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Full Length Research Paper

Antioxidant activity of plants methanolic extracts containing phenolic compounds

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The presence of natural antioxidant in plants is well known. This paper reports the antioxidative activities of some methanolic plant extracts namely 'ulam raja' (*Cosmos caudatus*), 'kesum' (*Polygonum minus*), 'selom' (*Oenanthe javanica*), 'pegaga' (*Centella asiatica*) and 'curry leaf' (*Murraya koenigii*). The analysis carried out was total phenolic content, ferric reducing power, ferric thiocyanate (FTC) and thiobarbituric acid (TBA) tests. From the analyses, *M. koenigii* had the highest yield extraction (1.65%), highest total phenolic content (38.60 mg TAE/ 100 g fresh weight) and antioxidant activity (70.60%) using FTC method. Increasing the concentration of the extracts resulted in increased ferric reducing antioxidant power for all methanolic extracts tested. TBA analysis showed that *C. caudatus* extract had the highest antioxidant effect. Total phenolic content had positive correlation with antioxidant capacity (r = 0.451). This shows that the plants, especially *M. koeniigi*, may be potent source of natural antioxidants.

Key words: Antioxidative activities, plants, total phenolic content, reducing power, thiobarbituric acid.

INTRODUCTION

In recent years much attention has been devoted to natural antioxidant and their association with health benefits (Arnous et al., 2001). Plants are potential sources of natural antioxidants. It produces various antioxidative compounds to counteract reactive oxygen species (ROS) in order to survive (Lu and Foo, 1995). ROS, which include free radicals such as superoxide anion radicals ($O^{\cdot}_2^-$), hydroxyl radicals ($OH^{\cdot}$) and non free-radical spesies such as H_2O_2 and singled oxygen ($^1O_2$), are various forms of activated oxygen. These molecules are exacerbating factors in cellular injury and aging process (Gülçin et al., 2003). In foods, ROS can cause lipid peroxidation, which leads to the deterioration of the food (Miller and Rice-Evans, 1997). The oxidative deterioration of the lipid-containing food is responsible for the rancid odours and flavours during processing and storage, consequently decreasing the nutritional quality and safety of foods, due to the formation of secondary, potentially toxic compounds. The addition of antioxidant is a method for increasing the shelf life of foods (Cook and Samman, 1996).

Plant phenolics are commonly found in both edible and non-edible plants, and have been reported to have multiple biological effects, including antioxidant activity. The antioxidant activity of phenolics is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donators, and singlet oxygen quenchers. In addition, they have a metal chelation potential (Rice-Evans et al., 1995). The phenolic compounds are increasingly of interest in the food industry because they retard oxidative degradation of lipids and thereby improve the quality and nutritional value of food (Kähkönen et al.,

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1999). The importance of natural phenolic compounds from plants materials is also raising interest among scientists, food manufacturers, and consumers due to functional food with specific health effects (Löliger, 1991).

Several studies had been conducted to evaluate the correlation between phenolic compounds and antioxidant activity. The antioxidant activity of Du-Zhong (*Eucomnia ulmoides*) (Yen and Hsieh, 1998), ear mushrooms (Chao, 2001) and anise (*Pimpenella anisum* L.) seed (Gülçin et al., 2003) were found to correlate with the phenolic compounds. Studies on local plants such as turmeric (*Curcuma domestica*), betel leaf (*Piper betel*), pandan leaf (*Panadanus odorus*), asam gelugur (*Garnicia atroviridis*), mengkudu (*Morinda citrifolia*), pegaga (*Centella asiatica*), ginger (*Zingiber officinale*), cassava shoot (*Manihot asculenta*), kesum (*Polygonum minus*) and selom (*Oenathe javanica*) (Huda-Faujan et al., 2007; Jayamalar and Suhaila, 1998; Mohd. Zin et al., 2002; Noriham et al., 2004; Zainol et al., 2003) also exhibit good antioxidant activity. The antioxidative properties of some vegetables and fruits are partly due to the low molecular weight phenolic compounds, which are known to be potent as antioxidants (Wang et al., 1999).

In Malaysia, herb is commonly eaten fresh as a vegetable (salad and *ulam*), especially among the Malay communities. Most of these herbs are believed to be associated with antioxidant activities and have many beneficial effects. Recently, there are numerous methods that have been developed to evaluate antioxidant activities of compounds and of complex mixtures such as plant extracts (Anatolovich et al., 2002). Despite the existence of these various methods, just one procedure cannot identify all possible mechanisms characterizing an antioxidant activity (Frankel and Meyer, 2000). Therefore, the aim of this study is to evaluate the antioxidative activity of several Malaysia plants using three different methods, and to evaluate the relationship between the antioxidative activity and total phenolic content of the plants.

MATERIALS AND METHODS

Chemicals

Ammonium thiocyanate and ferrous chloride were purchased from Merck (Merck KGaA, Darmstadt, Germany). Methanol, ferric chloride, linoleic acid (99.5%), potassium ferricyanide, butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), thiobarbituric acid (TBA) and trichloroacetic acid (TCA) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Commercial rosemary (Rosemary Herbalox ®) was obtained from Kalsec UK Ltd. (Suffolk, UK).

Materials and extraction method

All plants were purchased from a local wet market in Bandar Baru Bangi, Selangor, Malaysia. The plants were ulam raja (*Cosmos caudatus*), kesum (*Polygonum minus*), selom (*Oenanthe javanica*), pegaga (*Centella asiatica*) and curry leaf (*Murraya koenigii*). The edible portion of fresh samples was cleaned and washed under running tap water. The samples were dried in the oven at 37°C overnight. Then, the samples were weighed and blended with Waring blender and soaked with methanol [in ratio methanol : plant (6:1)] for seven days and filtered using Whatman No. 1 paper. The methanol was completely removed by vacuum evaporator at 50°C to give viscous mass. The crude extracts were weighed and stored at 0 – 4°C before analysis.

Determination of total phenolic contents

The amount of total phenolics in extract was determined with Folin–Ciocalteu reagent according to the method of Singleton and Rossi (1965) with slight modification using tannic acid as a standard. Briefly, 1.0 ml of extract solution (5 mg/ml) was added in a 100 ml volumetric flask that contained about 60 ml distilled water. Then, 5.0 ml of Folin–Ciocalteu reagent was added and the content of the flask was mixed thoroughly. After 1 - 8 min, 15.0 ml Na_2CO_3 (20 %) was added and the volume was made up to 100 ml using distilled water. The mixture was allowed to stand for 2 h with intermittent shaking. The absorbance was measured at 760 nm using a UV-Vis spectrophotometer (Jenway 6100, Dunmow, Essex, U.K). The total phenolic content was determined as mg of tannic acid equivalent (TAE) using an equation obtained from the standard tannic acid calibration graph.

Reducing antioxidant power

The reducing antioxidant power of plant methanolic extracts was determined by the method of Oyaizu (1986). Different concentrations of plant extracts (200 – 1200 ppm) in 1 ml of distilled water were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 ml, 1%). The mixture was incubated at 50°C for 20 min. Then, 2.5 ml of trichloroacetic acid (10%) was added to mixture, which was then centrifuged for 10 min at 3000 rpm. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1%). The absorbance was measured at 700 nm against a blank using UV-Vis spectrophotometer (Jenway 6100, Dunmow, Essex, U.K). Increased absorbance of the reaction mixture indicates increase in reducing power.

Ferric thiocyanate (FTC) method

The antioxidant activities of plant methanolic extracts were determined according to the FTC method (Osawa and Namiki, 1981) with slightly modification. Four milligrammes of each extract samples were dissolved in 4.0 ml ethanol (99.5 %) and kept in dark bottle (d = 40.0 mm, t = 75.0 mm). Each mixture were mixed with 4.1 ml linoleic acid (2.5% in ethanol 99.5%), 8.0 ml phosphate buffer (0.02 M, pH 7.0) and 3.9 ml distilled water to make up the volume to 20.0 ml. BHT was used as a positive control while the another bottle without sample was used as a negative control. The mixture was incubated at 40 – 45°C. After incubation, 9.7 ml ethanol (75 %) and 0.1 NH_4SCN (30%, as a colour reagent) was added to 0.1 ml of the mixture. Precisely 3 min after the addition of 0.1 ml of $FeCl_2$ (0.002 M) in HCl 3.5% to the reaction mixture, the absorbance of the resulting red colour was measured at 500 nm using spectrophotometer (Jenway 6100, Dunmow, Essex, UK) every 24 h until a day after the absorbance of the control reached maximum value (day seven). The inhibition of lipid peroxidation was calculated as follows:

$$\% \text{ Inhibition} = 100 - [(A_1/A_o) \times 100]$$

Where A_o is the absorbance of the control reaction and A_1 is the

Table 1. Extraction yields and total phenolic content of plant extracts.

Plant	Yield of extracts (%)	Total phenolic (mg TAE /100 g fresh weight)
Cosmos caudatus	1.13[ab]	18.83[b]
Centella asiatica	0.71[b]	7.79[c]
Murraya koenigii	1.65[a]	38.60[a]
Polygonum minus	0.80[b]	16.73[b]
Oenanthe javanica	0.66[b]	7.41[c]

Values with the same lowercase within each column are not significantly different (P>0.05).

absorbance in the presence of the sample extracts (Duh et al., 1999).

Thiobarbituric acid (TBA) test

The TBA test was conducted according to the combined method of Kikuzaki and Nakatani (1993) and Ottolenghi (1959). A milliliter of sample from the previous FTC method was added with 2 ml of trichloroacetic acid and 2 ml of thiobarbituric acid solution. This mixture was then placed in a boiling water bath at 100°C for 10 min. After cooling, it was centrifuged at 3000 rpm for 20 min and absorbance of the supernatant was then measured at 532 nm using UV-Vis spectrophotometer (Jenway 6100, Dunmow, Essex, UK).

Statistical analysis

All analyses were run in triplicates. Data were analysed by an analysis of variance (ANOVA) (P<0.05) and the means separated by Duncan's multiple range test using Statistical Analysis System 6.21 package (SAS 1995).

RESULTS AND DISCUSSIONS

The Extraction yield and phenolic content of the extracts

The yield of plant methanolic extracts and the concentration of total phenolic content (mg/100 g fresh weight) are shown in (Table 1). From the table, *M. koenigii* shows the highest extraction yields (1.65%) and total phenolic content (38.6%) among the samples. The percentage of extraction yields of *C. caudatus, P. minus, C. asiatica* and *O. javanica* were 1.13, 0.80, 0.71, and 0.66 mg TAE, respectively. Based on Herodeź et al. (2003), the percentage of extraction yields will increase with the particle size of sample, temperature extraction and the ratio of solvent and sample extraction. From the analysis, it also showed that *O. javanica* had the lowest total phenolic content.

Studies on total phenolic content had been published in several papers. Yen and Hsieh (1998) reported that the total phenolic content in Du-Zong (*Eucomnia ulmoides*) ranged from 8700 to 21000 mg GAE/ dry weight. Total phenolic content of *Moringa oleifera* in three different climates (India, Nicaragua and Niger) ranged from 2940 - 4250 mg GAE/ dry weight (Siddhuraju and Becker, 2003) and water plants extracts studied by Noriham et al. (2004) ranged from 257 - 3234 mg TAE/100 g dry weight. In addition, Jerez et al. (2007) evaluated the total phenolic from the bark of two kinds of pine, *Pinus pinaster* and *Pinus radiate*. Different levels reported in these studies may be attributed to the different plants, procedures and standards used to express as total phenolic contents used by individual groups of investigator. The usage of Folin-Ciocalteu reagent also was measured based on the colour measurement which was non-specific on phenol. Perhaps there were other components that can react with the reagent such as ascorbic acid (Shahidi and Naczk, 1995). Besides, various phenolic compounds have different response to this assay (Singleton and Rossi, 1965). However, the measurement of colour changes after two hours storage could be used to determine the existence of phenol in samples. This may due to the antoxidant properties of plant extract that react as reductant agent which known as redox action.

Reducing power

Reducing power is to measure the reductive ability of antioxidant, and it is evaluated by the transformation of Fe (III) to Fe (II) in the presence of the sample extracts (Gülçin et al., 2003). The reducing power of methanolic plant extracts are summarized in (Figure 1). From the figure, reducing power increased with an increased in extracts concentration. The data show that all the samples increased their reducing ability when the concentration of extracts was increased. This result is similar to that reported by Gülçin et al. (2003) and Noriham et al. (2004), who demonstrated antioxidative activity on *Pimpinella anisum* seeds extracts and four types of Malaysian plants. At 200 ppm, BHA had the highest ability to reduce Fe (III) and had no significant difference with *P. minus* (P >0.05). The ability of reducing power of methanolic extract of *P. minus* showed almost similar with synthetic antioxidant, BHA at 600, 800, 1000 and 1200 ppm. At 1200 ppm, all methanolic plant extracts have higher ability than BHT to reduce Fe (III) to Fe (II) (P <0.05). The ability to reduce Fe (III) may be attributed

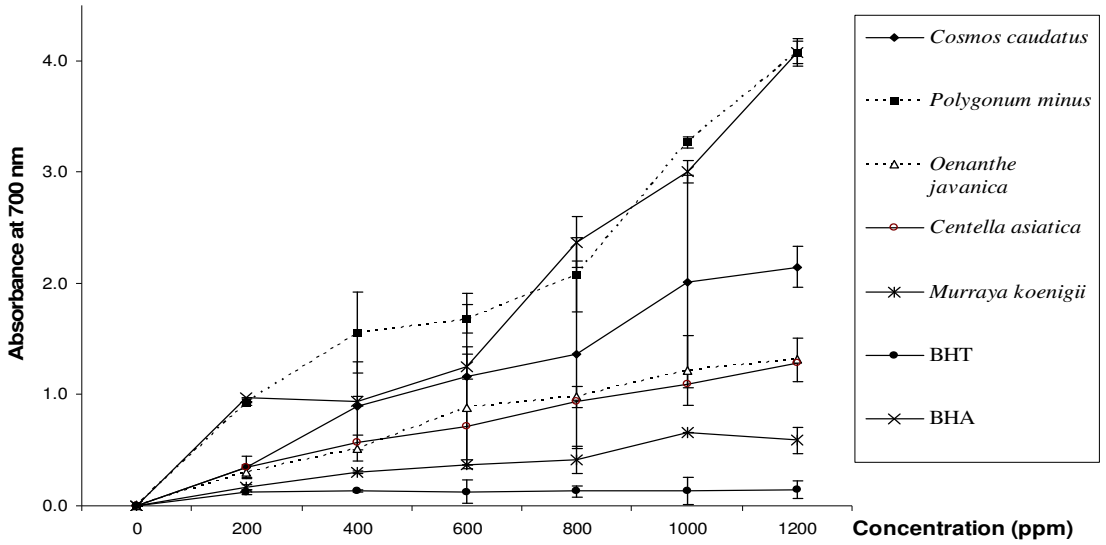

Figure 1. Reducing power of methanolic plant extracts

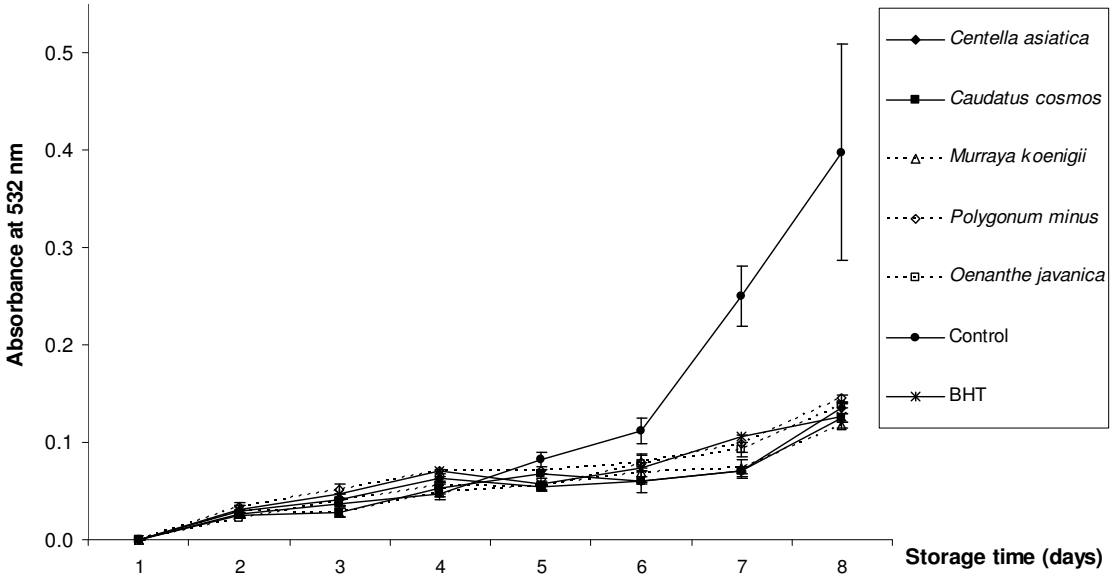

Figure 2. Analysis of FTC of methanolic plant extracts.

from hydrogen donation from phenolic compounds (Shimada et al., 1992) which is also related to presence of reductant agent (Duh, 1998). In addition, the number and position of hydroxyl group of phenolic compounds also rule their antioxidant activity (Rice-Evans et al., 1995).

Antioxidant capacity in linoleic acid emulsion

(Figure 2) shows the FTC of various types of mathanolic plant extracts studied. The FTC method measures the amount of peroxide value in the beginning of the lipid peroxidation, where ferric ion was formed upon reaction of peroxide with ferrous chloride. The ferric ion will then unite with ammonium thiocyanate producing ferric thiocyanate, a red-coloured substance. The darker the colour, the higher will be the absorbance.

From the analysis, it shows that all samples had been oxidized when stored for seven days at 40-45^{o}C. Initially, the absorbance of *O. javanica* was the lowest (0.022). After seven days storage, all samples exhibited good effect in inhibiting linoleic acid oxidation compared to control. The percentage of inhibition of linoleic acid of *M. koenigii, C. caudatus, C. asiatica, O. javanica,* and *P. minus* were 70.60, 68.67, 66.17, 65.41, and 63.66%, res-

Table 2. Analysis of TBA of methanolic plant extracts.

Samples	Absorbance (A=532 nm) at 7 days of storage
Cosmos caudatus	0.074^{bc}
Centella asiatica	0.064^{bc}
Murraya koeniigi	0.063^{bc}
Polygonum minus	0.086^{b}
Oenanthe javanica	0.066^{bc}
BHT	0.065^{bc}
Control	0.172^{a}

Values with the same lowercase within each column are not significantly different ($P < 0.05$).

pectively, with no significant difference compared to BHT (P >0.05). he absorbance of control was 0.399 after seven days storage. We have previously reported the percentage of inhibition of linoleic acid of these samples using water extraction was in the order of *M. koenigii* > *P. min*us > *C. caudatus* > *C. asiatica* > *O. javanica* with percentage values of 67.7, 63.2, 59.7 , 53.1 and 52.1 %, respectively (Huda-Faujan et al., 2007).

Noriham et al. (2004) reported the percentage of linoleic acid inhibition at 200 ppm of *P. minus*, *Zingiber officinale*, *Melicope lunu-ankenda* and *Manihot esculenta* were 85.5, 68.0, 68.4 and 54.7%, respectively. The antioxidant activities also increased with increasing the concentration of the plant extracts (Gülçin et al., 2003; Nagai et al., 2003). Gülçin et al. (2003) reported that the percentage of linoleic acid inhibition of *Pimpinella anisum* seeds at 100, 200 and 500 ppm were 56.5, 77.5 and 91.2%, respectively. The correlation between total phenolic content and antioxidant capacity in linoleic acid emulsion in this study was found positive (r = 0.451). These phenolic compounds may donate hydrogen and can terminate the free radical reaction chain by changing it to the stabile compounds (Amarowicz et al., 2000).

Thiobarbituric acid (TBA) test

FTC is used to measure the production of peroxide compound at the initial stage of oxidation while TBA test is used to measure the secondary product of oxidation such as aldehyde and ketone (Farag et al., 1989). (Table 2) shows the TBA analysis of the methanolic plant extracts at seventh day storage. The percentage of antioxidative activity for *C. caudatus*, *C. asiatica*, *M. koeniigi*, *P. minus* and *O. javanica* were 57, 63, 63, 50, 62 and 63%, respectively (Table 2). These results were not significantly different from BHT, where the percentage of antioxidant activity was 63%. The absorbance of control sample obviously showed the highest reading (P <0.05). This result was in line with our previous study (Huda-Faujan et al., 2007; Noriham et al., 2004) that the control sample was the highest absorbance reading in TBA after seven days storage. This could be indicated that the amount of peroxidation was greater than that in secondary stage. Secondary product such as malonaldehyde is not stable for a long period of time. It would be turned into alcohol and acid, which cannot be detected by a spectrophotometer (Asmah et al., 2000).

Conclusions

In conclusion, among all the local plants extract analysed, curry leaf (*M. koenigii*) showed the highest yield (1.65%), total phenolic content (38.60 mg TAE/ 100 g fresh weight) and antioxidant activity (70.60%) using FTC model. However, analysis of TBA shows that pegaga (*C. asiatica)* showed the highest antioxidant activity during inhibition of lipid oxidation. This mean value was not statistically different (P<0.05) from *M. koeniigi* and BHT. From the correlation analysis, it had been shown that total phenolic content had positive correlation with antioxidant capacity in acid linoleic emulsion (r = 0.451). This shows that Malaysian plants may be potent sources of natural antioxidants.

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