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Research Paper

Total Phenolic Content, Flavonoid Concentration and Antioxidant Activity of Leaves and Bark Extracts of *Celtis australis* L.

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ABSTRACT

In this study, total phenolic content, concentration of flavonoids and *in vitro* antioxidant activity of the methanolic 80% extracts of the leaves and the bark of *Celtis australis* L. (Ulmaceae) were determined using spectrophotometric methods in an effort to validate the medicinal potential activity of these parts. Leaves extract had a considerable larger amount yield of extract than the bark one using maceration as a method of extraction. Total phenols were calculated using Folin-Ciocalteu method whereas flavonoids concentrations were determined using $AlCl_3$ method. Antioxidant activity was determined depending on the extracts abililty to scavenge the radical DPPH• and to reduce Fe^{3+} to Fe^{2+} (FRAP ferric reducing ability power). Total phenols found in the leaves extract (16.89 ± 0.73 mg GA/g dry plant) were higher than the phenols found in the bark one (5.53 ± 0.19 mg GA/g dry plant). Flavonoids concentration was (14 ± 0.19 mg RU/ g dry plant) in the leaves extract and (0.17 ± 0.003 mg RU/g dry plant) in the bark one. DPPH radical scavenging activity of the two parts was almost the same, whereas the ferric reducing ability power test showed different results depending on the extract type and concentration. In two distinct tests of antioxidant evaluation, the extracts showed less values compared with BHT as a standard antioxidant. This study, has to some extent, validated the medicinal potential of the leaves and bark of *Celtis australis.*

KEYWORDS: *Celtis australis*; phenols; flavonoids; antioxidant activity; DPPH; FRAP.

Introduction

Antioxidants are believed to play a very important key role in the body defense system against reactive oxygen species (ROS), which are the harmful byproducts generated during normal cell aerobic respiration. Many of the recent researches have accepted that antioxidants are radical scavengers, which protect the human body against free radicals that may cause pathological conditions such as ischemia, anaemia, asthma, arthritis, inflammation, neurodegeneratation, and ageing process (Ravichandran and Panneerselvam, 2013). Plant-derived antioxidants are increasingly found beneficial in protecting against these diseases (El-Alfy et al., 2011). The antioxidant acitivity in plant refers mostly to phenolic compounds. The antioxidant potential of phenolic compounds has been shown in a number of *in vitro* studies. They are capable of direct chain-breaking antioxidant action by radical scavenging: in addition to being capable of scavenging of several non-physiological radicals such as $DPPH^{\bullet}$. Also, polyphenols have been suggested to spare essential antioxidants. For example, selected flavonoids (one branch of phenolic compounds) have been shown to be able to reduce the ascorbyl radical, *i.e.* to protect vitamin C (Nurmi, 2008).

Presently, much attention has been focused on the use of natural antioxidants to protect the human body from the oxidant damage caused by free radicals (Dasari et al., 2013). That is because recent reports revealed that synthetic antioxidants may be implicated in many health risks, including cancer and carcinogenesis (Samarin et al., 2012).

Celtis australis L. (Ulmaceae) is a deciduous tree. It is about 25 m high, bark pale-ashy grey or brown, often with white specks, branchlet drooping, leaves ovate-elliptical, flowers greenish, polygamous, 4, 5 merous, drupes ellipsoids, glabrous, purplish black etc. The bark of the plant gives yellow dye and wood used for making small articles. The paste obtained from the bark is an effective remedy for bone fracture and is also applied on pimples, contusions, sprains and joint pains (Semwal R and Semwal D, 2012). Bark decoctions are used as antiallergic (Akhtar et al., 2013), whereas leaves decoctions have been used to astringe the mucous membrane in peptic ulcers, diarrhea, and dysentery and as a remedy for heavy menstrual bleeding and colic (El-Alfy et al., 2011). Previously, three phenolics, acacetin 7-*O*-glucoside, isovitexin and cytisoside have been isolated from the leaves of *C. australis*. Recently, a novel sulphonated phenolic celtisanin and a bacteriohopanoid

3β-hydroxy-35-(cyclohexyl-5'-propan-7'-one)-33-ethyl-34-methyl-bactereo-hopane, along with three known compounds apigenin, quercetin and quercetin glucosides have been isolated from the plant, in addition to four triterpenoids (9β,31R)-9,25-cyclo-30-propylhopan-31-ol; (3β)-3-hydroxy-30-propylhopan-31-one; (3β)-oleanan-3-ol and (3β,9β)-9,25-cycloolean-12-en-3-yl β-D-glucofuranoside; a steroid (3β,9β,14β)-14-hydroxy-9,19-cychlocholan-3-yl β-Dglucopyranoside, and an anthraquinone 6-hydroxy-5,7,8-trimethoxy-9,10-dioxo-9,10-dihydroanthracen-2-yl acetate (Semwal R and Semwal D, 2012). A new flavonoid C-glycoside 8-(4-*α*-rhamnosyl-2″-*O*-β-d-galactopyranosylvitexin) with a considerable antioxidant and cytotoxic activity against defined carcinoma cells has been isolated from the leaves of this plant (El-Alfy et al., 2011).

The main objective of the research was to determine the total phenolic contents and the concentrations of flavonoids in methanolic 80% of the leaves and bark of *Celtis australis* using spectrophotometric methods, as well as to examine the antioxidant activity of plant extracts using *in vitro* model system. That was in order to give *Celtis australis*, which is known for its traditional therapeutic uses, a chance to be used in new therapeutic fields.

Materials and Methods

Plant material

Fresh leaves and bark of *Celtis australis* were collected from different places of Aleppo, Syria. The collection was done during April, 2014. The samples were identified by Dr. Ahmad Jaddouh (B.Sc. in Agricultural Science (speciality of Horticulture)) and confirmed by comparing them with the herbarium of Faculty of Agriculture, University of Aleppo.

Chemicals

Methanol GR (Eurolab,UK), Gallic acid (Prolab, Spain), Folin-ciocalteu (Sohariab SL, Spain), anhydrous sodium carbonate (Pareac quimica sau medien, Spain), Rutin (Extrasynthese Genay,France), Aluminum chloride hexa hydrate (Merck, Germany), 1,1-Diphenyl-2-picrylhydrazyl (DPPH) (Sigma-Aldrich, USA), Butylated hydroxytoluene (BHT) (Sigma -Aldrich, USA), Sodium phosphate (Reidel-haen-seelze, Germany), Disodium phosphate (Merck, Germany), Potassium ferricyanid (Merck, Germany), Trichloroacetic acid (Sigma- Aldrich, Germany), Ferric chloride (Merck, Germany). All used chemicals were of analytical grade.

Equipments

Many equipments have been used to complete this reaserch including: Rotatory evaporator (Heidolph, Germany), Sensitive balance (Sartorius TE214, Germany), Electrical mill (Moulinex, Syria), UV spectrophotometer (Shimadzu, Japan), Centrifuge (Heraeus, Germany), Titrimetric (Crison, Spain).

Preperation of plant extracts

Both bark and leaves were air dried at room temperature to constant weights. The dried plant materials were ground separately to powder. Twenty grams of each ground plant materials were extracted separately using methanol (80%) for 48 hours by maceration. The extracts were then filtrated through Whatman No 1 filter paper. This procedure was repeated thrice. After filtration the solvent was evaporated to dryness using rotary evaporator at 40 ºC (Adedapo et al., 2009). The obtained extracts were kept in a refrigerator at 4 C° until further use (Pieme et al., 2014). The yeild of extracts was calculated.

Determination of total phenolic contents in the extracts

The concentration of phenolics in plant extracts was determined using spectrophotometric method (Stanković, 2011) with a little bit modification. Methanolic solution of the extracts in the concentration of 0.5 mg/ml was used in the analysis. The reaction mixture was prepared by mixing 0.5 ml of methanolic solution of extract, 2.5 ml of 10% Folin-Ciocalteu's reagent diluted in water and 2.5 ml 7.5% Na_2CO_3. Blank was concomitantly prepared, containing 0.5 ml methanol, 2.5 ml 10% Folin-Ciocalteu's reagent diluted in water and 2.5 ml of 7.5% of Na_2CO_3. The samples were thereafter incubated in a thermostat at 45 ºC for 45 min. The absorbance was determined using spectrophotometer at λ_{max} = 765 nm. The samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained. The same procedure was repeated for the standard solution of gallic acid and the calibration line was construed. Based on the measured absorbance, the concentration of phenolics was read (mg/ml) from the calibration line; then the content of phenolics in extracts was expressed in terms of gallic acid equivalent (mg of GA/g of dry plant).

Determination of flavonoid concentrations in the plant extract

The content of flavonoids in the examined plant extracts was determined using spectrophotometric method (Stanković, 2011). The sample contained 1 ml of methanol solution of the extract in the concentration of 0.5 mg/ml for the leaves and 2 mg/ml for the bark and 1 ml of 2% $AlCl_3$ solution dissolved in methanol. The samples were incubated for an hour at room temperature. The absorbance was determined using spectrophotometer at λ_{max} = 415 nm. The samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained. The same procedure was repeated for the standard solution of rutin and the calibration line was construed. Based on the measured absorbance, the concentration of flavonoids was read (mg/ml) on the calibration line; then, the content of flavonoids in extracts was expressed in terms of rutin equivalent (mg of RU/g of dry plant).

Determination of antioxidant activity

DPPH radical scavenging assay

This assay measures the free radical scavenging capacity of the investigated extracts. DPPH is a molecule containing a stable free radical. In the presence of an antioxidant, which can donate an electron to DPPH, the purple color typical of free DPPH radical decays, and the absorbance change at λ = 517 nm is measured (Pieme et al., 2014). The effect of the extracts on DPPH radical was estimated using a standard method (Adedapo et al., 2009). A solution of 0.135 mM DPPH in methanol was prepared and 1 ml of this solution was mixed with 1 ml of different concentrations of extracts in methanol (The stock solution of extracts were prepared in methanol to achieve the concentration of 0.26 mg/ml for leaves and 0.15 mg/ml for bark). The reaction mixture was left in the dark at room temperature for 30 min. The absorbance of the mixture was measured spectrophotometrically at 517 nm. BHT was used as a reference. Control sample contained all the reagents except the extract. The ability to scavenge DPPH radical was calculated by the following equation:

DPPH radical scavenging activity (%) = $[(Abs_{control} - Abs_{sample}) / (Abs_{control})] \times 100$, where $Abs_{control}$ is the absorbance of DPPH radical + methanol; Abs_{sample} is the absorbance of DPPH radical + sample extract /standard. IC50 values were estimated from the % inhibition versus concentration plot, using a non-linear regression algorithm (Stanković, 2011). All the analysis was performed in triplicate.

Reductive ability (FRAP)

The reducing ability of tested plant parts was determined according to Dasari et al. (2013) method. To 1 ml of four increased methanolic extract concentrations (0.025-0.2 mg/ml) of both of leaves and bark, 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of potassium ferricyanide $[K_3Fe(CN)_6]$ (1%) were added. The mixture was incubated at 50°C for 20 min, after that 2.5 ml of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min, 2.5 ml of upper layer of the solution was mixed with 2.5 ml of distilled water and 0.5 ml of $FeCl_3$ (0.1%). Absorbance was measured at 700 nm. BHT was used as reference compound, all the analysis was performed in triplicate.

Statistical analysis

The experimental results were expressed as mean ± standard deviation (SD) of three replicates. Where applicable, the data were subjected to T test using the Statistical Analysis System (SPSS 17) programme. *P* Values < 0.01 were regarded as significant.

Results

Yeilds of plant extracts

Leaves had higher yeild of extract than bark. The percentage of dry yeild of extract for leaves was (27.52%), whereas it was (6.96%) for bark i.e., the ratio exceeded three folds.

Total phenolic content

The total phenolic content of the examined plant extracts was determined using the Folin-Ciocalteu's reagent. Figure 1 represents the calibration line for gallic acid (the measured absorbance versus the concentration). Based on the equitation got from it, total phenolic content of extracts was expressed in terms of gallic acid equivalent (mg of GA/g of dry plant) (Table 1). As illustrated in the table 1, the measured absorbance of the tested bark extract was higher than the leaves one, but total phenolic content in 1 g of dry plant was in leaves higher than it in bark (based on the yeilds of plant extract difference).

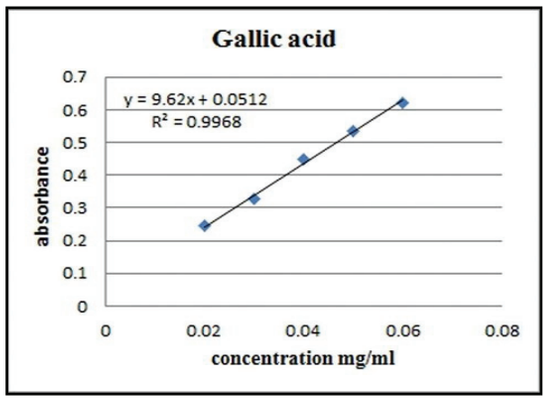

Fig. 1 The calibration line for gallic acid.

TABLE 1

Total phenolic content of leaves and bark of *C.australis* (n=3, x ± SD).

	The measured absorbance of plant extract *	Total phenolic content (mg GA/g dry plant)
Leaves	0.35 ± 0.01	16.89 ± 0.73**
Bark	0.43 ± 0.01	5.53 ± 0.19**

*The extract concentration was 0.5 mg/ml for both leaves and bark

**Indicates that these values are significantly different from each other at P < 0.01 using T test.

Flavonoid concentrations

The flavonoid concentration of the examined plant extracts was determined using Aluminum chloride reagent. Figure 2 represents the calibration line for rutin (the measured absorbance versus the concentration). Based on the equitation got from it, flavonoid concentration of extracts was expressed in terms of rutin equivalent (mg of RU/g of dry plant) (Table 2). As illustrated in Table 2, both of the measured absorbance and the flavonoid concentration of leaves extract were higher than bark extract ones.

Antioxidant Properties

DPPH radical scavenging assay (IC50 values)

According to the logarithmic curve got from the % inhibition versus the concentration plot (Figure 3 and Figure 4), IC 50 values for the both extracts of leaves and bark were estimated and compared with the one of BHT (Figure 5). Both leaves and bark had almost the same values ($IC50_{leaves}$= 0.1169±0.003 mg/ml, $IC50_{stem\ bark}$ =

0.117 ± 0.005 mg/ml). BHT had lower IC50 value than the plant extract ones ($IC50_{BHT}$ = 0.016 ± 0.001 mg/ml) which means that its radical scavenging activity is much more than it is in the plant extracts.

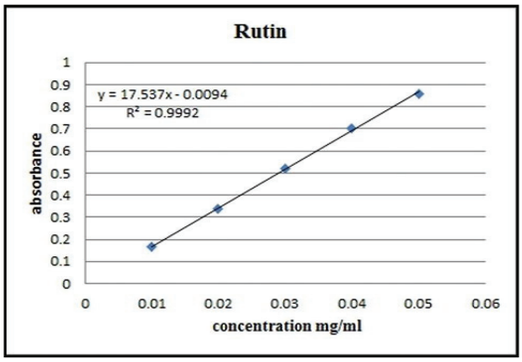

Fig. 2 The calibration line for rutin.

TABLE 2

Flavonoid concentration of leaves and bark of *C.australis* (n=3, x ± SD).

	The measured absorbance of plant extract*	Falavonoid concentration (mg RU/g dry plant)
Leaves	0.44 ± 0.01	14.00 ± 0.19**
Bark	0.08 ± 0.002	0.17 ± 0.003**

*The extract concentration was 0.5 mg/ml for leaves and 2 mg/ml for bark
**Indicates that these values are significantly different from each other at P<0.01 using T test.

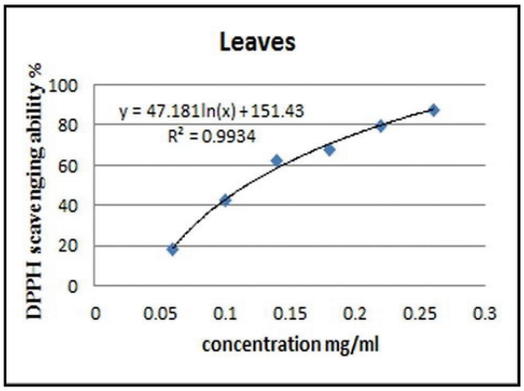

Fig. 3 DPPH scavenging acivity of leaves extract.

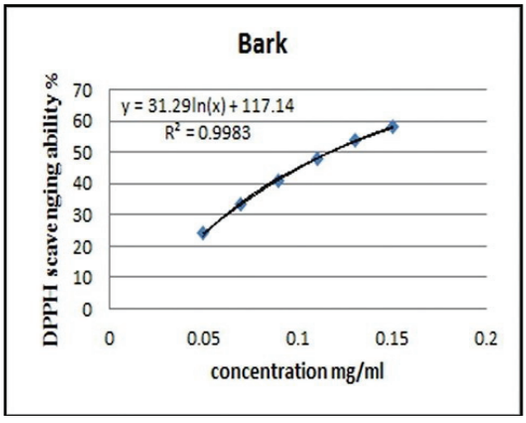

Fig. 4 DPPH scavenging acivity of bark extract.

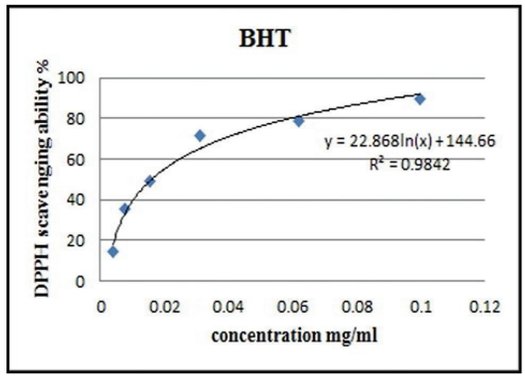

Fig. 5. DPPH scavenging acivity of BHT.

Reducing ability (FRAP)

Four concentrations (0.025, 0.05, 0.1, 0.2) mg/ml of each of the two extracts and the control were tested for their ability to reduce Fe^{3+} to Fe^{2+} using the illustrated spectrophotometric method. The results were expressed in the diagram (Figure 6) and compared with the BHT values as a control compound. As the figure 6 shows, the methanolic 80% extract of leaves and bark had almost a same reducing ability at low concentrations, but at higher concentrations bark extract showed a bit higher reducing ability than the leaves one. The reducing ability of the two extracts was lower than the BHT one.

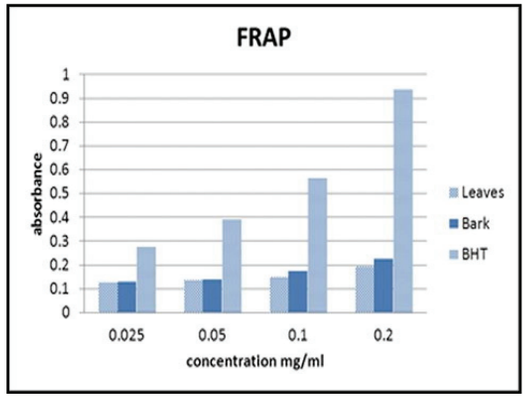

Fig. 6 Ferric reducing ability power of the tested samples.

Discussion

Polyphenolic compounds are known to have antioxidant activity and it is likely that the activity of the extracts is due to these compounds (Adedapo et al., 2009). Plant polyphenols have drawn increasing attention due to the antioxidant properties and their marked effects in the prevention of various oxidative stress associated diseases such as cancer (Dai and Mumper, 2010).

Results obtained from the present study revealed that the levels of phenols and flavonoids of the methanol (80%) extracts of the leaves and the bark of *C. australis* were considerable, even though that they were not the same for the two parts of the plant. Total phenolic content and flavonoid concentration of leaves extract were higher than bark one when calculated as mg

equivalent of standard/ 1 g of dry plant. The difference between the dried yeild of extracts for both leaves and bark has a considerable effect on the total amounts of phenols and flavonoids which are calculated in 1 g of dry plant. These amounts of total phenols and flavonoids found in *C.australis* leaves extract were higher than those found in *C.africana* ones, and the opposite was for the bark i.e., *C.africana* bark extract was higher in phenolic content and flavonoid concentration than *C.australis* bark extract according to the study done by Adedapo et al. (2009).

DPPH radical is widely used as a model to investigate the scavenging potential of several natural compounds such as phenolic and crude extract of plants (Hooda et al., 2013). The ability of the two extracts to scavenge the radical DPPH was almost the same. It could be logically explained by the similarity of the phenolic content of the extracts (mg/ml) at the same concentration not the phenolic content which is attributed to the dry weight of the plant. Even though this ability was lower than the ability of positive control (BHT). This study shows that the methanolic 80% extracts of the leaves and the bark of *Celtis australis* have the proton-donating ability and could serve as free radical inhibitors or scavengers.

For the reducing ability of the two studied extracts, the results show that this ability depends on the concentration of the extracts. The difference between the two extracts in their reducing ability was noticed at higher concentrations (when tested at different concentrations). The superiority of bark extratct reducing ability on the leaves extract one could be explained depending on the type of phenols found in each of two extracts and on the time of the reaction (Prior et al., 2005).

Conclusions

Based on these results it can be concluded that *Celtis australis* leaves and bark contain phytochemicals with exploitable antioxidant, free radical scavenging and could be considered as primary antioxidants.

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