg/mL, while the sequoyitol displayed a moderate inhibitory activity (IC_{50} = 463.8 µM) relative to that of galanthamine hydrobromide (IC_{50} = 2.92 µM). de França *et al.* (2015) reported a mild anticholinesterase activity of ethanol extract from *B. forficata*, while a remarkable anti-acetylcholinesterase activity was observed for betulin (IC_{50} = 28.4 µM) isolated from the bark of *Garcinia hombroniana* (Jamila *et al.*, 2015).

CONCLUSION

The methanol extract of *B. rufescens* stem bark afforded a cyclitol identified as 3-*O*-methyl-D-inositol. In the TLC bioautographic assay, the same extract was found more active against AChE than petroleum ether and ethyl acetate extracts of the plant. While the isolated compound had demonstrate a moderate activity in Ellman's method against the enzyme. Therefore, the compound could serve as a potential lead compound for synthesis of more potent derivatives.

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