

Antioxidant Properties and Total Phenolic Content of Three Varieties of Carob Tree Leaves from Morocco

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Abstract: The in vitro antioxidant activity and the total phenolic content (Folin–Ciocalteu method) of three successive extracts of three varieties of *Ceratonia siliqua* L. leaves (grafted female, spontaneous female, spontaneous male) grown in Morocco were investigated by using in-vitro antioxidant models including 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging assay, reducing power and total antioxidant capacity. The global polyphenols concentration ranged from 0.45 to 2.64 (g/L GAE) in the three categories of the extracts. In each variety, ethyl acetate fraction exhibited the highest antioxidant activity compared to other fractions. Grafted female trees globally showed a higher polyphenols concentration than the spontaneous female and spontaneous male ones. Our results clearly demonstrate that all extracts have antioxidant capacity. Among the categories, the ethyl acetate extracts of carob tree leaves exhibited strong scavenging effect on 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) than the diethyl ether and dichloromethane extracts. Carob leaf extracts contain high amounts of polyphenols with strong antiradical, antioxidant capacity and reducing properties which might constitute an important source of natural antioxidants.

Keywords: Antioxidant activity; Phenolic content; *Ceratonia siliqua* L.

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1. Introduction

The commercial development of plants as sources of antioxidants to enhance health and food preservation is of current interest [1]. Epidemiological studies have suggested positive associations between the consumption of phenolic-rich foods or beverages and the prevention of diseases [2].

These effects have been attributed to antioxidant components such as plant phenolics, including flavonoids and phenylpropanoids [3]. Antioxidants are compounds that neutralize chemically active products of metabolism, such as free radicals which damage the body. Sources of natural antioxidants are primarily phenolics that may occur in all products and parts of a plant such as fruits, vegetables, nuts, seeds, leaves, roots, and bark. Due to their potential antioxidant action, plant phenols and polyphenols, with their potential to act as antioxidants; play a major role in the prevention of various pathological conditions such as cancer, cardiovascular and neurodegenerative diseases believed to be associated with oxidative stress [4].

Carob tree (*Ceratonia siliqua* L.) is a rich plant in phenols and polyphenols and has been widely grown under the Mediterranean Climate for a long time. It is a perennial leguminous (Caesalpinioideae) that grows as an evergreen shrub or tree up to 10 m high, with a broad semi spherical crown and thick trunk with brown rough bark and sturdy branches. It belongs to the Caesalpinaceae sub family of the family Leguminosae (= syn Fabaceae). The Carob tree has been grown since antiquity in most countries of the Mediterranean basin, usually in mild and dry places with poor soils. It is an important component of the Mediterranean vegetation, and its adaptation in marginal soils of the Mediterranean regions is important environmentally and economically [5, 6]. The Arabs disseminated it along the North African coast and north into Spain and Portugal. The Carob tree matures slowly and bears pods in the 5th - 6th year. The flowers of the carob are very small, inconspicuous and unisexual. Leaves are 3-7 cm long, alternate, pinnate, with or without a terminal leaflet. Carob does not shed its leaves in the autumn but only in July every second year, and it only partially renews leaves in spring. The production of carob pod in the world is estimated at about 315,000 tonnes per year, Morocco is the fourth producer [7]. The production in Morocco, based on wild populations which is very variable. It has increased during the last 15 years, and it is estimated to be about 26,000 tonnes. The main spontaneous populations are concentrated in the regions of Tafelma in the north and Ait Ishaq in the south. However, three areas are commercially known Fès, Marrakech and Agadir. Acreage of spontaneous carob in Morocco is estimated to 30,000 ha. Carob tree thrives together with a number of other species such *Pistacia* spp., *Olea* spp. *Quercus* spp., etc.

The two main carob pod constituents are pulp (90%) and seed (10%). Chemical composition of the pulp depends on cultivar, origin and harvesting time [8]. Carob pulp has a high content in total sugar, consisting of mainly sucrose, glucose, fructose and maltose. In addition, it contains about 18% of cellulose and hemicellulose. Constituents of the carob seed are coat (30-33%), endosperm (42-46%) and embryo or germ (23-25%) [9, 10]. The seed coat contains antioxidants [11]. The endosperm is the galactomannan carob bean gum (CBG). Carob pod meal contained high levels of carbohydrates (45%), appreciable amounts of protein (3%), and low levels of fat (0.6%). Germ and seed meal contained more fat and less carbohydrates compared to the carob pod [12]. The butanol soluble fraction of the methanol extract of the carob cotyledons contain five C-glycosylflavones including schaftoside, isoschaftoside, neoschaftoside, isoschaftoside-4'-O-glucoside and schaftoside-4'-O-glucoside [13]. Flavonoids represented 26% of the polyphenols and the major components were identified as the glycosides myricetin and quercetin-3-O- α -L-rhamnoside [14, 15]. Phenolic contents of pulps and leaves from carob tree (*Ceratonia siliqua* L.), have been reported [12, 16-19]. M. Balaban has demonstrated that carob wood is characterized by its richness in gallotannins and in proanthocyanidins [20]. New flavonol glycoside, 4'-*p*-hydroxybenzoylisorhamnetin-3-O- α -L-rhamnopyranoside named ceratoside, together with the known kaempferol-3-O- α -L-rhamnopyranoside (afzelin), quercetin-3-O- α -L-arabinofuranoside (auriculain), quercetin-3-O- α -L-rhamnopyranoside, β -sitosterol and β -sitosterol-3-O- β -D-glucoside were isolated for the first time from *Ceratonia siliqua* L. seeds [21]. D. Z. Botega et al. showed that Exxenterol, a non-extractable tannin rich fiber, can be successfully employed as an additive to significantly prolong sunflower oil

frying-life, and thus decrease the potential toxicity of the heated oil [22]. Vaya & Mahmood show that the carob leaves are rich in flavonoids; and more than nine compounds were identified [14]. Recently, investigators isolated and identified the major polyphenols in carob fibers [18, 23], and other studies are on the variation and composition of phenolic compounds of carob pods [24]. Everywhere in Morocco, pods or only seeds are used to fight diarrhea in infants, children and adults. Infusion of carob leaves is used as an emetic for acute poisoning [25]. In Turkish folk medicine, leaves and barks of carob tree are used as an antidiarrheal and diuretic [26, 27]. The fruits of this plant are traditionally used as an antitussive and against warts [28, 29].

Some studies have shown the antioxidant activity of *Ceratonia siliqua* L. These studies were conducted only on carob pods and carob fruits. For example, S. Kumazawa et al. reported that carob pod crude polyphenol had high antioxidant activity comparable to that of authentic polyphenol compounds. Especially, it is apparent that carob pod crude polyphenol has strong effect against the discoloration of β -carotene. [30]. Similarly, M. Papagiannopoulos et al. showed that carob fiber and carob flours have high antioxidative activity expressed with a high DPPH radical scavenging activity [23]. The antioxidant activity of the carob pod is attributed to the presence of phenolic compounds [18, 31].

To the best of our knowledge, there are no such reports concerning Moroccan cultivars, so the present work has focused to study the antioxidant activity and the total phenolic content with Folin-Ciocalteu of the extracts of leaves of spontaneous male, spontaneous female, and grafted female carob trees growing in Morocco.

2. Materials and Methods

2.1. Plant Material

Ceratonia siliqua L. leaves used in this experiment were sampled on productive Dkar (spontaneous female), unproductive Dkar (spontaneous male) and Lanta (grafted female) trees from the province of Chafchaouen (NW of Morocco) [32]. Vouchers specimens (INP211, INP212, INP213) were identified by Professor A. Ennabili and have been deposited in the Herbarium of National Institute of Medicinal and Aromatic Plants, Sidi Mohamed Ben Abdellah University, Fès, Morocco.

2.2. Preparation of the extracts

The air-dried leaves of *Ceratonia siliqua* L. were extracted in a Soxhlet apparatus with hexane for 24 hours. The residue of the plant was dried, and then extracted with methanol/water 8:2 (v/v) by exhaustive maceration (3,500 ml). The methanol/water extract obtained from a sample was evaporated in order to remove the methanol, and the aqueous phase was extracted with diethyl ether (Et₂O), dichloromethane (CH₂Cl₂) and ethyl acetate (EtOAc), successively.

2.3. Total phenolic content

The total phenolic content was determined spectrophotometrically using the Folin–Ciocalteu method. This test is based on the oxidation of phenolic groups by phosphomolybdic and phosphotungstic acids (FC reagent). This reagent, based on the Slinkard and Singleton method [33], and the early work of Singleton & Rossi [34] is a colorimetric oxidation/reduction method for phenolic compounds. The products of the metal oxide reduction have a blue color that exhibits a broad light absorption with a maximum at 764 nm. The intensity of light absorption at that wavelength is proportional to the concentration of phenols. Briefly, a 20 μ L of the diluted sample was added to 100 μ L of Folin–Ciocalteu reagent. After 8 min, 300 μ L of saturated sodium carbonate solution (25%) was

added. The absorbance was measured at 764 nm. The calibration curve was prepared with gallic acid solutions ranging from 0 to 500 mg/L, and the results are given as gallic acid equivalents (GAE).

2.4. Antioxidant studies

2.4.1. Determination of free radical scavenging activity by DPPH method

Free radical scavenging activity of the sample extracts was determined spectrophotometrically using the method of Blois [35]. This method is based on the measurement of the reducing ability of antioxidants toward the DPPH radical. Briefly, 100 μ L of various concentrations of the extract in methanol were added to 10 mL of a methanol solution of DPPH ($1.01 \cdot 10^{-2}$ M). The mixture was vigorously shaken and then allowed to stand at room temperature for 30 min in the dark. The absorbance of the mixture was measured at 517 nm by using a double-beam UV-Visible Camspec M550 spectrophotometer. A mixture of 100 μ L of methanol and 10 mL of DPPH solution was used as the control. The scavenging activity on the DPPH radical was expressed as inhibition percentage using the following equation:

$$\% \text{ Inhibition} = [(A_B - A_S)/A_B] \times 100 \quad [36]$$

where A_B is the absorbance of the control reaction (containing all reagents except the test compound), and A_S is the absorbance of the test compound. Butylatedhydroxytoluene (BHT) was used as positive control. The tests were carried out in triplicate. The extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph of inhibition percentage plotted against extract concentration (4.0, 2.0, 1.0, 0.5 and 0.25 mg/L).

2.4.2. Reducing power assay (Iron reducing activity)

The reducing power of *Ceratonia siliqua* leaves extracts was determined according to the method previously described by Oyaizu [37]. Different concentrations of *Ceratonia siliqua* leaves extracts (0-1 mg) in 1 ml of distilled water were mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 mL, 1%). The mixture was incubated at 50°C for 20 min. A portion (2.5 ml) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 mL) was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1%) and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. Ascorbic acid, tannic acid and gallic acid were used as standards. Phosphate buffer (pH 6.6) was used as blank solution. All analyses were run in triplicate and results averaged.

2.4.3. Evaluation of total antioxidant capacity by phosphomolybdenum method

The antioxidant activity of the extract was evaluated by the phosphomolybdenum method according to the procedure described by Prieto et al. [38]. The assay is based on the reduction of Mo (VI)–Mo (V) by the extract and subsequent formation of a green phosphate/Mo (V) complex at acid pH. A 0.3 ml extract (25 μ g/ml, 50 μ g/ml, and 100 μ g/ml) was combined with 3 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). In case of blank 0.3 mL of methanol was used in place of extracts. The tubes containing the reaction solution were capped and incubated in a boiling water bath at 95°C for 90 min. After cooling to room temperature, the absorbance of the solution was measured at 695 nm using a spectrophotometer. The antioxidant capacity of each sample was expressed as ascorbic acid (A.A) equivalent using the following linear equation established using ascorbic acid as standard: $[A = 0.0037C + 0.0343; R^2 = 0.991]$ where A is

the absorbance at 695 nm and C the concentration as ascorbic acid equivalent ($\mu\text{g/ml}$). The values are presented as the means of triplicate analysis.

3. Results and Discussion

3.1. Total phenolic contents

Diethyl ether, dichloromethane, and ethyl acetate extracts of three varieties of *Ceratonia siliqua* L. leaves (grafted female, spontaneous female, spontaneous male) grown in Morocco were studied for their contents of total phenols. Table 1 shows the total phenol contents that were measured by Folin Ciocalteu reagent in terms of gallic acid equivalent (GAE). The results showed that the total phenolic content from different extracts of three categories of *Ceratonia siliqua* L. leaves ranging from 0.45 to 2.64 (g/L GAE).

Table 1. Total phenolic content of carob tree-categories leaves.

Categories	Extracts	Total phenolic content (g/L GAE)
Spontaneous female	EtOAc	2.60
	Et ₂ O	0.95
	CH ₂ Cl ₂	0.77
Spontaneous male	EtOAc	2.50
	Et ₂ O	0.80
	CH ₂ Cl ₂	0.45
Grafted female	EtOAc	2.64
	Et ₂ O	1.52
	CH ₂ Cl ₂	0.82

The highest total phenolic content was observed in ethyl acetate extract of the three categories, and the lowest was observed in dichloromethane extracts in all carob tree categories. The results showed that carob tree leaves fractions contained a mixture of phenolic compounds at different levels according to the polarity of solvent used in the extraction process, in the following order: ethyl acetate > ethyl ether > dichloromethane. Category affected the phenolic profile with the grafted female being generally richer in phenols. Phenolic compounds and flavonoids have been reported to be associated with antioxidative action in biological systems, acting as scavengers of singlet oxygen and free radicals [1].

3.2. Antioxidant studies

In the present study, three commonly used antioxidant evaluation methods such as DPPH radical scavenging activity, reducing power assay and phosphomolybdenum method were chosen to determine the antioxidant potential of the three varieties of *Ceratonia siliqua* L. leaves extracts.

3.2.1. Free radical scavenging activity

The antioxidant activity of plants is mainly due to the active compounds present in them. In this study, nine leaves samples from three *Ceratonia siliqua* L. categories were investigated for their

radical scavenging activity and the results are shown in Figures 1-3. DPPH radical scavenging activities of plant extracts varied from 1.17 to 61.17 %. All of the extracts tested possess radical-scavenging activity. This activity was increased by increasing the concentration of the sample extract. The highest antioxidant activity was observed in the ethyl acetate extract of the three categories of Carob tree leaves than other extracts studied. The data obtained showed that the ethyl acetate extracts presented a high activity. The lowest activity was shown by the leaves extract in dichloromethane. It was also found that the free-radical-scavenging activity of ethyl acetate extract of grafted female category ($IC_{50} = 0.41 \text{ g/L}$) was stronger than that of ethyl acetate extract of spontaneous female category ($IC_{50} = 0.45 \text{ g/L}$) and ethyl acetate extract of spontaneous male category ($IC_{50} = 1.50 \text{ g/L}$) (Table 2).

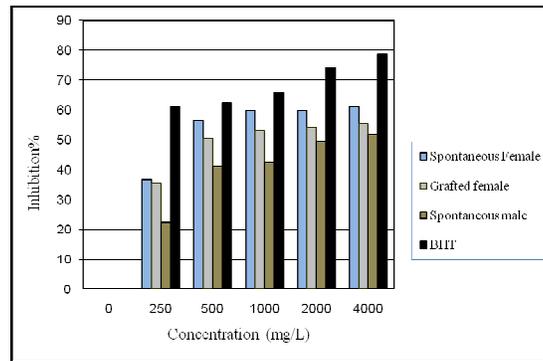


Figure 1. Antioxidant activity of ethyl acetate extracts.

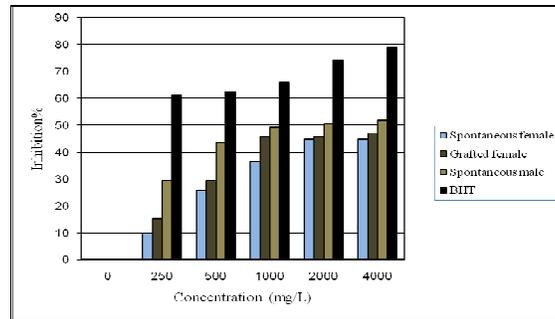


Figure 2. Antioxidant activity of ethyl ether extracts.

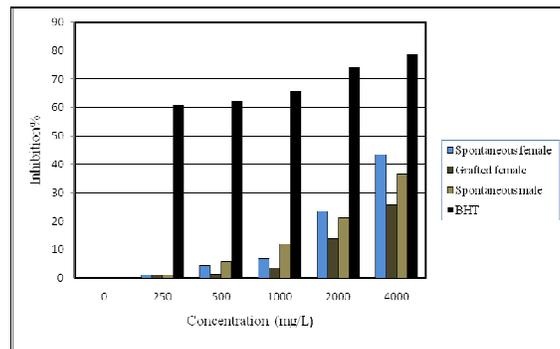


Figure 3. Antioxidant activity of dichloromethane extracts.

From these results it can be concluded that the antioxidant activity of three categories of Carob tree leaves is affected by the extracting solvent and the genotype [39, 40]. The ethyl acetate extract of *Ceratonia siliqua* L. leaves is promising starting material for the isolation of compounds with antioxidant activities. There is a lack of information available on the chemical composition of *Ceratonia siliqua* L. leaves inducing antioxidant activity. Further phytochemical investigations on these extracts including fractionation are needed to isolate active constituents and subsequent pharmacological evaluation.

Table 2. IC₅₀ (g/L) values of three fractions of carob tree-categories leaves.

Categories	Sample	IC ₅₀ (g/L)
Spontaneous female	EtOAc extract	0.45
	Et ₂ O extract	-
	CH ₂ Cl ₂ extract	-
Spontaneous male	EtOAc extract	1.40
	Et ₂ O extract	1.50
	CH ₂ Cl ₂ extract	-
Grafted female	EtOAc extract	0.41
	Et ₂ O extract	-
	CH ₂ Cl ₂ extract	-
-	BHT	0.21

3.2.2. Reducing power

The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides [41]. For the measurements of the reductive ability, it has been found that the Fe³⁺-Fe²⁺ transformation occurred in the presence of extract samples which was postulated previously by Oyaizu [37]. Tanaka et al. have observed a direct correlation between antioxidant activities and reducing power of certain plant extracts [42]. The reducing properties are generally associated with the presence of reductones [43], which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom [44]. Reductones are also reported to react with certain precursors of peroxide, thus preventing peroxide formation. In this assay, depending on the reducing power of antioxidant compounds, the yellow color of the test solution changes into various shades of green and blue. Therefore, by measuring the formation of Perl's Prussian blue at 700 nm, we can monitor the Fe²⁺ concentration. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. Reducing power of nine different extracts of *Ceratonia siliqua* L. and standards (ascorbic acid, gallic acid, tannic acid) using the potassium ferricyanide reduction method were depicted in Figures 4, 5 and 6.

The reducing power of the three varieties of carob tree leaves fractions increased and correlated well with the increasing concentration. However, the reduction power of gallic acid, tannic acid and ascorbic acid was relatively more pronounced than that of the three varieties of carob tree leaves fractions. The reducing power of *Ceratonia siliqua* L. leaves ranged from 0.40 to 2.63 Abs for 0.2 mg/mL to 1 mg/mL of extract (Figures 4-6). The ethyl acetate extract displayed a higher reducing activity compared to the ethyl ether and dichloromethane extracts. Category significantly affected the antioxidant activity. The results of the reducing activity for the ethyl acetate extract demonstrated that

the higher activity was found in the grafted female, followed by respectively spontaneous male and spontaneous female. In the ethyl ether extract and the dichloromethane extract, spontaneous male exhibited the highest reducing power following by grafted female and spontaneous female.

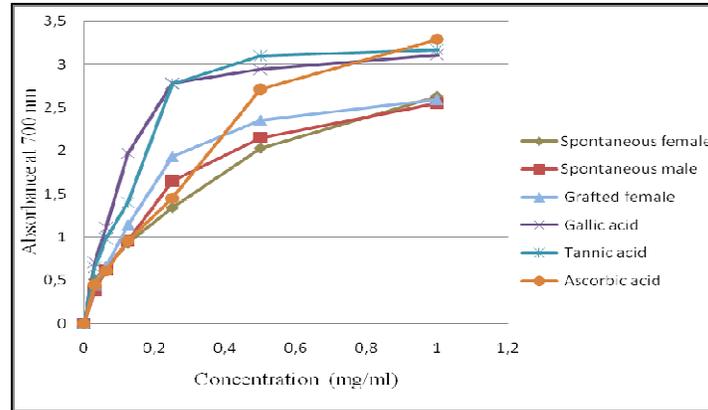


Figure 4. Total reducing power of the ethyl acetate extracts.

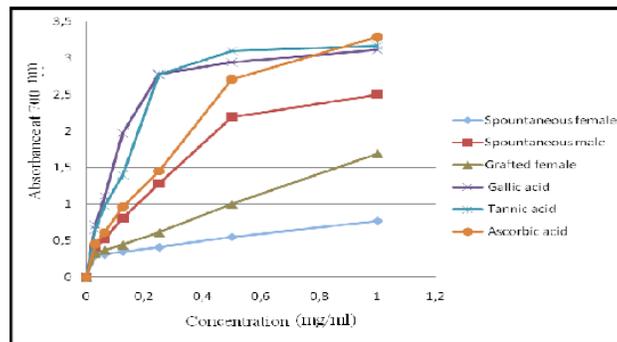


Figure 5. Total reducing power of the dichloromethane extracts.

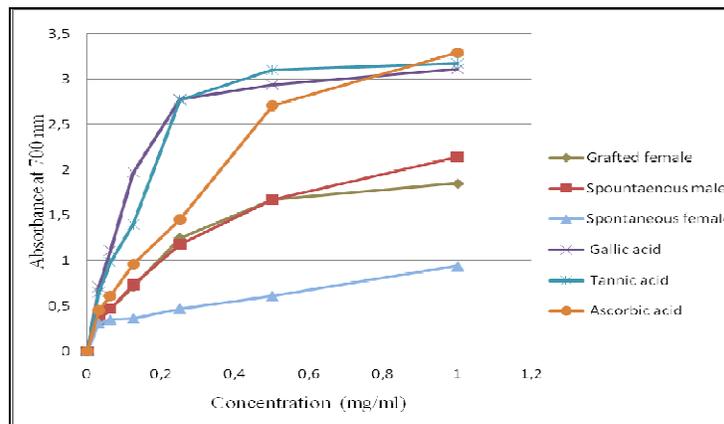


Figure 6. Total reducing power of the ethyl ether extracts.

3.2.3. Total antioxidant capacity

The antioxidant activity for the different extracts of *Ceratonia siliqua* L. leaves was evaluated by using phosphomolybdate method. It determines the total antioxidant capacity. This assay is based on the reduction of Mo(VI) to Mo(V) in presence of the antioxidant compounds and the subsequent formation of a green phosphate/Mo(V) complex at acidic pH, which is measured at 695 nm. Total antioxidant capacity of *Ceratonia siliqua* L. leaves extracts, expressed as equivalents of ascorbic acid ($\mu\text{g/mL}$ of extract), is shown in Figures 7, 8 and 9. The antioxidant capacity of *Ceratonia siliqua* L. leaves extracts was found to decrease in the order ethyl acetate extract > ethyl ether extract > dichloromethane extract. All the extracts showed an increase in antioxidant capacity with an increase in dose. The ethyl ether extract of spontaneous female showed a lower antioxidant capacity respectively than the dichloromethane and ethyl acetate extracts. Total antioxidant capacity of ethyl acetate extracts in the three varieties was found to be 100.00 μg , 106.81 μg and 107.30 μg ascorbic acid equivalents at 100 $\mu\text{g/mL}$ extract concentration, respectively for spontaneous male, spontaneous female and grafted female. This good antioxidant activity might be attributed to the presence of high amounts of polyphenols in these extracts.

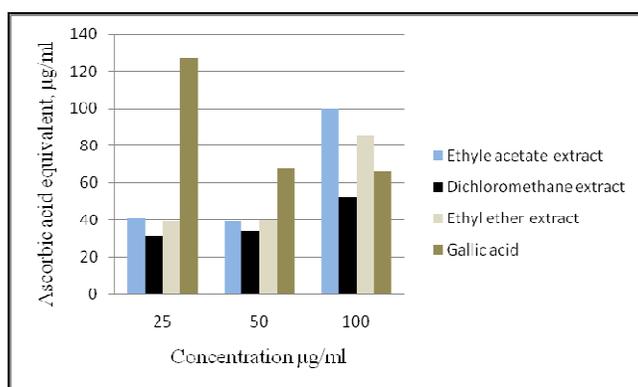


Figure 7. Antioxidant capacities of spontaneous male.

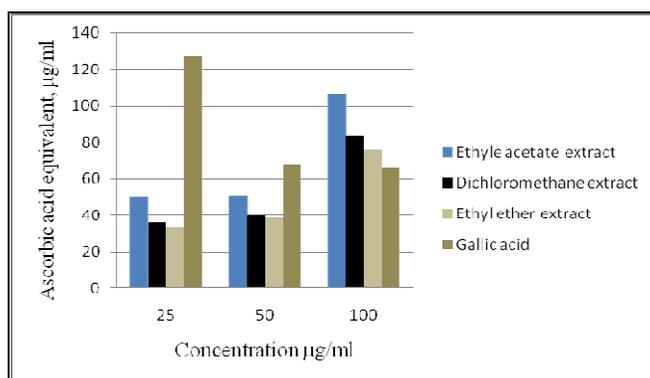


Figure 8. Antioxidant capacities of spontaneous female.

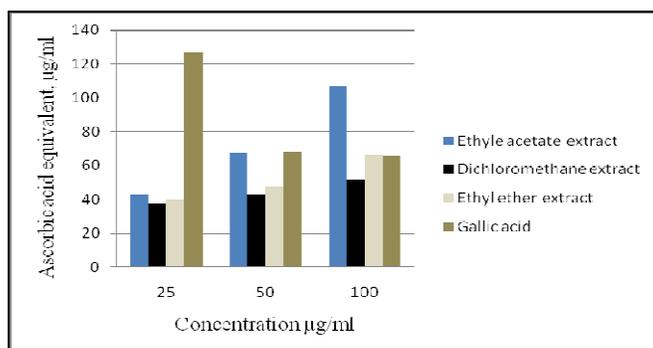


Figure 9. Antioxidant capacities of grafted female.

4. Conclusions

This paper deals with antioxidant activity and phenolic content of the three varieties of *Ceratonia siliqua* L. leaves grown in Morocco. Leaves of carob tree contain high amounts of polyphenol compounds. The extract showed significant activities in all antioxidant assays compared to the reference antioxidant butylated hydroxytoluene (BHT) and ascorbic acid (AA) in a dose dependent manner. In DPPH scavenging assay the IC_{50} value of the ethyl acetate extract of grafted female was found to be 0.41 g/L while the IC_{50} value of the reference standard BHT was 0.21 g/L. Total antioxidant activity was also found to increase in a dose dependent manner. Moreover, *Ceratonia siliqua* L. leaves extracts showed good reducing power. The ethyl acetate extract of leaves of the three carob tree categories showed potent antioxidant properties and contained significant amounts of phenolic compounds. The low antioxidant activity in dichloromethane and diethylether extracts can be attributed to fact that the compounds are polar in nature and are not completely extracted in dichloromethane and diethylether. These results suggest that *Ceratonia siliqua* L. leaves may act as a chemopreventative agent, providing antioxidant properties and offering effective protection from free radicals and support that *Ceratonia siliqua* L. is a promising source of natural antioxidants.

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