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Antioxidant and Cytotoxic Activities of Carob Tree Fruit Pulps Are Strongly Influenced by Gender and Cultivar

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Supporting Information

ABSTRACT: Extracts from fruit pulps of six female cultivars and two hermaphrodite Portuguese carob trees [(*Ceratonia siliqua* L., Fabaceae)] exhibited strong antioxidant activity and were rich in phenolic compounds. The extracts decreased the viability of different human cancer cell lines on a dose- and time-dependent manner. Gender and cultivar significantly influenced the chemical content and the biological activities of the extracts. Extracts from hermaphrodite trees had a higher content of phenolic compounds, and exhibited higher antioxidant and cytotoxic activities. Among females, cv. Aida had the highest radical scavenging activity and total content of phenolics, Mulata the highest capacity to inhibit lipid oxidation and Gasparinha the strongest cytotoxic activity on HeLa cells. The decrease in cell viability was associated with apoptosis on HeLa and MDA-MB-231 lines. (+)-Catechin and gallic acid (GA) were the main compounds identified in the extracts, and GA contributed to the antioxidant activity. Our results show that the antioxidant and cytotoxic activities of carob tree fruit pulps are strongly influenced by gender and cultivar, and provide new knowledge about the advantages of hermaphrodite trees over female cultivars, namely, as a source of compounds with biological interest, which may represent an increase of their agronomic interest.

KEYWORDS: apoptosis, cell cycle, cytotoxicity, HeLa cells, polyphenol, ROS

INTRODUCTION

Oxidative stress results from the imbalance between the generation of free radicals, namely, reactive nitrogen species (RNS) and reactive oxygen species (ROS), and the antioxidant defenses of the organism. It is considered an underlying basic etiology associated with many diseases such as cancer, diabetes, inflammation, and neurological disorders such as Alzheimer's.[1,2] Antioxidant compounds have a crucial role in protecting DNA, lipids, and proteins from the damage caused by oxidative stress and in the regulation of gene expression.[3] However, there is evidence that some synthetic antioxidants such as butylated hydroxytoluene (BHT, E320) and butylated hydroxyanisole (BHA, E321) have severe side effects, namely, carcinogenicity.[4] Therefore, the search for natural antioxidants as alternatives to synthetic compounds is of great interest among researchers.[3,4] Moreover, dietary antioxidants from plants, such as phenolic compounds, have a crucial role in protecting the organism against oxidative stress, and there is evidence that their intake can prevent aging and reduce the risk of many degenerative diseases due to their radical scavenging activity.[5−7]

Carob bean or St. John's bread, the brown pod of the carob tree [(*Ceratonia siliqua* L., Fabaceae)], is a fruit rich in carbohydrates which has been used for centuries by humans as a source of nutrition. The carob tree is mainly found in the Mediterranean area but also grows in certain regions of the USA, South and Central America, and Australia. The main producers of carob bean are Spain, followed by Italy, Morocco, Portugal, and Greece. Carob fruits are used industrially to produce locust bean gum (LBG, E410) extracted from the seeds and with a wide application in foods as a thickening agent and stabilizer. The pulp, comprising about 90% of the fruit, stands out due to its high content of carbohydrates, minerals, protein, insoluble dietary fibers, and different types of polyphenolic compounds.[8−12] The fine ground powder from the pulp (carob powder) is used in the production of confectioneries, beverages, bread, or pasta. Carob powder is

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often used as a cocoa substitute since it exhibits a number of advantages relative to that product, namely, a lower fat content, fewer calories, and more fiber and calcium. Moreover, it is an ingredient free of caffeine, theobromine, and cholesterol.

There are several reports on the biological activity of carob pulps. Carob powder is used as an antidiarrheal, especially in infants.[13] Water extracts show high antioxidant and free-radical scavenging activities in vitro[10] and antiproliferative activity in liver cells.[9] Carob fiber is a product obtained by water extraction of the pulps in a process that removes the majority of soluble carbohydrates.[14] In humans, the consumption of carob fiber has a high antioxidant effect,[10] lowers serum cholesterol and triglycerides, and modulates the blood lipid profile.[15] Moreover, carob fiber has an antiproliferative activity on colorectal cancer cells.[16−18] In spite of the potential of carob pulp in the promotion of human health, in part due to the presence of two important dietary factors in the prevention of chronic diseases, namely, dietary fiber and antioxidants,[3] most of the pulps are discarded and not effectively used, and are regarded as byproducts of LBG production in the carob industry.

Carob trees may be males, females, or hermaphrodites, which led some authors to consider trioecy as one of the most outstanding biological features of this species.[19] In Portugal, only female trees are grouped in cultivars and used in commercial orchards, and the hermaphrodites are mainly used as pollinators. In a recent work, a high genetic diversity was found in terms of morphologic, agronomic, and technologic characters between Portuguese female carob cultivars, which also differ greatly from carob cultivars from other countries of the Mediterranean basin.[20] To our knowledge, there are no reports on the influence of this diversity on the bioactivity of carob tree fruit pulps. In this context, this study aimed to determine the antioxidant and the in vitro cytotoxic activities of fruit pulp methanol extracts from six female cultivars (Mulata, Galhosa, Aida, Costela/Canela, Gasparinha, and Preta de Lagos) and two hermaphrodite carob trees growing in Algarve (Portugal). A phytochemical analysis of the phenolic fraction of the extracts was made, and the effect on cancer cell viability was evaluated on a panel of human cancer cell lines, namely, breast (MDA-MB-231), cervical (HeLa), prostate (DU-145), and on a colon cell line (HCT-116). The extract effect on intracellular ROS production by HeLa cells was assessed, and the induction of apoptosis was evaluated on HeLa and MDA-MB-231 cells. Additionally, the effect of the main phenolic compounds identified in the extract was investigated on HeLa cells.

MATERIALS AND METHODS

Chemicals. All chemicals were of analytical grade. Cell culture media and additives were purchased from Biological industries (Kibbutz Beit Haemek, Israel); fetal bovine serum (FBS) was from PAA laboratories (Pasching, Austria) and *n*-hexane from Riedel-de Haën (Buchs, Switzerland). Sigma Aldrich (Steinheim, Germany) supplied 1,1-diphenyl-2-picrylhydrazyl (DPPH), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), teophylline, (+)-catechin, chloroform, β-carotene, vanillin, myricetin, and ferulic, caffeic, syringic, gentisic, chlorogenic, linoleic, and cinamic acids. 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium monosodium salt (WST-1) was purchased from Roche (Barcelona, Spain). Folin−Ciocalteu (F−C) reagent, (−)-epicatechin, gallic acid (GA), methanol, Tween 40, *p*-dimethylaminocinnamaldehyde (DMACA), aluminum chloride ($AlCl_3$), and sodium carbonate (Na_2CO_3) were from Fluka (Steinheim, Germany). Rutin was from Dr. Ehrenstorfer GmbH (Augsburg, Germany), quercetin from Alfa Aesar (Karlsruhe, Germany), and kaempferol from Extrasynthèse (Genay Cedex, France). Cell lines were obtained from American Type Culture Collection (Rockville, MD). Other reagents were purchased from VWR International (Leuven, Belgium).

Plant Material. Mature fruits were collected in August of 2005 from six female Portuguese cultivars, namely, Mulata, Galhosa, Aida, Costela/Canela, Gasparinha, and Preta de Lagos, and from two hermaphrodite trees (H1 and H2). The trees are growing in a cultivar collection field from the Ministry of Agriculture, Rural Development and Fisheries (Direcção Regional de Agricultura do Algarve, Tavira, Portugal), and were identified by J. Graça. The fruits were manually deseeded, the pulps dried at 40 °C for 2 days, milled, and stored in the dark at −20 °C until use.

Preparation of the Extracts, GA and (+)-Catechin. The methanol extracts were prepared by Soxhlet extraction as described elsewhere.[21] Briefly, dried plant material (10 g) was defatted with *n*-hexane (400 mL) and then extracted with methanol (400 mL) in a Soxhlet apparatus (∼60 °C) for 5 h. For the cell experiments, the extracts were dried in a rotary-evaporator in vacuum (−0.4 to −0.6 bar) at 40 °C and resuspended in the appropriate culture medium. The stock solutions of GA and (+)-catechin were prepared on phosphate-buffered saline (PBS, pH 7.4) and diluted with culture medium immediately before use to concentrations corresponding to the amounts quantified in the crude extracts. All the other assays were performed with the original extracts.

Phytochemical Evaluation by Spectrophotometric Assays. Total phenolics content (TPC) was determined by a derived method of the F−C assay,[22] and the results were expressed as mg gallic acid equivalents (GAE)/g extract (dry weight, DW). Total condensed tannins content (TTC) was determined by the *p*-dimethylaminocinnamaldehyde (DMACA) assay,[23] and the results were expressed as mg catechin equivalents (CE/g extract DW). Total flavonoids content (TFC) was assessed by the aluminum chloride ($AlCl_3$) method,[24] and the results were expressed as mg rutin equivalents (RE/g extract DW). All the analyses were made on samples at the concentration of 2 mg/mL.

HPLC Analysis and Identification of the Main Compounds. HPLC analysis was made on an Agilent 1100 Series liquid chromatograph equipped with a UV−vis diode array detector (Agilent Technologies, Germany) and a Tracer excel 120 ODS-A column (150 mm × 4.0 mm, 5 μm particle size, Teknokroma, Spain). Analyses were performed at 25 °C on the extracts at the concentration of 10 mg/mL, as described previously.[25] In brief, the conditions used were as follows: injection volume, 40 μL; draw speed, 200 μL/min; mobile phase, 2.5% acetic acid in water (A) + methanol (B); applied gradient, 0−50 min, 30−80% B, 50−55 min, 80−30% B, and hold for 5 min; and flow rate, 0.5 mL/min. The levels of the different compounds were extrapolated from calibration standard curves.

Antioxidant Activity. Antioxidant activity was determined by two complementary methods: determination of the radical scavenging activity (RSA) by the DPPH assay[26] and inhibition of lipid peroxidation (ILP) by the β-carotene-linoleate bleaching model[27] as described previously.[21] The extracts were tested at concentrations ranging from 2.5 to 20 mg/mL, and the results were expressed as IC_{50} values (inhibitory concentration 50, mg/mL). GA and (+)-catechin were used as positive controls at the concentration of 1 mg/mL. This concentration was used because it provided antioxidant activities higher than 90% on preliminary results using the DPPH assay.

Cell Culture. HeLa and MDA-MB-231 cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) with 1000 mg/mL of glucose and 10% (v/v) FBS, DU-145 cells in Ham's F10 nutrient mixture supplemented with 5% (v/v) FBS, and HCT-116 cells in RPMI medium with 10% (v/v) 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),

Table 1. Total Phenolic Content (TPC), Total Tannin Content (TTC), Total Flavonoid Content (TFC), Radical Scavenging Activity (RSA, IC_{50}, mg/mL), and Inhibition of Lipid Peroxidation (ILP, %) of Fruit Pulp Extracts from Carob Trees[a]

	phenolics content			antioxidant activity	
	TPC[b]	TTC[c]	TFC[d]	RSA	ILP
female					
Mulata	15.6 ± 1.3 d	3.1 ± 0.6 c	1.4 ± 0.0 b	15.3 ± 0.5 f	74.4 ± 4.7 ab
Galhosa	14.5 ± 1.5 de	1.9 ± 0.0 d	0.5 ± 0.2 f	10.4 ± 0.4 e	46.7 ± 13.3 c
Aida	29.0 ± 2.0 c	1.4 ± 0.3 e	0.8 ± 0.0 e	4.1 ± 0.1 b	22.3 ± 1.8 d
Gasparinha	16.6 ± 1.2 de	8.8 ± 0.3 ab	1.8 ± 0.0 b	8.4 ± 0.1 d	80.9 ± 16.6 ab
Costela/Canela	11.6 ± 1.3 f	10.0 ± 0.3 a	1.4 ± 0.1 c	8.6 ± 0.0 d	61.1 ± 2.1 bc
Preta de Lagos	20.7 ± 2.3 d	1.1 ± 0.1 e	1.4 ± 0.0 c	7.5 ± 0.2 c	47.4 ± 0.5 c
mean	*18.0 ± 6.1*	*4.4 ± 3.9*	*1.2 ± 0.4*	*9.1 ± 3.7*	*88.9 ± 67.1*
hermaphrodite					
H1	45.9 ± 3.4 a	8.0 ± 1.0 b	1.2 ± 0.0 d	<2.5	87.9 ± 1.1 a
H2	36.7 ± 3.0 b	2.5 ± 0.3 d	2.6 ± 0.0 a	<2.5	81.0 ± 13.4 ab
mean	*41.3 ± 6.4*	*5.2 ± 3.9*	*1.9 ± 0.9*		*84.4 ± 4.8*
GA				0.2 ± 0.0 a	75.0 ± 2.5 ab
(+)-catechin				0.1 ± 0.0 a	37.0 ± 6.2 cd

[a] Data are reported as means ± SEM. For each column, statistical analysis was made between cultivars/trees. Values followed by different letters (a–f) are significantly different at $P < 0.05$ (one-way ANOVA, Duncan's new multiple range test). [b] mg GAE/g extract (DW). GAE, gallic acid equivalents. [c] mg CE/g extract (DW). CE, catechin equivalents. [d] mg RE/g extract (DW). RE, rutin equivalents.

and were grown in an incubator at 37 °C and 5.1% CO_2 in a humidified atmosphere.

Effect of the Extracts on Cell Viability. For the cell viability determination, cells were seeded on 96-well plates at a density of 7×10^3 cell/well for HeLa and 10×10^3 for the other cell lines, allowed to attach for 24 h, and treated with different concentrations of the extracts (2.5, 5, 10, 20, and 40 mg/mL) for 24, 48, and 72 h. Cell viability was determined by the 4-(3-4-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio)-1,3-benzenedisulfonate (WST-1) colorimetric assay in HeLa cells as previously described[21] and by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium inner salt (MTS) method[28] in the other cell lines. The results were expressed in terms of cell viability (%), half maximal inhibitory concentration (IC_{50}, mg/mL), and maximal degree of inhibition (%).

Effect of the Main Phenolic Compounds on Cell Viability. To determine the effect on cell viability of the major phenolic compounds in the extracts, (+)-catechin and GA, HeLa cells were treated with those compounds for 24, 48, and 72 h in concentrations corresponding to the amounts quantified in the crude extracts (GA, 3–33 μg/mL; and (+)-catechin, 40–120 μg/mL). In another experiment, cells were incubated with culture medium containing concentrations corresponding to the mean value of GA (13.9 μg/mL) and (+)-catechin (69.8 μg/mL) present in all samples. Cell viability was determined by the MTT assay, as described elsewhere.[25]

Cell Cycle Analysis. HeLa and MDA-MB-231 cells were incubated with the extract from female cv. Mulata at the concentrations of 20 and 40 mg/mL for 24 h (HeLa) and 48 h (MDA-MB-231), trypsinized, washed with ice-cold PBS and centrifuged (4 °C, 8 min, 1000 rpm). The cultivar Mulata was chosen because it is the main female cultivar present in commercial orchards in Portugal. The pellet was resuspended in 1 mL of ice-cold PBS and fixed overnight at −20 °C with ice-cold 70% (v/v) ethanol. Cells were centrifuged (4 °C, 10 min, 1000 rpm) and washed with PBS, and the pellet was incubated with 10 mL of Triton X-100 (0.1%, v/v) and 2 mg of RNase (Ribonuclease A, DNase-free). Finally, propidium iodide (PI, 1 mg/mL in water, w/v) was added to the cell suspension. The fluorescence intensity was measured by Fluorescence-Activated Cell Sorter (FACS) in a flow cytometer (Coulter XL). Data from 12 000 cells per sample were collected and analyzed by a Cell Fit Cell analysis program.

Morphological Observations. Morphological changes induced by the extract from Mulata were evaluated on HeLa cells by a TUNEL assay using DeadEnd Fluorimetric TUNEL System (Promega), according to the manufacturer. Observations were made by using confocal fluorescence microscopy (Leica TCS 4D).

Statistical Analysis. The data are presented as the mean ± standard error of mean (SEM) of at least three independent experiments. The values were evaluated by one-way analysis of variance (ANOVA) followed by a post hoc Duncan's multiple-comparison test ($P < 0.05$) using SPSS V15. Correlations were determined using the correlation and regression program of Microsoft Excel. The IC_{50} values were calculated by sigmoidal fitting of the data in the GraphPad Prism V4.

RESULTS AND DISCUSSION

Phytochemical Contents. Results of TPC, TTC, and TFC are depicted in Table 1. It is clear that gender and cultivar significantly influenced the phenolic contents of carob fruit pulps, with hermaphrodites being richer on those compounds. The mean values for TPC ranged from 18.0 mg GAE/g in female cultivars to 41.3 mg GAE/g in hermaphrodite trees. In the female cultivars, Aida (29.0 mg GAE/g) and Costela/Canela (11.6 mg GAE/g) presented the highest and the lowest values, respectively. As for total phenols, both gender and cultivar significantly influenced TFC ($P < 0.001$). The extracts from hermaphrodites were richer in those compounds (1.9 mg RE/g, Table 1). In the female cultivars, Gasparinha (1.8 mg RE/g) presented the highest content of flavonoids (Table 1). The cultivar/tree significantly influenced TTC, which was highest in Costela/Canela (10.0 mg CE/g) and Gasparinha (8.8 mg CE/g). This is in accordance with previous findings in the same species, where different amounts of antioxidant compounds were identified in different carob tree cultivars.[25] In fact, cross-varietal screening tests have repeatedly shown that certain genotypes within a

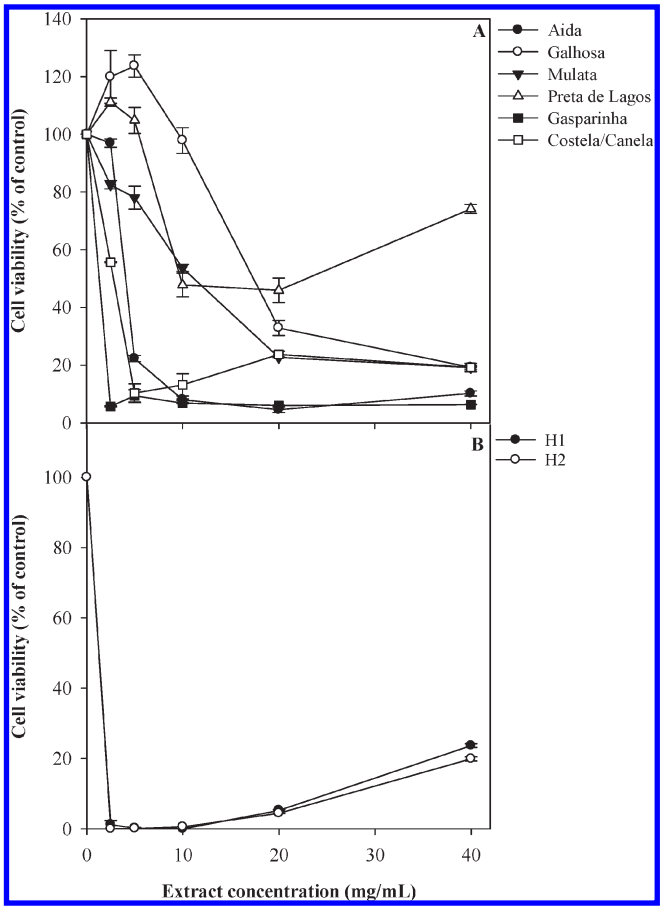

Figure 1. Effect of the application of fruit pulp extracts of female carob cultivars (A) and hermaphrodite trees (B) on HeLa cell viability after a period of incubation of 24 h. Values represent the means ± SEM of 3 replicate analyses.

plant species can have widely divergent levels of inherent antioxidants.[29−31]

The phenolic fraction of carob fruit pulps consists of water-soluble and insoluble tannins, flavonol glycosides, and high contents of different forms of GA: free GA, gallotannins, and methyl gallate.[5] The phenolic constituents of an aqueous carob extract have been previously characterized and consist mainly of GA.[9,11] However, in this work the major compound in the extracts was (+)-catechin, with mean values of 5.5 mg/g in females and 10.3 mg/g in the hermaphrodites. GA was detected in all samples, with mean values of 1.1 mg/g in females and 1.8 mg/g in hermaphrodites. The alkaloid theophylline was present in all samples except in Mulata and Gasparinha. Teophylline has been previously detected in aqueous extracts of carob pods[9] and was identified as the main compound in methanol extracts of carob tree germ flour.[25] Other compounds seemed to be related with particular extracts, such as gentisic acid detected only in the hermaphrodite tree H1; caffeic acid, only in Galhosa and Costela/Canela; and catechol, present only in Aida, Preta de Lagos, and in the hermaphrodite trees. The differences observed between our study and other reports in the same species can be due to different sample preparations and both the extraction and method of analysis. Moreover, the variations in polyphenol contents among extracts from the same species of different geographical origins can be attributed to genetic and geographical differences, and cultural conditions.[1]

Table 2. Half Maximal Inhibitory Concentration (IC_{50}, mg/mL) and Maximal Degree of Inhibition (Max, %) of Fruit Pulp Extracts of Carob Trees on HeLa Cells after 24 h of Incubation

cultivar/tree	IC_{50}	max
female		
Mulata	14.4 ± 4.4	83.3 ± 2.4
Galhosa	15.9 ± 2.9	75.7 ± 8.7
Aida	4.5 ± 0.3	94.2 ± 2.3
Gasparinha	<2.5	100.0 ± 0.0
Costela/Canela	4.2 ± 0.1	88.9 ± 7.6
Preta de Lagos	11.4 ± 2.3	67.0 ± 24.3
hermaphrodite		
H1	<2.5	100.0 ± 0.0
H2	<2.5	100.0 ± 0.0

Antioxidant Activity. For the evaluation of the antioxidant activity of extracts from natural sources, it is recommended to use complementary in vitro methods.[32] Among the different assays described in the literature, both in terms of free radical scavenging ability and inhibition of lipid peroxidation, there are two methods commonly used due to their high throughput and feasibility: DPPH and β-carotene−linoleic acid assays.[33] Having this in mind, these methods were used in this work to characterize the antioxidant potential of the methanol extracts of carob tree fruit pulps. The extracts showed a significant RSA which was gender- and cultivar/tree-dependent, and the best results were obtained with the hermaphrodite trees (IC_{50} < 2.5 mg/mL, Table 1). Among the female cultivars, Aida exhibited the highest activity (IC_{50}, 4.1 mg/mL; Table 1).

The results on the antioxidant activity of the extracts measured by the β-carotene-linoleate bleaching method are summarized in Table 1. The ILP confirmed the results obtained by the DPPH assay: indeed, Table 1 shows that ILP was both gender ($P < 0.05$)- and cultivar/tree-dependent ($P < 0.01$). The application of the extracts from hermaphrodites led to higher percentages of inhibition, and no significant differences were observed between trees ($P > 0.05$, Table 1). Among the female cultivars, Gasparinha and Mulata had the highest ILP when compared with those of Galhosa, Aida, and Preta de Lagos (Table 1). No correlation was found between the ILP and the total amount of phenolic compounds ($P > 0.05$, data not shown).

The antioxidant activity of the carob pulp extracts was generally lower than that obtained for the standards, GA and (+)-catechin (Table 1). However, the mean values for total phenolics in the extracts suggest that these should have the highest activity when compared at the same concentration of the individual phenolic compounds. The maximum lawful level for synthetic food antioxidants is established from different toxicological parameters that need to be applicable to naturally occurring compounds.[34] Therefore, the phenols from the pulp extracts could be used at higher levels than the synthetic phenolic, thereby increasing their antioxidant effectiveness. Moreover, the individual phenolic compounds can be present at low concentrations in the crude extract, which can be enriched in the compounds of interest by a bioactivity-guided fractionation.

Effect of the Extracts on Human Cancer Cell Viability and Apoptosis Induction. The extracts had a concentration- and time-dependent effect on cell viability, and as expected from the antioxidant assays, there was a significant variation in bioactivity between genders (Figure 1, Table 2). The extracts from

Table 3. Modulation of Cell Cycle Progression and Apoptosis in HeLa and MDA-MB-231 Cells by Extract of Pulps of Carob Trees[a]

		% cells in			
cells	extract (mg/mL)	G0/G1	G2/M	S	sub-G1
HeLa	0	63.1 ± 10.5	11.0 ± 5.9	25.7 ± 4.5	3.8 ± 0.9
	20	60.9 ± 1.8	14.4 ± 2.9	24.6 ± 4.1	4.8 ± 1.4
	40	53.7 ± 21.9	5.1 ± 3.1	41.0 ± 8.4*	12.8 ± 0.9**
MDA-MB-231	0	61.0 ± 3.9	17.1 ± 1.4	22.0 ± 2.7	1.4 ± 0.1
	20	51.9 ± 4.7	10.6 ± 6.6	37.4 ± 11.1*	3.9 ± 0.7
	40	44.9 ± 5.8*	22.5 ± 1.1	32.5 ± 7.0*	29.7 ± 9.8***

[a] Cells were treated with 20 or 40 mg/mL of methanol extract from pulps of female cv. Mulata for 24 h (HeLa) or 72 h (MDA-MB-231). Cell cycle distribution was assessed by FACS. Values represent the means ± SEM of 3 assessments. Significant difference with untreated group: *$P < 0.05$; **$P < 0.01$; and ***$P < 0.001$.

hermaphrodite trees exhibited the strongest activity at concentrations below 10 mg/mL (Figure 1B and Table 2). However, an increase in cell viability was observed with concentrations up to 40 mg/mL, although cell viability remained significantly lower than that of the control (Figure 1B). Among the female cultivars, Gasparinha presented the highest inhibitory capacity with an IC_{50} value bellow 2.5 mg/mL and a maximum degree of inhibition of 100% (Figure 1A and Table 2).

On HeLa cells, the effect of the extracts on cell viability became evident after 24 h, and regardless of the concentration applied, the maximum effect was achieved after 48 h. On the MDA-MB-231 cell line, we observed a promotion of cell viability with increasing concentrations of the extracts, for the 24 and 48 h periods of incubation (data not shown). The response in terms of cellular viability with increasing concentrations and periods of incubation was similar for colon (HCT-116) and prostate (DU-145) cells, but they were less susceptible to the extract than the HeLa cell line (data not shown). No correlation was found between the cytotoxic activity and the total amount of phenolic compounds ($P > 0.05$; data not shown). Our results indicate that the cytotoxic effect of pulp extracts from female cultivars and hermaphrodite trees on human cell lines varies according to the extract concentration, period of incubation, and target tumor cells, similar to reports on the same species[25] and in other plant species.[35] On HeLa and MDA-MB-231 cells, a mechanism involved in the cytotoxic effect of the extract could be related to the induction of apoptosis. As can be seen in Table 3, the application of the extract at 40 mg/mL caused a significant increase in cells corresponding to the sub-G1 population in both cell lines. Moreover, the extract induced cell arrest in the S phase, at 40 mg/mL for HeLa and at both concentrations tested for MDA-MB-231 cells. Untreated cells were polygonal in normal shape (Figure 3A), and the exposure to the extract from female cv. Mulata at the concentration of 20 mg/mL for 24 h resulted in cell retraction and rounding (Figure 3B). Cells with nuclear morphology changed; apoptotic bodies (membrane surrounded fragment) and chromatin condensation (cells which can be seen to be more illuminated) can be observed in Figure 3Ba, b, and c. Our results are similar to the ones obtained in the same species,[9,21,25] and are in accordance with other studies, where the application of plant extracts rich in phenolic compounds resulted in an inhibition of cell proliferation, linked to the induction of apoptosis.[36] Since the induction of apoptosis in cancer cells is considered one useful strategy for anticancer drug development,[36]

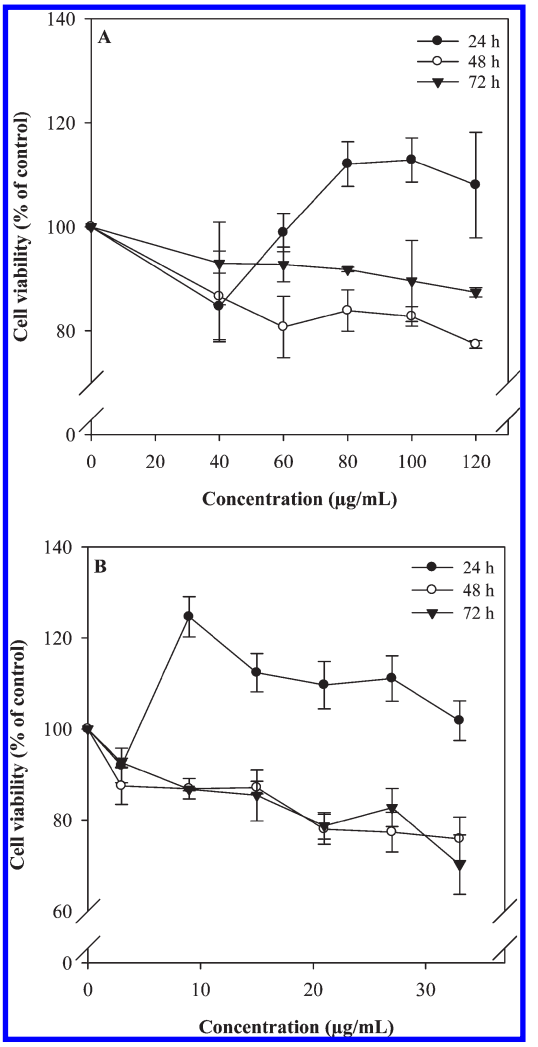

Figure 2. Effect of the application of (+)-catechin (A) and GA (B) on the viability of HeLa cells after 24, 48, and 72 h of incubation. Values represent the means ± SEM of 3 replicate analyses.

our results indicate methanol carob pulp extracts as sources of useful chemotherapeutic compounds.

Effect of the Main Phenolic Compounds on Cancer Cell Viability. Aiming to find if the effect of the crude extracts on

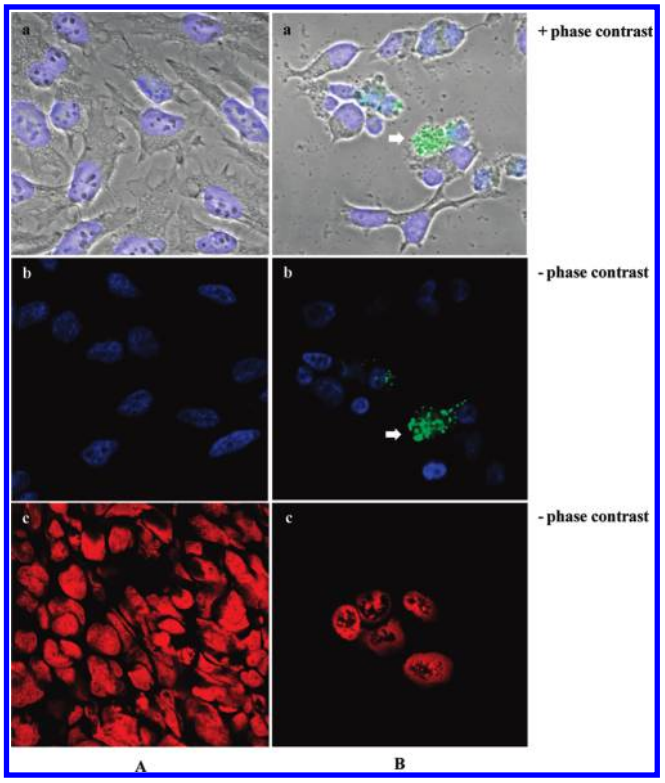

Figure 3. Morphological alterations of HeLa cells following exposure to extracts of carob pulps. Cells were stained by using a TUNEL assay (Aa and Ab) or with PI (Ac and Bc) and examined by fluorescence microscopy. (A) Control and (B) cells treated with 20 mg/mL of methanol pulp extract for 24 h. Images show nuclei at 630× magnification. Arrows indicate chromatin condensation and nuclear fragmentation.

cancer cell viability could be explained by the activity of its main components, the cytotoxic activity of GA and (+)-catechin was evaluated on HeLa cells at concentrations corresponding to the amounts contained in extracts. As can be seen in Figure 2, GA and (+)-catechin had similar effects on cell viability: after 24 h of incubation, a slight promotion of cell viability was observed for both compounds, while for the 48 and 72 h of incubation, no cytotoxic effects were observed (Figure 2). However, an increase in cell viability was observed after a 24 h treatment with the concentration of 13.9 μg/mL of GA (Figure 2). For the 48 and 72 h of incubation, only the combination of both compounds significantly decreased HeLa cell viability (data not shown). There are several reports on the antitumoral activity of these compounds: GA is ubiquitously distributed in vegetable food and shows antiapoptotic,[37] pro-apoptotic,[38] and antiproliferative[39] activities in cell based systems. Catechin and other related procyanidin compounds or extracts containing such compounds have antitumor activity.[40] However, our results indicate that under the conditions of our study, (+)-catechin and GA may not be responsible for the in vitro antitumoral activity of the crude extract since no cytotoxic activity was observed after the application of these compounds. In fact, only the application of a combination of both compounds resulted in a decrease in cell viability. Moreover, no correlation was found between the total amount of phenolics and the antioxidant and cytotoxic activity of the extracts. Altogether, these results indicate that apart from phenols, other compounds may be responsible for the biological activities of carob tree pulp extracts. Carob tree, a plant with high-antioxidant activity, for which phenolic content versus antioxidant activity falls above the regression line, is a plant from which novel antioxidants may be found.[41]

In conclusion, the results presented here indicate that the antioxidant and cytotoxic activities of carob tree fruit pulps are strongly influenced by gender and cultivar and that extracts from carob fruit pulps, especially those from hermaphrodite trees, have potential as sources of antioxidant and antitumor compounds. Besides male trees, hermaphrodites are sometimes used as pollinators in carob orchards, and since they are both pollinators and pod producers, they could be the preferred ones. Studies on the mineral content of female and hermaphrodite trees growing in Portugal showed that hermaphrodites have a more efficient use of mineral resources,[42] which may have important economic implications in commercial orchards. Our results reinforce the view that hermaphrodite trees may have several advantages over female cultivars, namely, as a source of compounds with biological interest, which may represent an increase of their agronomic interest. Our results also indicate that on cervical and breast cancer cells, the reduction of cancer cell viability occurred via apoptosis. Although GA was found to contribute to the antioxidant activity of the extracts, the specific phenolic compounds or other phytonutrients responsible for the antioxidant and cytotoxic activities remain unidentified.

■ ASSOCIATED CONTENT

Supporting Information. Tables containing the main results on the HPLC-DAD analysis of phenolic compounds contents in carob tree fruit pulp extract and half maximal inhibitory concentration and maximal degree of inhibition of fruit pulp extracts of female cv. Mulata on different cell lines after different periods of incubation and figures summarizing the effect of the period of incubation with pulp extracts from female cv. Mulata on the viability of different cancer cell lines and the effect of the application of (+)-catechin, GA, or a combination of both compounds on the HeLa cells viability. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

CE, catechin equivalents; DPPH, 1,1-diphenyl-2-picrylhydrazyl; DU-145, prostate cancer cells; FBS, fetal bovine serum; GA, gallic acid; GAE, gallic acid equivalents; HCT-116, colon cancer cells; HeLa, cervical cancer cells; MDA-MB-231, breast

cancer cells; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; RE, rutin equivalents; RSA, radical scavenging activity; TPC, total phenolics content; TTC, total condensed tannins content; WST-1, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium monosodium salt.

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