Scavenging Effect of Methanolic Extracts of Peanut Hulls on Free-Radical and Active-Oxygen Species

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The scavenging effect of methanolic extracts of peanut hulls (MEPH) on free-radical and active-oxygen species was investigated. MEPH showed marked activity as a radical scavenger in the experiment using 1,1-diphenyl-2-picrylhydrazyl radical, indicating that MEPH has effective activities as a hydrogen donor and as a primary antioxidant to react with lipid radicals. MEPH also possessed antioxidative activity toward hydrogen peroxide (H_2O_2) and superoxide ($O_2^{\cdot-}$), indicating that MEPH has a scavenging activity on H_2O_2 and $O_2^{\cdot-}$. The scavenging effect of MEPH on hydroxyl radical was investigated by means of electron paramagnetic resonance spectrometry. MEPH exhibited a marked scavenging effect on hydroxyl radical, and the scavenging activity of MEPH depended on its concentrations. These results indicate that MEPH is also active as an oxygen scavenger and as a secondary antioxidant. The overall antioxidant effect of MEPH on lipid peroxidation might be attributed to its properties of scavenging free-radical and active-oxygen species.

INTRODUCTION

Oxidative deterioration readily occurs spontaneously when materials containing lipids or lipid-containing foods are exposed to air. Oxidation interferes seriously with the efficiency of the processing steps: it can result in organoleptic rancidity in the finished products, making them unacceptable to consumers, and it can cause other degrading effects such as vitamin destruction, nutritional losses, and discoloration (Sherwin, 1978).

The addition of antioxidants to foods is effective in retarding the oxidation of fats. The possible toxicity of synthetic chemicals used as antioxidants has been long guestioned (Imaida et al., 1983), stimulating investigation of the effectiveness of naturally occurring compounds with antioxidative properties (Wu et al., 1982).

Antioxidants are classified into four types according to the mechanism of action: chain breaker (or free-radical inhibitor), peroxide decomposer, metal inactivator, or oxygen scavenger (Yagi, 1970; Dziezak, 1986). Much literature on the mechanism of action of antioxidants is available (Chimi et al., 1991; Okamoto et al., 1992). For example, Hayase et al. (1989) speculated that melanoidin showed a greater rate of scavenging hydroxyl radicals as a result of containing reductones, enamines, or pyrrole-like structures. The inhibition of autoxidation by hydroxytyrosol may be related to the ability to scavenge the peroxyl radical (Chimi et al., 1991).

Although the investigation of methanolic extracts of peanut hulls (MEPH) on antioxidative activity is reported (Duh et al., 1992; Yen et al., 1993; Yen and Duh, 1993), there is nothing on the mechanism of action of the antioxidative activity by MEPH. Thus, the objective of our work was to elucidate the mechanism of action of the antioxidative effect by MEPH.

MATERIALS AND METHODS

Chemicals. Catechin, dihydronicotinamidadenine dinucleotide (NADH), butylated hydroxyanisole (BHA), and 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) were obtained from Sigma Chemical Co. (St. Louis, MO). Hydrogen peroxide, tetramethyl murexide (TMM), hexamethylenetetramine, nitro blue tetrazolium (NBT), and phenazine methosulfate were purchased from E. Merck (Germany). 1,1-Diphenyl-2-picrylhydrazyl (DPPH) was obtained from Fluka Chemie AG (Switzerland).

Materials. Peanuts of Tainan select no. 11, Spanish type, were obtained from the Tainan District Agriculture Improvement Station, Taiwan, Republic of China. After harvest, the peanuts were hand-shelled. Peanut hulls were ground into a fine powder in a mill (Tecator Cemotec 1090 sample mill, Hoganas, Sweden), sealed in a plastic bottle, and stored at 4 °C until used.

Extractions. Peanut hull powder (5.0 g) was extracted with methanol (50 mL) overnight in a shaking incubator at ambient temperature. The extracts were filtered; the residue was re-extracted under the same conditions. The combined filtrates were evaporated in vacuum below 40 °C on a rotary evaporator to a final volume of 5 mL. The concentration of the extracts in the solvent was 44.5 ± 1.98 mg/mL, which was the averge of triplicate analyses.

Quantitative Analysis of Luteolin. The luteolin in peanut hulls was determined by HPLC performed with a Hitachi liquid chromatograph (Hitachi, Ltd., Tokyo) consisting of a Model L-6200 pump, a Rheodyne Model 7125 syringe loading sample injector, a Model L-4200 UV–vis detector set at 254 nm, and a Model D-2500 integrator. A LiChrosphere 100 RP-18 reversed-phase column (5 μm, 125 × 4 mm i.d., E. Merck) was used for analysis. The volume injected was 10 μL. The elution solvents were water–acetic acid (99:1 v/v) (A) and methanol (B). The gradient elution program was set at 1.6 mL/min, starting with 80% A and 20% B linearly to 60% A and 40% B in 35 min (Yen et al., 1993).

To determine the luteolin in the methanolic extracts, an authenic sample of luteolin was prepared; dilutions were made so that the range of concentration correlated with the estimated content of luteolin in the samples. The content of luteolin in the methanolic extracts of peanut hulls was calculated from the standard curve of luteolin. Triplicate samples were run for each set.

Determination of Total Phenolic Compounds. The total phenolic compounds present in the peanut hulls was determined spectrophotometrically using Folin–Denis reagent (AOAC, 1984). The methanolic extract (0.1 mL) of peanut hulls in a volumetric flask was diluted with glass-distilled water (75 mL). Folin–Denis reagent (5 mL) was added, and the contents of the flask were mixed thoroughly. After 3 min, Na_2CO_3 solution (10 mL; concentration 10 g/100 mL) was added and finally quantified to 100 mL with glass-distilled water; the mixture was allowed to stand

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for 30 min with intermittent shaking. The blue color was measured with a spectrophometer (Hitachi U-2000). The concentration of total phenolic compounds in the peanut hulls was determined by comparison with the absorbance of standard catechin at different concentrations.

Determination of the Effects on DPPH Radical. Four milliliters of methanolic extracts of peanut hulls (final concentration 1.92 mg/mL) decolorized with cartridges (Sep-Pak C_{18}, Waters) was added to a methanolic solution (1 mL) of DPPH radical (final concentration of DPPH was 2.0×10^{-4} M). The mixture was shaken vigorously and left to stand for 30 min; the absorbance of the resulting solution was measured at 517 nm with a spectrophotometer (Hitachi U-2000) (Blois, 1958). All tests and analyses were run in three replicates and averaged.

Measurements of Chelating Activity on Metal Ions. The chelating activity of MEPH on Fe^{2+} and Cu^{2+} was measured according to the method of Shimada et al. (1992). To prevent the color of MEPH from interfering with spectrometrical analysis, MEPH was decolorized with cartridges (Sep-Pak C_{18}, Waters); the final concentration was 0.192 mg/mL. No contamination of Fe and Cu ions was found in the test solution determined by inductively coupled plasma mass spectrometer (ICP-MS, Perkin-Elmer Elan 5000). Decolorized solution (2.0 mL) was added to hexamine buffer (2.0 mL, 10 mM) containing KCl (10 mM) and $FeSo_4 \cdot 7H_2O$ (3 mM) or $CuSO_4 \cdot 5H_2O$ (3 mM), and then tetramethyl murexide [0.2 mL, 1 mM (TMM)] was added. Absorbance at 480 nm was measured with a spectrophotometer (Hitachi U-2000). The measurement was carried out at 20 °C to prevent oxidation of Fe^{2+}.

Determination of the Effects on Hydrogen Peroxide. The ability of MEPH to scavenge hydrogen peroxide was determined spectrophotometrically (Ruch et al., 1989). A solution (2 mM) of hydrogen peroxide was prepared in phosphate-buffered saline [(PBS) pH 7.4] at 20 °C. Hydrogen peroxide concentration was determined spectrophotometrically at 230 nm using a molar extinction coefficient for hydrogen peroxide of 81 M^{-1} cm^{-1} (Beers and Sizer, 1952). Methanolic extracts of peanut hulls decolorized with cartridges (Sep-Pak C_{18}, Waters) were added to the hydrogen peroxide solution at final concentrations of 0, 0.06, 0.6, or 1.2 mg/mL at 20 °C. Absorbance of hydrogen peroxide at 230 nm was determined 10 min later in a spectrophotometer (Hitachi U-2000) against a blank solution containing MEPH (0, 0.06, 0.6, or 1.2 mg/mL) in PBS without hydrogen peroxide. All tests and analyses were run in replicates and averaged.

Determination of the Effects on Superoxide. Superoxide was determined by means of spectrophotometric measurement of the product on the reduction of nitro blue tetrazolium (Nishikimi et al., 1972). Superoxide was generated in a nonenzymic (phenazine methosulfate–NADH) system (Nishikimi et al., 1972). The nonenzymic generation of superoxide was measured in samples that contained MEPH (0.48 mg/mL) decolorized with cartridges (Sep-Pak C_{18}, Waters), phenazine methosulfate (20 μM), NADH (156 μM), and nitro blue tetrazolium (50 μM) in phosphate buffer (0.1 M, pH 7.4). After 5 min of incubation at ambient temperature, the color was read at 560 nm against blank samples. All tests and analyses were run in replicates and averaged.

Detection of Hydroxyl Radical by EPR Spectrometry. The hydroxyl radical rapidly reacts with the nitrone spin trap 5,5-dimethylpyrrolidine *N*-oxide (DMPO) (Sigma), and the resultant DMPO–OH adduct is detected by electron paramagnetic resonance (EPR) spectrometry (Rosen and Rauckman, 1984). The EPR spectrum was recorded 2 min after MEPH (0.5 mL) was mixed with H_2O_2 (0.2 mL, 10 mM), Fe^{2+} (0.2 mL, 10 mM), and DMPO (0.2 mL, 0.3 M) using an EPR spectrometer (Bruker ER 200 D 10/12) set at the following conditions: 3480-G magnetic field, 1.0-G modulation amplitude, 0.5-s time constant, and 200-s scan period (Shi and Dalal, 1991).

RESULTS AND DISCUSSION

Chemical Characteristics of MEPH. The contents of luteolin and total phenolic compounds in MEPH were 2.58 and 7.70 mg/g of hulls, respectively. The high content of total phenolic compounds in MEPH is responsible for inhibition of lipid peroxidation (Yen et al., 1993). Since the lipid peroxidation is induced and developed by free radicals and active oxygens (Sherwin, 1978; Namiki, 1990), free-radical scavengers and oxygen quenchers may inhibit lipid peroxidation (Torel et al., 1986). Phenolic compounds that exhibit scavenging efficiency on free radicals and active oxygens are numerous and widely distributed within the plant kingdom (Chimi et al., 1991; Namiki, 1990). Due to the high content of phenolic compounds found in MEPH, therefore, the investigation of the reactivity of MEPH with free radicals or with active oxygens will be helpful in understanding the mechanism of antioxidative function of MEPH.

Table 1. Scavenging Effects of Methanolic Extracts of Peanut Hulls (MEPH) and Other Compounds on 1,1-Diphenyl-2-picrylhydrazyl Radical

compd[a]	absorbance at 517 nm[b]	inhibition[b] (%)
control	0.224 ± 0.0087A	0C
MEPH	0.024 ± 0.0036B	89.3 ± 1.65B
catechin	0.016 ± 0.0003C	92.7 ± 1.04A
BHA	0.017 ± 0.0016C	92.6 ± 0.67A

[a] The concentrations of MEPH, catechin, and butylated hydroxyanisole (BHA) were 1.5 mg/mL, 8 μM, and 240 μM, respectively. [b] Each value is the mean ± standard deviation of three replicate analyses. Values within a column with the same upper case letters are not significantly different at $p > 0.05$.

Table 2. Chelating Effect of Different Compounds on Fe^{2+} and Cu^{2+}

	absorbance[b] (480 nm)	
compd[a]	Fe^{2+}	Cu^{2+}
control	0.778 ± 0.001A	1.047 ± 0.005A
MEPH	0.773 ± 0.008A	1.042 ± 0.012A
citric acid	0.484 ± 0.001B	0.968 ± 0.001B
EDTA	0.230 ± 0.002C	0.370 ± 0.012C

[a] The concentrations of methanolic extracts of peanut hulls (MEPH), citric acid, and ethylenediaminetetraacetic disodium salt dihydrate (EDTA) were 0.096/mL, 0.5 M, and 0.5 M, respectively. [b] Each value is the mean ± standard deviation of three replicate analyses. Values within a column with the same upper case letters are not significantly different at $p > 0.05$.

Measurement of Radical-Scavenging Activity. The scavenging activity of MEPH on the DPPH radical is shown in Table 1. Methanolic extracts of peanut hulls decolorized with cartridges (Sep-Pak C_{18}, Waters) at a concentration 1.5 mg/mL exhibited about 90% scavenging activity. Hence, methanolic extracts of peanut hulls mixed with DPPH decolorized DPPH due to its hydrogen-donating ability (Blois, 1958; Shimada et al., 1992). Catechin and butylated hydroxyanisole (BHA) at concentrations of 8 and 240 μM, respectively, also exhibited marked scavenging activity (>90%). These results show that MEPH is a free-radical inhibitor, a primary antioxidant that reacts with free radicals, particularly the hydroperoxide radical, which is the major propagator of the chain autoxidation of fats, so breaking the chain (Lea, 1958).

Measurement of Chelating Activity on Metal Ions. Although chelating agents are not antioxidants, they play a valuable role in the stabilization of fatty foods against rancidity. The ability of MEPH to form complexes with metal ions is demonstrated in Table 2. EDTA and citric acid showed chelating effect, but decolorized MEPH showed no chelating activity on metal ions. Luteolin containing 3′,4′-dihydroxy, 5,7-dihydroxy, and 4-CO groups was identified as an antioxidative component from MEPH (Duh et al., 1992) and reported to form a ligand with copper ion (Hudson and Lewis, 1984). However, in the present work, MEPH may lose chelating components as a result of decolorizing treatment by cartridges (Sep-Pak C_{18}, Waters), and it may be the major cause for suppression of chelating metal ions.

Table 3. Scavenging Effects of Methanolic Extracts of Peanut Hulls (MEPH), Butylated Hydroxyanisole (BHA), and Catechin on Hydrogen Peroxide

compd	concn	hydrogen peroxide[a] (mM)	inhibition[a] (%)
MEPH	0 (mg/mL)	2.00A	0E
	0.06 (mg/mL)	1.98 ± 0.07AB	1.0 ± 3.50E
	0.60 (mg/mL)	1.17 ± 0.05D	41.8 ± 2.36B
	1.20 (mg/mL)	0.22 ± 0.04E	89.1 ± 1.88A
BHA	43 (μM)	1.89 ± 0.08B	8.7 ± 1.16D
catechin	14 (μM)	1.72 ± 0.08C	13.8 ± 4.16C

[a] Each value is the mean ± standard deviation of three replicate analyses. Values within a column with the same upper case letters are not significantly different at $p > 0.05$.

Table 4. Influence of Investigated Compounds on Superoxide Generation by Phenazine Methosulfate and NADH[a]

compd[b]	absorbance at 560 nm[c]	compd[b]	absorbance at 560 nm[c]
control	0.331 ± 0.039A	BHA	0.244 ± 0.009B
catechin	0.248 ± 0.016B	MEPH	0.178 ± 0.013C

[a] Samples containing phenazine methosulfate (20 μM), NADH (156 μM), and nitro blue tetrazolium (50 μM) in 0.1 M phosphate buffer (pH 7.4) were incubated for 5 min at room temperature and read at 560 nm. [b] The concentrations of methanolic extracts of peanut hulls (MEPH), butylated hydroxyanisole (BHA), and catechin were 0.48 mg/mL, 75 μM, and 2.5 μM, respectively. [c] Each value is the mean ± standard deviation of six replicate analyses. Values within a column with the same upper case letters are not significantly different at $p > 0.05$.

Scavenging of Hydrogen Peroxide by MEPH. The scavenging activity of MEPH on hydrogen peroxide is shown in Table 3. MEPH was capable of scavenging hydrogen peroxide in a concentration-dependent fashion after 10 min of incubation. With MEPH (1.2 mg/mL), the concentration of hydrogen peroxide was diminished from 2 to 0.22 mM, representing a hydrogen peroxide reduction to 1.4833 μmol of H_2O_2/mg of MEPH. Namiki (1990) indicated that hydrogen peroxide has only a weak activity to initiate lipid peroxidation, but its activity as an active-oxygen species comes from its potential to produce the highly reactive hydroxyl radical through the Fenton reaction (Cohen and Heikkila, 1974). Therefore, the ability of MEPH to scavenge hydrogen peroxide may contribute to inhibition of the peroxidation of lipid.

Scavenging of Superoxide by MEPH. The ability of MEPH to scavenge superoxide generated by phenazine methosulfate–NADH (PMS–NADH) is shown in Table 4. The addition of MEPH (0.48 mg/mL) to the PMS–NADH system significantly ($p < 0.05$) diminished production of superoxide. Catechin (2.5 μM) and BHA (75 μM) also inhibited superoxide. Ilan et al. (1976) noted that superoxide acts as an oxidizing or reducing agent, depending on the substrate oxidation potential. Superoxide also decomposes to form stronger oxidative species such as singlet oxygen, hydroxyl radical, and hydrogen peroxide that initiate the peroxidation of lipid (Dahl and Richardson, 1978). Superoxide indirectly initiates lipid oxidation as a result of superoxide and hydrogen peroxide that serve as precursors of singlet oxygen and hydroxyl radicals (Kellogg and Fridovich, 1975; Aurand et al., 1977). Some reseachers (Lavelle et al., 1973; Tyler, 1975; Goldstein and Weissmann, 1977) reported that superoxide directly initiated lipid peroxidation. Robak and Gryglewski (1988) reported that antioxidant properties of flavonoids are effected mainly via scavenging of superoxide; the marked antioxidant activity of MEPH may be concerned with its higher scavenging of superoxide.

Scavenging of Hydroxyl Radical by MEPH. To investigate the reaction of hydroxyl radical, the well-known Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + {}^{\bullet}OH$) was the source of hydroxyl radical. Figure 1 shows that the reaction of Fe^{2+} with H_2O_2 in the presence of spin trapping agent DMPO generated a 1:2:2:1 quartet with hyperfine coupling parameters (a^n and a^h = 14.9 G). According to Figure 1b, MEPH (24.0 mg) in the reaction system of Figure 1a markedly scavenges the DMPO–OH spin adduct. Table 5 shows the EPR signal intensity of DMPO spin adducts formed by Fenton reaction. Methanolic extracts of peanut hulls at amounts of 4.8, 14.4, and 24.0 mg scavenged 29.9, 53.4, and 71.2% of hydroxyl radicals, respectively. Apparently, the activity to scavenge hydroxyl radicals by MEPH that we tested depended upon its concentrations. The hydroxyl radical is an extremely reactive free radical formed in biological systems (Hochstein and Atallah, 1988) and reacts rapidly with molecules of almost every type found in living cells, such as sugars, amino acids, phospholipids, DNA bases, and organic acid (Halliwell and Gutteridge, 1984). The hydroxyl radical found in biological systems is involved as an active intermediate in physiological processes (Czapski, 1984). Hydroxyl radicals are known to be capable of abstracting hydrogen atoms from membrane lipids (Fong et al., 1973) and bring about peroxidic reactions of lipids (Kitada et al., 1979). Methanolic extracts of peanut hulls have been demonstrated to exhibit markedly antioxidative activity (Duh et al., 1992). Apparently, the ability to quench the hydroxyl radical by

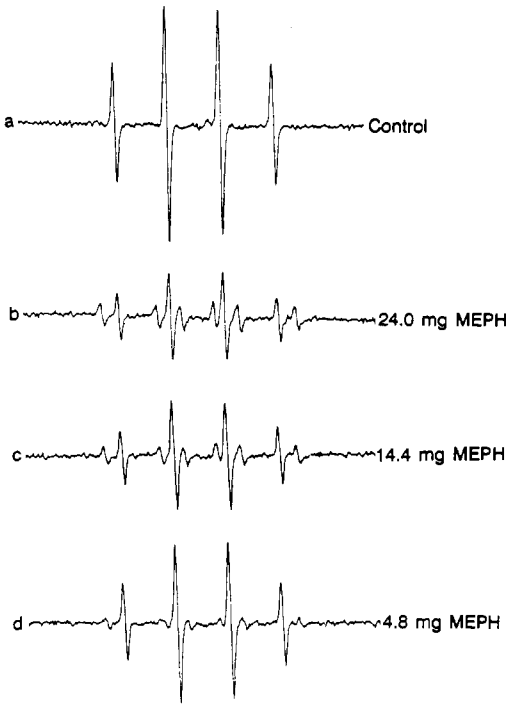

Figure 1. Scavenging of hydroxyl radical by methanolic extracts of peanut hulls (MEPH): (a) EPR spectrum recorded 2.5 min after mixing in a phosphate buffer solution (pH 7.2) of 0.055 M DMPO with 1.82 mM Fe^{2+} and 1.82 mM H_2O_2; (b) as for (a), but with 24.0 mg of MEPH; (c) as for (a), but with 14.4 mg of MEPH; (d) as for (a), but with 4.8 mg of MEPH. Spectrometer settings: receiver gain, 8 × 10; modulation amplitude, 1.0 G; scan time, 200 s; field, 3480 ± 50 G; time constant, 0.5 s.

Table 5. Effect of Methanolic Extracts of Peanut Hulls (MEPH) on EPR Signal Intensity of DMPO–OH Spin Adduct[a]

amt (mg)	rel EPR signal intensity	amt (mg)	rel EPR signal intensity
control	100.0	14.4	46.6
4.8	70.1	24.0	28.8

[a] Treatment and other details are the same as for Figure 1.

MEPH as we tested seems to relate directly to prevention of propagation of the process of lipid peroxidation. Husain et al. (1987) reported that the overall antioxidant effect of flavonoids on lipid peroxidation is the result of scavenging of hydroxyl radicals and superoxide anions at the stage of initiation and termination of peroxyl radicals. BHA and butylated hydroxytoluene function as antioxidants due to termination of reactions of free radicals of the chain type that lead to lipid peroxidation, whereas citric acid functions via quenching of heavy-metal-catalyzed autoxidation (Dziezak, 1986). The antioxidative properties of rosemary oleoresin in sausages are attributed to termination of free-radical reactions and quenching of reactive oxygen species (Richardson and Dahl, 1983).

In conclusion, we demonstrated methanolic extracts of peanut hulls have a strong hydrogen-donating ability and are good scavengers of active oxygen species, including hydroxyl radical, superoxide, and hydrogen peroxide. This property seems to be important in explaining how the antioxidative activity of MEPH arises.

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