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Sucrose supplementation during traditional carob syrup processing affected its chemical characteristics and biological activities

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ABSTRACT

The addition of sucrose is optional during carob syrups traditional processing. In this work, the polyphenolic profiles of carob syrups supplemented (CSS) or not with sugar (CS) were analyzed using RP-HPLC–ESI-MS. Quantitative data showed that adding of sucrose significantly ($p < 0.05$) decreased the polyphenolic amounts of about 58.6%, from 4.5 ± 0.32 to 1.86 ± 0.26 mg/g of CS and CSS, respectively. Gallic acid was the most abundant compound in both syrups representing 60.93% (CS) and 69.26% (CSS) of the total phenolics. Moreover, the adding of sugar decreased ($p < 0.05$) the antiradical potentials of 28% and 74%, as determined, respectively, by the ABTS and DPPH assays. CS was found to exhibit stronger antibacterial and antifungal activities than CSS. *Bacillus cereus* was the most sensitive strain to the extracts with CMI ≈500 (CS) and 622 (CSS) μg/ml. Both syrup extracts were cytotoxic to human neuroblastoma (SH-SY5Y) and fibroblast (3T3) cell lines as well as to mouse embryonic stem cells (D3). Tumoral SH-SY5Y cells were the most susceptible to the extracts with IC50 = 311.7 ± 23.65 (CS) and 390.6 ± 34.97 μg/ml (CSS). This study provides, for the first time, new analytical insights into traditionally made carob syrups and highlights the negative effect of sugar supplementation during processing.

1. Introduction

The carob tree (*Ceratonia siliqua* L.) is a worldwide evergreen species. Particularly, the Mediterranean region has been one of its domestication centers (Sidina et al., 2009). Carob has been cultivated for millenaries not only as a forage crop or as food for human consumption but also to cure various diseases (Sidina et al., 2009). Recently, this species attracted great attention and became socio-economically important for its multipurpose uses (Sandolo, Coviello, Matricardi, & Alhaique, 2007). Pods and seeds are used as row material in food, pharmaceutical and cosmetic industries, especially for gum extraction (Barracosa, Osorio, & Cravador, 2007). Bark and leaves have been used in folkloric medicine as laxative, diuretic, antidiarrhoeal and for the treatment of gastroenteritis in lactating babies and children (Kivçak, Mert, & Ozturk, 2002). The experimental and clinical studies performed on *C. siliqua* showed that most of its pharmacological actions were attributed to the antioxidant activity, which scavenges free radicals and/or inhibits lipid peroxidation (Custodio et al., 2011).

Processed syrups are generally used for softening and conserving seasonal fruits or for the preparation of cakes, cookies and homemade confectionery. These food byproducts are widely consumed in Tunisia, known locally as “Rub”, especially during the cold periods of the year for its high energetic sugar content (Dhaouadi et al., 2011, 2013). This is also the case for many North African and Arabic countries where these fruit syrups are poured on cooked dough (“asseeda”) on specific occasions, such as the celebration of religious festivities e.g. the Muhamed Prophet’s birthday (FAO, 2004). In these countries, the main fruit derived syrup products are prepared from date, barbary-fig and carob pods. Optionally, sucrose is supplemented to the fruit juice to prepare sweet syrup and reduce water activity for conservative purposes. Despite the high sucrose content of the carob pods, sugar is sometimes added as a traditional practice.

During the last decades, food trends are focused on healthful products. Thus, many scientific reports attempted to emphasize the advantages and benefits of particular foods, especially traditional

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and folkloric ones. Accordingly, these nutritional products are more and more drawing local and international market tendencies. Nowadays, the consumption of fruit and vegetables is regarded as important and good for health. Indeed, recent scientific studies indicated that a high intake of fruit and vegetables is associated with reduced risk for a number of chronic and degenerative diseases (Mansouri, Embarekb, Kokkalouc, & Kefalasa, 2005). In this respect, the recent explosion of interest in phytochemicals is attributed to the potential health benefits of plant antioxidants, especially, polyphenols. These latters exhibit several biological activities acting as antimicrobials, anti-carcinogenic, anti-inflammatories, antivirals, anti-allergic and immune-stimulators (Parr & Bolwell, 2000). The antioxidant activity of phenolics is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers. In addition, they have a metal chelation potential (Atoui, Mansouri, Boskou, & Kefalas, 2005).

To our knowledge, there is no report available on chemical composition of carob syrups and their biological activities. Thus, the aim of this work was to investigate traditionally made carob syrups and determine the effect of sucrose addition, a common practice during processing, on some functional properties, particularly (i) polyphenolic quantitative and qualitative content, (ii) antioxidant and antimicrobial capacities, and (iii) cytotoxic potentials using tumoral as well as non-tumoral human and mouse cell lines. This study should bring new knowledge about the adding of sugar during carob syrup preparation in order to support or oppose to this practice.

2. Material and methods

2.1. Carob syrup preparation

Carob (*C. siliqua* L.) fruit samples were purchased from local markets. The fruits were immediately washed with water and dried in the oven for 30 min at 40 °C. The fruits are then stored in dry environment at room temperature until used. The carob syrup is prepared according to the traditional method used by Tunisian families (Fig. 1). Pods pulps were ground using a blender (MOLINEX A 327R1/APO-2210R) and filtration was achieved through cloth. The obtained final syrups were divided into two batches (50 g, each) and kept in dark bottles at room temperature similarly to traditional practice.

2.2. Polyphenols extraction

The syrup (10 g) was mixed and extracted with distilled water (1:3, w:v) in a sonicator apparatus for 30 min. Following a centrifugation at 10,000×*g* for 15 min, cold acetone (−20 °C) was added to the supernatant (ratio 7:3, v:v). The precipitate was discarded following a centrifugation at 12,000×*g* for 10 min. The supernatant was collected and concentrated using a rotary evaporator (60 °C). The extracts were then stored at −20 °C until use. Immediately before the *in vitro* or cellular assays, samples were sterile-filtered through 0.22 μm filters.

2.3. Total phenolic quantitative estimation

Total phenolic content (TPC) was quantified following the method previously described by Li, Wong, Cheng, and Chen (2008) with slight modifications. One hundred microliter of syrup extract (diluted 20 times in water) was mixed with 400 μl of 10% Folin–Ciocalteu reagent (Sigma–Aldrich, France). Following incubation for 15 min in the dark at room temperature, 500 μl of 7.5% sodium bicarbonate (Na_2CO_3) were added to the mixture. After incubation for 30 min, the absorbance at 765 nm was recorded and gallic acid (Sigma–Aldrich, France) was used as a standard. TPC was expressed as gallic acid equivalent (GAE) per 1 g of syrup.

2.4. HPLC-DAD–MS instrumentation and conditions

The different extracts were analyzed and quantitated using an Agilent LC1100 series (Agilent Technologies, Inc., Palo Alto, CA, USA) controlled by the Chemstation software and equipped with pump, autosampler, column oven and UV–vis diode array detector. The HPLC instrument was coupled to an Esquire 3000+ (BrukerDaltonics, GmbH, Germany) mass spectrometer equipped with an ESI source and ion-trap mass analyzer, and controlled by Esquire Control and Data Analysis software. Analytical assays were performed using Merck Lichrospher 100 RP-18 (5 μm, 250 × 4 mm) column. The mobile phase comprised 0.5% formic acid in water (A) and methanol (B). Phenolic compounds separation was achieved using the previously described liquid chromatography technique (Fattouch et al., 2007) with minor modifications. The solvent gradient started at 95% A and 5% B, reaching 35% B at 20 min, 50% B at 25 min, 95% B at 40 min, 5% B at 42 min, and 5 more minutes for re-equilibration. The flow rate was 0.5 ml/min, and the injection volume was 10 μl. Diode-array detection was set at 280, 320, 340 and 360 nm. Mass spectrometry operating conditions were optimized in order to sensitivity values. The ESI source was operated in negative mode to generate [M−H] ions using the following conditions: desolvation temperature at 250 °C and vaporizer temperature at 400 °C, dry gas (nitrogen) was set at 4.5 l/min, probe voltage 4.5 kV, fragmentor voltage 20 V. The MS data were acquired as full scan mass spectra at 50–800 *m*/*z* by using 200 ms for collection of the ions in the trap. Identification of the main compounds was performed by HPLC-DAD analysis, comparing the retention time, UV spectra and MS data of the peaks in the samples with those of authentic standards or data reported in the literature. Quantitation of *p*-coumaric acid, epigallocatechin gallate, epigallocatechin, kaempferol, catechin, syringic acid, quercetin, caffeic acid, gallic acid, catechin gallate, myricetin and cinnamic acid was performed using commercial standards (Sigma–Aldrich, France). The software Chemstation for LC 3D (Agilent Technologies Life Sciences and Chemical Analysis, Waldbronn, Germany) was used for quantitation purposes. Quantitative evaluation of the compounds was performed by means of a five points regression curve ($R^2 > 0.996$) in a concentration range between 25 μg/ml and 1.2 mg/ml, using external standards and evaluated at 280 nm, which is the monitoring wavelength.

2.5. In vitro antioxidant activity

2.5.1. DPPH assay

The DPPH free radical-scavenging activity of each sample was determined according to the method described by Yang et al. (2012) with slight modifications. Briefly, 50 μl of sample solution or ascorbic acid standard at different concentrations were added to 2 ml of 40 μM DPPH (Sigma–Aldrich, France) in methanol. The mixture was shaken vigorously and left to stand for 1 h at room temperature in the dark. The radical-scavenging activity was calculated as follows:

$$\text{Inhibition}(\%) = [1 - (\text{Ai} - \text{Aj})/\text{A0}] \times 100$$

where, A0 is the absorbance of the blank sample, Ai is the absorbance in the presence of the test compound at different concentrations and Aj is the absorbance of the blank reagent. The IC50 (concentration providing 50% inhibition) was calculated graphically

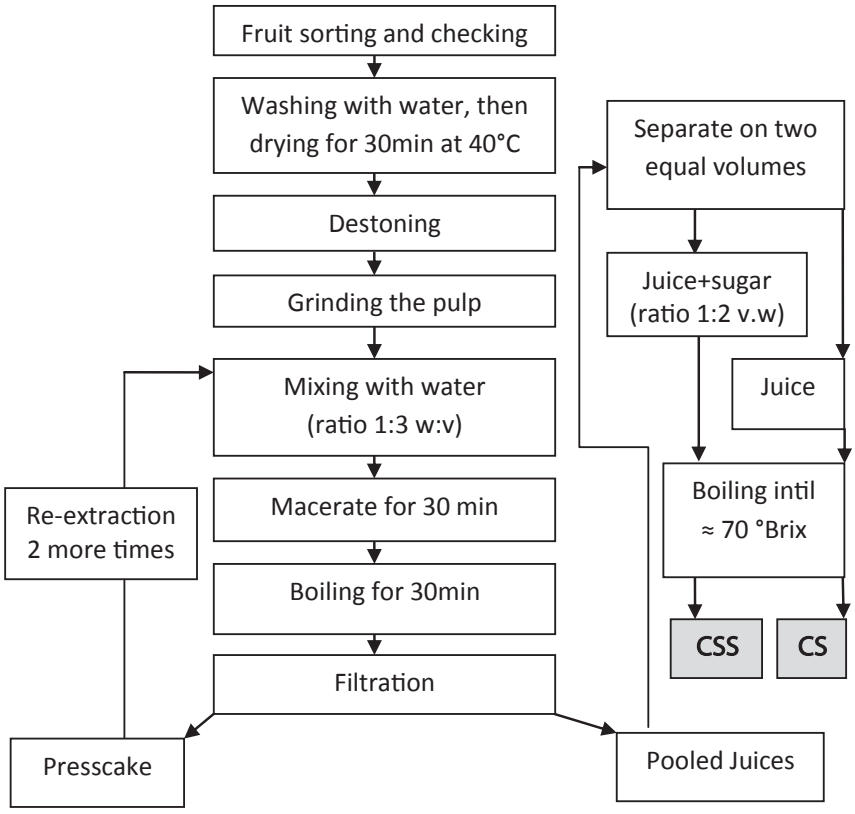

Fig. 1. Preparation process of carob syrups.

using a calibration curve in the linear range by plotting the extract concentration. Ascorbic acid was used for comparison.

2.5.2. ABTS assay

The ABTS assay was performed according to the method described by Maksimovića et al. (2013) with slight modifications. ABTS radical cation (ABTS+) was produced by mixing the 7 mM ABTS solution with 2.45 mM potassium persulfate aqueous solution, leaving them in the dark at room temperature for 12–16 h. The ABTS+ solution was diluted to get the absorbance of 0.70 ± 0.02 at 734 nm. Ten minutes after adding 100 μl of sample aqueous solution to 1.9 ml of diluted ABTS+ solution, the absorbance was measured at 734 nm. For the blank, 100 μl of water was used instead of the sample. All tests were carried out in triplicate.

2.6. Antimicrobial activity

2.6.1. Microorganisms and growth conditions

Bacteria and fungi were obtained from international culture collections (ATCC). They included Gram-positive bacteria: *Staphylococcus aureus* (ATCC 6538), *Staphylococcus epidermidis* (ATCC 12228), *Bacillus cereus* (ATCC 11778), *Bacillus subtilis* (ATCC 6633) and *Streptococcus faecalis* (ATCC 10541) and Gram-negative bacteria: *Pseudomonas aeruginosa* (ATCC 9027), *Escherichia coli* (ATCC 8739), and *Salmonella* sp. strain isolated from food. The following fungal strains were also tested, *Aspergillus brasiliensis* (ATCC 16404), *Fusarium oxysporum* (CTM10402) and *Fusarium culmorum* (ISPAVE 21w). The bacterial strains were cultivated in Muller–Hinton agar (MH) at 37 °C. Working cultures were prepared by adjusting the bacterial suspension in broth to 0.5 Mc Farland turbidity and confirmation using a serial 10-fold dilution method. The culture was spread plated on count agar Muller–Hinton (Oxoid Ltd, UK) medium in order to give a population of 10^8 colony-forming units (cfu) per plate. Fungal spore suspensions were collected from the surface of fungal colonies by gently scraping with a loop and suspended in 10 ml Potato Dextrose Broth (PDB). This suspension was mixed vigorously by vortexing for 10 min. The spore suspension stock was diluted to obtain a concentration of 10^6 spores/ml (measured by Malassez blade).

2.6.2. Agar diffusion assay

Antimicrobial activities of the carob syrup extract were evaluated by means of agar-well diffusion assay according to Fattouch et al. (2007). One hundred microliters of each bacterial culture were spread onto the surface of the agar plates of MH agar for bacteria and PDB for fungi. Once the plates had been aseptically dried, Ø6 mm wells were punched into the agar with a sterile Pasteur pipette. Following, 60 μl of each extract were placed into the wells and the plates were incubated at 37 °C for 24 h for bacterial strains and 72 h for fungi at 28 °C. Ampicillin (10 μg/wells) was used for positive control and sterile water was the negative control. Antimicrobial activity was evaluated by measuring the diameter of the inhibition zones around the well. Tests were performed in triplicate.

2.6.3. Determination of MIC

The minimum inhibitory concentration (MIC) was determined using the broth microdilution method. The test was performed in sterile 96-well microplates with a final volume in each well of 200 μl. One hundred microliters of a given dilution of the polyphenolic extract and 100 μl of the bacterial suspensions (5×10^5 cfu/ml) were added in the micro-wells. The plates were incubated aerobically at 37 °C for 24 h. Bacterial growth was revealed by the presence of turbidity and a "pellet" on the well bottom. MICs were determined as the first well in ascending order that did not produce a pellet.

2.7. Eukaryotic cytotoxicity assay

2.7.1. Cell lines and cultures

Human tumourigenic neuroblastoma SH-SY5Y and non-tumourigenic 3T3 fibroblast were obtained from the European collection cell. Neuroblastoma cells were cultivated in Dulbecco's modified Eagle's medium (DMEM): HAM (1:1 v/v) supplemented by 10% heat-inactivated fetal bovine serum (FBS, Cultex, Spain) in the presence of 1% of mixed antibiotics (streptomycin, penicillin and neomycin). Fibroblast cells were cultured in DMEM (Invitrogen, Karlsruhe, Germany), 10% and 1% of mixed antibiotics. Embryonic stem line D3 cells (American Type Culture Collection, Rockville, Maryland, USA) were grown in an undifferentiated state on 75 mm gelatin 0.1% in phosphate-buffered saline [PBS] (Sigma Chemical Co, St Louis, Missouri) treated surfaces in DMEM medium supplemented with 15% FBS, 1% non-essential amino acids, 50 U/ml penicillin, 100 mg/ml streptomycin, 0.1 mmol/l b-mercaptoethanol (all from Invitrogen), and 10^3 U/ml LIF (Chemicon, Temecula, CA, USA). Cells were grown in cell culture flasks at 37 °C in a humidified atmosphere containing 5% CO_2 in air (Lantto, Colucci, Zavadova, Hiltunen, & Raasmaja, 2009) for SH-SY5Y and 3T3 cells, the culture medium was changed every 2 days. For the stem cells, the growth was carried in a dry atmosphere to block cell proliferation and stem cell medium has been changed every day. When they had reached confluence, the cells were trypsinized with 0.1% trypsin and 0.02% EDTA in PBS.

2.7.2. Determination of cytotoxic activity

Cell viability was determined through the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide) assay. Cells were plated in 96-well plates and the cytotoxicity assay was performed when a density 80–95% confluence was reached. Mediums were removed and the cells were treated with different doses of the extracts diluted in the medium for 24 h at 37 °C. Control samples were treated only with buffer following extract removal and washing twice with PBS, 200 μl of MTT (1 mg/ml) were added to the cells and incubated at 37 °C for 3 h. Then, MTT was removed and the colored formazan was dissolved in 100 μl of DMSO (Barrajon et al., 2010). Using a Bio-Rad 3350 microplate reader, the absorbance (Abs) was determined at 540 nm and at 690 nm as a background. The optical density obtained was directly correlated with cell quantity. Cell viability was expressed as percentage (%) of the untreated control as follows:

$$\%\ \text{cell viability} = (\text{Abs of treated cells}/\text{Abs of untreated cells}) \times 100$$

2.8. Statistical analysis

All tests and analyses were run in triplicate and averaged. Quantitative presented data are means ± standard deviations. One-way analysis of variance with Dunnett's post-test was performed using GraphPad Prism version 5.04 for Windows (GraphPad Software, San Diego, CA, USA). Differences of $p < 0.05$ were considered significant.

3. Results and discussion

3.1. Polyphenolic extraction and estimation

As the first step in food analysis, the extraction method is determinant in order to obtain high yields of the analytes starting from complex matrices. The choice of the different parameters, especially polarity of the solvent, temperature, and time, largely determines the extraction selectivity and efficiency of the different compounds present in the sample. Due to their stable antioxidant properties and reactivity of phenolic compounds, the extraction procedure has to be as rapid and as careful as possible. In the work of Najjaa, Zerria, Fattouch, Ammar, and Neffati (2011), it is reported that the choice of solvent was a key factor for extraction efficiency. Previous studies on carob pods mainly used water as extraction solvent. Menelaos, Hans, Annett, Bernd, and Rudolf (2004) have worked on carob using different solvents: water, methanol, acetone and mixtures of solvent methanol/water and acetone/water, and found that acetone–water mixtures showed the highest efficiencies, whereas methanol/water mixtures had lower extraction yields. These authors found that the impact of the acetone content in the extraction solvent depends on the polarity of the compounds, and they reported that acetone 70% was the best extraction solvent to obtain highest yields of phenolics. In addition, aqueous-acetone (3/7, v/v) solvent had been previously shown to provide a good extraction of the main polyphenols from quince peel and pulp material (Fattouch et al., 2007). This simple procedure permitted to remove interfering compounds, especially precipitates proteins and peptides by cold acetone, which could absorb at 280 nm in HPLC analysis. The use of other extraction solvents (methanol, ethanol, water) is usually followed by preparative chromatographic SPE or SPME steps. The reduced number of the cold aqueous-acetone extraction steps might preserve the bioactive phenolics in their native forms. For this reason, in the present study, we preferentially used water–acetone at 3/7 (v/v) to prepare carob syrup polyphenols.

Phenolic compounds have been generally associated to various biological activities, including antioxidant, antimicrobial and cytotoxic potentials. *C. siliqua* syrups were investigated for their TPC by the Folin–Ciocalteu assay, and the values were expressed as mg gallic acid equivalents/g of syrup. Aqueous-acetone extracts from carob syrups supplemented with sugar (CSS) showed significantly ($p < 0.05$) lower polyphenolic content than those of carob syrups without sugar (CS). The obtained values for each syrup ranged between 4.37 ± 0.85 and 6.99 ± 0.52 mg GAE/g of CSS and CS, respectively. The results are in agreement with those reported by Sharma, Kumar, and Rao (2008) who investigated the influence of milk and sugar on antioxidant potential of black tea. These authors found that sugar addition to black tea brew powders decreased the phenolic content from 24.1 ± 1.57 to 19.1 ± 1.08 mg/g gallic acid equivalent for black tea and black tea with sugar, respectively.

3.2. Chromatographic identification and quantification

In order to identify their major compounds, the acetone extracts from CSS and CS were analyzed by using HPLC-DAD–ESI-MS technique. Typical chromatograms were obtained at 280 nm for CS (Fig. 2A) and CSS (Fig. 2B). Several peaks were identified (Table 1) using a local library of phenolic compounds and comparing their retention time, UV spectra and MS data with those of authentic commercial standards and/or those reported in literature. Twelve observed peaks, matching with the standard compounds used in this work, were detected in both carob syrups. By comparing mass spectra with those of standards: peak 1 (tR = 2.1 min) was identified as *p*-coumaric acid with a [M–H]– at *m/z* 163. The second peak (tR = 3.49 min and [M–H]– of 457), was identified as epigallocatechin gallate. Peak 3 (tR = 3.85 min) showed [M–H]– of 305 and was identified as epigallocatechin. The fourth peak (tR = 4.96 min, [M–H]– = 286) was identified as kaempferol. The fifth peak (tR = 5.87 min, [M–H]– of 289) was identified as catechin. Peak 6 (tR = 6.46 min) was identified as syringic acid and the [M–H]– peak was at *m/z* 197. Peak 7 (tR = 7.07 min and [M–H]– of 465), was identified as quercetin glycoside. Peak 8 (tR = 7.91 min) showed [M–H]– of 179 and was identified as caffeic acid. Peak 9

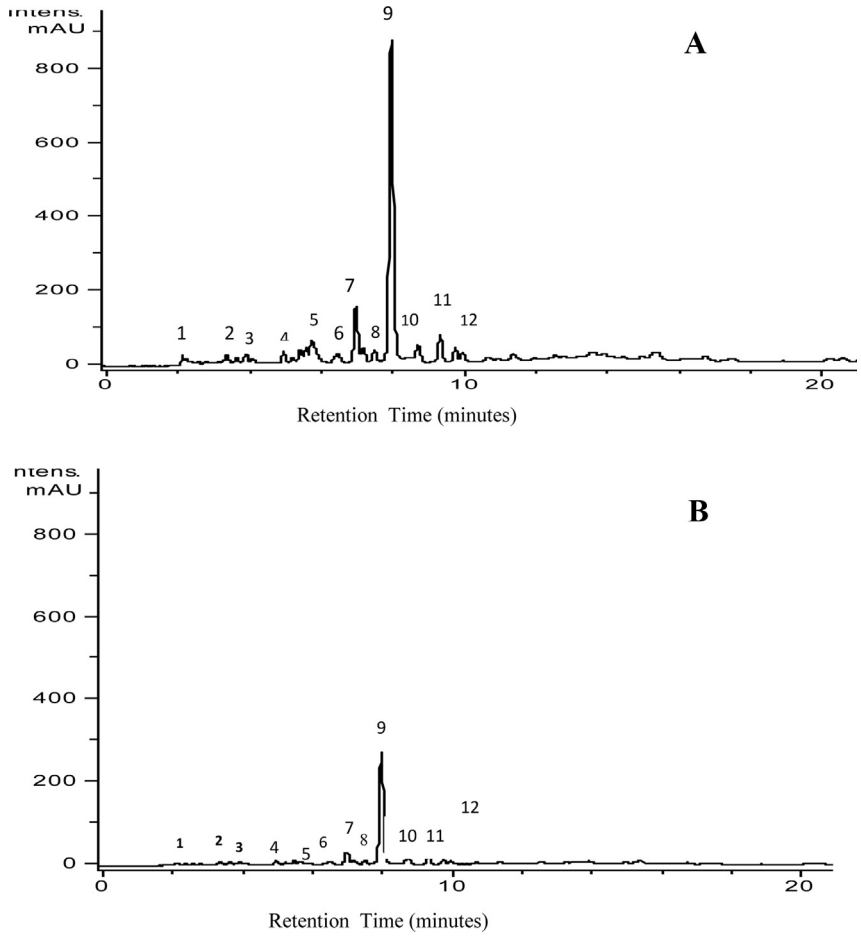

Fig. 2. HPLC profiles ($\lambda = 280$ nm) of polyphenolic extracts of carob syrups prepared without (A) or with (B) sucrose. Identified peaks as compared to standard compounds: 1, *p*-coumaric acid; 2, epigallocatechin gallate; 3, epigallocatechin; 4, kaempferol; 5, catechin; 6, syringic acid; 7, quercetin glycoside; 8, caffeic acid; 9, gallic acid; 10, catechin gallate; 11, myricetin 3-glycoside; 12, cinnamic acid.

(tR = 8.05 min) was identified as gallic acid and the [M−H]− peak was at *m*/*z* 169 which was the main polyphenol. Peak 10 (tR = 8.82 min) was identified as catechin gallate and the [M−H]− peak was at *m*/*z* 441. Peak 11 (tR = 9.17 min) was identified as myricetin 3-glycoside and the [M−H]− peak was at *m*/*z* 479. The last peak (tR = 9.84 min, [M−H]− of 148) was identified as the cinnamic acid. All these results are in agreement with those reported in the literature by Menelaos et al. (2004) who worked on carob fruit phenolics. The total amounts of the CSS and CS polyphenols were respectively 1.86 ± 0.28 and 4.53 ± 0.32 mg/g. Gallic acid was the major component (60.98 ± 2.04% and 69.26 ± 0.16% of total phenolics for CS and CSS, respectively), followed by quercetin glycoside (9.14 ± 0.01% and 9.93 ± 0.83% of total phenolics for CSS and CS, respectively) and syringic acid with 5.14 ± 0.02% for CSS and 8.94 ± 0.27% of total phenolics for CS. These data were in agreement with the results obtained by Fadel et al. (2012) in carob pulps and those reported by Corsi et al. (2002) who investigated polyphenols in carob pods and leaves infusions. These previous works also found that gallic acid was the major compound in carob fruit, leaves and seeds.

Table 1
LC−ESI-MS characteristics of the identified polyphenols in carob syrup CS and CSS aqueous-acetone extracts. Data presented are means ± SD ($n = 3$).

Peak *N*	RT	λ (nm)	[M−H]	Phenolic compounds	Carob syrup without sugar			Carob syrup with sugar		
					Content (μg/g)	SD	%	Content (μg/g)	SD	%
1.00	2.10	310.00	163.00	*p*-Coumaric acid	13.88	1.61	0.31	10.19	0.99	0.55
2.00	3.49	274.00	457.00	Epigallocatechin gallate	106.37	1.51	2.36	43.65	2.42	2.34
3.00	3.85	270.00	305.00	Epigallocatechin	104.67	0.78	2.32	27.08	2.35	1.45
4.00	4.93	246.00	286.00	Kaempferol	40.16	3.03	0.89	8.47	1.18	0.45
5.00	5.87	278.00	289.00	Catechin	263.34	9.08	5.84	39.76	1.39	2.13
6.00	6.46	278.00	197.00	Syringic acid	403.10	9.05	8.94	96.03	5.62	5.15
7.00	7.07	278.00	465.00	Quercetin glycoside	447.64	27.92	9.93	170.69	3.58	9.15
8.00	7.91	242.00	179.00	Caffeic acid	39.77	4.14	0.88	12.33	0.81	0.66
9.00	8.05	274.00	169.00	Gallic acid	2746.83	68.12	60.94	1292.72	37.96	69.26
10.00	8.82	278.00	441.00	Catechin gallate	184.52	14.53	4.09	88.36	8.67	4.73
11.00	9.17	258.00	479.00	Myricetin 3-glycoside	71.27	6.17	1.58	29.48	2.08	1.58
12.00	9.84	274.00	148.00	Cinnamic acid	109.11	2.56	2.42	47.65	3.19	2.55
Total					4530.67	322.40	100.00	1866.42	280.12	100.00

3.3. Antiradicals potentials

In this study, the antioxidant properties of both CSS and CS polyphenolic extracts of carob syrups were tested, and then compared with the activity of the well-known antioxidant, ascorbic acid. Moreover, the IC50 values (Fig. 3), which refer to the lowest concentration of the antioxidants necessary for 50% of radical scavenging, were also calculated. Using anionic DPPH• radical, the IC50 for the different carob syrup extracts were 47.25 ± 2.32 and 184.14 ± 3.83 μg/ml for CS and CSS, respectively; whereas, for Vitamin C, the IC50 was 10.29 ± 1.7 μg/ml. The free radical anionic DPPH• is known to accept an electron or hydrogen radical and become a stable diamagnetic molecule. It has been shown that many antioxidants, such as ascorbic acid (Vitamin C), tocopherol (Vitamin E), and flavonoids, reduced DPPH• due to their hydrogen donating ability. Our results are comparable to those determined by Ben Hsouna et al. (2011) in carob leaves extracts. Moreover, the cationic ABTS•+ radical-scavenging effects showed that the CS aqueous-acetone exhibited higher (IC50 = 168.71 ± 4.46 μg/ml) antiradical potential than CSS (IC50 = 236.4 ± 4.25 μg/ml). Ascorbic acid presented the lowest IC50 (20.3 ± 1.2 μg/ml), a finding similar to what reported by Yang et al. (2012) for this Vitamin C antioxidant compound. For all the antioxidant evaluation tests, the high repeatability of the experience was verified and no significant differences ($p > 0.05$) have been found between the repetitions.

3.4. Antimicrobial activities

The *in vitro* antibacterial activity was measured using eight different bacterial strains which are widely used as models for Gram-positive and Gram-negative bacteria. Most of the herein tested microorganisms are known as opportunistic human and animal pathogens and/or cause food contamination and quality deterioration. As shown in Table 2, carob syrups exhibited varying degrees of antibacterial activity against all tested strains. In general, for both CS and CSS polyphenolic extracts, the inhibition zones, as determined using agar diffusion assay, were found in the range of 9 and 26 mm; whereas, the microplate-determined MICs ranged between 500.6 ± 10.8 and 1502 ± 10.8 μg/ml. The Gram-positive bacteria were more susceptible to the carob syrup extracts than Gram-negative ones. Carob syrups prepared without sugar (CS) exhibited higher antimicrobial activities than the ones prepared with sugar (CSS). Bagamboula, Uyttendaele, and Debevere (2004) attributed the low sensibility of Gram-negative bacteria to the complexity of their double membrane-containing cell envelope compared to the single membrane structure of Gram-positive ones. The ability of plant extracts to disrupt the permeability barrier of cell membrane structures and the accompanying loss of chemiosmotic control are the most likely reasons for its bacteriostatic and/or bactericide action. CS was highly active against *B. cereus* showing an important growth inhibition at lower concentrations (500 ± 10.8 μg/ml). *B. subtilis* and *S. epidermidis* have a higher sensibility to CS phenolics than *S. aureus* and *S. faecalis* (Table 2). CSS inhibited only the growth of *B. cereus* (ATCC 11778) with MICs of about 622 ± 7 μg/ml. The obtained results are of great importance, particularly in the case of *B. cereus* and *S. aureus* which are known for the production of several types of enterotoxins that cause gastroenteritis (Halpin & Marth, 1989). The strong antimicrobial activity of the CS against the tested microorganisms was significantly ($p < 0.05$) attributed to the presence of high content of polyphenols in carob syrup extract. With regard to this, gallic acid and epigallocatechin gallate, which are the main compounds of the extract, may contribute significantly to this antibacterial capacity as reported by Kang, Oh, Kang, Hong, and Choi (2008). The low gallic acid and epigallocatechin gallate content of CSS could explain its lower antibacterial potentials.

In addition, CS has an important antifungal potential. *F. oxysporum* is considered as a second *Fusarium* species responsible for infections in humans (Anaissie et al., 2001). It is the most sensitive fungus (26 ± 0.7 mm) in the carob syrup extract prepared without sugar. CSS has no antifungal potential. The synergistic effects and the diversity of major and minor constituents present in the carob syrup should be taken into account for their biological activity, a statement worthy of deep investigation.

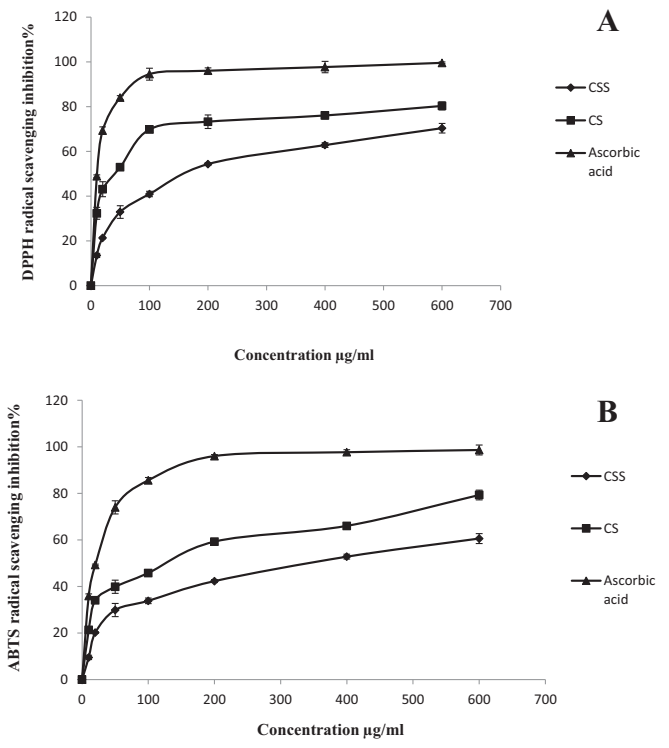

Fig. 3. DPPH•– (A) and ABTS•+ (B) free radical-scavenging properties of the CS and CSS polyphenolic extracts. Ascorbic acid was used as a reference. Data were presented as means ± SD ($n = 3$).

3.5. In vitro cytotoxicity

In order to evaluate the cytotoxic effects of carob syrup extracts, cell lines (SH-5YSY, 3T3 and D3) were treated with various concentrations of carob syrup for 24 h and then subjected to the MTT test. Fig. 4 shows the dose–response survival curves for the treatments of the different cell lines in the presence of CS or CSS aqueous-acetone extracts. The result shows that carob syrup extracts reduce tumoral human cell viability in a dose dependent manner. Moreover, the 50% inhibit concentration (IC50) values deduced from the plots shown in Fig. 4 were determined. Our data are comparable to those obtained in a recent study on the anticancer activity of several tea extracts of high polyphenolic contents against several cancer cell lines, where IC50 values ranged from 100 to 500 μg/ml (Friedman et al., 2007). The cytotoxic effect was significantly stronger ($p < 0.05$) in tumorigenic SH-SY5Y cells treated with CS (IC50 of 311.7 ± 23.65 μg/ml) than treated with CSS (390.6 ± 34.97 μg/ml). Same trends were observed for non-tumorigenic 3T3 cell line where CS (IC50 = 586.28 ± 20.3 μg/ml) is more effective to reduce cell viability than CSS (IC50 = 628.9 ± 42.6 μg/ml). In addition, carob syrup extracts

Table 2
Antibacterial and antifungal activities of the CS and CSS polyphenolic extracts and minimum inhibitory concentration (MIC) values expressed in μg/ml.

		CS[a] extract		CSS[b]extract		Ampicillin[c]
		Inhibition zone[d]	CMI	Inhibition zone[d]	CMI	Inhibition zone[d]
Bacterial strains						
Gram-positive	*S. aureus* (ATCC 6538)	22.5 ± 0.7	*	14.5 ± 0.7	*	11.2 ± 0.5
	S. epidermidis (ATCC 12228)	24.5 ± 0.7	1502 ± 10.8	NA	*	13.5 ± 0.3
	B. cereus (ATCC 11778)	26.5 ± 0.7	500 ± 10.8	15.5 ± 0.7	622 ± 7	19.2 ± 0.7
	B. subtilis (ATCC 6633)	23.3 ± 1.4	1502 ± 10.8	10.5 ± 0.7	*	16.3 ± 0.5
	S. faecalis (ATCC 10541)	14.5 ± 1.4	*	NA	*	15.6 ± 0.3
Gram-negative	*P. aeruginosa* (ATCC 9027)	9.5 ± 0.7	1502 ± 10.8	NA	*	17.5 ± 0.5
	E. coli (ATCC 8739)	13.3 ± 0.56	*	NA	*	12.7 ± 1.5
	Salmonella sp.	15.5 ± 0.7	1502 ± 10.8	NA	*	15.7 ± 0.3
Fungal strains						
A. brasiliensis (ATCC 16404)		15 ± 0.7	–	NA	–	–
Fusarium oxysporum (CTM10402)		26 ± 0.7	–	NA	–	–
Fusarium graminearum (ISPAVE 21w)		16 ± 0.7	–	NA	–	–

Values are given as means ± SD of triplicate experiment. (NA): Not active, (−): not tested, (*): No inhibition at the highest reached concentration.
[a] CS carob syrup prepared without sucrose.
[b] CSS carob syrup prepared with sucrose.
[c] Ampicillin concentration = 10 μg/well.
[d] Diameter of inhibition zones including the diameter of Ø6 mm well.

inhibited the growth of non-tumorigenic embryonic mouse stem cells (D3). In recent years, this latter cellular model was used in numerous areas of science because of its special properties such as unlimited cell proliferation capability and plasticity to potentially generate all cell lineages, thus, providing important endpoints for studying the embryological processes, the toxicology of development and to evaluate the potential embryotoxic effects of many chemicals (Pamies, Nestor, Sogorb, Roche1, & Reig, 2010). Carob syrup not supplemented with sucrose (CS) presented slightly higher effects on mice D3 cells (IC50 = 476.81 ± 21.77 μg/ml) than CSS (503.11 ± 29.49 μg/ml) which remain statistically not significant ($p > 0.1$). While all the tested concentrations of the CSS and CS were found cytotoxic, it was clear that carob syrup exhibits low toxicity to non-cancerous cell in comparison to tumorigenic cell lines and seems to have potential selectivity to cancer cell lines. In a previous work (Dhaouadi et al., 2013) we reported a differential effect of *Opuntia ficus-indica* syrup extract on *in vitro* cell viability, which was found dependent on the cell incubation time in the presence of the *O. ficus-indica* syrup extract. In that work, using the same concentration of the extract (149 μg/ml), after 1, 3 or 6 h of incubation, non-tumorigenic fibroblast cells maintained viability more than 60%; in contrast, the viability of tumorigenic SH-SY5Y neuroblastoma cells reached 15% after 6 h of incubation and practically no viability was detected after 24 h. In the present work, we investigated cell viability following incubation with CS or CSS extracts only for one time, 24 h. In future work, we might analyze the effect of incubation time in presence or not of CS or CSS extract on *in vitro* cell viability. We have to determine the minimal incubation time that leads to tumorigenic cells death but let non-tumorigenic cells alive, thus supporting potential applications of the extracts. Ben Hsouna et al. (2011) reported effects of carob essential oils on the *in vitro* viability of eukaryotic cells, suggesting that carob plant could be a natural source of cytotoxic compounds useful to control tumorigenic cells. It was previously reported by Yang, Yue, Runwei, and Guolin (2010) that the general cytotoxicity mechanism of plant extracts against many cancer cells was mediated through induction of apoptosis. In addition, the cytotoxicity of CS and CSS polyphenols might be attributed to the high hydrophobic properties of some compounds present in carob syrup, particularly those of low molecular weight (Yang et al., 2010), including gallic acid ([M−H]− = 169), the major compound in both CS and CSS extracts, bearing in mind that the observed cytotoxicity potentials may also involve synergic mechanisms and different bioactive substances. Indeed, hydrophobic and low molecular weight compounds could easily cross and/or interact with the membrane to cause a loss of structural integrity (Yang et al., 2010). In addition, it is accepted that the chemopreventive and tumor-inhibitory effect associated to some dietary polyphenols could be due to their antioxidant capacity (Menendez et al., 2007). A large number of studies evidenced the ability of the dietary polyphenols to modulate uncontrolled

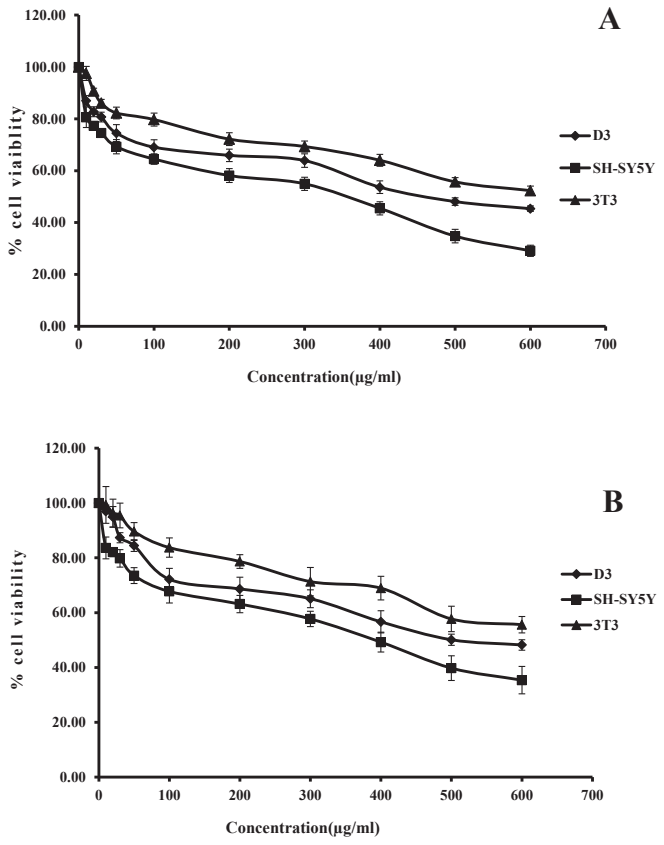

Fig. 4. Effects of the CS (A) and CSS (B) polyphenolic extracts on human cells: non-tumorigenic 3T3 fibroblast and tumorigenic SH-SY5Y neuroblastoma and mouse non-tumorigenic D3 stem cells, as determined by MTT assay. The data are presented as means of three independent experiments and are different from the untreated control cells at a level of $p < 0.05$.

proliferation pathways or protooncogenes expression (Menendez et al., 2007). In previous works on date and barbary-fig, polyphenolic extracts prepared from traditionally made syrups exhibited differential anti-carcinogenic activities (Dhaouadi et al., 2013), thus illustrating the usefulness of these fruit byproducts as source of bioactive compounds.

4. Conclusion

Our study is the first detailed document about the antioxidant, antibacterial, antifungal activities and cytotoxicity of *C. siliqua* traditionally-processed syrups supplemented or not with sucrose. This local (Tunisian) and regional (North African and Arabian) food could potentially be a good source of natural phenolics with health promoting properties that could be worldwide produced, since its plant source is already largely cultivated. The data presented in this paper suggested that carob syrups exhibit bioactive properties which are affected by sugar supplementation during syrup processing. Avoiding sugar addition during syrup preparation not only preserves carob syrup bioactivities, but also prevents nutritional negative attributes, knowing that sucrose is always associated with physiological disorders (mainly, diabetes and obesity). Thus, our results could have practical implications in biotechnological fields, including food processing and nutritional properties of natural fruit derived products.

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