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MEDICINAL PLANT COMMUNICATIONS

Med Plant Commun
3 (4): 68 - 73 (2020) - https://doi.org/10.37360/mpc.20.3.4.13

Short Communication

Comparison of health-promoting metabolite antioxidant effects in *Sclerocarya birrea* (A. Rich.) Hochst extracts

[Comparación de los efectos antioxidantes de metabolitos que promueven la salud en extractos de *Sclerocarya birrea* (A. Rich.) Hochst]

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Abstract: *Sclerocarya birrea* (A. Rich.) Hochst (Anacardiaceae) is a savannah tree that has long been used in sub-Saharan Africa as a medicinal remedy for numerous ailments. The purpose of this study was to increase the scientific knowledge about this plant by evaluating the total content of polyphenols, flavonoids, and tannins in the extracts of the root, leaves and bark, as well as their *in vitro* antioxidant activit. The chemical characterization was carried out by HPLC-MS/MS. Results reported the presence of glycoside flavonoids in *Sclerocarya birrea* leaves and procyanidins in roots and bark. The presence of procyanidins conferred the highest antioxidant activity of root extract form *Sclerocarya birrea*.

Keywords: *Sclerocarya birrea*; Antioxidant activity; Chemical profile; Procyanidins; Glycoside flavonoids

Resumen: *Sclerocarya birrea* (A. Rich.) Hochst (Anacardiaceae) es un árbol de la sabana que se ha utilizado durante mucho tiempo en el África subsahariana como remedio medicinal para numerosas dolencias. El propósito de este estudio fue incrementar el conocimiento científico sobre esta planta mediante la evaluación del contenido total de polifenoles, flavonoides y taninos en los extractos de raíz, hojas y corteza, así como su actividad antioxidante *in vitro*. La caracterización química se realizó mediante HPLC-MS/MS. Los resultados informaron la presencia de flavonoides glucósidos en hojas de *Sclerocarya birrea* y procianidinas en raíces y corteza. La presencia de procianidinas confiere la mayor actividad antioxidante del extracto de raíz de *Sclerocarya birrea*.

Palabras clave: *Sclerocarya birrea*; Actividad antioxidante; Perfil químico; Procianidinas; Flavonoides glucósidos

Received: 26 de octubre de 2019
Accepted: 20 de diciembre de 2019
Published online: 30 de noviembre de 2020
This article must be cited as: Faraone I, Russo D, Milella L. 2020. Comparison of health-promoting metabolite antioxidant effects in *Sclerocarya birrea* (A. Rich.) Hochst extracts. **Med Plant Commun** 3 (4): 68 – 73.
Conferencia dictada en VII Congreso Latinoamericano de Plantas Medicinales, Cuenca, Ecuador, 4 al 6 de Septiembre de 2019.

INTRODUCTION

Sclerocarya birrea (A. Rich.) Hochst, known as marula in English, belongs to the Anacardiaceae family, the same family as well known edible species such as mango (*Mangifera indica*), pistachio (*Pisticia vera*) and cashew (*Anacardium occidental*). It is an indigenous and fruit-bearing tree of sub-Saharan Africa, introduced to Australia, India, Israel, Mauritius, Oman and Réunion, that grows mostly in low altitudes and can reach up to 20 m in height and 1.2 m in diameter. The Marula tree has attracted enormous attention since 1906 and continues to do so today. In fact it has been identified as one of five fruit tree species that should be integrated in the domestication process as food and medicinal source for rural communities. The fruits are eaten or processed to make a beer or a jam, the kernels are eaten or the oil extracted, the leaves are used as forage for livestock and the wood is carved into utilitarian items such as spoons and plates (Wynberg et al. 2002). Beside, the bark, the leaves and root of *S. birrea* have also received attention because, traditionally they are used to treat an array of human ailments such as dysentery, fevers, malaria, diarrhea, stomach ailments, rheumatism, sore eyes, gangrenous rectitis, infertility, headaches, toothache and body pains. Scientific studies have reported antibacterial, antifungal, and astringent properties and anticonvulsant, antihyperglycaemic, and anti-infiammatory activity of *S. birrea* (Braca et al. 2003). Some these curative properties could be attributed to the high content of polyphenols. Previous studies on *Sclerocarya birrea* detected the presence of phenolic compounds in the leaves and pulp fruit by HPLC-UV/PDA and HPLC-ESI/MS (Braca et al. 2003). The aim of the present study was to determine the phenolic compounds, from three different parts of *Sclerocarya birrea*, bark, leaf and root, by HPLC/MS/MS and their antioxidant activity.

MATERIAL AND METHODS

Samples collection and preparation of extracts

Leaves, bark and root of *Sclerocarya birrea* (Anacardiaceae family) were collected from Senegal. The different parts of the plant were cleaned removing foreign particles, cut into a small pieces and dried for a few days. Leaves, shade dried at room temperature, were powdered, while bark and root of *S. birrea* were further broken up, using a mortar. Bark, leaves and root, respectively, 215, 150 and 160 grams of *S. birrea* were extracted with 5 volumes (v/w) of *n*-hexane, chloroform, chloroform:MeOH 9:1 and MeOH, at room temperature, in a dark bottles for 6 days with occasional shaking and stirring and changing the solvent three times. The extracts were filtered through filter paper and concentrated in a rotary evaporator at 37°C. All the samples were stored at 4°C until the use.

Total phenolic content (TPC)

The total phenolic content was determined using Folin–Ciocalteu Reagent (FCR) as previously described. For the analysis, 100 µL of the extracts, 100 µL methanol, 100 µL FCR and finally 700 µL Na_2CO_3 (20%) were added together and vortexed. Methanolic gallic acid solutions (10–400 mg/L) were used as standards. The mixture was incubated for 20 min in the dark and room temperature. After incubation the mixture was centrifuged at 13,000 rpm for 3 min. The absorbance of the supernatant was measured at 735 nm by spectrophotometer. The total phenolic content was expressed as gallic acid equivalent (GAE)/100 g dry weight (DW) of the sample (Russo et al. 2013).

Total Flavonoid Content (TFC)

An aliquot (150 µL) of each extract was added to 45 µL of 5% $NaNO_3$ into microcentrifuge tube. In the fifth and in the sixth minute, respectively, 90 µL of 10% $AlCl_3$ and 300 µL of 1 M NaOH solution were added. The final volume of the mixture was then brought to 1.5 mL by adding distilled water. The absorbance was measured against blank reagent at 510 nm after 10 minutes of incubation at room temperature (Armentano et al. 2015). Quercetin was used as standard to plot the regression curve. The total flavonoid content (TFC) was expressed as mg of quercetin equivalent/g of dried extract (mg QE/g of extract).

Total Tannin Content (TTC)

To 250 µL of each extract, 500 µL of bovine serum albumin solution in 0.2 M acetic buffer, pH 5.0 with 0.17 M NaCl was added and mixed carefully (Russo et al. 2018). After 15 min, samples were centrifuged at 5000 g for 15 min. The

supernatant was removed, and the pellet was dissolved in 1 mL of aqueous solution containing 1% SDS and 4% triethanolamine. Then 250 μL of 0.01 M $FeCl_3$ in 0.01 M HCl was added. After 30 min, the absorbance was recorded at 510 nm. Total tannin content (TTC) was expressed as mg of tannic acid equivalent/g of dried extract (mg TAE/g of extract), in this case tannic acid was used to construct a regression curve.

Antioxidant activity

ABTS Assay. The free radical scavenging capacity of each plant extract was also studied using the 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt ($ABTS^{\bullet}$) radical assay (Mezrag etal. 2017). ABTS was dissolved in deionized water to a 7 mM concentration and its radical cation ($ABTS^{+\bullet}$) was produced by reacting ABTS solution with 2.45 mM potassium persulfate and allowing the mixture to stand in the dark at room temperature for 12–16 h before use. Each extract (75 μL) was added to 1425 μL of $ABTS^{+\bullet}$ solution and the absorbance was measured after 2 h of incubation in the dark. All solutions were fresh prepared for the analysis. Results are expressed as percentage of radical inhibition. Trolox was used as reference standard.

Beta-Carotene Bleaching Assay. The antioxidant activity was evaluated by the β-carotene-linoleic acid bleaching method (BCB) as previously described (Milella et al. 2014). The absorbance was measured at 470 nm. Results are expressed as percentage of antioxidant activity (% AA).

Nitric Oxide ($NO^{\bullet}$) Radical Scavenging Activity. The antiradical activity was determined spectrophotometrically, according to a previously described procedure (Ferreres at al. 2012). IC50 was calculated from three independent assays, performed in triplicate. Results are expressed as percentage of radical inhibition. Ascorbic acid was used as positive control.

Superoxide Anion ($O_2^{\bullet-}$) Scavenging Activity. The effect of each extract on the superoxide radical-induced reduction of NBT was monitored at 560 nm. Superoxide radicals were generated by the NADH/PMS system, as previously reported (Ferreres at al. 2012). For each extract, different concentrations were tested. Results are expressed as percentage of radical inhibition. Ascorbic acid was used as positive control.

HPLC-ESI-MS/MS

The analysis was carried out by Alliance 2695 HPLC system (Waters Corp., Milford, MA) coupled to a Q-Tof of Premier mass spectrometer (Waters Corp., Micromass Ms Technologies, Manchester, UK). The Q-Tof is equipped with a lockspray source where an internal reference compound (leucine-enkephalin) was introduces simultaneously with the analyte for accurate mass measurements. The column, Atlantis T3 C18 column (waters Corp., Milford, MA; 100 mmx 2.1 mm; 3 μm particle size) was used to separate the compounds, maintained at 40°C and using 0.5% aqueous formic acid (solvent A) and 0.5% formic acid in 50:50 v/v acetonitrile:methanol (solvent B). The gradient program was set as follows:0-15%B in 1min, 60-40% B in 5 min, 50-50% B in 2 min, 30-70% B in 6 min, 20-80% B in 4 min, 80-20 % B in 7 min, applying a flow of 0.2 mg/mL. Electrospray mass spectra data were recorded on a negative ionization mode for a mass range from m/z 100 to 1600. Capillary voltage and cone voltage were set at 3kV and 30 V, respectively. The data were acquired using MassLynx version 4.1 (Russo et al. 2013).

RESULTS AND DISCUSSION

Total secondary metabolite content and antioxidant activity. In this present study, all extracts of *Sclerocarya birrea* leaf, bark and root have been evaluated for their total phenolic, flavonoid and tannin content and antioxidant activity by *in vitro* assays. Particularly root showed the highest content of polyphenols and tannins, whereas the leaf tissue reported the highest cintent of flavonoids (**Table No. 1**). Extracts reported antioxidant activity in dose-dependent manner and root showed the highest antioxidant activity (**Figure No. 1**).

Antioxidant activity was assayed by 4 different tests. The reduction of ABTS radical in the presence of such hydrogen-donating antioxidants was calculated and IC50 value was 12.54 ± 0.47 μg/mL for root, 18.7 ± 1.5 for leaves and 15.0 ± 0.9 μg/mL for bark. Trolox was used as the standard and its IC_{50} was 12.8 ± 0.9 μg/mL, evidencing the strong antioxidant potential of the extracts.

Inhibition of lipid peroxidation was carried out by the β-carotene bleaching (BCB) assay and butylated hydroxytoluene (BHT) was used as the standard (IC_{50} = 11.3 ± 0.2 μg/mL). The ability of extract to inhibit *β*-carotene bleaching expressed as IC_{50} was root showed an IC_{50} value of 151.02 ± 4.72 *μ*g/mL for root, 197.1 ± 14.2 μg/mL for bark extract, whereas MLE was not able to overcome the IC_{50} even at the highest tested concentrations.

Superoxide anion and nitric oxide are ROS normally produced inside the human body. The extracts are found to be an efficient scavenger of superoxide radical and nitric oxide radical. The scavenging effect of the root extract measured by IC_{50} was 21.21 ± 2.14 *μ*g/mL and 32.18 ± 3.24 *μ*g/mL for superoxide anion and nitric oxide, respectively. Bark extract showed IC_{50} values of 19.9 ± 2.9 and 15.6 ± 1.0 μg/mL for nitric oxide and superoxide anion, respectively. MLE showed an IC_{50} value of 31.1 ± 3.3 μg/mL for superoxide anion, but a low inhibition of nitric oxide reaching 39.9 ± 0.5% inhibition at 200 μg/mL. In both assays, ascorbic acid was used as the standard showing IC_{50} values of 35.6 ± 2.4 and 199.3 ± 20.1 μg/mL for nitric oxide and superoxide anion, respectively. In both assays, ascorbic acid was used as the standard showing IC_{50} values of 35.6 ± 2.4 and 199.3 ± 20.1 μg/mL for nitric oxide and superoxide anion, respectively.

Table No. 1
Total phenol content and antioxidant activity of methanol extracts

	TPC (mg GAE/g)	TTC (mg TAE/g)	TFC (mg QE/g)
Methanol leaf extract	62.6 ± 0.8	90.2 ± 4.7	132.7 ± 10.4
Methanol bark extract	241.3 ± 8.5	949.5 ± 29.7	57.7 ± 3.5
Methanol root extract	861.94 ± 12.25	1109.68 ± 21.59	95.47 ± 8.27

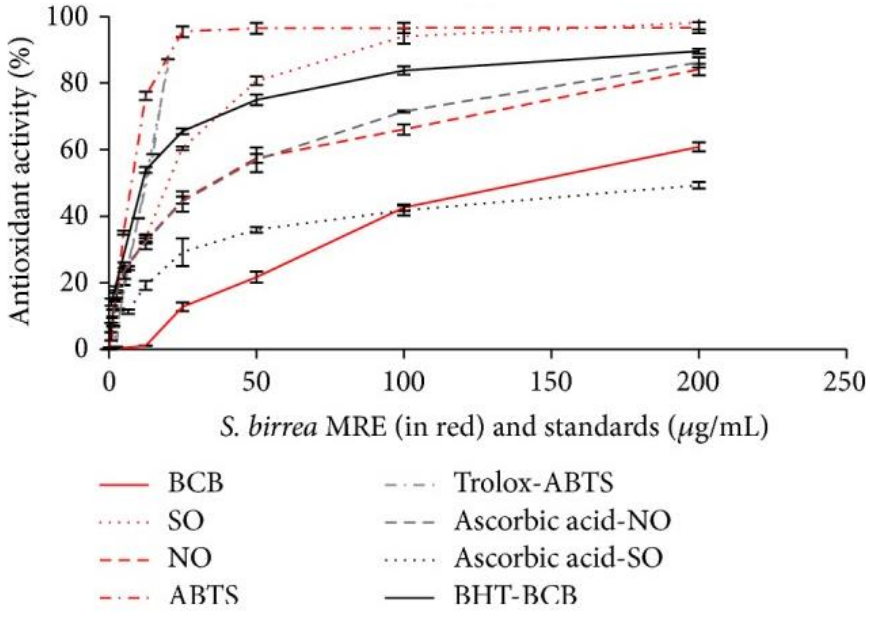

Figure No. 1
Antioxidant activity of *Sclerocarya birrea* methanolic root extract (MRE) compared with the reference standards. Antioxidant activity was measured by 4 different tests and in each one it is demonstrated to be dose-dependent. ABTS, nitric oxide (NO), superoxide anion (SO), and *β*-carotene bleaching (BCB) assays

LC-ESI-MS/MS.

In order to characterize chemical compounds, simultaneous experiments of LC/ESI/MS in negative ionization mode and LC/ESI/MS/MS were performed. This latest is extremely useful for peak assignment because the availability of the reference standards is limited. The LC/MS analysis results showed the major phenolic constituents in methanol extracts. A total of 20 compounds were detected, and they are shown in **Table No. 2.**

Table No. 2
Compounds identified by HPLC-ESI-MS/MS (L= leaf; B= bark; R= root).

Peak	Compound	Formula	m/z calc.	m/z obs.	detected in
1	Gallic acid	$C_7H_5O_5^-$	169.0137	69.0126	L, B, R
2	Dihydroxybenzoic acid Pyranoside	$C_{12}H_{13}O_8^-$	285.0610	285.0605	L
3	Quercetin 3-O-β-D-(6"-galloyl) glucopyranoside	$C_{28}H_{23}O_{16}^-$	615.0986	615.0982	L
4	Quercetin 3-O-β-D-(6"-galloyl)galactopyranoside	$C_{28}H_{23}O_{16}^-$	615.0986	615.0982	L
5	Myricetin 3-O-α-L-rhamnopyranoside	$C_{21}H_{19}O_{12}^-$	463.0877	463.0877	L
6	Quercetin 3-O-β-D-glucopyranoside	$C_{21}H_{19}O_{12}^-$	463.0877	463.0887	L
7	Quercetin 3-O- arabinoside	$C_{20}H_{17}O_{11}^-$	433.0771	433.0776	L
8	Kaempferol 3-O-β-D-(6"-galloyl)glucopyranoside	$C_{28}H_{23}O_{15}^-$	599.1037	599.1041	L
9	Quercetin 3-O-α-L-rhamnopyranoside	$C_{21}H_{19}O_{11}^-$	447.0928	447.0941	L
10	Kaempferol 3-O-arabinoside	$C_{20}H_{17}O_{10}^-$	417.0822	417.0838	L
11	Kaempferol 3-O-α-L-rhamnopyranoside	$C_{21}H_{19}O_{10}^-$	431.0978	431.0975	L
12	Catechin	$C_{15}H_{13}O_6^-$	289.0712	289.07	B, R
13	Epicatechin	$C_{15}H_{13}O_6^-$	289.0712	289.07	B
14	(-)-Epicatechin 3-O-galloyl ester	$C_{22}H_{17}O_{10}^-$	441.0822	441.083	L, B, R
15	(-)-Epigallocatechin 3-O-galloyl ester	$C_{22}H_{17}O_{11}^-$	457.0771	457.0763	R
16	ProcyanidinP2	$C_{30}H_{25}O_{12}^-$	577.1462	577.1429	L
17	Procyanidin P2G1	$C_{37}H_{29}O_{16}^-$	729.1456	729.1442	L, B
18	Procyanidin P2G2	$C_{44}H_{33}O_{20}^-$	881.1565	881.1535	B, R
19	Procyanidin P3G3	$C_{66}H_{49}O_{30}^-$	1321.2940	1321.2859	B
20	Disaccaride	$C_{12}H_{21}O_{11}^-$	341.1084	341.1075	L, B, R

The major part of this compounds were identified as flavonoid glycosides and galloylated procyanidins. Flavonoids glycoside, compounds from 3 to 11 (**Table No. 2)**, were identified exclusively in the leaf of *S. birrea*. A previous study (Braca et al., 2003) has already described eight of ten compounds found by LC-ESI-MS analysis (compounds 1, 3, 4, 5, 6, 8, 9 and 11). Two new compounds, quercetin 3-O- arabinoside (compound 7) and Kaempferol arabinoside (compound 10), were revealed and their structure was confirmed by LC-ESI-MS/MS.

Procyanidins, a group of flavonoids ubiquitous in the plant kingdom, are a mixture of flavan-3-ol monomers, epicatechin and/or catechin commonly bounded through C4-C8 linkage. This class of phenol compounds was found mainly in the bark of *S. birrea.* The analysis revealed the presence of catechin, epicatechin, epigalloacatechin and

epigallocatechin gallate and they were identified by comparing their respective retention time with the corresponding standards. The highest polymer of procyanidin founded was the compound 32 that showed a negative molecular ion $[M-H]^-$ at an m/z of 1321, which presents a degree of polymerization and a degree of galloylation of 3; this compound can could correspond to a trigallate procyanidin trimer P3G3.

The compounds 16, 17 and 18 have a molecular ion $[M-H]^-$ at an m/z of 577, 729 and 881, respectively. The MS/MS spectrum of the molecular ion at m/z 881 is similar at that showed above and it was identified as P2G2. The loss of gallic acid residue (152 Da), gallic acid residue + a molecule of water (m/z 152+18), the opening of the C ring and the cleavage the linkage of the oligomer leads at the formation of ions at m/z to 729, 559, 407, 289 and 125 Da, respectively.

The ion at m/z 729, identified as P2G1, once fragmented, gave the product at an m/z of 577, 407, 289 and 125 Da, the same of the others procyanidins cited above. The last compound belonging at this group having an m/z of 577 was identified as P2.

Besides two hydroxybenzoic acid derivates were identified, gallic acid and dihydroxybenzoic acid pyranoside. The gallic acid was identified by comparing the retention time with the standard. The determination of dihydroxybenoic acid pyranoside (m/z 285Da) was confirmed by LC-ESI-MS/MS. This ion produced a fragment at an m/z of 153 Da that corresponds at hydroxybenoic acid and the presence of a fragment at an m/z of 109 indicates a subsequent cleavage of the CO_2 (-44Da).

CONCLUSIONS

The presence of flavonoids glycoside was investigated in a previous study in *Sclerocarya birrea* leaves, but procyanidins were never reported. Only an epicatechin derivate was found in the roots. Oligomeric procyanidins have been particulary investigated due to their potential health-promoting effects observed in vitro and in vivo. Most prominently, procyanidin oligomers showed an important antioxidant activity and the ability to scavenge reactive oxygen and nitrogen species. The LC-ESI-MS/MS analysis allowed us to identify the oligomers in *S. birrea* and to characterize the different polymerization degree. In particulary, were found the procyanidin oligomers in *S. birrea* roots and bark explaining the high phenol content and in antioxidant activity.

REFERENCES

Armentano MF et al. 2015. **BioMed Res Int** 2015, Article ID 561589. **https://doi.org/10.1155/2015/561589**
Braca A et al. 2003. **J Agric Food Chem** 51: 6689-6695. **https://doi.org/10.1021/jf030374m**
Ferreres F et al. 2012. **Food Chem** 134: 894-904. **https://doi.org/10.1016/j.foodchem.2012.02.201**
Mezrag A et al. 2017. **Nat Prod Res** 31: 507-514. **https://doi.org/10.1080/14786419.2016.1195381**
Milella L et al. 2014. **Food Chem** 160: 298-304. **https://doi.org/10.1016/j.foodchem.2014.03.097**
Russo D et al. 2013. **ISRN Chromatogr** 2013, Article ID 283462. **https://doi.org/10.1155/2013/283462**
Russo D et al. 2018. **Int J Mol Sci** 19: 186. **https://doi.org/10.3390/ijms19010186**
Wynberg R et al. 2018. **South Afr Forest J** 196: 67-77. **https://doi.org/10.1080/20702620.2002.10434620**