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Supercritical fluid extractive fractionation – study of the antioxidant activities of propolis

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Abstract

Propolis was mixed with ethanol at a ratio of 1:10 (v/w) for 24 h to yield propolis ethanol extract (E). Extract E was further fractionated with supercritical carbon dioxide (SC-CO_2) into four fractions (R, F1, F2 and F3). To evaluate the selectivity of the fractionation, extracts corresponding to four fractions were characterized in terms of total flavonoid contents, antioxidant abilities and antioxidant mechanisms. Experimental results indicated that fractionation altered the composition distributions of fractions, e.g., by reducing total flavonoid contents. The antioxidant ability, metal chelating capacity, reducing power, and scavenging capacity of DPPH, $O_2\cdot^-$or ·OH radicals of propolis extract and fractions increased with propolis concentrations. The effects of scavenging on DPPH of propolis extract and fractions, at 2 mg/ml were R (93%), E (75%), F1 (56%), F2 (47%), and F3 (27%). At a concentration of 1 mg/ml, propolis fractions scavenged $O_2\cdot^-$ by over 73% and all the fractions trapped around 65% of the ·OH groups. This in vitro study of antioxidant effects showed that R and F1 were the best fractions, followed by F2.

Keywords: Propolis; Supercritical fluid extractive fractionation; Antioxidant activities

1. Introduction

The propolis of honeybee hives, frequently used in folk medicine, exhibits a wide spectrum of activity, including antimicrobial, anti-inflammatory and antitumor activities (Chen, Shiao, Hsu, Tsai, & Wang, 2001; Crocnan, Greabu, & Olinescu, 2000; Keskin, Hazir, Baser, & Kurkcuoglu, 2001). Some investigations have also addressed the antioxidant activity of propolis. Propolis ethanol extracts can scavenge DPPH and hydroxyl radicals (Banskota et al., 2000; Burdock, 1998). Many protective effects, related to the antioxidant activity of propolis, including effects against doxorubicin-induced myocardiopathy (Chopra, Pillai, Husain, & Giri, 1995), carbon tetrachloride-induced liver damage (Merino, Gonzalez, Gonzalez, & Remirez, 1996), galactosamine-induced hepatitis, and γ-irradiation (Banskota et al., 2001), were demonstrated.

Traditional approaches, including simple steam distillation and vacuum distillation, are adopted to yield fractions. However, these fractionation methods generally involve a high final distillation temperature and are inappropriate for heat-sensitive products (Yang & Wang, 1999). A method of separation, supercritical fluid extraction and fractionation (SFEF), resembles true boiling point (TBP) distillation except in that the variable is the total pressure of the separation system rather than the temperature of the TBP distillation (Yang & Wang, 1999). Fractionation is performed by changing pressure and/or temperature. A two-step SFEF successfully divides the oleoresin of rosemary leaves into two fractions with different antioxidant activities and essential oil contents on an industrial scale (Senorans, Ibanez, Cavero, Tabera, & Reglero, 2000). Esquivel, Ribeiro, and Bernardo-Gil (1999) used the fractional separation of a supercritical extract of savory oil to minimize the co-extraction of unwanted compounds. Therefore, the ethanolic extract of

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propolis was subjected to a series, successively partitioned with SC-CO_2 operated under the following operating conditions: 60 °C and a pressure of 20, 15, 10 or 5 MPa for R, F1, F2 or F3 fractions.

The intention of this experiment was to further examine the effects of large-scale fractionation, with supercritical carbon dioxide (SC-CO_2), on the antioxidant effect and antioxidant characteristics of these four fractions.

2. Materials and methods

2.1. Materials

Crude propolis was from Brazilian sources. 1,1-Diphenyl-2-picryl hydrazyl (DPPH), rutin, xanthine, xanthine oxidase, 1,1,3,3-tetramethoxy propane (TMP), ascorbic acid (Vit. C), α-tocopherol (Vit. E), 5,5-dimethyl pyrroline-N-oxide (DMPO), and quercetin were purchased from Sigma Chemical Co. (St Louis, MO, USA). Tris (hydroxymethyl) aminomethane hydrochloride (Tris-HCl) and thiobarbituric acid (TBA) were from Acros Chemical (NJ, USA). Sodium dodecylsulphate (SDS) was from TCI Co. (Tokyo, Japan). All other reagents used were obtained, either from Merck Co. (Darmstadt, Germany), or Fisher Co. (NJ, USA).

2.2. Propolis fractionation using supercritical carbon dioxide

Propolis was mixed with ethanol at a ratio of 1:10 (v/w) for 24 h to yield propolis ethanol extract (E). Extract E was further fractionated using supercritical carbon dioxide (SC-CO_2) under the following operational conditions: 60 °C and a pressure of 20, 15, 10 or 5 MPa, in three separators operated in series, to yield the residual (R), fraction 1 (F1), fraction 2 (F2) or fraction 3 (F3), respectively.

2.3. Determination of total flavonoid concentration

Flavonoid concentration was determined by following the method of Jia, Tang, and Wu (1999). The absorbance at 415 nm was measured after thoroughly mixing 500 μl propolis samples, 1.5 ml ethanol, 100 μl $Al(NO_3)_3$(10%), 100 μl CH_3COOK (1 M) and 2.8 ml H_2O, before leaving the mixture to sit for 40 min. Total flavonoid concentration was determined using quercetin as standard.

2.4. Estimating extent of anti-$FeCl_2$-ascorbic acid-stimulated lipid peroxidation in rat liver homogenate

Male Sprague–Dawley (SD) rats weighing 240 g (eight weeks old) were used. One gram of liver tissue was sliced and then homogenized with 10 ml of 150 mM KCl–Tris-HCl buffer (pH 7.2) and centrifuged at 500 g for 10 min to yield a liver homogenate supernatant. Lipid peroxidation was measured by the methods of Kimura, Kubo, Tani, Arichi, and Okuda (1981) and Yoden, Iio, and Tabata (1980). A mixture that contained 0.25 ml of liver homogenate, 50 μl of Tris-HCl buffer (pH 7.2), 50 μl of 0.1 mM ascorbic acid, 50 μl of 4 mM $FeCl_2$ and 50 μl of test compounds was incubated for 1 h at 37 °C. After incubation, 0.5 ml of 0.1 N HCl, 0.2 ml of 0.9% SDS and 0.9 ml of H_2O were added and shaken before 2 ml of 0.6% TBA were added. The mixture was heated for 30 min in a boiling water bath. After cooling, 5 ml of *n*-BuOH were added and the mixture was shaken vigorously. The *n*-BuOH layer were separated by centrifugation at 1000*g*. The fluorescence of the samples was measured using a FLUostar OPTIMA (BMG Labtech., Germany) with excitation at a wavelength of 515 nm and emission at a wavelength of 553 nm against a blank that contained all the reagents except liver homogenate. The extent of lipid peroxidation was given in nmol MDA/mg protein. Concentrations of MDA were determined from a standard curve prepared using 1,1,3,3-tetraethoxypropane. Protein content was determined by the method of Lowry, Rosebrough, Farr, and Randall (1951). The inhibition effect (%) was calculated as (%) inhibition $= [1 - (\text{absorbance of sample})/(\text{absorbance of control})] \times 100$. α-Tocopherol (1 mg/ml) was assayed for comparison of the results.

2.5. Chelating metal ions

The degree of chelating of ferrous ions by propolis was estimated by the method of Dinis, Madeira, and Almeida (1994). Each sample was incubated with 0.05 ml of $FeCl_2 \cdot 4H_2O$ (2 mM). The reaction was initiated by adding 5 mM ferrozine (0.2 ml). After equilibrium was obtained (after 10 min), the absorbance at 562 nm was measured. An untreated sample served as the control. Chelating activity (%) $= [1 - (\text{absorbance of sample})/(\text{absorbance of control})] \times 100$.

2.6. Reducing power

The extract or fractions of propolis (0–10 mg) in a phosphate buffer (2.5 ml, 0.2 M, pH 6.6) were added to potassium ferricyanide (2.5 ml, 10 mg/ml) to determine the reducing power (Shi & Dalal, 1991). After incubation at 50 °C for 20 min, trichloroacetic acid (2.5 ml, 100 mg/ml) was added to the mixture and then centrifuged at 650*g* for 10 min. The absorbance of the supernatant (2.5 ml) mixed with distilled water (2.5 ml) and ferric chloride (0.5 ml, 1.0 mg/ml) was measured at 700 nm.

2.7. Scavenging DPPH radicals

The scavenging of DPPH radicals was assayed following the method of Hatano et al. (1989). A mixture of

the extract or a fraction dissolved in MeOH with an equal volume of DPPH solution (0.1 mM) was shaken vigorously. The mixture was incubated at room temperature for 50 min before the absorbance at 517 nm was read. The scavenging activity was determined by comparing the absorbance with that of the blank (100%) that contained only DPPH and solvent.

2.8. Scavenging superoxide anions

Superoxide scavenging activity was assayed by a cytochrome c reduction method, as described by McCord and Fridovich (1969). Xanthine buffer solution was prepared by dissolving 3.04 mg of xanthine in 100 ml of 50 mM KH_2PO_4 buffer solution (pH 7.8), with gentle heating and sonic shaking until it was completely dissolved. It was then diluted to a final concentration of 0.1 mM. Two millilitre KH_2PO_4 (50 mM), 2 ml EDTA (0.1 mM), 2 ml cytochrome c (0.1 mM) and 40 ml xanthine solution (0.1 mM) were mixed to form a working solution. Volumes of 50 μl sample solution, 400 μl working solution and 530 μl H_2O were thoroughly mixed and 20 μl of 1 unit/ml xanthine oxidase were then added to the mixture. Then, the absorbance was measured at a wavelength of 550 nm for 70 s against the blank samples. Rutin (0.4 mg/ml) was used as a positive control for comparison.

2.9. Scavenging hydroxyl radicals

Hydroxyl radical reacts quickly with nitrone spin trap DMPO, and the resultant DMPO-OH adducts can be detected by an electron paramagnetic resonance (EPR) spectrometer (Togashi et al., 2000). The EPR spectrum was recorded 2.5 min after an extract or fraction of propolis (0–12.5 mg/ml) was mixed with DMPO (0.3 M, 0.2 ml), Fe^{2+} (10 mM, 0.2 ml) and H_2O_2 (10 mM, 0.2 ml) in a phosphate buffer solution (pH 7.2) using an EPR spectrometer (Bruker EMX 10, Germany) set to the following conditions: receiver gain, 8×10^5; modulation amplitude, 1.0 G; scan time, 200 s; field, 3461.3 ± 50 G, and time constant, 0.5 s.

2.10. Statistical analysis

Data were expressed as means ± SD determined from triplicate analysis. ANOVA procedures analyzed the variance. Duncan's multiple range tests, at $P < 0.05$, determined significant differences among means.

3. Results and discussion

Fractionation has been demonstrated to minimize the co-extraction of unwanted compounds and yield fractions with different compositions and thus different properties (Yang & Wang, 1999). The fractionation associated with the SC-CO_2 method is amenable to performance on a large scale and, moreover, is non-toxic. Under certain temperature and pressure ranges, carbon dioxide becomes liquid and displays non-polar characteristics. Following processing, temperature and pressure returned to the normal condition, in which carbon dioxide changed from a liquid to a gaseous form and then evaporated. The fractions thus obtained remained free of toxicity. Additionally, the process procedure was less complicated and quicker to complete, making it easier to obtain most of the active ingredients. Flavonoids and their esters in the propolis, such as galangin and caffeic acid phenethyl ester (CAPE), were pharmacologically active molecules and have been hypothesized to influence the antioxidant activity of propolis (Russo, Longo, & Vanella, 2002). This investigation characterized propolis extracts that correspond to the residue (R) and three fractions (F1, F2, and F3) according to total flavonoid contents and antioxidant activities. The total flavonoid content was expressed as the quercetin equivalent per gram ml dry weight (dw) of the propolis sample. The measured results reveal flavonoid quantities in E, R, F1, F2 and F3 of 98, 137, 90, 83 and 76 mg quercetin/g dw, respectively. The strong antioxidant activity of propolis mainly depends on the presence of several phenolic compounds, such as flavonoids (Bankova, Christov, Kujumgiev, Marcucci, & Popov, 1995). The high total flavonoid contents imply continued strong antioxidant activity for propolis fractions.

Flavonoids have been reported to display an antioxidative effect. Flavonoids are known to inhibit lipid peroxidation in model systems, such as the autoxidation of linoleic acid, methyl linolenate or lecithin liposomes and in biological systems, such as liver microsomes treated with Fe^{2+}(Brown, Khodr, Hider, & Rice-Evans, 1998; Ferrali, Signorini, & Caciotti, 1997; Sugihara, Arakawa, Ohnishi, & Furuno, 1999). Flavonoids vary among samples of propolis from different countries, such as Austria, Brazil, Bulgaria, China, England, France, Germany and Spain (Banskota et al., 1998; Hegazi, Abd, Hady, & Abd Allah, 2000; Tazawa, Warashina, Noro, & Miyase, 1998). Rich flavonoid contents in propolis fractions may protect humans from deleterious oxidative processes. Rat liver homogenate served as a substrate for assessing the formation and behaviour of lipid peroxides in a biological system. Fig. 1 shows the effect of propolis extract and fractions on lipid peroxidation, expressed as the ability to inhibit malondialdehyde (MDA) production in rat liver homogenate induced by $FeCl_2$-ascorbate. MDA values (data not shown), except F3 at concentrations below 1 mg/ml, were significantly lower than those of the control group that underwent $FeCl_2$-ascorbate induction, suggesting that propolis extract or fractions were strongly antioxidant. The antioxidant activity of propolis extract

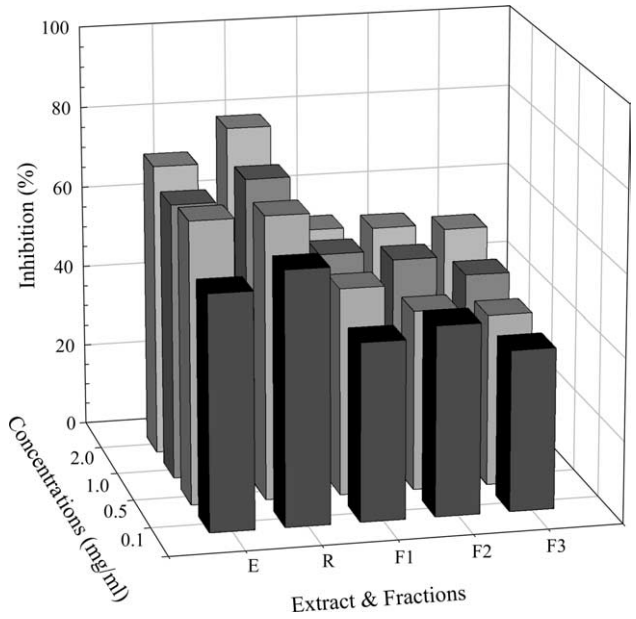

Fig. 1. Inhibition effect (%) of propolis extract and fractions on lipid peroxidation induced by $FeCl_2$-ascorbate in rat liver homogenates.

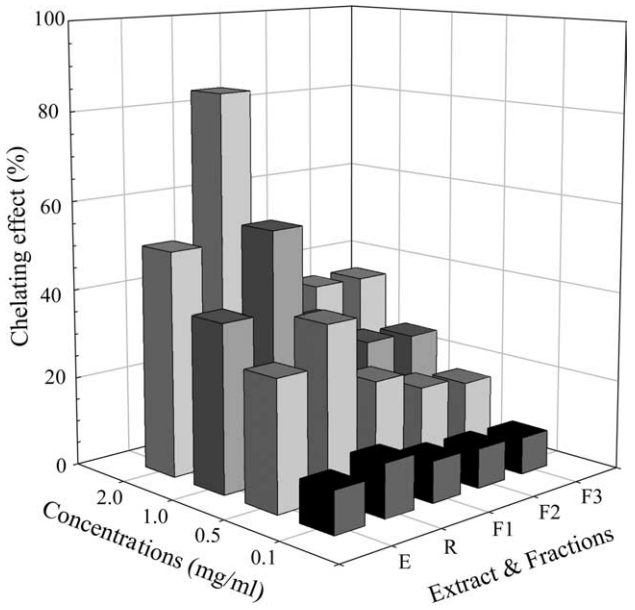

Fig. 2. Chelating effect (%) of various concentrations of propolis extract and fractions on ferrous ions.

and fractions increased with propolis concentration (Fig. 1). At a concentration of 2 mg/ml, the formation of MDA was markedly inhibited in all propolis samples, and lipid peroxidation inhibition followed the pattern: α-tocopherol (90–92%) > R (79–80%) > E (70–72%) > F1 (52–54%) > F2 (51–53%) > F3 (50–51%). The antioxidant capacity of F1 or F2 was slightly lower than that of E and R; however, 2.0 mg/ml of F1 or F2 still can inhibit over 50% of lipid peroxidation. These results demonstrate that various fractions have different effects in inhibiting lipid peroxidation and helping protect biological systems from damage caused by lipid peroxidation.

Flavonoids are antioxidant since they chelate trace metals or scavenge radicals (Pietta, 1999). Several studies have reported that the constituents of propolis extract can scavenge free radicals, including oxygen free radicals, in vitro (Basnet, Matsuno, & Neidlein, 1997; Matsushige, Kusumoto, Yamamoto, Kadota, & Namba, 1995; Matsushige, Basnet, Kadota, & Namba, 1996). Lipid peroxides are well known to be produced through a free radical chain process on autoxidation of lipids containing polyunsaturated fatty acids (Porter, Caldwell, & Mills, 1995). Moreover, lipid peroxidation in cell membranes is also known to occur in the presence of trace quantities of transition metal ions, owing to oxygen free radicals attacking polyunsaturated fatty acids present in and/or released from the cell membrane phospholipids (Halliwell & Gutteridge, 1990). Fig. 2 illustrates the chelating effect of propolis extract and fractions on ferrous ions. Like the antioxidant effect, the chelating capacity increased with the concentration of propolis extract and fractions. The reducing power

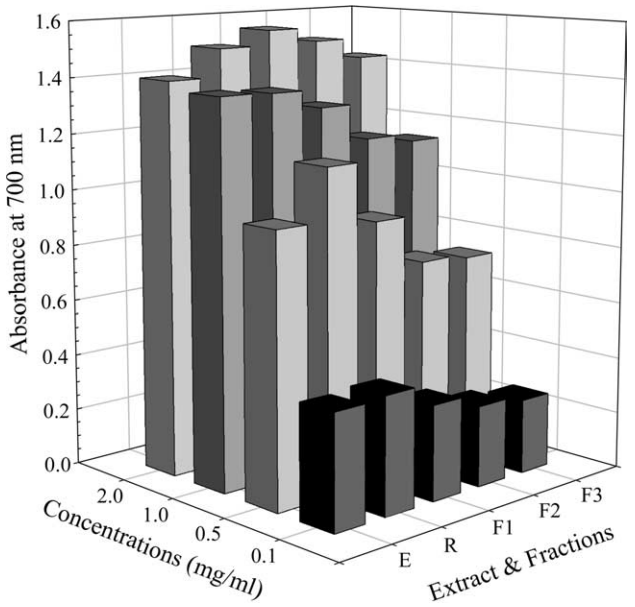

Fig. 3. Reducing power (absorbance at 700 nm) of various concentrations of propolis extract and fractions.

(absorbance at 700 nm) also increased with the concentration of propolis extract and fractions (Fig. 3). The absorbances of E, R, F1, F2 and F3 were between 1.40 and 1.56 at a concentration of 2 mg/ml. No significant differences were noted between propolis extract and fractions. Absorbance at 517 nm decreases when DPPH· accepts an electron or hydrogen radical; thus, DPPH· is used as a substrate to assess the scavenging activity of free radicals (Hatano et al., 1989). Fig. 4 illustrates the scavenging effect of various concentrations of propolis

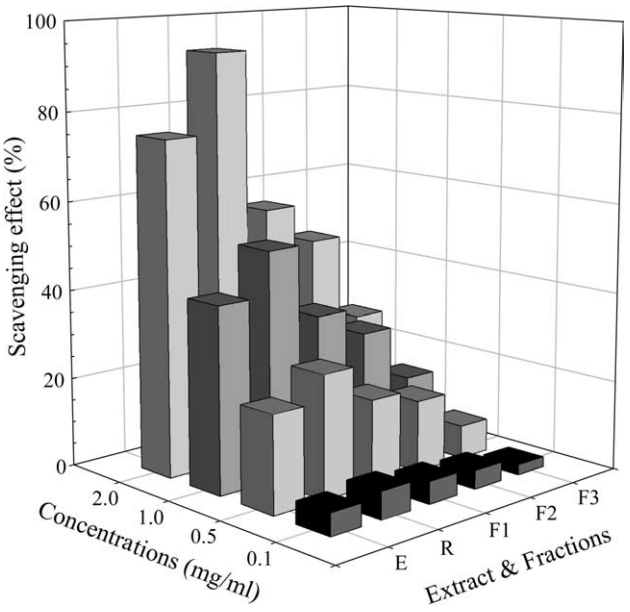

Fig. 4. Scavenging effects (%) of various concentrations of propolis extract and fractions on 1,1-diphenyl-2-picryl hydrazyl (DPPH) radical.

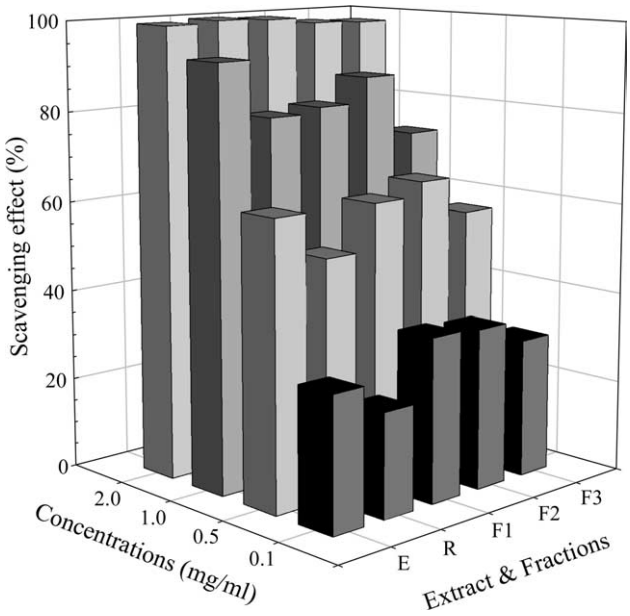

Fig. 5. Scavenging effects (%) of various concentrations of propolis extract and fractions on superoxide ($O_2\cdot^-$) radicals.

extract and fractions on DPPH. The ability to scavenge DPPH radicals increased with propolis extract and fraction concentrations. Moreover, the scavenging effects of propolis extract and fractions on DPPH at 2 mg/ml were: 93% (R), 80% (tocopherol), 75% (E), 56% (F1), 47% (F2), and 27% (F3). The free radical scavenging activity of R was significantly higher than that of tocopherol. Although F1 displayed less scavenging activity, 2 mg/ml F1 still displayed a scavenging effect of over 50%. Flavonoid compounds can donate hydrogen to break chain-reactions and thus protect lipids against oxidation damage (Pietta, 1999). The low concentration of propolis extract and fractions for DPPH scavenging indicated that propolis samples were effective free radical scavengers.

Reactive oxygen species (ROS) are generated very rapidly under aerobic conditions. Various types of ROS exist, including radicals, such as superoxide ($O_2\cdot^-$) and hydroxyl ion (·OH). Superoxide and hydroxyl ion have been implicated in the regulation of cell proliferation (Burdon & Rice-Evans, 1989). About 1–3% of the O_2 has been estimated to be converted to $O_2\cdot^-$ (Halliwell, 1996). Although SOD can dismutate $O_2\cdot^-$ in vivo, SOD is destroyed in the stomach following ingestion and thus cannot be absorbed by the intestine. Therefore, investigations have focussed on identifying $O_2\cdot^-$ scavengers, generally known as SOD-like materials or SOD activators. The extent of scavenging of superoxide anion ($O_2\cdot^-$) was measured by the increase in reduced cytochrome c, which displays maximal absorption at 550 nm. The scavenging effect of propolis extract and fractions on superoxide radicals also depends on dose (Fig. 5). Fractions F1, F2 and F3 displayed at least 50% scavenging at a concentration of 0.5 mg/ml. The scavenging effects of superoxide declined in the order E (93%) > F2 (87%) > rutin (85%) > F1 (81%) > R (80%) > F3 (73%) at 1 mg/ml. The above data indicate that the propolis fractions displayed the strongest scavenging effect on $O_2\cdot^-$; thus, propolis might be a good SOD-like material. The hydroxyl radical (·OH) is the most active free radical. EPR was used to test the scavenging effect on ·OH of the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH- + \cdot OH$). Furthermore, DMPO reacted with hydroxyl radicals to form DMPO-OH spin adducts. Fig. 6 reveals that propolis extract and fractions scavenged DMPO-OH spin adducts. All the samples displayed similar scavenging effects (65%) on hydroxyl radicals at a concentration of 1 mg/ml. Namiki (1990) observed that the hydroxyl radical is the major active oxygen species causing lipid oxidation. Therefore, the antioxidant activity of propolis fractions may also result from its scavenging effect on the hydroxyl radical.

The SFFE procedure examined here is suitable for performance on a large scale and, moreover, the preparation process involves no toxic solvents. Other studies have used methanol, hexane, or passing of extracts though separation columns, with CH_2Cl_2 or $CHCl_3$, to obtain active propolis fractions, but the extracts thus obtained contained hazardous solvents. Fractionation using SC-CO_2 on a pilot-plant scale yielded various total flavonoid contents and thus involved various antioxidant mechanisms. Overall, R and F1 had the strongest antioxidant capacities, followed by F2. This study demonstrates how various products can be obtained for various applications based on their compositions.

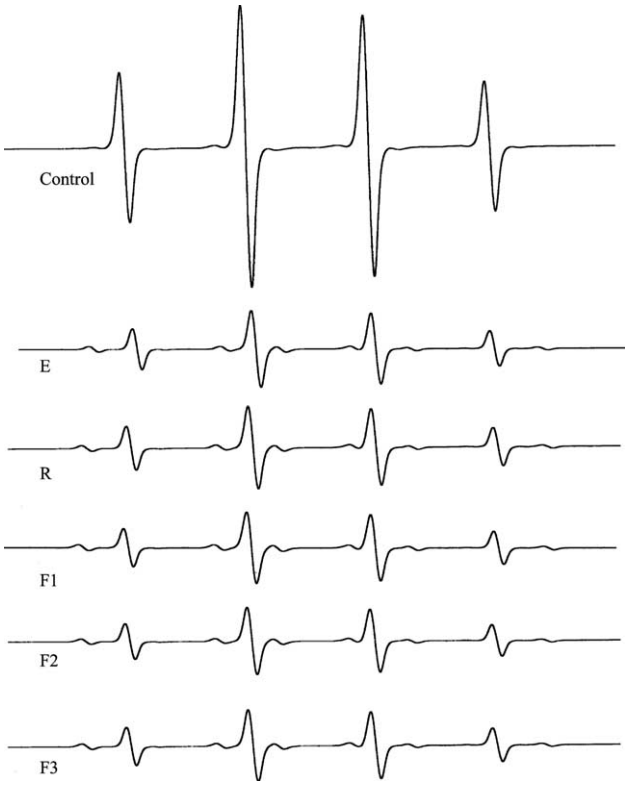

Fig. 6. Scavenging activity against hydroxyl (·OH) radical by propolis extract and fractions at the concentration of 1 mg/ml.

However, other biological functions of fractions, besides antioxidant activity, also deserve further study.

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