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TWO ANTIOXIDANT CAFFEIC ACID DERIVATIVES FROM THE ETHYL ACETATE FRACTION OF THE METHANOL LEAF-EXTRACT OF *ALSTONIA BOONEI* DE WILD

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ABSTRACT

The use of *Alstonia boonei* De Wild (Apocynaceae) leaves in the management of a host of ailments (such as rheumatic and muscular pains as well as hypertension and malaria) in several parts of West Africa (including Nigeria) and Asia is very common however yet to be fully validated. This study is aimed at the isolation of some phytoconstituents responsible for its anti-oxidant activity. The ethyl acetate fraction of the methanol leaf-extract of *Alstonia boonei* was subjected to a series of chromatographic separation giving rise to a number of sub-fractions. The chemical structures of the two compounds obtained from one of the sub-fractions were elucidated by a combination of UV, HPLC-MS, 1 D and 2 D NMR spectroscopy. The antioxidant activity of the isolated compounds was determined by DPPH free radical scavenging model. The compounds were elucidated as 5-caffeoylquinic acid (Chlorogenic acid)) and 4,5-dicaffeoylquinic acid**.** Both compounds showed good antioxidant activities (IC_{50} < 25 µg/mL) on DPPH free radical scavenging model. The ethnomedicinal use of the leaves of *A. boonei* De Wild in the management of inflammatory diseases and other disorders associated with oxidative stress is justified by the strong antioxidant activity of the isolated compounds.

KEYWORDS: Antioxidant, *Alstonia boonei*, caffeic acid, Phenolic compounds

INTRODUCTION

The chemistry of the leaves of *Alstonia boonei* De Wild (Apocynacae) has so far been poorly investigated, most of the reported works being limited to crude methanol extracts. *A. boonei* belongs to a wide spread genus of evergreen trees and shrubs consisting of about 50 species widely distributed in Africa, Asia and America [1].

The fresh leaves, stem bark and root bark have been widely used in the treatment of various ailments ranging from malaria to inflammatory diseases (including rheumatic pains) as well as hypertension [2]. Some pharmacological studies aimed at validating some of these ethnomedicinal uses are well documented [3-6]. There are also a few reports of chemical investigation of *A. boonei* including a report by Moronkola and Kunle, [7] on the extraction of volatile oils from the leaf fraction of the plant. These volatile oils in a more recent work [8] were shown to possess good insecticidal potentials against malaria vectors. Alkaloids isolated from the plant (mainly Echitamine), were also shown to possess good diuretic and hypotensive properties [9]. Our group also reported the isolation of beta amyrin and alpha amyrin acetate from the stem bark of *A. boonei*, which showed profound anti-inflammatory activity [6].

Most edible plant materials (including herbal remedies) with a range of pharmacological activities have been shown to contain a number of compounds which display good antioxidant activity. These antioxidant substances are known to block the action of free radicals which have been

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implicated in the pathogenesis of various ailments especially inflammatory diseases [10]. In our earlier study, we isolated and characterized eight flavonoid glycosides [11] which exhibited varying degrees of antioxidant activity. In this study, we report the isolation and structure elucidation of two caffeic acid derivatives and the investigation of their antioxidant activity.

MATERIALS AND METHODS

Plant Material

The leaves of *Alstonia boonei* De Wild were collected in Nsukka, Enugu State, Nigeria and identified by Mr. Alfred Ozioko, a taxonomist at the Centre for Ethno-medicine and Drugs Development, Nsukka, Enugu State, Nigeria. A voucher specimen of the leaf was deposited at the herbarium (Number INTERCEDD/024) section of the Centre.

Extraction and isolation of the compounds

About 500 g of clean, dried and pulverized leaves of *A. boonei* were extracted for 48 h by cold maceration in 2.5 L methanol with continuous stirring using a magnetic stirrer. The solvent was changed after every 24 h, filtered at 40°C under reduced pressure and the extract dried to obtain ME.

About 20 g of the methanol extract was dispersed in 200 ml of 10% methanol in distilled water and sonicated for 10 min. This dispersion was defatted by partitioning using n-hexane (500 ml x 3) and subsequently partitioned in ethyl acetate (500 ml x 3) to obtain the ethyl acetate fraction (EF). The ethyl acetate fraction was subjected to vacuum liquid chromatography on a 5-litre VLC column eluting in succession with 500 ml each of gradient mixtures of hexane : ethyl acetate and dichloromethane : methanol. Altogether, a total of 14 pool fractions were obtained (EF 1-14).

The progress of the ethyl acetate fractions were all through the separation process, monitored using TLC plates precoated with silica gel 60 F254 and using DCM : methanol (4:1) as mobile phase. The resulting band separation describing the separation of the compounds was detected under UV.

The VLC fraction of EF14 was directly processed with semi-preparative HPLC to obtain compounds **1** and **2.**

Table 1: The ^{1}HNMR Data of Compounds 1 and 2

Position	Compound 1	Compound 2
	δ_H (*J* in *Hz*)	δ_H (*J* in *Hz*)
1	-	-
2	1.94 (d, *J*=14.7)	2.18
	2.15 (d, *J*=14.7)	2.28#
3	4.11	4.44 m
4	3.67	5.14 d
5	5.38	5.71
6	2.00 (d, *J*=11.9)	2.28#
	2.10 (d, *J*=12.6)	2.37
7	-	-
1'	-	-
2'	7.05 (d, *J* = 1.9)	7.00
3'	-	-
4'	-	-
5'	6.77 (d, *J* = 8.2)	6.73
6'	6.95 (d, *J* = 1.9, 8.2)	6.86#
7'	7.57 (d, *J*=15.9)	7.58
8'	6.29 (d, *J*=15.9)	6.27
9'		-
1"		-
2"		6.96
3"		-
4"		-
5"		6.7
6"		6.86#
7"		7.49
8"		6.18
9"		-

NMR was measured at 500 MHz (^{1}H) (CD_3OD), #=overlapping signals

Structure elucidation of the isolated compounds

HPLC/ESI-MS was carried out on compounds **1** and **2** using a thermo Finnigen LCQ - mass spectrometry connected to a UV detector.

The H-NMR spectra were recorded at 300k on Bruker ARX 500 NMR Spectrometre. 1D and 2D spectra were obtained using the standard Bruker software.

DPPH free radical scavenging assay

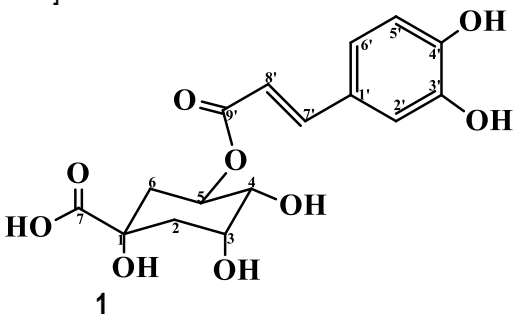
The antioxidant activity of compounds **1** and **2** were assessed using the DPPH model with Ascorbic acid as standard as previously reported [11](Okoye and Okoye, 2016). The analysis was carried out using a 96 well Enzyme Linked ImmunoSorbent Assay (ELISA) plate reader in order to subject all samples to the same conditions. The antioxidant activity of the compounds was expressed as IC_{50} (the concentration of the test compound showing 50% inhibition of DPPH.

Table 2: IC_{50} values of the DPPH free radical scavenging activity of the extract, fractions and isolated compounds

Test Compounds	IC_{50} (µg/ml)
Compound 1	22
Compound 2	10
Vitamin C	49

RESULTS AND DISCUSSION

Compound **1** was isolated as a brown amorphous solid and showed UV absorption maxima at λ_{max} 218 nm, 242 nm, and 326 nm , which is typical for caffeic acid derivatives. Its molecular mass was deduced as 354 g/mol based on the pseudomolecular ion peaks found in ESIMS spectrum at *m*/*z* 355 $[M + H]^+$ in the positive mode and *m*/*z* 353 $[M -H]^-$ in the negative mode. The ^1H NMR spectrum of compound **1**(Table 1) displayed three oxymethine protons at δ_H 5.38, 4.11 and 3.67 assigned to H-5, H-3 and H-4 respectively, together with two pairs of sp^3 diastropic methylene protons at δ_H 2.15/1.94 and 2.10/2.00 assigned to $H_{2A/B}$-2 and $H_{2\ A/B}$ -6, respectively. These signals belong to the quinic acid unit of the molecule. The ^1H NMR spectrum also shows signals of three coupled aromatic protons of ABX pattern at δ_H 7.05 (d, *J* = 1.9 Hz), 6.77 (d, *J* = 8.2 Hz) and 6.95 (d, *J* = 8.2 Hz), corresponding to H-2', H-5' and 6' respectively. A pair of doublet at δ_H 7.57 and 6.29 with coupling constants of 15.9 Hz observed corresponds to the two trans olefinic protons found in hydroxy cinnamic acids (H-7' and H-8' respectively). Compound **1** was, thus, identified as 5-caffeoylquinic acid (chlorogenic acid) (Fig. 1)based on the analysis of the UV, ^1H NMR and MS spectra and comparison of data with those previously reported [12] (Pauli *et al.*, 1998].



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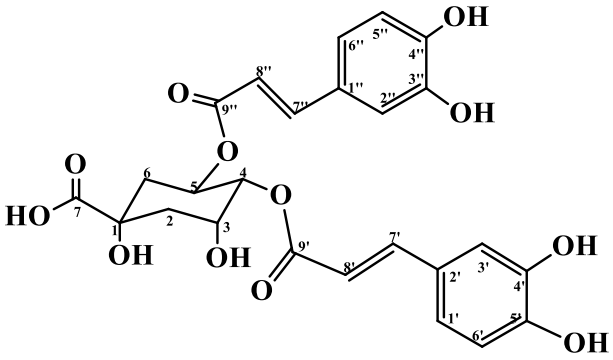

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Figure 1: Chemical structures of the isolated compounds

Compound **2** was obtained as a yellowish brown amorphous solid with UV absorption maxima at 219, 244 and 329 nm. Its molecular mass, 516 g/mol was deduced from the ESIMS spectrum revealing peaks at *m*/*z* 516.9 $[M+1]^+$, 539.0 $[M+Na]^+$ in the positive mode and 515.3 $[M-1]^-$ in the negative mode. The MS spectrum also showed a fragment peak at *m*/*z* 354.9 $[M-162+1]^+$ which is associated to the loss of a caffeic acid unit. The ^1H NMR spectrum of **2** (Table 1)is similar to that of compound **1.** The major difference is the presence of signals corresponding to extra three coupled aromatic proton signals of the ABX pattern. The ^1H NMR spectrum of **2**, thus, showed resonances for two ABX systems δ_H 7.05 (d, *J* =1.9 Hz), 6.77 (d, *J* = 8.2 Hz) and 6.95 (dd, *J* = 8.2 Hz); and δ_H 7.09 (d, *J* = 2.2 Hz), 6.80 (d, *J* = 8.2 Hz) and 6.95 (d, *J* = 8.2 Hz)] which were assigned to two 1,3,4-trisubstituted phenyl units. The ^1H NMR spectrum of **2**, thus, exhibited signals which can be associated to one quinic acid and two caffeoyl moieties. The assignments of signals and the configuration of the quinic acid moiety were determined by the analysis of ^1H-^1H COSY. The

proton resonances at 5.14 and 5.71 were assigned to H-4 and H-5 respectively. When ^{1}H NMR spectrum of compound **2** was compared with that of compound **1** (chlorogenic acid), a downfield shift of H-4 signal by 1.47 ppm and that of H-5 signal by 0.33 ppm were observed. This suggested the attachment of the second caffeoyl unit at C-4. From these data obtained and comparing with earlier reported data, Compound **2** was identified as 4,5-dicaffeoylquinic acid (Fig. 1).

Compound **1** and **2** were subjected to antioxidant assay using the DPPH free radical scavenging model. The results obtained showed that both compounds exhibited a dose dependent free radical scavenging capacity with IC_{50} values of 22 µg/ml and 10 µg/ml respectively (Table 2). The strong antioxidant activity observed for these compounds can be rationalized by the presence of two ortho positioned OH groups in their aromatic ring systems. Presence of two ortho positioned OH groups in aromatic ring was previously reported as a requirement for good antioxidant activity in phenolic compounds [11, 13, 14] (De Martino *et al*., 2012; Okoye and Okoye 2016, Okoye et al., 2015). The stronger antioxidant activity displayed by Compound **2** (which has two aromatic rings containing two 'ortho' positioned OH) compared to compound **1** (which has only one aromatic ring containing two 'ortho' positioned OH) in this study further lend credence to this proposition. Hou et al (2005) [15] earlier proposed that caffeic acid derivatives show good antioxidant activities probably due to their proton donating ability. A poly caffeoyl substitution of the quinic acid moiety is, thus, expected to increase such proton donating ability as observed in Compound **1**.

CONCLUSION

Two potent antioxidant caffeic acid derivatives were isolated from the ethyl acetate fraction of the leaves of *A. boonei* De Wild. These compounds displayed stronger antioxidant activity than the standard drug (ascorbic acid) used in this study. Their presences are likely to largely contribute to the obvious pharmacological properties as well as justify the wide range of folkloric use of the leaves of *A. boonei* De Wild.

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CONFLICT OF INTEREST

The authors declare no conflict of interest. The authors alone are responsible for the content and writing of this article.

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