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Research Paper

Determination of Phenolic Composition of Carob pods Grown in Different Regions of Morocco

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ABSTRACT

The phenolic contents of ethanolic extracts of carob pods from six regions in Morocco were measured in this work. The identification and quantification of individual target polyphenolic compounds were performed by high performance liquid-chromatography electrospray-ionisation mass spectrometry (HPLC-ESI-MS) and gas-chromatography mass spectrometry (GC-MS). A total of fifty two phenolic compounds were identified with a yield ranged from 9.15 mg/gm to 55.73 mg/gm. The predominant polyphenolic compounds in all analyzed carob pods extracts were gallic acid, gallate glucoside and gallic acid glucoside. Gallic acid was the principal free phenolic acid in carob pods accounting for 17.96 to 32.92% of the total phenolics present. Gallate glucoside was detected only in two regions (Essaouira and Beni-Mellal) with higher concentration (22.02% and 36.70%). Content of gallic acid glucoside ranged from 23.07 to 57.03% of the total phenol level in carob pods. Flavonol glycosides constituted 6.41 to 21.16% of the polyphenols, and the major components were identified as the glycosides myricetin rhamnoside and quercetin rhamnoside.

Keywords: Phenolic compounds; Carob pods; HPLC-ESI-MS; GC-MS.

INTRODUCTION

The carob tree (*Ceratonia siliqua* L.), also called algarroba, locust bean and St. John's bread, is a leguminous evergreen tree which grows throughout the Mediterranean region, mainly in Spain, Morocco, Italy and Portugal. The fruit pod (containing sweet pulp) gives, after removal of the seeds, carob powder (Yousif and Alghzawi, 2000) often used as a chocolate or cocoa substitute (Nyerges, 1978; Brand, 1984). The advantage of using carob as a chocolate substitute, resides in that carob is an ingredient free from caffeine and theobromine. In Europe several commercial carob products are available as Carovit™ (Alimcarat S.L., Spain). Carovit™ is roasted carob flour, used as a cocoa substitute in baking, cereal bars, chocolate confectionery, ice creams and light products. Carob pods are characterized by a high

sugar content (more than 50%) mainly composed of sucrose. However, (Avallone, et al., 1997) determined carob pods composition. High content of carbohydrates (45%, sucrose at more than 30%), appreciable amounts of protein (3%) and low levels of fat (0.6%) were detected. High tannin content is also present in carob pods composition, which limits the consumption by cattle because of reduced digestibility (Priolo, 2000). Nevertheless, the isolation and quantification individual of phenolic compound of carob pods from which the carob fiber is obtained has not been studied in depth. Most previous studies have concentrated on the polyphenolic content of carob pods with little in the way of definitive structure elucidation. (Corsi, et al., 2002) studied the phenolic fraction of carob pods by infusion with boiling water (2-15 min). A total of 1.36 mg/kg was detected dominated by gallic acid (88%) with relevant contributions by the catechins, epigallocatechin and epicatechin gallate. (Sakakibara, et al., 2003) using methanol as solvent, but with batch extraction and sonication for a very short period (3-1 min) gave similar data to that of (Corsi, et al., 2002) but with a significantly lower yield (0.23 gm/kg). The profile was again dominated by gallic acid (91%) with minor contributions from quercetin glycosides, catechins and ellagic acid. (Owen, et al., 2003) reported individual polyphenolic contents in carob fibre extracted with methanol in a Soxhlet apparatus using HPLC coupled with electrospray ionization tandem mass spectrometry (HPLC–ESI-MS/MS) and gas-chromatography mass spectrometry (GC-MS). A total of 24 polyphenol compounds were identified with a yield of 3.94 gm/kg (dry weight). The profile was dominated by gallic acid in various forms: free gallic acid (42% of polyphenols by weight), gallotannins (29%), and methyl gallate (1%), while simple phenols, mainly cinnamic acid, made up about 2% of the total. Flavonoids represented 26% of the polyphenols, and the major components were identified as the glycosides myricetin- and quercetin-3-*O*-α-L-rhamnoside (ca. 9% and 10%, respectively). Recently, (Faik, et al., 2007) studied identification and quantification of polyphenols in carob fruits of Turkey, This study reports that total phenolics compounds were obtained in the yield of 17.49 mg/gm (dry weight). Gallic acid (3.27 mg/gm dry weight) was the most abundant phenolic acid present in all three phenolic fractions (free, ester and glycoside) isolated from pods.

However, to date, studies on the isolation and identification of phenolic compounds from Moroccan carob pods has not been published. The objective of this study was to determine the variation and composition of phenolic compounds in ethanol extracts of six samples of carob pods collected in different regions of Morocco.

MATERIALS AND METHODS

Samples: Samples of carob were collected during August and September 2005 from different regions of Morocco where they grow naturally, in the following areas: Essaouira (south-west of Morocco), Beni-Mellal (Center of Morocco), Tafraout (Center-southern of Morocco), Nador (North of Morocco), Fes (West of Morocco), Taza (Western-north of Morocco. Samples were stored at room temperature in our laboratory and analyzed within three months of collection. Pods were randomly selected and prepared by removing the seeds and grinding them in a ball mill.

Extraction of carob pods: Carob pod (10 gm) was extracted in a Soxhlet apparatus, first with hexane (4 h) to remove lipids and then with ethanol (5 h). The extract obtained was evaporated to dryness on a rotary evaporator at 35°C.

Column chromatography on silicic acid: The dried residue was suspended in methanol (50 ml), immobilized on silicic acid by lyophilisation, and subjected to

column chromatography (CC), using a 38 x 4.5 cm glass column filled with silica gel 60 (mesh size: 70–230) in DCM to a level ca. 5 cm from the top. The immobilised extract was added to the free volume at the head of the column. After bedding down of the gel material, fractionation was conducted by successive applications of methanol (1, 2, 5, 10, 20 and 30%) in DCM (1 L). Fractions (200 ml) were collected, and the solvent was removed by rotary evaporation in vacuo at 35 °C. Dried fractions were suspended in methanol (5.0 ml) and diluted when necessary prior to analytical high-performance liquid chromatography (HPLC). Phenolic compounds in the relevant fractions were purified by semi-preparative HPLC for spectroscopic analysis.

Analytical HPLC: Analytical HPLC was conducted on a Hewlett-Packard (HP) 1090 liquid chromatograph fitted with a C-18, reversed-phase (5 mm) column (25 cm x 4 mm I.D.; Latex, Eppelheim, Germany). For the separation of individual compounds in methanolic extracts of carob pods and in fractions obtained by column chromatography, the mobile phase consisted of 2% acetic acid in doubly distilled water (solvent A) and methanol (solvent B), utilising the solvent gradient published previously (Owen et al., 2000a; 2000b; 2003). Phenolic compounds in the eluant were detected with a UV diode-array detector (HP 1040M) set at 250, 278 and 340 nm. Extracts of carob pods were dissolved in methanol (5.0 ml) and diluted, when necessary, prior to injection (20 µl) into the HPLC. The flow rate of the mobile phase was 1 ml/min. The amounts of phenolic compounds in fractions obtained by column chromatography were determined using calibration curves generated with authentic standards in duplicate by measuring the UV absorption at □max as a function of concentration in the range 0.025–4.0 mM.

Semi-preparative HPLC: Semi-preparative HPLC was conducted on an Agilent 1100 liquid chromatograph fitted with a reversed-phase C18 column (10 mm I.D.; Latek, Eppelheim, Germany) similar to that used for analytical HPLC. Acetonitrile was used instead of methanol as mobile phase with a flow rate of 3 ml/min. Peaks eluting from the column were collected on an Agilent HP 220 Microplate Sampler. Each purified fraction was pooled, and solvent was removed by lyophilisation.

Alkaline hydrolysis: From each fraction collected by column chromatography, 50 µl of the methanolic suspension were dried under a stream of nitrogen and 10% KOH in methanol (100 ml) was added. The solutions were incubated at 100 °C for 1 h. Doubly distilled water (1.0 ml) was added, and the solutions were acidified by addition of conc. HCl (20 µl) and extracted twice with diethyl ether (2.0 ml). Samples were dried over anhydrous sodium sulphate, and diethyl ether was removed under a stream of nitrogen. The residue was suspended in methanol (500 µl). Samples (100 µl) of these solutions were again dried under a stream of nitrogen prior to the formation of the trimethyl silyl ethers using BSTFA (100 µl, 37 °C for 30 min), and these derivatives were analysed with GC-MS.

Acid hydrolysis: The workup was similar to that described for alkaline hydrolysis, except that H_2SO_4 (0.5 M) in doubly distilled water replaced KOH (10%) in methanol and incubation was for 1 h at 37 °C. Doubly distilled water (1.0 ml) was added and the samples were extracted twice with diethyl ether (2.0 ml).

GC–MS: Analyses were performed exactly as described by (Owen et al., 2003). Prior to GC–MS, dried methanolic extracts (10 ml) were derivatized by addition of BSTFA (100 ml) at 37 °C for 30 min.

HPLC–ESI-MS: HPLC–ESI-MS was conducted on an Agilent 1100 HPLC, coupled to an Agilent single quadrapole mass-selective detector (HP 1101; Agilent Technologies, Waldbronn, Germany). Chromatographic separation was conducted using a C18, reversed-phase (5 µm) column (250 x 4 mm I.D.; Latek, Eppelheim,

Germany). The mobile phase was 2% acetic acid in double distilled water (solvent A) and methanol (solvent B), utilising the solvent gradient published previously (Owen et al., 2008). The flow rate of the mobile phase was 0.5 ml/min. HPLC-ESI-MS was performed in the negative- ion mode under the following conditions: drying gas (nitrogen) flow = 10 l/min; nebulizer pressure = 30 psi; drying gas temperature = 350 °C; capillary voltage = 2500 V; fragmenter voltage = 100 V; mass range = 50-3000 D.

RESULTS AND DISCUSSION

Fifty-two phenolic compounds were identified in the ethanol extracts of six samples of carob pods from different regions in Morocco. The analyses of the samples from Essaouira, Béni-Mellal, Tafraout, Nador, Fès, Taza showed the presence of 41, 32, 18, 28, 20 and 39 identified phenolic compounds accounting for 3.28 mg/gm, 4.77 mg/g, 16.5 mg/gm, 9.15 mg/gm, 18.75 mg/gm and 55.73 mg/gm of the whole extracts, respectively. There are some quantitative differences in the phenolic composition of the carob pods between the six regions (Tables 1 and 2). From the data obtained, we observed that contents gallic acid, gallate glucoside and gallic acid glucoside were the highest of all phenolic compounds. The gallic acid and gallic acid glucoside compounds from Taza presents higher concentration (10.43 mg/gm and 16.63 mg/gm) than other regions. Gallate glucoside content (17.51 mg/gm) was highest in Beni-Mellal, it was only detected in two regions. On the other hand, gallate derivatives such as digallate, trigallate and tetragallate were found in means quantities in Moroccan carob pods. The highest concentration of digallate (4.8 mg/gm), trigallate (5.7 mg/gm) and tetragallate (9.9 mg/gm) was detected in Taza region. Some other compounds were identified in the class of simple phenols such as *p*-coumaric acid, cinnamic acid, ferulic acid, methoxy-*p*-OH-benzaldehyde, vanilic acid, *p*-hydroxybenzoic acid, ethylgallate and *cis*-*p*-coumaric acid with smaller quantities flavonol glycoside (above 0.5 mg/gm). In the case of flavonoids, some glycosides of flavonols are present in all samples of carob pods, but they are more abundant in Essaouira (5.92 mg/gm) and in Taza (5.54 mg/gm) than in the other regions, with significant difference. The major flavonol glycoside components identified were myricetin rhamnoside (0.6-1.7 mg/gm) and quercetin rhamnoside (0.6-2.3 mg/gm). Smaller quantities of (below 2.9% of the total polyphenols) the hydroxyflavone derivatives were found in the class of flavones.

Our study shows the variability on the level of the yield and significant difference of the phenolic composition in the six samples of carob pods. This variability of phenolic composition can be explained by several factors. Among these factors, the geographical origin and the nature of the cultivar are certainly those that have a pronounced influence on the composition.

CONCLUSION

This is the first extensive study of polyphenolic content in carob pods grown in different regions of Morocco included determination of fifty two phenolic compounds. The results of the study showed that in the all analysed samples the most abundant polyphenols were Gallic acid, Gallate glucoside and acid Gallic glucoside. Gallate glucoside was detected only in two regions (Essaouira and Beni-Mellal) with higher concentration. Among all the ethanolic extracts analysed, the samples of Essaouira, Béni-Mellal and Taza exhibited a significantly higher phenolic content than other samples. A relatively high variation in their contents was found.

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Table-1: **Concentration of phenolic compounds in carob pods of six regions of Morocco.**

Compound	Essaouira	Béni-Mellal	Tafraout	Nador	Fès	Taza
	Yield µg/gm extract					
3,4-DHBA	30.3	-	-	-	-	25.2
Apigenin	3.7	7.4	4.7	-	8.7	-
3,4,7-Trihydroxyflavone	-	11.6	-	-	-	22.6
7,4'-dihydroxyflavone	-	-	-	15.2	18.7	-
Benzoic acid	203.2	154.3	-	168.9	90.5	73.3
Chrysoeriol	36.5	-	-	-	-	21.3
Cinnamic acid	6.9	38.1	-	68.5	44.3	111.0
cis-p-coumaric acid	16.4	-	-	-	-	-
Digallate	2334.7	3114.3	908.8	361.5	2052.1	4774.4
Dihydoxyflavone	5.8	18.3	-	-	-	-
Dimethoxy-p-OH-benzaldehyde	2.1	-	-	-	-	-
Dimethoxybenzaldehyde	-	-	-	-	-	8.4
Eriodictyol glucoside	424.8	-	-	13.8	40.5	259.6
Eriodictyol	2.2	9.4	-	15.7	-	-
Ethylgallate	215.6	173.4	15.5	17.7	198.0	210.2
Ellagic acid	-	331.8	36.6	14.4	26.9	205.8
Ferulic acid	5.2	20.0	-	27.9	-	38.9
Gallate glucoside	7227.9	17517.4	-	-	-	-
Gallic acid	8869.4	8573.6	3118.1	3011.5	3840.4	10434.0
Gallic acid glucoside	132.3	-	9424.8	2109.9	8232.2	16629.2
Genistein	16.5	-	-	5.1	-	4.5
Genistein glucoside	106.7	53.3	-	38.7	105.1	114.9
Genistein-4,7-dimethyl ether	15.9	-	-	-	-	-
Genistein dimethylether	-	6.6	-	-	-	-
Gallic acid diglucoside	-	-	-	-	-	928.3
Isorhamnetin	237.1	200.6	35.2	-	289.9	224.2
Kaempferol rhamnosise	-	-	-	-	-	82.8
Liquiritigenin	10.4	-	-	-	-	-
Methoxy Genkwanin	145.1	97.2	-	73.3	-	57.5
Methoxy-p-OH-benzaldehyde	24.7	9.6	2.7	-	-	23.2
Myricetin glucoside	1411.2	644.5	369.5	276.9	-	1082.2
Myricetin rhamnoside	1451.2	1160.4	568.7	712.2	546.9	1672.6
Methylgallate	-	40.4	-	-	-	25.7
Methoxybenzaldehyde	-	-	-	4.7	-	-
Methoxy Genkwanin glucoside	-	-	-	-	-	59.1
Naringenin	34.1	10.5	5.1	18.9	-	14.0
Naringenin glucoside	240.0	58.6	-	-	43.9	146.6
p-coumaric acid	60.7	30.5	11.3	29.7	11.6	31.2
p-HBA	2.0	18.6	-	5.7	-	22.2
p-OH-benzaldehyde	33.1	8.5	5.5	6.9	-	6.1
Quercetin	31.2	-	-	9.8	-	50.2
Quercetin arabinoside	162.0	-	-	-	-	138.6
Quercetin glucoside	578.7	-	123.7	115.5	118.6	462.9
Quercetin rhamnoside	2314.8	1254.8	714.0	830.6	634.5	1950.5
Syringic acid	1.3	6.1	-	-	-	15.0
Taxifolin	38.3	23.6	-	-	-	39.4
Tetragallate	3803.1	9689.9	470.6	487.5	1335.8	9975.0
Tricetin-3'5'-dimethylether	83.3	-	-	-	79.2	-
Trigallate	2466.7	4348.0	693.8	516.6	1037.1	5683.8
Trihydroxy trimethoxy flavone	23.8	-	-	-	-	-
Tricetin	-	81.3	16.3	174.4	-	82.8
Vanillic acid	7.0	15.8	-	15.4	-	28.8
Total	32816.3	47728.2	16524.9	9146.9	18755	55735.8

Table-2: **Percentage of phenolic compounds in carob pods of six regions of Morocco.**

Compound	Essaouira	Béni-Mellal	Tafraout	Nador	Fès	Taza
	% of total of phenolic					
3,4-DHBA	0.09	-	-	-	-	0.04
Apigenin	0.01	0.02	0.03	-	0.05	-
3,4,7-Trihydroxyflavone	-	0.02	-	-	-	0.04
7,4'-dihydroxyflavone	-	-	-	0.17	0.10	-
Benzoic acid	0.62	0.32	-	1.85	0.48	0.13
Chrysoeriol	0.11	-	-	-	-	0.04
Cinnamic acid	0.02	0.08	-	0.75	0.24	0.20
Cis-p-coumaric acid	0.05	-	-	-	-	-
Digallate	7.11	6.52	5.50	3.95	10.94	8.57
Dihydoxyflavone	0.02	0.04	-	-	-	-
Dimethoxy-p-OH-benzaldehyde	0.01	-	-	-	-	-
Dimethoxybenzaldehyde	-	-	-	-	-	0.02
Eriodictyol glucoside	1.29	-	-	0.15	0.22	0.47
Eriodictyol	0.01	0.02	-	0.17	-	-
Ethylgallate	0.66	0.36	0.09	0.19	1.06	0.38
Ellagic acid	-	0.70	0.22	0.16	0.14	0.37
Ferulic acid	0.02	0.04	-	0.30	-	0.07
Gallate glucoside	22.02	36.70	-	-	-	-
Gallic acid	27.03	17.96	18.87	32.92	20.48	18.72
Gallic acid glucoside	0.40	-	57.03	23.07	43.89	29.84
Genistein	0.05	-	-	0.05	-	0.01
Genistein glucoside	0.32	0.11	-	0.42	0.56	0.21
Genistein-4,7-dimethyl ether	0.05	-	-	-	-	-
Genistein dimethylether	-	0.01	-	-	-	-
Gallic acid diglucoside	-	-	-	-	-	1.66
Isorhamnetin	0.72	0.42	0.21	-	1.55	0.40
Kaempferol rhamnosise	-	-	-	-	-	0.15
Liquiritigenin	0.03	-	-	-	-	-
Methoxy Genkwanin	0.44	0.20	-	0.80	-	0.10
Methoxy-p-OH-benzaldehyde	0.07	0.02	0.02	-	-	0.04
Myricetin glucoside	4.30	1.35	2.24	3.03	-	1.94
Myricetin rhamnoside	4.42	2.43	3.44	7.79	2.92	3.00
Methylgallate	-	0.08	-	-	-	0.05
Methoxybenzaldehyde	-	-	-	0.05	-	-
Methoxy Genkwanin glucoside	-	-	-	-	-	0.11
Naringenin	0.10	0.02	0.03	0.21	-	0.03
Naringenin glucoside	0.73	0.12	-	-	0.23	0.26
p-Coumaric acid	0.18	0.06	0.07	0.32	0.06	0.06
p-HBA	0.01	0.04	-	0.06	-	0.04
p-OH-benzaldehyde	0.10	0.02	0.03	0.07	-	0.01
Quercetin	0.09	-	-	0.11	-	0.09
Quercetin arabinoside	0.49	-	-	-	-	0.25
Quercetin glucoside	1.76	-	0.75	1.26	0.63	0.83
Quercetin rhamnoside	7.05	2.63	4.32	9.08	3.38	3.50
Syringic acid	0.01	0.01	-	-	-	0.03
Taxifolin	0.12	0.05	-	-	-	0.07
Tetragallate	11.59	20.30	2.85	5.33	7.12	17.90
Tricetin-3'5'-dimethylether	0.25	-	-	-	0.42	-
Trigallate	7.52	9.11	4.20	5.65	5.53	10.20
Trihydroxy trimethoxy flavone	0.07	-	-	-	-	-
Tricetin	-	0.17	0.10	1.91	-	0.15
Vanillic acid	0.02	0.03	-	0.17	-	0.05