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Total Polyphenols and Gallic Acid Contents in Domesticated Carob (*Ceratonia siliqua* L.) Pods and Leaves

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ABSTRACT

Extraction condition efficiency of total polyphenols was studied on carob pods (Ceratonia siliqua L.) by performing extractions with various solvent systems, in order to evaluate and optimize the conditions of the recovery of polyphenols. Maximum amounts of polyphenols were found in 80% acetone extracts versus water inefficiency. Total polyphenol contents, determined by Folin-Ciocalteu method, were estimated at 11.19 mg/g (dry weight) and the chromatographic analyses demonstrated that gallic acid is the major polyphenol compound of the extracts (45% of polyphenols by dry weight material).

Carob leaves and different parts of fruits were also analyzed for their total polyphenols and gallic acid content. It was found that the mature leaves of the year contain more polyphenols and gallic acid (45.26 mg/g of total polyphenols and 17.01 mg/g of gallic acid). Tegument extracts contained lesser amounts of polyphenols and gallic acid (26.30 mg/g of total polyphenols and 2.51 mg/g of gallic acid), while only traces were detected in germ and endosperm (1.33 and 0.80 mg/g of total polyphenols; 0.99 and 0.72 mg/g of gallic acid, respectively). It should be noted that there are some additional phenolic compounds present whose structures should be precised.

Key words: *Carob tree, Ceratonia siliqua, Extraction, Total polyphenols, Gallic acid.*

Abbreviations: *GAE: Gallic acid equivalents; DM: Dry matter; HPLC-DAD: High-performance liquid chromatography with a diode-array detector.*

INTRODUCTION

Phenolic compounds, bioactive secondary metabolites well-known as antioxidants are widely distributed in the Plant Kingdom and are present in many plant-based foods and beverages. The acceptability of fruit and vegetables for human consumption may be affected by their content of phenolic compounds[1].

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The potential use of polyphenols in human health has lead to researches focused on the separation and characterization of active phenolic compounds in various plant derived foods[2,3,4].

Carob tree (*Ceratonia siliqua* L.) is one of the most original trees of Moroccan biodiversity; it is leguminous forage of the subfamily of Caesalpinioideae, generally dioecious, rarely monoecious and sometimes hermaphrodite, typically Mediterranean. In Morocco, it is present throughout the country in the form of natural or artificial plantations. It is considered as one of the best performing fruit and forest trees, with many valuable socio-economic and ecological interests, which has been the subject of numerous national reforestation plans and projects (Tadla region, Northern Morocco)[5].

All parts of the tree are useful and exploited for their great wealth and added value in several fields. The leaves are a popular fodder and have dietary and medicinal properties (against diarrhea, bactericides). Also, carob fruits find applications in nutritional, dietary, pharmaceutical and cosmetic fields because they contain large amounts of polysaccharides, proteins, fats, sugars, tannins, fibers and mineral salts[6,7]. Ecologically, the carob tree generally grows in arid zones and on poor soils thanks to its capacity to develop adaptation strategies to the different degrees of water stress[8,9].

The aim of the present study is to find the best extraction conditions to quantitatively recover polyphenols from various parts of carob tree. Moreover, we also focus on the determination of both total polyphenols and gallic acid content of carob pods (pulp and seeds) and leaves of different ages.

MATERIAL AND METHODS

Plant material

Both carob mature pods and leaves of different ages (mature leaves, young leaves of the year and old leaves) were collected between August and September 2012 from a domesticated female tree located 30 km on Tetouan-Chefchaoun road, Amtel Region, Moroccan Rif.

To separate the different constituents of the seeds (tegument, endosperm and germ), they were previously soaked for 48 h after scarification according to the technique of Correia and Martin-Louçào[10], with 36N sulfuric acid for one hour, and washed thrice with sterile distilled water for 10 to 15 min.

Before their use, the leaves at different ages and the different constituents of the pods (pulp and seeds) were dried in an oven at 70°C for one week and then ground using a mill balls apparatus. The resulted powder was used for the following analysis.

Extraction procedure

Three extraction methods were used with different solvents: maceration with 80% aqueous acetone, maceration with 80% aqueous methanol and 10% (v/v) aqueous decoction. We made three extracts from pulps, obtained by the three extraction methods mentioned above (Fig. 1).

1. Extraction with 80% aqueous acetone

10 g of pulp powder was strongly stirred with 50 ml of 80% aqueous acetone for 20 min at 30°C. The extraction media was filtered and dried under vacuum.

The entire extraction procedure was repeated 2 additional times. Then, the filtrates were combined before its concentration by evaporation under vacuum at 40°C.

2. Extraction with 80% aqueous methanol

10 g of pulp powder was defatted with 500 ml n-hexane in a Soxhlet apparatus for 3 h. The residue was dried under vacuum at 30°C, immerged into 50 ml of 80% methanol and strongly stirred at 30°C for 20 min. After paper filtration, the filtrate was concentrated to dryness under vacuum at 35°C.

3. 10% aqueous decoction

A 10% aqueous decoction was carried out as follow: 10 g of pulp powder was immerged into 100 ml distilled water and boiled for 1 h. The mixture was then cooled, filtered and concentrated under vacuum.

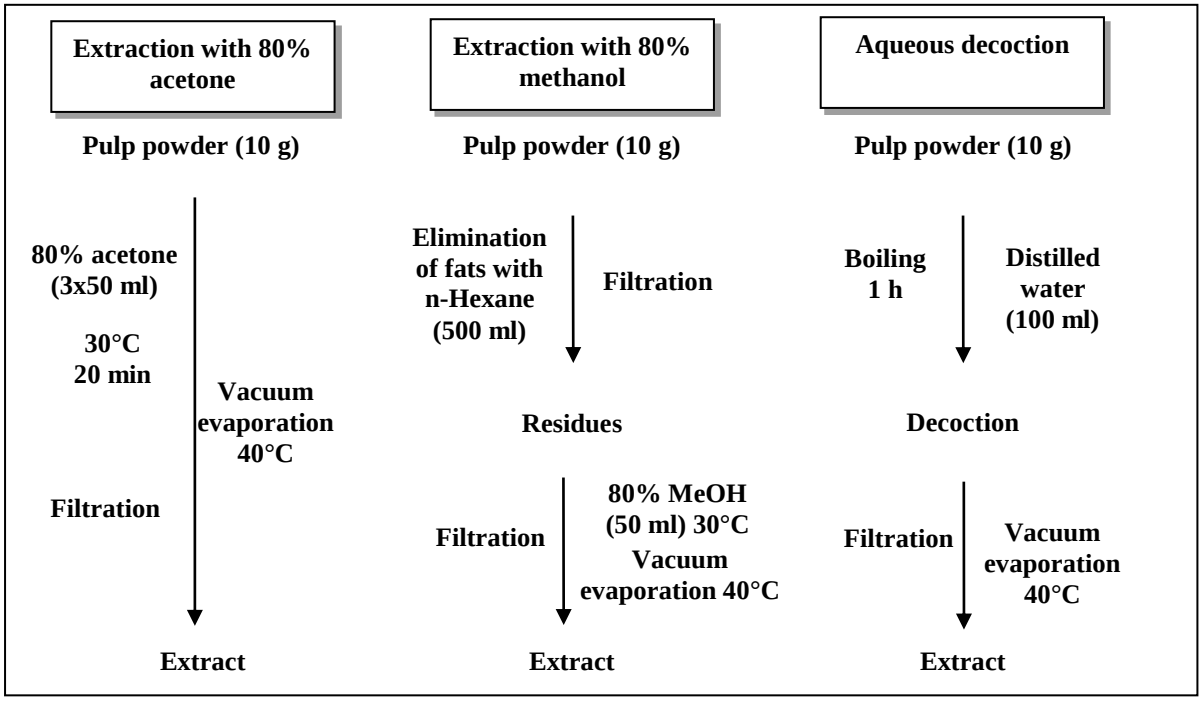

Fig. 1: Schematic representation of the preparation of pulp extracts by three different extraction methods

They were then diluted with 80% methanol until a volume of 25 ml was reached. The extracts were lyophilized and stored until use.

Determination of total polyphenols content

The total polyphenols contents of the extracts of various samples were estimated by the Folin-Ciocalteu method[7]. This biochemical analytical method allows the quantification of phenolic compounds by a spectrophotometric measurement of the extracts.

In a 4 ml tube, 2370 µl of distilled water and 150 µl of Folin-Ciocalteu reagent were added to 30 µl of the sample to be assayed. The mixture was stirred by vortex to homogenize and 450 µl of a 20% saturated sodium carbonate Na_2CO_3 solution was added. The mixture was stirred and incubated at room temperature for 2 h. The yellow color of the reagent turned blue and the absorbance of the sample was measured at 750 nm using a spectrophotometer.

Polyphenols were quantified as gallic acid equivalent (GAE). For this reason, an external calibration was performed using different concentrations of gallic acid (0, 50, 100, 200, 300, 400, 600, 800, 900 and 1000 mg/l).

Sample extracts highly concentrated in polyphenols were diluted 10 times before each assay.

Determination of gallic acid content by HPLC-DAD

Polyphenols were separated on a C18 column (250 mm × 4.6 mm). The solvent was delivered at a flow rate of 0.5 ml/min and consisted of a mixture of acetonitrile and a 2% aqueous acetic acid solution (94:6). The injection volume was fixed at 20 µl.

For the gradient, the acetonitrile passed in 40 min from 0 to 15% and then in 40 min to 45%, followed by 100% acetonitrile for in 40 min. The detection was performed using a diode array detector with a wavelength fixed at 280 nm.

Dry extracts of the different samples were dissolved in 80% aqueous methanol (25 ml) and filtered with a syringe (SRP15, 0.45 µm) prior to injection. Samples being too concentrated in polyphenols were diluted 6 times before filtration and injection by HPLC.

The quantitative analysis consisted in the evaluation of gallic acid concentration in the extracts using external calibration.

A series of standard gallic acid (Sigma, Deisenhofen, Germany) solutions consisting of eight concentration points (0, 50, 100, 200, 400, 600, 800, and 1000 µg/ml) was prepared and injected into the HPLC analyzer with

identical volumes. The graph of the peak area of the standards was plotted as a function of their concentration.

All results in gallic acid and polyphenols were given in mg/g of dry starting carob material.

RESULTS

Solvent extraction efficiency

Three extraction solvent have been tested on polyphenol extraction on carob pulp (Fig. 2).

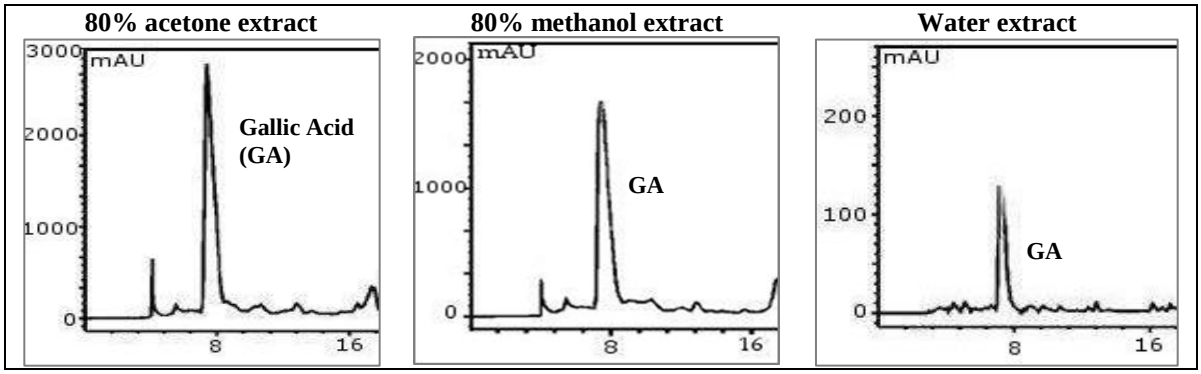

Fig. 2: Chromatograms recorded by HPLC-DAD analysis (280 nm) of carob pulp extracts, obtained with three various solvents (80% acetone, 80% methanol and distilled water)

There is almost no significant qualitative difference between acetone and methanol extraction showing a lot of signals, but with gallic acid being the major one. However, extraction with 80% aqueous acetone leads to higher contents of gallic acid than for methanol-based extraction and even more than water decoction. The phenolic profile of carob pulp extracts was dominated by gallic acid, identified by comparing its retention time (7.3 min) with that of pure injected gallic acid as control.

Extraction with 80% aqueous acetone gives the best results in term of gallic acid content, while extraction with 80% aqueous methanol is half time less efficient, and water decoction is almost inefficient (Table 1).

Table 1: Extraction efficiency of gallic acid according to three extraction solvents

Solvent	Gallic acid (mg/g DM)
80% Acetone	5.04
80% Methanol	2.51
Distilled water	0.48

For this reason, the 80% aqueous acetone method was chosen for extraction of gallic acid from carob by-products.

Total phenolic content of carob leaves and pods

Total polyphenols content, expressed as gallic acid equivalents in the 80% acetone extracts of different samples, using the Folin-Ciocalteu method shows that carob pulp contains a high level of total polyphenols (11.19 mg GAE/g DM) (Table 2).

Table 2: Total polyphenols and gallic acid contents in carob pulp, seed and leaves of domesticated carob tree

Samples	Total phenolic content (mg GAE/g DM)[a]	Gallic acid (mg/g DM)[b]
Pulp	11.19	5.04
Seed:		
Tegument	26.30	2.51
Endosperm	0.80	0.99
Germ	1.33	0.72
Leaves of the year:		
Mature leaves	45.26	17.01
Young leaves	26.27	7.08
Old leaves	32.01	7.04

a: Results expressed in mg gallic acid equivalents (GAE) per g of dry matter of the sample

b: Results expressed in mg of gallic acid per g of dry matter of the sample

It is noted that polyphenols content of the tegument (26.3 mg GAE/g DM) is very high in comparison with endosperm and germ (0.80 mg GAE/g DM and 1.33 mg GAE/g DM, respectively). Mature leaf extract of the year reveals also to be an important source of total polyphenols (45.26 mg GAE/g DM).

Identification and determination of gallic acid content in carob pods and leaves

Gallic acid which was identified in pulp extract (5.04 mg/g DM) was also found in the extracts of other samples analyzed, from 0.72 to 17.01 mg/g DM (Table 2). Mature leaves extract of the year shows a higher content of gallic acid than the extracts of old and young leaves (17.01 vs. 7.04 and 7.08 mg/g DM, respectively).

Seed tegument is richer in gallic acid (2.51 mg/g DM) than the other constituents of the seed, where it is present in traces: 0.72 and 0.99 mg/g DM in germ and endosperm, respectively.

DISCUSSION

Extraction with 80% acetone makes it possible to recover the majority of polyphenols from carob pulp. For the extraction of polyphenols, several solvents may be used. Hexane or dichloromethane are used beforehand to remove fats and chlorophyll[11]. According to the literature, acetone at different dilutions is the most used and most efficient solvent for the extraction of polyphenols. Avallone et al.[12], Sebai et al.[13] and Dhaouadi et al.[14] tested pure acetone and other authors used 80% acetone[7,15,16]. 70% acetone is the most common solvent[12,17-22] and 50% acetone was also used[23,24,25]. Similarly, methanol (pure, 80, 85, 70%) was used for the extraction of polyphenols[7,12,13,26-32], as well as ethanol (pure, 75, 50, 30, 25, 10 and 5%)[13,16,33-35]. Several authors have performed extraction with distilled water at room temperature or in decoction[13,34,36-45]. Roseiro et al.[21] tested the extraction with supercritical CO_2 as solvent and 80% ethanol as co-solvent.

We obtain a content of 11.19 mg GAE/g of dry matter. Total polyphenols contents of carob pulp mentioned in the literature range from 0.0003 to 198.2 mg GAE/g DM. The minimum content is reported by Sebai et al.[13] after extraction of an immature pulp powder with petroleum ether. Extraction with distilled water yielded the best results: 186.10 mg GAE/g DM[39]; 192 mg GAE/g DM[36]; 198.2 mg GAE/g DM[44]. For acetone extraction, total polyphenols contents, obtained by other studies, range from 3.50 mg GAE/g DM[25] to 42.9 mg GAE/g DM[34].

The phenolic profile was dominated by gallic acid with a content of 5.04 mg/g DM (45% polyphenols by weight). Gallic acid content of pulp, determined by HPLC/MS or HPLC/DAD ranges from 1.20 to 10.44 mg/g DM[14,17,19,26,30,32,33,37].

Carob leaves extract was also found to be very rich in phenolic compounds compared to pulp. Polyphenols and gallic acid contents in mature leaves of the year (45.26 mg GAE/g DM and 17.01 mg/g of gallic acid) are higher than those of young and old leaves. Studies on total polyphenols and gallic acid contents of carob leaves are few. Corsi et al.[37] quantified 6.28 mg of total polyphenols per g of carob leaves and 4.30 mg/g of gallic acid. Custódio et al.[46] found 261.1 mg/g total polyphenols and 26.9 mg gallic acid /g DM, determined by HPLC-DAD. Recently, a study recorded polyphenols contents of successive extracts of *Ceratonia siliqua* L. leaves, varying between 91.2 and 680 mg GAE/g DM in the following order: ethyl acetate fraction> dichloromethane fraction> hexane fraction[47]. Also, extraction with acetone yielded 21.41 mg GAE/g[16]. In addition, Ghanemi et al.[48] carried out extraction with methanol-acetone-water (7:7:6, v/v/v) and obtained 9.215 mg GAE/g DM. Indeed, HPLC analysis showed that organic extract contained high contents of m-coumaric acid (2192.38 µg/g DM) and gallic acid (1445.38 µg/g DM)[48].

The tegument shows a high level of phenolic compounds as well as gallic acid (26.3 mg GAE/g DM and 2.51 mg/g of gallic acid), while the endosperm and the germ contain only traces of these compounds. The highest total polyphenols content has also been found in the germ, from 24.85 to 40.8 mg GAE/g

DM[12,32,46,49], whereas these compounds have been reported in traces (0.0834 mg/g DM[23]) in the endosperm. In general, total polyphenols content of the whole seed oscillates between 0.66 and 22.05 mg GAE/g DM[12,49], with gallic acid as one of the main compounds, according to the results of Mahtout et al.[32] (0.84 mg/g DM).

CONCLUSION

Polyphenols contents and composition varies from one author to another. The fluctuations obtained must be due to various factors such as the variety of carob tree, the producing country, the analyzed part (pulp, seed, fiber, soluble part or insoluble residual part), methods used for the extraction or the determination of polyphenols[50].

The determination of total polyphenols and gallic acid contents in different parts of carob pods and in the leaves of different ages, reveals differences in the content of these compounds between the different structures analyzed, suggesting a variation in the biosynthesis of polyphenols in carob tree. The present study shows that these metabolites are produced more intensely in the leaf than other organs, and that gallic acid is the main phenolic compound in carob leaves, pulps and seeds.

Carob pods remain a non-negligible source of polyphenols and a potential natural antioxidant that could contribute to the prevention of various pathologies.

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