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Research Paper

Phytochemical constituents and biological activities of *Ceratonia siliqua* L. leaves grown in western Algeria

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Abstract

***Ceratonia siliqua* L. belongs to the *Leguminosae* family. It is native to the Middle East and has spread throughout the Mediterranean Basin, western America, South Africa and southern Australia. Phytochemical profile and biological activities of this plant leaves were evaluated in the present work. Findings show that the leaves contain useful nutrients and interesting amount of phenolics, flavonoids and condensed tannins. Antioxidant activities were determined using DPPH and FRAP. An IC_{50} of 7 and 30 µg/mL was obtained by DPPH test for organic and aqueous extracts, respectively. Subsequently, the organic extract of carob leaf polyphenols (CLP) was assessed for biological activities. This extract exhibited considerable anti-α-amylase activity with IC_{50} of 0.09 mg/mL. Indeed, an advantageously low hemolytic activity was noted towards human erythrocytes. Further, the antimicrobial activity was evaluated using disc diffusion method against Gram positive bacteria namely *S. aureus, B. cereus, B. subtilis* and *M. luteus* and Gram negative bacteria namely *P. aerugenosa, E. coli* and *K. pneumoniae*. Also the *fungus C. albicans* was tested. Interestingly, *S. aureus* expressed the greatest sensitivity to treatment with minimum inhibitory concentrations (MIC) of 2.5 µg/mL. Results obtained highlight the panoply of biological activities of *C. siliqua* leaves as antioxidant, hypoglycemic, antimicrobial agent with low toxicity.**

Keywords: *Ceratonia siliqua* L., phytochemicals, extracts, antioxidant, biological activities.

Introduction

Almost 50–70 % of the active substances commonly used in human medicine come from natural products, either after exploring their traditional use or through drug repurposing. Knowledge conjugated with traditional medicine has conducted promising research of several medicinal plants as potential herbal medicines and has allowed to the isolation of numerous active compounds[1]. However, most African people believe that herbal medicines are usually safe with no serious adverse effects. The overlap between conventional and traditional or herbal medicine is an obvious reality in countries in health systems transition[2]. Constant and reemerging public interest in alternative and complementary medicine is justified by the high cost of new drugs, increased side effects, microbial resistance and lack of curative treatment for several chronic diseases[3]. Sound knowledge of the mechanisms of herb–drug interactions is essential for clinical risk assessment, which is in turn necessary to healthcare practitioners in their efforts to minimize risk and to guarantee that taking herbal medicinal products is as safe as possible[4]. Carob (*Ceratonia siliqua*) is an evergreen tree that belongs to the legume family (Fabaceae). Its original habitats are the western parts of Asia, but after

its domestication, it spread to all Mediterranean Basin and then to the western shores of the Americas, South Africa and southern regions of Australia[5]. This plant is characterized by its ecological, industrial and ornamental importance. It is recognized for its medicinal and therapeutic virtues[6]. Carob tree cultivars quality is influenced by variations in the morphological features which include environment and geographical locations. Effectively, pods of carob trees from Tlemcen region in western Algeria have the particularity to possess fleshy pulp and high sugar content[7]. Additionally, it has been reported that *C. siliqua* has various properties including anti-inflammatory, antimicrobial, anti-diarrheal, antioxidant, anti-ulcer, anti-constipation and anti-absorptive of glucose activities gastrointestinal tract[8]. An exhaustive review reported by Azab[9] summarized modern research findings and recommendations for future research subjects on this plant. To the best of our knowledge, there is few works on the pharmaceutical quality of phenolic compounds in Algerian *C. siliqua* leaves. Consequently, and in continuation of our previous work[10], this study aims to evaluate the phytochemical content and to investigate the antioxidant and some biological properties (antimicrobial, hemolytic and alpha-amylase inhibiting activity) of *Ceratonia siliqua* leaves cultivated in Algeria.

Material and Methods

Plant material

Carob leaves were collected in Mars (2014) from Nedroma region, Tlemcen (Algeria). The plant was identified by Prof. Benabadji Nouri, Tlemcen University (Algeria). A voucher specimen (CS 1724) was deposited at Herbarium Center of the Faculty of Pharmacy (Tlemcen).

Determination of primary metabolites

Procedures described by AOAC were used to determine moisture, crude fiber, ash and protein-Kjeldahl nitrogen[11]. Fat and carbohydrates were determined following ISO 659[12] and Dubois *et al.*[13], respectively. All experiments were conducted in triplicate to ensure the reproducibility of the results.

Determination of polyphenols, flavonoids and condensed tannins

Carob leaf polyphenols (CLP) extraction and determination protocols were described previously in Ghanemi *et al.*[10], this reported method from Liyana-Pathirana and Shahidi[14] has the particularity of using an extraction with three mixed solvents, without heating. The flavonoids content was evaluated spectrophotometrically according to a method based on the formation of a complex flavonoid-aluminium[15]. The ethyl acetate and butanol fractions containing flavonoids were prepared according to Bekkara *et al.*[16]. Tannins content was estimated using vanillin-HCl method as reported by Julkunen-Titto[17] with slight modifications. The results of flavonoids and tannins content were expressed in mg catechin equivalent per 100 g dried weight (mg CE/ 100 g DW).

DPPH radical-scavenging activity

Evaluation of the scavenging activity against the stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) was measured according to the method described in Sanchez-Moreno *et al.*[18]. A volume of 50 µL of various concentrations of the extracts was mixed with 1950 µL of DPPH methanol solution (0.025% p/v).The mixture was shaken vigorously and incubated at room temperature. The absorbance of the samples was measured by spectrophotometer at 515 nm. Ascorbic acid, Gallic acid, BHA and BHT as DPPH scavenging compounds were used as positive controls. DPPH-free radical scavenging activity was expressed as a percentage (%) according to the following equation:

$$\%\text{DPPH scavenging activity} = [(A_{blank} - A_{sample})/ A_{blank}] \times 100$$

Where A_{blank} is the absorbance of the control, A_{sample} is the absorbance of the compound tested.

Reducing power test

Reducing power of CLP was determined as reported by Belyagoubi-Benhammou *et al.*[19]**.** Different concentrations of CLP extract were mixed with 2.5 mL phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of 1 % potassium ferricyanide. The mixture was incubated at 50 °C for 20min and 2.5mL of 10% TCA (trichloroacetic acid) was added, followed by centrifugation at 3000 rpm for 10 min. The resulting supernatant solution (2.5 mL) was mixed with 2.5 mL of distilled water and 0.5 mL of 1% (w/v) $FeCl_3$. The absorbance was measured at 700 nm by a spectrophotometer. A higher absorbance value of the reaction mixture determined increased reducing power. For each sample, three replicates were made and the average result was noted. Gallic acid (GA) was used as standard.

α-amylase inhibitory activity

In this assay, the protocol of Thalapaneni *et al.*[20] was adopted with slight modifications. A total of 500 µl of test samples (0, 12.5, 25, 50 and 100 µg/mL of extract and acarbose as positive control at 1 mg/mL) were added to 500 µl of a 1 % soluble starch solution in 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M sodium chloride).Then 500 µl of the enzyme solution (α-amylase from porcine pancreas (EC 3.2.1.1)) with final concentration of (0.3 mg/mL), was added to each tube. The reaction mixtures were then incubated at 37˚C for 15 min. Finally, the reaction was stopped with 1 mL of 3,5-dinitrosalicylic acid color reagent. The test tubes were then incubated in a boiling water bath for 8 min, and rapidly cooled in an icewater bath. The absorbance was measured at 540 nm. The α-amylase inhibition was expressed as percentage of inhibition and calculated by the following formula:

$$\text{Inhibitory effect} = [(OD_{control} - OD_{sample})/ OD_{control}] \times 100$$

Where $OD_{control}$ and OD_{sample} are the optical density of the control and the sample tested, respectively.

Hemolytic activity

In vitro hemolytic activity was followed by spectrophotometer method[21]. Five milliliters of blood were collected from a healthy individual. The blood was centrifuged at 1500 rpm for 3 min. The pellet was washed three times with sterile phosphate buffer saline solution (pH 7.2±0.2) by centrifugation at 1500 rpm for 5 min. The cells were re-suspended in normal saline to 0.5 %. A volume of 0.5 mL of the cell suspension was mixed with 0.5 mL of varying dilutions of the extract. The mixtures were incubated for 30 min and 60 min at 37˚C then centrifuged at 1500 rpm for 10 min. The free hemoglobin in the supernatants was measured using UV-Vis spectrophotometer at 540 nm. Phosphate buffer saline and distilled water were used as minimal and maximal hemolytic controls, respectively. Each experiment was performed in triplicates at each concentration. The level of percentage hemolysis by the extracts was calculated according to the formula:

$$\text{Percentage hemolysis} = [(A_s - A_n)/ A_c] \times 100$$

Where A_s is the absorbance of the sample, A_n is the absorbance of the saline control and A_c is the absorbance of the water control. All tests were conducted in triplicate and analyzed statically using ANOVA one-way.

Antimicrobial screening

Microbial strains

Antimicrobial activity of CLP was evaluated using eight different microorganisms. Four Gram-positive strains *(Staphylococcus aureus* ATCC 6538, *Bacillus subtilis* ATCC 6633, *Bacillus cereus* ATCC 25921, *Micrococcus luteus* ATCC 9341) and three Gram negative bacteria (*Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 8739 and *Klebsiella pneumoniae* IBMC Strasbourg). We also evaluated the antifungal activity on the yeast *Candida albicans* (CIP 444 / ATCC 10231).

Preparation of test sample

The extract of CLP was evaporated to eliminate methanol and the residue was dissolved in 5 % dimethyl sulfoxide (DMSO) then sterilized by microfiltration. Ampicillin (10 µg/disc, Biomaxima S.A.) for bacteria and nystatin (100 µg/disc, Sigma) for fungus were used as standard antimicrobial drugs. The discs (6 mm in diameter) were impregnated with 10 µL of each test sample.

Preparation of inoculum

Bacteria inocula were prepared in Mueller Hinton Broth (MHB) after incubation for 24 h at 37°C while *Candida albicans* were incubated for 24-48 h at 30 °C. The suspensions were adjusted to 0.5 McFarland standard turbidity (10^6 CFU/mL by MHB for bacteria and 10^5 CFU/mL for *Candida* by SDB). All bacterial strains were maintained previously with nutrient agar media (Sigma-Aldrich) while *C. albicans* were grown in Sabouraud Dextrose Agar Oxoid.

Agar disc diffusion method

Inhibition zones were determined against the selected microbial strains according to the disc diffusion method recommended by the Clinical Laboratory Standards Institute (CLSI) guidelines[22]. Petri dishes were thus maintained 1–2 hours at 4°C to permit CLP extracts diffusion. Then, plates were stored at 37°C and maintained in incubator 24 h for bacteria and 24-48 h for fungus, depending on the

incubation time required for a visible growth. At the end of the incubation period, inhibition zone diameters were measured, including paper disk (in mm). All assays were repeated in triplicate.

Determination of MIC

MIC of CLP extracts was determined using the microbroth dilution method, according to the Clinical and Laboratory Standards Institute (CLSI) protocols[22]. The test was performed in sterile 96-well microplates with a final volume in each microplate well of 100µL. Briefly, the stock solution of CLP in DMSO was serially diluted to give two-fold dilutions which were added to each medium. The tests were carried out in triplicate and values of MIC are expressed in µg/mL. The microplate was covered and incubated for 24 h at 37°C. The MIC of CLP extract was recorded as the lowest dilution that inhibited growth of microorganisms.

Results and Discussion

Phytochemical analysis of *Ceratonia siliqua*

The overall results of the primary metabolite assays are summarized in Table 1. They showed that carob leaves contained a relatively high percentage of protein and carbohydrates compared to the other components of these leaves. This phytochemical profile indicated that the leaves of the carob tree contain nutrients that could be useful in nutrition. Findings of total phenolics CLP, total flavonoids and condensed tannins contents of organic extracts of *C. siliqua* are presented in Table 1.

Table 1: Phytochemical composition of *C. siliqua* leaves

Metabolites	Values
Primary metabolites	%
moisture	12.38% ±0.12
fat	4.255% ±0.14
ash	4.608% ±0.20
carbohydrates	16.625% ±0.024
Fibres brutes	11.77% ±0.06
Protein	22.25% ± 0.08
Secondary metabolites	**mg/g**
Polyphenols	1.72±0.02 mg GAE/g
Flavonoids (ethyleacetate phase)	1.056 ±0.05 mg CE/g
Flavonoids (n-buthanol phase)	1.132±0.03mg CE/g
Condensed tannins	6.69±0.01 mg CE/g

Phenolics are secondary plant metabolites well known for their high level of antioxidant capacity[23]. Moreover, several studies have shown the relationship between their anti-oxidant and biological activities[24,25]. It has been shown that metabolites such as total polyphenols and gallic acid are produced more intensely in the leaf than other organs. Gallic acid is the major phenolic compound in carob leaves, pulps and seeds[26]. Results of the polyphenols content in *C. siliqua* leaves are in adequacy with those reported in our previous study[10], where we detected phenolic compounds by HPLC in this decreasing order: *m*-Coumaric acid > Gallic acid > Syringic acid >*p*-Coumaric acid > Quercetin >Quercetin 3-Orutinoside > Chlorogenic acid, thus suggesting the synergistic effect between polyphenols in CLP activity.

Biological activities of *Ceratonia siliqua* extracts

Antioxidant activity

The scavenging effects of the CLP extracts with the DPPH and FRAP tests are carried out at gradual concentrations and expressed in IC_{50}.The extract of CLP showed the highest activity compared to all the standards (Table 2). As the aqueous extract is less active than the organic extract, subsequent analysis will be carried out with the organic extract only. The best antioxidant activity was exhibited on the organic extract with the lowest IC_{50} value (0.007 mg/mL), a low IC_{50} value being an indicator of high activity antioxidant[27].

Table 2: Antioxidant and anti-α-amylases activities of *C. siliqua* extract

Sample	Antioxidant		anti-α-amylase
	DPPH	FRAP	
Polyphenols aqueous extract	0.03±0.001[a]	0.05±0.005[a]	-
Polyphenols organic extract	0.007±0.0001	0.01±0.004	0.09±0.001[a]
Ascorbic acid[b]	0.020±0.004	-[c]	-
Gallic acid[b]	0.026±0.005	0.04±0.002	-
BHA[b]	0.022±0.007	-	-
BHT[b]	0.022±0.005	-	-
Acarbose[b]	-	-	0.05±0.006

[a] activities were expressed in IC_{50}(mg/mL) (mean±SD, n=3).
[b] positive control.
[c] not tested.

α-amylase inhibitory activity

The CLP extract was tested for its inhibitory effect on the α-amylase enzyme (Table 2). The α-amylase is a vital enzyme for type-II diabetes. The inhibitory action of pancreatic α-amylase can be considered as effective strategy for the control of diabetes by reducing the absorption of glucose through the intestinal mucosa[28]. The CLP displayed a fairly potent potential with an IC_{50}=0.09 mg/mL compared to the positive control represented by acarbose with an IC_{50}=0.05 mg/mL. The hypoglycemic effect of the aqueous extract of carob leaves was recently demonstrated by Rtibi *et al.*[29], who showed that the aqueous extract reduced in vivo and in vitro the absorption of glucose. The α-amylase inhibitors have also been widely prescribed for obesity-related pathologies such as type-II diabetes. Indeed, acarbose as the most common α-amylase inhibitor which improves postprandial lipemia and reduces the remains of atherogenic chylomicrons and very low-density lipoproteins[30].

Hemolytic activity

It appeared that the hemolytic percentage increased in a dose-proportional manner. After one hour (T_{60}) of incubation, the CLP extract exhibited maximum hemolytic activity against blood cells with a level of 27.31 % at a concentration of 326µg/mL (Figure 1). This finding indicated that the CLP exerted a weak to moderate hemolytic effect towards human erythrocytes. Based on the results obtained, it is worth noting that the CLP might possess low hemolytic activity, which could be explained by a weak disruptive effect on erythrocytes. This inhibitory effect was previously shown by Ghanemi *et al.*[10] against the viability of cancer cells (HCT-116, SW-480, HT-29 and CT-26). Besides, CLP exerted a dose-dependent cytotoxic effect through the induction of apoptosis on colorectal cancer cell lines, with an IC_{50} around 20 µg/mL.

The parameters studied

Precociousness of germination: It is expressed by the rate of the first seeds germinated corresponding to the time interval between the seedling and the beginning of the emergence of the radicle through the membrane[2].

Final germination rate: It is expressed by the ratio of seed germination to total seed number.

Germination kinetics: It most often represents the evolution of cumulative germination percentages over time. This kinetics is based on the cumulative germination rates, that is to say, the variation in germination rates with time expressed in days under all test conditions[3].

The length of the radical: Measurements of the length of the radicle are made on the last day of germination using millimeter paper.

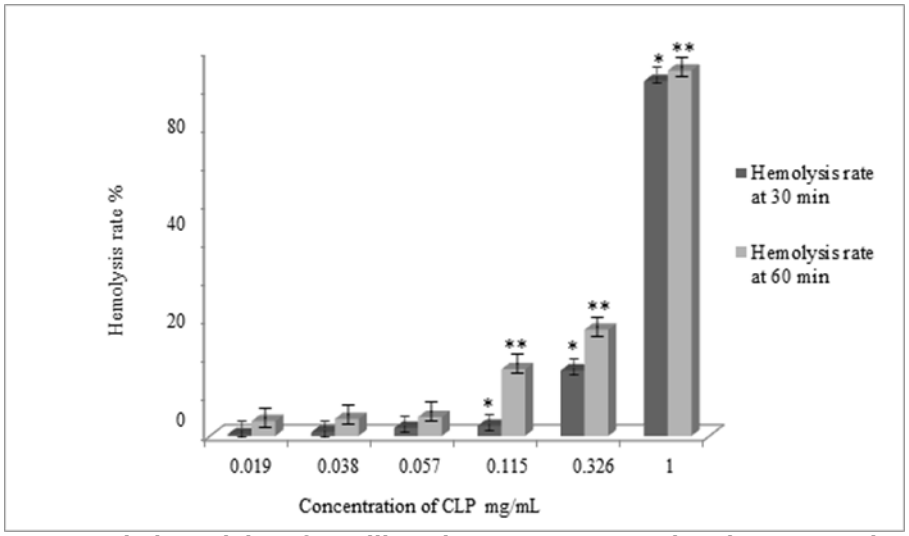

Figure 1: Hemolytic activity of *C. siliqua* leaves extract against human erythrocytes

All values represent the mean ± standard deviation (n=3), * and ** represent $p< 0.05$ and $p< 0.01$ respectively as compared to T_0.

Antimicrobial activity

The antibacterial activity of the extracts investigated against the Gram positive and Gram negative bacteria used (microdilution method) is presented in (Table 3).

Table 3: Antimicrobial activity of *C. siliqua* extract

	Code	Inhibition zone diameter's (mm)	MIC (mg/mL)
Gram-positive bacteria			
Staphylococcus aureus	ATCC 6538	11	0.0025
Bacillus subtilis	ATCC 6633	7	0.011
Bacillus cereus	ATCC 25921	8	0.011
Micrococcusluteus	ATCC 9341	9	0.005
Gram-negative bacteria			
Pseudomonas aeruginosa	ATCC 27853	-	-
Escherichia coli	ATCC 8739	7	0.022
Klebsiellapneumoniae	IBMC Strasbourg	-	-
Fungi			
Candida albicans	CIP 444	-	-
Candida albicans	ATCC 10231	-	-
Ampicillin*	-	25-33	-
Nystatin*	-	30	-

* represent antibacterial and antifungal drug standards as positive control.

Overall, the data have shown that the leaf material is ideal for antimicrobial uses, and can be used without any damaging effects on the plant[31]. According to the results obtained, the CLP extract had a MIC of 0.0025 mg/mL against *S. aureus* whilst *B. cereus* and *B. subtilis* exhibited a MIC of 0.011 mg/mL. For the Gram negative bacteria, only *E. coli* was sensitive with a MIC of 0.022 mg/mL. The antibacterial activity was higher against the Gram positive *S. aureus* compared to the Gram negative strains like *E. coli*, this activity is related to the presence of the total phenolic content of the extract[32].

Our results agree with Al-Seeni[33] where n-butanol extract of C. siliqua leaves was the most effective extract against *S. aureus* and *E. coli*. However, contrary to our findings, antifungal activity with ethyl-acetate and methanol has been observed against *C. albicans*. The MICs of the aqueous extract of *C. siliqua* varied between 30 mg/mL for *S. aureus* and 50 mg/mL for *E. coli* and *C. albicans*. These

results are corroborated with our sensitive strains. The evolution of *S. aureus* during the modern era of antibiotics has been delineated by distinct strain emergence events, many of which include the acquisition of antibiotic resistance. The relatively high burden of methicillin-resistant *S. aureus* (MRSA) in healthcare and community settings is a major concern worldwide[34]. On the other hand, Aissani et al.35 showed high antimicrobial activity against the reference strain L. monocytogenes (ATCC 35152) and isolated strains with a MIC of 28.12 µg/mL. The differences in results regarding antioxidant and antibacterial activities with other studies are attributed to the difference in geographic distribution, climate, harvest season, chemical composition and soil type which varies depending on the origin of the species[25].

Conclusion

Our findings suggest that the extract of *Ceratonia siliqua* L. leaves, rich in polyphenols, exhibits promising biological activities (especially as antioxidant, hypoglycemic, and antibacterial with low toxicity). The combination of those properties in a single extract could find application in the pharmaceutical industry and in the phyto-pharmaceutical herbal-type products.

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