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ISOLATION OF TWO FLAVONOIDS FROM *BAUHINIA MONANDRA* (KURZ) LEAVES AND THEIR ANTIOXIDATIVE EFFECTS

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Abstract

Bauhinia monandra (Kurz), traditionally use in the treatment of diabetes with established significant anti-diabetic activity was investigated for its antioxidant constituents since the activity demonstrated can be linked to the presence of antioxidant compounds. Bioassay directed fractionation of the ethyl acetate soluble leaves extract has led to isolation of two active compounds identified as: Quercetin-3-O-rutinoside (**1**) and Quercetin (**2**). The molecular structures elucidations of both compounds were carried out using spectroscopic studies (^{1}H NMR, ^{13}C NMR and MS). These compounds are reported from this species for the first time. A DPPH spectrophotometric assay was used to evaluate the antioxidant potential of the compounds. Compound **2** had higher antioxidant activity while Compound **1** had lower activity than L-ascorbic acid which was used as standard.

Key words: *Bauhinia monandra*; antioxidant activity; flavonoid; DPPH.

Introduction

Flavonoids constitute a major group of phenolic compounds in plants. They provide pigmentation for fruits, flowers and seeds to attract pollinators and seeds dispersers. They assist in plant defense against pathogenic microorganism (Schijlen et al., 2004, Parr and Bowell, 2000). The number of flavonoids is constantly increasing due to the structural variation associated with these compounds. It is well known that

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antioxidant activity in higher plants has often been associated with phenolic compounds (Thabrew et al., 1998).

Bauhinia monandra (Kurz) belongs to the family Fabaceae. It is traditionally used for the treatment of diabetes and as a diuretic (Argolo et al., 2004). Freshly crushed leaves are also used to treat stonefish stings (Hansworth, 1990). Pharmacological reports have shown that the ethanol extract of the leaves exhibited hypoglycaemic activity (Coelho and Silva 2000). Subsequent fractionation of the ethanol extract led to isolation of one of the active compounds which was identified as galactose-specific lectin. (Coelho and Silva, 2000). Oral administration of the stem bark extract in rats (1g/kg), exhibited significant anti-diabetic activity (Abo and Jimoh, 2004). Methanol leaf extracts had no antimicrobial activity against *B. substilis*, *E. coli*, *S. aureus* and *P. aeruginosa* (Binutu, 1986).

Many plant constituents are effective as remedy for some diseases and accounts for large number of pharmaceutical important compounds in Western Pharmacopoeia and a number of important drugs. For example, taxol and artermisinin were reported from plants (Tshibangu *et al.*, 2002). In our quest to finding a novel antioxidant agents from plants, we have carried out bioactivity directed phytochemical study on the leaves extract of *B. monandra* to isolate and identify the antioxidant constituents because anti-diabetic activity has been linked to antioxidant compounds (McCune and Johns, 2002). Antioxidant potential of the isolated compounds was also evaluated using a DPPH free radical scavenging assay.

Material and Methods

General

Spectroscopic data were obtained from the following instruments: UV- Versa-max® microplate reader, NMR – Varian (^{1}H 300 MHz, ^{13}C 75 MHz),
Electron impact mass spectra (EI-MS**) –** Shimadzu 2010 gcms. L-ascorbic acid (Merck), DPPH 2, 2-diphenyl-1-picryl hydrazyl (Sigma) and absolute methanol (Fluka).
All other chemicals used were of analytical grade obtained from BDH Chemicals Ltd, Poole England and Sigma chemical Co. USA.

Plant material

Leaves of *B. monandra* were collected at the Obafemi Awolowo University, Ile-Ife, Nigeria, in February 2005. Collected leaves were air dried at room temperature for three weeks. Dr. H.C. Illoh of the Department of Botany, Obafemi Awolowo University, Ile-Ife, authenticated the plant. A voucher specimen was deposited at the Herbarium of the Faculty of Pharmacy, Obafemi Awolowo University with herbarium number FPI 107.

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Extraction

Finely ground leaves (700 g) were extracted with methanol (MeOH). The extract was concentrated to dryness *in vacuo* at 40 ^{0}C to remove the methanol. The aqueous extract was made and successively partitioned with hexane (Hex.), dichloromethane (DCM), ethyl acetate (EtOAc) and butanol (BuOH). The combined organic layer of each partition was evaporated to dryness *in vacuo* at 40 ^{0}C using rotary evaporator to afford Hex., DCM, EtOAc and BuOH fractions, Table 1.

Isolation of the compounds

The EtOAc fraction (5.40 g) was fractionated on silica gel column chromatography using an increasing gradient of EtOAc in chloroform ($CHCl_3$) up to 100%, followed by an increasing gradient of MeOH up to 100%. This gave five pooled fractions A_1 – E_1. Purification of E_1 (40.0 mg) on Sephadex column chromatography starting with DCM - MeOH (7.5:2.5) followed by increasing gradient of MeOH up to 70% afforded compound **1** (19.0 mg). Combination of fractions B_1 and C_1 (330.0 mg) and subsequent fractionation on Sephadex LH-20 using toluene (Tol.) -MeOH (4:1) gave compound **2** (92.0 mg).

Fractionation of BuOH fraction (8.00g) on Sephadex LH-20 column starting with Tol.-EtOH (1: 1) followed by increasing gradient of EtOH up to 100% also afforded compound **1** (240.0 mg).

Structure Elucidation

Quercetin-3-O-rutinoside (**1**). ^{13}C-NMR (75 MHz, DMSO- d_6). 156.4 (C2), 133.3 (C3), 177.4 (C4), 161.2 (C5), 98.7 (C6), 164.2 (C7), 93.6 (C8), 156.6 (C9), 103.9 (C10), 121.2 (C1`), 115.2 (C2`), 144.8 (C3`), 148.4 (C4`), 116.3 (C5`), 121.6 (C6`), 101.2 (C1``), 74.1 (C2``), 76.4 (C3``), 70.6 (C4``), 75.9 (C5``), 67.0 (C6``), 100.8 (C1```), 70.4 (C2```), 70.0 (C3```), 71.8 (C4```), 68.3 (C5```), 17.8 (C6```).

^{1}H NMR (300 MHz, DMSO-d_6): δ 3.03 - 3.71 {m, rhamnoglucosyl (rutinosyl) - Hs}, 4.37 (1H, s, rhamnosyl, H-1``), 5.34 (1H, d, J = 6.9 Hz, glucosyl, H-1```), 6.18 (1H, d, J = 1.2 Hz, H-6), 6.37 (1H, bs, H-8), 6.84 (1H, d, J = 8.1 Hz, H- 5'), 7.54 (2H, d, H-2' and H-6'), 12.59 (1H, s, 5-OH). EI-MS: m/z 302 {[MH - rutinosyl]$^+$, 100%}, due to loss of sugar (Markham 1982). The spectra data were in agreement with that of quercetin-3-O-rutinoside reported in the literature (Harborne and Mabry, 1982; Markham 1982).

Quercetin **(2).** ^{13}C-NMR (75 MHz, DMSO- d_6). 146.8 (C2), 135.8 (C3), 175.9 (C4), 160.7 (C5), 98.2 (C6), 163.9 (C7), 93.4 (C8), 156.2 (C9), 103.0 (C10), 122.0 (C1`), 115.1 (C2`), 145.1 (C3`), 147.7 (C4`), 115.6 (C5`), 120.0 (C6`).

^{1}H NMR (300 MHz, DMSO-d_6): 6.18 (1H, d, J = 1.8 Hz, H-6), 6.40 (1H, d, J = 2.1 Hz, H-8), 6.89 (1H, d, J = 8.4 Hz, H- 5'), 7.54 (1H, dd, J = 2.1 and 8.7 Hz, H-6'), 7.67 (1H, d, J = 2.1 Hz, H- 1'), 12.49 (1H, s, 5-OH). EI-MS: m/z 302 {[M]$^+$, 100%}. The

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spectra data were in agreement with that of quercetin reported in the literature (Markham, 1982).

Antioxidant activity

Qualitative assay screening entailed spraying the TLC chromatograms of the partitioned fractions and the crude extract with 0.2% DPPH in MeOH. This revealed the antioxidant behaviour of the extracts. This was also repeated for the two compounds isolated.

Quantitative antioxidant activity was determined spectrophotometrically as described by Mensor et al., 2001, with some modifications. Briefly, the reactions were carried out in 96-well microtitre plates and each compound was tested at 100.0, 50.00, 25.00, 12.50, 6.25 and 3.13 μM. Twenty micro liters of 0.3 mM DPPH in methanol was added to 50 μL of each concentration of sample tested and allowed to react at room temperature in the dark for thirty minute. Blank solutions were prepared with sample solution (50 μL) and 20 μL of methanol only while the negative control was DPPH solution, 20 μL plus 50 μL methanol. The decrease in absorbance was measured at 515 nm on a microplate reader. Values obtained were converted to percentage antioxidant activity (AA%) using the formula:

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

(Abs_{sample} is the absorbance of the sample, Abs_{blank} is the absorbance of the blank and $Abs_{control}$ is the absorbance of the control). L-ascorbic acid (vitamin C) was used as a positive control (antioxidant agent).

The EC_{50} value, defined as the concentration of the sample leading to 50% reduction of the initial DPPH concentration, was calculated from the linear regression of plots of concentration of the test compounds (μM) against the mean percentage of the antioxidant activity obtained from the three replicate assays.

Statistical analysis

The results were expressed as mean ± SEM and the EC_{50} values obtained from the regression plots (Sigma Plots^R 2001, SPSS Science) had a good coefficient of correlation, ($r^2 \geq 0.955$).

Result and Discussion

Immediate bleaching of the purple DPPH colour by some constituents of EtOAc and BuOH fractions was observed on spraying TLC chromatograms of the solvent fractions of the crude extract with 0.2% DPPH in MeOH. The bleaching effect was also observed with the two compounds isolated.

The chemical structures of the two antioxidants: quercetin-3-O-rutinoside (**1**) and quercetin (**2**) were established by comparison of their spectral data with those reported in the literature (Harborne and Mabry, 1982; Markham 1982).

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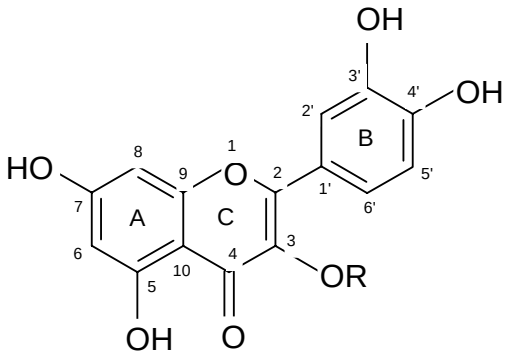
These compounds were different in their antioxidant activity in quantitative assay, (Table 2). Compound **2** had higher antioxidant activity while Compound **1** had lower activity than L-ascorbic acid which was used as standard.
Conditions for effective radicals scavenging activity in flavonoids observed from structure activity relationship studies include the presence of catechols group (3′-OH and 4′-OH) on ring B, the 3-OH group in combination with a C2 C3 double bond and keto group in position 4 (Gilbert et al., 2003, Harborne and Williams, 2000 and Saskia et al., 1996). These structural units fortify the antioxidant activity by increasing the stability of the flavonoids radical after donating phenolic hydrogen. The higher antioxidant activity of compound **2** compared to compound **1** could be due to the presence of free hydroxyl group in position 3 as both compounds have catechol group (3` and 4` di-OH) on ring B. It has been demonstrated that substitution of 3-OH reduces activity, (Op de Beck, 2003).

Conclusion

Many flavonoids have shown strong antioxidant properties (Harborne and Williams, 2000; Raj and Shalini, 1999). Quercetin-3-O-rutinoside (rutin) and quercetin have been established as strong antioxidant principles and had been used as standards in antioxidant experiments (Braca, et al., 2003; Mensor *et al.*, 2003; Thabrew et al., 1998).



R

1. rutinosyl
2. H

Figure 1: Antioxidants from *B. monandra*

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Table 1: Weights of the *B. monandra* leaves extracts

Extract	Weight (g)
Methanol extract	91.7
Hexane fraction	5.3
Dichloromethane fraction	4.1
Ethyl acetate fraction	5.6
n-BuOH fraction	29.2

Table 2: Antioxidant potential of *B. monandra* constituents

Sample	EC_{50} ± SEM (µM)
Quercetin-3-O-rutinoside (**1**)	16.11 ± 0.581
Quercetin (**2**)	10.64 ± 0.087
L-ascorbic acid	12.52 ± 0.187

The presence of these compounds in abundance in the leaves extracts of *B. monandra* could provide rationale for the use of this plant in folk medicine.

Our results confirmed the earlier qualitative antioxidant analysis of *B. monandra* leaves extracts in which three major antioxidant compounds (flavonoids and steroid) were identified, (Argolo et al., 2004). We have now isolated, characterised and evaluated the antioxidant potential of the flavonoid constituents.

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