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Carob Pods (*Ceratonia siliqua* L.) as a Source of Polyphenolic Antioxidants

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Summary

The possibility of utilising chopped and deseeded carob pods (kibbles) as a source of polyphenolic antioxidants was examined by performing extractions with various solvent systems, in order to evaluate and optimize the conditions for the recovery of polyphenols. Maximum quantities of polyphenolic components were found in 80 % acetone extracts, as evaluated by measuring total polyphenol and total flavanol content. By contrast, ethyl acetate was inefficient in extracting polyphenols. The assessment of the antioxidant potency of carob pod extracts employing two characteristic *in vitro* models showed that carobs contain polyphenols with appreciable antiradical and reducing properties. The values obtained were compared to the data on red wines and pure polyphenolic antioxidants.

Key words: antioxidants, antiradical activity, carobs, polyphenols, reducing power

Introduction

Carob tree (*Ceratonia siliqua* L., Leguminosae family), which is widely cultivated in the Mediterranean area, is considered to be an important component of vegetation for economic and environmental reasons (*1*). World production is estimated at about 310 000 tonnes per year, produced from about 200 000 hectares with very variable yields depending on the cultivar, region, and farming practices.

Carob pod is the fruit of the carob tree (*Ceratonia siliqua* L.), and is mostly used in the food industry for carob bean gum and locust bean gum, which are polysaccharides (galactomannans) contained in the endosperm of the seeds (*1,2*). However, carob pod mainly consists of pulp (90 %), which is rich in sugars (48–56 %), but it also contains a large amount of condensed tannins (16–20 %) (*1,3–5*), although lower tannin values have been reported (*6*). Carob leaves have been reported to contain considerably lower values of 0.7 % on dry matter basis (*7*).

In recent years interest in carobs as a cheap source of various products has been increasing. Some investigations explored carob pods as a readily available and inexpensive material for the production of bioethanol (*8,9*), and as a substrate for citric acid production (*10*), while carob extract have been a subject of studies for their influence on central and peripheral benzodiazepine receptors (*11*). However, data on carob pod antioxidant properties related to its polyphenolic composition are very limited (*12*). In this study a first approach to the efficiency of various solvents for satisfactory polyphenol extraction was attempted, and the extracts obtained were subjected to some representative *in vitro* tests, in order to obtain an insight into the antioxidant functions of carob pod polyphenols.

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Materials and Methods

Chemicals

Catechin, gallic acid, tannic acid, Folin-Ciocalteu reagent, *p*-(dimethylamino)-cinnamaldehyde (DMACA), quercetin, caffeic acid, ascorbic acid, 2,2-diphenyl--β-picrylhydrazyl (DPPH•) radical, EDTA (disodium salt), and 2,4,6-tripyridyl-*s*-triazine (TPTZ) were from Sigma Chemical Co. (St. Louis, MO). Citric acid and Trolox™ were from Aldrich (Steinheim, Germany).

Plant material

Deseeded and chopped carob pods (kibbles), approximately 1.5–2 mm in diameter, were obtained from a carob-processing factory (Chania, Crete). Kibbles were stored in a cool and dry place, and analysed shortly after the receipt.

Polyphenol extraction

A general scheme illustrating the extracting procedure may be seen in Fig. 1. A lot of 10 g of kibbles was placed in a round-bottomed flask and 50 mL of solvent were added. The flask was attached to a rotary evaporator and the extraction was performed by spinning the flask at maximum speed without vacuum for 20 min at 30 °C. Following this, the extract was filtered through filter paper, and the whole procedure was repeated twice. Extracts were pooled and concentrated under vacuum ($t \leq 40$ °C), and then brought up to 25 mL with 80 % MeOH. The solvent systems used for extraction can be seen in Table 1.

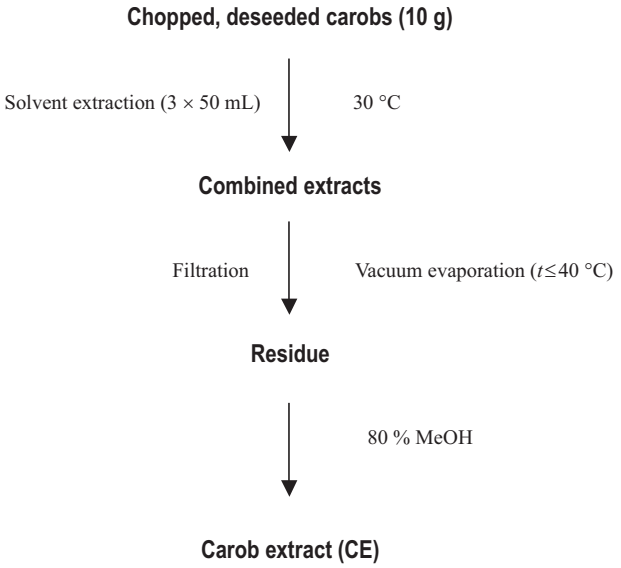

Fig. 1. Schematic representation of carob extract (CE) preparation

Table 1. Extraction efficiency of total polyphenols (TP) and total flavanols (TF) from carob pods, using various solvent systems

Solvent system	Total polyphenols (TP)[a]	Total flavanols (TF)[b]	TP/TF
Ethyl acetate	0.19±0.04	> 0.01	–
Methanol	2.20±0.34	0.27±0.07	8.1
80 % methanol	3.38±0.52	0.48±0.07	7.0
80 % acetone	9.28±0.61	1.00±0.23	9.3
80 % acetonitrile	3.22±0.47	0.70±0.12	4.6

a) Expressed as gallic acid equivalents, b) Expressed as catechin equivalents
Results are mean values of triplicate determinations of three individual procedures (n=3)±SD, and expressed as mg/g

Determination of total polyphenol and total flavanol content

Total polyphenols and total flavanols were determined by using the Folin-Ciocalteu and DMACA methodology, respectively, as described previously (*13*).

Evaluation of antioxidant potency

The antioxidant capacity of carob pod extracts was assessed using two different tests, including the measurement of the antiradical activity (A_{AR}), and the reducing power (P_R). All examinations were carried out according to the protocols described elsewhere (*13*). For the A_{AR} and P_R tests, extracts were diluted accordingly with MeOH and distilled water, respectively.

Statistical analyses

All measurements were run in triplicates (n=3), unless elsewhere specified, and the values were averaged and given along with the standard deviation (±SD). Analyses were performed with Microsoft Excel™ 2000.

Results and Discussion

Early examinations on carob pod polyphenols showed that carob tannins lack solubility in solvents such as ethyl acetate, methanol and ethanol. It was presumed that catechins and leucoanthocyanidins found in green (unripe) carobs may be regarded as possible precursors, and it was also reported that gallic acid occurs in higher amounts in ripe than in green carobs (*14*). Other investigations also found that carob pod tannins are highly polymerized (molecular weight up to 32 000), insoluble, and occur in carob pods as non-porous granular forms (*3*). More recent studies indicated that carob pods contain 1.9 mg/g of total polyphenols, 0.28 mg/g of proanthocyanidins, and 0.1 mg/kg of hydrolysable tannins (gallo- and ellagitannins), located mainly in germs, whereas seeds contained only traces of these components (*15*). Another examination of carobs showed their contents in total phenols and total flavanols to be 19.2 and 4.37 g per 100 g, respectively (*12*). Furthermore, carob pods were reported to contain 6.1 % of total polyphenols, and chemical degradation of tannins produced flavanols including catechin, epicatechin, epigallocatechin, epigallocatechin gallate, and epicatechin gallate, along with simpler phenolics such as phloroglucinol, pyrogallol, catechol, and gallic acid (*16*).

The assessment of the solvent systems used was based on two representative indices, the total polyphenol and total flavanol content. Total polyphenol determination was accomplished using the well established and widely used Folin-Ciocalteu method, while in the case of flavanols the determination was carried out fol-

lowing derivatisation with DMACA, which provides higher sensitivity and specificity compared to the vanillin assay (*17*). This method was successfully employed for the determination of total flavanols in white (*18*) and red wines (*13,19,20*). The results illustrated in Table 1 indicated that ethyl acetate, the most nonpolar solvent employed, was highly unsuitable for the extraction of polyphenols. By contrast, very efficient extraction could be performed employing 80 % acetone, which gave the highest total polyphenol and total flavanol values. However, 80 % aqueous acetonitrile extracted relatively high amounts of flavanols, as indicated by the ratio TP/TF. Methanol alone extracted low amounts of TP and TF, while the addition of water at 20 % did not alter its extracting efficiency to any significant extent. The values found for the extract obtained with 80 % acetone are lower than those reported when using 70 % acetone (*15*). It appears, therefore, that slight modifications in the extracting medium may have a prominent impact on the amount and nature of the compounds recovered, and therefore particular emphasis should be given to the selection of solvent system. This fact is clearly illustrated by comparing 80 % methanol and 80 % acetonitrile (Table 1). With both systems almost equal amounts of TP were extracted, but with the acetonitrile system notably higher flavanol amount was obtained.

The extracts obtained with the most efficient solvent system (80 % aqueous acetone) were further considered for testing the antioxidant characteristics. The investigation of the antioxidant potential of carob pod extracts was based on two different parameters, the antiradical activity (A_{AR}), and the ferric-reducing power (P_R). For a more descriptive and reliable evaluation, five well-known antioxidants were also tested in order to obtain comparative data, including gallic acid, caffeic acid, catechin, quercetin, and tannic acid. Comparisons were also made with selected data from previous examinations of red wines (*13*), which have been assessed using exactly the same methodology.

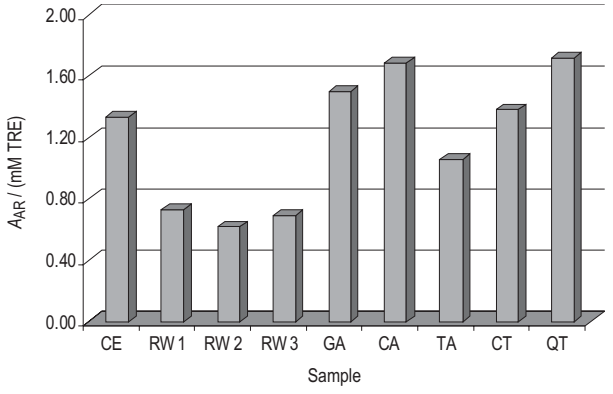

Fig. 2. Comparative diagram showing antiradical activity (A_{AR}) values of carob extract (CE) obtained with 80 % aqueous acetone, red wines (RW 1, 2 and 3), gallic acid (GA), caffeic acid (CA), tannic acid (TA), catechin (CT) and quercetin (QT). For the determination of A_{AR} and P_R of carob pod extracts and wines, the concentration was adapted at 100 mg/L of GAE. All pure compounds were tested at a final concentration of 100 mg/L. Results are the mean values of triplicate determinations (n=3)±SD. Data for red wines were from Arnous *et al*. (*13*). GAE – gallic acid equivalent; TRE – Trolox™ equivalent

As can be seen in Fig. 2, carob extract (CE) exhibited higher A_{AR} than the aged red wines and tannic acid (TA), comparable to that of catechin (CT), but lower than that of gallic acid (GA), quercetin (QT), and caffeic acid (CA). On the basis of reducing power (P_R), however, CE was almost 4-fold more efficient than red wines and catechin, and as potent reducing agent as GA and CA (Fig. 3).

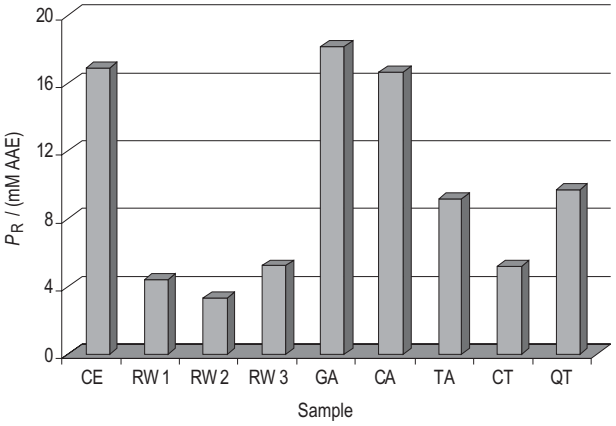

Fig. 3. Comparative diagram illustrating the ferric reducing power values of carob pod extract, red wines and pure polyphenols. Column assignment is as in Fig. 2. AAE – ascorbic acid equivalents

The interpretation of the antioxidant behaviour of carob pod extract is a rather complicated issue, considering that the antioxidant characteristics examined represent, in essence, the integration of actions of more than one polyphenolic classes. It can be claimed that extracts containing the same total polyphenol content as aged red wines appear significantly more potent, but it is obscure which compounds this potency may be attributed to. The evidence from the red wines, which may be considered similar complex matrices, consisting of a plethora of polyphenols, indicated that flavanols are likely to account for increased antiradical activity (*13,20–25*). Moreover, the high correlation of antiradical activity and reducing power in aged red wines (*26*) raised the assumption that compounds which express antiradical activity meet the criteria for exhibiting reducing effects as well (redox-active polyphenols). In this context, it would be reasonable to hypothesise that the antioxidant capacity seen in carob extracts reflects their high content in flavanols, particularly proanthocyanidins. This assumption is further supported by the high A_{AR} value observed for catechin (Fig. 2). On the other hand, the reducing ability, which was found comparable to that of gallic acid (Fig. 3), could be linked to the gallotannin fraction and/or free gallic acid, since carobs have not been reported to contain hydroxycinnamate derivatives similar to caffeic acid. It should be noted that, employing the DPPH• assay, Kumazawa *et al*. (*12*) also found that quercetin and gallic acid are more potent antioxidants than carob extracts, which was also true for catechin. However, in that study extractions were performed with water, which eventually resulted in extracts with different polyphenolic compositions. Therefore, the results from the two investigations are not absolutely comparable.

Conclusions

Carob pods may actually be regarded as by-products in the carob-processing procedure, because the seeds are considered the most valuable part of the fruit, containing polysaccharides, which are widely used in the food industry. They are, therefore, a cheap source of natural polyphenolic phytochemicals, whose nature and importance is, as yet, poorly investigated. The study presented here indicated that efficient polyphenol extraction from carob pods might be achieved employing aqueous acetone. The extracts obtained with this procedure exhibit appreciable antioxidant capacity, an evidence for the high potential of carobs as a cost-effective source of value-added polyphenolic phytochemicals. Currently, a work studying the isolation and structure elucidation of the bioactive constituents in carobs is in progress.

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Mahune rogača (*Ceratonia siliqua* L.) kao izvor polifenolnih antioksidanasa

Sažetak

Da bi se utvrdili i optimirali uvjeti za dobivanje polifenolnih antioksidanasa, ispitana je mogućnost ekstrakcije razmrvljenih mahuna rogača bez sjemenki raznim otapalima. Maksimalna je količina polifenolnih sastojaka, određena mjerenjem količine ukupnih polifenola i ukupnih flavonola, nađena u 80 %-tnim acetonskim ekstraktima. Etil-acetat nije bio djelotvoran za ekstrakciju polifenola. Rogači sadržavaju polifenole sa znatnim antiradikalnim i redukcijskim svojstvima, što je utvrđeno određivanjem polifenola reagensom Folin-Ciocalteu, a flavonola postupkom DMACA. Dobivene vrijednosti uspoređene su s podacima o antioksidansima u crvenim vinima te sa čistim polifenolnim antioksidansima.