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In vitro studies of polyphenol compounds, total antioxidant capacity and other dietary indices in a mixture of plants (Prolipid)

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Abstract
The best health and nutrition results can be achieved not only from the consumption of fruits and vegetables with high antioxidant capacities, but also from medicinal plants and herbs. Therefore, in the present investigation, the bioactive compounds (polyphenols and flavonoids) and the radical scavenging capacities of Prolipid, a mixture of herbs, were studied. Water extracts showed relatively high capacity of about 61.5% inhibition with the β-carotene–linoleic acid assay. In order to support the data obtained with β-carotene–linoleic acid assay, three different antioxidant assays were used: ferric-reducing/antioxidant power, trolox equivalent antioxidant capacity, and 1,1-diphenyl-2-picrylhydrazyl radical with prolonged time of their reactions. It was found that the amounts of polyphenols in water and methanol extracts were 22.849 ± 2.267 and 3.241 ± 0.325 mg gallic acid equivalent/g dry weight, and the antioxidant capacities in same extracts as determined by the 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) test after 120 min were 275.81 ± 27.58 and 62.25 ± 6.11 micromole Trolox equivalents (μMTE) g dry weight, respectively. The correlation coefficients between polyphenols and antioxidant capacities of Prolipid with 1,1-diphenyl-2-picrylhydrazyl radical and β-carotene–linoleic acid assay were about 0.97 and 0.98. In conclusion, the bioactivity of Prolipid was high and the total polyphenols were the main contributors to the overall antioxidant capacity. The results of our investigation *in vitro* are comparable with other medicinal plants and fruits widely used in the treatment of humans without known side effects. Prolipid, a mixture of herbs, can therefore be used as a supplement for nutritional and healthy purposes.

Keywords: *Prolipid, main antioxidants, antioxidant capacity, antioxidant assays*

Introduction

Medicinal plants, herbs and fruits with a high content of bioactive compounds and related antioxidant capacity are inversely associated with morbidity and mortality from atherosclerosis in general, and coronary atherosclerosis in particular (O'Hara et al. 1998; Heber 2001; Holt and Chandra 2002; Gorinstein et al. 2005; Park et al. 2006). It has been shown that the best health and nutrition results can be achieved from the

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consumption of plants with high antioxidant activities (Proteggente et al. 2002; Halliwell and Whiteman 2004). Dietary bioactive compounds and microelements from different functional foods, herbs and nutraceuticals (ginseng, ginkgo, nuts, grains, tomato, soy phytoestrogens, curcumin, melatonin, polyphenols, antioxidant vitamins, carnitine, carnosine and ubiquinone) can ameliorate or even prevent diseases (Ferrari 2004; Szentmihalyi et al. 2005). Most of the *in vivo* experiments performed support this statement. Choi and Hwang (2005) showed that the intake of medicinal plants in rat diets results in an increase in antioxidant enzyme activity and high-density lipoprotein-cholesterol of serum, and in a decrease of malondialdehyde, which may reduce the risk of inflammatory and heart disease. Feeding of three herbs changed the cholesterol metabolism and increased antioxidant activity in plasma of steers (Miller et al. 2004; Hosoda et al. 2006).

Most researchers investigate traditional plants. However, we are now witnesses of interest in plants growing in tropical and subtropical areas. Ahmad and Holdsworth (2003) investigated 50 plant species used by Kadazan/Dusun communities in Sabah, Malaysia, as traditional herbal medicine. The antioxidant capacity of 14 herbs/spices from Cameroon has been evaluated, and it was found that the herbs contain high amount of antioxidants (Agbor et al. 2005). The antioxidant activity of sequential extracts obtained from *Chaerophyllum hirsutum* (Dall'Acqua and Innocenti 2004) was tested using only the reaction with the stable radical 1,1-diphenyl-2-picrylhydrazyl (DPPH). The bioactivity of traditional Japanese herbs was also reported in a recent investigation (Xiufen et al. 2004).

A special place is reserved for a mixture of some Indonesian herbs with the trade name Prolipid that has been successfully used as a plasma lipid-lowering remedy for a long time. There are no registered side effects from the use of this herbal mixture; however, scientifically it has not been investigated in connection with bioactive compounds.

Therefore, in the present study we decided to determine not only the amount of bioactive compounds in Prolipid, but also the antioxidant capacities of its methanol and water extracts. In order to receive the most reliable data, four different antioxidant assays—the Trolox equivalent antioxidant capacity (TEAC), ferric-reducing/antioxidant power (FRAP), DPPH radical, and β-carotene–linoleic acid assays—were applied.

As far as we know, there are no such published investigations.

Materials and methods

Chemicals

Trolox (6-hydroxy-2,5,7,8,-tetramethyl-chroman-2-carboxylic acid), butylated hydroxyanisole, 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\cdot+}$), $FeCl_3 \times 6H_2O$, Folin–Ciocalteu reagent, DPPH radical and β-carotene were obtained from Sigma Chemical Co. (St Louis, MO, USA). 2,4,6-tripyridyl-*s*-triazine (TPTZ) was purchased from Fluka Chemie (Buchs, Switzerland). All reagents were of analytical grade. Deionized and distilled water were used throughout.

Sample preparation

According to the producing Company's data (Perum Indofarma, Bekasi, Indonesia) the raw material used in Prolipid was a mixture of the following plants: *Sonchus*

arvensis L. from the *Compositae* (*Asteraceae*) family, *Guazuma ulmifolia* L. from the *Sterculiaceae* family and *Murraya paniculata* L. from the *Rutaceae* family. Prolipid contains extracts of *G. ulmifolia* (20% w/w), *M. paniculata* (10% w/w) and *S. arvensis* (10% w/w). The rest are supporting substances. One Prolipid capsule contains extracts of the following herbs and supporting substances: Guazuma, 86 mg; Murraya, 43 mg; Souchi, 43 mg; colloidal silicon dioxide, 20 mg; amylum maydis, 237.14 mg; and methylparaben–flopylparaben, 0.86 mg.

Prolipid capsules were obtained as a gift from the drug importer COWIK (Warsaw, Poland).

Extraction of polyphenols

Prolipid samples were taken from capsules, defatted with acetone and then extracted from 1 g with 20 ml of 80% methanol for 3 h three times at room temperature, and the solvent was then removed by vacuum distillation for polyphenols (methanol-soluble extract). Decoction was prepared from 1 g dried plant material from the capsules of Prolipid in 20 ml distilled water for 20 min by boiling for soluble water polyphenols (water fraction). The samples were centrifuged for 5 min at 4,000 $\times g$ with a benchtop centrifuge to remove solids. Aliquots of 1 ml water extracts were frozen and used when necessary for the antioxidant tests.

Ultraviolet–visible spectophotometric analysis

The spectra of water and methanol extracts in concentrations of 1 mg/ml were measured on a Uvikon 930 spectrometer (Bio-Teck-Kontron, Kontron Instruments, Watford, UK) and were recorded from 180 to 300 nm. The solution of standard catechin was prepared in methanol at a concentration of 50 μM (Sarni-Manchado et al. 2000).

Polyphenols

The Folin–Ciocalteu method was used and the measurement was performed at 765 nm with gallic acid as the standard (Singleton et al. 1999). The results were expressed as milligrams of gallic acid equivalent (GAE) per gram of dry weight (DW).

Flavonoids

Flavonoids (extracted with 5% $NaNO_2$, 10% $AlCl_3 \times 6H_2O$ and 1 M NaOH) were measured at 510 nm with a known (+)-catechin concentration as a standard. The results were expressed as milligrams of catechin equivalents (CE) per gram of DW (Singleton et al. 1999).

Antioxidant capacities

The β-carotene–linoleic acid assay was performed according to Ferreira et al. (2006). A stock solution of β-carotene and linoleic acid was prepared by dissolving 0.5 mg β-carotene in 1 ml chloroform and adding 25 μl linoleic acid together with 200 mg Tween 40. The chloroform was evaporated. One hundred millilitres of aerated water were added to the residue. To 2.5 ml of this mixture were added 300 μl of each extract.

The samples were incubated in boiling water for 120 min together with two blanks, one containing the antioxidant butylated hydroxyanisole (BHT) and the other one without antioxidant. The absorbance was measured at 470 nm.

For the TEAC assay, the $ABTS^{\cdot+}$ radical cation was generated by the interaction of ABTS (250 μM) and $K_2S_2O_8$ (40 μM). After the addition of 990 μl $ABTS^{\cdot+}$ solution to 10 μl Prolipid extracts or Trolox standards (final concentration 0–20 μM) in methanol or 20 mM acetate buffer (pH 4.5), the absorbance was monitored. The percentage decrease of the absorbance at 734 nm was calculated and plotted as a function of the concentration of the extracts and of Trolox for the standard reference data (Ozgen et al. 2006).

The FRAP assay measures the ability of the antioxidants contained in the samples to reduce ferric-tripiridyltriazine (Fe^{3+}-TPTZ) to a ferrous form (Fe^{2+}) that absorbs light at 593 nm. The ferro-iron and ferric-iron form complexes with TPTZ reagent that are the main products of this reaction. The FRAP level was calculated by plotting a standard curve of absorbance against concentration of Trolox (Ozgen et al. 2006).

In the DPPH assay, the volume of Prolipid extracts in different test tubes was adjusted to 100 μl by adding MeOH. A 0.1 mM methanolic solution of DPPH was added (5 μl) to these tubes. The control was prepared as above without any extract, and MeOH was used for the baseline correction. Changes in the sample's absorbance were measured at 517 nm. Butylated hydroxyanisole was used for comparison (Ozgen et al. 2006).

The three antioxidant assays (DPPH, ABTS and FRAP) were compared at the same periods of time duration (10, 30, 60, and 120 min) and the same concentration of the investigated Prolipid water and methanolic extracts of 10 mg/ml. For each individual antioxidant assay, a trolox aliquot was used to develop a standard curve (Ozgen et al. 2006). All data were then expressed as trolox equivalents (TE).

Statistical analyses

The values are the mean ± standard deviation of three measurements. Where appropriate, data were tested by two-way analysis of variance using GraphPad Prism, version 2.0 (GraphPad Software, San Diego, CA, USA), following by Duncan's new multiple range test to assess differences between-group means. Differences of $P < 0.05$ were considered significant.

Results

There were various contents of phenolic compounds in the extracts, depending on the extraction solvent. Water extracts (Figure 1) had maximum absorptions of their ultraviolet spectra in a narrow range between 198.3 and 205.4 nm, and the methanolic extract was very similar to catechin (standard) and was between 198.4 and 206.2 nm, which indicated that flavonoids predominated in the phenolic compounds. The absorption units on the spectra were higher in the water extract than in the methanol extract, showing higher yield during the decoction in boiling water than in methanol solvent.

The amounts of polyphenols in water and methanol extracts were estimated as 22.849 ± 2.267 and 3.241 ± 0.325 mg GAE/g DW, respectively (Figure 2). The

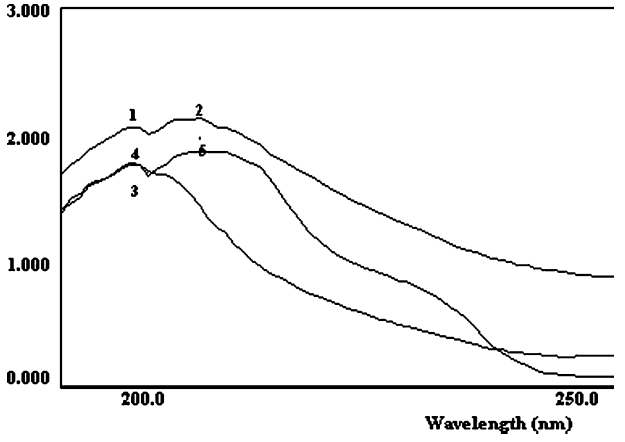

Figure 1. Ultraviolet–visible spectrum of the polyphenols and flavonoids in water and methanol extracts of Prolipid detected at the corresponding wavelength at 1 mg/ml with major peaks: (1), 198.3 nm, absorption units (Abs), 2.061; (2), 205.4 nm, Abs, 2.136; (3) = (4), 198.4 nm, Abs, 1.791; (5), 206.2 nm, Abs, 1.881. Peaks 1 and 2, water extract; peaks 3 and 4, methanol extract; peaks 4 and 5, 50 μM catechin.

contents of flavonoids in water and methanol extracts were 2.295 ± 0.212 and 0.583 ± 0.058 mg CE/g DW, respectively. The antioxidant capacity of the water extracts was also higher than in methanol extracts as determined by the three other studied methods: for ABTS after 120 min, 275.81 ± 27.58 and 62.25 ± 6.11 μMTE/g DW, respectively (Figure 3); for FRAP after 120 min, 74.98 ± 7.21 and 9.87 ± 0.93 μMTE/g DW, respectively; and for DPPH after 120 min, 62.44 ± 4.65 and 8.24 ± 0.71 μMTE/g DW, respectively (Figure 3). In Prolipid the water-soluble polyphenols are probably the main contributors that influence the antioxidant capacity. The antioxidant methods with the prolonged time showed the increase in the percentage of inhibition by ABTS, DPPH and FRAP scavenging assays during 120 min. The kinetic data of the antioxidant capacity in these three methods demonstrated an increase by 1.6, 2.0 and 1.8 times, showing that all radical scavenging reactions are a factor of time (Figure 3). The obtained results proved that the same patterns of water and methanol extracts occurred during the prolonged time of scavenging reaction, but the increase of the antioxidant capacity was slightly different with the three methods used (Figures 2 and 3).The increase of the antioxidant capacity only by ABTS during 120 min was slightly higher in methanol extracts than in water extracts during the same reaction time and the same concentration of the sample. For the plant extracts the routine time of the ABTS, DPPH and FRAP assays is not enough to complete the reaction, and therefore the prolonged time was used (Ozgen et al. 2006).

In the water extract the ratio of the antioxidant capacities by ABTS/FRAP after 10 min was 2.02, and after 120 min was 1.83; in methanol extract after 10 min the ratio was 2.44, and after 120 min it was 3.15. The correlation coefficients in the water fraction between the polyphenols and the antioxidant capacities were relatively high. The best results were obtained between the percentage of inhibition and the antioxidant capacities determined by DPPH and β-carotene with the same concentration (20 mg/ml) and the same time of incubation (120 min); these were 0.9662 and 0.9784, respectively (Figure 4).

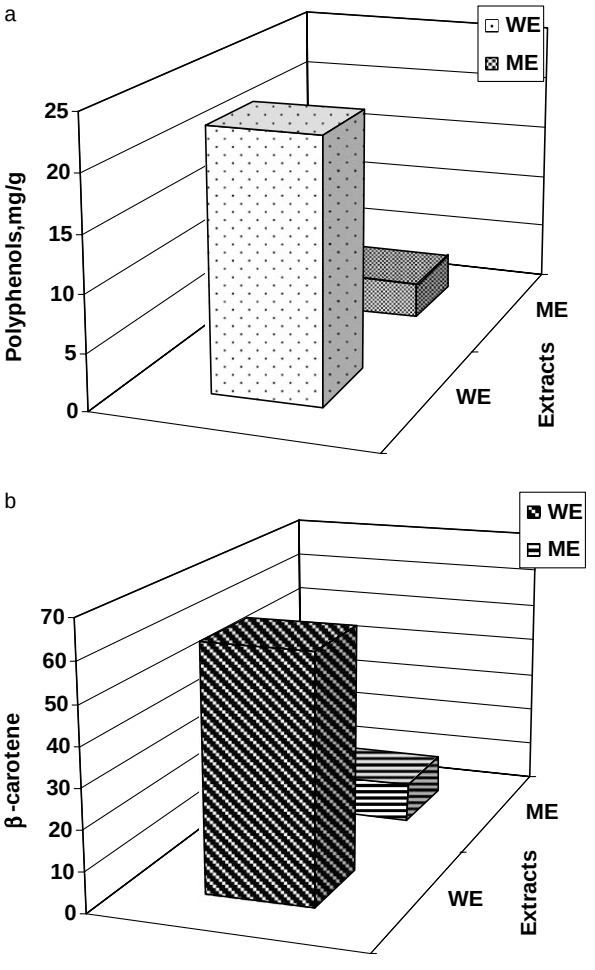

Figure 2. (a) Polyphenols (mg GAE/g) in water (WE) and methanol (ME) extracts of Prolipid. (b) Antioxidant capacity determined by β-carotene–linoleic acid assay (% inhibition).

Discussion

Free radicals and other reactive species are thought to play an important role in many human diseases. Establishing their precise role requires the ability to measure them and the oxidative damage that they cause (Halliwell and Whiteman 2004). Plants have long been regarded as having considerable health benefits, due to their main antioxidant compounds—phenolics (Sarni-Marchado et al. 2000; Caballero-George et al. 2002; Gorinstein et al. 2005). In this manuscript, we reviewed some of the most commonly used biologically based approaches, including herbs, supplements, and other medicinal plants, which are encountered in prevention of cardiovascular disease, focusing on potential and adverse effects, and treatment interactions (Miller et al. 2004). It was shown that plants could prevent a wide range of illnesses; therefore, in recent years many investigations of plants *in vitro* were conducted. According to the US Recommended Dietary Allowances, *Agrimoniae herba* for chromium, *Betulae folium* for manganese, *Taraxaci radix* for copper and chromium, and *Urticae folium* for

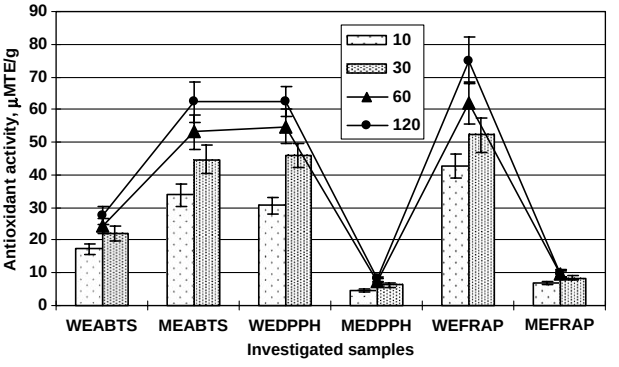

Figure 3. Antioxidant activity (AA) of methanol (ME) and water (WE) extracts from Prolipid determined by the three different antioxidant methods (µM TE/g DW).

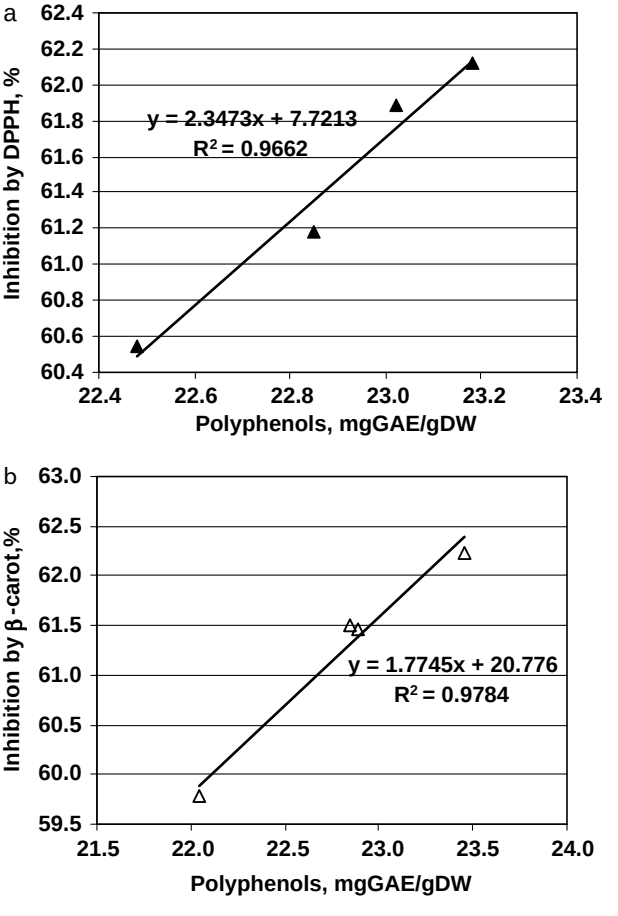

Figure 4. Correlation coefficients between (a, ▲) polyphenols (mg GAE/g DW, x) and the antioxidant capacity by DPPH of Prolipid water extract (% inhibition, y), and (b, Δ) polyphenols (mg GAE/g DW, x) and the antioxidant capacity by β-carotene–linoleic acid assay of Prolipid water extract (% inhibition, y).

potassium and calcium were recommended as good nutritional sources in teas (Szentmihalyi et al. 2005). It was found that plants contain different quantities of antioxidant compounds and have different levels of antioxidant capacity (Ahmad and Holdsworth 2003; Agbor et al. 2005; Choi and Hwang 2005). Therefore a mixture of herbs (Prolipid), which is used as an additive for decreasing lipids in blood, was investigated in this study.

The antioxidant capacity is mainly derived from the water-soluble antioxidants and has a high correlation coefficient with polyphenols (0.97), which corresponds with other works (Caballero-George et al. 2002). The radical scavenging assays were used to show the ability of the prolipid extracts to scavenge free radicals *in vitro* (expressed as the TEAC value). The results were higher because of the prolonged time of the ABTS, DPPH and FRAP application. The dry substance of the capsule was prepared as sometimes consumed, as a boiled medicinal tea/soup or medicinal bolus. Both aqueous and methanolic extracts of Prolipid were used for comparison. As it was shown, the ABTS values of Prolipid were higher than those with the FRAP assay in water-soluble as well as water-insoluble extracts. This can be explained by the water-soluble extracts containing a high proportion of antioxidants with a higher activity in the ABTS method than in the FRAP assay. According to Nilsson et al. (2005) the highest ratio of ABTS/FRAP was about 2.6 for catechin. As shown in Figure 1, the ultraviolet spectra of catechin and the methanol extract of Prolipid were closed to each other and showed that catechin was one of the main bioactive compounds responsible for the high antioxidant capacity of the studied mixture of herbs. Such interpretation of our results corresponds also with the data of Caballero-George et al. (2002), who reported that 70% acetone extract of the bark of *G. ulmifolia* Lam. consisted mainly of epicatechin, which contributes to the very broad spectrum of biological activities of the condensed tannins. Another possibility for explanation of the antioxidant capacity can be that the synergy effects are higher in water than in acetone extracts (Nilsson et al. 2005). A large screening study (Halvorsen et al. 2002) of the antioxidant capacity in dietary plants reported the data of methanol extracts.

Our results are in correspondence with others (Cai et al. 2004) showing that the total phenolics (g GAE/100 g DW) in aqueous extracts were similar to *Pyrrosia lingua* (Thunb.) Farwell at 2.04, in comparison with a slightly higher amount in Prolipid of 2.28, but the methanolic extracts in Prolipid were weaker by as much as five times (Prolipid versus *P. lingua* = 0.324 versus 1.65). The TEAC by ABTS (μMTE/100 g DW) of Prolipid in water fraction is about 17,260 in comparison with 17,674 for Anacardiaceae *Rhus chinensis* Mill. or *R. potaninii* Maxim. (the Gall is medically used part). Major types of phenolic compounds in this medicinal plant were hydrolysable tannins (gallotannin) and phenolic acids (gallic acid). The disagreement between the phenolic compounds and the antioxidant activity in the study of Cai et al. (2004) with our results can be explained by different variables such as the geographical area, seasonal time of collection, different type of medicinal plants and the conditions of extraction and time of the antioxidant assay, as it is known that the antioxidant assay is a function of the time used. The minimum time of the ABTS in this report was 10 min in comparison with the 6 min used by Cao et al. (2004). The results of the antioxidant value in the present report therefore have to be higher than in Cao et al. (2004). According to the water extract, the antioxidant capacity of Prolipid has one of the highest values among the traditional Chinese medicinal plants (Cai et al. 2004).

Hot water extraction used in this study was a useful method with an extracting efficiency of 80.38% for antioxidant capacity and 85.82% for total phenols, as compared with 80% in the methanolic extract. These results were similar to others (Cai et al. 2004) showing about 83.7% for antioxidant activity and 77.4% for total phenolic content.

The antioxidant capacity by DPPH of Prolipid water extracts can be compared with some Japanese herbs. Our results with the DPPH assay have shown that, at a concentration of 10 mg/ml, in the water extract after 10 min the reaction was about 14.82%, and 2.11% in the methanol extract. These data correspond to Xiufen et al. (2004), who showed inhibition of 28.03% of one of the investigated Japanese herbs Senbuti at a concentration of 0.67 mg/ml. The results of the degree of inhibition by DPPH and β-carotene (Figures 2 and 4) can also be compared with the data of Ferreira et al. (2006), where medicinal plants from Portugal were screened. Decoction extracts of DPPH scavenging were similar for *Laurus nobilis* of about 61%, our results being 61.18%; and the β-carotene was similar to another plant, *Mentha suaveolens*, at about 65% in comparison with our data of 61.5%.

The amount of polyphenols for Prolipid varied between 3.24 in methanol and 22.85 in water extracts, respectively, and corresponded with the data of Prakash et al. (2007), who showed the variation of the investigated medicinal plants from 2.8 to 107.8 mg/g. The correlation coefficient between the content of polyphenols and their free radical scavenging activities was high and differed from the data of Prakash et al. (2007). These authors show that fruits of *Cassia fistula* with high phenol content (107.8 mg/g) have poor reducing and antiradical powers. In contrast, the bark of *Casuarina equisetifolia* and fruits of *Lawsonia inermis* with comparatively lower phenol contents (72.1 and 75.8 mg/g, respectively) exhibited good reducing and antiradical powers.

The antioxidant activity of Prolipid can be compared with fruits such as *Actinida chinensis* (kiwifruit), *Citrus sinensis* (orange), persimmon and grapefruit (Gorinstein et al. 2005; Park et al. 2006). The antioxidant capacities of kiwifruit and orange methanol extracts by ABTS were about 48.9 and 67.2 versus Prolipid of 3,387 μMTE/100 g DW, and the polyphenols were 0.22 and 0.51 versus Prolipid of 0.324 g GAE/100 g DW (Cai et al. 2004). The ratio of ABTS/FRAP in our research is equal to orange (1.83–2.02), corresponding with Nilsson et al. (2005), with the ABTS result of 283 μM TE/g DW and FRAP of 142.4 μM Fe^{2+}/g DW. These data were similar to ABTS Prolipid of 276 and to FRAP of 150 during the prolonged time of the applied assays in our report. FRAP in grapefruit was 51 μM Fe^{2+}/g DW (25.5 μM TE/g DW) in kiwifruit and persimmon 56.9 μM Fe^{2+}/g DW(28.5 μM TE/g DW), and these data were similar to some dietary plants and Prolipid (Halvorsen et al. 2002; Szeto et al. 2002; Nilsson et al. 2005).

The results reveal that the extracts of Prolipid showed remarkably higher antioxidant capacity and contained significantly more phenolics in comparison with some fruits.

Conclusions

Prolipid has high amounts of bioactive substances, mostly such as polyphenols and flavonoids, and a corresponding high antioxidant capacity. Prolipid can be

compared with other medicinal plants, herbs and fruits that are widely used for medicinal and nutritional purposes.

Functional foods and nutraceuticals constitute a great promise to improve health and prevent aging-related chronic diseases (Ferrari 2004; Szentmihalyi et al. 2005) and Prolipid can be used for these purposes.

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