

Full Length Research Paper

Identification of phenolic compounds by high performance liquid chromatography/mass spectrometry (HPLC/MS) and *in vitro* evaluation of the antioxidant and antimicrobial activities of *Ceratonia siliqua* leaves extracts

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Ceratonia siliqua is a typical plant of the Mediterranean area, which is mainly used as animal and human food and in folk medicine for treating some diseases such as antidiarrheal and diuretic. The present study was planned to evaluate the potential of antimicrobial and antioxidant activities of *C. siliqua* leaves extract and the identification of bioactive compounds by high performance liquid chromatography/mass spectrometry (HPLC/MS) in the active extract. The antioxidant activities of the different organic extracts of *C. siliqua* were assayed by 2,2-diphenyl-1-picrylhydrazyl (DPPH) and β -carotene tests. Among the tested extracts, results showed that the ethyl acetate extract displayed the greatest DPPH scavenging ability with an IC_{50} of 1.8 μ g/ml and a strong β -carotene bleaching inhibition after 120 min of incubation with an IC_{50} of 24.01 μ g/ml. The investigation of the phenolic and flavonoids content showed that the ethyl acetate extract of *C. siliqua* revealed the highest phenolic contents. Only the ethyl acetate extract of *C. siliqua* (EACs) showed antimicrobial activity with a broad-spectrum microbiocide with diameter inhibition zones ranging from 12 to 24 mm and MIC values of 0.312 to 1.25 mg/ml. The HPLC finger print of EACs active extract showed the presence of six phenolic compounds. They included (1) 1,6-Di-galloyl-glucose, (2) 1,2,6-Tri-galloyl-glucose, (3) Myricetin glucoside, (4) 1,2,3,6-Tetra-galloyl-glucose, (5) Myricetin rhamnoside and (6) Syringic acid. These results are a good agreement of the popular use and experimentally observed effects of *C. siliqua* and would promote the reasonable usage and exploitation of the biomolecules of this important plant.

Key words: *Ceratonia siliqua*, Chemical composition, antimicrobial activity, antioxidant activity, phenolic compounds.

INTRODUCTION

In recent years, there has been considerable interest in the finding of antioxidants and antimicrobial compounds

from natural sources to control human and plant diseases (Tepe et al., 2005). Natural antioxidant inhibited oxidative

damage of food products and may prevent inflammatory conditions (Khanna et al., 2007), ageing and neurodegenerative disease (Fusco et al., 2007).

The market constantly addresses its attention to secondary metabolites produced by plants to check their properties and to evaluate their possible use in the industry. Also, the scientific interest in these metabolites has been increased today with the search of new antimicrobial agents, due to the increasing development of the resistance pattern of microorganisms to most currently used antimicrobial drugs. A number of natural products such as phenolics, flavonoids, coumarins, curcuminoids or terpenes have been characterised by these metabolites which allows easy transport across cell membranes to induce different biological activities, including antioxidant, anti-inflammatory and anticholinesterase effects (Loizzo et al., 2007).

Tunisian flora is remarkable for its diversity of medicinal plants. *Ceratonia siliqua* commonly known as Carob, belongs to the family of Leguminosae. The tree attains a mature height and spread of 6 to 12 m, and sometimes more than 20 m, with branches extended to ground level (Shigenorik et al., 2002). The leaves and fruits of this plant are used to treat a variety of diseases. Carob pods have traditionally been used as animal and human food and currently the main use is the seed for gum extraction. Bark and leaves have been used in Tunisian folk medicine as laxative, diuretic, antidiarrheal and for the treatment of gastroenteritis of lactating babies (Kivçak and Mert, 2002). Furthermore, recent studies have confirmed the presence of antioxidant, hypocholesterolemic activities in pods of *C. siliqua* (Dimitris and Makris, 2004) and antiproliferative effects on T1 cell line and substances acting on peripheral benzodiazepine receptor in its leaves (Avallone et al., 2002; Corsi et al., 2002).

The aims of this study were to screen the different fraction of *C. siliqua* for its antioxidant, antimicrobial activities and to identify its major phenolic compounds for the first time. The antimicrobial activity was evaluated against different microorganisms, including Gram-positive and negative bacteria. The antioxidant potential was studied by two distinct assays: scavenging of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals and β -carotene bleaching assay. Moreover, this study characterized the active extract by high performance liquid chromatography/mass spectrometry (HPLC/MS) and discussed their possible health benefits.

MATERIALS AND METHODS

Chemicals

DPPH and butylated hydroxytoluene (BHT) were purchased from

Sigma Chemical France. Folin-Ciocalteu reagent, sodium carbonate (Na_2CO_3) and other solvents were of analytical grade and were freshly prepared in distilled water.

Preparation of *C. siliqua* leaf extracts

Fresh leaves of *C. siliqua* were collected from Chebba (Mahdia, Tunisia, latitude 35.23° and longitude 11.11°) and a voucher specimen (LBPes C.S. 15.01) was deposited in the laboratory of Biopesticides of the Centre of Biotechnology of Sfax. The dried leaves were ground to fine powder using a grinder and the resulted material (100 g of powder) was extracted by maceration in ethanol-water 80% with shaking, at room temperature. 3.5 g of the dried hydroethanolic crude extract was suspended in 100 ml distilled water and was sequentially partitioned with n-hexane (3 × 250 ml), dichloromethane (3 × 250 ml) and ethyl acetate (3 × 250 ml). The resulting three fractions were evaporated under vacuum to dryness to give the hexane (m = 20 mg), the dichloromethane (m = 15 mg) and the ethyl acetate (m = 1.5 g) fractions. The remaining aqueous layer was lyophilised to give water fraction (m = 150 mg). The stock solutions were kept at 4°C in the dark until further analysis.

Total phenolic content

Total phenolic content (TPC) was determined using the Folin-Ciocalteu method (Waterman and Mole, 1994) adapted to a microscale. Briefly, 10 μl of diluted sample solution were shaken for 5 min with 50 μl of Folin-Ciocalteu reagent. Then 150 μl of 20% Na_2CO_3 were added and the mixture was shaken once again for 1 min. Finally, the solution was brought up to 790 μl by the addition of distilled water. After 90 min, the absorbance at 760 nm was evaluated using a spectrophotometer SmartSpecTTM3000 (Bio-Rad; Hercules, CA, USA). Gallic acid was used as an internal standard for the calibration curve. The phenolic content was expressed as mg of gallic acid equivalent per gram of dry sample (mg GAE/g) using the linear equation based on the calibration curve.

Determination of total flavonoids content

The flavonoids content in extracts was determined spectrophotometrically according to Quettier-Deleu et al. (2000), using a method based on the formation of a complex flavonoid-aluminium, having the maximum absorption at 430 nm. The flavonoids content was expressed in mg of quercetin equivalent per gram of dry plant extract (mg QE/g).

Antioxidant testing assays

DPPH radical scavenging activity

Radical scavenging activity of the different fractions was determined using DPPH radical as a reagent according to the method of Kirby and Schmidt (1997) with some modifications. Briefly, 1 ml of a 4% (w/v) solution of DPPH radical in ethanol was mixed with 500 μl of sample solutions in ethanol (different concentrations). The mixture was incubated for 20 min in the dark at room temperature. Scavenging capacity was read spectrophotometrically by monitoring the decrease of the absorbance at 517 nm. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity. Ascorbic acid was used as

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standard. The percent DPPH scavenging effect was calculated using the following equation: DPPH scavenging effect (%) = $(A_{\text{control}} - A_{\text{sample}} / A_{\text{control}}) \times 100$. Where A_{control} is the absorbance of the control reaction where the sample is replaced by 500 μl ethanol. Tests were carried out in triplicate.

β -Carotene bleaching assay

The antioxidant activity was determined according to the β -carotene bleaching method described by Pratt (1980). A stock solution of β -carotene/linoleic acid mixture was prepared as follows: 0.5 mg of β -carotene was dissolved in 1 ml of chloroform with 25 μl of linoleic acid and 200 mg of Tween-20. Chloroform was completely evaporated, using a vacuum evaporator. Then, 100 ml of distilled water, saturated with oxygen (30 min), were added and the obtained solution was vigorously shaken. 4 ml of this reaction mixture were dispensed into test tubes and 200 μl of each sample, prepared at different concentrations, were added. The emulsion system was incubated for 2 h at 50°C. The same procedure was repeated with BHT as positive control, and a blank as a negative control. After this incubation period, the absorbance of each mixture was measured at 490 nm. Antioxidant activity in β -carotene bleaching model in percentage (A %) was calculated with the following equation: $A\% = 1 - (A_0 - A_t / A'_0 - A'_t) \times 100$, where A_0 and A'_0 are absorbances of the sample and the blank, respectively, measured at zero time, and A_t and A'_t are absorbances of the sample and the blank, respectively, measured after 2 h. All tests were carried out in triplicate.

Antimicrobial activity

Microorganisms and growth conditions: Authentic pure cultures of bacteria were obtained from international culture collections (ATCC) and the local culture collection of the Center of Biotechnology of Sfax, Tunisia. They included Gram-positive bacteria: *Bacillus subtilis* ATCC 6633, *Bacillus cereus* ATCC 14579, *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228, *Enterococcus faecalis* ATCC 29212, *Micrococcus luteus* ATCC 1880 and Gram-negative bacteria: *Klebsiella pneumoniae* ATCC 10031, *Escherichia coli* ATCC 8739 and *Pseudomonas aeruginosa* ATCC 9027.

The bacterial strains were cultivated in Muller-Hinton agar (MH) (Oxoid Ltd, UK) at 37°C except for *Bacillus* species which were incubated at 30°C. Working cultures were prepared by inoculating a loopful of each test bacteria in 3 ml of Muller-Hinton broth (MH) (Oxoid Ltd, UK) and were incubated at 37°C for 12 h. For the test, final inoculum concentrations of 10^6 CFU/ml bacteria were used.

Agar diffusion method: Antimicrobial activities of the *C. siliqua* were evaluated by means of agar-well diffusion assay according to Güven et al. (2006) with some modifications. Fifteen milliliters of the molten agar (45°C) were poured into sterile petri dishes (\varnothing 90 mm). Working cell suspensions were prepared and 100 μl was evenly spread onto the surface of the agar plates of Mueller-Hinton agar (Oxoid Ltd, UK) for bacteria. Once the plates had been aseptically dried, 06 mm wells were punched into the agar with a sterile Pasteur pipette.

The extract was dissolved in dimethylsulfoxide/water (1/9; v/v) to a final concentration of 50 mg/ml. Thus, 80 μl were placed into the wells and the plates were incubated at 37°C for 24 h for bacterial strains. Gentamicin (10 μg /wells) was used as a positive control. Negative control consisted of 10% dimethyl sulfoxide (DMSO) which is used to dissolve the extract. Antimicrobial activity was

evaluated by measuring the diameter of circular inhibition zones around the well. Tests were performed in triplicate.

Determination of minimal inhibitory concentration (MIC) and minimal biocide concentration (MBC)

MIC of extract of *C. siliqua* were determined according to Gulluce et al. (2007) with minor modifications. The test was performed in sterile 96-well microplates with a final volume in each microplate well of 100 μl . For susceptibility testing, 100 μl of Mueller-Hinton broth or potatoes dextrose broth was distributed from the second to the twelfth test wells. A stock solution of the extract was prepared by dissolving 100 μl of the extract in dimethyl sulfoxide and then adjusted to a final concentration of 50 mg/ml by Mueller-Hinton broth. The first well of the microplate was prepared by dispensing 160 μl of the growth medium and 40 μl of the tested extract to reach a final concentration of 10 mg/ml and then 100 μl of scalar dilutions were transferred from the second to the ninth well. Thereafter and from each well, 10 μl of the suspension were removed and replaced by the bacterial suspensions to final inoculum concentrations of 10^6 CFU/ml. The final extracts concentrations adopted to evaluate the antimicrobial activity were 0.039 to 10 mg/ml.

The tenth well was considered as positive growth control containing Mueller-Hinton media for bacterial strains, since no extract solution was added. Another well containing 10% dimethylsulfoxide (v/v), without extract, was used as negative control. The plates were then covered with the sterile plate covers and incubated at 37°C for 24 h. The MIC was defined as the lowest concentration of the total extract tested at which the microorganism does not demonstrate visible growth after incubation. As an indicator of microorganism growth, 25 μl of 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (0.5 mg/ml) dissolved in sterile water were added to the wells and incubated at 37°C for 30 min (Eloff, 1998). Where microbial growth was inhibited, the solution in the well remained clear after incubation with MTT. For the determination of MBC, a portion of liquid (5 μl) from each well that showed no change in colour was plated on nutrient agar and incubated at 37°C for 24 h. The lowest concentration that yielded no growth after this sub-culturing was taken as the MBC, indicating that >99.9% of the original inoculum was killed. The determinations of MIC and MBC values were done in triplicate.

HPLC-MS analysis of phenolic compounds

Reverse phase high performance liquid chromatography was used to analyse phenolic compounds present in the active fraction of *C. siliqua* (EACs), using the separation module (Knauer Analogy) equipped with a C18 column (Zorbax, 2.6 \times 250 mm, 3.5 μm particle size) and a diode array detector (DAD). The samples were eluted with a gradient system consisting of solvent A (water, 0.1% formic acid) and solvent B (Acetonitrile, 0.1% formic acid), used as the mobile phase, with a flow rate of 500 $\mu\text{l}/\text{min}$. The temperature of the column was maintained at 25°C and the injection volume was 10 μl . The gradient system started from 90% A at 1 min, to 20% B at 4 min, 80% B at 30 min, 100% B at 32 min, 100% B at 36 min and 20% B at 38 min.

The peaks of the phenolic compounds were monitored at 280 nm. Electrospray ionisation mass spectroscopic (ESI-MS) analysis of phenolic compounds in ethyl acetate fraction was performed using an Applied Biosystems (LC/MSD TRAP \times CT). Mass spectra were achieved by electrospray ionisation in both positive and negative modes. The capillaries 4500 (negative) and 3500 V (positive) were used in this study. The electrospray probe-flow was adjusted to 8 ml/min. Continuous mass spectra were obtained by

Table 1. Amounts of total flavonoid, total phenolic compounds and evaluation of the IC₅₀ values of the DPPH free radical scavenging assay of the different fractions of *C. siliqua* and Ascorbic acid was used as standard. Each value represents the mean ± S.D. of three experiments.

Extract/Fraction	Phenolic content (mg GAE/g)	Flavonoid content (mg QE/g)	DPPH IC ₅₀ (µg/ml)
Hexane	91.20 ± 3.94	ND	ND
Dichloromethane	139.58 ± 6.55	75.94 ± 7.68	41.01
Ethyl acetate	680 ± 8.33	193.30 ± 3.07	1.80
Water	130 ± 5.62	21.71 ± 8.71	8.65
Ascorbic acid	-	-	3.50

mg GAE /g): mg of gallic acid equivalent per g of dry plant extract; mg QE/g: mg of quercetin equivalent per g of dry plant extract. ND: not detected.

scanning from 50 to 900 m/z. Identification of the phenolic compounds of each fraction was achieved by comparison with ESI-MS spectra comparisons with literature reports.

Statistical analysis

Experimental results concerning this study were expressed as means ± standard deviation of three parallel measurements. The significance of difference was calculated by Student's *t* test, and values $p < 0.05$ and $p < 0.001$ were considered to be significant and highly significant, respectively.

RESULTS AND DISCUSSION

Total phenolics (TPC) and flavonoids

C. siliqua leaves fractions were investigated for their TPC by the Folin–Ciocalteu assay and for their flavonoids by AlCl₃ reagent. As shown in Table 1, the TPC values expressed as mg gallic acid equivalents/g of dry extract (mg GAE/g) of the successive *C. siliqua* leaves extracts ranged between 91.2 to 680 mg GAE/g and were in the following order: ethyl acetate fraction > dichloromethane fraction > water fraction > hexane fraction.

Ethyl acetate fraction of *C. siliqua* leaves (EACS fraction) have a total flavonoid content of 193.3 mg of quercetin equivalents/g of dried extract, while the water fraction have the lowest concentration. It was observed that the amount of flavonoids in the analyzed plant extracts showed a high correlation with the total amount of phenolics.

Total antioxidant capacity

DPPH test

The DPPH, a stable free radical, has been widely used to evaluate the free radicals scavenging ability of various natural products (Porto et al., 2000). The effect of the different *C. siliqua* fractions on DPPH radical scavenging showed a dose-dependent activity that can be evaluated

by the determination of the IC₅₀ values corresponding to the amount of the fraction required to scavenge 50% of DPPH radicals present in the reaction mixture. High IC₅₀ values indicate low antioxidant activity. As shown in Table 1, the most potent radical scavenger extract was EACS fraction (IC₅₀ = 1.8 µg/ml), followed by water fraction (IC₅₀ = 8.65 µg/ml) and dichloromethane fraction (IC₅₀ = 41.01 µg/ml). Therefore, it can be concluded that these extracts were able to reduce the stable free radical DPPH to the yellow-colored diphenylpicrylhydrazine. Additionally, the IC₅₀ of this fraction was better than the reported IC₅₀ values obtained for gallic acid (IC₅₀ = 64 µg/ml) and BHA (IC₅₀ = 114 µg/ml) (Ozsoy et al., 2009). The antioxidant activity of the EACS fraction could be attributed to its high total phenolic content. The key role of these compounds as scavengers of free radicals was reported in several studies (Komali et al., 1999; Moller et al., 1999).

Based on these results, EACS fraction have been chosen rich in phenolic compounds, to identify its chemical composition and investigate its antioxidant and antimicrobial activities.

β-carotene bleaching method

The inhibitory effect of the ethyl acetate fraction of *C. siliqua* on lipid peroxidation was determined by the β-carotene/linoleic acid bleaching test. The antioxidant activity of *C. siliqua* was evaluated using different concentrations of extracts and was compared with BHT used as reference. Results are presented in Table 2. The addition of the EACS fraction and the BHT at a concentration of 5 and 20 µg/ml prevented the bleaching of β-carotene with different degrees. Antioxidant activities of 38.95 and 75.79% were obtained using 20 µg/ml of the EACS fraction and BHT, respectively. The *C. siliqua* fraction was found to hinder the extent of β-carotene bleaching by quenching peroxide radicals to terminate the peroxidation chain reaction. This fraction possessed better antioxidant activity than other extracts such as water extract of chestnut fruit as described by Barreira et

Table 2. Inhibition of lipid peroxidation obtained by ethyl acetate fraction from *C. siliqua* leaves and BHT as assessed by the coupled oxidation of β -carotene and linoleic acid over 120 min. Data represent the means \pm SD (n=3).

Concentration ($\mu\text{g/ml}$)	Inhibition peroxidation lipidique	
	Ethyl acetate extract	BHT
1	6.26 \pm 1.03	52.71 \pm 0.46
2.5	13.78 \pm 0.49	58 \pm 1.25
5	18.63 \pm 1.80	62.98 \pm 1.30
20	38.95 \pm 3.50	75.79 \pm 1.50
IC ₅₀	24.01 \pm 2.24	5.01 \pm 1.08

Table 3. Antimicrobial activities of ethyl acetate extract of *C. siliqua* against food borne and spoiling bacteria and the determination of the minimal inhibition concentration (MIC) and the minimal biocidal concentration (MBC) in $\mu\text{g/ml}$.

Strains	Ethyl acetate fraction			Gentamicin ^d IZ (mm)
	IZ ^a (mm)	MIC ^b (mg/ml)	MBC ^c (mg/ml)	
Gram positive bacteria				
<i>Bacillus cereus</i>	20	1.25	5	23
<i>Bacillus subtilis</i>	22	1.25	5	20
<i>Staphylococcus aureus</i>	24	1.25	5	19
<i>Enterococcus faecalis</i>	17	0.625	5	23
<i>Staphylococcus epidermidis</i>	20	1.25	5	20
<i>Micrococcus luteus</i>	23	0.312	0.625	20
Gram negative bacteria				
<i>Escherichia coli</i>	12	1.25	1.25	18
<i>Pseudomonas aeruginosa</i>	18	0.312	10	14
<i>Klebsiella pneumoniae</i>	15	1.250	10	18

Data are reported as the mean \pm SD from three different experiments. Gentamicin was used as a standard antibiotic at a concentration of 10 $\mu\text{g/well}$. ^aInhibition zones. ^bMinimal inhibitory concentration measured by the broth microdilution using 96-well microplates method. ^cMinimal bactericidal concentration by subculture on nutrient agar at 37°C. ^dGentamicin (10 $\mu\text{g/well}$).

al. (2008). The inhibition of lipid peroxidation by addition of EACS leaves fraction can be used to improve the quality and stability of food products. This antioxidant activity could be attributed to its high phenolic content. The significant correlation between the phenolic content and the antioxidant activity of various vegetable extracts has been previously observed (Velioglu et al., 1998).

Antibacterial activity

The antibacterial activity of the different fractions of *C. siliqua* was quantitatively assessed by measuring the diameter of the inhibition zone around the well and the determination of the MIC and MBC. As shown in Table 3, among the tested extracts, only the EACS fraction showed antimicrobial activities. This fraction inhibited the growth of various species belonging to both Gram-positive and Gram-negative bacteria. The diameter of the

inhibition zones were in the range of 12 to 24 mm. The largest growth inhibition "halo" was observed from *S. aureus* (24 mm) and *M. luteus* (23 mm). The inhibition zone diameter of the EACS fraction was comparable with that of gentamicin used as standard antibiotic and positive control. The observed differences in the inhibition zones within pathogenic bacteria could be probably due to cell membrane permeability or other genetic factors. Earlier reports have shown that Gram positive-bacteria are more sensitive than Gram negative form (El-Astal et al., 2005).

The MIC values of the EACS fraction ranged from 0.312 to 1.25 mg/ml and the MBC were from 0.625 to 10 mg/ml (Table 3). Furthermore, EACS fraction showed the most potent inhibition for *P. aeruginosa* and *M. luteus* (MIC = 0.312 mg/ml). Infections caused by *P. aeruginosa*, especially those with multi-drugs resistance, are among the most difficult to treat with conventional antibiotics. In this study, the growth of *P. aeruginosa* was remarkably

Table 4. LC–ESI–MS data for phenolic compounds in ethyl acetate fraction of *C. siliqua* leaves.

Peak	HPLC retention time (min)	Molecular mass (M)	Major fragment ions m/z	Compounds
1	10	484	331	1,6-Di-O-galloyl-glucose
2	12.4	636	465	1,2,6-Tri-O-galloyl-glucose
3	14.9	480	316	Myricetin glucoside
4	15.1	788	617.1	1,2,3,6-Tetra-O-galloyl-glucose
5	16.2	464	316	Myricetin rhamnoside
6	17.4	198	177	Syringic acid

inhibited by the leaves of EACS fraction (MIC = 0.312 mg/ml). These results show that *C. siliqua* fraction can be used to minimize problems of drug resistance and protect foods against multiple pathogenic bacteria.

To the best of our knowledge, this is the first study that demonstrates that EACS fraction contains antimicrobial substances or to the high level of phenolic components in leaves used by plants as defence mechanisms against pathogenic microorganisms (Cowan, 1999).

Identification of phenolic compounds by HPLC-MS

The EACS fraction was analyzed by HPLC-MS in order to identify its chemical composition. The identification of the different peaks was based on their retention times in HPLC, their characteristic UV/Visible and mass spectra with positive and negative ionization at different fragmentation voltages, in comparison to authentic standards. Close examination of the MS and MS² spectra obtained from the LC-UV profile of the EACS fraction confirmed the presence of individual components from several classes: simple phenol, free flavonol, glycosylated flavonol and isoflavone (Table 4). By comparing mass spectra with those of literature data given by Owen et al. (2003), Peak 1 ($t_R = 10$ min) was identified as 1,6-Di-galloyl-glucose and the $[M-H]^+$ peak was at m/z 484.

The second peak ($t_R = 12.4$ min and $[M-H]^-$ of 635), was identified as 1,2,6-Tri-galloyl-glucose. Peak 3 ($t_R = 14.9$ min) showed $[M-H]^-$ of 480 and was identified as myricetin glucoside. The value of the characteristic fragment ion of the latter peak ($m/z = 316$), was also identical with the reported in the literature. The fourth peak ($t_R = 15.1$ min, $[M-H]^- = 787$ and a fragment ion value of 617) was identified as 1,2,3,6-Tetra-galloyl-glucose which is the main polyphenol. The fifth peak ($t_R = 16.2$ min, $[M-H]^-$ of 463) was identified as myricetin rhamnoside. The value of its fragment ion, found to be of 316, was similar to that reported in carob fibre. The last peak ($t_R = 17.4$ min, $[M-H]^-$ of 197) was identified as the syringic acid. All these results are in agreement with those reported in the literature by Owen et al. (2003) in carob fibre.

This is the first report on detection of phenolic

compounds in *C. siliqua* leaves to the best of our knowledge. Our data showed that in comparison to the carob pod itself, leaves of *C. siliqua* contains higher concentrations of phenolic compounds than detected in earlier report (Corsi et al., 2002; Porto et al., 2000). Corsi et al. (2002) have demonstrated that the phenolic fraction of carob pod by infusion was dominated by gallic acid with minor contributions of catechins, epigallocatechin and epicatechin gallate. As part of our investigation on the constituents of *C. siliqua* leaves, we have demonstrated that the actively ethyl acetate fraction contained mainly syringic acid, myricetin glycosides and gallic acids derivatives.

Further screening of the reported phenolics failed to identify catechin, epicatechin, quercetin and kaempferol. Therefore, the proximate analysis of Tunisian *C. siliqua* constituents, compared to those obtained in previous work, showed some variation in the composition. This variation in composition can be attributed to the diversity of geographical environments (soil, sunlight, temperature, precipitation, etc). Custódio et al. (2009) have reported previously that gender significantly affected the phenolic profile with the hermaphrodites being generally richer in phenols

Conclusion

Conclusively, this study is the first report dealing with the *in vitro* biological activities of the ethyl acetate fraction of *C. siliqua* leaves. It exhibited a high content of total phenolic compounds and the strongest antioxidant activity. In addition, this fraction inhibited the peroxidation of linoleic acid, and acted strong hydrogen-donating agents in the DPPH assay. The antioxidant ability of this fraction was strongly correlated with the phenolic and flavonoid content. Furthermore, six compounds were identified from *C. siliqua* leaves, namely 1,6-di-galloyl-glucose, 1,2,6-tri-galloyl-glucose, myricetin glucoside, 1,2,3,6-tetra-O-galloyl-glucose, myricetin rhamnoside and syringic acid.

The results of this study demonstrated that *C. siliqua* leaves might be a good candidate for employment as antimicrobial activities and an excellent food preservative against bacteria growth. These two properties of plant

fraction are of great interest for food industry, since their possible use as natural additives emerged from a growing tendency to replace synthetic antioxidants by natural ones. Further investigation is required to examine the cytotoxicity effects of leaves of EACS fraction.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

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