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 AlCl₃ method. Antioxidant activity was determined depending on the extracts ability to scavenge the radical DPPH• and to reduce Fe³⁺ to Fe²⁺ (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, neurodegeneration, 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 activity 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*. 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 ova
to-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 celtisianin and a bacteriohopanoid...
3β-hydroxy-35-(cyclohexyl-5'-propan-7'-one)-33-ethyl-34-
methyl-bactereto-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-cyclolean-12-en-3-yl β-D-glucourano-
side; a steroid (3β,9β,14β)-14-hydroxy-9,19-cylocholan-
3-yl β-Dglucopyranoside, and an anthraquinone 6-
hydroxy-5,7,8-trimethoxy-9,10-dioxo-9,10-dihydroan-
thracen-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
research 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 °C (Adedapo et al.,
2009). The obtained extracts were kept in a refrigerator
at 4 C° until further use (Pieme et al., 2014). The yield
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% Na2CO3. 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 Na2CO3.
The samples were thereafter incubated in a thermostat
at 45 °C 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% AlCl3 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) from 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 $\lambda = 517$ 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:

$$ \text{DPPH radical scavenging activity (\%)} = \left( \frac{\text{Abs} \text{control} - \text{Abs} \text{sample}}{\text{Abs} \text{control}} \right) \times 100 $$

where Abscontrol is the absorbance of DPPH radical + methanol; Abssample 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.

**Reducive 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**

**Yields of plant extracts**

Leaves had higher yield of extract than bark. The percentage of dry yield 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 yields of plant extract difference).

![Fig. 1 The calibration line for gallic acid.](image)

**TABLE 1**

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

<table>
<thead>
<tr>
<th></th>
<th>The measured absorbance of plant extract *</th>
<th>Total phenolic content (mg GA/g dry plant)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaves</td>
<td>0.35 ± 0.01</td>
<td>16.89 ± 0.73**</td>
</tr>
<tr>
<td>Bark</td>
<td>0.43 ± 0.01</td>
<td>5.53 ± 0.19**</td>
</tr>
</tbody>
</table>

*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 (IC50leaves = 0.1169±0.003 mg/ml, IC50stem bark =
0.117 ± 0.005 mg/ml). BHT had lower IC50 value than the plant extract ones (IC50_bark = 0.016 ± 0.001 mg/ml) which means that its radical scavenging activity is much more than it is in the plant extracts.

**TABLE 2**
Flavonoid concentration of leaves and bark of *C. australis* (n=3, x ± SD).

<table>
<thead>
<tr>
<th></th>
<th>The measured absorbance of plant extract</th>
<th>Flavonoid concentration (mg RUG dry plant)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaves</td>
<td>0.44 ± 0.01</td>
<td>14.00 ± 0.19**</td>
</tr>
<tr>
<td>Bark</td>
<td>0.08 ± 0.002</td>
<td>0.17 ± 0.003**</td>
</tr>
</tbody>
</table>

*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.

**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 yield 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 extract 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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References


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