SHORT REPORT

A C G publications

Rec. Nat. Prod. 7:3 (2013) 225-229

records of natural products

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

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(Received November 7, 2011; Revised April 1, 2012; Accepted May 17, 2013)

Abstract: Methanol (ME) and hot water extracts (WE) of carob tree sapwood (*Ceratonia siliqua* L.) exhibited high antioxidant activity and were rich in phenolic compounds, with the main compounds identified by HPLC/DAD as gentisic acid and (-)-epicatechin. The ME displayed a high *in vitro* antitumor activity against human tumoural cell lines and reduced intracellular ROS production by HeLa cells after treatment with H_2O_2. (-)-Epicatechin was shown to contribute to the cytotoxic activity of the ME. This is the first report on the biological activity of carob tree sapwood.
Keywords: Antioxidant activity; cytotoxic; gentisic acid; phenolic compounds; ROS.

1. Plant Source

Carob tree (*Ceratonia siliqua* L.) is an evergreen plant from the Mediterranean area. Carob fruits are mainly used for locust bean gum (LBG, E410) extraction from the seeds, widely used in food industry as a thickening agent and stabilizer. Sapwood samples, which were identified by J. Graça, were obtained from mature female trees of cultivar Mulata in August of 2005, a cultivar collection field, Ministry of Agriculture and Rural Development and Fisheries (Direcção Regional de Agricultura do Algarve, Tavira, Portugal).

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The article was published by Academy of Chemistry of Globe Publications
www.acgpubs.org/RNP © Published 5/28/2013 EISSN: 1307-6167

2. Previous Studies

Fruits and leaves of carob tree contain proteins and phenolic compounds, and exhibit antioxidant, antiproliferative and antimicrobial activities [1-16]. In a previous work Balaban [17] used common spectroscopic (Folin–Ciocalteu and Rhodanin assays) and chromatographic (GC-MS) methods to determine the chemical composition of methanol / water extracts of heartwood and sapwood of carob tree from Turkey. Those extracts were fractionated by organic solvents and hydrolysed with hydrochloric acid to determine bound hydrolysable tannins. Before hydrolysis the main phenolics of diethyl ether and diethyl ether / methanol fractions of sapwood were gallic acid (GA) and chalcone (4 × OH), whereas after hydrolysation, the diethyl ether / methanol fraction was mainly composed by methyl inositol, GA and methyl GA. To our best knowledge, there are no reports on the biological activities of carob tree sapwood.

3. Present Study

Samples were dried at 40ºC for 2 days, milled and stored in dark at -20ºC. ME was prepared by Soxhlet extraction [8] and WE was obtained by boiling samples (1 g) in 100 mL of distilled water for 10 min, which was cooled and filtered (Whatmann nº 4). For the cell experiments, methanol was removed under reduced vacuum at 40°C and the dry extract resuspended in the culture medium. Stock solutions of the phenolic compounds were prepared in a phosphate-buffered saline (PBS, pH 7.4), and diluted with culture medium immediately before use in the cell assays to the concentrations corresponding to the amounts quantified in the ME.

Total content of phenolic compounds (TPC), condensed tannins (TTC) and flavonoids (TFC) were evaluated as described previously [8]. The HPLC analysis and identification of the main phenolic compounds in the extracts were conducted according to [9-11].

The antioxidant activities of the extracts at different concentrations (125 to 1000 µg/mL) were evaluated by their radical-scavenging activity (RSA) on the DPPH [18, 19] and ABTS [20, 21] radicals and reducing power [22, 23]. Butylated hydroxytoluene (BHT, E321) was used as a positive control.

Four human tumour cell lines (cervical: HeLa, prostate: DU-145, breast: MDA-MB-231 and colon: HCT-116 cells) were used to determine cytotoxicity of ME, as it exhibited the highest antioxidant activity. Extracts, dissolved in culture medium, were applied to cells for 72h at different concentrations (25-400 µg/mL), and the cell viability was determined by WST-1 assay in HeLa cells [16] and MTS method [17, 18] in other cell lines. Phenolic compounds were applied to HeLa cells for 72h in concentrations corresponding to the amounts quantified in the ME, and cell viability was determined by the MTT assay [9, 11]. The capacity of the ME to scavenge intracellular ROS generation as a result of an oxidative stress induction by H_2O_2 treatment of HeLa cells was evaluated by 2',7'-dichlorodihydrofluorescin diacetate (DCFH-DA) method [9, 10].

4. Results and Discussion

ME had the highest level of TPC, almost 5 times higher than the level present in the WE (4.8 mg GAE/g, Table 1). In a previous study [17], it was reported that an aqueous extract of sapwood from carob tree had a TPC of 4.2 mg GAE/g dried material, as determined by F-C method, which is similar to the value obtained in this work for WE. The TTC was 2.5 mg CE/g for the ME and 1.2 mg CE/g for the WE, while flavonoids were present in trace amounts in both samples (Table 1). Our results indicated that the sapwood of carob tree has a higher TPC than leaves, pulps and germ flour, but lower concentrations of TTC and TFC [8, 18]. Avallone *et al*. [9] also observed different amounts of phenolic compounds in different organs (pods, germ and seeds) of female carob trees.

Gentisic acid was identified as a major compound, followed by (-)-epicatechin, GA, (+)-catechin and chlorogenic acid (Table 1, supporting information). Minor amounts of syringic acid, vannilin, rutin and kaempferol were also observed (Table 1, supporting information). Balaban [17] studied the phenolic composition of aqueous / methanolic extracts of heartwood and sapwood from carob tree, after fractioning using organic solvents, before and after hydrolysis with hydrochloric acid.

Before hydrolysis, the main compounds identified on diethyl ether and diethyl ether / methanol fractions of sapwood were GA and chalcone (4 × OH), while after hydrolysis, the diethyl ether / methanol fraction was mainly composed by methyl inositol, GA and methyl GA. Our results differ from that earlier reported, since gentisic acid was identified as the main compound in carob tree sapwood.

Table 1. Phytochemical evaluation and radical scavenging activity (RSA) of carob tree sapwood extractives.

	Phenolics content			**RSA (IC_{50}, µg/mL)**	
Sample	**TPC**	**TTC**	**TFC**	**DPPH**	**ABTS**
Methanol extract	23.8 ± 0.9[a]	2.5 ± 0.2[a]	0.5 ± 0.0	281.1 ± 6.9[a]	253.2 ± 0.0[a]
Hot water extract	4.8 ± 0.1[b]	1.2 ± 0.0[b]	ta	476.6 ± 46.4[b]	341.2 ± 0.1[b]
BHT[*]	-	-	-	258.1 ± 18.2[a]	758.0 ± 0.0[c]

TPC: Total phenolic content, mg GAE/g extract DW; TTC: total tannin content, mg CE/g extract, DW; TFC: total flavonoid content, mg RE/g extract, DW; GAE: gallic acid equivalents; CE: catechin equivalents; RE: rutin equivalents; ta: trace amounts; (-) not tested. [a, b, c]Different letters in the same column indicates significant differences by Duncan's New Multiple Range Test at $p < 0.05$; *Reference compound, 1 mg/mL.

The extracts showed similar RSA on DPPH, and even higher on ABTS radicals than BHT (Table 1), and higher capacity of reducing iron (Fig. 1, supporting information). Antioxidant activity of carob tree sapwood was found to be higher than the reported earlier for leaf, pulps and germ flour extracts of the same species [16, 18], which could be due to its higher content of phenolic compounds [24].

Treatment with ME resulted in the decrease in cell viability, for all cell lines tested (Fig. 1). However, only in HeLa cells the application of increasing concentrations of the extract was accompanied by the corresponding decrease of cellular viability, following a clear concentration-dependent pattern (Fig. 1). The IC_{50} values obtained for different cell lines were as follows: HeLa, 41.3 µg/mL; MDA-MB-231, 21.8 µg/mL and HCT-116, 101.5 µg/mL.

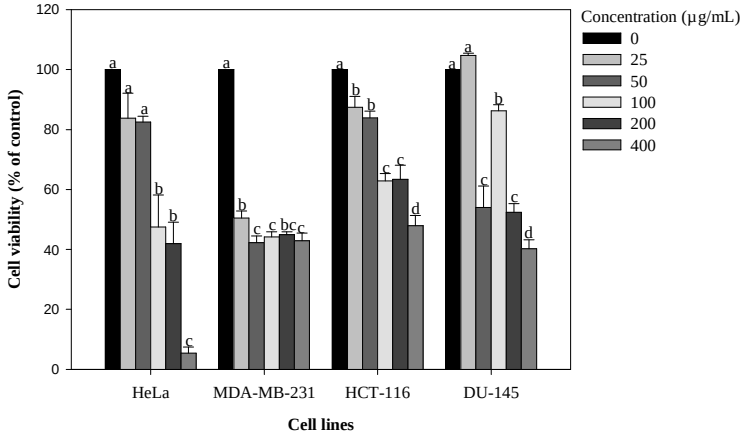

Figure 1. Effect of treatment with the ME from carob tree sapwood on the cell viability of human cancer cell lines after 72h of incubation. Different letters in the same cell line indicates significant differences between concentrations by Duncan's New Multiple Range Test.

The ME had a higher *in vitro* antitumoral activity against breast and colon cell lines than leaf extract of the same species [9], possibly due to the higher content in phenolic compounds. Phenolics display *in vitro* antiproliferative and cytotoxic activities through different mechanisms, such as apoptosis and cell cycle arrest [25]. There are also evidences that their cytotoxicity can be due to their oxidative activity, since phenolics can be either antioxidants or pro-oxidants [25]. In fact, their effect on the *in vitro* viability and proliferation of tumour cells is highly dependent on their structural characteristics (reviewed in [25]).

Gentisic acid had a protumoural activity on HeLa cells (cell viability: 125.9%, Fig. 2, supporting information). Gentisic acid is an active metabolite of salicylic acid degradation and displays analgesic, anti-inflammatory, antirheumatic, antiarthritic, cytostatic and antioxidant activities

[21]. However, our results indicated that under the experimental conditions of this study, gentisic acid was not the responsible for the cytotoxic activity of the crude extract, since it caused a significant increase in cell viability. Besides the structural characteristics of phenolic compounds, other features, such as dose, target molecule, and environment can modify their *in vitro* antitumoral activity [26]. (-)-Epicatechin exhibited the strongest cytotoxic activity, reducing cell viability to 59.4% (Fig. 2, supporting information). Epicatechin, which is one of the main components of green tea, is a flavonoid [27] and together with epigallocatechin gallate, epigallocatechin, and epicatechin gallate, is responsible for the antitumoral activity of green tea [27]. A significant reduction in cell viability was observed after treatment with all the remaining phenolic compounds, except for chlorogenic acid, which had no cytotoxic effect (Figure 2, supporting information). However, since we tested the activity of the total extract, we cannot exclude that other compounds might contribute to the inhibition of cell viability. The biological activity of a crude extract cannot be attributed to a single compound but also to other components present in the extract, often resulting from their synergistic effects.

The application of the ME at the concentration of 400 µg/mL resulted in a significant decrease of ROS production compared with control cells (Fig. 3, supporting information). ROS are the products of normal cellular metabolism, and are extremely reactive and potentially damaging transient chemical species to several macromolecules, such as DNA. The human body is equipped with enzymatic and nonenzymatic antioxidant systems to protect cells against ROS induced damage. Nevertheless, those systems may not be enough in cases involving severe or continued oxidative stress, and certain amounts of exogenous antioxidants are constantly required for the maintenance of adequate level of antioxidants in order to balance the ROS in human body [28].

As a conclusion, carob tree sapwood can be a source of antioxidant and cytotoxic compounds, and (-)-epicatechin can be one of its active constituents.

Acknowledgments

This work was partly supported by CICYT (CTQ2006-03794/BQU), Instituto de Salud Carlos III (CB06-01-0074 and PI060624), the Generalitat de Catalunya (2005SGR 00662), the Institute for Research in Biomedicine, and the Barcelona Science Park. L. Custódio thanks to the Portuguese Foundation for Science and Technology (FCT) for a post-doctoral grant (grant SFRH/BPD/20736/2004).

Supporting Information

Supporting Information accompanies this paper on http://www.acgpubs.org/RNP

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