Isolation and Structure Elucidation of Phenolic Compounds of Carob Leaves Grown in Egypt

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Abstract: Carob leaves (Ceratonia siliqua L.), grown in Egypt have not fully identified in Egypt. Therefore, eight major phenolic compounds were isolated from the aqueous ethanolic extract using different methods of chromatography; gallic acid, quercetin 3-O-C7,B-D-glucoside (Isoquercitin), Kaempferol 3-O-C7,α-L-rhamnoside (Afzelin), Quercetin 3-O-C7,α-L-rhamnoside (Quercitin), 1,2,6 tri-O-galloyl-B-D-C7-glucopyranose, (-)Epigallocatechin-3-O-gallate, kaempferol and quercetin. Their structures established by conventional methods of analysis. This is the first phytochemical study of polyphenolic content of carob leaves grown in Egypt.

Keywords: Aqueous ethanolic extract, carob leaves, ceratonia siliqua, phenolics

INTRODUCTION

The tree of carob is widely cultivated in the Mediterranean area. It is considered as an important component of vegetation for economic and environmental reasons (Batle, 1997). Carob fruits are rich in phenolic compounds. The main polyphenolic constituents isolated from carob pods are insoluble, highly polymerized condensed tannins containing a flavon nucleus (Würsch et al., 1984). Degredation experiments with hydrochloric acid yielded (+)-catechin, (-)-epicatechin gallate ester, (+)-epicatechin gallate, (-)-epigallocatechin gallate and (-)-epigallocatechin or delphinidin, pelargonidin and cyaniding, respectively, indicating that the polymers are composed of flavan-3-ol and flavan-3, 4-diol subunits (Tamir et al., 1971; Marakis et al., 1993, 1997).

Water extractable polyphenols of carob pulp were found to include gallic acid, (-)-epigallocatechin, (+)-catechin, (-)-epicatechin gallate, and (-)-epigallocatechin gallate with gallic acid as the main detectable components (Manson et al., 1992; Avallone et al., 2002), while extraction with 90% methanol containing acetic acid (0.5%) afforded, in addition, quercetin glycosides and ellagic acid (Sakakibara et al., 2003). HPLC analysis of carob pod and leaf extracts revealed the presence of gallic acid, (-)epigallocatechin 3-gallate and (-)epigallocatechin 3-gallate. These compounds are well known to exert antiproliferative effects in a concentration reached 6.28 mg/g in carob leaves and 1.36 mg/g in carob pods extract (Corsi et al., 2002).

Recent study was performed on carob pods extract grown in Morocco where the predominant polyphenolitics were gallic acid, gallate glucoside and gallic acid glucoside (Rakib et al., 2010).

Carob leaves grown in Egypt have not been subjected to an in-depth phytochemical investigation. From this standpoint, the study describes herein the isolation and structure elucidation of eight phenolics from the aqueous ethanolic extract of carob leaves.

MATERIALS AND METHODS

Plant source: Carob leaves were collected from Orman garden in Giza, Egypt, 2006. They were authenticated by Prof. Dr. Abdel Salam El Noyehy, Prof. of Taxonomy, Faculty of Science, Ain Shams University, Cairo, Egypt. Voucher specimen was deposited at the herbarium of Pharmacognosy Department, Faculty of Pharmacy, Ain Shams University, Cairo, Egypt. The leaves were dried in shade and reduced to a fine powder.

Chemicals: Kaempferol, quercetin, apigenin and luteolin (from NMR department, NRC, Cairo), glucose, rhamnose and glucuronic acid (from E.Merk, Darmstadt Germany), (-)-Epigallocatechin-3-O-gallate from Pharmacognosy Department, Faculty of Pharmacy, Ain Shams University, Sheets of Whatman paper No.1 and No.3 MM Paper Chromatography (Whatman Ltd. Maidstone, Kent, England), Sephadex LH-20 (25-100μm) (Pharmacia-Fine Chemicals, Sweden), Polyamide powder polyamide 65 for CC, Riedel-De Haen-AG, Sheeze, Seelze-Hannover, Germany.

Extraction protocols: Powders of air dried leaves of carob (1 kg) were extracted by 70 % ethanol on cold till exhaustion. The solvent was distilled of in rotary evaporator at 55°C till dryness. The extract was concentrated till constant weight 80 g and kept in vacuum desiccator over anhydrous calcium chloride.

Ultraviolet spectrophotometric analysis: Chromatographically, pure materials dissolved in
analytically pure methanol (definite concentration), were subjected to UV spectrophotometric investigation in 4 mL capacity quartz cells (1 cm thick) using a Carl Zeiss spectrophotometer PMQ II. AICl3, AICl/HCl, fused NaOAc/H3BO3 and NaOMe reagents were separately added to the methanolic solution of investigated material and UV measurements were then carried out.

Nuclear magnetic resonance spectroscopic analysis: The NMR spectra were recorded on a Varian Mercury VX-300 NMR spectrometer. H spectra run at 300 and 500 MHz and 13C spectra were run at 75.46 MHz in deuterated dimethylsulphoxide (DMSO-d6). Chemical shifts are quoted in δ and were related to that of the solvents. The mass spectra were recorded on a Shimadzu GCMS-QP-1000EX mass spectrometer at 70 eV.

Isolation and identification: The isolation and purification of the phenolic components were achieved through the application of the concentrated extract (60 g) to a Sephadex LH-20 column (150x4.5 cm) and eluted with water followed by H2O-MeOH mixtures of decreasing polarities to yield five fractions (I-V). Compound (1) 22.0 mg and (2) 35.0 mg were isolated from fraction I (eluted with water) by polyamide column fractionation using H2O-MeOH (decreasing polarity) for elution then preparative paper chromatography (PPC) using HOAc-15%. Compound (3) 8.3 mg and (4) 10.0 mg from fraction II (eluted with 50% MeOH). Compound (5) was separated from fraction III (eluted with 80%) by Sephadex LH-20 column using saturated water butanol 11.7 mg. Purification of the compound achieved through PPC. Compound (6) 15.2 mg, (7) 8.0 mg and (8) 7.7 mg from fraction IV paper were chromatographically analyzed using preparative paper chromatography on Whatmann paper No. 3 and 6% acetic acid as a developing system.

RESULTS AND DISCUSSION

The result of this study shows that carob leaves contain varieties of individual compounds from several classes: simple phenols, polyphenols, free flavonoids, glycosylated flavonoids and gallotannins. Altogether, eight major individual structures were identified via conventional methods of analysis, these are gallic acid, quercetin 3-O-’C,-B-D-glucoside (Isoquercetin), Kaempferol 3-O-’C,-a-L-rhamnoside (Azelin), Quercetin 3-O-’C,-a-L-rhamnoside (Quercitin), 1,2,6 tri-O-galloyl-B-D-’C,-glucopyranose, (-)-Epigallocatechin-3-O-gallate, kaempferol and quercetin.

CONCLUSION

The aqueous ethanolic extract of carob leaves showed antioxidant activity via scavenging the reactive oxygen species (Eldahshan, 2006). Furthermore, polyphenols act as agonists and/or antagonist with carcinogen-related receptors such as epidermal growth factor (Ashida et al., 2000), aryl hydrocarbon receptor (An et al., 2001), and oestrogen receptors (Agullo et al., 1997), and modulate the expression of protein kinases in tumour cell populations (Kobuchi et al., 1999). There is a marked cytotoxic activity of crude aqueous extract of carob on both mammalian cell lines (Vero and HEP-2) and the effect is more obvious on the human cell line (HEP-2) (Nagib et al., 2010).

Carob is an important source of food in tropical regions, but at present the leaves are discarded. These waste products which contain a lot of phenolics appear to have real potential low-cost source of antioxidant as well as anticancer drug.

Studies are needed to obtain a more complete profile of their anticancer potential. This may be a useful area for future study.

Gallic acid (1): off white amorphous powder, λmax (MeOH) 272 nm, H-NMR (DMSO-d6) 6.91 (H, s, H-2, 6), 13C-NMR (DMSO-d6): 121.0 (C-1), 109.0 (C-2 & C-6), 145.9 (C-3 & C-5), 138.3 (C-4), and 168.0 (C-7).

Quercetin 3-O-’C,-B-D-glucoside (Isoquercetin) (2): amorphous yellow powder, λmax (MeOH) 258, 355 nm, H-NMR (DMSO-d6) 6.20 (1H, d, J = 2.0 Hz, H-6), 6.41 (1H, d, J = 2.0 Hz, H-8), 6.82 (1H, d, J = 8.5 Hz, H-5’), 7.54 (1H, dd, J = 8.5 and 2.5 Hz, H-6’), 7.76 (1H, d, J = 2.5 Hz, H-2’), 5.345 (d, J = 7.5 Hz, H-1 glucose), 3.08-3.88 (m, six sugar protons), 13C-NMR(DMSO-d6)156.164 (C-2), 133.344 (C-3), 177.78 (C-4), 161.241 (C-5), 98.935 (C-6), 164.606 (C-7), 93.628 (C-8), 156.40 (C-9), 103.787 (C-10), 121.106 (C-1’), 115.254 (C-2’), 144.864 (C-3’), 148.549 (C-4’), 116.212 (C-5’), 121.625 (C-6’), 100.991 (C-1”), 61.027 (C-6”), 69.992 (C-4”), 74.158 (C-2”), 76.561 (C-3”), 77.557 (C-5”).

Kaempferol 3-O-’C,-a-L-rhamnoside (Azelin) (3): pale yellow amorphous powder, λmax (MeOH) 267, 353, H-NMR (DMSO-d6) 0.9 (d, J = 6 Hz, CH3), 5.29 (1H, d, J = 1.5 Hz H-1”), 6.21 (1H, d, J = 2.5 Hz, H-6), 6.42 (1H, d, J = 2.5 Hz, H-8), 6.81 (2H, d, J = 8.4 Hz, H-3’ and H-5’), 7.74 (2H, d, J = 8.4 Hz, H-2’ and H-6’), 13C-NMR(DMSO-d6) 17.53 (C-6’”), 70.11 (C-5’”), 70.35 (C-2’”), 70.69 (C-3’”), 71.13 (C-4’”), 93.88 (C-8), 98.92 (C-6), 101.77 (C-1’”), 103.98 (C-10), 115.48 (C-3’” and C-5”), 120.99 (C-1”), 130.64 (C-2”’) and (C-6’”), 134.15 (C-3”), 156.56 (C-9), 157.24 (C-2”), 160.14 (C-4”), 161.28 (C-5’), 164.80 (C-7), 177.70 (C-4’).

Quercetin 3-O-’C,-a-L-rhamnoside (Quercitin) (4): pale yellow amorphous powder, λmax (MeOH) 259, 297sh, 348, H-NMR (DMSO-d6) 0.9 (d, J = 6 Hz, CH3), 5.2d (d, J = 1.5 Hz, H-1”), 6.15 (d, J = 2.2 Hz, H-6), 6.3d (d, J =
Gallic acid (1)  
Quercetin 3-O-B-C6-D-glucoside (2)  
Kaempferol 3-O-C1-α-L-rhamnoside (3)

Quercetin 3-O-1-C6-α-L-rhamnoside (4)  
1, 2, 6-tri-O-galloyl-B-D-glucose (5)

(-)-Epigallocatechin-3-O-gallate (6)

1,2,6 tri-O-galloyl-B-D-C6-glucopyranose (5): Cream colored amorphous powder, $\lambda_{\text{max}}$(MeOH) 277, $^1$H-NMR (DMSO-$d_6$) 5.88(d, $J = 8.5$ Hz, H-1), 5.02(d, $J = 8.5$ Hz, H-2), 3.7(t, $J = 8.5$ Hz, H-3), 3.5(t, $J = 8.5$ Hz, H-4), 3.83(m, H-5), 4.43(d, $J = 13$ Hz, H-6), 4.3(dd, $J = 13, 5$ Hz, H-6'), 6.88(s), 6.92(s), 6.98(s) aromatic galloyl protons, $^1$C-NMR(DMSO-$d_6$) 62.9 (C-6 gluc.), 69.7(C-4 gluc.), 72.8(C-5 gluc.), 73.75(C-3 gluc.), 73.8(C-2 gluc.), 92.16(C-1 gluc.), 118.0, 119.2, 119.3 (C-1 galloyl), 108.7, 108.8, 109.0 (C-2, 6 galloyl), 145.4, 145.5, 145.6 (C-3, 5, galloyl), 138.63, 138.69, 139.30(C-4 galloyl), 164.2, 165.0, 165.8 (C = O).

(-)-Epigallocatechin-3-O-gallate (6): off-white amorphous powder, $\lambda_{\text{max}}$(MeOH) 269, $^1$H-NMR (DMSO-
d_{4}) 4.82 (1H, d, J = 7.5 Hz, H-2), 5.03 (1H, m, H-3), 2.30 (ax.H, dd, J = 16.4, 4.4 Hz, H-4), 1.91 (eq.H, dd, J = 16.4, 3.5 Hz, H-4), 5.73 (1H, d, J = 1.8 Hz, H-8), 5.62 (1H, d, J = 1.8 Hz, H-6), 6.07 (2H, s, H-2', H-6'), 6.67 (2H, s, H-2'', H-6''), 13-C-NMR Spectral data, 165.69 (C-7'', [C = O]), 157.30 (C-7), 156.32 (C-9), 155.04 (C-5), 146.5 (C-3'', C-5''), 145.83 (C-3', C-5'), 139.07 (C-4''), 133.1 (C-4), 128.9 (C-1'), 119.36 (C-1''), 109.0 (C-2', C-6''), 105.3 (C-2', C-6'), 97.78 (C-10), 95.77 (C-8), 94.39 (C-6), 77.325 (C-2), 69.31 (C-3), 29.27 (C-4).

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