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Full Length Research Paper

Antioxidant properties of crude and fractionated extracts of *Alpinia mutica* rhizomes and their total phenolic content

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In the present study, the antioxidant potential of the crude methanol and its fractionated extracts (hexane, ethyl acetate and water) of the rhizomes of *Alpinia mutica* was investigated, employing four different assays. The antioxidant assays used were 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, β-carotene bleaching, superoxide dismutase (SOD) and the reducing power assays while the total phenolic content was assessed using the Folin-Ciocalteu method. The ethyl acetate fraction showed the highest antioxidant property in comparison to the other extracts against all assays conducted. The ethyl acetate fraction displayed better antioxidant activity in comparison to the standard compound, butylated hydroxyanisole (BHA) and ascorbic acid in the β-carotene linoleate model system and the SOD assay. The ethyl acetate fraction was also found to have the highest phenolic content among the extract and fractions. The phenolic compounds present in the ethyl acetate fraction may have contributed to the good antioxidant properties of the ethyl acetate fraction. In conclusion, the rhizome extract of *A. mutica* especially the ethyl acetate fraction possesses good antioxidant activity and can be potentially used as natural antioxidants.

Key words: *Alpinia mutica*, antioxidant, radical scavenger, flavonoid, total phenolic content.

INTRODUCTION

A free radical is a molecule containing one or more unpaired electrons in the outermost shell. Some examples such as the reactive oxygen species (ROS) and reactive nitrogen species (RNS) are the most common dangerous species among the free radicals (Victor et al., 2006). These free radicals are generated through the endogenous processes such as respiration, metabolism and phagocytosis. It can also be generated by exogenous systems such as pesticides, some pollutants, organic solvents and during radiation (Cadenas, 1989; Davies, 1995). However, the production of free radicals is balanced by an equivalent synthesis of antioxidants through our antioxidant defence systems, which are the enzymatic antioxidants (superoxide dismutase, glutathione peroxidase, quinone reductase and catalases) and the non-enzymatic antioxidant (ascorbic acid, α-tocopherol, melatonin, β-carotene) obtained from the diet (Rice-Evans and Burdon, 1993; Halliwell, 1996; Davies, 2000; Valko et al., 2000).

Despite its harmful effects, ROS and RNS play an important role in the physiological functions of our body when present in moderate or low concentrations such as in the signal transduction pathway, smooth muscle relaxation, defense against infectious agents and cell growth (Halliwell, 1987; Mate´s and Perez-Gomez, 1999; Dröge, 2002). However, accumulation of free radicals in our body causes a phenomenon called oxidation stress which is defined as imbalance between oxidants and antioxidants in favour of oxidants, potentially leading to cellular damage (Aruoma, 1994; Sies, 1997; Gulcın et al., 2003). When oxidative stress occurred, it eventually leads to several deteriorating effects to our cellular

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Table 1. Preparation of different concentrations of gallic acid solution for calibration plot.

Gallic acid (mg/l)	Gallic acid stock solution (ml)	Methanol (ml)
0	0.000	1.000
25	0.005	0.995
50	0.010	0.990
75	0.015	0.985
100	0.020	0.980
150	0.030	0.970
200	0.040	0.960
250	0.050	0.950
500	0.100	0.900
1000	0.200	0.800

bio-macromolecules such as DNA damage, lipid peroxidation, tissue injury and protein degradation (Dasgupta et al., 1997; Valko et al., 2004; Poli et al., 2004). Therefore, free radicals are increasingly recognized for their contribution to a number of diseases such as cancer, arthritis, neurodegenerative disorders, atherosclerosis and aging (Halliwell and Guteridge, 1984; Young and Woodside, 2001).

Alpinia mutica, which belong to the family Zingiberaceae, are perennial plants that grow wild in east of peninsular Malaysia. The rhizomes have been used by village folks to treat flatulence whilst the fruits to reduce swelling. Only two biological investigations were reported for this plant, which were the inhibitory effect on platelet activating factor receptor binding and inhibitory activity towards lipid oxidation (Jantan et al., 2004; Habsah et al., 2000). Thus, there is limited information on the antioxidant potential of this plant. To our knowledge, there is only one report on the inhibitory activity towards lipid oxidation of methanol and dichloromethane extracts of the rhizomes of *A. mutica*. There has been considerable interest to develop natural antioxidants from botanical sources, especially edible medicinal plants due to the negative consumer perception towards synthetic antioxidants

A study was thus conducted to investigate the antioxidant activity of the crude and fractionated extracts of *A. mutica* using four different antioxidant assays. The total phenolic content was conducted by the Folin-Ciocalteu method in order to determine the correlation between the phenolic content and the antioxidant activity in the assay conducted. This paper reports the result of the above investigations.

MATERIALS AND METHODS

Plant material

The rhizomes of *A. mutica* were collected from Perak, Malaysia. This species was authenticated by Professor Halijah Ibrahim, from Faculty of Science, University of Malaya and a voucher specimen (No. KLU 46200) was deposited at the herbarium of the University of Malaya.

Chemicals and Reagents

Butylated hydroxyanisole (BHA), ascorbic acid, Folin-Ciocalteu's phenol reagent, β-carotene, linoleic acid, tween 80, gallic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH) and potassium ferricyanide were acquired from Sigma-Aldrich Company. Methanol, hexane, ethyl acetate, superoxide dismutase (SOD) assay Kit-WST and trichloroacetic acid were obtained from Merck Company. Solvents used were of analytical grade.

Extraction and fractionation

The extracts were prepared as previously described (Sri Nurestri et al., 2011). Briefly, the rhizomes were cleaned, cut into small pieces then dried in the oven at 40°C and ground into fine powder. The powdered rhizomes (200.00 g) were soaked in 80% aqueous methanol for 3 days at room temperature. After filtration, the filtrate was evaporated in vacuo to give a crude methanol extract (21.56 g, 10.78%). The percentage yield of crude methanol extract is based on the weight of dried and ground plant materials. The crude methanol extract was fractionated using hexane to give the hexane fraction (0.56 g, 6.10%). The hexane-insoluble fraction was partitioned between ethyl acetate and water (1:1) to give ethyl acetate fraction (2.52 g, 6.75%) and water fraction (16.00 g, 59.30%). The percentage yield of fractionated extracts (hexane, ethyl acetate and water) were calculated based on the weight of the crude methanol extract.

Determination of total phenolic content

The total phenolic content was determined according to the Folin-Ciocalteu method described by Cheung et al. (2003). The concentrations of phenolic compounds in the extract of *A. mutica*, expressed as milligram gallic acid equivalents (GAEs)/g of extract using an equation obtained from the gallic acid calibration plot.

Preparation of gallic acid calibration plot

A calibration plot, using gallic acid with concentrations ranging from 25 to 1000 mg/l was prepared. Gallic acid stock solutions(5mg/ml) in volumes ranging from 0.005 to 0.2 ml were pipetted out into test tubes. The final volume was made to 1 ml with methanol in each test tube (Table 1). Different concentrations of the resultant gallic acid solution (0.02 ml) and negative control (methanol was used instead of gallic acid) were mixed with 1.58 ml of distilled water. Folin-Ciocalteu's phenol reagent (0.1 ml) was added to each test tube. After 3 min, 0.3 ml of saturated sodium carbonate (Na_2CO_3) solution (~35%) was added to the mixture. The reaction mixtures were incubated at 40°C for 30 min. The blank contained only methanol. The absorbance was determined at 765 nm with a spectrophotometer. The gallic acid calibration plot was obtained by plotting the absorbance against concentration of gallic acid (mg/l).

Determination of total phenolic content in extract and positive reference standard

BHA and ascorbic acid were used as positive reference standard in the study. All the test extracts and standards were prepared at

Table 2. Preparation of each solution for sample, blank 1, blank 2 and blank 3.

Components	Wells			
	Sample (µl)	Blank 1 (µl)	Blank 2(µl)	Blank 3 (µl)
Sample solution	20	-	20	-
ddH_2O	-	20	-	20
WST working solution	200	200	200	200
Enzyme working solution	20	20	-	-
Dilution buffer	-	-	20	20

concentration of 20 mg/ml in methanol as stock extracts.

The extracts/standards were prepared at a concentration of 1.0 mg/ml; 0.02 ml of each extract/standard was mixed with 1.58 ml of distilled water in test tubes. Folin-Ciocalteu's phenol reagent (0.1 ml) was then added to each test tube. After 3 min, 0.3 ml of saturated Na_2CO_3 solution (~35%) was added to the mixture. The reaction mixtures were incubated at 40°C for 30 mins. The blank contained only methanol. In the control, the extracts/standards were replaced by methanol. The absorbance was determined at 765 nm with a spectrophotometer (Hitachi U2000). All extracts were assayed in triplicate.

DPPH radical scavenging activity

The scavenging effect of the aqueous methanol and fractionated extracts of *A. mutica* rhizomes were measured by the method described by Cheung et al. (2003) with some modifications. DPPH solution in methanol (0.02 µM) was prepared and the extracts at different concentrations were added to 0.8% of DPPH solution. The resultant reaction mixtures were incubated at room temperature for 30 min. The absorbance of the reaction mixtures was measured at 520 nm. Methanol was used as blank and DPPH solution without addition of extract was used as control. BHA and ascorbic acid were used as positive control. The scavenging activity was calculated by the following formula:

$$\text{Scavenging activity (\%)} = \frac{A_1 - A_0}{A_0} \times 100\,\%$$

where A_0 is the absorbance of the control and A_1 is the absorbance of the extract/reference standard. IC_{50} values were determined from the graph of percentage of inhibition plotted against the concentration of extracts. IC_{50} is defined as the amount of extract needed to scavenge 50% of DPPH radicals.

Reducing power test

The reducing power of the prepared extracts was determined according to the method of Oyaizu (1986). Briefly, each extract in varying concentrations of 4, 8, 16 and 20 mg/ml were prepared and dissolved in 1.0 ml of methanol to which was added 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of 1% (w/v) solution of potassium ferricyanide. The mixture was incubated at 50°C for 20 min. Then, 2.5 ml of 10% (w/v) trichloroacetic acid solution was added and the mixture was centrifuged at 1000 rpm for 10 min. 2.5 ml of aliquot supernatant was mixed with 2.5 ml of distilled water and 0.5 ml of 0.1% (w/v) solution of ferric chloride. Absorbance of the reaction mixture was read using a spectrophotometer (Hitachi, U2000) at 700 nm. Increased absorbance of the reaction mixture indicates greater reducing power. Mean values from three independent samples were calculated for each extract. Ascorbic acid and BHA were used as positive reference standards.

β-carotene bleaching assay

The antioxidant activity of the aqueous methanol and its fractionated extracts were determined using the β-carotene-linoleate model system according to method described by Cheung et al. (2003) with slight modification. A reagent mixture of 1.0 ml ß-carotene solution (0.2 mg/ml in chloroform), 0.02 ml linoleic acid and 0.2 ml Tween 80 was prepared in the round bottom flask. After chloroform was removed under vacuum, 50 ml of oxygenated water was added into the flask and shaken vigorously. Aliquots (5.0 µl) of the emulsion were transferred into test tubes each containing 0.2 ml of extracts at different concentrations (4, 8, 16 and 20 mg/ml). After the emulsion was added into each test tube, the absorbance at zero time was measured immediately at 470 nm using a spectrophotometer. The test tubes were then incubated at 50°C and the absorbance was measured again at time intervals of 20 min for 2 h. The methanol (instead of extract) was used as control while the blank contained all the earlier chemicals (0.02 ml of linoleic acid and 0.2 ml of Tween 80 in 50 ml of oxygenated distilled water) except β-carotene solution. BHA was used as standard. The rate of ß-carotene bleaching (R) was calculated according to the equation.

$$R = \frac{\ln (A_0/A_t)}{t}$$

where ln is natural logarithm, A_0 is absorbance at time 0, A_t is absorbance at time t, and t is 20, 40, 60, 80, 100 or 120 min. The antioxidant activity percentage was calculated in terms of percentage inhibition relative to the control, using the equation.

$$\text{Antioxidant activity (\%)} = \left(\frac{R_{control} - R_{sample}}{R_{control}} \right) \times 100\%$$

SOD (superoxide dismutase) inhibition activity assay

The SOD activity was measured using SOD assay Kit-WST purchased from Sigma-Aldrich. This assay is based on the colorimetric assay for the measurement of total antioxidant capacity of crude aqueous methanol and its fractions. This was done using 96 wells microtiter plate. A sample solution (20 µl) was added to sample and blank 2 wells, and 20 µl of doubled distilled water (ddH_2O) was added to blank 1 and blank 3 wells. WST working solution (20 µl) was added to each well and enzyme working solution (20 µl) was added to sample and blank 1 wells which was then mixed thoroughly. The plate was then incubated at 37°C for 20 min. The absorbance was read at 450 nm using Elisa microplate reader. Table 2 summarizes the preparation of reaction mixture for sample, blank 1, 2 and 3 wells. The SOD activity was calculated according to the following equation:

Table 3. Extraction yields and total phenolic content of the crude and fractionated extracts of *A. mutica*.

Extracts/fraction	Weight of extract (g)	Concentration of total phenolics (mg of GAEs/g of crude methanol and fractionated dry extract)	Yield of phenolics (g of GAEs)	% yield of phenolics
Methanol 80%	21.56	0.44 ± 0.05^{a}	9.49×10^{-3}	0.04
Hexane	0.56	0.75 ± 0.15^{b}	4.20×10^{-4}	0.08
Ethyl acetate	2.52	1.55 ± 0.16^{c}	3.91×10^{-3}	0.16
Water	16.00	0.54 ± 0.02^{ab}	8.64×10^{-3}	0.05

Values expressed are mean ± standard deviation of triplicate measurements. Means with different letters in the same column are significantly different ($p < 0.05$, ANOVA), mg/g: mg of gallic acid equivalent (GAEs)/g of extract or fractions.

SOD activity (inhibition rate, %) = $\{[(A_{blank1} - A_{blank3}) - (A_{sample} - A_{blank2})] / (A_{blank1} - A_{blank3})\} \times 100$

Where A_{blank1}, A_{blank2}, A_{blank3} and A_{sample} were the absorbances of blank 1, 2, 3, and sample wells. One unit of SOD activity was defined as the amount of enzyme having a 50% inhibitory effect on WST-1 (Xing et al., 2010).

Statistical analysis

Experimental results were conducted in triplicate and the results were expressed as means ± SD of three parallel measurements. Analysis of variance was used to determine any significant difference between groups using STATGRAPHICS Plus software (version 3.0, Statistical Graphics Corp., Princeton, NJ, USA). Statistical significance was accepted at $p < 0.05$. Duncan's Multiple Range test (DMRT) was used to determine the significant differences between groups.

RESULTS AND DISCUSSION

Assays for assessment of antioxidant activity

The antioxidant activity of the aqueous crude methanol and fractionated extracts (hexane, ethyl acetate and water) were evaluated using various assays based on different mechanisms. In this study, four different assays were used, namely the radical-scavenging assay, β-carotene bleaching assay, reducing power assay and SOD inhibition assay. The total phenolic compounds content of the extract and the fractions were evaluated using the Folin-Ciocalteu method and the correlation between the total phenolic content and the antioxidant properties was also determined.

Determination of the total phenolic contents

Phenols are one of the major groups of non-essential dietary components present in fruits and vegetables and have been associated with health benefits including prevention of cancer and atherosclerosis (López-Vélez et al., 2003; Teissedre, 1996; Turkoglu et al., 2007). Phenolic compounds have been reported to have redox properties which allow them to act as radical scavengers, metal chelators, reducing agents, hydrogen donors, and singlet oxygen quenchers (Kähkönen et al.; 1999; Proestos et al., 2006). There are reports in the literature mentioning that there is a highly positive relation between total phenolic content and antioxidant activity in many plant species (Duh et al., 1999; Gulcın et al., 2004). Among these phenolic compounds, flavonoids have received much attention and have been widely studied for their antioxidant activity in the past few years (Rice-Evans et al., 1996). Therefore, the Folin Ciocalteu's method has become a routine assay in determining the phenolic content in a test sample. Table 3 shows the amount of phenolic content of the crude extract and its fractions determined by the Folin-Ciocalteu method. The concentration of total phenolic content in the crude methanol extract and fractions was measured as mg/g of gallic acid equivalent using the equation below which was derived from the standard gallic acid curve shown in Figure 1.

$$\text{Total phenolic content (mg/l of GAEs)} = \frac{(y - 0.0007)}{0.001}$$

*y: absorbance of test samples after substraction of the control.

The ethyl acetate fraction showed the highest phenolic content (1.55 ± 0.16 mg GAEs/g extract) among the extract and fractions. This is followed by the hexane fraction (0.75 ± 0.15 mg GAEs/g extract), water fraction (0.54 ± 0.02 mg GAEs/g extract) and methanol extract (0.44 ± 0.05 mg GAEs/g extract).

DPPH radical-scavenging activity (DPPH)

The DPPH radical scavenging method is the most popular and widely used method for screening the free radical scavenging ability of compounds. This assay is sensitive and easy to perform and offers a rapid way to screen radical scavenging activity as compared to other methods. DPPH is a stable radical, with a strong absorption maximum at 517 (purple color) in the UV spectrum. In the presence of an antioxidant which acts as a hydrogen donor, DPPH radical is reduced to 1,1-diphenyl-2-picryl hydrazine by accepting an electron from the antioxidant and accompanied by loss of purple color.

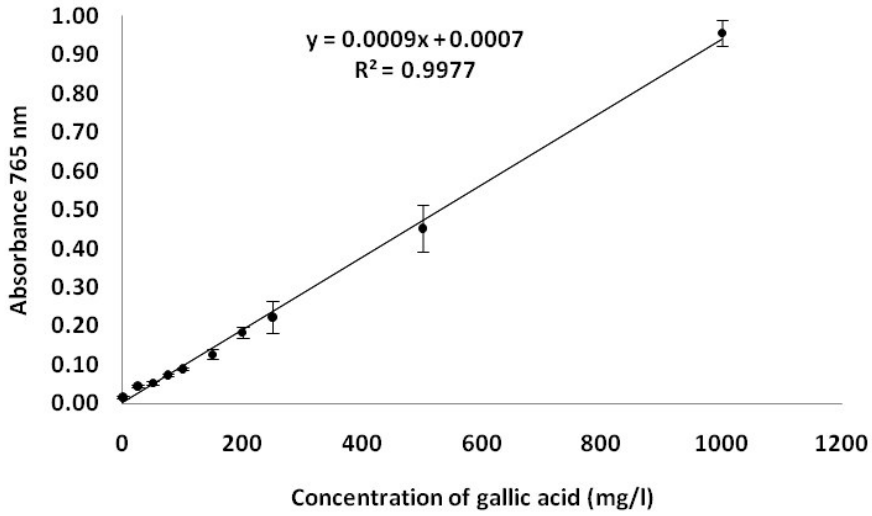

Figure 1. Calibration plot of gallic acid.

Table 4. The radical scavenging activity of *A. mutica* extract on DPPH radicals.

Extracts	EC_{50} values (mg/ml)
Methanol 80%	0.465 ± 0.022^{d}
Hexane	0.338 ± 0.007^{c}
Ethyl Acetate	0.215 ± 0.004^{b}
Water	0.676 ± 0.007^{e}
Ascorbic acid	0.015 ± 0.600^{a}
BHA	0.013 ± 0.600^{a}

Values expressed are mean ± standard deviation of three measurements. Means with different letters in the same column are significantly different ($p < 0.05$, ANOVA). E_{50} value is defined as efficient concentration of DPPH radical being scavenged by 50%.

Thus, this assay involves the measurement of hydrogen atom transfer or electron donation from the potential antioxidant to free radical molecules in an aqueous condition (Becker et al., 2004).

Table 4 shows the radical scavenging activity of the crude and fractionated extracts of *A. mutica*. The degree of discoloration indicates the scavenging potentials of the antioxidant (Yamaguchi et al., 1998; Jayaprakasha et al., 2004). The free radical scavenging activity was expressed as the effective concentration required for 50% of DPPH radical ($DPPH^{\bullet}$) reduction (IC_{50}) obtained from a plot of graph of scavenging activity against the concentration of the extract and its fractions. The ethyl acetate fraction demonstrated the significantly highest activity (EC_{50} 0.215 ± 0.004 mg/ml) compared to other extract and fractions. This was followed by the hexane fraction (EC_{50} 0.338 ± 0.007 mg/ml), the methanol extract (EC_{50} 0.465 ± 0.022 mg/ml) and the water fraction (EC_{50} 0.676 ± 0.007 mg/ml). Ascorbic acid and BHA was used as standards with EC_{50} values 0.015 ± 0.6 and 0.013 ± 0.6 mg/ml respectively.

β-carotene bleaching assay

The β-carotene bleaching method is based on the loss of the orange colour of β-carotene due to its reaction with radicals which are formed by linoleic acid oxidation in an emulsion. The rate of β-carotene bleaching can be slowed down in the presence of antioxidants. Peroxyl radical ($LOO^{\bullet}$) formed by linoleic acid in the presence of oxygen during incubation at 50˚C reacted with β-carotene to form a stable β-carotene radical (Moon and Shibamoto, 2009). The presence of different antioxidants can hinder the extent of β-carotene bleaching by neutralizing the linoleate-free radicals and other radicals formed in the system (Jayaprakasha et al., 2001). This spectrophotometric method is based on the reduction of the orange color of β-carotene with absorption at 470 nm.

The antioxidant activity of the extracts varied significantly with different concentrations ($p < 0.05$; Table 5, figure 2). The antioxidant activity of the extract and its fractions and the standard compound increased in the following order: Ethyl acetate > BHA > hexane > methanol > water. The ethyl acetate fraction showed significantly higher antioxidant activity compared to the other extracts, ranging between 89.61 to 94.53% when tested at concentrations of 4 to 20 mg/ml, and it also showed even better antioxidant activity in comparison to BHA. This is followed by hexane fraction which showed better antioxidant (82.79 to 86.39%) in comparison to BHA at the concentration range of 4 to 8 mg/ml but has lower antioxidant capacity at concentrations higher than 8 mg/ml. In comparison to BHA, the crude methanol extract showed better antioxidant activity (83.39 to 93.34%) at concentrations greater than 8 mg/ml. However, at the

Table 5. Antioxidant activity (%) of *A. mutica* extracts at various concentrations measured by β-carotene bleaching assay.

Concentrations (mg/ml)	Antioxidant activity of crude methanol extract and its fractions				
	Methanol 80% extract	Hexane fraction	Ethyl acetate fraction	Water fraction	BHA*
4	69.73±0.59^{ax}	82.79±1.29^{cx}	89.56±0.66^{dx}	74.05±1.21^{bx}	81.51±0.67^{cw}
8	86.39±0.76^{cy}	86.39±1.50^{cy}	91.66±0.48^{dx}	77.44±0.90^{ay}	84.50±0.21^{bx}
16	92.19±1.38^{cz}	87.98±0.88^{byz}	94.53±0.40^{dy}	80.18±1.17^{az}	89.54±0.27^{by}
20	93.34±0.30^{cz}	89.46±1.29^{bz}	95.30±2.25^{dy}	81.37±1.22^{az}	92.92±0.23^{dz}

Values expressed are mean ± standard deviation of triplicate measurements. For the same extract or standard with different concentrations, means in the same column with different letters (w-z) were significantly different ($p < 0.05$, ANOVA). For different extracts with the same concentration, means in the same row with different letters (a-d) were significantly different ($p < 0.05$, ANOVA). BHA was used as the standard.

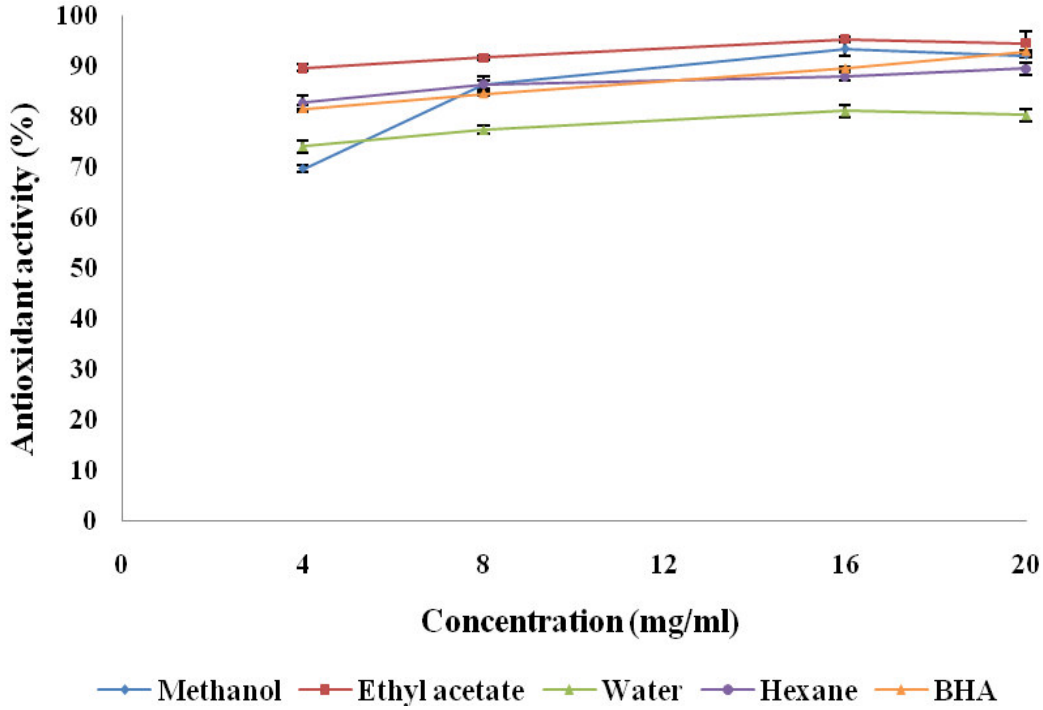

Figure 2. Antioxidant activity (%) of the crude methanol extract and fractionated extracts of rhizomes of *A. mutica* assayed by the β-carotene bleaching method.

lower concentration of 4 mg/ml, the crude methanol extract showed lower antioxidant activity. In contrast, the water extract showed lower antioxidant activity at all tested concentration range of 4 to 20 mg/ml in comparison to BHA. In this assay, ascorbic acid did not give any antioxidant activity and this phenomenon referred to as the "polar paradox" has been reported previously (Porter, 1993; Koleva et al., 2002; Huang et al., 2005). Figure 3 shows the decrease in absorbance of β-carotene in the presence of the crude methanol and its fractionated extracts, the control and BHA, at a concentration of 4 mg/ml. In the absence of antioxidant, β–carotene oxidized most rapidly (as shown by the graph of the control). In contrast, the oxidation rate of β–carotene decreases in the presence of plant extracts and the standard, BHA. The ethyl acetate fraction showed the highest ability in reducing bleaching of β-carotene in comparison to other extracts, even better than BHA, when observed over a period of 120 min.

Reducing power assay

In the reducing power assay, antioxidant acts as a reductant that can reduce the Fe^{3+}/ferricyanide complex to ferrous form (Fe^{2+}). Thus, ferrous ions (Fe^{2+}) can be

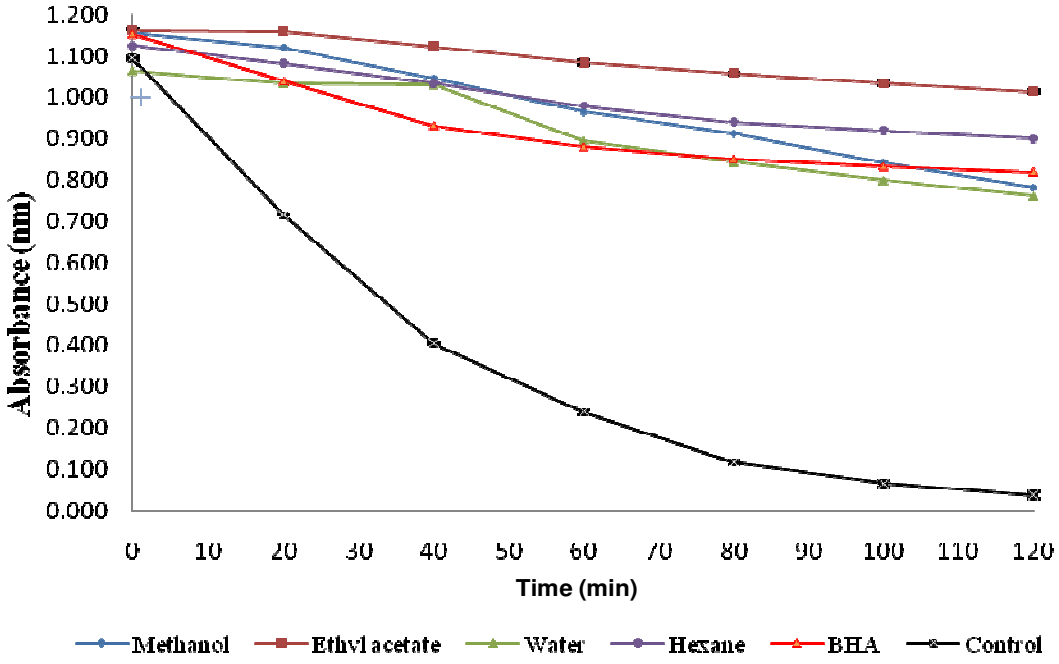

Figure 3. Degradation rate of crude and fractionated extracts of rhizomes of *A. mutica* assayed by β-carotene bleaching assay.

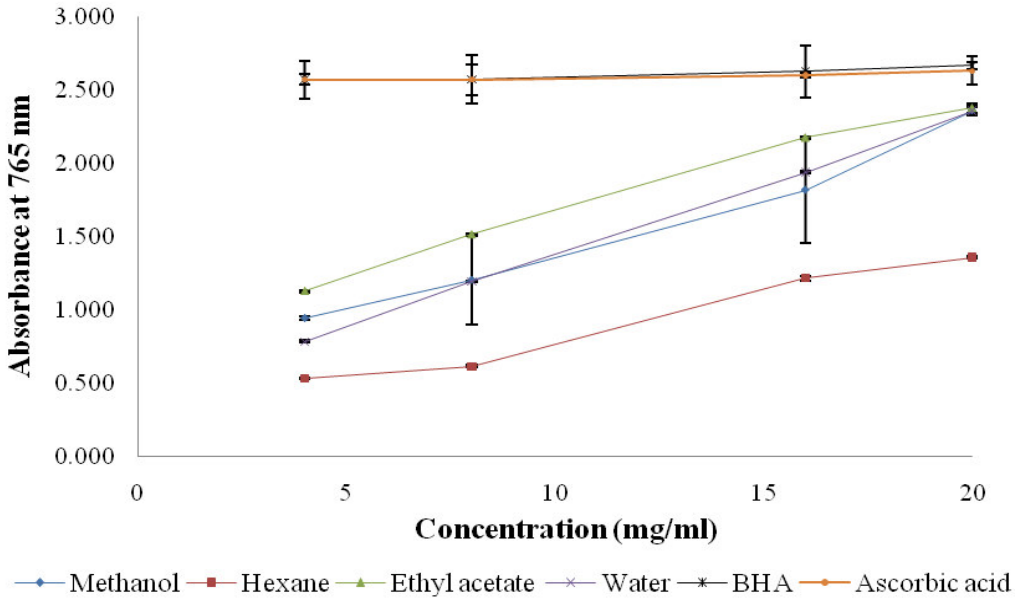

Figure 4. Reducing powers of the crude and fractionated extracts at different concentrations.

measured by the formation of Pearl's Prussian blue at 700 nm using spectroscopic (Lai et al., 2001). The greater the absorbance at 700 nm indicates the greater the reducing power of the extract. Figure 4 shows increasing reducing power with increasing concentration of the extracts. The reducing power is generally associated with the presence of reductones (Duh, 1998). Gordon (1990) reported that the antioxidant action of reductones is based on the breaking of the free-radical chain by donating a hydrogen atom.

The extracts that showed comparable absorbance values with ascorbic acid and BHA are considered to have high reducing power. The reductive capability of the extracts and its fractions decreases in the following order:

Table 6: Reducing powers of the extracts at various concentrations.

Extracts/fraction	Concentrations of extracts (mg/ml)			
	4	8	16	20
Methanol 80%	0.941±0.01^{cw}	1.202±0.71^{bw}	1.817±0.36^{bx}	2.359±0.03^{by}
Hexane	0.531±0.0002^{aw}	0.615±0.01^{ax}	1.215±0.01^{ay}	1.358±0.01^{az}
Ethyl acetate	1.127±0.01^{dw}	1.513±0.01^{cx}	2.174±0.01^{cy}	2.377±0.03^{bz}
Water	0.783±0.01^{bw}	1.195±0.005^{bx}	1.939±0.01^{bcy}	2.359±0.03^{bz}
Ascorbic acid*	2.573±0.04^{ew}	2.570±0.10^{dw}	2.602±0.01^{dw}	2.633±0.10^{cw}
BHA*	2.570±0.13^{ew}	2.572±0.17^{dw}	2.627±0.18^{dw}	2.666±0.02^{dw}

Absorbance values expressed are mean ± standard deviation of triplicate measurements. For the same extract or standard with different concentrations, means in the same column with different letters (a-d) were significantly different ($p < 0.05$, ANOVA). For different extracts with the same concentration, means in the same row with different letters (w-z) were significantly different ($p < 0.05$, ANOVA). *Positive reference standard.

Table 7: Inhibiton rate (SOD activity) of the crude and fractionated extracts

Extracts	Inhibiton rate (%)
Methanol 80%	64.84 ± 4.6^{a}
Hexane	69.57 ± 5.0^{a}
Ethyl acetate	83.56 ± 5.9^{b}
Water	61.19 ± 9.1^{a}
BHA	70.19 ± 2.9^{a}

Each value expressed was mean±standard deviation of triplicate measurements. Means with different letters in the same column are significantly different ($p < 0.05$, ANOVA). Data are expressed as % inhibition rate of SOD.

Ethyl acetate > water > methanol > hexane. The positive control, BHA and ascorbic acid, demonstrated much higher reducing power compared to the crude extract and its fractions. The reducing capability of the extract and its fractions increases with increasing concentrations of the extract as shown in Table 6.

The reducing power of the ethyl acetate fraction was found to be significantly higher in comparison to other fractions and showed highest absorbances values of 1.127, 1.513, 2.174, and 2.377 at concentrations of 4, 8, 16, and 20 mg/ml respectively. The hexane fraction showed significantly lowest reducing power among the extracts and fractions with absorbance values of 0.531, 0.615, 1.215 and 1.358 at the same concentrations. However, the positive control, BHA and ascorbic acid, demonstrated much higher reducing power compared to the extract and fractions.

Superoxide dismutase assay

In SOD assay, superoxide anion (O_2^-) radical is produced from the reaction between xanthine and oxygen (O_2) catalyzed by xanthine oxidase. The superoxide anionthen reduces WST-1 [(2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium)] to a water-soluble formazan dye that can be measured at the absorbance of 450 nm using a spectrophotometer. In the presence of an enzymatic antioxidant, the reduction of WST-1 can be inhibited by neutralizing O_2^-. Thus, the SOD activity can be quantified by measuring the decrease in the color development at 450 nm (Dudonné et al., 2009; Xing et al., 2010).

This assay is to determine the antioxidant activity of the test samples. Among the extracts, the ethyl acetate fraction exhibited the highest inhibition rate (64.84 ± 4.6%) and even higher than the inhibition rate of the standard, BHA (Table 7). The inhibition rate of the ethyl acetate fraction was also found to be significantly different ($p < 0.05$) from that shown by the standard (BHA). The water fraction showed the lowest inhibition rate (61.19 ± 9.1%). In comparison to the crude methanol extract and its fractions, the ethyl acetate fraction showed the highest antioxidant activity in the DPPH radical scavenging, β-carotene bleaching, reducing power and SOD assays.

This result correlated well with the total phenolic content which was found to be highest in the ethyl acetate fraction. It is well established that phenolic compounds have good antioxidant activity. Based on the result of the antioxidant assays, the ethyl acetate fraction of *A. mutica* posseses higher antioxidant activity than BHA in the β-carotene bleaching and the superoxide

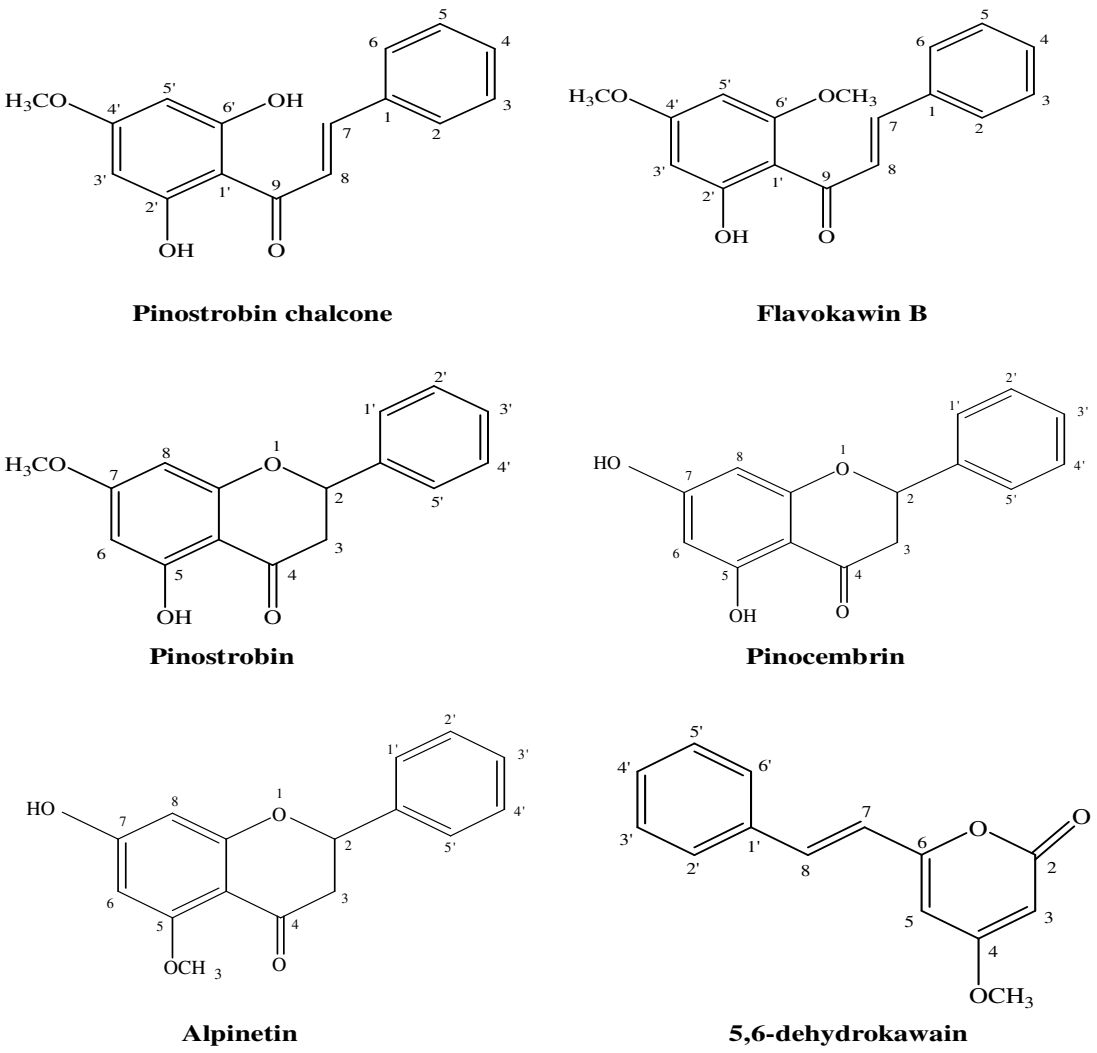

Figure 5. Structures of isolated compounds from *A. mutica*.

scavenging assays. In earlier studies, several phenolic compounds were reported from extracts of *A. mutica* rhizomes. These compounds were alpinetin, flavokawain B, pinostrobin chalcone, pinostrobin, 5,6-dehydrokawain and pinocembrin (Malek et al., 2011; Sirat et al., 1996 a,b); 5,6-dehydrokawain being the major compound (Figure 5). This explains the high total phenolic content and the antioxidant activity of the ethyl acetate fraction of *A. mutica*. These compounds may have contributed to the antioxidant activity of the ethyl acetate fraction of *A. mutica*. At this stage, it is not clear whether 5,6-dehydrokawain (the major component) contributes to the antioxidant activity as it is not a phenolic compound. However, Mohamad et al. (2004) reported that 5,6-dehydrokawain did not exhibit any significant antioxidant activity in the DPPH radical scavenging assay. Thus, this compound may not be responsible for the antioxidant activity. From our review of the current literature, this is the first report on the reducing power, β-carotene bleaching and SOD assays of *A. mutica* and the total content of phenolic compounds. The methods used in this investigation consists of the study on radical scavenging, reducing power, SOD and β-carotene bleaching activities. This makes it possible to screen different kinds of antioxidants in the extract based on their antioxidative mechanisms. Each of the method has their strengths and limitation in the evaluation of the anti-oxidant activity. Furthermore, the specificity and sensitivity are different for each method (Kulisic, 2004). A single method is not sufficient to provide a comprehensive picture of the antioxidant properties of the tested samples. For that reason, a combination of four assays was a better choice to evaluate the antioxidant activity of the extract and give more reliable results (Frankel et al., 1994; Koleva et al., 2002). The results of the current study showed that all the extract and fractions

of *A. mutica* exhibited different extent of antioxidant activity. The ethyl acetate fractions, which contain the highest amount of phenolic content, exhibited the greatest antioxidant activity even exceeding that of the synthetic antioxidants (BHA, ascorbic acid) in the β-carotene bleaching and superoxide scavenging assays. The antioxidant activity of the ethyl acetate fraction maybe contributed by the phenolic compounds present in it, namely flavokawin B, alpinetin, and pinostrobin chalcone. Thus, this study suggests that the ethyl acetate fraction of *A. mutica* may be a potential source of natural antoxidant.

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