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# Comparative characterization of carob pulp and seeds extracts: HPLC, antimicrobial, anti-inflammatory, and cytotoxic studies

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**Abstract**

HPLC profiling, antimicrobial, cytotoxic, and anti-inflammatory activities of carob (*Ceratonia siliqua* L.) pulp and seeds extracts were investigated. Gallic acid was the main identified phenolic. Ellagic acid, rutin, quercetin, and kaempferol were also tentatively identified. Carob seeds extract exhibited antimicrobial activity against *Pseudomonas aeruginosa* (6.25 and 25 mg $mL^{-1}$ for minimum inhibitory concentration and minimum bactericidal concentration, respectively). Carob extracts showed a weak dose-response inhibition of the lipopolysaccharide induced nitric oxide release in RAW macrophages. Carob extracts even in the high dose did not inhibit the cell growth either against MCF-7 or HT-29 cell lines. Carob pods may be used as a functional food or food additive based on these findings..

**KEY WORDS:** Carob; phenolics; HPLC; cytotoxicity; antimicrobial; anti-inflammatory

## 1. Introduction

Carob (*Ceratonia siliqua L.*) pods have traditionally been used as foods for animals and humans. Carob is a well-known member of the pea family. Carob pods are composed of pulp (90 %) and shiny seeds (10 %) [1]. The pods are composed principally of tannins, pectins, hemicellulose, and cellulose. Industrially, carob pulp is mainly used as syrups, cocoa substitutes (roasted powder), or antidiarrheal preparations (tannin-rich fractions). In traditional medicine, carob pulp is recommended for the treatment of GIT disorders as an antidiarrheal remedy [2], and as an antitussive agent [3]. Products of insoluble carob dietary fiber demonstrated cholesterol-lowering effects [4]. The preparations of carob fibers have been granted an European patent as anti-inflammatory and chemopreventive agents [5]. Antiproliferative [6], antioxidant, anticancer [7], antidiabetic, antimicrobial as well as antihyperlipidemic were reported activities of carob [1].

Carob pods have a high sugar content (up to 50%), but low lipid and protein contents (0.4-0.8 and 3-4 % respectively)[8]. Polyphenols, especially tannins were identified from carob pods accounting for their astringent taste [9]. Condensed tannins were present in carob with much higher levels versus hydrolysable ones. The total phenolic content (TPC) of carob pulps was 18 to 41.3 mg gallic acid equivalent (GAE)/g. Catechins and gallic acid contents ranged from 6-10 and 1-2 mg/g respectively [10]. The pods comprised 3.7 mg/g total tannins [4].

Gallic acid being the major phenolic acid followed by cinnamic, chlorogenic ellagic, ferulic, gentisic, p-coumaric, and syringic acids [11]. Carob is a rich source of flavonols glycosides viz. isorhamnetin, kaempferol, myricetin, and quercetin [11] [12]. Apigenin, chrysoeriol, luteolin, naringenin, and genistein were detected at lower levels [4] [13]. HPLC analysis of carob pods showed the presence of gallic acid and its derivatives including epicatechin gallate and epigallocatechin gallate [6].

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Carob is used as a functional food with a global production of roughly 310,000 tonnes per year [14]. The industry has discarded a considerable amount of carob seeds garbage. Carob seeds may be a low-cost, high-antioxidant, antiproliferative phytochemical source. In addition, few reports were done about the chemical composition and biological activities of carob. The purpose of our study was to highlight the potential health effects of carob seed by-products. Herein, the components of carob pulp and seed were profiled using HPLC/PDA. Furthermore, the antimicrobial, anti-inflammatory, and cytotoxic activities of carob pulp and seed extracts were also evaluated.

## 2. Material and Methods

### 2.1. Chemicals

Authentic phenolics and flavonoids were kindly supplied by Nawah Scientific Inc., (Cairo, Egypt) for the HPLC study. Dimethylsulfoxide (DMSO) was from Sigma Aldrich, St. Louis, MO, USA. Dent supplement (Oxoid H. pylori Selective Supplement), antibiotics discs were purchased from HiMedia (Mumbai, India). Other chemicals were of the highest purity.

### 2.2. Plant material and extraction

Dried carob (*Ceratonia siliqua L.*) pods were purchased (2020) at ripe stages from local market in Egypt. The pods' identity was confirmed in the pharmacognosy department, Faculty of Pharmacy, O6U, Giza, Egypt. Pulp and seeds were carefully separated, powdered (100 g each) and were extracted in Soxhlet (80 % methanol, 2x500 mL). Filtered extracts were evaporated individually (Rotavapor®, BÜCHI, Switzerland) [15] and were used for biological and chemical investigations.

### 2.3. Total phenolics and flavonoids contents and HPLC profiles:

Total phenolics content (TPC) as gallic acid equivalent (GAE g-1) and total flavonoids content (TFC) as catechin equivalent (CE g-1) were carried out. The colored complex formed between phenolics and flavonoids with folin-ciocalteu's phenol and ALCl3, respectively were measured using a spectrophotometer (Shimadzu, Japan), with reference to the standard calibration curves [16]. For the HPLC study, identification was performed using Waters 2690 Alliance HPLC system equipped with a Waters 996 photodiode array detector (280 nm) equipped with a C18 column (Kromasil: 4.6x150 mm, 5 µm). Gradient elution (1 mL/min) of 0.1 % phosphoric acid in water and methanol was proceeded. Extracts and a stock solution of standards were dissolved in methanol separately, filtered (0.2 µ syringe filter), and 10 µL were injected. Peaks assignments were confirmed in comparison to authentics.

### 2.4. Antimicrobial activity

#### 2.4.1. Inoculums preparation

Microbial strains were provided from Microbiology and Immunology Department, Faculty of Pharmacy, O6U, Giza, Egypt. Each bacterial isolate was sub-cultured aerobically on Mueller-Hinton agar (LAB M limited, Lancashire, UK) and incubated overnight (37◦C), except H. pylori was sub-cultured microaerophilic (5% CO2) (37°C, 72 h) in Columbia blood agar with Dent supplement. Fungal isolates also were sub-cultured in Sabouraud agar plate (25°C, 48 h.). The microbial growth was harvested using saline water and diluted to attain viable cell count of 108 CFU mL-1.

#### 2.4.2. Antimicrobial assays of carob pulp and seed extracts

Carob pulp and seed extracts (100 mg mL-1 in DMSO) were sterilized by membrane filter (0.22 mm) then loaded over wells (7 mm holes) on Petri dishes containing inoculums preparation (15 mL). The plates were incubated according to the microbial strain. After incubation, the zones of inhibition diameter (mm) were measured in triplets. Antibiotic discs were used as a positive control [17]. The minimum inhibitory concentration (MIC), that inhibits the microbial growth after 24 h of incubation, was determined for the effective seeds extracts using broth microdilution method in microtiter plates (Infinite F50 Tecan-Sweden, 620 nm) [18]. Serial two-fold dilutions from 50-0.976 mg mL-1 of both carob pulp and seeds were prepared. An inoculum (50 µL) of microbial isolates (106 CFU mL-1) in Mueller-Hinton broth, and of carob seeds extracts in different concentrations (50 µL) were mixed. For the determination of minimum bactericidal concentration (MBC), respective microbes (10 µL) and carob extracts were inoculated, and bacterial counts were determined (CFU mL-1) in triplicate.

### 2.5. In Vitro Cytotoxicity Screening

MCF-7 (breast adenocarcinoma) and HT-29 (colorectal cancer) cells were obtained from Nawah Scientific Inc., (Cairo, Egypt) and maintained in DMEM and RPMI media, respectively. Media were supplemented with streptomycin (100 mg mL-1), penicillin (100 units/mL), and heat-inactivated fetal bovine serum (10 %) in humidified, CO2 (5%) atmosphere at 37 °C. Cell viability was assessed by SRB assay [19] using only two high doses of the extracts (10 and 100 µg mL-1) following US National Cancer Institute (NCI) screening methodology [20]. Each concentration was performed in triplicates and the percentage of relative viability was calculated as follows: Cell viability % = [Absorbance of treated cells/Absorbance of control cells)] × 100.

### 2.6. In Vitro Anti-inflammatory Screening

Murine macrophages RAW264.7 cells (ATCC®) were maintained in complete Dulbecco's Modified Eagle's Medium supplemented with fetal bovine serum (10%), penicillin (100 U mL-1), streptomycin sulphate (100 µg mL-1) and L-glutamine (2 mM) in a humidified 5% CO2 incubator. RAW264.7 cell stock (0.5 × 106 cells/mL) was seeded into 96-well microwell plates and incubated overnight. On the next day, non-induced triplicate wells received medium with the sample vehicle (DMSO, 0.1%). Inflammation group of triplicate wells received the inducer of inflammation [ LPS as 100 ng mL-1 in complete culture media]. Sample groups of triplicate wells received both carob extracts (10 &100 µg mL-1 in DMSO separately) and diluted into culture media containing LPS (DMSO=0.1%). Caffeic acid phenecyl ester (CAPE, 5 µM) was used as an anti-inflammatory positive control. After 24 h of incubation, Griess assay [21] was used to determine nitric oxide (NO) in all wells. Equal volumes of culture supernatants and Griess reagent were mixed and incubated (10 min) to form the colored diazonium salt. The absorbance was measured on a Tecan Sunrise™ microplate reader (540 nm) (Austria). NO inhibition % of both carob extracts were calculated relative to the LPS-induced inflammation group, normalized to cell viability determined with Alamar Blue™ reduction assay [22].

## 3. Results and discussion

### 3.1. Total phenolic and flavonoids contents and HPLC profiling:

Aqueous methanol is a polar solvent that works well for extracting phenolics. TPC was 21.25±0.04 and 39.71±0.02 mg GAE g-1 for the pulp and the seeds extracts of carob, respectively. While TFC was 12.51±0.01 and 23.97±0.09 mg CE g-1, respectively. The TPC and TFC differed between pulp and seeds extracts as always TFC is lower than TPC. Carob pulp and seeds extracts were analyzed using HPLC-PDA (Fig. 1). Phenolic acids represent the major relative content in both the pulp and the seeds. In the pulp, gallic and ellagic acids were tentatively identified (Table 1, Fig. 1a). While in the seeds, gallic acid, rutin, quercetin, and kaempferol were detected (Table 1, Fig. 1b). Phenolics and flavonoids are strong antimicrobial agents.

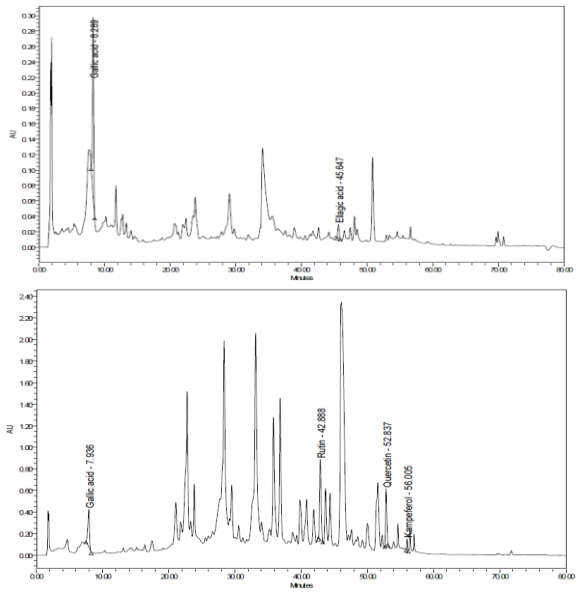


Figure 1: Analytical HPLC-PDA chromatograms of carob pulp (a) and seeds (b) extracts

**Table 1: Tentatively identified phenolics from carob pulp and seeds extracts**

| Peak no. | Compound | $R_t$ min | UV shifts | CP | CS | Ref |
|---|---|---|---|---|---|---|
| 1 | Gallic acid | 7.93 | 215.9, 269.3 | + | + | [12] |
| 2 | Rutin | 42.8 | 211.1, 282.4, 327.9 | | + | -- |
| 3 | Ellagic acid | 45.6 | 251.4, 367.3 | + | | -- |
| 4 | Quercetin | 52.8 | 253.8, 372.1 | | + | [12] |
| 5 | kaempferol | 56.0 | 361.5, | | + | [12] |

CP; carob pulp, CS; carob seeds

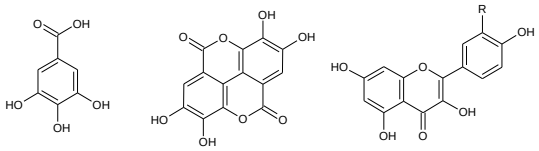

Figure 2. The major phenolics identified in carob pulp and seeds extracts.

### 3.2. Antimicrobial activity of carob extracts

Both carob extracts were potentially effective in suppressing microbial growth (Table 2). S. aureus, S. ventriculi, M. kristinae., P. aeruginosa, K. pneumonia (MDR) were susceptible to carob seeds than pulp with a zone of inhibition (ZOI) ranging from 18 to 25 mm, larger than those of reference standard antibiotics (resistant to 17 mm). While S. epidermidis, S. aureus (MRSA), M. phlei, B. subtilis, E. faecalis, E. coli, P. aeruginosa (MDR), H. pylori, C. albicans, A. niger, and P. notatum were moderated to carob seeds with a ZOI from 13-17 mm, Carob pulp failed to inhibit the growth of all microbial isolates except M. kristinae, M. phlei, E. faecalis, and P. aeruginosa with 10-11 mm ZOI. (Table 2). DMSO was used as negative control.

**Table 2: Sensitivity test, MICs, and MBC of carob pulp and seeds extracts (100 mg mL$^{-1}$ in DMSO) against microbial strains.**

| Bacterial Isolates | CS | CP | CS | |
|---|---|---|---|---|
| | Zone of Inhibition (mm) | | MIC (mg mL$^{-1}$) | MBC (mg mL$^{-1}$) |
| ***Gram-positive strains*** | | | | |
| ***Bacillus subtilis*** | 15±0.5 | - | 50 | >50 |
| ***Enterococcus faecalis*** | 15±30 | 10±0.6 | 12.5 | 50 |
| ***Micrococcus kristinae*** | 20±20 | 11±0.7 | 12.5 | 50 |
| ***Mycobacterium phlei*** | 17±1.6 | 10±0.8 | 25 | >50 |
| ***Sarcina ventriculi*** | 20±2 | - | 25 | >50 |
| ***Staphylococcus aureus*** | 22±0.2 | - | 25 | >50 |
| ***Staphylococcus aureus (MRSA)*** | 15±1.2 | - | 50 | >50 |
| ***Staphylococcus epidermidis*** | 15±0.5 | - | 25 | >50 |
| ***Gram-negative strains*** | | | | |
| ***Escherichia coli*** | 14±11 | - | 50 | >50 |
| ***Klebsiella pneumonia (MDR)*** | 18±0.0 | - | 50 | >50 |
| ***Pseudomonas aeruginosa*** | 25±2.5 | 11±0.9 | 6.25 | 25 |
| ***Pseudomonas aeruginosa (MDR)*** | 13±1.5 | - | >50 | ND |
| ***Helicobacter pylori*** | 12±1.5 | - | >50 | ND |
| ***Yeast strains*** | | | | |
| ***Candida albicans*** | 17±0.7 | - | 25 | >50 |
| ***Aspergillus niger*** | 13±0.6 | - | >50 | ND |
| ***Penicillium notatum*** | 15±1.5 | - | >50 | ND |

CP; carob pulp, CS; carob seeds, (MDR): multidrug-resistant, Standard antimicrobial agents: nitrofurantoin, amoxacillin/clavulanic acid, cefepime, metronidazole and fluconidazole showed zones of inhibition ranging from 11 to 34 mm.

Carob seeds displayed the lowest MIC values of 6.25, 12.5 and 12.5 mg mL-1 against P. aeruginosa, M. kristinae., and E. faecalis, respectively (Table 2). Consequentially, MBC of carob seeds extract was determined with the most effective inhibition by disc diffusion and MIC assays. Carob seeds have antibacterial activity against P. aeruginosa opportunistic pathogen which known to cause respiratory infections, dermatitis, bacteremia, gastrointestinal infections, and a variety of systemic infections.

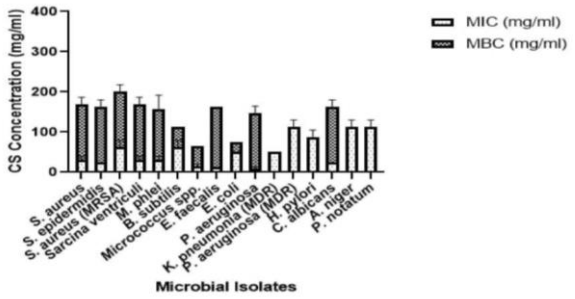


Figure 3: MICs and MBC of carob seeds extract (100 mg mL−1 in DMSO) against microbial strains

### 3.3. Anti-inflammatory and Cytotoxicity assays

Concerning the screening of the anti-inflammatory activity, carob pulp or seeds showed limited inhibition of the LPS-induced NO release in RAW macrophages (Table 3). Being an edible pod, both carob extracts even in the high dose did not inhibit the cell growth either against MCF-7 or HT-29 cell lines. Based on these results as expected, no need for progress to the full 5-dose assay (Table 3). The antiproliferative effect is noticed with the consumption of carob products contingent on their polyphenolic composition [11] [28].

**Table 3: Anti-inflammatory and cytotoxic activities of carob pulp and seed extracts**

| Assay | CP | | CS | |
|---|---|---|---|---|
| | **10 $\mu$g mL$^{-1}$** | **100 $\mu$g mL$^{-1}$** | **10 $\mu$g mL$^{-1}$** | **100 $\mu$g mL$^{-1}$** |
| **Anti-inflammatory** | 10.95±1.1 | 24.45±1.6 | 17.34±2.4 | 29.40±2.9 |
| **Cytotoxicity** | | | | |
| MCF-7 | 98.06 ± 0.06 | 97.06 ± 0.07 | 97.63 ± 0.33 | 97.06 ± 0.51 |
| HT-29 | 99.52 ± 0.42 | 94.98 ± 0.50 | 99.52 ± 0.52 | 90.35 ± 1.49 |

Values are represented as Mean± SD, Caffeic acid phenecyl ester (CAPE) 5 µM (a standard anti-inflammatory agent) exhibits 82.88±2.7

### Conclusion

Gallic and ellagic acids were the major phenolic compounds found in carob pulp and seeds. Owing to the phenolic content, the by-product of carob seeds can serve as a dietary source of natural antioxidants and antimicrobials for the food industry.

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**Conflicts of Interest**: no conflict of interest was declared by the authors

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