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Antioxidant and Antidiabetic Activities of Flavonoid Derivative Compounds Isolated from *Sclerocarya birrea* Leaves

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Sclerocarya birrea is one of many medicinal plants used in African traditional medicine for treatment of diabetes. Bioassay-guided fractionation of the ethanolic crude extract of the leaves of the plant species led to the isolation of a pure compound (**CMP-1**). The pure compound showed antioxidant and antidiabetic activities with average IC_{50} values of 1.04 mg/mL and 46 µg/mL, respectively that were well comparable to the crude extract and known standards compounds, ascorbic acid and quercetin. The spectroscopic profiling of the purified compound revealed a flavonoid derivative structural skeleton. The findings of the study suggested that the flavonoid derivative compounds play a major role towards the medicinal value of *Sclerocarya birrea*.

Keywords: *Sclerocarya birrea*, Antioxidant activity, Antidiabetic activity, Flavonoid derivatives.

INTRODUCTION

The scourge of metabolic diseases such as diabetes mellitus has of recent increased to an alarming rate, the world over. It is estimated that about 422 million global adults were living with diabetes in 2014, a rise from about 108 million in 1980. Diabetes mellitus, is generally characterized by elevated blood glucose levels above the accepted norm that mainly emanate from defects in insulin secretion, insulin uptake, or even both [1]. Several medicinal plants of the world are reportedly used in the management of the condition and their efficacy has been linked to their antioxidant properties [2]. Unchecked hyperglycaemia is linked to the promotion of glucose auto-oxidation, which results in the generation of free radicals that exceeds the scavenging capacities of the macro/microvascular antioxidant defence mechanism [3].

Antioxidant compounds such as vitamin C and α-lipoic acid, as well as many phenolic compounds in particular flavonoids were found to be effective in the alleviation of diabetic associated complications [3,4]. Medicinal plants are a good source of natural antioxidant compounds. As such, their consumption or intake of the extracts from their parts is beneficial as it provide a dietary supplementation of the body with natural antioxidants [5]. One such medicinal plant with reported anti-hyperglycaemic properties is *Sclerocarrya birrea*. Both its stem-bark and leaves have been shown to possess glucose lowering properties in several *in vitro* studies [5-8].

Sclerocarya birrea, a member of the Anacardiaceae family that is commonly known as the 'marula' tree, is one of the highly valued medicinally important plants with a lot of different pharmacological actions been attributed to it [7]. It is an indigenous tree of southern Africa and its medical effects are attributed to its phytochemical contents such as polyphenols, tannins, coumarins, avonoids, triterpenoids and phytosterols. Furthermore, pharmacological studies of the plant have shown it to possess anti-diarrheal, antidiabetic, anti-inammatory, antimicrobial, anti-plasmodia, antihypertensive, anticonvulsant, anti-nociceptive and antioxidant properties [7,9]. It was reported that the antidiabetic effect of 'marula' tree could be associated with stimulation of insulin secretion and the antioxidant activity of its phytoconstituents [8,10]. However, information on the compounds that contribute to the antidiabetic and other biol-

ogical activities of the plant species remains scanty. The current study was aimed at the bioassay-guided purification of compound(s) from the leaves of *Sclerocarya birrea* as contribution to understand the nature of natural products that potentially contribute to the antidiabetic activity of the plant species.

EXPERIMENTAL

Sample collection, preparation, extraction and storage The leaves of *Sclerocarya birrea* were collected at the grounds of Sefako Makgatho Health Sciences University (coordinates: 25.6212º S, 28.0169º E) during the summer season using convenience sampling method based on availability. The leaves were rinsed with distilled water, dried at room temperature and ground to fine powder. The fine powder of *S. birrea* leaves (450 g) was then extracted with ethanol employing a bulk cold-maceration extraction procedure. The resultant extract was filtered, concentrated using a rotary evaporator. The remaining solvent was evaporated to dryness under the stream of air and resultant dry extract was stored in the dark until further usage.

Chacterization: The ^{1}H & ^{13}C NMR profiles were obtained on an Oxford 300 Hz Nuclear Magnetic Resonance instrument using DMSO-d_6. The structural characterization of the fraction was achieved through the comparison of the NMR profile with those reported in the literature. The UV-Vis analysis of the purified compound fraction from the ethanolic leaf extract of *S. birrea* was obtained using a Nanocolor UV/Vis spectrophotometer (Macherey-Nagel, USA).

GC-MS analysis: The purified fraction was derivertized by adding N,O-*bis*(trimethylsilyl)trifluoroacetamide (BSTFA) + trimethylchlorosilane (TMCS) solution and boiling at 60 ºC for 24 h prior to GC-MS analysis. The GC-MS analysis of the purified fraction was done using a Shimadzu QP2010 SE gas chromatograph-mass spectrometer(GC-MS) with an inert cap 5MS/SIL, silica capillary column (30 mm × 0.25 mm ID × 1 µmdf, composed of 100% dimethyl-polysiloxane). An electron ionization system with ionizing energy of 70 eV was used for detection. Helium gas was used as the carrier gas at constant flow rate of 1 mL/min with an injection volume of 2 µL; injection temperature of 260 ºC and ion-source temperature of 230 ºC. The oven temperature was set from 50 ºC (isothermal for 1 min), with an increase of 20 ºC/min to 180 ºC (isothermal for 5 min), then increase to 240 ºC/min, with an increase of 20 ºC/min to 280 ºC (isothermal for 5 min). Mass spectra were taken at 70 eV; scan interval of 0.3 s and fragments from 50 to 700 *m/z*. Software adopted to handle mass spectra and chromatogram was a GC-MS SOLUTUION version 2.6.

Column bioassay-guided fractionation

Extract fractionation: A sample of the ethanolic extract of leaves (45.6 g) was fractionated on a silica gel packed vacuum liquid chromatography with different solvents of different polarities (dichloromethane, ethyl acetate, methanol) following an order from lower polarity to higher polarity. Different fractions were collected from each solvent based on differences in elution colour and all fractions were screened for free radical scavenging activity against DPPH.

Column chromatography elution of methanol fraction (fraction 8): An amount of 6.58 g from one of the methanol fractions (fraction 8) was eluted on silica gel column using the solvent system of chloroform:methanol (8:2 v/v) as the mobile phase and fractions were collected in different test tubes. Separation of components within fractions from different test tubes was monitored through TLC fingerprinting using DPPH and vanillin-sulphuric acid staining as resolution indicators. After column elution, fractions in test tubes 13 to 40 were pooled together and dried in a pre-weighed beaker. The resultant dry fraction was re-eluted using the same solvent system as the mobile phase. Fractions in test tubes 26 to 54 of the re-elution were then pooled together and in a similar procedure subjected to a further re-elution. After, the second re-elution round precipitation was noted in several test tubes and the precipitate was collected through filtration of the contents in test tubes 13 to 24. The precipitate was washed with chloroform and dissolved in methanol. The dissolved precipitate was then run on a TLC plate and visualized under UV light and after sprayed with both DPPH and vanillin-sulphuric reagent to monitor the purification. The precipitate was then stored as the purified fraction.

Evaluation of antioxidant activity strength: The antioxidant activity strength of the ethanolic extract of leaves, the methanolic fraction (**F8**) and the purified fraction was evaluated through the micro-dilution DPPH free radical scavenging and the H_2O_2 scavenging assays, as outlined below:

DPPH free radical scavenging assay: The free radical scavenging activity strength of the samples was evaluated against DPPH as reported by Abdille *et al.* [11] with slight modification. All wells of the micro-titer plate were filled with 150 µL of distilled water. Then serial dilution (0-150 µg/mL) of the ethanolic leaf extract, methanolic fraction (**F8**) and the purified fraction was done and the same was done with positive control standards, ascorbic acid and quercetin. Then, 50 µL methanol solution of DPPH (0.2 mg/mL) was added into all the wells and incubated at room temperature for 30 min. Absorbance was then measured at 517 nm using SpectraMax iD3 microtiter plate reader (Molecular devices, USA). The percentage inhibition of the extracts was calculated as follows:

$$H_2O_2 \text{ inhibition } (\%) = \frac{A_{control} - A_{extract}}{A_{control}} \times 100 \quad (1)$$

Hydrogen peroxide scavenging assay: The scavenging activity of the samples towards H_2O_2 was determined using the method as described by Ngonda [12] with minor modifications. A solution of H_2O_2 (40 mM) was prepared in phosphate buffer saline (PBS), pH 7.4. About 0.1 mg/mL of the sample was added to H_2O_2 solution and absorbance measured at 560 nm against a blank solution containing phosphate buffer without hydrogen peroxide. Ascorbic acid and quercetin were used as positive controls. The percentage of hydrogen peroxide scavenging by the extracts, compound and standard reagents was calculated using the following formula:

$$\text{Inhibition } (\%) = \frac{A_{control} - A_{extract}}{A_{control}} \times 100 \quad (2)$$

Evaluation of antidiabetic activity: The antidiabetic activity of the purified compound (**CMP 1**) was evaluated by α-amylase and α-glucosidase inhibition assays and compared to those of the ethanol crude leaf extract, methanolic fraction (**F8**). Quercetin was used as a positive standard [13-15], as outlined below:

α-Amylase inhibitory activity: α-Amylase inhibitory activity was evaluated by Kim *et al*. [16] with minor modifications. In a 96-well plate, reaction mixture containing 50 µL phosphate buffer (100 mM, pH = 6.8) with α-amylase and 100 µL of varying concentrations of samples and quercetin standard (12.5, 25, 50 and 100 µg/mL) was pre-incubated at 25 ºC for 15 min. Then, 50 µL of soluble starch with phosphate buffer pH 7 was added as a substrate and incubated further at 25 ºC for 15 min; 100 µL of the DNS colour reagent was then added and the mixture boiled for 10 min. The absorbance of the resulting mixture was measured at 540 nm using microplate reader SpectraMax ID3 (Molecular devices, United States). The results were expressed as percentage inhibition, which was calculated as follows:

$$\text{Inhibition } (\%) = \frac{A_{control} - A_{extract}}{A_{control}} \times 100 \quad (3)$$

α-Glucosidase inhibitory activity: α-Glucosidase inhibitory activity was also evaluated by Kim *et al*. [16] with minor modifications. In a 96-well plate, reaction mixture containing 50 µL phosphate buffer with α-glucosidase and 100 µL of varying concentrations of samples and quercetin standard (6.25, 12.5, 25, 50 and 100 µL/mL) was pre-incubated at 25 ºC for 15 min. Then, 100 µL α-NPG was added as a sub-strate and incubated further at 25 ºC for 20 min. The reaction was stopped by adding 100 µL Na_2CO_3 (0.1 M). The absorbance of the released *p*-nitrophenol was measured at 405 nm microplate reader SpectraMax ID3 (Molecular devices, USA). The results were expressed using eqn. 4 as percentage inhibition.

$$\text{Inhibition } (\%) = \frac{A_{control} - A_{extract}}{A_{control}} \times 100 \quad (4)$$

RESULTS AND DISCUSSION

The crude ethanolic extract of *Sclerocarya birrea* leaves was fractionated on a column with three solvents (DCM, ethyl acetate and methanol) and several fractions were collected based on differences in elution colours. Both the crude ethanolic extract and its fractions were screened for antioxidant activity and have all demonstrated the presence of DPPH free radical scavenging activity. One of the methanolic solvent fractions (**F8**) was eluted and re-eluted on a silica elution until a purified compound fraction (**CMP 1**) with free radical scavenging activity and reactivity to vanillin-sulphuric reagent was obtained as a yellowish powder.

Structural characterization of the purified sample was achieved through spectroscopic techniques (GC-MS, UV-Vis and ^{1}H & ^{13}C NMR) and the results are shown in Table-1. While the NMR profile of the purified compound was compared with those reported in the literature and that of the quercetin standard and the results are shown in Table-2.

TABLE-1
SPECTROSCOPIC PROFILE OF THE PURIFIED COMPOUND FRACTION FROM THE ETHANOLIC LEAF EXTRACT OF *S. birrea*

Technique	Spectral profile
^{1}H NMR (δ_H)	12.49; 7.49; 7.46; 7.43; 6.84; 6.40; 6.17; 5.46; 4.26; 4.12; 3.91; 3.68; 3.59; 3.28; 2.48
^{13}C NMR (δ_C)	178.3; 164.8; 161.7; 157.7; 157.0; 149.1; 145.6; 134.0; 121.6; 104.6; 82.5; 61.2
GC-MS (*m/z*)	73; 147; 487; 540; 559; 575; 647
UV-Vis (λ_{max})	260, 360 nm

TABLE-2
COMPARISON OF THE NMR DATA OF THE PURIFIED COMPOUND FRACTION (**CMP 1**), STANDARD QUERCETIN AND THOSE REPORTED IN THE LITERATURE FOR QUERCETIN GLYCOSIDE COMPOUND

Peak number	**CMP 1**		Quercetin standard		Quercetin 3-0-β-D-glucopyranoside [17]	
	^{1}H NMR	^{13}C NMR	^{1}H NMR	^{13}C NMR	^{1}H NMR	^{13}C NMR
1	12.5	178.3	12.7	176.5	12.6	179.6
2		164.8	10.8	164.5	10.8	165.6
3		161.7	9.65	161.4	9.65	162.2
4		157.7	9.35	156.8	9.14	159.1
5	7.49	149.1	7.65	148.3	7.61	150.2
6	7.43	145.6	7.50	145.7	7.58	145.0
7	6.84	134.0	6.84	136.4	6.86	136.2
8	6.40	121.6	6.36	122.6	6.41	122.0
9	6.17	116.0	6.16	116.3	6.21	116.0
10		104.6		103.7		104.6
11		99.0		99.0		100.6
12		95.0		94.2		
13	5.46	82.5			5.46	77.9
14	4.27					
15	3.91	77.8			3.10-3.60	76.0
16	3.69					
17	3.54	61.2				

Values in italics are those of peaks present but not detected for labeling.

The ^{1}H NMR profile of the purified compound showed peaks with chemical shifts of δ_H 12.49, δ_H 7.49, δ_H 7.43, δ_H 6.84 and δ_H 6.17 are consistent with several reported flavonoids [18]. Peaks shown at δ_H 5.46, δ_H 4.26, δ_H 4.12 and δ_H 3.91 are also consistent with those for anomeric protons of sugar moieties [19]. In addition, the absence of peaks between δ_H 0.50 and δ_H 1.8 suggested the absence of the methyl group in the structure of the purified compound. The ^{13}C NMR profile of the purified compound also confirms its flavonoid skeletal characteristics as shown by the presence of the peaks at δ_C 178.3, δ_C 157.7 and δ_C 121.6. The ^{13}C NMR analysis data also confirms the presence of sugar moieties as indicated by peaks between δ_C 61.2 and δ_C 82.5 [20,21]. Therefore, the NMR profile of the purified compound is consistent with those of flavonoids derivatives.

Furthermore, the GC-MS profile of both the purified compound fraction and the quercetin standard were obtained. The mass spectrum of the purified fraction showed a fragment of *m/z* 647 [M+H] that fragmented into *m/z* 73, 147, 487, 540, 559, 575 which resonate with some fragmentation pattern of some flavonoid derivatives [22]. The mass spectrum of the quercetin standard showed similar fragmentation pattern to that of the purified compound fraction with the exception of the fragment peak with *m/z* of 147 that was present only in mass spectrum of the purified compound fraction which, is consistent with the 5-C sugar moiety or a *p*-coumaroyl [23]. Therefore, the substitution of one or more hydroxyl groups of the flavonoid aglycone with either a sugar molecule or a *p*-coumaroyl molecule is also supported by the mass spectrum of the purified compound.

The UV spectrum showed peaks at 260 and 360 nm that is consistent with the two UV band for flavonol compounds [24]. Therefore, based on the NMR, GC-MS and UV-visible spectral profile of the purified fraction and the comparison with those from the literature, the purified compound is deemed to be a flavonoid derivative whose aglycone part is consistent with that of quercetin. The tentative structure of the purified compound arrived at upon comparison of the spectroscopic data with those of known standard and of compounds reported in the literature is shown in Fig. 1.

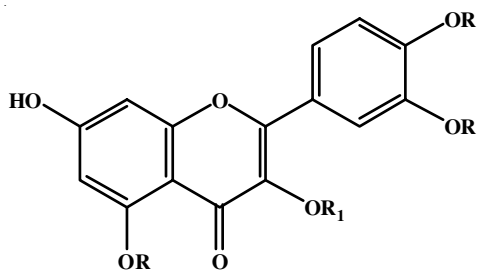

Fig. 1. Tentative structure of the purified compound fraction from the ethanolic extract of *Sclerocarya birrea* leaves based on the comparison of its spectroscopic data to that reported in the literature (R1; R: possible varied substituents)

DPPH and H_2O_2 scavenging activity: The antioxidant activity of the purified compound fraction was evaluated using the DPPH and the H_2O_2 assays and compared to those of the ethanolic extract and the parent methanol fractions. The 50% inhibition concentration (IC_{50}) of the samples against the two test radical reagents were determined and the results are shown in Table-3. The purified compound (**CMP 1**) fraction showed average IC_{50} values of 1.04 mg/mL against DPPH and H_2O_2, respectively that compared well to those of the parent methanol fraction of the ethanolic leaf extract of *S. birrea*, as well as the two positive control standards, quercetin and ascorbic acid.

TABLE-3
DPPH AND H_2O_2 INHIBITION ACTIVITY OF THE PURIFIED COMPOUND (**CMP 1**) FROM THE ETHANOLIC EXTRACT OF *S. birrea* LEAVES

Sample	EC_{50} (mg/mL) DPPH	EC_{50} (mg/mL) H_2O_2	Average EC_{50}
Ethanolic extract leaves	0.70	0.68	0.69^{a}
F8 fraction	1.20	1.34	1.27^{b}
CMP 1	0.58	1.50	$1.04^{b,c}$
Quercetin	0.38	1.50	0.94^{c}
Ascorbic acid	0.38	1.50	0.94^{c}

^{a,b,c}Values with similar letters are not significantly different and those with different letters are significantly different, $p < 0.05$)

α-Amylase and α-glucosidase inhibition activity: The potential antidiabetic activity of the isolated compound was evaluated using the α-amylase and α-glucosidase inhibition assays and compared to those of the parent methanolic fraction (**F8**) and crude ethanolic leaf extract. The 50% inhibition concentration (IC_{50}) of the samples against α-amylase and α-glucosidase were determined and the results are shown in Table-4. The purified compound (**CMP 1**) fraction showed average IC_{50} value of 46 µg/mL against α-amylase and α-glucosidase, respectively that compared well to those of the parent methanol fraction of the ethanolic extract of *S. birrea* leaves as well as the positive control standard, quercetin.

TABLE-4
α-AMYLASE AND α-GLUCOSIDASE INHIBITION OF THE PURIFIED COMPOUND (**CMP 1**) ISOLATED FROM THE ETHANOLIC EXTRACT OF *S. birrea* LEAVES

Sample	IC_{50} (µg/mL)		Average IC_{50} (µg/mL)
	α-Amylase	α-Glucosidase	
Ethanolic extract leaves	44	93	68.5^{b}
F8 fraction	35	65	50.0^{a}
CMP 1	80	12	46.0^{a}
Quercetin	120	12.5	66.3^{b}

^{a,b}Values with similar letters are not significantly different and those with different letters are significantly different, $p < 0.05$)

Conclusion

In this study, a purified compound fraction was obtained from one of the methanolic fractions of the ethanolic extract of *Sclerocarya birrea* leaves through the antioxidant activity guided column fractionation procedure. The purified compound showed antioxidant activity strength similar to those of the known standards quercetin and ascorbic acid. The activity strength of the purified compound was also more similar to that of the parent methanolic fraction (**F8**), which suggested

that the purified compound is the major bioactive compound in the parent methanolic fraction. However, the ethanolic extract of *S. birrea* leaves showed even higher relative antioxidant activity strength indicating the role of other compounds in synergy to the purified compound. The purified compound also showed inhibition activity against diabetic associated enzymes, α-glucosidase and α-glucosidase, with activity strength that compared well with that of the positive standard, quercetin.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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