Scholars Academic Journal of Pharmacy (SAJP) **ISSN 2320-4206**
Sch. Acad. J. Pharm., 2013; 2(5):375-380

(An International Publisher for Academic and Scientific Resources)
www.saspublisher.com

Research Article

Simple and fast method for the extraction of polyphenol and the separation of proanthocyanidins from carob pods

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Abstract: Carob tree (*Ceratoniasiliqua*) is widely cultivated in the Mediterranean area and is considered to be an important component of vegetation for economic and environmental reasons. The possibility of utilizing carob podsas a source of polyphenol antioxidants was examined by performing extractions with various solvent systems, in order to evaluate and optimize the conditions for the recovery of polyphenols and proanthocyanidins., such as water, ethanol, acetone and ethyl acetate have been used for the extraction of phenolics from plant materials, often with different proportions of water. Maximum quantities of polyphenol components were found in 10 % aqueous ethanol, as evaluated by measuring total polyphenol and maximum content of proanthocyanidins was found in 70% aqueous acetone. By contrast, ethyl acetate was inefficient in extracting polyphenols. The assessment of the antioxidant potency of carob pod extracts employing DPPH assay showed that carobs contain polyphenols with appreciable radical scavenging properties. A simple method for the separation of proanthocyanidins from other polyphenols was accomplished by sequential liquid extraction using pure ethanol and 70% aqueous acetone, this method needs further procedures for the purification of separated proanthocyanidins.
Keywords: *Ceratoniasiliqua*; DPPH assay; Polyphenol; Proanthocyanidins; Separation

INTRODUCTION

Antioxidants preventing free-radical reactions attract intense scientific and economic interest in human health[1, 2]and food industry [3]. Antioxidants, at low concentrations, delay or prevent molecular deterioration by adverse radical reactions and radical-related oxidation [2, 3] and protect the human body against damage by reactive oxygen species [1]. Food industryaims to minimize radicalinduced deterioration (e.g., rancidity) using antioxidants during the manufacturing process [4]. That way, foods can maintain their nutritional quality over a defined shelf life [5].

Among natural antioxidants, polyphenols successfully scavenge free radicals via their OH groups [6, 7]. There is a high correlation between content of phenolic substances and total antioxidant activity of various plants extracts [8]. These phenolic substances include more than 8000 compounds with great structural diversity.Currently, proanthocyanidins areoligomeric and polymeric end products of the flavonoid biosynthetic pathway andare believed to be contributors to the health benefits of fruits and vegetables [9]. Studiesshowed that proanthocyanidin antioxidant capabilitiesare 20 times more powerful than vitamin C and 50 times more potent than vitamin E [10]. Thesecomplex flavonoids have been linked to concentration dependentanti-carcinogenic activity [11] and inhibition of bacterial growth.[12]Proanthocyanidin oligomers demonstrated immune-modulatoryeffects.[13]Several investigations showed improved vascular healthafter short- or long-term consumption of proanthocyanidinsor foods and supplements that contained them [14]. These effects includedvasodilation (presumably as a result of increased NO production), decreased platelet aggregation[15], reduced sensitivity of low-density lipoproteins (LDL) to oxidization [16] and modulation of several reactions associated with inflammation [17].

Carob tree (*Ceratoniasiliqua*), which is widely cultivated in the Mediterranean area, is considered to be an important component of vegetation for economic and environmental reasons.[18]World production is estimated at about 310000 tons per year, produced from about 200000 hectares with very variableyields depending on the cultivar, region, and farming practices [19].

Carob pod is the fruit of the carob tree, and is mostly used in various processes of food technology,medicine, and other industrial processes. At the industriallevel, the pod was employed in the production of ethanolby *Saccharomyces cerevisiae* [20], theproduction of citric acid by *Aspergillusniger* [21], the production of mannitol by lactic acid bacteria.[22] In the food industry, carob pods were used for the production of carob bean gum and locust bean gum, which are polysaccharides contained in the endosperm of the seeds [19]. The pod consistsmainly of pulp

(90%), which is rich in sugars (48–72%), but also containsa large amount of proanthocyanidins [23]. Lowerproanthocyanidins values have been reported in some cases [24].

Although carob trees are found in great abundance in Mediterranean forests, investigations on its polyphenol composition are very rare. Therefore, the objective was to study the efficiency of various solvents for satisfactory polyphenol and proanthocyanidins extraction, and the extracts obtained were subjected to a representative in vitro test, in order to obtain an insight into the antioxidant functions of carob pod polyphenols. In this study, a first approach to the separation of proanthocyanidins from other polyphenols from the extract was attempted by sequential extraction.

MATERIALS AND METHODS

Chemical and reagents

Folin-Ciocalteu reagent 2N (Sigma-Aldrich, Switzerland), gallic acid (Sigma-Aldrich, China), 2,2-Diphenyl-1- picrylhydrazyl (DPPH) (Sigma-Aldrich, USA), sodium carbonate anhydrous (Surechem, England),ferric ammonium sulfate (Carl Roth, Germany),methanol and ethanol were obtained from Sharlau (Spain), ethyl acetate, acetone, 1-butanol and hydrochloric acid were obtained from Surechem (England).

Equipment

Micropipette 100-1000 µl (Iso lab, Germany), sensitive balance (Sartorius, Germany), ultra sonic bath, electric stirrer and heater,moisture analyzer balance (Precisa, Switzerland), centrifuge (Shanghai surgical instruments factory, China), spectrophotometer (Jasco V-530, USA.

Samples preparation

The dried carob pods were purchased from local markets and powdered in a blender. They were stored at -18°C until analysis.

The moisture content was determined by using a moisture analyzer balance.

Selection of optimal extractionfactors

The extraction of bioactive compounds from plant materials is the first step in the utilization ofphytochemicals in the preparation of dietary supplements or nutraceutical, food ingredients,pharmaceutical, and cosmetic products.

In general, efficiency of the extraction of a compound is influenced by multiple parameters as temperature, time, solid/liquid ratio and solvent polarity. Before the development of the study, a first set of tests were performed to select the extraction factors (temperature, time and ratio) which are effective on phenolic extraction yield.

According to our previously published paper on the extraction of phenolic compounds,the best yield of phenolic compounds was obtained when extraction was done at 50°C for 20 minutes [25].

Solvents, such as methanol, ethanol, acetone, ethyl acetate, and their combinations have been used for the extraction of phenolics from plant materials, often with different proportions of water. Selecting the right solvent affects the amount and rate of polyphenols extracted. In this study, different solvents with different polarities (water, ethanol,aqueous mixtures of ethanol, acetone/water mixture (70/30), ethyl acetate) were investigated on the total phenolic extraction from carob pods.

Extraction procedure

1 g of dried and ground pods was placed in a thermostatic water bath shaker with 100 ml of extraction solvent at 50°C for 20 min. The liquid extract was separated from solids by centrifugation at 2000 rpm for 10 min. The supernatant was transferred to a 100 ml flask, and the extraction solvent was added to make the final volume 100 ml [25].

4.6. Total Polyphenol Content

The total polyphenol content in the extract was determined by the Folin-Ciocalteu method as described by the International Organization for Standardization (ISO) [26]. 250 µl of the extract was diluted with distilled water to 10 ml. Aliquots of 1 ml of sample was mixed with 5 ml of 10-fold-diluted Folin-Ciocalteu reagent. After 3 min, 4 ml of 7.5% sodium carbonate was added. The mixturewas allowed to stand for 30 min at 40°C temperature (water bath) before the absorbance was measured at 734 nm. The total polyphenol content in the extract was calculated and expressed as gallic acid equivalents (GAE; g/100 g dry mass) using a gallic acid (0–120 mg/l) standard curve.

Proanthocyanidins Content

The proanthocyanidin content in the extract was determined by the Acid Butanol assay according to the method of Porter et al.[27]A sample of 200 µl extract diluted with 300 µl of acetone 70% was pipetted into a 100 x 12 mm test tube. 3.0 ml of butanol–HCL reagent (95:5) and 0.1 ml of 2% ferric acid prepared in HCl 2N were added. The tube was vortexed and then the mouth of the tube was covered with a glass marble and put in the heating block at 97 to 100 °C for 60 minutes. The tube was then allowed to cool and absorbance was recorded at 550 nm. The formula for calculating percentage of proanthocyanidins as leucoanthocyanidin equivalent is (absorbance 550 nm x 78.26 x dilution factor)/(%dry matter) .

DPPH radical–scavenging activity

The antioxidant activity was measured in term of hydrogen donating or radical scavenging ability using the stable DPPH method according to the method

proposed by Brand-Williams *et al.* [28]. 250 µl of the extract was diluted with distilled water to 10 ml. Aliquots of 200µl of sample was mixed with 2 ml of 100 µM DPPH methanolic solution. The mixture was placed in the dark at room temperature for 60 min. The absorbance of the resulting solution was then read at 520 nm. The antiradical activity was expressed in terms of the percentage reduction of the DPPH. The ability to scavenge the DPPH radical was calculated using the following equation:

DPPH scavenging effect (%) = $[(A_0-A_1)/A_0]*100$

Where A_0 is the absorbance of the control at 60 min, and A_1 is the absorbance of the sample at 60 min.

RESULTS AND DISCUSSION

Total polyphenols and proanthocyanidins content

The assessment of the solvent systems used was based on two representative indices, the total polyphenol and total proanthocyanidins content.

Table1: Total polyphenol and proanthocyanidins content of carob pods in different solvent extracts

Solvent	Polyphenol content (g GAE/100g)*	Proanthocyanidins content (g/100g)*
Water	3.54±0.003	0.12±0.004
Acetone 70%	4.29±0.015	0.33±0.007
Ethanol 100%	2.72±0.003	0
Ethanol 75%	3.94±0.011	0.11±0.002
Ethanol 50%	4.20±0.006	0.11±0.002
Ethanol 25%	4.43±0.010	0.12±0.002
Ethanol 10%	**4.96±0.005**	0.27±0.008
Ethanol 5%	3.74±0.002	0.11±0.008
Ethyl acetate	0.34±0.009	0

* The values are mean ± standard deviation (n = 3).

The results illustrated in Table 1 indicated that ethyl acetate, the most nonpolar solvent employed, was highly unsuitable for the extraction of polyphenols and proanthocyanidins. This result is in agreement with Makris *et al.* [19]. By contrast, very efficient extraction of total polyphenols could be performed employing 10% ethanol, while 70% acetone gave the highest proanthocyanidins values.

It appears, therefore, that slight modifications in the extracting medium or concentration 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.

In the present study, the total polyphenol content was 4.96±0.005 (g/100 g GAE) and proanthocyanidins content was 0.33±0.007 (g/100 g). In Sicilian carob pods, content of total polyphenols was found at level of 0.19 g/100g dry weight [29]. Another examination of carobs showed their contents in total polyphenols and total proanthocyanidins to be 19.2 and 4.37 g/100g, respectively [30]. Furthermore carob pods were reported to contain 6.1% of total polyphenols [31].

Variations within and among carob pod origins in polyphenol and proanthocyanidins content can be probably due to the geographical, variety, cultural conditions or degree of maturation in origins. This relation was well documented in Sicilian carob pods by Avallone *et al.* and Glew *et al.* who found large variations in the content of total polyphenols (1.58–2.44 g/100g) and proanthocyanidins (0.21–0.39 g/100g dry weight) collected from eight different locations in one geographical region [29, 32].

Antioxidant activity

The interpretation of the antioxidant behavior of extracts is a rather complicated issue, considering that the antioxidant characteristics examined represent the integration of actions of more than one phenolic class. The carob pods extracts obtained with different solvents were further considered for testing the antioxidant characteristics.

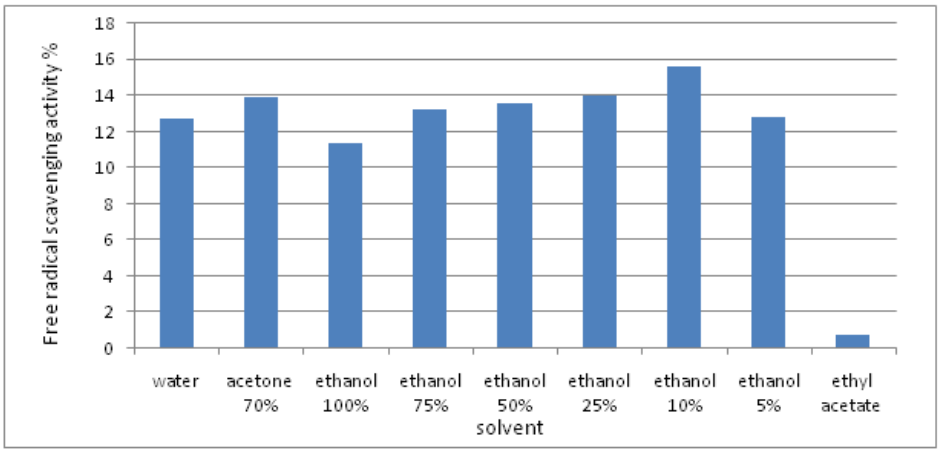

Fig. 1: Comparative diagram illustrating the DPPH assay values of carob pod extracts in different solvents

As presented in Figure.1, ethyl acetate extract showed the lowest radical scavenging activity. Whereas, ethanol:water (10:90) extract showed the highest radical scavenging activity. Based on these results, the DPPH radical scavenging activity of carob pods extracts is related to the polyphenols yield.

Separation of proanthocyanidins

To concentrate and obtain proanthocyanidins rich fractions before analysis, strategies including sequential extraction, liquid-liquid partitioning or solid phase extraction (SPE) based on polarity and acidity have been commonly used.

An example of sequential extraction was provided by extraction of phenolic compounds from tissues of cider apples as reported by Guyot *et al.* [33].The freeze-dried apple tissue powder was extracted sequentially with hexane (to remove lipids, carotenoids and chlorophyll), methanol (sugars, organic acids and phenolic compounds with low molecular weight) and aqueous acetone (polymerized polyphenols).

As illustrated in Table.1, when pure ethanol was used as a solvent, proanthocyanidins were not detected in the extract. While acetone:water mixture (70:30) was the most useful solvent for the extraction of proanthocyanidins from carob pods. Separation of proanthocyanidins from other polyphenolic compounds in the final extract was based on these results. Five sequential extractions were conducted using pure ethanol for the first three fractions and acetone:water mixture (70:30) for the last two fractions, results were given in Table 2.

Table 2: Separation of proanthocyanidins fromcarob podsby sequential extraction

Fraction number	Sequential solvent extraction	Polyphenol content (g GAE/100g)*	Proanthocyanidins content (g/100g)*
1	Ethanol 100%	2.72±0.003	0
2	Ethanol 100%	1.15±0.007	0
3	Ethanol 100%	0.11±0.003	0
4	Acetone 70%	0.09±0.005	0.30±0.004
5	Acetone 70%	0.01±0.003	0.08±0.004

* The values are mean ± standard deviation (n = 3).

As noticed proanthocyanidins were well separated from other phenolic compounds but further purification procedures should be conducted to remove non-phenolic compounds such as sugars, organic acids and lipids.

CONCLUSION:

Carob pods may actually be regarded as a cheap source of naturalphenolic phytochemicals, whose nature andimportance is, as yet, poorly investigated. The studypresented here indicates that efficient polyphenol extractionfrom carob pods might be achieved employingaqueous ethanol. The extracts obtained with this procedureexhibit appreciable antioxidant capacity, an evidencefor the high potential of carobs as a cost-effectivesource of natural antioxidants. Proanthocyanidins present in the extract could be separated from other phenolic compounds by a simple and fast sequential extraction using two different organic solvents.

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