Seasonal variations in phenolics and antioxidant properties of leaves extract from *Celtis australis*

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**Abstract:** Seasonal variations have an influence on the availability of secondary metabolites of plants, which impacts their therapeutic efficacy. For some plants, the bioavailability of secondary metabolites, and thus the harvest period, is better in the spring, while for others it is summer. *Celtis australis* (Cannabaceae), a deciduous tree, is used in the treatment of various diseases in many traditional systems around the world. In the present work, seasonal influence on total polyphenols, flavonoids and condensed tannins contents as well as on the antioxidant potential of leaves hydro-methanolic extract from *Celtis australis* have been evaluated. Plant leaves were harvested during spring and summer which are its flowering and fruiting seasons respectively. The total phenols, flavonoids and condensed tannins contents were quantified by spectrophotometry using Gallic acid, Quercetin and Catechin as standards respectively. The antioxidant potential of the plant extracts was evaluated using three complementary methods: DPPH radical scavenging activity, conjugated dienes and TBARS inhibition during linoleic acid peroxidation. Our results showed that the levels of all quantified compounds increased significantly during the growing season until midsummer and this increase is positively correlated with the antioxidant
potential of the plant extract. It can be concluded that leaves harvesting should occur in the middle of the summer to obtain a better medicinal efficacy of the plant.

**Key words:** *Celtis australis*, Condensed tannins, Flavonoids, Polyphenols, Antioxidant, Seasonal impact

## INTRODUCTION

It is well known that plants are an important source of natural and health care compounds. Several studies have reported that diet rich in antioxidants is directly associated with the prevention of various human diseases, such as cardiovascular diseases, neuro-pathologic disorders, cancer, diabetes, among others\(^1\)\(^,\)\(^2\). All of these disorders have in common to be associated with an overproduction of free radicals in the body. These chemical species are generally very reactive because of their chemical instability due to the presence of one or more unpaired electrons. They are normally produced during aerobic metabolism. However, under certain abnormal conditions (drugs, industrial pollutions, radiations, smoking etc…) their production exceeds the capacity of the body to eliminate them causing oxidative stress\(^3\). This disorder can be prevented or delayed by the intake of natural and synthetic antioxidant compounds. Several studies have reported that plant constituents with antioxidant activity exerted a protective effect against oxidative stress\(^4\). Otherwise, several studies have reported the impact of environmental factors such as light, temperature, soil water, soil fertility and salinity, on biosynthesis and accumulation of secondary metabolites of plants, and therefore their pharmacological efficacy\(^5\)\(^,\)\(^6\).

*Celtis australis,* commonly known as the European nettle tree, Mediterranean hackberry or honeyberry, is a deciduous tree distributed in many parts of the world including southwestern Asia, and Mediterranean region. This plant is used in the treatment of various diseases in several traditional systems around the world. In Indian traditional medicine, the bark of leaves is considered as an important remedy for bone fracture and also applied to pimples, contusions, sprains and joint pains\(^7\). The decoction of both leaves and fruits is used in the treatment of amenorrhea, heavy menstrual and inter-menstrual bleeding, diarrhea, dysentery and peptic ulcers\(^8\). In Moroccan traditional medicine, *C. australis* commonly called “Taghzaz” is also used to treat various gastro-intestinal ailments\(^9\). Phytochemical investigations have shown the plant to contain several chemical species of secondary metabolites such as terpenoids, amides, lignin glycosides and steroids\(^10\), flavonoids\(^11\), sulphonated phenolic\(^12\), bacteriophananoid\(^13\), terpenoids, steroids and anthraquinones\(^14\), glycosyl-flavonoids\(^15\), phenolic amides\(^16\), \(\beta\)-sitosterol, \(\beta\)-sitosterol-glucoside and vanilic acid\(^17\), among others.

The purpose of the present study was to evaluate the seasonal effect on both phenolics contents (total phenols, flavonoids and condensed tannins) and antioxidant potential of leaves hydro-methanolic extract from *C. australis*.

## MATERIAL AND METHODS

**Sample preparation:** Plant material was authenticated by a specialist and a voucher specimen (reference CA1/13) is kept on file in our laboratory.

The study on seasonal impact has been conducted by collecting leaves of *C. australis* every two weeks from Mars to September which corresponds to two different seasons: Spring (Mars-Mai), wet period and summer (June–September), dry period. All the samples were collected from the same site (El Jadida city, Morocco).
The leaves of each sample (50g) were washed, dried at 35°C, powdered mechanically and sieved using a fine muslin cloth. The obtained powder was then exhaustively extracted three times with hydro-methanolic solution (1/5, V/V), by maceration in a hermetically closed glass vessel for 24 h, at room temperature (22°C), under occasional shaking. After filtration, and centrifugation at 500g for 30min, the resultant supernatant was concentrated under reduced pressure at 40°C. The yields varied from 11 to 25%. The crude extracts were stored at +4°C until use.

Chemicals: 1,1-Diphenyl-2-picrylhydrazyl (DPPH), butylated hydroxytoluene (BHT), catechin, gallic acid, quercetin, vanillin, Folin-Ciocalteu’s phenol reagent, sodium carbonate, sodium nitrite, linoleic acid, cupric sulphate, EDTA (ethylene diamine tetracetic), Tween 20, trichloracetic acid (TCA), thiobarbituric acid, butanol and other chemicals used were of analytical grade.

Quantitative analysis

Total polyphenols content (TPC) was quantified by the method previously described18.

Total flavonoids content (TFC) was measured using the method previously described18.

Total condensed tannins content (TCT) was performed by the method previously described18.

Assessment of antioxidant activity

DPPH radical scavenging activity: Radical scavenging activity (RSA) of the plant extracts was evaluated as the method previously described19.

Measurement of TBARS was monitored according to the method as previously reported18.

Measurement of conjugated dienes (CDs) formation was measured according to the method as previously described18.

Statistical analysis: The experimental results are expressed as mean ± standard deviation (SD) of three replicates. Where applicable, the data were subjected to one way analysis of variance (ANOVA). P Values < 0.05 were considered as significant.

RESULTS AND DISCUSSION

As shown in figure 1, TPC ranged from 45.76±7.13 to 85.84±8.03mg of Gallic acid equivalent/g of dry matter (mg GAE/g). TPC increased significantly (P<0.05) until midsummer (March 26 to August 13) and hold steady after. During this period, TPC raised up by 188%. This increase could be explained by the fact that, during the studied period (March to September), weather becomes gradually hotter with lengthening day. Several studies using different plants reported similar results. Ercisli et al.19 observed that the level of total phenolics of Camellia sinensis leaves was higher at second harvest time (July 15) than first and third harvest times (May 15 and September 15). According to the authors, the effect of temperature and day length could explain these results; the second fresh leaves harvest was made in warmer while the first and third harvests leaves were made in cooler months. Comparable results were obtained by Varga et al.20 who showed that phenolics content of Glechoma hederaea leaves harvested in July was higher than those harvested in April and October. Likewise, Bhandari and Kwak21 reported that the levels of phenols of commercial broccoli cultivars were generally higher in leaves and stems during the fall than the spring. To our knowledge, only one study focused on C. australis reporting the seasonal variation (spring to mid-autumn) in phenolic compounds. The obtained results, different from ours, showed that young leaves harvested in early spring contain highest amount of phenolics which rapidly decreases until mid-May. After this, phenolic content fluctuates but shows no discernible trend in either direction22. The geographical and therefore climatic
characteristics of the two sites could explain this difference. Our study site is located in coastal zone at 15 meters altitude with a temperate oceanic climate (mild and wet winter and cool summer). Precipitation is scarce (425 mm/year), and is concentrated from October to April; in the summer, it hardly rains. The other site is located in a mountainous area at 230m altitude with a semi-continental climate (cold winter, hot summer). The rains are moderate, (700 mm/year); the rainiest season is summer, because of the afternoon storms, while the driest season is winter.

This hypothesis is supported by Yang et al.23, who reported the effect of latitude and climate on the biosynthesis of different phenolic compounds in various currant cultivars. The results showed that the phenolic compounds contents of currants growing in northern latitude were 10 to 19% higher than those of currants growing in southern latitude. High radiation and temperature were associated with low contents of the major phenolic compounds in all the cultivars studied while high humidity correlated with low levels of hydroxycinnamic acid conjugates in green and white currants.

![Figure 1: Seasonal effect on total polyphenolic content (TPC) of leaves extract from C. australis](image)

TFC of leaves extracts from *C. australis* are reported in figure 2, where the results are expressed as mg of Quercetin equivalent per g of dried matter (mg QE/g). The rate of TFC increased significantly until midsummer (P>0.05) by 288%, from 22.69±6.04mg QE/g (early spring) to 64.04±7.95 mg QE/g (midsummer). The part of flavonoids related to total polyphenols also enhanced by 152%, from 49.6% in early spring to 75.4% in midsummer. Our results are corroborated by several studies using different plants. Dildar et al.24 reported the increase of flavonoids of leaves of *Melilotus indicus* from February to April. Same results were observed by Chaves et al.25 who observed that the flavonoid content of aerial parts of *Pseudobonbax marginatum* was higher in summer than in winter. Using different broccoli cultivars, Bhandari and Kwak21 showed that the levels of flavonoids were generally higher in florets in the spring than in the fall, but were higher in leaves and stems during the fall than the spring. All of these studies attribute the increase of flavonoid contents to the changes in ecological parameters, mainly the sunlight and day length.
Figure 2: Seasonal effect on total flavonoids content (TFC) of leaves extract from *C. australis*

*Figure 3* reported seasonal variation of CTC, expressed as mg of Catechin equivalent/g of dry matter (mg CE/g), that increase from 1.34±0.35 mg CE/g (early spring) to 2.79±0.48mg CE/g (midsummer). As for TPC and TFC, significant increase of CTC was observed until midsummer (P<0.05), reaching 208%. The part of condensed tannins related to total polyphenols increased also during the study period by 197%, from 2.93% (early spring) to 4.13% (midsummer). These results are similar to that obtained with wild bush tea leaves (*Athrixia phyllicoides*) whose condensed tannins increased from 2.66% in spring to 4.8% in autumn. According to the authors, this difference may reflect a response of drought stress during the dry autumn. The same trends were reported by Zhang et al. who showed that CTC of both sun and shade leaves of *Aegiceras corniculatum* increased during the growing seasons but CTC of sun leaves were significantly higher than those of shade ones in the same seasons. The authors assume that the content of carbon-based secondary metabolites tannins in the summer should be higher than that in the other seasons due to the stronger photosynthesis.

*Figure 3*: Seasonal effect on condensed tannins content (CTC) of leaves extract from *C. australis*
The importance of light in the growth of plants and the synthesis of secondary metabolites, via photosynthesis, is well known. Our results showed seasonal impact on all quantified metabolites that increased significantly, reaching their highest levels toward midsummer. Sunshine and day's length seem to be the main environmental factors of this increase. This phenomenon could be consistent with the photodamage hypothesis proposed by Close and McArthur\(^28\). According to the authors, when an unbalance between energy and photosynthesis capacity exists, the excess of absorbed energy by leaves might increases concentration of reactive oxygen species in plants and leads to destructive oxidative processes of biomolecules (lipids, proteins, nucleic acids) and enzymes. To protect themselves from oxidative stress, plants synthetize more antioxidant metabolites such as phenolic compounds. In these conditions it seems interesting to analyze seasonal effect on antioxidant potential of leaves extracts of the studied plant.

The antioxidant activity was determined by widely used and convenient methods: DPPH radical scavenging assay, TBARS and conjugated dienes inhibitions during linoleic acid peroxidation.

The DPPH (1,1-diphenyl-2-picrylhydrazyl), a stable free radical, can accept a hydrogen free radical or an electron to form diamagnetic species\(^29\). The reaction can be monitored spectrophotometrically at 517nm. Results, expressed as percentage of radical scavenging activity (RSA %), using different concentrations of leaves extract (10, 30, 60, 120, 200µg/ml) are reported in figure 4. The plant extract exhibited significant dose-dependent effect (P<0.05) associated with seasonal influence. During growing seasons, RSA increased reaching its maximum toward midsummer. Otherwise, statistical analysis indicated positive correlation (R²=0.99) between the phenolic contents and RSA seasonality, suggesting that environmental impact elicits important metabolic responses. This observation was reported by Harbowy and Balentine\(^30\) who observed that exposure of plants to stronger sunlight for a longer duration induces the synthesis of phenolic compounds.

Other studies using different plants corroborated seasonal influence on both total phenolic contents and RSA\(^31,32\).

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**Figure 4:** Seasonal effect on radical scavenging activity (RSA) of leaves extracts from *C. australis*.
In presence of oxygen, lipids are oxidized leading to the occurrence of chain reactions with the formation of coupled double bonds primary oxidation products such as conjugated dienes. The seasonal impact on the antioxidant potential of the plant extract, used at different concentrations (16, 32, 48, 64 and 80µg/ml), was assessed during the primary oxidation process of linoleic acid. The results are expressed as percentage of CDs inhibition. As shown in figure 5, significant and concentration-effect (P<0.05) was observed associated with seasonal impact. Highest effect was reached at midsummer. As for RSA, positive correlation between phenolic contents and antioxidant capacity was observed (R²=0.99). Similar results were reported with *Melilotus indicus* showing seasonal variations of both biosynthesis of phenolic contents and lipid peroxidation inhibitory activity that increase from colder to hotter seasons when plant receive stronger sunlight for a longer duration\(^\text{24}\). In the same way, Varga et al.\(^\text{20}\) reported that meteorological factors (temperature and illumination) affect the level of phenolic compounds in *Glechoma haderaceae*.

![Figure 5: Seasonal effect on conjugated dienes (CD) inhibition of leaves extract from C. australis](image)

The formation of secondary lipid oxidation products, such as aldehydes and other volatiles compounds was quantified by TBARS method using different concentrations of leaves extract (16, 32, 48, 64 and 80µg/ml). The method is monitored spectrophotometrically and the results are expressed in percentage of inhibition of TBARS. As shown in figure 6, the capacity of the plant extract to inhibit TBARS production increased significantly and dose-dependently associated with seasonal influence until midsummer. Otherwise, the antioxidant potential of plant extracts was positively correlated with the phenolic contents (R²=0.98). These results are corroborated by Cardenosa et al.\(^\text{33}\) who reported seasonal impact on TBARS inhibition that is positively correlated with increase of both ascorbic acid and tocopherols contents in different variety of *Citrus sinensis* fruits.
CONCLUSION

The results obtained in this work proved that the phenolic contents and antioxidant activity are overcome by the effects of the harvesting season. The biosynthesis of total polyphenols, flavonoids and condensed tannins increased until midsummer when the plant received stronger sunlight for a longer duration. The increase of phenolic compounds contents is positively correlated with antioxidant potential of the plant extract, supporting their role in the defense against free radicals. For better pharmacological efficiency of the plant, the leaves must be harvested in midsummer when phenolics content was highest.

REFERENCES


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