Supporting Information

Sapwood of Carob Tree (*Ceratonia siliqua* L.) as a Potential Source of Bioactive Compounds

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Luísa Custódio[1*], Ana Luísa Escapa[1], João Patarra[1], Rosa Aligué[2], Fernando Alberício[3,4,5,6], Nuno Rosa Neng[7], José Manuel Florêncio Nogueira[7] and Anabela Romano[1]

[1]Institute for Biotechnology and Bioengineering, Centre of Genomics and Biotechnology (IBB/CGB), Faculty of Sciences and Technology, Ed. 8, Campus of Gambelas, 8005 139, Faro, Portugal
[2]University of Barcelona, School of Medicine, Department of Cell Biology, Casanova 143, E-08036, Barcelona, Spain
[3]Institute for Research in Biomedicine, Barcelona Science Park, Baldiri Reixac 10, 08028, Barcelona, Spain.
[4]CIBER-BBN, Networking Centre on Bioengineering, Biomaterials and Nanomedicine, Barcelona Science Park, Baldiri Reixac 10, 08028 Barcelona, Spain.
[5]University of Barcelona, Department of Organic Chemistry, Martí i Franqués 1-11, 08028 Barcelona, Spain.
[6]School of Chemistry, University of KwaZulu-Natal, 4001-Durban, South Africa.
[7]University of Lisbon, Faculty of Sciences, Chemistry and Biochemistry Department and Centre of Chemistry and Biochemistry Campo Grande Ed. C8, 1749-016 Lisbon, Portugal

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1. Spectrophotometry studies

The total content of phenolic compounds (TPC) was determined by the Folin-Ciocalteu (F-C) assay. Aliquots of the extracts (0.1 mL, 10 mg/mL) were added to 5 mL of distilled water (dH_2O) and 0.5 mL of F-C reagent, vigorously shaken and incubated for 3 min at room temperature (RT). Then, 1 mL of a saturated solution of sodium carbonate (Na_2CO_3) was added and the volume made up to 10 mL with dH_2O. The mixtures were allowed to stand for 60 min at RT, and the total phenols were determined by colorimetry at 720 nm. TPC was calculated as a gallic acid equivalent (GAE) from the calibration curve of gallic acid standard solutions, and expressed as gallic acid equivalents (GAE) in milligrams per gram of initial dry plant material.

Total condensed tannins content (TTC) was determined by the p-dimethylaminocinnamaldehyde (DMACA). Samples (0.4 mL, 10 mg/mL) were mixed with 2 mL of DMACA solution (0.1% in 1 N HCl in methanol), vortexed and incubated at RT for 10 min. Absorbances were read at 640 nm on a Shimadzu UV-160A spectophotometer. TTC was estimated from a calibration curve, constructed by plotting known solutions of (+)-catechin and results were expressed as catechin equivalents (CE) in milligrams per gram of initial dry plant material.

Total flavonoid content (TFC) was estimated by the aluminium chloride ($AlCl_3$) method. Briefly, 1 mL of the samples (10 mg/mL) was mixed with 1 mL of 2% methanol $AlCl_3$. $6H_2O$ and the absorbance was measured 10 min later at 430 nm (Shimadzu UV-160A). TFC was expressed as rutin equivalents (RE) in milligrams per gram of initial dry plant material by comparison with the values obtained from standard rutin treated in the same conditions.

2. HPLC analysis and identification of the main phenolic compounds

Samples were analysed in a liquid chromatograph (Agilent 1100 Series LC system, Germany) equipped with a diode array detection and a Tracer excel 120 ODS-A column (150 mm × 4.0 mm, 5 µm particle size, Teknokroma, Spain). A volume of 40 µL of the filtered extracts (0.45-µm nylon filters, Corning Inc., U.S.A.) at the concentration of 10 mg/mL were injected and analysed using a mobile phase consisting of solution A (mixture of 2.5% acetic acid in water) and B (methanol) (B), and the following gradient: t = 0-50 min: 30-80% B; t =50-55 min: 80-30% B. The flow rate was 1.0 mL/min and the analyses were performed at 25ºC. For identification, the retention parameters of each assay were compared with the standard controls and the peak purity with the UV-visible spectral reference data. Identification of the phenolic compounds was achieved by comparison of retention times with those of commercial pure compounds.

2. Antioxidant activity assays

2.1. Radical-scavenging activity (RSA) on the DPPH radical: Samples (22 µL) at concentrations ranging from 125 to 1000 µg/mL, methanol or dH_2O (blanks) were mixed with 200 µL of a methanol DPPH solution (120 µM) in 96-well flat bottom microtitration plates, and incubated in darkness at room temperature (RT) for 30 min. The absorbance was measured at 515 nm and RSA (%) calculated according to the following equation:

$$RSA\ \% = (A_{blank}-A_{sample})/A_{blank} \times 100$$

where A_{blank}, and A_{sample} are the absorbance of the control reaction (a reaction with all the reagents except the test extract) and the absorbance of the test extract, respectively. Results were expressed as half maximal inhibitory concentration (IC_{50}, µg/mL). Butylated hydroxytoluene (BHT, E321) was used as a positive control.

2.2. RSA on the ABTS radical: The ABTS radical was produced by reacting 7.4 mM ABTS in dH_2O and 2.6 mM potassium persulfate in the dark at RT for 16 h. Before use, the $ABTS^+$ solution was diluted with ethanol to get an absorbance of 1.4 units at 414 nm. The reaction mixture contained 200 µL of $ABTS^+$ solution and 50 µL of the sample solution, methanol or dH_2O (blanks). The absorbance of the resulting solution was measured after 6 min at 420 nm on a 96-well plate reader (Infinite M200 spectrophotometer, Tecan). RSA was calculated using the same formula presented for DPPH, and the results were expressed as IC_{50} values (µg/mL). BHT was used as a positive control.

2.3. Reductive activity: Samples (250 µL), sodium phosphate buffer (250 µL, pH 6.6) and 1% potassium ferricyanide (250 µL) were mixed and incubated in a water bath at 50ºC for 20 min. Then, 250 µL of 10% trichloroacetic acid (w/v in dH_2O) were added to the mixture and centrifuged (1000 rpm, 10 min.). The supernatant (500 µL) was then mixed with equal volume of dH_2O and ferric chloride solution (0.1%, w/v). Absorbances were measured at 700 nm (Infinite M200 spectrophotometer, Tecan). Increased absorbance of the reaction mixture indicates increased reducing power. BHT was used as a positive control.

3. In vitro antitumoral activity

3.1. Cell lines and culture conditions: HeLa cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) with 1000 mg/mL of glucose, supplemented with 10% (v/v) Fetal Bovine Serum (FBS). DU-145 prostate cancer cells were maintained in Ham's F10 nutrient mixture supplemented with 5% (v/v) FBS; MDA-MB-231 breast carcinoma cells were grown in DMEM supplemented with 10% (v/v) FBS and HCT-116 colon cancer cells were maintained in Roswell Park Memorial Institute 1640 medium (RPMI) supplemented with 10% FBS. All the cell lines were maintained in culture medium supplemented with L-glutamine (2 mM), sodium pyruvate (111 mg/L), penicillin (50 U/mL) and streptomicin (50 µg/mL), and were grown in an incubator at 37ºC, 5.1% CO_2 in humidified atmosphere.

3.2. Effect of the methanol extract on cell viability: Exponentially growing cells were seeded on 96-well plates (10×10^3 cells/well), incubated (24h, 37ºC, 5.1% CO_2 in humidified atmosphere) and treated with the extract (25-400 µg/mL dissolved in culture medium) during 72h. Cell viability was determined by the WST-1 colorimetric assay while the MTS method was used in the other cell lines. Results were expressed as cell viability (%) and IC_{50} (µg/mL), which is the amount of extract able to inhibit cell viability by 50%.

3.3. Effect of the main phenolic compounds on cell viability: Hela cells (7×10^3/well) were seeded in 96-well plates and incubated for 24h. Then, culture medium was replaced by 100 µL of fresh medium containing phenolic compounds in concentrations corresponding to the amounts quantified in complete extract. The phenolic compounds and concentrations used were gentisic acid: 652.8 µg/mL, (-)-epicatechin: 450.9 µg/mL, (+)-catechin: 102.6 µg/mL, chlorogenic acid: 62.4 µg/mL and GA: 30.1 µg/mL. After 72 h of incubation cell viability was determined by the MTT assay.

4. Measurement of ROS

The capacity of the extract to scavenge intracellular ROS generation as a result of a oxidative stress induction was evaluated by the 2',7'-dichlorodihydrofluorescin diacetate (DCFH-DA) method. Cells were exposed to the extract at the concentrations of 200 and 400 µg/mL, trypsinized and washed with PBS. Treated and control cells were resuspended in PBS containing 10 µM DCFH-DA at 37ºC for 30 min and then incubated with 4 mM H_2O_2 as ROS inducer for 30 min at 37°C. Relative amount of intracellular ROS (%) was subjected to evaluation by Fluorescence Activated Cell Sorting (FACS) in a flow cytometer (Coulter XL) and calculated according to the following equation:

$$\text{ROS (\%)} = (FI_1 / FI_0) \times 100$$

where FI_0 was the fluorescence intensity of the negative control and FI_1 the fluorescence intensity in the presence of the extract at an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

5. Statistical analysis

Data were reported as the mean ± standard error of mean (SEM) for at least three replicates and were subjected to analysis of variance (ANOVA) to assess treatment differences using the SPSS statistical package for Windows v.16.0. Significance between means was tested by Duncan's New Multiple Range Test ($P = 0.05$). The IC_{50} values were calculated with GraphPad Prism v.4.

Table 1. HPLC analysis of the phenolic compounds contents (mg/g DW) of carob tree sapwood extractives.

RT[a] (min)	**Compound[b]**	**Amount[c]**	
		Methanol extract	**Hot water extract**
2.9	gallic acid	3.0	n.d.
4.2	(+)-catechin	10.2	0.3
5.3	chlorogenic acid	6.2	0.3
6.8	(-)-epicatechin	45.0	n.d.
7.3	gentisic acid	65.2	1.9
7.8	vanillin	0.2	n.d.
8.1	syringic acid	0.8	n.d.
17.7	rutin	0.2	n.d.
21.9	kaempherol	0.2	n.d.
	Total	*131.0*	*2.5*

[a]Retention time; [b]Identified by comparison of the retention parameters with the standard controls and peak purity with the UV-vis spectral reference data; [c]Determined with calibration plots; n.d.: not detected.

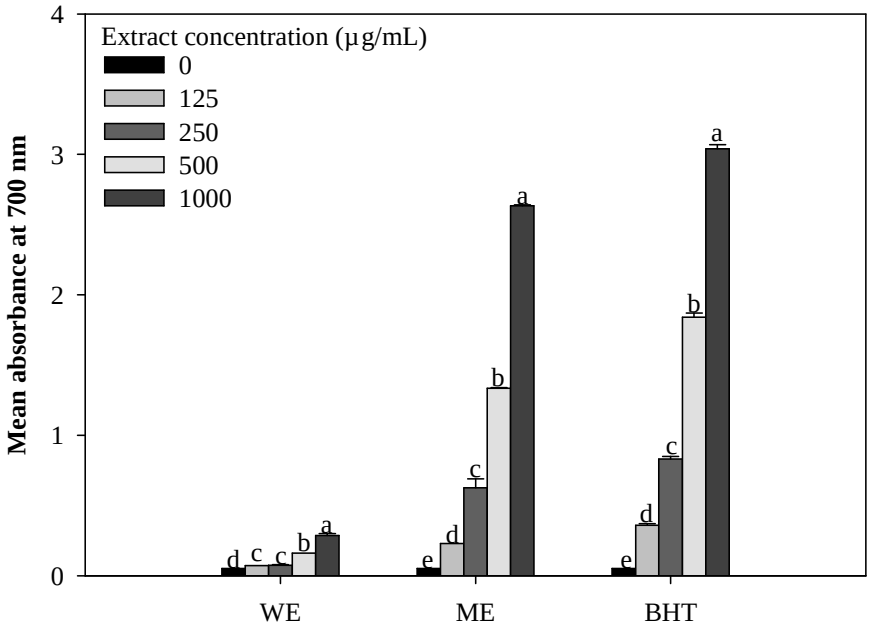

Figure 1. Reducing activity of methanol extract (ME) and hot water extract (WE) of sapwood from carob tree. Different letters in the same sample indicates significant differences between concentrations by Duncan's New Multiple Range Test.

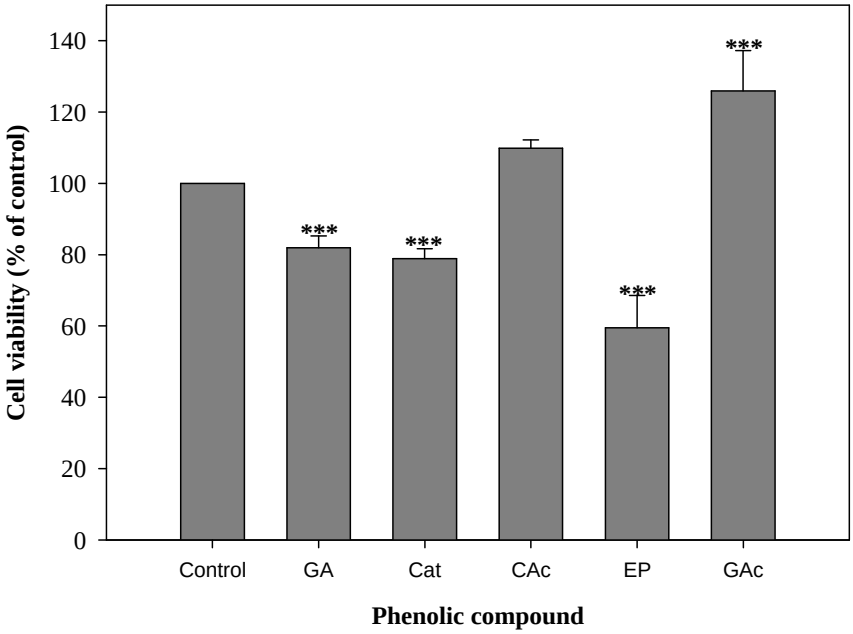

Figure 2. Effect of the application of gentisic acid (GAc), (-)-epicatechin (EP) (+)-catechin (C), chlorogenic acid (ChAc) and gallic acid (GA) on HeLa cells viability after 72 h of incubation. The phenolic compounds and concentrations used were gentisic acid: 652.8 μg/mL, (-)-epicatechin: 450.9 μg/mL, (+)-catechin: 102.6 μg/mL, chlorogenic acid: 62.4 μg/mL and GA: 30.1 μg/mL. Significant differences with untreated cells: *$p < 0.05$, **$p < 0.01$, *** $p < 0.001$.

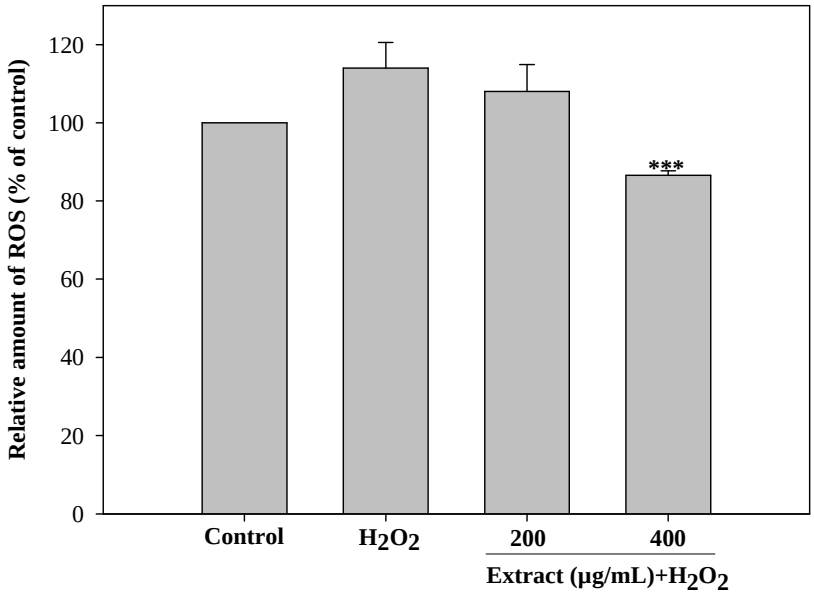

Figure 3. Effect of the application of the methanol extract of sapwood on intracellular ROS production in HeLa cells. Control cells were cultured without extract and H_2O_2 exposure, whereas treated cell were grown in the presence of H_2O_2 without extract, or in the presence of extract and H_2O_2. Significant differences with untreated cells: *$p < 0.05$, **$p < 0.01$, *** $p < 0.001$.