Assessment of antioxidant potential of phenolic compounds fractions of Algerian *Ceratonia siliqua* L. pods during ripening stages


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**Abstract**

The consecutive extraction via different solvents of increasing/decreasing polarity, which allowed a pre-fractionation of the extracted compounds, is widely used as major method for extraction of polyphenols. Thus, the phenolic compounds of *Ceratonia siliqua* L., at unripe and ripe stages, were extracted by four solvents of increasing polarity (hexane, chloroform, ethyl acetate, and acetone/water (70:30, v/v), consecutively. The extracts were evaluated for their total phenolic and flavonoid contents, while the antioxidant capacity was assessed in vitro using DPPH radical and hydrogen peroxide (H$_2$O$_2$) scavenging assays. The phenolic profile has been carried out by HPLC-MS/MS. The results showed that the phenolic contents and antioxidant capacity varied with the nature of extracts and the ripening stage. The 70% aqueous acetone and the ethyl acetate extracts contained the highest phenolic and flavonoid contents, and subsequently showed a pronounced scavenging activity on DPPH and hydrogen peroxide at unripe and ripe stages. The HPLC-MS/MS allowed the identification of five free phenolic acids (gallic, syringic, cinnamic, p-coumaric and ellagic acid for the first time) and five flavonoids (apigenin, naringenin, kaempferol, quercetin rhamnoside and myricetin rhamnoside) at both ripening stages.

**Keywords**

*Ceratonia siliqua*

Ripening stage

Extraction

Antioxidant capacity

Phenolic profile

**Introduction**

The carob tree (*Ceratonia siliqua* L.), belonging to the family of Fabaceae, is since ancient times a typical plant of the Mediterranean area (Batlle and Tous, 1997). Then, it has been introduced and grown in many dry regions of the world. According to the data, world-wide carob pod production amounts has been estimated at about 400,000 tons per year (Roukas, 1999; Karababa and Coskuner, 2013) depending on the cultivar, region, and farming practices (Makris and Kefalas, 2004). Their cultivation and production are centered mainly in Spain, Portugal, Greece and Morocco. Algeria is presently the sixth major world producer of carob, with a production of around 3,53 tons in 2014 (FAO, 2015).

Carob pod has been used as feed and food mainly due to its sugary pulp contents (40-60% of sugars). Nevertheless, only the seeds (~10% of the pod) are used industrially for carob bean gum extraction (Morton, 1987; Batlle and Tous, 1997). The pod is light to dark brown, flattened, straight or slightly curved, with a thickened margin. The unripe pod is green, moist and very astringent, but the ripe pod is sweet (Morton, 1987). Recently, many studies on carob extracts has been demonstrated to have various health promoting effects mainly attributed to its high phenolic contents, such as, antioxidant properties (Makris and Kefalas, 2004; Custodio et al., 2011; Sebai et al., 2013; Benchikh et al., 2014), and anti-proliferative activity (Corsi et al., 2002; Roseiro et al., 2013).

The phenolic composition of carob pulp is quite different among the literature, depending not only on the carob variety, geographical origin, weather conditions, harvesting and storage, but also on technological factors such as the extraction methodologies (Bernardo-Gil et al., 2011). Moreover, the structural diversity of polyphenols is responsible for the great variability of the physicochemical properties thus influencing their extraction. Amongst other things, the solubility of phenolic compounds is affected by the nature of solvent used (Naczk and Shahidi, 2004).
Numerous techniques have been used for extraction of carob phenolic compounds including supercritical fluid, ultrasound and conventional extractions (Bernardo-Gil et al., 2011; Roseiro et al., 2013; Benchikh et al., 2014). Among these, the consecutive extraction using different solvents of increasing/decreasing polarity, which allowed a pre-fractionation of extracted compounds, have been widely considered as major method for extraction of polyphenols from plants (Baravalia et al., 2009; Bekir et al., 2013; Jan et al., 2013). However, to our knowledge, there is only the report of El Hajaji et al. (2011) on the extraction of phenolic compounds of carob barks using a consecutive extraction via different solvents of increasing polarity. Moreover, in the present work, carob fruit was studied as widely grown in Algeria from trees localized in a sub-littoral Mediterranean area (Bejaia) and there are very few investigations regarding the evaluation of its antioxidant potential and phenolic compounds during ripening stages (Benchikh et al., 2014, 2016). In addition, our work highlights the bioactive potential of fractioned extracts of carob pods at different stages of fruit ripening. This approach was chosen in order to obtain rich phenolic extracts with high biological activities of carob pods at green and ripe stages. Moreover, qualitative and quantitative differences are clearly expected and studied.

In summary, the aims of the present work were:
(1) Extraction of phenolic compounds of Algerian carob pods at unripe and ripe stages with different solvents of increasing polarity, consecutively. (2) Determination of total phenolic (TPC) and total flavonoid contents (TFC) and evaluation of their antioxidant activity (DPPH and \( \text{H}_2\text{O}_2 \) scavenging assays) of different extracts. (3) Identification and characterization of the phenolic compounds of the most important extract by HPLC-MS/MS.

Materials and Methods

Chemicals

Folin-Ciocalteu reagent, sodium carbonate anhydrous, hexane, acetone and methanol (99.8% purity) were from Biochem, Chemopharma (Montreal, Canada); aluminium chloride hexahydrate (≥97.0% purity) was from Biochem, Chemopharma (Georgia, USA); gallic acid was from Prolabo (Montreuil, France); 2,2-diphenyl-1-picrylhydrazyl (DPPH), Ethyl-acetate and chloroform were from Sigma-Aldrich GmbH (Sternheim, Germany); ethanol (≥ 99.5% purity) was from VWR prolabo (EC). Acetic acid and methanol (grade HPLC-MS) were from Scharlau S.L. (Barcelona, Spain). Ellagic acid and quercetin were obtained from Sigma-Aldrich (Germany).

Plant material

Fresh carob pods (Lahlou variety) typically cultivated in Bejaia (Algeria) were collected from different points of the tree and in various parts of the parcel. Fruits were harvested in 2014 in good sanitary conditions at different dates during ripening (20 June: unripe stage (Green pods); 30 July: ripe stage (Brown pods)). The sample was randomly selected and washed carefully; seeds were removed and pulps were lyophilized (Alpha1-4 LD Plus, Christ, Osterode am Harz, Germany), ground with a crusher (IKA®, A 11 basic, Staufen, Germany) and passed through a 500 µm sieve before analyses.

Preparation of extracts

Four solvents having different polarity, including hexane, chloroform, ethyl acetate, and acetone-water (70:30, v/v) were used successively to extract phenolic compounds of \( \text{C. siliqua} \) pods. The extraction solvents used in this study were chosen to represent a full range of solvent polarity (from low to high polarity).

The first extraction was carried out by mixing 10 g of carob pulp powder in hexane (100 mL×3 times) in a shaking water bath (WB22, Memmert, Germany) at 40°C for 60 min. The mixture was centrifuged at 1700 g for 5 min and filtered through filter paper (Whatman no. 4). The three filtrates were pooled and the obtained extract was stored at 4°C. The retentate was used for the subsequent extractions performed respectively with chloroform, ethyl acetate, and acetone 70%, in the same way as performed with hexane. The four extracts were evaporated and dried under vacuum (heating bath no higher than 40°C). The dried sample of each extract was weighed and the yield of soluble constituents was calculated from the following equation:

\[
yield (\%) = \left( \frac{W1}{W2} \right) \times 100
\]

where W1 was the weight of the dry extract after evaporation of different solvents and W2 was the dry weight of the carob powder (10 g). The extracts were redissolved in ethanol (1 mg/mL) to measure the total phenolic and flavonoid contents as well as the antioxidant activity. The experiment was repeated in triplicate.

Total phenolic content (TPC)

Total phenolic content was determined using Folin-Ciocalteu reagent according to the method described by Singleton and Rossi (1965). One
hundred microliters of sample were mixed with 1 mL of Folin-Ciocalteu reagent and 0.8 mL of 7.5% sodium carbonate solution. The test tubes were allowed to stand in the dark at room temperature for 30 min. Absorbance was measured at 765 nm (spectrophotometer Uviline 9400, France). Gallic acid was used for external standardization, from 0.05 g/L to 0.2 g/L. Total phenolic content was expressed as milligrams of gallic acid equivalents per gram of dry extract (mg GAE/g).

**Total flavonoid content (TFC)**

Total flavonoid content was determined according to the method of Quettier-Deleu et al. (2000), based on the formation of flavonoids-aluminum complex. Equal volumes of extract and aluminum chloride solution (2%) were mixed. The absorbance of the reaction mixture was measured at 430 nm after 15 min of incubation. Quercetin was used for external standardization, from 0.005 g/L to 0.025 g/L. Total flavonoid content was expressed as milligrams of quercetin equivalents per gram of dry extract (mg QE/g).

**DPPH radical scavenging assay**

Radical scavenging activity of the different extracts was measured according to the procedure described by Brand-Williams et al. (1995). One milliliter of DPPH solution (60 µM) was mixed with 100 µL of sample at different concentrations. The decrease in absorbance was determined at 517 nm, after 30 min of incubation. The DPPH radical scavenging activity was calculated according to the following equation:

\[
\text{% inhibition} = \left[ \frac{(\text{Ac}-\text{As})}{\text{Ac}} \right] \times 100
\]

where Ac is the absorbance of the control and As is the absorbance of the sample.

IC\text{50} was calculated as the concentration of extract causing a 50% inhibition of DPPH radical; a low IC\text{50} value corresponds to a high antioxidant activity of sample.

**Hydrogen peroxide scavenging activity**

The H\textsubscript{2}O\textsubscript{2} scavenging capacity of different extracts was determined by the method of Ruch et al. (1989). One hundred and fifty microliters of various extracts were mixed with 500 µL of H\textsubscript{2}O\textsubscript{2} (40 mM prepared in phosphate buffer (0.1 M, pH 7.4)) and 1850 µL of phosphate buffer solution (0.1 M, pH 7.4). After 10 min of incubation, the absorbance was recorded at 230 nm. The scavenging activity was calculated according to equation (1). The concentrations of sample required to scavenge 50% of H\textsubscript{2}O\textsubscript{2} (IC\textsubscript{50}) were determined.

**HPLC-MS/MS analysis.**

Identification of phenolics in carob pods extracts was carried out on a HPLC-MS/MS system consisting of an Agilent 1100 Series HPLC (Agilent Technologies, Santa Clara, CA, USA) equipped with a micro-well plate autosampler and a capillary pump, and connected to an Agilent Ion Trap XCT Plus mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) using an electrospray interface (ESI). Samples were centrifuged at 14000 rpm during 10 min and the supernatants were filtered through 0.22 µm. Forty microliters of each filtered sample were injected into a Agilent Zorbax SB-Aq HPLC column (5 µm, 150 × 4.6 mm), thermostatted at 40°C and eluted at a flow rate of 200 µL/min. Mobile phase A, consisting of water + 0.1% acetic acid and mobile phase B, consisting of pure methanol, were used for the chromatographic separation. The elution program consisted in 0 - 5 min 5% B; 5 – 30 min 50% B; 40 - 45 min 100% B; 50 - 60 min 5% B.

UV chromatograms were recorded at 210, 275 and 340 nm. The mass spectrometer was operated in the negative mode with a capillary spray high voltage of 3500 V, and a scan speed of 22000 (m/z)/sec from 50-500 m/z, with the target mass located at 200 m/z. The Smart ICC target was set to 200.000 counts, whereas the maximum accumulating time was 20 milliseconds, and 3 spectra were averaged in each scan. The nebulizer gas pressure was set to 30 psi, whereas the drying gas was set to a flow of 8 L/min at a temperature of 350°C. The selected ions were extracted and analyzed both in scan (MS) and in MRM mode (MS/MS). The selected ions were sequentially fragmented using helium collision-induced dissociation (CID) with an isolation width of 1 m/z and a relative collision energy of 35%.

Mass spectra were obtained using the Data Analysis program for LC/MSD Trap Version 3.2 (Bruker Daltonik, GmbH, Germany). Quantification of phenolic components (relative amounts) was expressed as a percentage. The percentage was calculated from the area of individual phenolic compound relative to the sum of identified phenolics areas in the sample, which was considered as 100%.

**Statistical analyses**

All data reported were expressed as means ± standard deviation (n = 6: two repetitions for three independent extractions). The analysis of the variance with one factor (ANOVA) was calculated using STATISTICA® 5.5 software in order to determine
significant differences between results. Differences between the means at 5%, 1% and 1‰ levels (p < 0.05 or 0.01 or 0.001) were considered statistically significant.

Results and Discussion

Extraction yields of soluble constituents

Plant tissues contain various antioxidants, which are hard to quantify each one separately. Therefore, distinct intermediate extractions ensuring a maximum extraction of the present antioxidants were used (Dorman and Hiltunen, 2004). In this study, the extractive yield of *C. siliqua* varied depending on the different solvents used and the recovery percentage of extractable compounds ranged from 0.28% to 53.06% (Table 1). The yields of polar extracts (acetone 70% and ethyl acetate) of carob pods at both ripening stages are higher than the less-polar extracts (hexane, chloroform). The acetone 70% extract had the highest extractive yield from unripe and ripe carob pods with values of 41.72% and 53.06%, respectively. On the contrary, the lowest yield was recorded with the chloroform extract (0.50%) at the unripe stage and hexane extract (0.28%) at the ripe stage. Differences of yield extracts can be explained by the difference in the polarity of solvent used and the chemical nature of the sample. Indeed, our previous studies indicated that the extraction yield depends on solvent, time and temperature as well as chemical composition of the sample (Chaalal *et al*. 2012; Benchikh and Louaileche, 2014; Bachir Bey *et al*. 2014). Moreover, under the same time and temperature conditions, the solvent used and the chemical property of sample are the two most important factors (Baravalia *et al*., 2009). It can consequently be argued that the carob pods are constituted mainly with polar extractable compounds at both ripe and unripe stages.

Total phenolic contents

The phenolic compounds solubility is dependent on the polymerization degree, the solvent used, and the interactions of phenolics with other sample components as well as formation of insoluble complex. Thus, in the present study, successive extractions using solvents of increasing polarity were used: hexane, chloroform, ethyl acetate and acetone 70%. The total phenolic contents (TPC) of carob pods extracts at two ripening stages are presented in Figure 1 (A). The results showed that the acetone 70% extract contained the highest total phenolic content with value of 162.55 ± 5.44 mg GAE/g at unripe stage, which is very higher than those reported by Makris and Kefalas (2004) when studying an aqueous acetone extract (9.28 ± 0.61 mg GAE/g). However, at ripe stage the ethyl acetate extract recorded the highest amount with 64.76 ± 2.92 mg GAE/g. The statistical analysis revealed significant differences (p< 0.001) between TPC of different extracts at both ripening stages.

| Table 1. Yields, scavenging activity on DPPH radical and hydrogen peroxide of extracts |
|---------------------------------------------|---------------------------------------------|
| **Unripe stage** | **Ripe stage** |
| **Extracts** | **Yield (%)** | **IC$_{50}$ (µg/mL)** | **Yield (%)** | **IC$_{50}$ (µg/mL)** |
| Hexane | 0.97 ± 0.02 a | nd | nd | 0.28 ± 0.07 a | nd | nd |
| Chloroform | 0.50 ± 0.03 a | nd | nd | 0.45 ± 0.03 a | nd | nd |
| Ethyl-acetate | 1.45 ± 0.22 a | 792.54 ± 5.67 a | 832.97 ± 7.61 a | 0.80 ± 0.09 a | 992.04 ± 14.44 a | 801.29 ± 14.27 a |
| Acetone 70% | 41.72 ± 2.04 a | 538.80 ± 9.03 a | 744.54 ± 3.88 a | 53.06 ± 1.51 a | 785.32 ± 3.95 a | 840.48 ± 15.49 a |

Values are mean ± standard deviation (n = 6); the letters within the same row present significant difference at (p<0.001)

nd: not determined (The IC$_{50}$ were found to be greater than 1000 µg/mL)
Kriaa et al., 2013; Sebai et al., 2013). Kaneria et al. (2012) and Bekir et al. (2013) reported that the polarity of solvent had an influence on the extraction of phenolic compounds. The TPC for the different solvent extracts at both ripening stages investigated in this study was markedly higher than those found by Ouzounidou et al. (2012), for extracts of unripe carob grown in Greece (13.6 and 24.8 mg GAE/100 g). Besides, Benchikh et al. (2014) reported that the phenolic contents of carob pods of three varieties cultivated in Algeria were ranged from 17.55± 0.34 to 19.82± 0.48 g GAE/100 g DW at the green stage; likewise, at ripe stage the amounts were ranged from 1.35 ± 0.01 to 2.34 ± 0.01 g GAE/100 g DW. Sebai et al. (2013) reported that the phenolic contents of unripe carob cultivated in Tunisia were 1.27 ± 0.15 mg GAE/kg (acetone extract) and 2.58 ± 0.27 mg GAE/kg (hexane extract). The reduction of total phenolics at ripe stage could be due according to Gull et al. (2012) to the oxidation of phenolic compounds by polyphenol oxidase and/or to the conversion of soluble phenolics into insoluble compounds, which are bound to polysaccharides in the cell wall. Moreover, Khled khoudja et al. (2014) reported that the differences of phenolic contents in plant could be attributed to the biological factors (genotype, organ and ontogeny) and/or edaphic/environmental conditions (temperature, salinity, water stress and light intensity).

**Total flavonoid contents**

As shown in Figure 1 (B), the total flavonoid contents varied strongly with the solvent used. The acetone 70% and ethyl acetate extracts had the highest amounts with values of 3.93 ± 0.10 and 1.52 ± 0.14 mg QE/g at unripe and ripe stages, respectively. Whereas, the hexane and chloroform extracts showed the lowest contents. Polar extracts contained more total flavonoids than less-polar extracts. In the same way, Sebai et al. (2013) found that flavonoid contents of immature carob cultivated in Tunisia dependent on the solvent used. Thus, the founded hierarchy was ethanol (6.78 mg QE/kg) > water (6.14 mg QE/kg) > methanol (2.49 mg QE/kg) > hexane (1.58 mg QE/kg) > acetone (0.68 mg QE/kg) > Ether petroleum (0.12 mg QE/kg). Our results also revealed that flavonoid content of carob declined markedly with the ripening stage of the fruit. Likewise, Benchikh et al. (2014) reported that the flavonoid contents of carob pods of three varieties cultivated in Algeria were ranged from 270 ± 0.03 to 320.24 ± 0.06 mg QE/100 g DW at green stage; however, at ripe stage the amounts were ranged from 135 ± 0.01 to 181± 0.01 mg QE/100g DW. Furthermore, being important
molecules produced after the phenylpropanoid pathway, the high concentration of TFC at unripe stage indicates the strong occurrence of the flavonoid biosynthetic pathway.

**DPPH radical scavenging assay**

DPPH has been used extensively as a free radical to evaluate reducing substances and is useful reagent for investigating the free radical scavenging activities of a given compound. The DPPH radical-scavenging activity of each extract was assayed. As shown in Figure 2 (A) and (B), all extracts quenched DPPH radical and the scavenging activity increases with the compound concentration and the solvent polarity. Among all the tested extracts, the acetone 70% and ethyl acetate extracts from the ripening stage shows stronger activity than with the other solvents. The IC\(_{50}\) values are presented in Table 1. A high IC\(_{50}\) value corresponds to a low antioxidant activity. The DPPH radical scavenging activity of the acetone 70% extract was 536.80 ± 9.03 µg/mL and 795.32 ± 3.95 µg/mL followed by ethyl-acetate extract with values of 792.54 ± 5.67 µg/mL and 992.04 ± 14.44 µg/ mL for unripe and ripe stages, respectively. The IC\(_{50}\) values for the chloroform and hexane extracts were found to be greater than 1000 µg/mL. These differences in DPPH activity between extracts (different solvents and ripening stage) could be certainly due to the phenolic contents and the harvest time. Bekir et al. (2013) reported that the antioxidant propriety of Punica granatum leaves extracts, varied according to solvent polarity, and the hierarchy found was methanol> ethanol > ethyl acetate > dichloromethane > hexane. Moreover, our results after stage comparison are lined with those of Gull et al. (2012) who reported a decrease of antiradical activity with respect to different maturity stages of guava fruits.

**Hydrogen peroxide scavenging activity**

Hydrogen peroxide itself is not very reactive, but sometimes it is harmful to cells since it may rapidly decomposed in the body into oxygen and water resulting in hydroxyl radicals (OH\(^•\)) that can initiate lipid peroxidation and cause damage to cell membrane and DNA (Halliwell and Gutteridge, 1981). Therefore, eradication of H\(_2\)O\(_2\) is of primary importance for antioxidant defense in cell or food systems (Patel et al., 2011). As shown in Figure 2 (a) and (b), the percentage scavenging activity increased within the concentration of the extracts. The scavenging ability of carob pods extracts on H\(_2\)O\(_2\) radical was in the following order: acetone 70%> ethyl acetate> chloroform> hexane extracts at green stage and it was ethyl acetate > acetone 70%> chloroform> hexane extracts at ripe stage. It is also noticed that the hydrogen peroxide scavenging activity of carob pods extracts at both ripening stages increased with the solvent polarity. Thus, the best antioxidant activities correspond to the polar extracts (acetone 70% and ethyl acetate), as when DPPH was assayed. Similarly, Patel et al. (2011) obtained for Pedalium murex Linn fruits an important activity of ethyl acetate fraction in depleting H\(_2\)O\(_2\). Data
obtained from IC_{50} values (Table 1) indicates that the acetone 70% and ethyl acetate extracts showed moderate H_2O_2-scavenging activity. As previously demonstrated, differences in the antioxidant capacities of the studied extracts are probably related to their different phenolic compositions.

**HPLC-MS/MS analysis**

Using colorimetric methods, the acetone 70% extract was shown to have the main phenolic content and the highest antioxidant activity. Therefore, the phenolic profile of this extract was analyzed by HPLC-MS/MS. As seen from Table 2, five free phenolic acids (gallic, syringic, p-coumaric, cinnamic and ellagic acids) and five flavonoids (apigenin, naringenin, quercetin rhamnoside, kaempferol and myricetin rhamnoside) were identified and semi-quantified in both unripe and ripe carob pulp extracts. These results are in agreement with the findings of Owen *et al.* (2003) and Benchikh *et al.* (2016) on the analysis of phenolic profiles of carob fiber and pulp, respectively. Phenolic acids represent the major phenolic compounds at both ripening stages. Their total relative amount was found to be four times higher than those of flavonoids. Indeed, it was observed that gallic acid was the most abundant phenolic acid and it is very dominating in both unripe and ripe carob pods. The relative content of this acid was higher at unripe stage (43.15%). Whereas, the relative amounts of p-coumaric and ellagic acids were higher at ripe stage. The most flavonoids increased at ripe stage. Even if comparison with other studies on the carob phenolic composition is important, it remains difficult since the papers reported on the subject used different extraction conditions and plant material leading to different phenolic profiles (Corsi *et al.*, 2002; Owen *et al.*, 2003; Papagiannopoulos *et al.*, 2004; Bernardo-Gil *et al.*, 2011; Benchikh *et al.*, 2016).

Phenolic compounds represent a striking example of metabolic plasticity enabling plants to adapt to changing biotic and abiotic environments as protectants for plant bodies against herbivores and pathogens, as well as from physical stresses like ultraviolet light and heat (Bourgaud *et al.*, 2001; Samanta *et al.*, 2011). Therefore, the amounts of these compounds at green stage were higher than those at ripe stage, because the fruit, still at its growing stage, synthesizes many phenolic compounds to protect against biotic and/or abiotic environmental hazards.

Phenolic compounds are derived from cinnamic acid, hence, the presence of this acid at both ripening stages is expected. The presence of p-coumaric acid could be explained by further modification of cinnamic acid by subsequent hydroxylation and methylation steps (Gross, 1985). Similarly, gallic and syringic acids are synthesized via side-chain degradation and the conversion mechanism of cinnamic acid to benzoic acids (Gross, 1985). Moreover, the hydrolysis of tannins (ellagitannins, gallotannins) leads to the release of gallic and ellagic acids. The naringenin was synthesized from p-coumaroyl-CoA, which derived from p-coumaric acid. This compound has been considered as a central intermediate in the production of different flavonoids including apigenin and kaempferol (Crozier *et al.*, 2006). This fact could explain the presence of these flavonoids in the extract.

**Correlation between phenolic compounds and antioxidant activities**

Correlation coefficients (r) between different phenolic contents and in vitro antioxidant activities evaluated for carob pods extracts are presented in Table 3. The phenolic and flavonoid contents exhibited a positive correlation with DPPH and H_2O_2 scavenging activities. For the four extracts (hexane, chloroform, ethyl-acetate and acetone
70%) at both ripening stages, antioxidant activities (DPPH and \(H_2O_2\)) exhibited a significant (\(p < 0.05\)) correlation mainly with TFC than TPC; which means that flavonoids are important contributors to the antioxidant activity in carob fruit. Besides, the flavonoids, with some structures, can act as donors of protons or electrons, which lead to obtain a good correlation with them (Rice-Evans et al., 1996).

**Conclusion**

In the present study, we extracted for the first time the phenolic compounds from Algerian carob pulp at unripe and ripe stages, via a consecutive extraction using four solvents of increasing polarity. Hexane, chloroform, ethyl acetate and 70% aqueous acetone extracts were evaluated for their phenolic contents extraction power and antioxidant abilities. Among the four extracts tested, the 70% aqueous acetone and ethyl acetate extracts were found to possess the highest amounts of phenolics and flavonoids as well as the best antioxidant activity. The HPLC-MS/MS analysis of acetone 70% allowed the identification of five free phenolic acids (gallic, syringic, cinnamic, p-coumaric and ellagic acids) and five flavonoids (apigenin, kaempferol, naringenin, quercetin rhamnoside and myricetin rhamnoside), with gallic acid as the most abundant phenolics at unripe and ripe stages. The ellagic acid was identified for the first time in carob fruit extract. Hence, the antioxidant activities of the extracts were positively correlated (\(p< 0.05\)) with their TPC mainly with TFC. Thus, *C. siliqua* L. can be suggested as a potential natural source of antioxidants with a special focus on polar fraction extracted by efficient solvents at unripe stage. The extracts could be appropriate for use in nutritional/ pharmaceutical fields. In addition, it could be interesting to evaluate the biological activities (antibacterial, anti-inflammatory and anti-proliferative) of each fractioned extract in vitro and/ or in vivo for applied approaches.

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**References**


