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Investigation of the Antioxidant Activity of *Alchornea laxiflora* (Benth) and its Constituents

[1]A.O. Adeloye, [1]M.A. Aderogba, [2]T.O. Idowu, [3] E.M. Obuotor and [2]A.O. Ogundaini
[1]Department of Chemistry, Faculty of Science,
[2] Department of Pharmaceutical Chemistry, Faculty of Pharmacy,
[3]Department of Biochemistry, Faculty of Science,
Obafemi Awolowo University, Ile-Ife, Nigeria

Abstract: The antioxidant activity of the 50% aqueous ethanol leaf extract and solvent fractions of *Alchonea laxiflora* were evaluated using DPPH Spectrophotometric assay. The two fractions tested, ethyl acetate and n-butanol fractions exhibited a relatively high activity with EC_{50} of 12.97±0.30 μg mL^{-1} and 24.34±0.33μg mL^{-1}, respectively. The crude extract, exhibited the least activity (EC_{50}, 106.74±0.29). All tested samples were however less potent than ascorbic acid used as reference standard (EC_{50}, 4.78±0.25). Bioassay guided fractionation of the n-butanol fraction using a combination of Accelerated gradient Chromatography (AGC) and Sephadex LH-20 column chromatography led to the isolation of two flavonoids. These compounds were identified as taxifolin glycoside and quercetrin using spectroscopy techniques such as- MS, ^1H and ^{13}C NMR.

Key words: *Alchonea laxiflora,* antioxidant activity, DPPH, flavonoids

INTRODUCTION

The addition of natural antioxidants such as α-tocopherol and L-ascorbic acid to food and other related products, as preservatives, have gained wide acceptability owing to the fact that these agents are relatively safe and cause fewer adverse reactions[1,2] . However, their antioxidant potentials have been reported to be lower than those of synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxyl toluene (BHT)[3]. Consequently, there is a major interest in searching for safe but efficient antioxidants with high activity from natural sources as substitutes for these synthetic chemicals. Our natural environment is endowed with many biologically active plants that need to be exploited for the benefit of mankind [4,6]. One of such plant is *Alchornea laxiflora* (Benth) Pax and Hoffman, a plant used locally for the preservation of food items in Nigeria [6,7]. *A. laxiflora* is a shrub belonging to the family Euphobiaceae and is widely distributed throughout tropical Africa. The infusion of its leaves is often employed in folk medicine in the management of malaria infection, anti-inflammatory and other infectious disturbances[8]. Ogundipe, *et al.*, [8] in a comparative bioactivity study of the leaves and roots of *A. laxiflora* demonstrated the presence of potent anti-inflammatory and antimicrobial activities in the ethyl acetate soluble fraction of both extract. Subsequent investigation on the active ethyl acetate fraction led to the isolation of Quercetin and two of its disulphate derivatives along with quercetrin, rutin and quercetin-3,4′-diacetate. The antimicrobial activity of this plant were attributed to these compounds[7].

A co-generic species, *A. cordiflora* has also been extensively studied. Its been reported that decoction from the powdered leaves are used in the treatment of wounds, sores, ulcers and gonorrhoea [8]. Previous chemical and biological studies on the leaf extract of this specie have reported the occurrence of some flavonol glycosides and triisopentenylguanidine[10]. Of these compounds, only triisopentenylguanidine had appreciable antibacterial activity[11].

Antioxidant activity in higher plants have been linked with flavonoids, which are a group of polyphenolic compounds ubiquitously found in plants and one of the most important classes of phytocompounds with biological activity [12,14]. Antioxidants play a role in the maintenance of the pro/antioxidant balance by neutralizing the free radicals which are responsible for deleterious processes in biological systems[2]. Their presence in food or body at low concentrations compared with that of an oxidizable substrate markedly delay or prevent the oxidation of that substrate[15]. Excess free radicals generated in the body have been linked to

Corresponding Author: M.A. Aderogba, Department of Chemistry, Faculty of Science, Obafemi Awolowo University, Ile-Ife, Nigeria

oxidative stress related diseases like cancer, diabetes, inflammation and AIDS[16].

In this study, we report the antioxidant activity of the crude and the solvent fractions of *A. laxiflora*, along with the constituents of the butanol solvent fraction.This is in our continuous search for novel antioxidant agents from natural sources.

MATERIAL AND METHODS

Chemicals: All chemicals used were of analytical grade obtained from BDH Chemicals Ltd, Poole England and Fluka chemika.

Plant material: The leaves of *A. laxiflora* were collected in Obafemi Awolowo University, Ile-Ife, Nigeria. Dr. H.C. Illoh of Department of Botany, Obafemi Awolowo University, Ile-Ife, authenticated the plant, where a voucher specimen (Number: FHI 58417) was deposited.

Extraction: The collected plant material was air dried for three weeks and milled to obtain a powder (480.0g).

The powdered leaves was extracted with 50% aqueous ethanol for 48hrs and filtered. The filtrate was concentrated *in vacuo* to dryness on rotary evaporator, yielding a crude extract (63.2 g, 13.1%). Part of the crude extract was subsequently suspended in distilled water and partitioned with ethyl acetate. The ethyl acetate fraction was collected and concentrated to dryness. The aqueous fraction was further extracted with butanol. This afforded the butanol fraction on concentration *in vacuo* at 40°C. The crude extract and the solvent fractions were tested for antioxidant activity.

Evaluation of Antioxidant activity: The antioxidant activity of the crude and solvent fractions from *A. laxiflora* were determined using the method of Mensor *et al.*, [17] with some modification. The samples were tested at 250.0, 125.0, 50.0, 25.0, 10.0, 5.0 and 2.5μg mL^{-1}. The solution of 0.3 mM DPPH (1,1-diphenyl-2-picrylhydrazyl) in methanol was prepared and 1.0mL of this solution was added to 2.0mL of each sample solutions of different concentrations. The reaction mixture was shaken gently and placed in the dark for 30min at room temperature. The absorbance was then measured at 515nm on a visible spectrophotometer (Pharmacie Biotech, Novaspec II). These were converted to percentage antioxidant activity (AA%) using the formula :

$$AA\% = 100 - \{[(Abs_{sample} - Abs_{blank}) \times 100] / Abs_{control}\}$$

Where Abs_{sample} was the absorbance of the sample, Abs_{blank} was the absorbance of the blank and $Abs_{control}$ was the absorbance of the control.

The EC_{50} value, defined as the amount of the sample sufficient to elicit 50% reduction of the initial DPPH concentration, was calculated from the linear regression of plots (SigmaPlot® 2001, SPSS Science) of concentration of test compounds (μg mL^{-1}) against the mean percentage of antioxidant activity obtained from the three replicate tests. The free radical scavenging activity of ascorbic acid (Vitamin C) was also measured under the same condition to serve as a reference standard (positive control).

Data handling: The results are expressed as mean±SEM (Standard error of mean) of the EC_{50} values obtained from three determinations. The plots used showed a good coefficient of determination ($r^2 \geq 0.829$).

Isolation of the antioxidant constituents of the BuOH Fraction of *A. laxiflora*: Accelerated Gradient Chromatography was used for the fractionation of the BuOH fraction on silical gel (230-400 mesh) using an increasing gradient of dichloromethane in toluene; up to 100% of dicholoromethane. Followed by an increasing gradient of EtOAc in dicholoromethane up to 100% and lastly with an increasing gradient of MeOH in dicholoromethane up to 100%. This afforded six fractions coded (4A-4F). Further fractionation of 4E on AGC using an increasing gradient of acetone in toluene up to 100% acetone. This yielded two factions coded (7A and 7B). Purification of 7A on Sephadex LH-20 column chromatography involved isocratic elution with a mixture of dichloromethane –toluene (3:7). This was followed by an increasing gradient of acetone on the mixture up to 100%. The fractions obtained were bulked into seven fractions coded (8A-8G). Fractions 8C and D have the same TLC profile and demonstrated activity with DPPH on spraying. These were bulked together and further purified on AGC using increasing gradient of EtOAc in hexane up to 100%. 37 tubes of 15ml each were collected and analysed by TLC using dichloromethane –acetone(2:3). Tubes containing only one spot were bulked together and concentrated to dryness *in vacuo*. This yielded white powder (compound 1).

Fraction 8E which also demonstrated considerable activity on spraying with DPPH was further fractionated on AGC using increasing gradient of EtOAc in hexane up to 100%. 33 tubes of 15ml each were collected and analysed by TLC using dichloromethane-acetone (2:3). Tubes 26-33 had only one spot, these were concentrated *in vacuo* and afforded a yellow powder compound 2. Both compounds rapidly bleached DPPH on spraying.

Table] 1: EC_{50} Values of *A. laxiflora* extract

Sample	EC_{50} ($\mu g\ mL^{-1}$)±SEM
Crude extract	106.74±0.29
Ethyl acetate fraction	12.97±0.30
Butanol fraction	24.34±0.33
Ascorbic acid	4.78±0.25

Identification of the active compounds isolated

Compound 1: TLC characteristics: (R_f values), 100% EtOAc (0.3), dichloromethane-acetone 2:3 (0.4). It showed purple and brown colours under UV light at 254 and 366nm respectively. It appeared yellow on TLC silica plate and when sprayed with vanillin – sulphuric acid, it gave a pink colour. ^{1}H-NMR and ^{13}C-NMR (Table 2). M/Z = 304 $[MH]^+$. It was established as taxifolin glycoside.

Compound 2: TLC characteristics: (R_f values), 100%EtOAc (0.4), toluene-MeOH 2:1 (0.6). A deep yellow colour was observed when sprayed with vanillin – sulphuric acid on silica TLC plate. ^{1}H-NMR and ^{13}C-NMR see Table 2. M/Z = 302 $[MH]^+$. It was established as quercitrin.

RESULTS AND DISCUSSION

As shown in Table 1, the three test samples showed radical scavenging activity. The scavenging activity order of the test samples was ethyl acetate > butanol > crude. This trend could be attributed to the total concentration of polyphenolic constitiuents present in the different test samples. Earlier studies by Ogundipe *et al.*, [7] have led to the isolation of six bioactive flavonoid compounds: rutin, quercetrin quercetin and three quercetin derivatives from the ethyl acetate soluble fraction of the methanolic extract of *A. laxiflora*. Thus the various substituted isolated flavonoids presumably contributed to the observed higher antioxidant activity of this fraction. The lower activity, compared to ethyl acetate, observed in the more polar butanol fraction activity could be adduced to the lower concentration of flavonoid namely taxifolin and quercetrin present in the butanol fraction. Free radical scavenging is one of generally accepted mechanisms against lipid oxidation. The effect of antioxidants on DPPH radical scavenging was thought to be due to their hydrogen donating ability

Table 2: NMR Data for Compound 1 and 2 (300MHz ^{1}H-NMR, 75 MHz ^{13}C- NMR MeOH-d_4)

	Compound 1 (taxifolin glycoside)			Compound 2 (quercitrin)	
Position	^{1}H	HMBC correlation	^{13}C	^{1}H	^{13}C
2	5.14 (d, 1H, J=10.60Hz)	H-2', H-3	83.8		157.5
3	4.82 (d, 1H, 10.60Hz)	H-2	76.3		135.2
4		H-2, H-3	196.1		178.6
5			165.4		158.3
6	5.92 (d, 1H, J=2.00Hz)	H-8	97.4	6.38 (d,1H,J=2.05)	98.8
7		H-6, H-8	169.0		164.9
8	5.94 (d, 1H,J=2.00Hz)	H-6	96.4	6.21(d,1H,J=2.05)	93.74
9		H-2, H-6, H-8	164.3		162.2
10		H-6, H-8	102.4		104.9
1′		H-6', H-3, H-2	128.9		122.0
2′	6.87 (dd, 1H, J=1.70,8.00Hz)	H-5', H-6', H-2	115.7	7.35 (d,1H,J=2.0Hz)	115.4
3′	6.87 (dd,1H, J=8.00Hz)	H-2', H-5', H-6'	146.5		145.4
4′		H-5', H-6', H-2'	147.4		148.8
5′			116.3	6.93 (d,1H,J=8.2Hz)	116.0
6′	6.99 (d,1H,J=1.70Hz)	H-2', H-2	120.7	7.32(dd,1H,J=2.0, 8.2 Hz)	121.9

Coupling constants are between parentheses

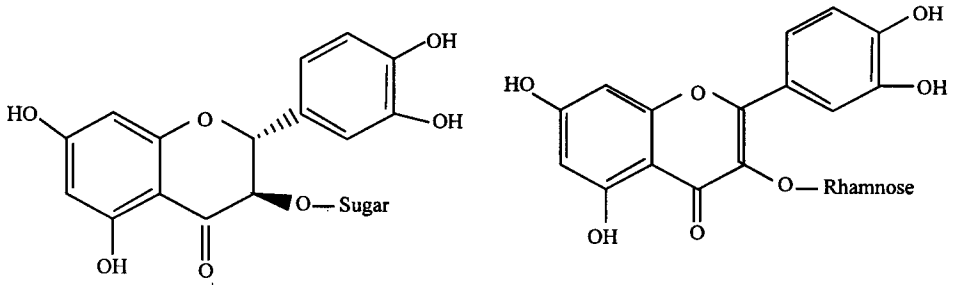

Fig. 1: Structures of the isolated compounds

[18]. Numerous studies on structure-activity relationship of flavonoids have shown that the antioxidative activity of group of compounds is related to the substitution pattern of their hydroxyl groups and the presence of a second hydroxyl group in the *ortho* or *para* position is known to increase the antioxidative activity due to additional resonance stability and *o*-quinone or *p*-quinone formation[19,20]. Thus the higher antioxidant activity of the ethyl acetate fraction could possibly be adduced to the fact that the fraction contains higher preponderance of free radical scavenging agents, quercetin, quercetrin rutin which are all have the catechol moiety in the B whereas in the butanol fraction there are only two constituents with this property and the absence of C2C3 double which enhances activity [22] in one of the two constituents may be responsible for the lower activity observed. In may be argued therefore that the occurrence of compounds with high free radical scavenging ability in the ethyl acetate [7] and butanol fractions of *A. laxiflora* leaf extract as observed in the present study justifies its local use as a preservative for food products as well as its potential use in ethnomedicine in the management of inflammatory and other oxidative stress induced perturbations. However, it will be necessary to carry out further study in order to evaluate the synergistic effect of the different phenolic compounds contained in *A. laxiflora* leaf extract.

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