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Antioxidant Activity of *Ardisia crispa* (Mata pelanduk) (Aktiviti Antioksidasi daripada *Ardisia crispa* (Mata pelanduk))

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ABSTRACT

The leaf and fruit crude extracts of hexane, chloroform, methanol and water of *Ardisia crispa* were screened for their antioxidant activity using DPPH radical scavenging, ferric reducing power, and metal chelating antioxidant assay. The methanol crude extract of fruits showed higher antioxidant activity ($90.16 \pm 0.01\%$) than the methanol crude extract of leaves ($82.24 \pm 0.02\%$) in the DPPH radical scavenging assay. In the ferric reducing power assay methanol fruit extract showed the highest absorbance indicating high antioxidant activities than leaf extract. In the metal chelating antioxidant assay fruit methanol extract gave 40% antioxidant activities than the leaf. Thin Layer Chromatography of the fruit methanol crude extract showed that it contained phenolic compounds when it was detected with folin reagent. HPLC analysis revealed that the fruit methanol extract contained gallic acid. This indicated that the high antioxidant activities of the fruits were due to the presence of gallic acid in the fruits of *Ardisia crispa*.

Keywords: Antioxidants; DPPH radical scavenging activity; gallic acid; metal chelating activity; reducing power assay

ABSTRAK

Ekstrak mentah heksan, kloroform, metanol, air daripada daun dan buah *Ardisia crispa* telah disaring untuk menentukan aktiviti antioksidan menggunakan asai antioksidan penyahbebas radikal DPPH, kuasa penurunan ferik, dan logam pengkelat. Ekstrak metanol buah menunjukkan aktiviti antioksidan yang lebih tinggi daripada ekstrak daun dengan peratus perencatan penyahbebas DPPH $90.16 \pm 0.01\%$ and $82.24 \pm 0.02\%$ masing-masing. Dalam asai kuasa penurunan ferik ekstrak metanol buah menunjukkan serapan yang tertinggi menandakan aktiviti antioksidan yang tinggi daripada ekstrak daun. Dalam asai logam pengkelat ekstrak metanol, buah memberikan 40% aktiviti antioksidan daripada daun. Kromatografi lapisan nipis ekstrak mentah buah menunjukkan, terdapat kandungan sebatian fenolik. Analisis HPLC menunjukkan ekstrak mentah mengandungi asid galik. Ini menunjukkan aktiviti antioksidan yang tinggi di dalam buah adalah disebabkan kehadiran asid galik dalam buah *Ardisia crispa*.

Kata kunci: Aktiviti pengkelat logam; antioksidan; asai kuasa penurun; asid galik; penyahbebas radikal DPPH

INTRODUCTION

The genus *Ardisia* Sw. is the largest in the family Myrsinaceae and comprises approximately 500 species distributed throughout subtropical and tropical regions of the world (Kobayashi & de Mejía 2005). Several species of the genus possess interesting biological activity such as the extract of *Ardisia japonica* exhibited antioxidant activity (Ryu et al. 2002), anti-HIV (Piacente et al. 1996), and anticancer (Nikolovska-Coleska et al. 2004). de-Mares et al. (2001) reported that *Ardisia compressa* leaves possess a high antioxidant capacity. In China the roots of *Ardisia crispa* (bai liang jin/coralberry) are used to reduce fever and to stop excessive salivation and in Malaysia the juice expressed from the leaves is used to treat scurvy (Christophe 2002). Moreover, Kang et al. (2001) have isolated AC7-1 compound from *Ardisia crispa* which showed inhibition of B16-F10 melanoma cell invasion and effectively suppression of pulmonary metastasis and

tumour growth. However, no study of the antioxidant activity of *Ardisia crispa* has been reported.

Free radicals are constantly produced within the human body in response to both internal and external stimuli (Matés et al. 1999). In small amount these products play an important role as growth regulator, signal transducers, and as part of the immune defense system (Atmani et al. 2009). However, excess generation of free radicals and other oxidants will cause oxidative stress. Nowadays, it is well known that oxidative stress is associated with several diseases such as cancer, arteriosclerosis, neurodegenerative diseases, and ageing processes (de Oliveira et al. 2009). Our cells are well protected against free radicals damage either by endogenous antioxidative enzymes such as superoxide dismutase (SOD) and catalase (CAT) or by exogenous chemicals such as α tocopherol, ascorbic acid, carotenoids, polyphenol compounds and glutathione (Cheesman & Slater 1993).

Many studies have suggested that the intake of fruits and vegetables is correlated with a low risk of cancer and cardiovascular disease (Cazzi et al. 1997). In recent years, there is a vast interest in the use of natural antioxidants derived from medicinal plants in medical field to promote human health and in industry to replace the synthetic additives such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) that might be carcinogenic (Whysner et al. 1994).

So far, no studies of the antioxidant activity from the *Ardisia crispa* have been carried out. Thus, in this study the antioxidant capacity of the fruit and crude extracts of the *Ardisia crispa* was determined using DPPH, reducing power and metal chelating assays.

MATERIALS AND METHODS

PLANT COLLECTION

The leaves and fruits of *Ardisia crispa* were collected from Rimba Ilmu, Botanical Garden, University of Malaya, Kuala Lumpur, Malaysia. The leaves and fruits were dried under shaded area and grinded to fine powder for extraction purposes.

PREPARATION OF PLANT EXTRACT

The dry powder of leaves and fruits of *Ardisia crispa* at 20 mg each was extracted with 200 mL hexane, chloroform, methanol, and water. The mixture was incubated in water bath at 40 °C for 2 h. Then the mixture was filtered with Whatman filter paper and dried using a vacuum rotary evaporator. The dried extract was placed in sample bottle and kept in the dark at -20 °C until further use.

DETERMINATION OF ANTIOXIDANT ACTIVITY DPPH ASSAY

The free radical scavenging activity of crude extracts on 2,2-diphenyl-1-picrylhydrazyl (DPPH[•]) radical was measured according to the method described by (Sanchez-Moreno et al. 1999 a,b) with slight modification. 975 µL of crude extract dissolved in methanol (1.0, 2.0, 3.0, 4.0 and 5.0 mg mL⁻¹) was mixed with 25 µL of DPPH[•] radical solution (8 mg mL⁻¹). After 30 min, the optical density was read at 517 nm, methanol was used as a blank. Each measurement was made in triplicate. Ascorbic acid was used as standard. The percentage of DPPH free radical quenching activity was determined by the formula:

$$\% \text{ inhibition DPPH} = [(A_{\text{control}} - A_{\text{sample}})/A_{\text{control}}] \times 100\%$$

where A_{control} refers to the absorbance at 517 nm of the control sample and A_{sample} refers to the absorbance at 517 nm for the sample at different extract concentration.

FERRIC REDUCING POWER ASSAY (FRAP)

The reducing power of the *Ardisia crispa* extracts was determined using the method of Oyaizu (1986). The extract solution (0.5 mL) at different doses was mixed with phosphate buffer (0.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (0.5 mL, 1%). The mixture was then incubated at 50 °C for 20 min. A portion of trichloroacetic acid (0.5 mL, 10%) was added to the mixture, which was then centrifuged for 10 min at 3000 rpm (1000 g). The upper layer of solution (0.5 mL) was mixed with distilled water (0.5 mL) and $FeCl_3$ (0.1 mL, 0.1%) for 10 min, and then the absorbance was measured at 700 nm in a spectrophotometer, with higher absorbance indicating greater reducing power. Butylated hydroxyanisole (BHA) was used as a standard.

METAL CHELATING ACTIVITY ASSAY

The chelation of ferrous ions by *Ardisia crispa* extracts was estimated according to the method of Dinis et al. (1994). 0.95 mL of crude extracts at different doses was added to a solution of 50 µL $FeCl_2$ (2 mM). The reaction was initiated by the addition of 200 µL ferrozine (5 mM), and then the mixture was shaken vigorously and left standing at room temperature for 10 min. After equilibrium had been reached, absorbance of the solution was measured spectrophotometrically at 562 nm. The percentage of inhibition of ferrozine- Fe^{2+} complex of each sample was calculated according to the following formula:

$$\% \text{ inhibition} = [(Abs_{\text{control}} - Abs_{\text{sample}})/Abs_{\text{control}}] \times 100,$$

where, Abs_{control} is the absorbance reading of control and Abs_{sample} is the absorbance reading of sample

DETERMINATION OF TOTAL PHENOLIC CONTENT

The total phenolic content was measured according to the method described by Velioglu et al. (1998). 0.5 mL of each extract was added to 5 mL of Folin-Ciocalteu reagent followed by addition of 4 mL of 1M Na_2CO_3 , the mixture was and allowed to stand in water bath at 45 °C for 15 min. The solution was mixed well and absorbance was measured using spectrophotometer at 765 nm. A calibration curve using gallic acid with concentrations ranging from 0.5 to 2.5 mg/mL was prepared. The results were mean values of triplicates expressed as mg of GAE (gallic acid equivalents)/g extracts.

DETERMINATION OF TOTAL FLAVONOID CONTENT

The total flavonoid content was determined according to the method described by Zhishen et al. (1999). 1 mL of each extract was added to 0.3 mL of 5% $NaNO_3$. After 5 min. incubation in water bath at 37°C, 0.3 mL of 10 % $AlCl_3$ was added and the mixture was incubated at 37 °C for 6 min. Two milliliter of 1M NaOH 2mL was added to the mixture followed by 10 mL of distilled water. The

solution was mixed well, and absorbance was measured using spectrophotometer at 510 nm. A calibration curve, using quercetin with concentrations ranging from 0.5 to 2.5 mg/mL was prepared. The results were mean values of triplicates expressed as mg of QE (Quercetin equivalents)/g extracts.

SEPARATION AND ISOLATION OF CHEMICAL COMPOUNDS USING THIN LAYER CHROMATOGRAPHY (TLC) AND HIGH PRESSURE LIQUID CHROMATOGRAPHY (HPLC)

The crude extract was spotted on the TLC plate (silica gel 60, aluminium plate) and developed using the chloroform:methanol (90:10) solvent system. The HPLC analysis was performed as described by Chen et al. (2001) using the HPLC system from model Shimadzu consisted of LC-10AT pump system (Shimadzu Co. Japan) and an SPD-M10A DIODE array detector. The chemical compounds were detected at both 280 and 360 nm. The column used was a Thermo scientific Hypersil BDS-C₁₈ (250 × 4.6 mm², 5 μm). A gradient solvent system consisting of solvent A (water-acetic acid, 97:3, v/v) and solvent B (MeOH) was used. The solvent gradient is presented in Table 1. Standard of gallic acid was purchased from Sigma. The solvents were purchased from Fisher Scientific. All solutions were

filtered through 0.45 μm membranes before HPLC analysis, and the mobile phase solvents were degassed before use.

RESULTS AND DISCUSSION

TLC performed in this study showed that the fruit methanol crude extract contained phenolic compounds. The presence of the phenolic compounds was detected using folin reagent that gives blue or purple colours.

The HPLC separation which was carried out from the methanol extracts showed the presence of gallic acid in the fruits of *Ardisia crispa*. The identification of gallic acid in the fruit methanol extract was based on the retention time of a standard of gallic acid as shown in Figures 1, 2 and 3. The presence of gallic acid in *Ardisia* species has been reported previously. Sumino et al. (2002) reported the presence of gallic acid in the fruits of *Ardisia colorata*. de Mejía et al. (2006) also mentioned that *Ardisia compressa* contains gallic acid. Natural phenolic acid gallic is well known antioxidant found in green tea (Lu et al. 2006) and is commonly used in food, drug and cosmetics. However, there is no report on the presence of gallic acid in *Ardisia crispa* and its antioxidant activity. This is the first primary study on the antioxidant activity of *Ardisia crispa* fruits.

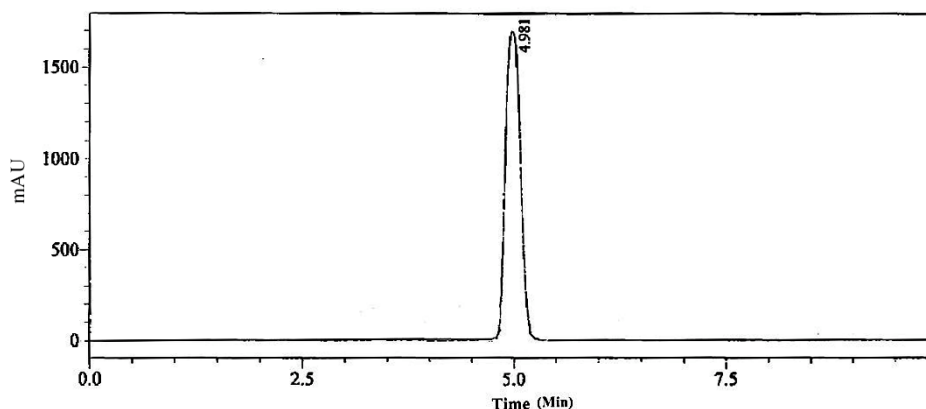


FIGURE 1. HPLC chromatogram of standard gallic acid.

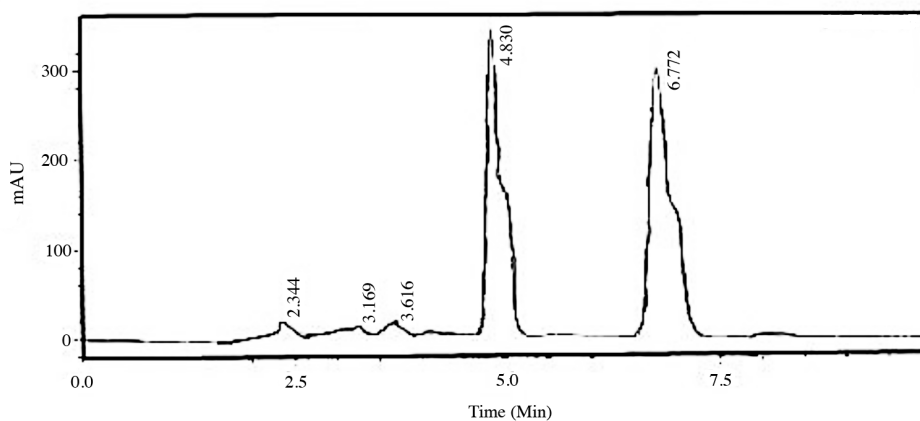


FIGURE 2. HPLC chromatogram of fruit methanol extracts of *Ardisia crispa*

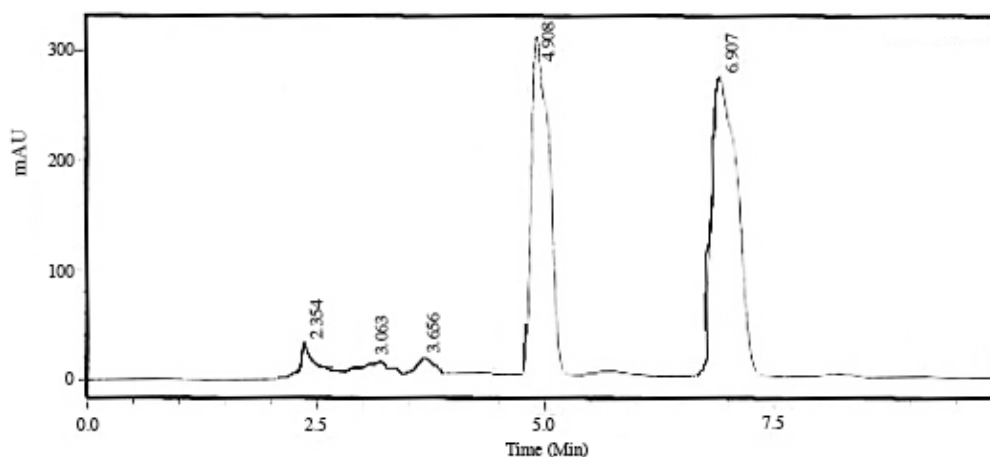


FIGURE 3. HPLC chromatogram of leaf methanol extracts of *Ardisia crispa*.

Several methods have been developed for the assessment of the antioxidant efficiency. Because many active species and reaction mechanisms are involved in oxidative stress process, no simple universal method which can be applied for accurate and quantitative measurement of antioxidant capacity (Frankel & Finley 2008). Generally, in these methods a radical is generated and the antioxidant capability of a sample against the radical is evaluated (Erel 2004). In the present study, the antioxidant activity of crude extracts of *Ardisia crispa* was determined using DPPH radical scavenging assay, reducing power assay, and metal chelating assay.

DPPH assay has been widely used to determine the free radical scavenging activity of various plants (Villano et al. 2007). *Ardisia crispa* extracts exhibit DPPH radical scavenging activity (Figure 4). All of the crude extract except leaf hexane extract shows DPPH scavenging activity

more than 50%. The fruit extracts showed higher inhibition activity than leaf extracts. Fruit and leaf methanol extracts showed the highest scavenging effects of $90.16 \pm 0.01\%$ and $82.24 \pm 0.02\%$, respectively at concentration of 5 mg/mL, with IC_{50} value of 0.9 mg/mL and 1.5 mg/mL, respectively. The lowest scavenging activity was shown by leaf hexane extract with inhibition activity of $45.77 \pm 0.01\%$ at 5 mg/mL. The high inhibition of DPPH in fruit extract could be due to the acid gallic as it was detected in TLC and HPLC. Gow-Chin et al. (2002) have shown that gallic acid has scavenging effect on DPPH radicals. Study by Vilapakkam et al. (2011) also showed that gallic acid has antioxidant effect on induced diabetic winster rats.

FRAP assay was frequently used to study the antioxidant activity of plants (Li et al. 2006). It measures the ferric to ferrous ion reduction in the presence of antioxidant. The reducing power of a sample may serve as important pointer

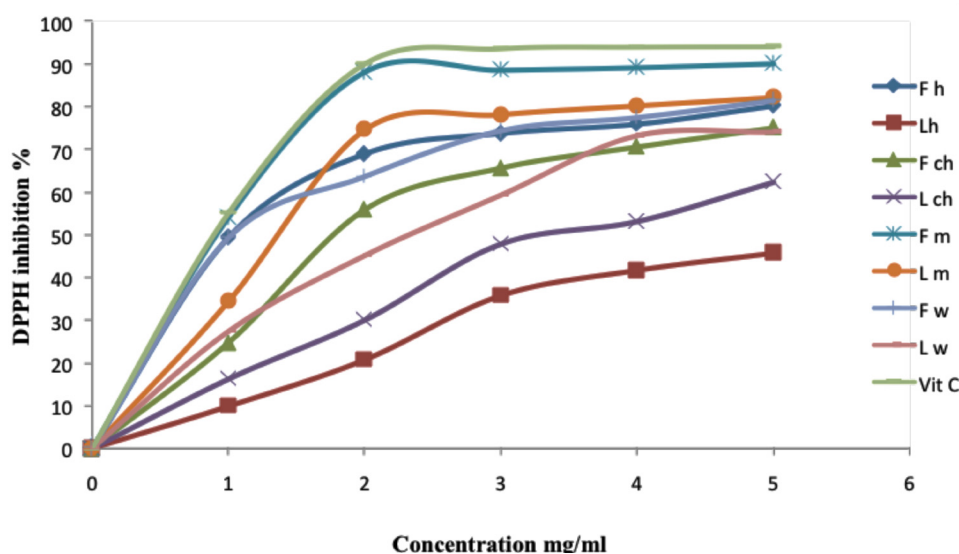


FIGURE 4. Scavenging activity of crude extracts from *Ardisia crispa* on DPPH radical, F h = fruit hexane extract, L h = leaf hexane extract, F ch = fruit chloroform extract, L ch = leaf chloroform extract, F m = fruit methanol extract, L m = leaf methanol extract, F w = fruit water extract, L w = leaf water extract.

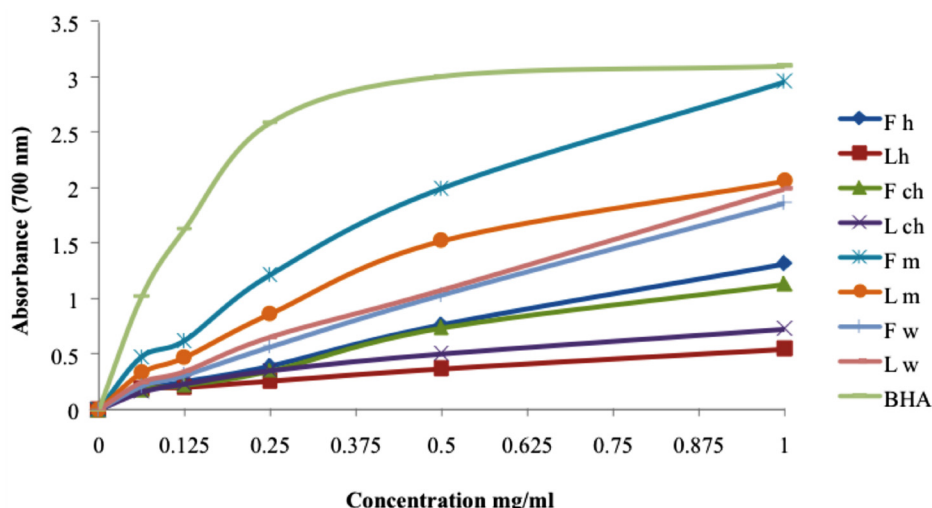


FIGURE 5. Reducing power activity of crude extracts from *Ardisia crispa*, F h = fruit hexane extract, L h = leaf hexane extract, F ch = fruit chloroform extract, L ch = leaf chloroform extract, F m = fruit methanol extract, L m = leaf methanol extract, F w = fruit water extract, L w = leaf water extract, BHA = butylated hydroxyanisole.

of its potential antioxidant capacity (Meir et al. 1995). It showed the reducing capacity of a compound and is able to act as a significant indicator for antioxidant activity. Figure 5 shows that all crude extracts showed reducing power increased with concentration of each sample. The highest reducing power was achieved with fruit and leaf methanol extracts. The reducing power assay correlated with the results obtained by the DPPH assay that the fruit extracts showed higher scavenging and reducing power activities than leaf extracts. Both fruit and leaf methanol extracts showed the highest scavenging and reducing

power capacities. The correlation between the DPPH radical scavenging activity and the reducing power activity shown by the extracts is due to the presence of gallic acid which has been detected in thin layer chromatography and HPLC. Sakagami and Satoh (1997) reported that gallic acid increases reducing power with its concentration.

Metal chelating activity is significant as it reduces the concentration of the catalysing transition metal in lipid peroxidation through the Fenton reaction (Hseu et al. 2008). In the present assay, all the crude extracts exhibited metal chelating activities lower than 50% with

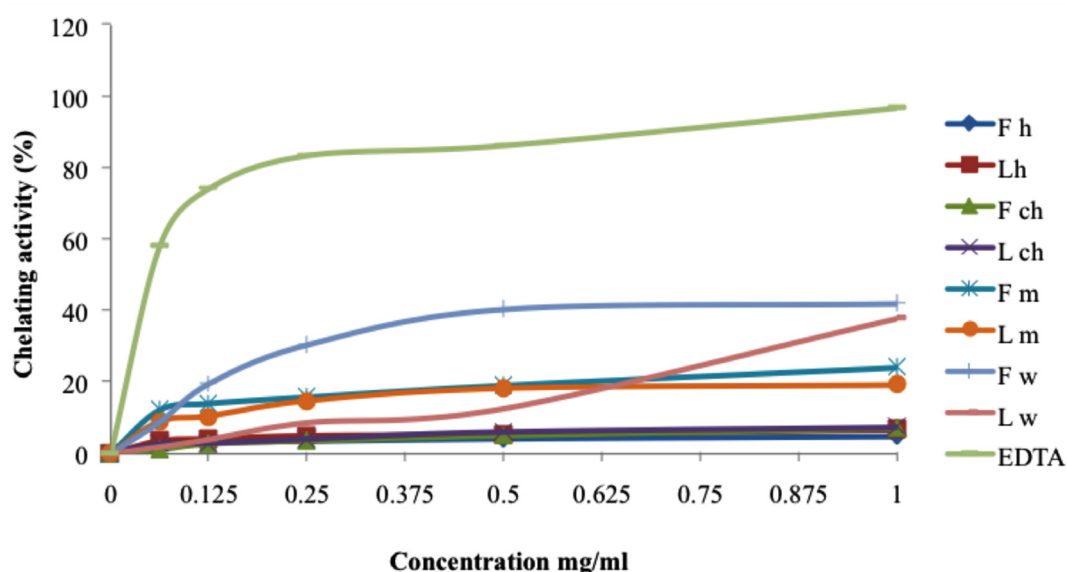


FIGURE 6. Metal chelating activity of crude extracts from *Ardisia crispa*, F h = fruit hexane extract, L h = leaf hexane extract, F ch = fruit chloroform extract, L ch = leaf chloroform extract, F m = fruit methanol extract, L m = leaf methanol extract, F w = fruit water extract, L w = leaf water extract, EDTA.

TABLE 1. Total phenolic and flavonoid content of crude extracts from leaves and fruits of *Ardisia crispa*

| Sample extracts | Total phenolic content mg GAE/g | | Total flavonoid content mg QE/g | |
|--------------------|------------------------------------|-----------|------------------------------------|----------------|
| | Fruits | Leaves | Fruit extracts | Leave extracts |
| Hexane extract | 0.95±0.14 | 0.67±0.02 | 1.87±0.01 | 1.61±0.04 |
| Chloroform extract | 2.33±0.02 | 1.78±0.06 | 2.50±0.01 | 2.00±0.03 |
| Methanol extract | 8.14±0.02 | 5.57±0.07 | 6.54±0.02 | 3.12±0.04 |
| Water extract | 1.86±0.03 | 1.34±0.04 | 0.52±0.06 | 0.47±0.07 |

fruit methanol extracts having high activity (Figure 6). The presence of gallic acids in the extracts is the possible explanation for the low antioxidant activity. Duh et al. (2001) also reported that gallic acid shows weak chelating activity towards ferum ions. Many authors have reported that metal chelating potency plays a minor role in the overall antioxidant activities of some polyphenols (Rice-Evans et al. 1996). Andjelkovic et al. (2006) reported that the ability of phenolic compounds to chelate iron was far lower than that of EDTA. The results in Table 1 show that fruit methanol extract showed the highest phenolic content at 8.14±0.02 mgGAE/g followed by the leaf 5.57±0.07 mgGAE/g. Similarly, the highest flavonoid content was in methanol fruit extract at 6.54±0.02 mgGAE/g. This finding is in agreement with several reports showed that the antioxidant capacity of plant materials is well correlated with their phenolic content (Velioglu et al. 1998). Li et al. (2008) have reported a high correlation between the antioxidant capacity and total phenols of methanol extracts from 45 medicinal plants. Yun et al. (2009) have also shown that phenolic and flavonoid content correlated with antioxidant activity. The flavonoids and related polyphenols have been found to contribute significantly to the total antioxidant capacity of many fruits and vegetables (Dastmalchi et al. 2008). This indicated that the high antioxidant activity of the fruit methanol extract is also due to the presence of high phenolic and flavonoid contents.

CONCLUSION

Overall, the methanol extracts of *Ardisia crispa* fruit revealed the highest DPPH radical scavenging, reducing power and metal chelating activity. Thus, the present study suggested that the fruit extract of *Ardisia crispa* is a useful nutrition for the food industry. The gallic acid could be the active compounds responsible for the high antioxidant activity in the fruit extracts. Further studies should be carried out to investigate in detail the presence of other chemical compounds that might involve in the contribution of the antioxidant properties of *Ardisia crispa*.

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