ORIGINAL ARTICLE

A C G publications

Rec. Nat. Prod. 3:1 (2009) 23-31

records of natural products

Antioxidant Properties of the Methanol Extracts of the Leaves and Stems of *Celtis africana*

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(Received September 11, 2008; Revised October 27, 2008; Accepted October 28, 2008)

Abstract: The antioxidant activities of the methanol extracts from the leaves and stems of *Celtis africana* (Ulmaceae) were assessed in an effort to validate the medicinal potential of the subterranean part of the herb. The antioxidant activity and phenolic contents of the stem as determined by the DPPH, proanthocyanidins, total phenols, the flavonoids, and total flavonols were higher than that of the leaves. On the other hand, the FRAP contents of the leaves were higher than that of the stem. The ABTS scavenging activities of both the stem and leaves were similar and comparable to that of BHT, the standard antioxidant used. This study, has to some extent, validated the medicinal potential of the leaves and stems of *Celtis africana.*

Keywords: *Celtis africana*; free radical scavenging activity; polyphenols; reducing capacity.

1. Introduction

Free radicals which have one or more unpaired electrons are produced in normal or pathological cell metabolism. Reactive oxygen species (ROS) react easily with free radicals to become radicals themselves. ROS are various forms of activated oxygen, which include free radicals such as superoxide anion radicals ($O_2^{\cdot-}$) and hydroxyl radicals ($OH^{\cdot}$), as well as non-free radical species (H_2O_2) and the singled oxygen ($^1O_2$) [1, 2, 3, 4]. Also, excessive generation of ROS, induced by various stimuli and which exceed the antioxidant capacity of the organism, leads to a variety of pathophysiological processes such as inflammation, diabetes, genotoxicity, and cancer [5, 6, 7].

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The article was published by Academy of Chemistry of Globe Publications
www.acgpubs.org/RNP © Published 01/13/2009 EISSN:1307-6167

Exogenous sources of free radicals include tobacco smoke, ionizing radiation, certain pollutants, organic solvents, and pesticides [3, 8, 9, 10]. Therefore, ROS can cause lipid peroxidation in foods, leading to their deterioration. In addition, these ROS can easily initiate the peroxidation of membrane lipids, leading to the accumulation of lipid peroxidation. The peroxidation products and their secondary oxidation products such as malondialdehyde and 4-hydroxyinonenal can react with biological substrates such as protein, amines, and deoxyribonucleic acid [7, 11]. As a result of this, much attention has been focused on the use of antioxidants, especially natural antioxidants to inhibit lipid peroxidation and to protect from damage due to free radicals. A great number of aromatic and other medicinal plants contain chemical compounds that exhibit antioxidant properties. Sources of natural antioxidants are primarily, plant phenolics that may occur in all parts of plants such as fruits, vegetables, nuts, seeds, leaves, roots and barks [12, 13].

Celtis africana Burm.f. (Ulmaceae) is a medium-sized tree whose trunk is usually single and upright; its crown is broadly upright or rounded; the foliage is dark green or yellowish green in diseased trees [14]. *C. africana* is common and widespread in South Africa. It occurs in a wide range of habitats from the coast up to 2100 cm, from the Cape Peninsula northwards through South Africa to Ethiopia, where it grows in dense forest, on rocky outcrops, in bushveld, in open grass, on mountain slopes, on coastal dunes, along river banks, and in kloofs [14, 15, 16, 17, 18, 19, 20]. The leaves are eaten by cattle and other domestic animals [14]. The bark of *C. africana* is also known to be a seasonal food for the Red Colobus monkey [21]. The wood of *Celtis africana* is white to yellowish in colour and of medium hardness. It is tough and strong, and polishes well, but is difficult to work. It is a good general timber suitable for making planks, shelving, yokes, tent-bows and furniture. The African people have always used it to make a variety of household articles. It is also thought to have magical properties. The wood is mixed with crocodile fat as a charm against lightning, and many people believe that it has the power over evil and that pegs of wood driven into the ground will keep witches away [15]. The family Ulmaceae is most often treated as a single family with two subfamilies: Ulmeae and Celteae. A flavonoid survey of 80 species of Ulmaceae shows that each of the 19 genera in the survey was characterized by the production of flavonols (Ulmoid) or glycoflavones (Celtoid), but not both [22].

In this paper, we present the results of the antioxidant activities of methanol extracts of the leaves and stems of *C. africana*. The findings from this work may add to the overall value of the medicinal potential of the herb.

2. Materials and Methods

2.1 Plant collection

Both the stems and leaves of the plant were collected in July 2006 from the Eastern Cape Province of South Africa. The area falls within the latitudes 30°00-34° 15´S and longitudes 22° 45´ - 30° 15´E. It is bounded by the sea in the east and the drier Karoo (semi-desert vegetation) in the west [23]. These areas consist of villages which are generally classified as rural and poor. The plant was identified by its vernacular name by Sam Boltina and later validated at the Department of Botany, University of Fort Hare by Professor Don Grierson, and voucher specimens (Aded Med 2007/1-10) were deposited in the Griffen Herbarium of the University.

2.2 Extract preparation

Both stems and leaves were air dried at room temperature to constant weights. The dried plant materials were ground separately to powder. Two hundred grams of each ground plant materials were shaken separately in methanol for 48 hrs on an orbital shaker. Extracts were filtered using a Buckner funnel and Whatman No 1 filter paper. Each filtrate was concentrated to dryness under reduced

pressure at 40ºC using a rotary evaporator. Each extract was resuspended in methanol to make 50 mg/ml stock solution [24].

2.3 Chemicals

1,1-Diphenyl-2-picrylhydrazyl (DPPH), 2,2′-azinobis-3- ethylbenzothiazoline-6-sulfonic acid (ABTS), 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4′,4″-disulfonic acid, potassium ferricyanide; catechin, butylated hydroxytoluene (BHT), ascorbic acid, catechin, tannic acid, quercetin and $FeCl_3$ were purchased from Sigma Chemical Co. (St. Louis, MO, USA), vanillin from BDH; and Folin-Ciocalteus's phenol reagent and sodium carbonate were from Merck Chemical Supplies (Damstadt, Germany). All the chemicals used including the solvents, were of analytical grade.

2.4 Determination of total phenolics

Using modified Folin-Ciocalteu method [25] total phenol contents in the extracts were determined. An aliquot of the extract was mixed with 5 ml Folin-Ciocalteu reagent (previously diluted with water at 1:10 v/v) and 4 ml (75 g/l) of sodium carbonate. The tubes were vortexed for 15 sec and allowed to stand for 30 min at 40ºC for color development. Absorbance was then measured at 765 nm using the Hewlett Packard UV-VS spectrophotometer. Samples of extracts were evaluated at a final concentration of 0.1 mg/ml. Total phenolic content were expressed as mg/g tannic acid equivalent using the following equation based on the calibration curve: $y = 0.1216x$, $R^2 = 0.9365$, where x was the absorbance and y was the tannic acid equivalent (mg/g).

2.5 Determination of total Flavonoids

Estimation of the total flavonoids in the plant extracts was carried out using the method of Ordon Ez et al. [26]. To 0.5 ml of sample, 0.5 ml of 2% $AlCl_3$ ethanol solution was added. After one hour at room temperature, the absorbance was measured at 420 nm. A yellow color indicated the presence of flavonoids. Extract samples were evaluated at a final concentration of 0.1 mg/ml. Total flavonoid content was calculated as quercetin (mg/g) using the following equation based on the calibration curve: $y = 0.0255x$, $R^2 = 0.9812$, where x was the absorbance and was the quercetin equivalent (mg/g).

2.6 Determination of total proanthocyanidins

Determination of proanthocyanidin was based on the procedure reported by Sun et al. [27]. A volume of 0.5 ml of 0.1 mg/ml of extract solution was mixed with 3 ml of 4% vanillin-methanol solution and 1.5 ml hydrochloric acid; the mixture was allowed to stand for 15 min. The absorbance was measured at 500 nm. Extract samples were evaluated at a final concentration of 0.1 mg/ml. Total proanthocyanidin content was expressed as catechin equivalents (mg/g) using the following equation based on the calibration curve: $y = 0.5825x$, $R^2 = 0.9277$, where x was the absorbance and y is the catechin equivalent (mg/g).

2.7 Determination of antioxidant activity

2.7.1 ABTS radical scavenging assay

To determine ABTS radical scavenging assay, the method of Re et al. [28] was adopted. The stock solutions included 7 mM ABTS solution and 2.4 mM potassium persulfate solution. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 h at room temperature in the dark. The solution was then diluted by mixing 1 ml ABTS solution with 60 ml methanol to obtain an absorbance of 0.706 ± 0.001 units at 734 nm using the spectrophotometer. Fresh ABTS solution was prepared for each assay. Plant extracts (1 ml) were allowed to react with 1 ml of the ABTS solution and the absorbance was taken at 734 nm after 7 min using the spectrophotometer. The ABTS scavenging capacity of the extract was compared with that of BHT and percentage inhibition calculated as ABTS radical scavenging activity (%) = $[(Abs_{control} - Abs_{sample})]/(Abs_{control})]$ x 100 where $Abs_{control}$ is the absorbance of ABTS radical + methanol; Abs_{sample} is the absorbance of ABTS radical + sample extract /standard.

2.7.2 DPPH radical scavenging assay

The effect of the extracts on DPPH radical was estimated using the method of Liyana-Pathiranan and Shahidi [29]. A solution of 0.135 mM DPPH in methanol was prepared and 1.0 ml of this solution was mixed with 1.0 ml of extract in methanol containing 0.02–0.1 mg of the extract. The reaction mixture left in the dark at room temperature for 30 min. The absorbance of the mixture was measured spectrophotometrically at 517 nm. Ascorbic acid and BHT were used as references. The ability to scavenge DPPH radical was calculated by the following equation: DPPH radical scavenging activity (%) = $[(Abs_{control} - Abs_{sample})]/ (Abs_{control})]$ x 100 where $Abs_{control}$ is the absorbance of DPPH radical + methanol; Abs_{sample} is the absorbance of DPPH radical + sample extract /standard.

2.7.3 Reducing ability (FRAP assay)

The determination of the total antioxidant activity (FRAP assay) in the extract is a modified method of Benzie and Strain [30]. The stock solutions included 300 mM acetate buffer (3.1 g $C_2H_3NaO_2{\cdot}3H_2O$ and 16 ml $C_2H_4O_2$), pH 3.6, 10 mM TPTZ (2, 4, 6-tripyridyl-*s*-triazine) solution in 40 mM HCl, and 20 mM $FeCl_3{\cdot}6H_2O$ solution. The fresh working solution was prepared by mixing 25 ml acetate buffer, 2.5 ml TPTZ, and 2.5 ml $FeCl_3{\cdot}6H_2O$. The temperature of the solution was raised to 37 °C before use. Plant extracts (150 µL) were allowed to react with 2850 µl of the FRAP solution for 30 min in the dark condition. Readings of the colored product (ferrous tripyridyltriazine complex) were taken at 593 nm. The standard curve was linear between 200 and 1000 µM $FeSO_4$. Results are expressed in µM Fe (II)/g dry mass and compared with that of BHT, ascorbic acid, quercetin and catechin.

2.9 Statistical analysis

The experimental results were expressed as mean ± standard deviation (SD) of three replicates. Where applicable, the data were subjected to one way analysis of variance (ANOVA) and the differences between samples were determined by Duncan's Multiple Range test using the Statistical Analysis System (SAS, 1999) programme. *P* Values < 0.05 were regarded as significant.

3. Results and Discussion

3.1 Total phenolic, flavonoids, and proanthocyanidin contents

Results obtained in the present study revealed that the level of these phenolic compounds in the methanol extracts of the leaves and stem of *C. africana* were considerable (Table 1). Polyphenolic compounds are known to have antioxidant activity and it is likely that the activity of the extracts is due to these compounds [31, 32]. This activity is believed to be mainly due to their redox properties, which play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides [33]. In fact, many medicinal plants contain large amounts of antioxidants such as polyphenols. Many of these phytochemicals possess significant antioxidant capacities that are associated with lower occurrence and lower mortality rates of several human diseases [34, 35]. The results strongly suggest that phenolics are important components of this plant, and some of its pharmacological effects could be attributed to the presence of these valuable constituents.

Table 1. Polyphenol contents of the methanol extracts of the leaves and stems of *Celtis africana.* (n= 3, X ± SEM).

Phenolics	**Leaves**	**Stems**
Total polyphenol [a]	14.0 ± 0.11	15.39 ± 0.28*
Flavonoids [b]	0.70 ± 0.02	1.07 ± 0.07*
Total Flavonol [c]	0.10 ± 0	0.10 ± 0
Proanthocyanidins [d]	0.37 ± 0.21	4.58 ± 0.25*

[a]Expressed as mg tannic acid/g of dry plant material.
[b]Expressed as mg quercetin/g of dry plant material.
[c]Expressed as mg quercetin/g of dry plant material.
[d]Expressed as mg quercetin/g of dry plant material
* indicates that this value is significantly different from the other at P<0.05

*3.2 **Reducing ability** (FRAP)*

The reducing ability of the extracts was in the range of 407.60 - 695.98 µm Fe (II)/g (Table 2). The antioxidant potentials of the methanol extracts of the leaves and stem of *C. africana* were estimated from their ability to reduce TPRZ-Fe (III) complex to TPTZ-Fe (II). The FRAP values for the methanol extracts of the leaves and stem of *C. africana* were significantly lower than that of ascorbic acid, quercetin and catechin, but higher than that of BHT. The ferric reducing/antioxidant power (FRAP assay) is widely used in the evaluation of the antioxidant component in dietary polyphenols [36]. Antioxidant activity increased proportionally to the polyphenol content. According to recent reports, a highly positive relationship between total phenols and antioxidant activity appears to be the trend in many plant species [37].

Table 2. Total antioxidant (FRAP) activity of the leaves and stem extracts of Celtis *africana.*

Extracts	FRAP[e]
Leaves	695.98 ± 33.47
Stem	407.60 ± 36.86
Ascorbic acid	1632.1±16.95
BHT	63.46 ± 2.49
Catechin	972.02 ± 0.61
Quercetin	3107.29 ±31.28

[e]Expressed in units of µmol Fe (II)/g.

3.3 DPPH radical scavenging activity

Figure 1 shows the dose-response curve of DPPH radical scavenging activity of the methanol extracts of the leaves and stems of *C. africana,* compared with BHT and ascorbic acid. It was observed that methanol extracts of the stem of *C. africana* had higher activity than that of the leaves. At a concentration of 0.1 mg/ml, the scavenging activity of methanol extract of the leaves reached 64.95%, while at the same concentration, that of the stem was 89.69%. The effect of antioxidants on DPPH is thought to be due to their hydrogen donating ability [38]. Though the DPPH radical scavenging abilities of the extracts were less than those of ascorbic acid (100%) and BHT (98.3) at 0.1 mg/ml, the study showed that the extracts have the proton-donating ability and could serve as free radical inhibitors or scavengers, acting possibly as primary antioxidants.

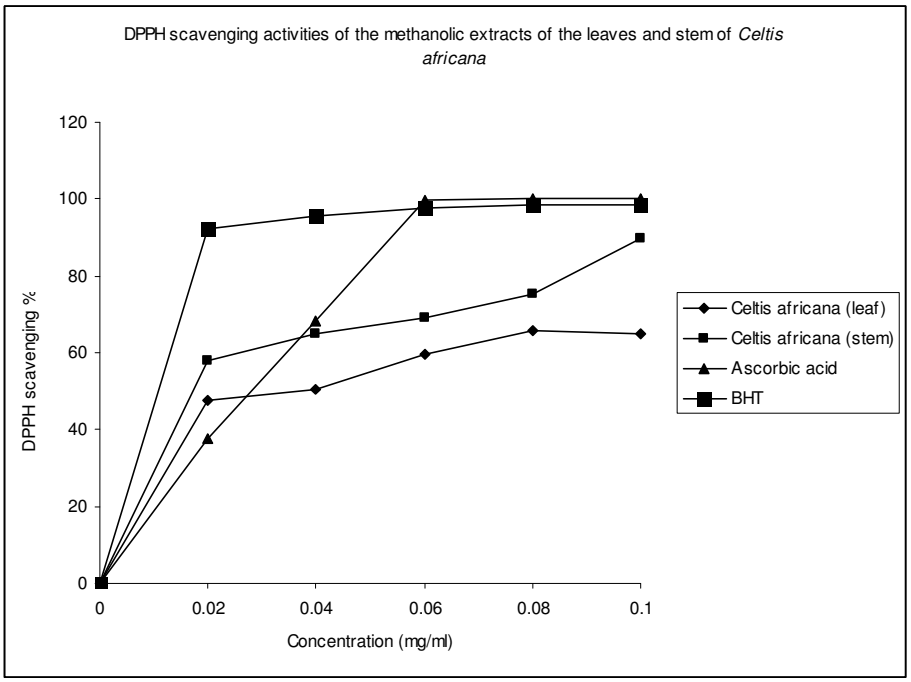

Figure 1. DPPH scavenging activities of the methanol extracts of the leaves and stem of *Celtis africana.*

3.4 ABTS radical scavenging activity

The methanol extracts of the leaves and stems of *C. africana* were fast and effective scavengers of the ABTS radical (Fig 2) and this activity was comparable to that of BHT. At 0.02mg/ml, the extracts exhibited higher activity than BHT, but at 0.1 mg/ml the activity of the extracts were similar to that of BHT. The percentage inhibition was 98.8, 98.8, and 99.3% for the leaf extract, stem extract, and BHT respectively at 0.1 mg/ml concentration. Proton radical scavenging is an important attribute of antioxidants. ABTS, a protonated radical, has characteristic absorbance maxima at 734 nm which decreases with the scavenging of the proton radicals [13].

The scavenging of the $ABTS^{+\bullet}$ radical by the extracts was found to be much higher than that of $DPPH^{\bullet}$ radical. Factors like stereoselectivity of the radicals or the solubility of the extract in different testing systems have been reported to affect the capacity of extracts to react and quench different radicals [39]. Wang et al. [40] found that some compounds which have $ABTS^{+\bullet}$ scavenging activity did not show DPPH scavenging activity. This is not the case in this study. This further showed the capability of the extracts to scavenge different free radicals in different systems, indicating that they may be useful therapeutic agents for treating radical-related pathological damage.

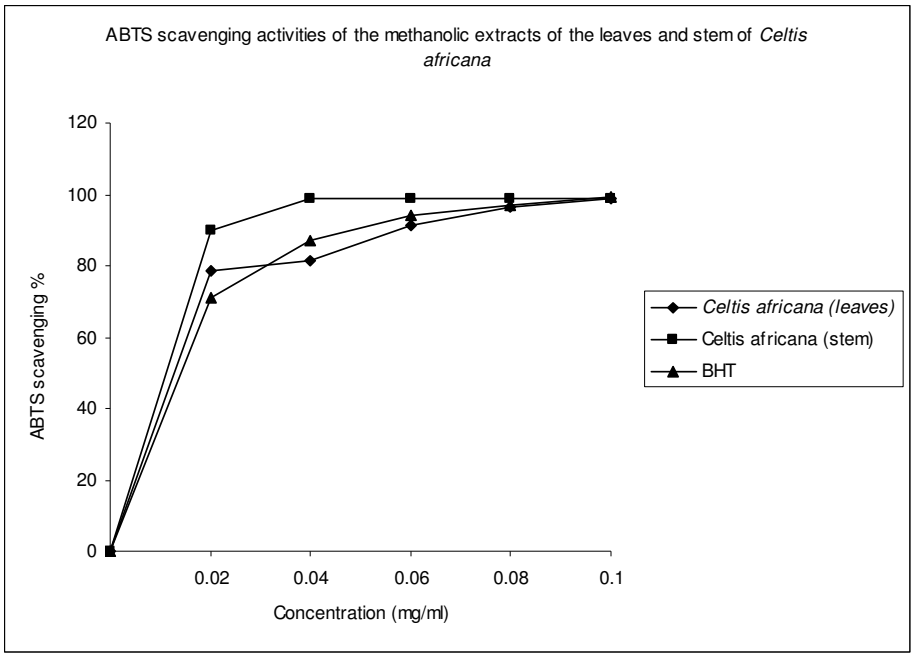

Figure 2. ABTS scavenging activities of the methanol extracts of the leaves and stem of *Celtis africana.*

Acknowledgements

The authors wish to acknowledge the financial support of Govan Mbeki Research and Development Center (GMRDC) of the University of Fort Hare for funding the research.

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