African Journal of Biotechnology Vol. 9 (27), pp. 4264-4271, 5 July, 2010
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2010 Academic Journals

Full Length Research Paper

Chemical composition and antioxidant properties of the essential oil and methanol extracts of rhizoma *Alpinia officinarum* from China in vitro

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Accepted 12 March, 2010

This study was designed to examine the in vitro antioxidant activities of the essential oil and methanol extracts of rhizoma *Alpinia officinarum* (small galanga) from China. The essential oil was analyzed by gas chromatography/ mass spectrometry (GC/MS) and 46 constituents were identified. Methanol extract from rhizoma *A. officinarum* was fractionated by chromatography of silica gel using ethyl acetate, acetone-methanol (8:1, V/V) and methanol, respectively. The antioxidant activities of essential oil and methanol extracts were evaluated with reducing power, diphenylpicrylhydrazyl (DPPH) assay and ferric thiocyanate test. In the above three assays, the essential oil and methanol extracts showed antioxidant potential to varying degrees; and acetone-methanol (8:1, V/V) subfraction exhibited better antioxidant potency than others. Owing to having antioxidative components, the essential oil exhibited satisfying antioxidant activities. A positive correlation was observed between the antioxidant activity potential and total phenolic contents of the extracts.

Key words: Rhizoma *Alpinia officinarum*, antioxidant activity, essential oil, methanol extracts, gas chromatography-mass spectrometry.

INTRODUCTION

Recently, increasing attention has been focused on the use of natural antioxidants, such as tocopherols vitamins, phenolic compounds including flavonoids and phenolic acids, and volatile compounds (Mascio et al., 1991; Rana and Agarwal, 2004; Singh et al., 2009; Obame et al., 2007) for preventing oxidation of biomolecules which can lead to cell injury and death (Freidovich, 1999; Ignarro et al., 1999; McCord, 2000) and for preservation of food materials. Numerous reports have described antioxidants and compounds with radical-scavenging activity present in fruits, vegetables, herbs and cereals extracts. In order to prolong the storage stability of foods, several synthetic antioxidants such as butylated hydroxyanisole (BHA), Butylated hydroxytoluene (BHT), and tert-butylhydroquinone (TBHQ) are commercially available and currently used. However, these synthetic substances may be inappropriate for chronic human consumption, because some reports have mentioned their possible toxic properties to human health and environment (Ito et al., 1986; Stich, 1991). For example, these substances can exhibit carcinogenic effects in living organisms (Ames, 1983; Baardseth, 1989). Hence, the development of alternative antioxidants from natural origin has attracted considerable attention and is thought to be a desirable development.

Alpinia officinarum Hance (small galanga) is a well-know pungent and aromatic plant whose rhizoma is frequently used as a medicinal herb in China. It is a member of the ginger family (*Zingiberaceae*) and its rhizome resemble ginger in shape. Its root contains volatile oil, resin, galangol, kaempferid, galangin and alpinin. The active components are the volatile oil and acrid resin. Galangin is dioxyflavanol and has been obtained synthetically. Many biological activities of galanga have been reported which include antitumor, antiulcer, antibacterial

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Abbreviations: BHA, Butylated hydroxyanisole; **BHT,** butylated hydroxytoluene; **TBHQ,** tert-butylhydroquinone; **GC/MS,** gas chromatography-mass spectrometry; **TCA,** trichloroacetic acid; **DPPH,** 1, 1-diphenyl-2-picrylhydrazyl; **FCR,** folin-ciocalteu reagent; **GAE,** gallic acid equivalents.

and antifungal properties. Some glycosides and antioxidative compounds in small galanga have been separated and identified (Ly et al., 2002, 2003). The properties of the essential oil in the rhizome of galanga have been reported by some researchers (Ly et al., 2001; Raina et al., 2002). They analyzed the compositions of different kinds of galanga (including great and small galanga).

As far as our literature survey could ascertain, antioxidant activities of essential oil of rhizoma *A. officinarum* has not been previously studied; and there are no literatures about systemic and multi-method evaluations on methanol extracts isolated from this species. So in the present study, the antioxidative capacities of the essential oil and methanol extracts of rhizoma *A. officinarum* were investigated. The antioxidant activities were determined by using three complementary in vitro assays: reducing power, inhibition of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical and linoleic acid peroxidation (ferric thiocyanate) method. The chemical composition of the essential oil was evaluated by using gas chromatography-mass spectrometry (GC/MS) analysis.

MATERIALS AND METHODS

Chemicals

Linoleic acid, tert-butylhydroquinone (TBHQ), ascorbic acid, gallic acid and DPPH were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents were purchased from Beijing chemical reagent factory and they were all of analytical grade.

Plant materials

Air-dried rhizoma *A. officinarum* (small galanga) was purchased from Yongxin drugstore in Changchun (China). The whole small galanga was dried in the shade and ground to pass a 1 mm screen and stored at 4℃ before experiment.

Extraction of the essential oil

The ground plant material processed as above were submitted for 3 h to steam distillation using a Clevenger apparatus to produce the essential oil in a yield of 2.05% (v/w) based on the dry weight of the sample. The obtained essential oil was dried over anhydrous sodium sulphate and after filtration, stored in the dark at 4℃ until used within a maximum period of one week.

Preparation of the methanol extracts

The air-dried and finely crushed sample (28.4 g) was extracted with methanol in a Soxhlet apparatus for 12 h. The fractionation of methanol extract was performed as described below. The methanol extract solution was evaporated under vacuum to dryness (5.38 g) and then separated by the chromatography of silica gel with ethyl acetate, acetone-methanol (8:1, v/v) and methanol, respectively. The resulting three extracts were evaporated under vacuum to dryness to give the ethyl acetate (fraction I), acetone-methanol (8:1, v/v) (fraction II) and methanol (fraction III) subfractions. They were quantitatively redissolved in methanol. The stock solutions were kept at 4℃ in the dark until further analysis.

GC/MS analysis conditions

The chemical composition of the essential oil was analyzed using GC/MS technique. The mass spectrometer was SHIMADZU GCMS-QP2010 Plus (Shimadzu Corporation, Japan) in the electron impact (EI) ionisation mode (70 eV) and HP-5MS (bonded and cross-linked 5% phenyl-methylpolysiloxane, 30 × 0.25 mm2, coating thickness 0.25 µm) capillary column (Restek, Bellefonte, PA). Injector and detector temperatures were set at 260℃. The oven temperature was held at 60℃ for 30 min, then programmed to 240℃ at rate of 5℃ /min. Helium (99.99%) was the carrier gas at a flow rate of 1 ml/min. Diluted samples(1/100 in hexane, v/v) of 1.0 ml were injected automatically. The identification of the components was based on the comparison of their mass spectra with those of NIST147.LIB and NIST27.LIB and as well as by comparison of their retention times.

Determination of reducing power

Total reducing power was determined as described by Oyaizu (1986), but modified slightly. Briefly, each 1 ml of the sample solution at different concentrations was mixed with 0.2 ml of phosphate buffer (0.2 mol/l, pH 7.2) and 1 ml of potassium ferricyanide (1%). The mixture was then incubated at 50℃ for 20 min. Afterwards, 0.2 ml of trichloroacetic acid (TCA, 10%) was added to the mixture, which was then centrifuged at 4000 g for 10 min. The upper layer of solution (2.0 ml) was mixed with 0.35 ml of ferric chloride (0.35%), and the absorbance was measured at 700 nm in a spectrophotometer. Increased absorbance of the reaction mixture indicated increased reducing power. Tests were carried out in triplicate. Ascorbic acid and TBHQ were used as positive control.

DPPH radical-scavenging activity

Radical scavenging activity was determined by a spectrophotometric method based on the reduction of a methanol solution of DPPH using the method of Blois (1958). One millilitre of various concentrations of the extracts (including essential oil and methanol extracts) in methanol was added to 1 ml of a 0.2 mM methanol solution of DPPH. The mixture was shaken vigorously and left to stand at room temperature for 30 min in the dark. Then the absorbance was measured at 517 nm against a blank by a spectrophotometer (Shimadzu□Tokyo, Japan). Absolute methanol was used to zero the spectrophotometer. The DPPH solution was freshly prepared daily, stored in a flask covered with aluminium foil, and kept in the dark at 4℃ between measurements. Inhibition of free radical, DPPH, in percent (I%) was calculated according to formula:

$$I\ \% = (A_{blank} - A_{sample}) / A_{blank} \times 100$$

Where, A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound) and A_{sample} is absorbance of the test compound.

Extract concentration proving 50% inhibition (IC_{50}) was calculated from the graph after plotting inhibition percentage against extract concentration. A lower IC_{50} value indicates greater antioxidant activity. Tests were carried out in triplicate. Ascorbic acid was used as positive control.

Antioxidant activity using linoleic acid peroxidation method

The antioxidant activity analysis using ferric thiocyanate was performed

according to the method reported by Osawa and Namiki (1981). 0.6 g of each test material was dissolved in 0.12 mL of 98% ethanol, and 2.88 mL of a 2.51% linoleic acid solution in ethanol and 9 mL of a 40 mM phosphate buffer (pH 7.0) were added. The mixture was incubated at 40℃ in a stoppered test tube in the dark for 3 days (72 h). During the incubation, a 0.1 mL aliquot was taken from the mixture, and diluted with 9.7 mL of 75% ethanol, followed by the addition of 0.1 mL of 30% ammonium thiocyanate. Precisely 3 min after adding the 0.1 mL of 20 mM ferrous chloride in 3.5% hydrochloric acid, the absorbance of the red color was measured at 500 nm. The level of lipid peroxidation inhibition by each fraction was calculated from the absorbance ratio to that of a blank without any sample. All tests and analyses were carried out in triplicate. TBHQ were used as positive control.

Determination of total phenolic contents

The total phenolic content of plant extracts was determined using Folin-Ciocalteu reagent (FCR) according to the procedure reported by Singleton et al. (1999) with some modifications. One milliliter of each plant extract solution, prepared in methanol at a concentration of 0.1 mg/ml was mixed with 7.5 ml of FCR which was diluted 10-fold with distilled water. After standing at room temperature for 5 min, 7.5 ml of 60 mg/ml of aqueous Na_2CO_3 solution were added. The mixture was kept at room temperature for 2 h and then the absorbance was measured at 760 nm. The results were expressed in gallic acid equivalents (GAE), determined utilizing a separately prepared absorbance versus concentration (5 - 100 µg/ml) curve for gallic acid. The concentrations of phenolic compounds were calculated according to the following equation that was obtained from the standard gallic acid graph:

$$\text{Absorbance} = 0.0066 \times \text{gallic acid} + 0.1059 \quad (R^2 = 0.9985)$$

Statistical analysis

The experimental data were expressed as means ± standard deviations. Statistical analysis was performed on the data by statistical package for the social sciences (SPSS) V13.0 (SPSS Inc., Chicago). Significant difference was considered at a level of $p < 0.05$.

RESULTS AND DISCUSSION

Chemical composition of the essential oil

About 46 (98.47% of the total oil) constituents were identified from small *galanga* essential oil. GC/MS analysis revealed that the major constituents of the essential oil were eucalyptol (28.11%), α-terpineol (9.17%), γ-muurolene (7.88%), α-farnesene (5.73%), caryophyllene (4.66%), α-bergamotene (4.18%) and γ-gurjunene (3.63%), respectively, as listed in Table 1. GC/MS analysis of the essential oil demonstrated the abundance of monoterpene hydrocarbons and oxygenated monoterpenes. The findings on the major components of *A. officinarum* Hance essential oil were partly in agreement with previous report (Ly et al., 2001; Raina et al., 2002). According to Ly et al. (2001), for example, β-bisabolene was the most major component of drying small *galanga* essential oil, whereas it was eucalyptol (1, 8-cineole) in the present study. These differences might have been derived from local, climatic and seasonal factors. So it is necessary to illuminate the components of essential oil before exploring its antioxidative activity.

Yield and total phenolic contents of fractions

The amount of crude methanol extract was 189 mg/g dry small *galanga* powder. Table 2 shows the yield and total phenolic content of different solvent fractions of the crude methanol extract and essential oil. The proportion of compounds in the fraction I (38.5%) was larger than that of fraction II and fraction III (25.02 and 23.37%, respectively). This might indicate that most of the eluted solutes were unpolar components.

The total phenolic contents of the methanol extracts and essential oil of small *galanga* as determined by Folin-Ciocalteu method were reported as gallic acid equivalents (Table 2). Among the five extracts, fraction II contained the highest (221.36 ± 2.79 mg GAE/g) amount of phenolic compounds followed by the fraction I (139.55 ± 0.58 mg GAE/g) , fraction III (128.94 ± 1.67 mg GAE/g), crude methanol extract (98.64 ± 1.35 mg GAE/g) and essential oil (31.5 ± 0.65 mg GAE/g). The levels of total phenolics determined in this way are not absolute measurements of the amounts of phenolic compounds, but are in fact based on their chemical reducing capacity relative to gallic acid.

Reducing power

Reducing power assay is often used to evaluate the ability of natural antioxidant to donate electron (Yildirim et al., 2000; Dorman et al., 2003). Many reports have revealed that there is a direct correlation between antioxidant activities and reducing power of certain plant extracts (Duh, 1998; Duh et al., 1999; Yildirim et al., 2001).

Figure 1 shows the reducing power (as indicated by the absorbance at 700 nm) of methanol extracts (including crude methanol extract and its separated three sub-fractions) and essential oil from *A. officinarum* Hance compared with ascorbic acid and TBQH as standards. The reducing power of all samples increased with the concentrations. The sequence of reducing power was as follow: fraction II > fraction III > fraction I > crude methanol extract > essential oil(corresponding absorbances at 700 nm were 1.984 ± 0.012, 1.335 ± 0.032, 1.072 ± 0.092, 0.922 ± 0.052 and 0.189 ± 0.039 at 100 µg/ml, respectively). However, at the same concentration, ascorbic acid and TBHQ showed excellent reducing powers of 5.000 ± 0.000 and 3.732 ± 0.059, respectively□which are significantly higher than those initially obtained (P□0.05). According to the results in the present study, fraction II has a remarkable potency to donate electron to reactive free radicals.

DPPH radical-scavenging activity

We chose the DPPH radical-scavenging method due to its

Table 1. Composition percentage of *Alpinia officinarum* Hance essential oil.

GC peak number	Retention time	Component	Relative percentage (%)
1	6.85	α-Pinene	1.65
2	7.275	Camphene	1.89
3	8.067	ß-Pinece	1.98
4	9.592	Limonene	1.75
5	9.667	Eucalyptol	28.11
6	10.492	γ-Terpinene	0.28
7	11.692	Linalool	0.30
8	13.092	D(+)-Camphor	2.29
9	13.742	Isoborneol	0.67
10	14.067	(+/-)-Terpinen-4-ol	2.25
11	14.450	α-Terpineol	9.17
12	15.308	Fenchyl acetate	0.54
13	15.942	Benzylacetone	0.21
14	16.717	(E)-Cinnamaldehyde	1.29
15	17.175	Isobornyl formate	0.18
16	18.308	Isobutyl benzoate	0.22
17	19.567	Ylangene	0.41
18	20.083	2-Phenylethyl 2-bromopropanoate	0.80
19	20.858	Caryophyllene	4.66
20	21.192	α-Bergamotene	4.18
21	21.292	α-Guaiene	0.67
22	21.642	(E)-beta-Farnesene	0.55
23	21.725	α-Caryophyllene	1.81
24	22.117	Bicyclo [2.2.1] heptane, 2-cyclopropylidene-1, 7, 7-trimethyl	0.21
25	22.258	Guaiene	0.83
26	22.358	Germacrene D	0.25
27	22.442	Phenethyl 2-methylbutyrate	0.53
28	22.558	Eudesma-4(14),11-diene	1.92
29	22.775	γ-Gurjunene	3.63
30	22.925	α-Farnesene	5.73
31	23.000	r-Himachalene	2.19
32	23.208	γ-Muurolene	7.88
33	23.400	δ-Cadinene	1.97
34	23.750	(-)-α- Gurjunene	1.08
35	23.900	ß-Panasinsene	1.19
36	24.283	γ- Elemene	0.93
37	24.983	Tricyclo[6.3.0.0(2,4)]undec-8-ene, 3,3,7,11-tetramethyl-	0.41
38	25.058	2,3,3-Trimethyloctane	0.23
39	25.617	Cubenol	0.60
40	26.008	Viridiflorol	0.23
41	26.192	T-cadinol	0.33
42	26.525	Epiglobulol	1.04
43	26.792	(Z,E)-α-Farnesene	0.35
44	27.317	2,8-Dimethy-undecane	0.21
45	27.475	α-Bulnesene	0.43
46	27.750	Z-α-Trans-Bergamotol	0.44
		Total identified	98.47

Table 2. Yield and phenolic content (as gallic acid equivalent) of methanol extracts and essential oil from small galanga.

Sample	Yield (%)	Phenolics (mg GAE/g)[a]
Crude methanol extract	18.9	98.64 ± 1.35
Fraction I	38.5	139.55 ± 0.58
Fraction II	25.0	221.36 ± 2.79
Fraction III	23.4	128.94 ± 1.67
Essential oil	2.05	31.5 ± 0.65

[a]The values of phenolics are means ± SD of three replicates.

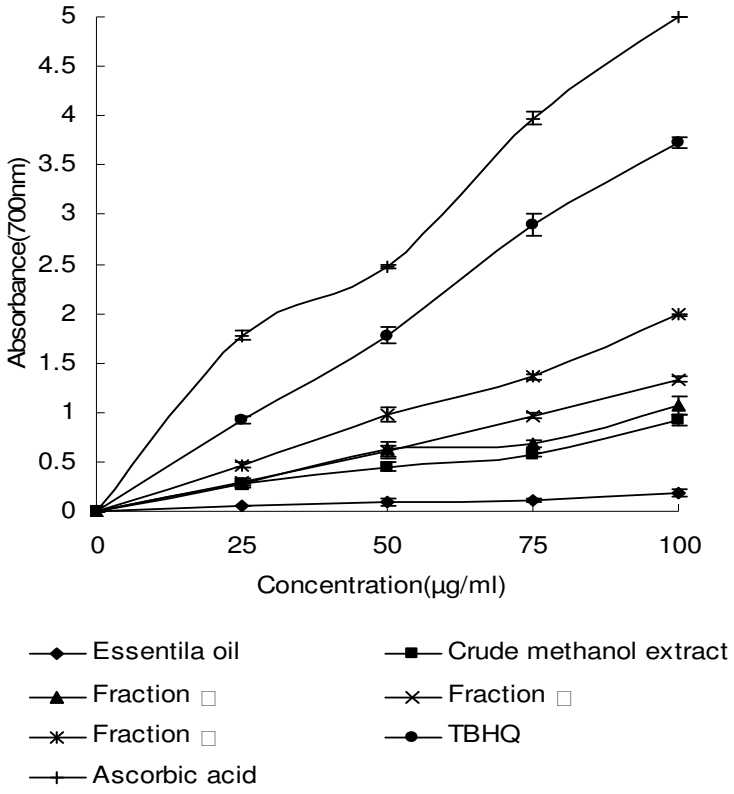

Figure 1. Reducing power of methanol extracts and essential oil from *Alpinia officinarum* Hance (mean ± S.D.; n = 3).

simplicity, rapidity, sensitivity and reproducibility (Koleva et al., 2002). This method is also very convenient for the screening of large numbers of samples with different polarity. The effect of antioxidant on DPPH radical scavenging was thought to be due to their hydrogen donating ability or radical scavenging activity. Assessed samples were able to reduce the stable violet DPPH radical to the yellow DPPH-H, reaching 50% of reduction with IC_{50} values. Lower IC_{50} value indicates higher antioxidant activity.

The antioxidant activity of the galangal essential oil had been evaluated by DPPH radical-scavenging test (Figure 2) whose IC_{50} value was IC_{50} = 1.75 ± 0.11 mg/ml. This value is comparable to *Rosmarinus officinalis* L. essential oil and 1, 8-cineole, whose antioxidant properties have been explored (Wang et al., 2008). Analyzing and comparing the results of DPPH assays, it was found that the galangal essential oil exhibited excellent antioxidant activities than R. officinalis L. essential oil, though 1, 8-cineole (eucalyptol) is the main component of the two kinds of essential oils. Besides, there is nearly the same proportion of 1, 8-cineole, as the main compound, in above essential oils. The galangal essential oil contains antioxidative compounds, namely eucalyptol, □-terpineol, γ-muurolene, α-farnesene, caryophyllene, α-bergamotene and γ-gurjunene, which was reported to possess a strong antioxidant activity (Wang et al., 2008; El-Ghorab et al., 2007; Özer et al., 2007; Zhang and Shu, 2003;

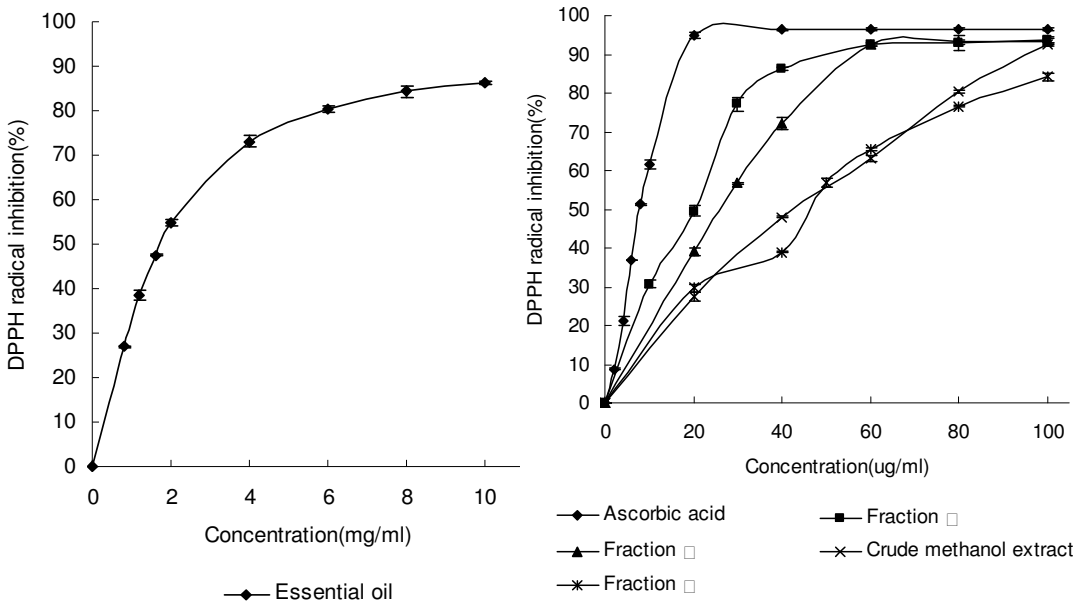

Figure 2. DPPH radical-scavenging activities of essential oil and methanol extracts from *Alpinia officinarum* Hance (mean ± S.D.; n = 3).

Table 3. Free radical-scavenging activity of small galanga essential oil and methanol extracts.

Sample	DPPH IC_{50} (μg/ml)
Crude methanol extract	41.88 ± 0.72
Fraction I	46.91 ± 0.69
Fraction II	20.98 ± 1.37
Fraction III	25.79 ± 0.47
Essential oil	1.75 ± 0.11 (mg/ml)
Ascorbic acid	7.91 ± 0.14

Jayaprakasha et al., 2003). This could be the reason why the galangal essential oil shows preferable antioxidant activity. From the research and literature above, it was also found that the small galanga essential oil showed greater antioxidant activity than its main components. So it is very difficult to attribute the antioxidant effect of the total essential oil to one or a few active principles. However, the antioxidant activity of the essential oil is the cooperating results of their compositions (Wang et al., 2008).

Free radical scavenging properties of the crude methanol extract and its three separated sub-fractions are presented in Table 3. It can be seen that the three sub-fractions prepared by different solvents exhibited varying degrees of scavenging capacities. Among them fraction II showed the highest scavenging activity with an IC_{50} value of 20.98 ± 1.37 μg/ml (Figure 2). The other two sub-fractions, fraction I and fraction III, showed significantly weaker scavenging potency ($P < 0.05$), with the IC_{50} values of 46.91 ± 0.69 and 25.79 ± 0.47 μg/ml, respectively. The results suggested that some components within fraction II were significantly strong radical-scavenging components. The crude methanol extract of small galanga exhibited moderate antioxidant activities among its three separated fractions (IC_{50} = 41.88 ± 0.72 μg/ml). The methanol extracts showed higher scavenging ability on DPPH radicals than the essential oil. At the same time, DPPH scavenging abilities of the methanol extracts and the essential oil were lower than that of ascorbic acid (IC_{50} = 7.91 ± 0.14 μg/ml).

Antioxidant activity using linoleic acid peroxidation method

The antioxidant effects of various extracts from small galanga, in preventing the peroxidation of linoleic acid (Singh et al., 2007), as measured by ferric thiocyanate method, are shown in Figure 3. The absorbances of the systems with various antioxidants and without any test material as control at 500 nm were plotted as a function of time. The incubation period was 72 h at 40°C. It is seen that a lower absorbance indicates a higher level of antioxidant activity. As expected, the highest absorbances are seen for the control system without any test material at all times.

Figure 3 shows the changes in the absorbance for each fraction during the 3 days of incubation at 40°C, in comparison with TBHQ. The absorbance of the control increased in proportion to the incubation time, and the absorbance of the other samples also increased with increasing incubation time. However, all the samples showed a significantly lower increment in rate compared to the control. Fraction II showed a slightly higher

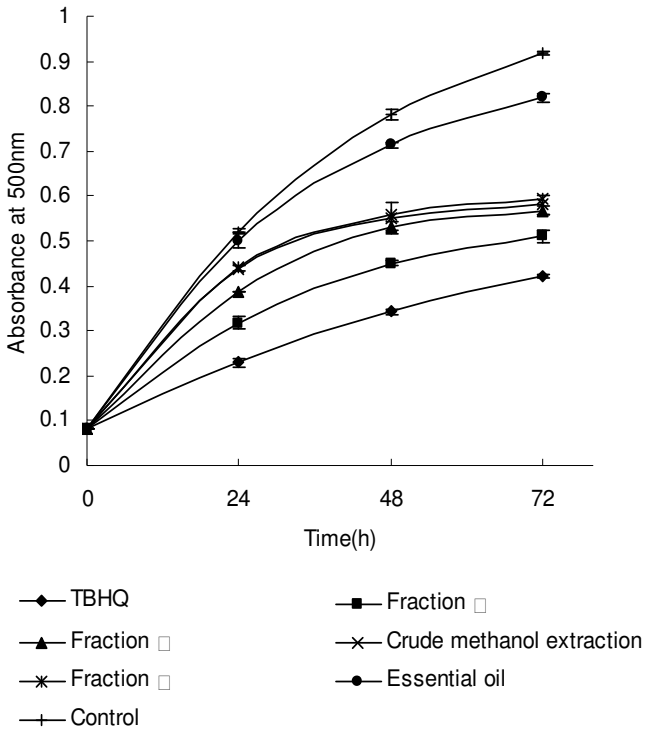

Figure 3. Absorbance of methanol extracts and essential oil from *Alpinia officinarum* Hance by FTC method with increasing incubation time (mean ± S.D.; n = 3).

inhibition effect after 72 h than the others ($P < 0.05$). The differences in the absorbance between the fractions and commercial antioxidants, TBHQ, also increased with the incubation time. Of the five extracts, the increasing orders of antioxidant activity may be given as essential oil < fraction I < crude methanol extract < fraction III < fraction II at the concentration of 0.1 mg/ml. And this result is partly in accordance with the results of reducing power, DPPH and total phenolic contents tests.

From the results of total phenolic contents and antioxidant tests above, we can conclude that there is a positive correlation between antioxidant activity potential and amount of phenolic compounds of the extracts. The amounts of total phenols found in the plant methanol extracts were more than that in the oil. This can explain why the methanol extracts exhibited higher antioxidant activities than the essential oil.

Conclusion

On the basis of results in this study, it can be concluded that acetone-methanol (8:1, V/V) fraction (fraction II) of methanol extract from *A. officinarum* Hance shows the strongest antioxidant properties in reducing power, DPPH assay and ferric thiocyanate test compared with ethyl acetate fraction (fraction I), methanol fraction (fraction III) and crude methanol extract. The results of present work indicate that acetone-methanol (8:1, V/V) fraction of methanol extract from small galanga might be potential antioxidant for application in food products. Research is in progress to isolate and identify the antioxidant components in acetone-methanol (8:1, V/V) fraction. When compared with other kinds of essential oils and its main components, small galanga essential oil showed satisfying antioxidant activities. It is very difficult to attribute the antioxidant effect of a total essential oil to one or a part of active principles, because there is always a mixture of different chemical compounds in essential oil. According to the results of this study, the essential oil or the methanol extracts of *A. officinarum* Hance may be suggested as a new potential source of natural antioxidant for the food industry.

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