JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Antioxidant Activity of Polyphenols in Carob Pods

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We extracted polyphenols from carob (*Ceratonia siliqua* L.) pods, and evaluated the in vitro antioxidant activity of the crude polyphenol fraction (CPP). The total polyphenol content in CPP determined by the Folin−Ciocalteu method was 19.2%. The condensed tannin content determined by the vanillin and proanthocyanidin assay systems was 4.37% and 1.36%, respectively. β-Carotene bleaching, 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging, inhibition of lipid peroxidation by the erythrocyte ghost, and microsomal assay systems were used to evaluate the antioxidant activity. CPP showed a stronger inhibitory effect against the discoloration of β-carotene than other polyphenol compounds such as catechins and procyanidins. CPP had weaker antioxidant activity in the DPPH free radical scavenging, the erythrocyte ghost, and microsomal systems than authentic polyphenol compounds at the same concentrations. The activity adjusted by the polyphenol concentration was, however, comparable to that of authentic polyphenol compounds. Considering most carob pods are discarded and not effectively utilized at present, these results suggested that carob pods could be utilized as a functional food or food ingredient.

KEYWORDS: Carob pods; polyphenol; antioxidant activity; condensed tannin

INTRODUCTION

The carob tree (*Ceratonia siliqua* L.) has been widely cultivated in Mediterranean countries for years. This tree was distributed by Arabs in the Mediterranean area. The tree attains a mature height and spread of 6−12 m, and sometimes more than 20 m, with branches extended to ground level. The carob fruit is brown in color and its size reaches, in some cases, 25 cm in length. The two principal components of the fruit of carob tree are the pods and the seeds. The seeds, which comprise about 10% of the weight of the fruit, are composed principally of gallactomannans (*1*). They are used as a growth medium for microorganisms and as a food stabilizer, and they have other applications in the textile, food, cosmetic, and pharmaceutical industries (*2*, *3*).

Carob pods, on the other hand, have a high soluble sugar content (about 40−50%), but low protein (3−4%) and lipid contents (0.4−0.8%). Carob pods also contain lots of polyphenols, especially highly condensed tannins (*2*, *3*). Nowadays, the main application of the pods is as animal feed. For humans, the carob pods have been used mainly as a cocoa substitute in a few countries because of its low price and the absence of caffeine (*4*, *5*). However, most carob pods are discarded and not effectively utilized at present.

Further, little information is available concerning the chemical composition and biological activities of carob pods. In particular, polyphenols in carob pods have not been well characterized. Marakis et al. reported that catechins and proanthocyanidins are the major types of polyphenols in carob pods (*6*, *7*). Conversely, Nishira et al. isolated nine hydrolyzable tannins from carob pods, and among them, two galloyl glucoses were identified (*8*). They have reported that many kinds of hydrolyzable tannins such as galloyl glucose compounds were present in carob pods in large amounts, but their structures were not completely elucidated (*9*).

In the present study, we investigated the in vitro antioxidant activity of crude polyphenol in the carob pods (CPP). The aims of this study were to (1) characterize CPP prepared from carob pods; (2) measure the antioxidant activity of CPP by various assay methods such as β-carotene bleaching, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging, and the inhibition of lipid peroxidation by the erythrocyte ghost and microsomal fractions; and (3) compare the antioxidant activity of CPP with those of other polyphenol compounds.

MATERIALS AND METHODS

Chemicals. Bovine serum albumin (BSA), (+)-catechin, linoleic acid, and *tert*-butylhydroxyperoxide (*t*-BHP) were purchased from Sigma Chemical Co. (St. Louis, MO) . (−)-Epicatechin (EC), (−)-epicatechin gallate (ECg), (−)-epigallocatechin (EGC), (−)-epigallocatechin gallate (EGCg), procyanidin B1, and procyanidin B2 were purchased from Funakoshi Co., Ltd., Japan. Thiobarbituric acid (TBA) and quercetin were purchased from Kanto Chemicals Co., Ltd., Japan. β-Carotene, dimethyl sulfoxide (DMSO), DPPH, EDTA·2Na, gallic acid, Tween 40, and vanillin were purchased from Wako Pure

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10.1021/jf010938r CCC: $22.00
Published on Web 12/13/2001

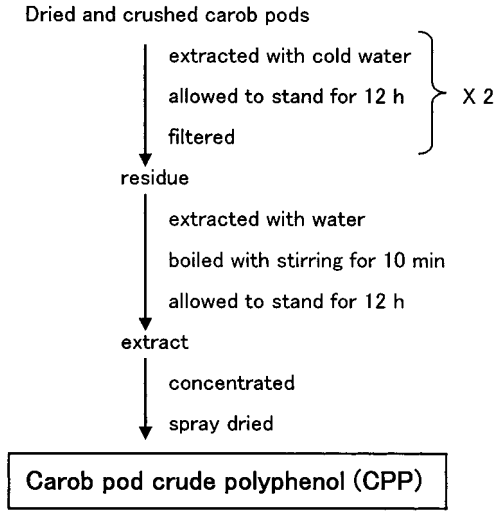

Figure 1. Scheme for preparation of the carob pod crude polyphenol (CPP).

Chemicals Industries Ltd., Japan. ADP and NADPH were obtained from Oriental Yeast Co., Ltd., Japan.

Preparation of Carob Pod Polyphenols. Carob pods were imported from Greece and ground at Somar Corporation Ltd., Japan. CPP was prepared by the following procedure (Figure 1): Dried and ground carob pods (carob pod powder) were extracted with cold water and allowed to stand for 12 h at 3 °C. The extract was filtered to remove sugars present in carob pods. This extraction procedure was performed twice. Then the residue of the carob pods was extracted with water at room temperature and boiled for 10 min with stirring. The sample was allowed to stand for 12 h at 25 °C. After filtration, the filtrate was concentrated and dried with spraying to give CPP.

Total Polyphenol Content. The total polyphenolic content of CPP was determined by the Folin−Ciocalteu colorimetric method (*10*). The CPP solution (0.5 mL of 10 μg/mL) was mixed with 0.5 mL of the Folin−Ciocalteu reagent (Kanto Chemicals Co., Ltd.) and 0.5 mL of 10% Na_2CO_3, and the absorbance was measured at 760 nm after 1 h incubation at room temperature. Total polyphenols were expressed as g/100 g gallic acid equivalents.

Condensed Tannin Content. *Vanillin Assay*. Catechins and proanthocyanidins reactive to vanillin were analyzed by the vanillin method (*11*−*13*). CPP solution (2 mL) was placed in a test tube together with 4 mL of vanillin (1% in 7 M H_2SO_4) in an ice bath and then incubated at 25 °C. After exactly 15 min, the absorbance of the solution was read at 500 nm. Concentrations were calculated as (+)-catechin (g/100 g) from a calibration curve.

Proanthocyanidin Assay. Total proanthocyanins were assayed by the proanthocyanidin method (*14*, *15*). A mixture of 0.25 mL of CPP solution and 3.5 mL of the butanol−HCl reagent (butanol−concentrated HCl (95:5) and 1.4% (w/v) $FeSO_4 \cdot 7H_2O$) was mixed well. The suspension was heated in capped tubes for 40 min in a boiling water bath. The mixture was allowed to cool, and the absorbance was measured at 550 nm.

Antioxidant Activity. *Linoleic Acid Oxidation.* This experiment was carried out by the method of Emmons et al. (*16*). β-Carotene (5 mg) was dissolved in 50 mL of chloroform, and 3 mL was added to 40 mg of linoleic acid and 400 mg of Tween 40. Chloroform was removed under a stream of nitrogen gas. Distilled water (100 mL) was added and mixed well. Aliquots (3 mL) of the β-carotene/linoleic acid emulsion were mixed with 40 μL of sample solution and incubated in a water bath at 50 °C. Oxidation of the emulsion was monitored spectrophotometrically by measuring absorbance at 470 nm over a 60-min period. Control samples contained 40 μL of solvent in place of the extract. The antioxidant activity is expressed as percent inhibition relative to the control after a 60-min incubation using the following equation:

$$AA = 100(DR_C - DR_S)/DR_C$$

Table 1. Contents of Total Polyphenol and Condensed Tannin in Carob Pod Crude Polyphenol (CPP)

	contents (g/100 g)
total polyphenol[a]	19.2 ± 0.3[c]
condensed tannin[b]	
vanillin assay	4.37 ± 0.10
proanthocyanidin assay	1.36 ± 0.01

[a] Total polyphenol content was determined by the Folin–Ciocalteu method using gallic acid as the standard. [b] The assays were performed as described in Materials and Methods. [c] Standard deviation.

where AA is the antioxidant activity, DR_C is the degradation rate of the control (= ln(*a*/*b*)/60), DR_S is the degradation rate in the presence of the sample (= ln(*a*/*b*)/60), *a* is the initial absorbance at time 0, and *b* is the absorbance at 60 min. CPP was evaluated at the final concentrations of 10 μg/mL and 50 μg/mL, and other polyphenol compounds at 10 μg/mL in the assay mixture.

Free Radical Scavenging Activity on DPPH. The reaction mixture contained 2 mL of ethanol, 125 μM DPPH, and test samples. After 2 min incubation at room temperature, the absorbance was recorded at 517 nm. All experiments were carried out in triplicate and repeated at least three times. Results were expressed as percentage decrease with respect to control values (*17*). CPP was evaluated at the final concentration of 5 μg/mL and 25 μg/mL, and other polyphenol compounds at 5 μg/mL in the assay mixture.

Erythrocyte Ghost System. Rabbit red blood was obtained from Japan Biotest Institute Co. Erythrocyte ghosts were prepared from rabbit red blood cell membranes by the method of Osawa et al. (*18*). The induction of lipid peroxidation was carried out with *t*-BHP according to the method of Ames et al. (*19*). Test samples were dissolved with DMSO and added to the erythrocyte ghosts. After incubation, the TBA-reactive substances (TBARS) were measured by reading its absorbance at 535 nm. CPP was evaluated at the final concentration of 100 μg/mL and 500 μg/mL, and other polyphenol compounds at 100 μg/mL in the assay mixture.

Microsomal Lipid Peroxidation. Rat liver S9 fraction (aroclor 1254-induced) was obtained from Oriental Yeast Co., Ltd. It was prepared each time at a concentration of 1 mg/mL protein by the Lowry method using BSA as the standard protein. Test samples dissolved in DMSO were added to the microsomal incubation system. The following additions were made, and the incubation was carried out at 37 °C for 30 min: 2 mM ADP, 0.1 mM EDTA, 0.1 mM $FeCl_3$, and 0.1 mM NADPH in 50 mM Tris−HCl buffer solution. After incubation, the formation of the TBARS was measured as described above. CPP was evaluated at the final concentration of 1 and 5 mg/mL, and other polyphenol compounds at 1 mg/mL in the assay mixture.

RESULTS

Carob Pod Polyphenol Sample. The procedure adopted for the preparation of polyphenols from carob pods is outlined in Figure 1. The sugar (about 40−50%) contained in carob pods (*3*) must be efficiently removed. We extracted carob pods with cold water twice to remove the contained sugars. Then the residues were extracted with hot water, and the extract was dried with spraying to give CPP. CPP was obtained at 10% weight yield from the original carob pod powder.

Table 1 shows the total polyphenol content and condensed tannin content in CPP. Total polyphenols in CPP determined by the Folin−Ciocalteu method was 19.2%. Except for polyphenols, CPP contained a 39.3% gum fraction, 19.8% sucrose, 5.69% fructose, and 3.39% glucose (data not shown). We have examined the interference of the sugars in CPP for the Folin reaction by adding a further 5% glucose to the CPP solution. The value increment by the addition of glucose was less than 5% of the original one (data not shown). This result suggests that the obtained value of the total polyphenol content of CPP

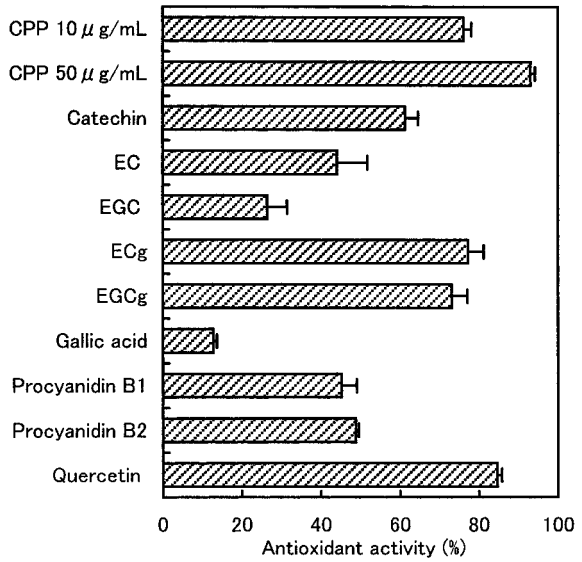

Figure 2. Antioxidant activity of CPP and well-known polyphenol compounds in the β-carotene–linoleic acid system. The compounds, except for carob, were used for the assay at the final concentration of 10 μg/mL. The values represent the percent inhibition of autoxidation of the linoleic acid/β-carotene emulsion. Measurements were carried out in triplicate. Means and standard deviation are indicated.

might be slightly overestimated. The condensed tannin content in CPP was determined by the vanillin assay (vanillin−H_2SO_4 method) and the proanthocyanidin assay (butanol−HCl method). The vanillin assay provides the quantity of flavanols including catechins and proanthocyanidins (*20*). On the other hand, the proanthocyanidin assay provides the degree of polymerization of proanthocyanidins, because this assay is a very specific reaction based on conversion to anthocyanidins by means of autoxidation following acid-catalyzed cleavage of the interflavonoid bonds (*14*). Therefore, the value obtained by the vanillin assay divided by that obtained by the proanthocyanidin assay is a ratio that provides a rough estimate of the degree of polymerization of flavanols. The values determined by the vanillin assay and the proanthocyanidin assay were 4.37 ± 0.1 and 1.36 ± 0.01 g/100 g of CPP, respectively, thus indicating that the degree of polymerization of flavanols was 31.1% (= 1.36/4.37).

Antioxidant Activity. Figure 2 shows the antioxidant activity of CPP determined by the β-carotene−linoleic acid system. The antioxidant assay using the discoloration of β-carotene is widely used, because β-carotene is extremely susceptible to free-radical-mediated oxidation. Because of its 11 pairs of double bonds, which are extremely sensitive to oxidation, β-carotene is discolorized easily with the oxidation of linoleic acid (*21*). Antioxidant activity of CPP was compared with those of the well-known polyphenol compounds, (+)-catechin, EC, EGC, ECg, EGCg, gallic acid, procyanidin B1, procyanidin B2, and quercetin. CPP was evaluated at the final concentration of 10 μg/mL and 50 μg/mL for the assay, and other polyphenol compounds were compared at 10 μg/mL under the same conditions. CPP showed high antioxidant activity at a concentration of 10 μg/mL. Its activity was equivalent to those of ECg, EGCg, and quercetin. Total polyphenol content of CPP is 19.2% as shown in Table 1. Therefore, CPP should have the highest activity when it is compared at the same concentration of the total polyphenols.

The DPPH free radical scavenging activity of CPP is shown in Figure 3. The radical scavenging activity of CPP at 5 μg/mL was 13% and was not as strong as those of other polyphenols.

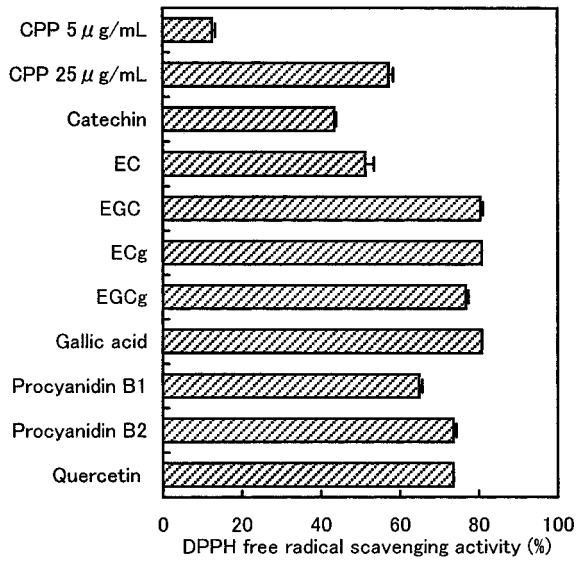

Figure 3. DPPH radical scavenging activity of CPP and well-known polyphenol compounds. The compounds, except for carob, were used for the assay at the final concentration of 5 μg/mL. The values represent the percent of the DPPH radical scavenging activity. Measurements were carried out in triplicate. Means and standard deviation are indicated.

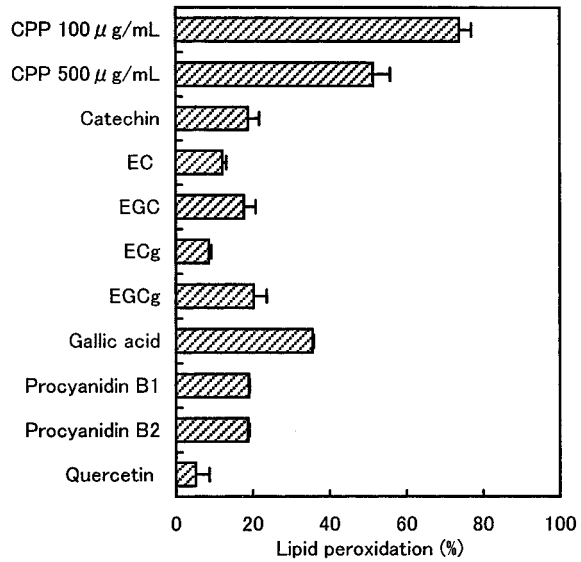

Figure 4. Antioxidant activity of CPP and well-known polyphenol compounds in the rabbit erythrocyte membrane ghost. The compounds, except for carob, were used for the assay at the final concentration of 100 μg/mL. The values obtained without polyphenol samples were regarded as 100% lipid peroxidation. Measurements were carried out in triplicate. Means and standard deviation are indicated.

However, the activity of CPP at 25 μg/mL was stronger than those of (+)-catechin and EC.

Figure 4 shows the antioxidant activity of CPP determined by the rabbit erythrocyte membrane ghost system. In Figure 4, the values obtained without samples were used for 100% lipid peroxidation. At concentrations of 100 and 500 μg/mL, CPP showed antioxidant activity inhibiting about 25% and 50% of the peroxidation, respectively. The antioxidant activity of CPP even at 500 μg/mL was less effective compared with other polyphenols in this assay system. The antioxidant activity of other polyphenol compounds inhibited 70−80% of the lipid peroxidation.

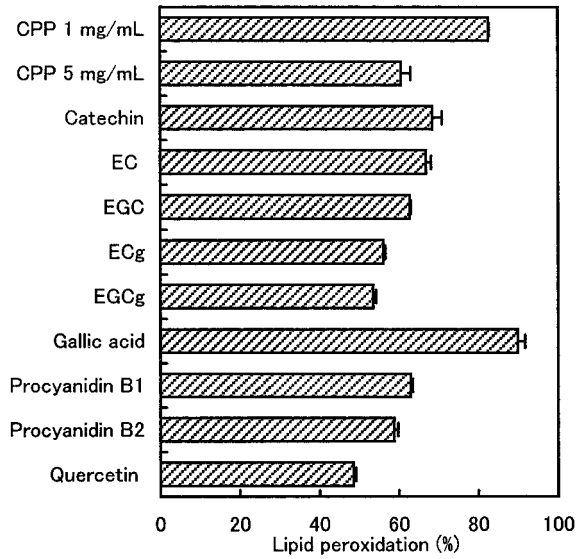

Figure 5. Antioxidant activity of CPP and well-known polyphenol compounds in the rat liver microsomal system. The compounds, except for carob, were used for the assay at the final concentration of 1 mg/mL. The values obtained without polyphenol samples were regarded as 100% lipid peroxidation. Measurement was carried out in triplicate. Means and standard deviation are indicated.

Figure 5 is the antioxidant activity of CPP evaluated by the rat liver microsomal system. As with Figure 4, the values obtained without samples were used for 100% lipid peroxidation also in Figure 5. In this experiment, CPP inhibited 10% of the lipid peroxidation at 1 mg/mL. However, 5 mg/mL of CPP inhibited 40% of the peroxidation, which was equivalent to the inhibition by catechins and procyanidins. Gallic acid, which has been reported to be included in carob pods (*9*), hardly showed antioxidant activity in this assay system.

DISCUSSION

The in vitro antioxidant activity of carob pod crude polyphenol (CPP) was evaluated. The total polyphenol content in CPP used in this study was 19.2% as shown in Table 1. The polyphenol content could be increased by thoroughly removing the sugar. The quantity of flavanols in CPP, determined by the vanillin assay, was 4.37% (Table 1). Thus, the quantity of flavanol was about 23% (= 4.37/19.2) of the total polyphenol. Further, the degree of polymerization of flavanol was found to be 31.1%. These results support the previous report that catechins and proanthocyanidins are present in carob pods (*6*, *7*). However, further studies are needed to elucidate the other polyphenol compounds contained in carob pods.

First, we evaluated the antioxidant activity of CPP by the β-carotene bleaching assay, because β-carotene shows strong biological activity and is physiologically an important compound (*22*, *23*). If β-carotene is decomposed before its intake, its biological functions in the body would not be observed. Furthermore, when β-carotene is used as a coloring agent for beverages its discoloration would markedly reduce the quality of those products. In the present study, we found that CPP has stronger effects against the discoloration of β-carotene than other polyphenol compounds such as catechins and procyanidins (Figure 2). Unten et al. reported that EGCg has stronger antioxidant activity than either (+)-catechin or EC (*21*). However, CPP with a polyphenol content of 19.2% showed discoloration of β-carotene equivalent with that of EGCg. Thus, carob pod polyphenol might be useful in preventing the discoloring of β-carotene.

Next, the DPPH free radical scavenging activity of CPP was investigated. The model system of scavenging DPPH free radicals is a simple method to evaluate the antioxidative activity of antioxidants. It is accepted that the DPPH free radical scavenging by antioxidants is due to their hydrogen-donating ability (*17*). CPP exhibited the DPPH free radical scavenging ability at the concentration of 25 μg/mL as compared with (+)-catechin and epicatechin at 5 μg/mL. The activity of CPP was slightly weaker than those of the other polyphenol compounds.

We used an assay method using the erythrocyte membrane ghost and another method using rat microsomes as a model of the in vivo system. Red blood cell membranes are prone to lipid peroxidation because of their high polyunsaturated lipid content. The antioxidant assay using the rabbit erythrocyte membrane ghost system has been reported to reflect the activity in vivo, because the permeabilities of the membranes of the compound are similar (*18*). CPP at 500 μg/mL inhibited about 50% of the peroxidation (Figure 4). However, the antioxidant activities of the other polyphenol compounds for the comparison were higher than that of CPP. The antioxidant assay using the rat microsome fraction is also considered to reflect the in vivo situation. CPP at a concentration of 5 mg/mL showed about 40% inhibition of the peroxidation, which was equivalent to those of catechins and procyanidins (Figure 5). These results suggest that carob pod polyphenol may possess antioxidative properties in vivo.

In conclusion, we have confirmed the antioxidant activity of crude polyphenol derived from carob pods. Especially, it was apparent that CPP has strong effects against the discoloration of β-carotene.

ABBREVIATIONS USED

CPP, carob pod crude polyphenol; DPPH, 1,1-diphenyl-2-picrylhydrazyl; EC, (−)-epicatechin; ECg, (−)-epicatechin gallate; EGC, (−)-epigallocatechin; EGCg, (−)-epigallocatechin gallate.

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Received for review July 19, 2001. Revised manuscript received October 9, 2001. Accepted October 9, 2001.

JF010938R