Isolation and characterization of novel antioxidant constituent of *Croton zambesicus* leaf extract

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A 1, 1-diphenyl-2-picrylhydrazyl (DPPH) activity directed fractionation was used to target antioxidant constituents of the ethyl acetate fraction obtained from a 20% aqueous methanol crude extract of *Croton zambesicus* leaf. Repeated column chromatography of the fraction on silica gel and Sephadex LH-20 led to the isolation of a new natural product, identified as quercetin-3-O-β-6"(p-coumaroyl) glucopyranoside-3'-methyl ether, helichrysoside-3'-methyl ether (**1**), along with kaempferol-3-O-β-6"(p-coumaroyl) glucopyranoside, tiliroside (**2**) and apigenin-6-C-glucoside, isovitexin (**3**) as the antioxidant constituents. The structures of the isolated compounds were elucidated using spectroscopic techniques, namely NMR (1D and 2D) and mass spectrometry. Compounds **1** and **2** are reported from this species for the first time. In the qualitative antioxidant assay, the three isolated compounds instantly bleached the DPPH (0.2% MeOH) purple colour indicating antioxidant activity. In the quantitative antioxidant assay, all the isolated compounds demonstrated weak antioxidant activity compared to quercetin and rutin used as positive control antioxidant agents. The compounds displayed little to no cytotoxicity against Vero cells in an *in vitro* assay. The presence of these antioxidant compounds in the leaf extract of *Croton zambesicus* could provide rationale for the ethnomedicinal use of the plant, in management of oxidative stress related diseases in folk medicine.

Keywords: *Croton zambesicus*; antioxidant activity; DPPH; cytotoxicity; quercetin-3-O-β-6" (p-coumaroyl) glucopyranoside-3'-methyl ether, helichrysoside-3'-methyl ether.

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1. Introduction

Croton zambesicus Müll.Arg is a shrub or small tree widely distributed in tropical Africa. It serves as an ornamental plant which is often cultivated in towns and villages in Nigeria, and belongs to the family Euphorbiaceae (Burkill, 1985; Okokon, Ofodum, Ajibesin, Danladi, & Gamaniel, 2005). Phytochemical investigations of its leaf extract have revealed the presence of flavone-C-glycosides (Wagner, Hoerhammer, & Kimly, 1970), diterpenoids from the leaves and stem bark of the plant (Block et al., 2004; Block et al., 2002; Ngadjui, Abegaz, Keumedjio, Folefoc, & Kapche., 2000; Ngadjui et al., 1999) and essential oils from the leaf (Menut et al., 1995).

Croton zambesicus is associated with the management of type 2 diabetes mellitus and hypertension in Nigerian folk medicine (Ofusori et al., 2007; Okokon, Bassey, & Obot, 2006). A pharmacological report has confirmed the antidiabetic activity of the ethanolic leaf extract in alloxan-induced rats, where the activity demonstrated by the leaf extract was comparable to that of the standard drug chlorpropamide (Okokon, Bassey, & Obot, 2006). Antiplasmodial activity of the root and stem bark extracts of *C. zambesicus* has also been reported (Okokon & Nwafor, 2009; Tsamoc**,** Zolloa, Gut, & Rosenthald, 2009), while a diterpene ent-18-hydroxy-trachyloban-3-one isolated from *C. zambesicus* demonstrated vasorelaxant activity in a concentration dependent manner (Martinsen et al., 2010).

Diabetes in humans is associated with oxidative stress, leading to the generation of free radicals due to hyperglycemia. Body cells and tissues are adversely affected by free radicals, which can eventually lead to cancer, diabetes and other diseases. Accumulated studies have demonstrated that antioxidants scavenge these dangerous free radicals from the body and ameliorate oxidative stress conditions (Arts, Dallinga, Voss, Haenen, & Bast, 2003; Johansen, Harris, Rychly, & Ergul, 2005). Thus, activity demonstrated by *C. zambesicus* leaf extract could be linked to the presence of antioxidant compounds in its extract (Argolo, Sant'Ana, Pletsch, & Coelho, 2004; McCune & Johns, 2002; Thabrew, Hughes, & McFarlane, 1998).

In our continuing search for potent, safe and new antioxidant compounds from natural sources (Teffo, Aderogba, & Eloff, 2010; Aderogba, McGaw, Ogundaini, & Eloff, 2008; Aderogba, McGaw, Ogundaini, & Eloff, 2007; Aderogba, Ogundaini, & Eloff , 2006), due to the inability of our endogenous antioxidant defense systems to completely protect the body against excess free radicals produced in some conditions such as cigarette smoke, air pollutants

and UV radiation (Pietta, 2000), an investigation was undertaken to evaluate the possible antioxidant constituents of *C. zambesicus* with a view of ascertaining whether its antidiabetic property is related to its possible antioxidant properties. Antioxidant compounds were targeted using a 1, 1-diphenyl-2-picrylhydrazyl (DPPH) activity guided fractionation. Cytotoxicity of the isolated compounds against a representative cell line (Vero African Green monkey kidney cells) was performed using a tetrazolium-based colorimetric assay.

2. Results and Discussion

Qualitative antioxidant activity screening of the four solvent fractions of *C. zambesicus* revealed that the antioxidant constituents in the leaf crude extract were extractable into the ethyl acetate and butanol fractions. A DPPH activity directed fractionation of these fractions led to the isolation of three compounds: quercetin-3-O-β-6"(p-coumaroyl) glucopyranoside-3'-methyl ether, helichrysoside-3'-methyl ether, (**1**), kaempferol-3-O-β-6"(p-coumaroyl) glucopyranoside, tiliroside (**2**) and apigenin-6-C-glucoside, isovitexin (**3**). Compounds **1**-**3** instantly bleached the DPPH purple colour indicating antioxidant potential (free radical scavenging activity). In the quantitative antioxidant assay, all the isolated compounds demonstrated weak antioxidant activity compared to quercetin and rutin used as standard antioxidant agents. Only compound **3** scavenged 50% of DPPH at the highest concentration tested (200 µM) with EC_{50} = 189.06 ± 2.65, Compounds **1** and **2** scavenged only 26.78% and 25.96% of the DPPH free radicals respectively. Quercetin and rutin had EC_{50} of 5.313 ± 0.07 and 9.986 ± 0.05 µM. Based on structural activity studies, good antioxidant activity (effective free radical scavenging activity) requires the presence of a catechol group (3′, 4′ -OH) on ring B, along with the presence of 2, 3 unsaturation with 3-OH and a keto group in position 4 (OP de Beck, Cartier, David, Dijoux-Franca, & Mariotte, 2003; Saskia et al., 1996). None of the isolated compounds possessed all these structural requirements, and this could explain the weak antioxidant activity demonstrated by all the isolated compounds. Quercetin fulfilled all these structural requirements hence demonstrated highest antioxidant activity followed by rutin with substituted 3-OH. Compounds **1** and **2** are reported from this species for the first time and compound **1** is a new natural product. Compound **1**(65 mg) is a yellow amorphous powder, ESI mass spectrum (positive mode) showed the molecular ion peak as the base peak at m/z = 647.1 $[M+ Na]^+$ that was consistent with the molecular formula $C_{31}H_{28}O_{14}$. This was confirmed with the base peak from the negative mode at

m/z = 623.1 $[M-H]^+$. Its IR (DMSO) exhibited absorption bands as follows: ν_{max} 3532, 3351 and 1654 cm^{-1}. UV (MeOH) spectrum showed the λ_{max} absorptions at 215, 255, 267, and 315. In the ^1H NMR analysis of the compound, a methoxy singlet appeared at δ 3.90, the splitting pattern signals of protons assigned to the ring-B indicated 3', 4' substitution. The spectrum showed signals at δ 7.89 (1H, d, J = 2.1 Hz, H-2'), 7.55 (1H, dd, J = 2.1, 8.4 Hz, H-6') and 6.84 (1H, d, J = 8.7 Hz, H-5') along with two meta related protons of ring-A at δ 6.13 (1H, d, J=2.1 Hz) and 6.28 (1H, d, J=2.1 Hz). The p-coumaroyl group showed an AA'BB' system as two doublets at δ 7.29 (2H, d, J=8.7 Hz, H-3''' and 5''') and 6.80 (2H, d, J=8.4 Hz, H-2''' and 6'''). Another two doublets occurred at δ 7.40 (1H, d, J= 15.9 Hz, H-7''') and 6.09 (1H, d, J= 15.9 Hz, H-8''') of the trans-disubstituted double bond of the p-coumaroyl group. ^{13}C NMR and DEPT spectra of this compound revealed the presence of 29 carbons. These were classified as one OCH_3, six were assigned to a glucopyranosyl group, fifteen other carbons were assigned to 3', 4', 5, 7-tetra substituted flavonol nucleus and the remaining seven signals were identified as that of the p-coumaroyl group. The positions of attachment of the methoxy, glucopyranosyl and p-coumaroyl groups were established by HMBC correlations (Figure 1). Anomeric proton (H-1'') of the glucose correlated with C-3 (cross peak H-1''/ C-3) of the flavonol nucleus and its CH_2 (C-6) correlated with the C=O at position 9''' (cross peak H-6''/ C-9''') of the p-coumaroyl group. A methoxy group was at C-3' in agreement with the HMBC correlation (cross peak OMe/ C-3') of the flavonoid nucleus. This was also confirmed by correlation of only H-2' and H-5' with methoxylated carbon (C-3') and the entire three ring B protons (H-2', 5' and 6') with C-4' of the flavonol nucleus. A trans-p-coumaroyl group was assigned based on the following cross peaks H-7'''/ C-2''', C-6''', C-8''', C-9''' and H-8'''/ C-1''', C-9'''. Compound **1** was determined to be quercetin-3-O-β-6'' (p-coumaroyl) glucopyranoside-3'-methyl ether, (helichrysoside-3'-methyl ether) a new natural product. The ^{13}C NMR spectrum (MeOD, 75 MHz) δ 157.3 (C-2), 133.9 (C-3), 177.9 (C-4), 161.5 (C-5), 98.5 (C-6), 164.5 (C-7), 93.5 (C-8), 156.8 (C-9), 104.2 (C-10), 122.5 (C-1'), 112.8 (C-2'), 146.8 (C-3'), 149.4 (C-4'), 114.5 (C-5'), 121.7 (C-6'), 55.3 (C-3'-OCH_3), 102.6 (C-1''), 74.5 (C-2''), 76.6(C-3''),70.4 (C-4''), 74.4 (C-5''), 62.9(C-6''), 125.6 (C-1'''),129.8 (C-2''', 6'''), 115.44 (H-3''', 5'''), 159.7 (H-4'''), 145.2 (C-7'''),113.3 (C-8'''),167.4 (C-9'''). ^1H NMR spectrum (MeOD, 300 MHz): δ 7.86 (1H, d, J=2.1 Hz, H-2'), 7.55 (1H, dd, J=2.1, 8.4 Hz, H-6'), 6.84 (1H, d, , J= 8.7, H-5'), 6.28 (1H, d, J=2.1 Hz, H- 8), 6.13 (1H, d, J=2.1 Hz, H-6), 7.28 (1H, d, J=8.7 Hz, C-2''', 6'''), 6.80 (1H, d, J=8.4 Hz, C-3''', 5'''), 7.40 (1H, d, J=15.9 Hz,

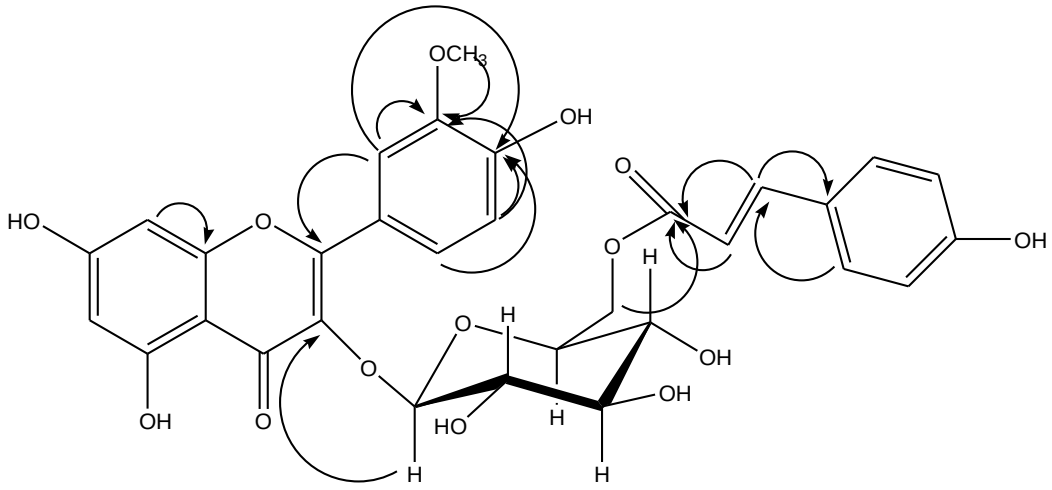
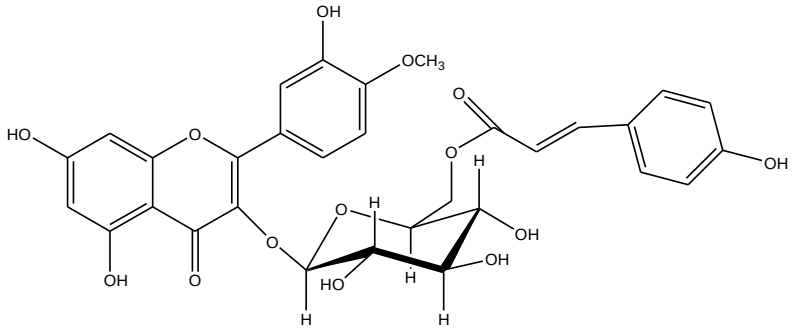


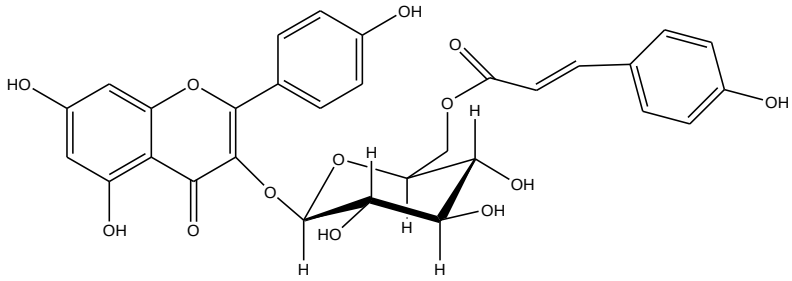
Figure 1: HMBC correlations of compound **1**

C-7'"), 6.09 (1H, d, J=15.9 Hz, C-8'"), 3.53-4.30 (glucosyl-H, m, H-2"- 6"), 5.37 (1H, d, J=7.8 Hz, H-1"). To the best of our knowledge, this natural product is reported for the first time, (Figures 1 and 2). Helichrysoside has been reported as a constituent of *Helichrysum stoechas* (Lavault & Richome, 2004) and *Helichrysum kraussii* (Candy, Laing, & Weeks, 1975).

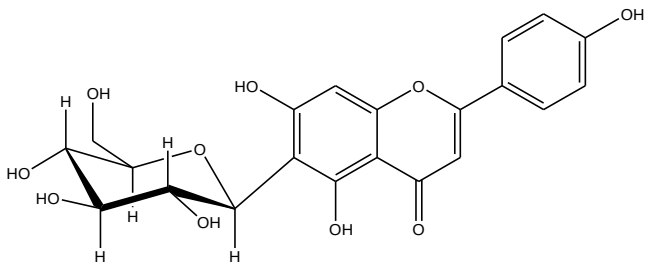
The cytotoxicity of the isolated compounds was measured using the commonly employed tetrazolium-based MTT assay. MTT added to the cells after incubation with test compound is reduced by mitochondrial succinate dehydrogenase to a water-insoluble blue formazan product, which can be dissolved in DMSO and measured spectrophotometrically. The number of viable cells is directly proportional to the amount of formazan (Mosmann, 1983). Owing to reports of some plant extracts and compounds such as flavonoids possessing the ability to non-specifically reduce MTT in the absence of cells (Bruggisser, Von Daeniken, Jundt, Schaffner, & Tullberg-Reinert, 2002; Shoemaker, Cohen, & Campbell, 2004), cell-free controls were included. The cells were also examined microscopically after incubation with the compounds and before addition of MTT. In this case, the cytopathic effect (CPE) evaluation under the microscope correlated well with the results of the MTT assay. Isovitexin was not cytotoxic in the MTT assay at the highest concentration tested (200 µg/ml), and also showed no visible effect on the cells. Tiliroside and helichrysoside-3'-methyl ether showed average LC_{50} values of 169.45 and 189.54 µg/ml respectively. These two compounds showed slight CPE only at the highest concentration of 200 µg/ml tested, confirming the MTT assay results. The presence of little to no cytotoxicity



Compound **1**



Compound **2**



Compound **3**

Figure 2: Isolated compound from *C. zambesicus*

of the isolated constituents may be confirmed with tests on a variety of cell types, and *in vivo* toxicity studies are important before further development.

Many studies have demonstrated beneficial effects of exogenous antioxidants in reducing free radical generation and removing free radicals from the body, hence, protecting antioxidant defenses in management of oxidative stress diseases like diabetes (Aruoma, Neergheen, & Bahorun, 2006-07; Kaneto et al., 1999; Pietta, 2000). In addition, exogenous antioxidants are also required to maintain good health, due to the inability of our endogenous defense systems to completely protect the body against excess free radicals produced in some conditions such as

cigarette smoke, air pollutants and UV radiation (Pietta, 2000). The presence of these antioxidant compounds in the leaf extract of *C. zambesicus* investigated could provide rationale for the ethnomedicinal use of the plant in management of oxidative stress related diseases in Nigerian folk medicine.

3. EXPERIMENTAL

3.1 General

All solvents used were laboratory grade reagents and were distilled prior to use. All thin layer chromatography analyses were performed at room temperature using pre-coated plates (MERCK, silica gel 60 F_{254} 0.2 thickness). Detection of spots was performed by viewing under Ultraviolet light (254 and 365 nm). Open column chromatography was carried out using silica gel 60 (MERCK). Infra red spectrum was obtained on a FT-IR Spectrometer, PerkinElmer Spectrum 100 Series and GBC UV/VIS 920 spectrophotometer for UV spectrum. Multiskan® spectrum (Thermo Electron Corporation) was used as microplate reader. Nuclear magnetic resonance (NMR) ^{1}H (300 MHz) and ^{13}C (75 MHz) spectra were recorded on a Bruker Ultra Shield spectrometer. Chemical shifts are expressed in parts per million (ppm). The Electron Spray Ionization (ESI) mass spectrum was recorded on a Finnigan LCQ Deca spectrometer.

3.2 Plant material

The leaves of *Croton zambesicus* were collected beside the main gate of Obafemi Awolowo University, Ile-Ife, Nigeria, in October, 2007. The plant was identified by Mr. O. Oladele of the Herbarium Section, Faculty of Pharmacy and the voucher specimen (UHI 16183) is deposited in the University Herbarium at the Department of Botany, Obafemi Awolowo University, Ile-Ife. The collected plant material was air dried for 2 weeks and powdered.

3.2.1 Extraction

The powdered leaves of *C. zambesicus* (700 g) were extracted with 20% aqueous methanol (10 L) at room temperature for 24 hours and filtered. The crude extract was concentrated *in vacuo* at 40 ºC to about ⅓ of the original volume. This afforded the crude extract of the plant.

3.2.2 Solvent partioning of the crude extract

Concentrated crude extract of *C. zambesicus* was in turn extracted separately with n-hexane (3 x 1 L), dichloromethane (3 x 1 L), ethyl acetate (3 x 1 L) and finally n-butanol (3 x 700 mL). The solvent fractions were concentrated to dryness *in vacuo* to afford four solvent extracts; hexane, dichloromethane, ethyl acetate and butanol fractions.

3.3 Qualitative screening for the antioxidant compounds

DPPH assay: The solvent fractions of the crude extract were spotted on TLC plates and developed with appropriate solvent systems as follows: 100% Hexane for hexane fraction, 100% dichloromethane for dichloromethane fraction, Chloroform/methanol (9:1) for ethyl acetate fraction and Chloroform/methanol (7.5:2.5) for butanol fraction. Each plate was subsequently sprayed with 0.2% DPPH solution in methanol. Some of the constituents of the more polar solvents (ethyl acetate and butanol) instantly bleached the purple colour of the DPPH which indicated antioxidant activity. These fractions were selected for phytochemical investigation.

3.4 Isolation of the antioxidant compounds

The ethyl acetate fraction (6.0 g) was fractionated on a silica gel column. The column was eluted with hexane followed by an increasing gradient of chloroform up to 100% and followed in turn by an increasing gradient of methanol up to 100%. The fractions collected were analysed on TLC plates using chloroform / methanol (9:1) as the solvent system. This afforded four fractions, A_1-D_1. Fraction A_1 (1.0 g) was purified on a Sephadex LH-20 column using chloroform / methanol (7.5:2.5). On analysis of the fractions on TLC plates using chloroform / methanol (9:1), four fractions (A_2-D_2) were collected. Fraction B_2 contained a single spot, which afforded compound **2** (80 mg). Sephadex LH-20 column fractionation of A_2 (500 mg) using ethyl acetate / methanol (9:1), followed by an increasing gradient of methanol up to 50%, yielded two fractions (A_3-B_3) after analysis by TLC using chloroform / methanol (4:1) as the solvent system. Fraction B_3 (380 mg) was further purified on a Sephadex LH-20 column using chloroform / methanol (9:1) followed by an increasing gradient of methanol up to 60%. Four fractions (A_4-D_4) were obtained after TLC analysis using chloroform / methanol (4:1). Fraction C_4 contained a single spot, and this afforded compound **1**. Fraction B_1 (270 mg) was fractionated on a Sephadex LH-20 column using chloroform / methanol (9:1) followed by an increasing gradient of methanol up to 100%.

Two fractions (A_5-B_5) were obtained after analysis on TLC plates using chloroform / methanol (4:1). Fraction B_5 (185 mg) was purified on a Sephadex LH-20 column using ethyl acetate / methanol (9:1) followed by an increasing gradient of methanol up to 100%. Three fractions (A_6-C_6) were obtained after TLC analysis using chloroform / methanol (7:3). Fraction B_6 contained a single spot which was taken as compound **3** (60 mg). Fractionation of the butanol fraction on a silica gel column and subsequent purification of the fractions obtained also yielded compound **3**.

3.5 Structure elucidation of isolated compounds

Structure elucidation of the isolated compounds was carried out using spectroscopic techniques: mass spectrometry, ^{1}H and ^{13}C NMR, and DEPT together with 2D experiments (COSY, HMQC and HMBC).

3.5.1 Compound **2** was identified as kaempferol-3-O-β-6" (p-coumaroyl) glucopyranoside, tiliroside (**2**). ESI mass spectrum (positive mode) showed the molecular ion peak as the base peak at m/z = 617.1 $[M+Na]^+$ that was consistent with the molecular formula $C_{30}H_{26}O_{13}$. This was confirmed with the prominent peak from the negative mode with m/z = 593.1 $[M-H]^+$. The ^{13}C NMR spectrum (CD_3OD, 75 MHz): δ 157.9 (C-2), 133.8 (C-3), 178.0 (C-4), 161.5 (C-5), 98.6 (C-6), 164.5 (C-7), 93.4 (C-8), 156.9 (C-9), 104.2 (C-10), 121.3 (C-1'), 130.9 (C-2', 6 '), 114.6 (C-3', 5'), 160.1 (C-4'), 102.6 (C-1"), 74.4 (C-2"), 74.3 (C-3"),70.3 (C-4"), 76.6 (C-5"), 62.9 (C-6"), 125.7(C-1'"), 129.8 (C-2'", 6'"), 115.4 (C-3'", 5'"), 159.8 (C-4'"), 145.1 (C-7'"),113.3 (C-8'"),167.4 (C-9'"). ^{1}H NMR spectrum (MeOD, 300 MHz): δ 7.89 (2H, d, J=9.0 Hz, H-2', 6'), 6.69 (4H, t, H-3', 5' and 3'", 5'"), 6.19 (1H, d, J=2.1 Hz, H- 8), 6.02 (1H, d, J=2.1 Hz, H-6), 7.18 (2H, d, J=9.0 Hz, H-2'", 6'"), 6.69 (4H, t, H-3', 5' and 3'", 5'"), 7.32 (1H, d, J=15.9 Hz, H-7'") , 5.99 (1H, d, J=15.9 Hz, H-8'"), 5.16 (1H, d, J=7.5 Hz, H-1"), 3.40-4.30 (glucosyl-H, m, H-2"-6"). Tiliroside has recently been reported from *Croton caudatus* (Zou et al., 2010). It was also identified as a minor phytochemical constituent of the ethyl acetate fraction of *Croton tonkinensis,* using reserved-phase high performance liquid chromatography (RP HPLC) coupled with a photodiode array (PDA) detector (Giang, Lee, & Son, 2004), (Figure 2).

3.5.2 Compound **3** was characterized as apigenin-6-C-glucoside, isovitexin. ESI mass spectrum (positive mode) showed the molecular ion peak as the base peak at m/z = 455.1

$[M+Na]^+$ that was consistent with the molecular formula $C_{21}H_{20}O_{10}$. This was confirmed with the peak from the negative mode at m/z = 431.0 $[M-H]^+$. The ^{13}C NMR spectrum (CD_3OD, 75 MHz): δ 164.7 (C-2), 102.4 (C-3), 182.6 (C-4), 160.6 (C-5), 107.7 (C-6), 163.4 (C-7), 93.8 (C-8), 157.2 (C-9), 103.8 (C-10), 121.6 (C-1'), 128.0 (C-2', 6 '), 115.6 (C-3', 5'), 161.3 (C-4'), 73.8 (C-1"), 71.2 (C-2"), 78.7(C-3"),70.4 (C-4"), 81.2(C-5"), 61.5(C-6"). ^1H NMR spectrum (MeOD, 300 MHz): δ 7.92 (2H, d, J=8.4 Hz, H-2', 6'), 6.92 (2H, d, J= 8.4 Hz H-3', 5'), 6.79 (1H, s, H- 3), 6.52 (1H, H-8), 4.93 (1H, bs, H-1"), 3.48-4.20 (glucosyl-H, m, H-2"- 6"). This compound had previously been reported from this species (Wagner, Hoerhammer, & Kimly, 1970). The spectroscopic data were in agreement with the literature (Haribal & Renwick, 1998), (Figure 2).

3.6 Evaluation of antioxidant potential of the isolated compounds

Quantitative antioxidant activity of the isolated compounds was carried out as previously reported (Aderogba et al., 2007). Different concentrations (200, 100, 50, 25, 12.5, 6.25, 3.125 and 1.563 µM) of each of the isolated compounds were prepared in triplicate in microtitre plates. Fifty microlitres of each compound were mixed with 20µL of 0.25mM DPPH in methanol. Absorbance was read at 515 nm after allowing the mixture to react in the dark for 30 minutes. Blank solutions were prepared with 50 µL of the sample solution and methanol (20 µL) only. The negative control was 20µL of DPPH solution and 50µL of methanol. Percentage antioxidant activity was calculated 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 negative control. Quercetin and rutin were used as the positive controls (antioxidant agents). EC_{50} values were calculated from the linear regression of plots of concentration of the compound against the mean percentage of antioxidant activity.

3.7 Cytotoxicity evaluation of the isolated compounds

The isolated compounds were tested for cytotoxic effects using a tetrazolium-based colorimetric assay (Mosmann, 1983). The compounds were incubated in quadruplicate at several concentrations with African green monkey (Vero) cells at 37 ºC in a 5% CO_2 incubator for 5 days (Aderogba, McGaw, Ogundaini, & Eloff, 2007; McGaw, Steenkamp, & Eloff, 2007). Each experiment was repeated three times. Untreated cells, cell-free controls and a positive control

(berberine chloride, Sigma) were included in each assay. After incubation, the cells were washed with PBS and fresh cell culture medium (Minimum Essential Medium, MEM) was added to the cells. Thirty µl MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide, Sigma, stock solution of 5 mg/ml in PBS) was then added to each well and the plates were incubated for 4 h at 37 ºC. Following incubation, the cell culture medium was aspirated from the cells. The MTT formazan crystals formed by actively growing cells were then dissolved by adding 50 µl DMSO. The amount of MTT reduction was measured by detecting absorbance in a microplate reader (VersaMax, Molecular Devices) at 570 nm. The wells in column 1, with medium and MTT but no cells, were used to blank the plate reader. LC_{50} values were calculated as the concentration of test compound causing a 50% reduction of absorbance compared to untreated cells. The cells were examined microscopically prior to washing and adding MTT to confirm the presence or absence of a cytopathic effect on the cells at the different concentrations of antioxidant compounds tested.

Acknowledgements

Aderogba is grateful to the Organisation for the Prohibition of Chemical Weapons (OPCW) for a Research Fellowship to the University of Botswana. McGaw acknowledges the University of Pretoria for a Research Fellowship.

References

Aderogba, M. A., McGaw, L.J., Ogundaini A.O., & Eloff, J.N. (2008). Cytotoxicity study of antioxidant flavonoids from *Bauhinia tomentosa* leaf extract. *Nigerian Journal of Natural Products and Medicine*, 12, 50-54.

Aderogba, M.A., McGaw, L. J., Ogundaini, O.A., & Eloff, J.N. (2007). Antioxidant activity and cytotoxicity study of the flavonol glycosides from *Bauhinia galpinii*. *Natural Product Research*, 21, 591- 599.

Aderogba, M.A., Ogundaini, O.A, & Eloff, J.N. (2006). Isolation of two flavonoids from *Bauhinia monandra* (Kurz) leaves and their antioxidative effects. *African Journal of traditional, Complementary and Alternative Medicine* 3, 59 – 65.

Argolo, A.C.C., Sant'Ana, A.E.G., Pletsch, M., & Coelho, L.C.B.B., (2004).Antioxidant activity of leaf extracts from *Bauhinia monandra*. *Bioresources Technology*, 95, 229–233.

Arts, M.J.T.J., Dallinga, J.S., Voss, H., Haenen, R.M.M., & Bast, A. (2003). A critical appraisal of the use of the antioxidant capacity (TEAC) assay in defining optimal antioxidant structures. *Food Chemistry* 80, 409 – 414.

Aruoma, O.I., Neergheen, V.S., Bahorun, T., & Jen. L-S. (2006-07). Free Radicals, Antioxidants and Diabetes: Embryopathy, Retinopathy, Neuropathy, Nephropathy and Cardiovascular Complications. *Neuroembryol Aging*, 4, 117–137.

Boyoma, F.F., Kemgnea, E.M., Tepongninga, R., Ngouanaa, V., Fon Mbachamb, W., Tsamoc**,** E**.,** Zolloa, P.H.A., Gut, J., & Rosenthald, P. J. (2009). Antiplasmodial activity of extracts from seven medicinal plants used in malaria treatment in Cameroon. *Journal of Ethnopharmacology*, 123, 483-488.

Block, S., Baccelli, C., Tinant, B., Van Meervelt, L., Rozenberg, R., Habib Jiwan, J-L., Llabrès, G., De Pauw-Gillet, M-C., & Quetin-Leclercq, J. (2004). Diterpenes from the leaves of *Croton zambesicus. Phytochemistry*, 65, 1165-1171.

Block, S., Stevigny, C., De Pauw-Gillet, M.C., De Hoffmann, E., Llabres, G., Adjakidle, V., & Quetin-Leclercq, J. (2002). ent-Trachyloban- 3b-ol, a new cytotoxic diterpene from *Croton zambesicus*. *Planta Medica*, 68, 647-649.

Bruggisser, R., Von Daeniken, K., Jundt, G., Schaffner,W., & Tullberg-Reinert, H., (2002). Interference of plant extracts, phytoestrogens and antioxidants with the MTT tetrazolium assay. *Planta Medica*, 68, 445–448.

Burkill, H.M. (1985). *The useful plants of west tropical Africa*, Royal Botanic Gardens, Kew Vol. 2.

Candy H.A., Laing, M., & Weeks, C.M. (1975). The crystal and molecular structure of Helichysoside, a new acylated flavonoid glycoside from *Helichrysum kraussii. Tetrahedron Letters*, 14, 1211-1214.

Giang, P. M., Lee, J. J., & Son. P. T. (2004). Flavonoid glucoside from the leaves of *Croton tonkinensis* Gagnep Euphobiaceae. *Vietnam Journal of Chemistry* 42, 125-128.

Haribal, M. & Renwick J. A. A. (1998). Isovitexin 6”-O-β-D-glucopyranoside: A feeding deterrent to *Pieris napi oleracea* from *Alliaria petiolata. Phytochemistry*, 47, 1237-1240.

Johansen, J.S., Harris, A.K., Richly, D. J., & Ergul, A. (2005). Oxidative stress and the use of antioxidants in diabetes: Linking basic science to clinical practice. *Cardiovascular Diabetology*, 4, 5.

Kaneto, H., Kajimoto, Y., Miyagawa, J., Matsuoka, T., Fujitani, Y., Umayahara, Y., Hanafusa, T., Matsuzawa, Y., Yamasaki, Y., & Hori, M. (1999). Beneficial Effects of Antioxidants in Diabetes: Possible Protection of Pancreatic b-Cells against glucose toxicity. *Diabetes*, 48, 2398-2406.

Lavault, M. & Richome, P. (2004). Constituents of *Helichrysum stoechas* variety *olonnense. Chemistry of Natural Compounds,* 40, 118-121.

Martinsen, A., Baccelli, C., Navarro, I., Abad, A., Quetin-Leclercq, J., & Morel, N. (2010). Vascular activity of a natural diterpene isolated from *Croton zambesicus* and of a structurally similar synthetic trachylobane. *Vascular Pharmacology*, 52, 63-69.

McCune, L.M. & Johns, T. (2002). Antioxidant activity in medicinal plants associated with the symptoms of diabetes mellitus used by the Indigenous people of the North American boreal forest. *Journal of Ethnopharmacology,* 82, 197 – 205.

McGaw, L.J., Steenkamp, V., & Eloff J.N. (2007). Evaluation of a widely used bush tea (*Athrixia phylicoides*) for cytotoxicity, antioxidant activity, caffeine content and presence of pyrrolizidine alkaloids. *Journal of Ethnopharmacology*, 110, 16-22.

Menut, C., Lamaty, G., Bassiere, J.M., Suleiman, A.M., Fendero, P., & Maidou, E. (1995). Aromatic plants of tropical Central Africa XXII, Volatile constituents of *Croton aubreville*, J Leonard and *C. zambesicus* Muell. Arg. *Journal of Essential Oil Research*, 7, 419-422.

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods,* 65, 55–63.

Ngadjui, B.T., Abegaz, B.M., Keumedjio, F., Gabriel N. Folefoc, G.N., & Kapche, G.W. F. (2002). Diterpenoids from the stem bark of *Croton zambesicus*. *Phytochemistry*, 60, 345-349.

Ngadjui, B. T., Folefoc, G.G., Keumedjio, F., Dongo, E., Sondengam, B.L., & Connolly, J.D. (1999). Crotonadiol, a labdane diterpenoid from the stem barkof *Croton zambesicus*. *Phytochemistry*, 51, 171-174.

Ofusori, D.A., Oluwayinka, O.P., Adelakun, A.E., Keji, S.T., Oluyemi, K.A., Adesanya, O.A., Ajeigbe, K.O., & Ayoka, A.O. (2007). Evaluation of the effect of ethanolic extract of *Croton zambesicus* on the testes of Swiss albino mice. *African Journal of Biotechnology*, 6, 2434-2438.

Okokon, J.E. & Nwafor, P.A.(2009). Antiplasmodial activity of root extract and fractions of Croton zambesicus. *Journal of Ethnopharmacology*, 121, 74–78.

Okokon, J. E., Bassey, A. L. & Obot, J. (2006). Antidiabetic activity of ethanolic leaf extract of *Croton zambesicus* Muell.(thunder plant) in alloxan diabetic rats. *African Journal of Traditional, Complementary and Alternative Medicine*, 3, 21-26.

Okokon, J.E., Ofodum, K.C., Ajibesin, K.K., Danladi, B., & Gamaniel, K.S. (2005) Pharmacological screening and evaluation of antiplasmodial activity of *Croton zambesicus* against *Plasmodium berghei berghei* infection in mice. *Indian Journal of Pharmacology*, 37, 243-246.

OP de Beck, P., Cartier, G., David, B., Dijoux-Franca, M-G., & Mariotte, A-M. (2003). Antioxidant flavonoids and phenolic acids from leaves of *Leea guineense* G. Don (Leeaceae). *Phytotherapy Research*, 17, 345 – 347.

Pietta, P-G. (2000). Flavonoids as Antioxidants. *Journal of Natural Products***,** 63, 1035-1042.

Saskia, A.B.E., Acker, V., Berg, D.V., Trop, N.J.L.M., Griffioen, D.H., Van Bennekom, W.P., Van Der Vijgh, W.J.F., & Bast, A. (1996). Structural aspects of antioxidant activity of flavonoids. *Free Radical Biology and Medicine*, 20, 331–342.

Shoemaker, M., Cohen, I., & Campbell, M. (2004). Reduction of MTT by aqueous herbal extracts in the absence of cells. *Journal of Ethnopharmacology*, 93, 381–384.

Teffo, L.S., Aderogba, M.A. & Eloff, J.N. (2010). Antibacterial and antioxidant activities of four kaempferol methyl ethers isolated from *Dodonaea viscosa* Jacq. var. *angustifolia* leaf extracts. *South African Journal of Botany*, 76, 25–29.

Thabrew, M.I., Hughes, R.D., & McFarlane, I.G. (1998). Antioxidant activity of *Osbeckia aspera*. *Phytotherapy Research*, 12, 288–290.

Wagner, H., Hoerhammer,L., & Kimly, I.C. (1970). Flavone-C-glycosides in *Croton zambesicus. Phytochemistry*, 9, 897.

Zou, G-A., Su, Z-H., Zhang, H-W., Wang, Y., Yang, J-S., & Zou. Z-M. (2010). Flavonoids from the Stems of *Croton caudatus* Geisel. var. *tomentosus* Hook, *Molecules*, 15, 1097-1102.