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ORIGINAL PAPER

Biochemical Characteristics of the Herb Mixture Prolipid as a Plant Food Supplement and Medicinal Remedy

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Abstract Prolipid a known mixture of herbs is used as a plasma lipid lowering medicine. No side effects were registered. However, the bioactive substances of Prolipid were not investigated. Therefore in this investigation Prolipids bioactive compounds and antioxidant activity were studied. The contents of polyphenols and flavonoids were 19.87±2.09 and 3.09±0.31 mg gallic acid equivalent GAE/g DW and 2.09±0.24 and 0.57±0.05 mg catechin equivalent CE/g DW in water and methanol fractions, respectively. Anthocyanins (0.02±0.001 mg/g DW) and flavanols (7.58±0.81 μg CE/g DW) were found only in water fraction. The antioxidant activity of Prolipid, as determined by four different antioxidant assays [ferric-reducing/antioxidant power (FRAP); cupric reducing antioxidant capacity (CUPRAC); trolox equivalent antioxidant capacity (TEAC); 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH)], was higher in water than in methanol fraction. The correlation coefficients between polyphenols, flavonoids and antioxidant activities of Prolipid water extracts with TEAC were 0.97 and 0.90, respectively. It can be concluded that the content of polyphenol compounds in Prolipid is very high and they are the main contributors to Prolipid's overall antioxidant activity. Prolipid is widely used in human treatment without known side effects on patients and is comparable to other medicinal plants, and as a strong antioxidant mixture could be used as a supplement to known atherosclerosis preventing diets.

Keywords Prolipid herb mixture · Plant food supplement · Antioxidants · Antioxidant activity · Radical scavenging assays

Abbreviations

AA	Antioxidant activity
ABTS	2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)
BHA	Butylated hydroxyanisole
CE	Catechin equivalent
CGE	Cyanidin-3-glucoside equivalent
CUPRAC	Cupric reducing antioxidant capacity
DPPH	1,1-diphenyl-2-picrylhydrazyl radical
EGCG	Epigallocatechin gallate equivalent
FRAP	Ferric reducing antioxidant power
GAE	Gallic acid equivalent
MF	Methanol fraction
TEAC	Trolox equivalent antioxidant capacity
TPTZ	2,4,6-tripyridyl-*s*-triazine
Trolox	6-hydroxy-2,5,7,8,-tetramethyl-chroman-2-carboxylic acid
WF	Water fraction

Introduction

Some medicinal plants are widely and successfully used in folk medicine for treatment of different diseases [1, 2]. Plants have long been regarded as having considerable health benefits, due to their main antioxidant compounds—phenolics [3–5]. It was shown that most of the herbs used possess antioxidant properties [6, 7]. Therefore, these natural

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products are successfully applied for treatments in which oxidative stress is prominent: cardiovascular disease [8–12], aging [13, 14], liver disease, jaundice and diabetes [6]. Among used herbs a special place is reserved for Prolipid. This herb mixture was investigated in vitro, in vivo and in humans. Prolipid contains extracts of guazumae (*Guazuma ulmifolia*), murraya (*Murraya paniculata*) and sonchus (*Sonchus arvensis*). Chemical analysis shows that the herbs contain some important bioactive compounds inter alia, cinnamates, coumarins, carotenoids, flavonoids, and tannins [15, 16]. In experiments on laboratory animals and in investigations of patients, these herbs lead to plasma hypolipidemic and hypoglycemic effects [17, 18]. It also must be mentioned that the herbs which are included in the Prolipid mixture have been widely used for many years in traditional medicine in tropical countries without any side effects [19]. However, the antioxidant activity of Prolipid has been practically not investigated. Therefore, the aim of this study was to determine the contents of Prolipid's polyphenols, flavonoids, anthocyanins and flavanols and the mixtures' antioxidant activity and to compare these values with other used herbs. It was shown that assessments of the bioactivity of individual compounds do not reflect the true antioxidant value of the studied antioxidant natural product [20]. Therefore, in addition to the investigation of individual bioactive compounds, the total antioxidant activity of Prolipid was also determined. There are many methods for determination of total antioxidant activity and every one has its limitations [21]. Some of these antioxidant assays give a variety of antioxidant activity trends [22]. In order to receive the most reliable data several different antioxidant assays were used: (a) TEAC; (b) FRAP; (c) DPPH; (d) CUPRAC and (e) Folin-Ciocalteau. As far as we know there are no published papers on this matter.

Materials and Methods

Chemicals

Trolox (6-hydroxy-2,5,7,8,-tetramethyl-chroman-2-carboxylic acid); butylated hydroxyanisole (BHA); ABTS|$^{+}$[2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)]; $FeCl_3 \cdot 6H_2O$; Folin-Ciocalteau reagent; DPPH; $CuCl_2 \cdot 2H_2O$ and neocuproine (2,9-dimethyl-1,10-phenanthroline) were obtained from Sigma Chemical Co., St. Louis, MO, USA. 2,4,6-tripyridyl-*s*-triazine (TPTZ) was purchased from Fluka Chemie, Buchs, Switzerland.

Plant Material

Prolipid is a mixture of different herb extracts and was obtained from the producing Company. According to the Company's data (Perum Indofarma, Bekasi, Indonesia) the bioactive material used in Prolipid is from 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 *Guazuma ulmifolia*—20% *w*/*w*, *Murraya paniculata*—10% *w*/*w* and *Sonchus arvensis*—10% *w*/*w*. The rest are supporting substances. Prolipid was provided by the drug (medicine) importer COWIK (Warsaw, Poland).

Extraction of Polyphenols

In order to obtain methanol-soluble polyphenols (methanol fraction), Prolipid samples were taken from capsules, defatted with acetone and then extracted from 1 g with 20 ml of methanol at room temperature for 3 h. The sample (1 g) was extracted with 20 ml of water at room temperature for 3 h for obtaining water-soluble polyphenols (water fraction).

UV-Visible Spectophotometric Analysis

The spectra of water and methanol fractions in concentration of 1 and 0.5 mg/ml were measured on an Uvikon 930 (Bio-Teck-Kontron) and were recorded from 180 to 300 nm. The solution of phenolic compounds was prepared in methanol [23].

Polyphenols Determination

The Folin-Ciocalteu method was used and the measurement was performed at 765 nm with gallic acid as the standard. The results were expressed as milligrams GAE per gram of DW [24].

Total Flavonoid Determination

Flavonoids, extracted with 5% $NaNO_2$, 10% $AlCl_3 \cdot 6H_2O$ and 1 M NaOH, were measured at 510 nm with known (+)-catechin concentration as a standard and expressed as mg CE/g DW [24].

Total Anthocyanins Determination

The total anthocyanins content was measured by a pH differential method [25]. Results were expressed as micrograms of cyanidin-3-glucoside equivalent (CGE) per gram of DW.

Total Flavanols Determination

Flavanol content was estimated using the *p*-dimethylaminocinnamaldehyde (DMACA) method. Results were expressed as micrograms of CE per gram of DW [26].

Determination of the Antioxidant Activity

As it was already mentioned the following tests for determination of the antioxidant activity were used:

1. TEAC was done using the ABTS|$^{+}$radical cation in methanol or phosphate-buffered saline (pH 7.4). For the modified assay, ABTS was dissolved in 20 mM acetate buffer (pH 4.5). The absorbance was measured at 734 nm [15].
2. FRAP assay measures the ability of the antioxidants contained in the samples to reduce ferric (Fe^{3+})-TPTZ to a ferrous form (Fe^{2+}) which absorbs light at 593 nm [14].
3. In DPPH assay the changes in the absorbance of the samples were measured at 517 nm. BHA was used for comparison [11, 12].
4. CUPRAC is based on utilizing the copper (II)-neocuproine [Cu (II)-Nc] reagent as the chromogenic oxidizing agent. The absorbance at 450 nm was recorded against a reagent blank [4].

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 methanolic and water fractions of 10 mg/ml [11]. All data were then expressed as TE.

Statistical Analysis

The results of the investigation are expressed as means±SD of five repetitions. Where appropriate, the analysis of variance (ANOVA) was used. P values of <0.05 were adopted as statistically significant.

Results

UV Spectra

There were different contents of phenolic compounds in the fractions, depending on the extraction solvent. Water and methanolic fractions had maximum absorptions of their UV spectra in a narrow range between 197.6 and 279.9 nm and very similar to gallic acid and epicatechin. The absorption units on the spectra were slightly higher in the water extract than in the methanol one, showing a higher yield during the extraction in water than in methanol solvent. The difference was not significant.

The Bioactive Compounds

The amount of polyphenols in water (WF) and methanol (MF) fractions was estimated as: 19.87±2.09 and 3.09± 0.31 mg GAE/g DW, respectively.

Flavonoids in WF and MF were as 2.09±0.24 and 0.57± 0.05 mg CE/g DW, respectively.

Anthocyanins and flavanols (μg CE/g DW) were found only in water fraction: 0.02±0.01 mg/g DW and 7.58± 0.81 μg CE/g DW, respectively.

The Antioxidant Activity

The antioxidant activity in WF was higher than in MF by all studied methods (μM TE/g DW): ABTS results obtained after 9 min were 117.79±12.12 and 16.64±1.73; CUPRAC results obtained after 60 min 34.72±4.03 and 4.96±0.52; FRAP results obtained after 4 min 37.79±4.42 and 4.65± 0.42; DPPH results obtained after 20 min 36.16±3.64 and 5.21±0.61, respectively (Fig. 1). Probably in Prolipid the water soluble polyphenols are the main contributors which influence the antioxidant activity. The antioxidant activity using ABTS assay was related to the inhibition time of the reaction and proportionally increased with the reaction time from 1 to 9 min of this reaction (Fig. 2a). Glutathione (GL) was used as a standard, and the antioxidant capacity of GL and MF were nearly equal, but the WF was about eight times more active (Fig. 2b). The antioxidant methods with the prolonged time showed the following results: the percentage of inhibition by ABTS and DPPH scavenging assays increased during 120 min (Fig. 2c), and the antioxidant activity by three methods (Table 1) has increased as well, showing that the scavenging reaction is a factor of time (Fig. 2a,c). For the plant extracts the routine time of ABTS, DPPH and FRAP assays is not enough to complete the reaction, therefore the prolonged time was used [11, 12, 15]. The obtained results proved that the same patterns of WF and MF occurred during the prolonged scavenging reaction time, but the increase of the antioxidant activity was slightly different with the three methods used (Table 1). In WF the increase in the antioxidant activity using three methods was the following: in ABTS, 248.31/

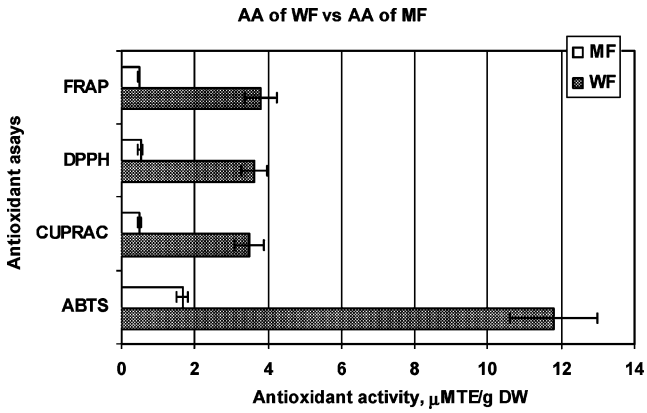

Fig. 1 Antioxidant activity of MF and WF from Prolipid determined by four different antioxidant methods (10× μM TE/g DW)

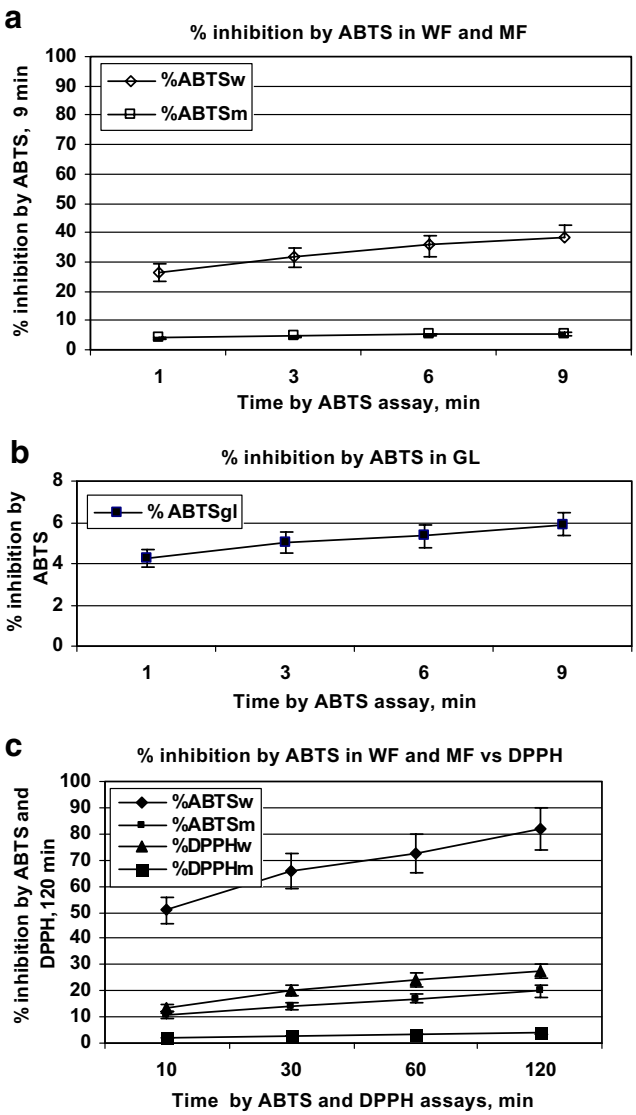

Fig. 2 Kinetics of ABTS scavenging effect of: **a** Prolipid WF and MF; **b** gluthatione (GL) during 9 min. The concentration of the samples was 0.25 mg/ml. ABTS was produced by reacting with potassium persulfate. **c** Kinetics of ABTS and DPPH scavenging effect of Prolipid extracts by prolonged assays during 120 min. The concentration of the samples was 0.1 mg/ml. *%ABTSw* Percent inhibition by ABTS test in WF; *%ABTSm* percent inhibition by ABTS test in MF; *%DPPHw*, percent inhibition by DPPH test in WF; *%DPPHm*, percent inhibition by DPPH test in MF

117.79=2.11; in FRAP, 64.91/37.79=1.72 and in DPPH, 56.44/36.16=1.56 times. In MF the following numbers of the increase in antioxidant activity were found: in ABTS, 60.25/16.64= 3.62; in FRAP, 9.27/4.65 =1.99 and in DPPH, 5.5/5.2=1.1 times higher than by normally used assays for routine analyses (Fig. 1 and Table 1). The increase of the antioxidant activity by ABTS and FRAP within the time was higher in methanol extracts than in the water ones during the same time of reaction and the same concentration of the sample. The antioxidant activity by

Table 1 Antioxidant activity (μM TE g^{-1} DW) by ABTS, DPPH and FRAP scavenging assays in water (WF) and methanol (MF) fractions

Sample/ Time	10 min	30 min	60 min	120 min
ABTS				
WF	154.46± 15.5[a]	199.37± 19.25[b]	219.81± 20.11[c]	248.31± 21.61[d]
MF	32.87±3.05[a]	42.72±4.21[b]	51.13±5.23[c]	60.25±6.11[d]
DPPH				
WF	27.58±2.54[a]	40.95±3.65[b]	49.56±5.21[c]	56.44±4.65[d]
MF	3.93±0.41[a]	6.04±0.72[b]	7.17±0.62[c]	7.84±0.71[d]
FRAP				
WF	35.67±3.81[a]	45.38±3.25[b]	53.46±5.41[c]	64.91±6.21 [d]
MF	5.94±0.63[a]	7.82±0.68[b]	9.06±0.91[c]	9.27±0.93 [d]

The values are means±SD of five repetitions. Means in rows without superscript letters in common differ significantly ($P<0.05$)
ABTS [2,2′-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid)]; *DPPH* 1,1-diphenyl-2-picrylhydrazyl; *DW* dry weight; *FRAP* ferric-reducing/ antioxidant power; *TE* trolox equivalent

DPPH was nearly the same in methanol extract during the prolonged time and in water fraction the increase was lower than by ABTS and FRAP. Therefore DPPH assay can be used for these types of herbs using the normal time of

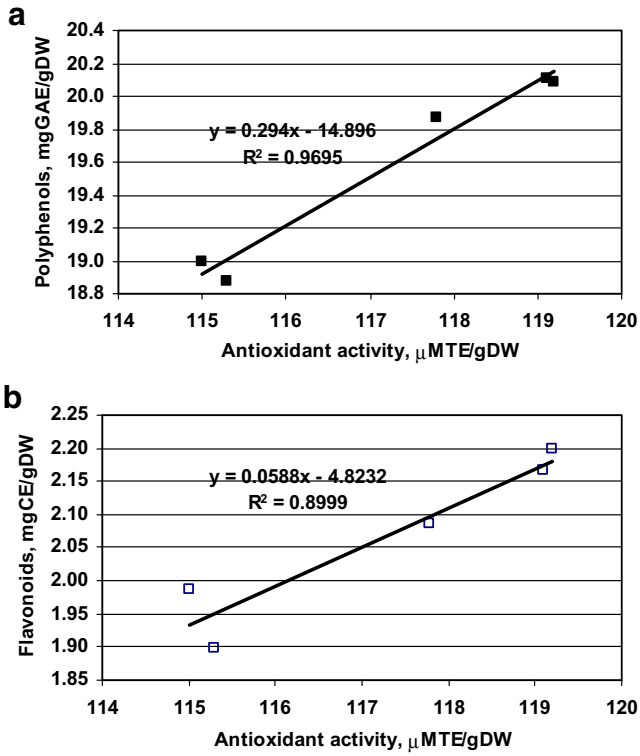

Fig. 3 Correlation coefficients between: **a** *closed squares* the antioxidant activity of WF from Prolipid (μM TE/g DW, X) and polyphenols (mgGAE/g DW, Y) and **b** *open squares* the antioxidant activity of WF from Prolipid (μM TE/g DW, X) and flavonoids (mgCE/g DW, Y)

reaction (20 min). The correlation coefficients in WF between the polyphenols, flavonoids and antioxidant activities were 0.9695 and 0.8999 (Fig. 3).

Discussion

Nowadays we are witnesses of increasing interest in plants growing in subtropical and tropical areas [8]. Indonesian herb mixture, named Prolipid, which has been successfully used for long time as a plasma lipid lowering remedy, is one of the most popular remedies. There are no registered side effects from the use of this herbal mixture in experiments with laboratory animals and in clinical investigation [19]. However, the antioxidant activity of Prolipid or of its components has not been scientifically investigated enough [8, 17]. Therefore, in this investigation the contents of the main bioactive compounds in this herb's mixture and its antioxidant activity were determined.

Plants contain a large number of structurally different antioxidant polyphenols. It is of great interest to know whether some of the most important ones such as gallic acid and epicatechin are similar in their UV spectra with the WF and MF.

The shifts in the wavelengths in gallic acids were fixed at 216, 218 and 270 mn and in epicatechin in the range of 216 nm which were similar to Friedman and Juergens [23]. A high content of polyphenols and flavonoids was registered: 19.869 and 3.087 mg GAE/g DW and 2.086 and 0.566 mg CE/g DW in WF and MF, respectively. Anthocyanins (0.018 mg/g DW) as well as flavanols (7.577 μg CE/g DW) were found only in water fraction. Also the antioxidant activity of Prolipid, as determined by four different antioxidant assays (FRAP, CUPRAC, TEAC and DPPH) was higher in water than in methanol fraction. Furthermore it was found that total polyphenols are the main contributor to Prolipid's overall antioxidant activity. The antioxidant activity is mainly derived from the water soluble antioxidants and has a high correlation coefficient with polyphenols (0.97). Our results are similar to Apak et al. [4], who reported that the correlation of the total phenolic content of herbal teas determined with Folin-Ciocalteau assay and their CUPRAC and ABTS total antioxidant capacities gave linear curves with correlation coefficients of 0.966 and 0.936, respectively. Our results are in accordance with others who investigated the total phenolic content (TPC) and related total antioxidant activity (AA) of 70 medicinal plant infusions [2]. TPC of medicinal plant water extractions at high temperature ranges from 9 to 2,218 mg GAE/l and AA by FRAP ranged from 0.06 to 25 mMTE/l. There was a significant linear correlation between TPC and AA by FRAP. According to Katalinic et al. [2] 70 medicinal plant extracts could be divided into five groups: (a) very low FRAP (<1 mM/l); (b) low FRAP (1–5 mM/l); (c) good FRAP (5–10 mM/l); (d) high FRAP (10–20 mM/l); and (e) very high FRAP (>20 mM/l). In order to compare our data with that cited by Katalinic et al. [2], the polyphenols in WF estimated as 19.87 mg GAE/g DW were equal to 583.5 mg CE/l or 2,000 μM CE/l. The AA by FRAP after 120 min was 64.91 μM TE/g DW which is equal to 6,491 μM Fe^{++}/l. The phenol antioxidant coefficient (PAC), calculated as a ratio of FRAP (μM Fe^{++}/l)/total phenolics (μM CE/l), is equal to 3.24. In comparison to results of Katalinic et al. [2], our data show that Prolipid is similar to *Solidaginis herba*, in which total phenolics are 686 mg CE/l, FRAP is 6,514 μM Fe^{++}/l and PAC is 2.8. Our overall results are slightly lower than in the cited study [2]. It can be explained by the conditions of infusion (temperature and different time of the boiling). According to the results of antioxidant activity by FRAP, Prolipid belongs to a good FRAP activity group: it means that the antioxidant activity of this product is relatively high. Our results correspond as well with Katsube et al. [3], which revealed high levels of LDL antioxidant activity in plant products for which such activity levels are underestimated in the DPPH radical scavenging assay and Folin–Ciocalteu assay. As shown above, in this report the polyphenol content of the WF was about 19.87 mg GAE/g DW which corresponds with 25.4 μM epigallocatechin-3 gallate equivalent (EGCG)/g DW. The DPPH with the prolonged method was 56.44 μM TE/g and equal to 30.87 μM EGCG /g and without prolonged time was 36.16 μM TE/g which corresponds to 19.78 μM EGCG /g. These data were similar to sarutoribara (*Smillax china*). The obtained results can be compared as well with Ivanova et al. [1] and Silva et al. [5], who screened different medicinal plants for polyphenols and antioxidant activity. It can be concluded that the bioactivity of Prolipid was high and the polyphenol compounds were the main contributors to its overall antioxidant activity. Prolipid is widely used in human treatment without known side effects on patients and is comparable to other medicinal plants, and as a strong antioxidant mixture could be used as a supplement to known atherosclerosis preventing diets.

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