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Research Article
Phytochemical Compounds and Antioxidant Activity of Two Extracts of Wild and Domesticated Carob Leaves

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Abstract

Background and Objective: Carob tree (*Ceratonia siliqua* L.) is a perennial leguminous species and is known as a medicinal importance tree. This species exhibits a myriad of biological effects including antibacterial, antidiarrheal and antidiabetic. To this end, current study evaluates the difference between the phytochemical composition of the leaves of two accessions of "wild" and "domesticated" hermaphroditic carob trees. **Materials and Methods:** The comparison between two carob accessions "wild" and "domesticated" was done according to methanolic extraction by the Soxhlet and aqueous extraction by maceration. The polyphenols, flavonoids, tannins and their antioxidant activity were measured. The ANOVA test was used for the analysis of results. **Results:** The total polyphenols in aqueous extract are 6.19±0.25 mg equivalent gallic acid/g dry weight (EGA/g DW) and 4.23±0.2 mg EGA/g DW) in carob fresh leaves for wild and domesticated trees, respectively. The flavonoid content was higher in methanolic extract (3.17±0.64 mg quercetin equivalent/g DW) than in aqueous extract (1.06±0.19 mg EQ/g DW) for wild trees. Wild accession recorded the highest concentration of condensed tannins in the methanolic extract (6.4±0.3 mg catechin equivalents/g DW) while low levels were recorded in aqueous extract (0.51±0.27 mg EC/g DW). **Conclusion:** Such knowledge is expected to be the key to understanding the biochemical composition of two different leaves of *C. siliqua* accessions and its various commercial food products.

Key words: Antioxidant activity, *Ceratonia silique*, flavonoid, polyphenols, tannins, wild carob tree

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The carob tree (*Ceratonia siliqua* L.), which belongs to the vegetable family, is xerophytic since it has a great capacity to adapt to water constraints[1], hence its distribution in arid and semi-arid -arid regions belonging to Mediterranean climate[2]. The genus *Ceratonia* belongs to Fabaceae (syn. Leguminosae) of the order Rosales. Vegetables are important members of tropical, subtropical temperate vegetation around the world. It is one of the largest families of flowering plants, comprising 650 genera and over 18,000 species is extremely variable in morphology and ecology[3]. The word "siliqua" derives from the Latin meaning a silique or pod and alludes to the hardness and shape of the fruit[4].

The carob tree presents a potential reservoir of bioactive natural molecules as well as phenolic compounds and for this reason, it has been cultivated for millennia, not only as a feeder or food for human consumption but also to treat various diseases[5].

The carob tree (*Ceratonia siliqua* L.), a species of great importance for human health its biochemical composition deserve to be evaluated. This medicinal plant, which belongs to the vegetable family, is widely cultivated in Mediterranean countries, particularly in Tunisia because it is characterized by adaptation to water constraints and is well distributed in arid zones while presenting great therapeutic interest.

The center of origin of the carob tree remains mysterious since there is great disagreement between the authors' hypotheses. Firstly, it was placed by Tous *et al.*[6] and Vavilov[7], in the Eastern Mediterranean Region (Turkey and Syria). However, Mahdad and Gaouar[8] considered that the carob tree originated in the southern highlands of Arabia (Yemen)[4]. More recently, Zohary *et al.*[9] reported that the carob tree originated in the Arabian Peninsula and from archaeobotanical evidence (most of it in Palestine) showed the existence of the carob tree in the eastern Mediterranean basin before the start of agriculture[3].

For millennia, fruit carob has been used to treat illnesses, while its bark and leaves have been used in traditional medicine as anti-diarrheals, antimicrobials, anti-inflammatories, etc.

Carob tree is a source of bioactive molecules which present several advantages in cosmetology, pharmacology and the food industry. According to El Hajaji *et al.*[10] carob leaves have a high amount of calcium (Ca), low amounts of magnesium (Mg), potassium (K), sodium (Na), iron (Fe), while copper (Cu), zinc (Zn), chlorine (Cl) and selenium (Se) are negligible. According to Dallali *et al.*[2], leaves are an important reservoir of macronutrients such as lipids (21.33±0.82 to 45.22±0.31 mg/g DW) of which unsaturated fatty acids have the highest proportions (62. 52 to 54.68%) to that of saturated fatty acids (45.32 to 37.48%). These compounds are of great importance in herbal therapy and as healthy food due to their capacity to reduce the risk of occurrence of a certain number of pathologies (cancers, cardiovascular or neurodegenerative diseases, etc.[11].

Polyphenols have well-established antioxidant properties, that is why they are considered key determinants of the sensory and nutritional quality of plants[5]. The study by El Hajaji *et al.*[12] estimated that carob leaves are very rich in phenolic compounds. The total polyphenol contents found in certain studies vary depending on the pedoclimatic conditions of the implantation environment.

The present work aims to assess the phenolic compounds as well as the antioxidant activity of two leaves of two accessions (cultivated and wild) in relation to the method of extraction.

MATERIALS AND METHODS

Study area: Leaves were collected from two accessions (wild and cultivated carob trees) planted in the plot of the National Research Institute of Rural Engineering Water and Forest (INRGREF) Tunis. The study was carried out during 2021-2022.

Plant materials: Freshly collected plant material (1 kg), was carefully cleaned with a wet cloth, dried in the greenhouse in the open air for 2 weeks, then reduced to powder using a blade grinder (Moulinex AR110830). The resulting powder is stored at -20°C in tightly closed vials for subsequent analyses.

Preparation of the extracts: The comparison between two carob accessions "wild" and "domesticated" according to methanolic extraction by the Soxhlet and aqueous extraction by maceration. The extraction process is carried out according to the method of Custódio *et al.*[13], in this step 90% methanol used as a solvent to extract the polyphenols for 5 hrs with the Soxhlet. Subsequently, the methanol solution was centrifuged at 3000 rpm for 10 min and then the solvent was evaporated at 65°C. The dry residues are weighed and taken up in 10 mL of 80% methanol and stored at -20°C until use. Maceration is carried out with 10 g of leaf powder placed in 500 mL of distilled water at room temperature for 24 hrs with magnetic stirring. After centrifugation, the solvent was evaporated to dryness at 50°C and then placed in an oven at 46°C for 24 hrs.

Measurement of total phenol content: The total phenolic content was determined spectrophotometrically using the Folin-Ciocalteu method[14]. This test is based on the oxidation of phenolic groups by phosphomolybdic and phosphotungstic acids (FC reagent). In volumetric flasks, a volume of 200 µL of each extract was added with a mixture of 400 µL of Folin-Ciocalteu reagent diluted (10×). After 3 min, added 800 µL of sodium carbonate solution. The vials were shaken and kept for 1 hr, the absorbance was read at 725 nm. The calibration curve was prepared with gallic acid solutions ranging from 0 to 500 mg/L the results are given as gallic acid equivalents (GAE).

Determination of total flavonoids: The total flavonoid contents in the extracts were determined by spectrophotometry using the aluminum chloride method described by Ahmed *et al.*[15]. This dosage is based on the formation of a very stable complex, between aluminum chloride and the oxygen atoms present on carbons 4 and 5 of the flavonoids[16]. A 400 µL of the plant extract is introduced into volumetric flasks with 120 µL of $NaNO_2$ (5×). After 5 min, we added 120 µL of $AlCl_3$ diluted to 10% and shook the vials. After 6 min, we added 800 µL of 40% NaOH.

Thus, a reading of the optical density at 510 nm makes it possible to determine the concentration of the flavonoids, by referring to a calibration curve drawn from a series of standard solutions of quercetin having different concentrations: 10, 20, 30, 40 and 50 mg/mL. The flavonoid contents are expressed in milligram quercetin equivalent per gram of dry plant weight (mg EQ/g Dw). The total flavonoid contents in the extracts were determined by spectrophotometry using the aluminum chloride method described by Ahmed *et al.*[15].

Dosage of condensed tannins: A dose of 50 µL of extract is suitably added to 1.5 mL of 4% vanillin and 750 µL of HCl. After homogenization, the mixture is incubated at room temperature for 20 min. The absorbance of this preparation is measured at 550 nm[16].

A calibration curve is performed in parallel under the same operating conditions using catechin at different concentrations of 50, 100, 150, 200, 250 and 300 µg/mL with 50 µL of vanillin. Condensed tannins were expressed as mg catechin equivalents (CE)/L g of dry weight (mg EC/g DW).

Determination of hydrolyzable tannins: A volume of 1 mL of each extract was added to 1 mL of 10% Ferric Chloride ($FeCl_3$), then mixed and left for 20 min at room temperature. An optical density reading at 660 nm allows the concentration of hydrolysable tannins to be determined by reference to a calibration curve drawn from a series of standard catechin solutions with different concentrations (50, 100, 150, 200, 250, 300, 350 and 400 µg/mL) and by adding 1 mL of ferric chloride.

Determination of antioxidant activity; DPPH radical scavenging activity: The tests were carried out in triplicate. The method of Perez Jimenez *et al.*[17] was used to determine the free radical scavenging activity by DPPH (2,2-Diphényl-1-Picrylhydrazyl) based on the spectrophotometer. The scavenging activity on the DPPH radical was expressed as an inhibition percentage using the following equation:

$$\text{Inhibition (\%)} = \frac{\text{Abs - Absc}}{\text{Abs}} \times 100$$

where, Abs is the absorbance of the control reaction (containing all reagents except the test compound) and Absc is the absorbance of the test compound. Ascorbic acid was used as positive control. The extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph of inhibition percentage plotted against extract concentration (200, 100, 50, 25 and 10 µg/mL). The IC_{50} was inversely linked to the antioxidant capacity of a compound, as it expresses the amount of antioxidants needed to decrease the concentration of free radicals by 50%. The lower the IC_{50} value, the higher the antioxidant activity of a compound.

Statistical analysis: All data were subjected to a One-way Analysis of Variance (ANOVA) with SPSS version 21.0 to determine the significance. Means and Standard Errors (SE) of all data were calculated based on three replicates (n = 3). A p-value under 5% was considered statistically significant. Duncan's multiple range test was used to perform means' comparisons.

RESULTS

Polyphenol contents: According to the extraction method, it is noticed that in the wild accession, the aqueous extract presented the highest content of polyphenols (6.19±1.25 mg EAG/g DW) than the methanolic extract (5.19±0.19 mg EAG/g DW). On the other hand, in the domesticated accession we found that the polyphenol content in the methanolic extract (4.88±0.25 mg EAG/g DW) was slightly higher than that in the aqueous extract (4.23±0.2 mg EAG/g DW) (Fig.1).

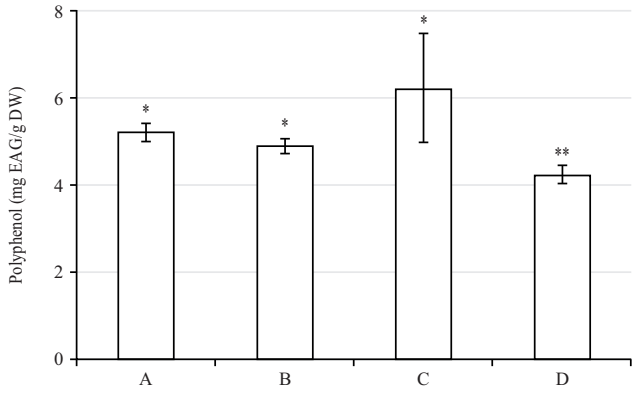

Fig. 1: Total polyphenol contents (mg EAG/g DW) of aqueous and methanolic extracts of *Ceratonia siliqua* L. leaves
A: Methanol extracts of wild leaves, B: Methanol extracts of domesticated leaves, C: Aqueous extract of wild leaves, D: Aqueous extract of domesticated leaves, *Significant at p = 0.5 and **,***Highly significant at p = 0.5

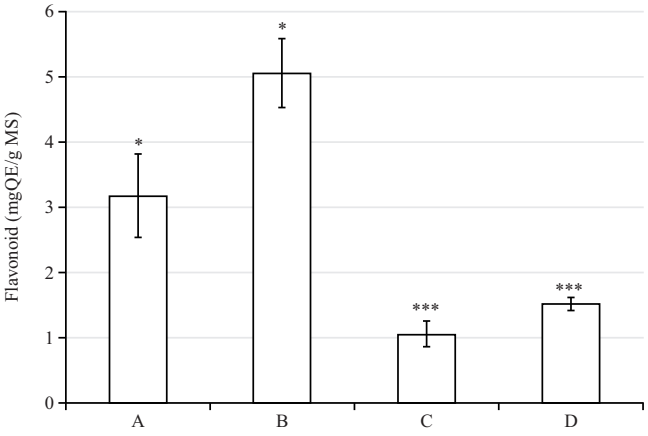

Fig. 2: Flavonoid content (mg QE/g MS) of aqueous and methanolic extracts of the leaves of *Ceratonia siliqua* L.
A: Methanol extracts of wild leaves, B: Methanol extracts of domesticated leaves, C: Aqueous extract of wild leaves, D: Aqueous extract of domesticated leaves, *Significant at p = 0.5 and **,***Highly significant at p = 0.5

Total flavonoids: A comparison using the extraction method showed that in wild carob leaves, the methanol extract had a content of (3.17±0.64 mg EQ/g DW) which is higher than the aqueous extract (1.06±0.19 mg EQ/g DW) with a very significant difference. Whereas, the cultivated accession found that the methanol extract (5.06±0.48 mg EQ/g DW) had a higher content than the aqueous extract (1.51±0.10 mg EC/g DW) (Fig. 2).

The comparison by accession showed that the leaves of the cultivated carob were richer in flavonoids than those of the wild carob.

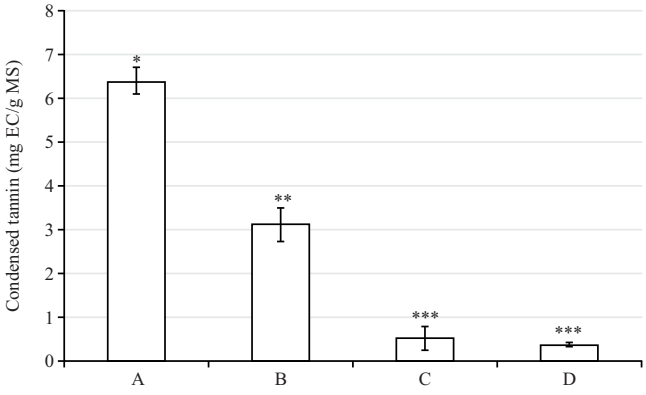

Fig. 3: Condensed tannins (mg EC/g DW) of aqueous and methanolic extracts of the leaves of *Ceratonia siliqua* L.
A: Methanol extracts of wild leaves, B: Methanol extracts of domesticated leaves, C: Aqueous extract of wild leaves, D: Aqueous extract of domesticated leaves, *Significant at p = 0.5 and **,***Highly significant at p = 0.5

Condensed tannins: Based on the obtained results based on a comparison between the two used extraction methods, for wild carob, the highest concentration of condensed tannins was recorded in the methanol extract (6.4±0.3 mg EC/g DW) whereas low concentrations are in the aqueous extract (0.51±0.27 mg EC/g DW). In addition, the cultivated accession had high levels of 3.1±0.39 mg EC/g DW in the methanolic extract and low levels of 0.36±0.038 mg EC/g DW in the aqueous extract (Fig. 3).

Hydrolysable tannins: These results showed that in wild accession both methanol extract (9.24±0.33 mg EC/g DW) and aqueous (8.7±0.011 mg EC/g DW) were very rich in hydrolyzable tannins. Although, in the domesticated carob we found that both methanolic (8.91±0.065 mg EC/g DW) and aqueous (8.32±0.028 mg EC/g DW) extracts were very rich in hydrolyzable tannins (Fig. 4).

Antioxidant capacity using DPPH: The study evaluated the antioxidant activity of ascorbic acid, which is a very potent anti-oxidant and used as a reference. The regression formula for obtaining the antioxidant power evaluation curve presented by the percentage of DPPH inhibition based on different concentrations of ascorbic acid is y = 301.175x+9.4183 with a determination coefficient (R^2 = 0.9916). The two accession extracts (wild and domesticated) combined with the two methods of extracts (aqueous and soxhlet-methanol) were further investigated using the DPPH assay to evaluate the antioxidant activity of leaf extracts. Results were summarized in Fig. 5. This

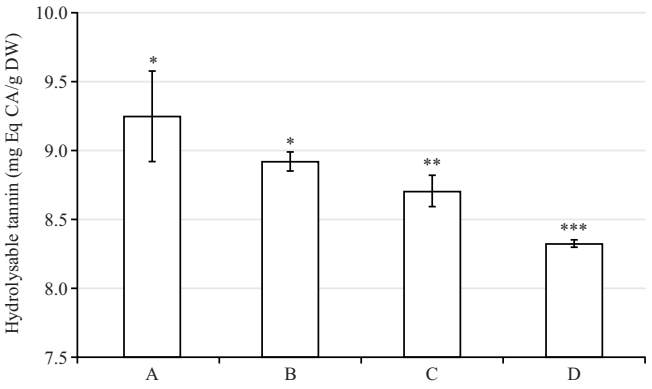

Fig. 4: Hydrolysable tannins (mg EC/g DW) of aqueous and methanolic extracts of *Ceratonia siliqua* L. leaves
A: Methanol extracts of wild leaves, B: Methanol extracts of domesticated leaves, C: Aqueous extract of wild leaves, D: Aqueous extract of domesticated leaves, *Significant at p = 0.5 and **,***Highly significant at p = 0.5

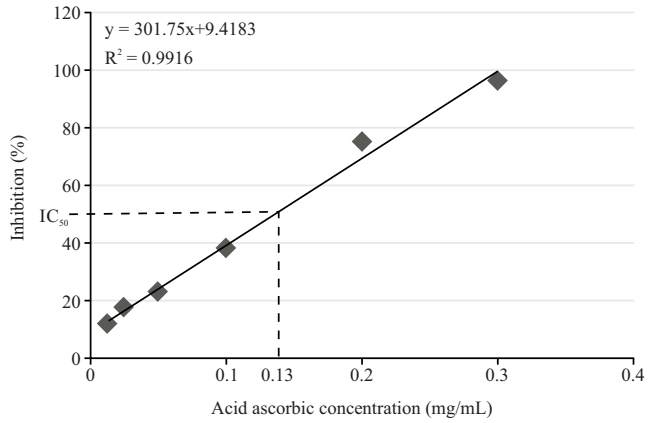

Fig. 5: Evaluation curve for the antioxidant activity of ascorbic acid

Table 1: IC_{50} of the different extracts

Extract	IC_{50} (mg/mL)
Wild leaves	0.16
Domesticated leaves	0.26
Ascorbic acid	0.13

antioxidant ability can be assessed by the determination of IC_{50} values related to the amount of the sample required to reduce 50% of free radicals.

The results of the DPPH inhibition test by the methanolic extracts to review this term of wild and domesticated carob were represented by the curve shown in Fig. 5.

The curve represented by (Fig. 5) showed an exponential appearance where the percentage of free radical inhibition increases with the increase in the concentration of the extracts.

The DPPH inhibition rate recorded in the presence of both types of extract was slightly lower than that of ascorbic acid. The concentration of the essential sample to inhibit 50% of radical DPPH was calculated by linear regression of the inhibition percentages calculated on the basis of different concentrations of prepared extracts (Table 1).

DISCUSSION

The two extraction methods as well as the two accessions (wild and cultivated) revealed different results. The aqueous extract revealed the higher quantity of polyphenol than the methanolic one. The current results were in accordance with the study Uysal *et al.*[18], who found a polyphenol content quantity of 154.72±3.76 mg EAG/g DW for the aqueous extract. Until now there has been no study that reveals the difference between these two types of solvent for domesticated carob leaves. These results concluded that the wild accession is richer in polyphenols than the domesticated accession, which was in contradiction with the results of El Hajaji *et al.*[12].

These results were in accordance with the work of Dallali *et al.*[2] and Corsi *et al.*[19] which showed that the polyphenol content can reach 6.45 mg EAG/g DW and 6.28 mg EAG/g DW, respectively. However, present results showed lower values than those found by Hsouna *et al.*[20], where the values are 91.2 to 680 mg EAG/g DW. This difference in results can be explained by the extraction method where these authors used ethanol as a maceration solvent. Variability in biochemical characteristics can also be due to genetic diversity.

The carob plants from current study site are hermaphroditic, which explains the difference with the results found by Custódio *et al.*[13] which were extracted from male feet (28.8±2.6 mg EAG/g DW) and female feet (12.3±1.1 mg EAG/g DW.)

It is therefore noted that the variations observed will probably be due to numerous factors including gender, origin, accession, extraction method and the used solvent.

Results of the flavonoid content were similar to those of Dallali *et al.*[2], who found that the flavonoid content ranges between 3.42 and 7.42 mg EQ/g DW. While another study found a high level of flavonoids compared to present results (193.3 mg EQ/g DW)[21].

Custódio *et al.*[13] showed that the analysis of cultivated Portuguese carob leaves showed flavonoid levels similar to our results. This can be explained by the fact that carob belongs to the Mediterranean region itself. The methanol

extract of the cultivated carob from this study contains a content (5.06±0.48 mg EQ/g DW) that is within the range of the content of the Portuguese methanolic extract (1.6±0.1; 7.0±1.0 mg EQ/g DW). Sengül *et al.*[21] found high levels of flavonoids in aqueous extract 42.89±0.35 mg EQ/g DW and in methanol extract 64.64±1.26 mg EQ/g DW. Moroccan carob leaf extract contains 25.35 mg EQ/g DW[22]. These values are very high as a result of current findings on genetic variability in the Mediterranean Region.

The results showed that the soxhlet extraction method is more efficient than maceration. Domesticated carob contains more flavonoids than wild carob. The differences observed can be explained by the genetic potential of the plant material and the extraction method[20]. Carob trees of this study are hermaphrodite which may explain the difference in concentrations of condensed tannins with results found in other studies. The study by Custódio *et al.*[13] showed that the male species recorded 4.4±1.4 mg EC/g DW and the female one recorded 9.7±0.6 mg EC/g DW. In contrast, the aqueous extract gives us very low concentrations of condensed tannins ranging between 0.3 and 0.5 mg EC/g DW. These results can be explained by the difficult extraction of this compound by cold maceration which requires other more efficient techniques.

These results show that the wild accession is very rich in condensed tannins than the cultivated accession. The Soxhlet extraction method is the most effective than maceration and methanol is the corresponding solvent by excellence. Results obtained were within the margins of the results carried out on carob in Tunisia by Rtibi *et al.*[23], when studying variability of hydrolysable tannins. The methanol extract of the wild carob leaves (IC_{50} = 0.16 mg/mL) has a higher antioxidant activity than the methanolic extract from the domesticated carob leaves (IC_{50}). This difference in finding results was due to the richness of polyphenols in the extracts of the wild carob compared to domesticated ones in the same extract process. The polyphenol content of the methanol extract of the wild species is 5.19 mg Eq AG/g DW, while that of the cultivated carob is 4.88 mg EQ AG/g DW (Table 1).

Compared to ascorbic acid (reference antioxidant) which has an IC_{50} value of 0.13 mg/mL, it was concluded that the two carob extracts are slightly less active than the reference. The samples in our study have a stronger antioxidant power than the Moroccan carob in the study of El Hajaji *et al.*[12]. A Portuguese study Custódio *et al.*[13] showed that the methanol extract of domesticated carob has an IC_{50} of 0.27 mg/mL which was the same result of current study for domesticate carob (IC_{50} = 0.26 mg/mL). Consequently, based on the high level of total phenols, flavonoids and tannins leaves and carob extracts (methanolic and aqueous) show a significant antioxidant capacity. Phenolic compounds contribute to the antioxidant propriety in a dose-dependent manner until a maximum of activity[24]. The studied results were in accordance with previous results[25]. Polyphenols, through their antioxidant activities in carob leaf extracts' neutralize free radicals. Therefore, it is suggested that carob may be used for the prevention of free radical-related diseases as a dietary natural antioxidant[26].

CONCLUSION

The total phenolic quantification and the antioxidant activity were assessed using two applied methods of extracts on two accessions of carob species. The result showed variability in the quantification of the finding phytochemical product. Polyphenol in the aqueous extract is higher than this in methanolic extract. So, extraction is a very important step in the isolation and recovery of compounds of interest. It can be concluded that the presence of polyphenols in carob leaves related to high antioxidant activity has a valuable effect on human health.

SIGNIFICANCE STATEMENT

Carob leaves contain phenolic compounds which give them different roles including antioxidant activity. These polyphenols are essentially tannins, flavonoids etc. So, to assess these compounds their extraction need with two methods from wild and cultivated accessions. According to the method of extraction, it is noticed that in the wild accession, the aqueous extract presented the highest content of polyphenols than the methanolic extract. The comparison by accession showed that the leaves of the cultivated carob were richer in flavonoids than those of the wild carob. The two accessions present almost the same quantity of hydrolysable tannins. The study showed that the carob leaves have antioxidant activity which may be proposed to be used in pharmacology and food industry to protect against damage from oxidative stress.

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