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SCREENING OF PHYTOCHEMICALS AND ANTIOXIDANT ACTIVITY OF *ARUM DIOSCORIDIS* SEEDS

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ABSTRACT

The aim of this paper was to evaluate the antioxidant properties of the different extracts from *Arum dioscoridis* seeds (methanolic, acetone and hexane extracts) and to correlate their antioxidant potential to the composition of phenolic compounds. The scavenging ability of free radicals was measured using β-carotene–linoleic acid model system and 2,2-diphenyl-1-picrylhydrazyl (DPPH·) analysis and the IC_{50} values of the extracts were also determined. The methanolic extract of *A. dioscoridis* seeds showed greater antioxidant activity than acetone and hexane extracts by β-carotene–linoleic acid model system and DPPH· analysis, respectively. Qualitative and quantitative analyses of major phenolic compounds were also performed. The main antioxidant compound from the methanol extract was found to be vitexin. Other identified phenolics in all extracts that are likely to contribute to the antioxidant potential are: ferulic acid, naringin, eriodictyol and p-coumaric acid.

PRACTICAL APPLICATIONS

Antioxidant properties of the different extracts from *Arum dioscoridis* seeds (methanolic, acetone and hexane extracts) were evaluated in this study. The scavenging ability of free radicals was measured using the β-carotene–linoleic acid model system and 2,2-diphenyl-1-picrylhydrazyl (DPPH·) analysis, and the IC_{50} values of the extracts were also determined. The methanolic extract of *A. dioscoridis* seeds showed greater antioxidant activity than acetone and hexane extracts, respectively. The main antioxidant compound from the methanol extract was found to be vitexin. Other identified phenolics such as ferulic acid, naringin, eriodictyol and p-coumaric acid in all extracts are likely to contribute to the antioxidant potential.

INTRODUCTION

Reactive oxygen species (ROS) are generated from normal function of tissue in biological system, which is the main reason of the oxidative stress. In the excess of ROS such as superoxide, hydrogen peroxide, hydroxyl and nitrogen radical, they can exert harmful compounds associated with many diseases including atherosclerosis, diabetes mellitus, immunodeficiency diseases and aging (Wada and Ou 2002; Fritz *et al*. 2003).

In recent years, the interest and the usage of antioxidants have increased for human diseases linking oxidative stress (Vaya and Aviram 2001). Therefore, most of the regulatory guidelines and pharmacopoeias suggest chemical analysis of botanical materials including their fractions and extracts for quality control and standardization. High performance liquid chromatography is routinely used as one of the valuable tools for qualitative and quantitative analysis of the plant extracts and antioxidant compounds (Proestos *et al*. 2006; Akinmoladun *et al*. 2007; Teow *et al*. 2007). Many medicinal plants contain a large amount of phenolic compounds that show strong antioxidant activity and reduce the oxidative stress by eliminating potential free radicals (Halliwell and Gutteridge 1989; Chu *et al*. 2000; Katalinić *et al*. 2004; Pourmorad *et al*. 2006).

Many Turkish medicinal plants are considered as potential sources of the antioxidant compounds (Ahmed *et al*. 1993; Sener 1994; Sokmen *et al*. 1999; Tuzlaci and Erol 2000). One of the species of the Araceae family, *Arum dioscoridis* is grown in Turkey. It is widely used as a traditional plant in Asia and

Europe for many years. However, there are only a few studies on *A. dioscoridis*. Ido Izhaki (1998) determined amino acid and protein content of *A. dioscoridis* grown in East Mediterranean. Janakat and Al-Thnaibat (2008) studied on antilipoperoxidative activity of the methanolic extract of *A. dioscoridis* leaves against lipid peroxidation induced by a rat liver homogenate model. They studied lipid peroxidation of their leaves on rat and have reported that *A. dioscoridis* may be used in food processing because of their high antioxidant capacity leading to antilipoperoxidative effect (Williams *et al*. 1999; Jaim and Kulkarni 1999; Hung *et al*. 2004). The objectives of our research were: (1) to compare the antioxidant activity of different extract of *A. dioscoridis* grown in Turkey using 2,2-diphenyl-1-picrylhydrazyl (DPPH·) and β-carotene–linoleic acid assay; (2) to identify the phytochemicals of *A. dioscoridis* using high-performance liquid chromatography (HPLC); and (3) to find out the proper solvent to extract antioxidants of *A. dioscoridis* as antioxidant activity could be affected by the extracting solvents (Sun *et al*. 2007).

MATERIAL AND METHOD

Chemicals

Rutin, quercetin, butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), sodium carbonate, aluminum chloride, β-carotene, Tween 20, linoleic acid, sodium acetate, chloroform, methanol, hexane and acetone were supplied by Sigma-Aldrich, St. Louis, MO. All chemicals and reagents were of analytical grade. Double distilled water was used in the experiments.

Plant Material and Extraction

A. dioscoridis was grown in Antalya in the South of Turkey. Plant was collected in June 2008 and seeds of the plant were separated carefully. The plant collected was identified at Akdeniz University, Department of Biology, Antalya, Turkey. The seeds were washed in distilled water five times until the water was clear and then wiped dry with absorbent paper. Cleaned seeds were dried in the sun and kept in tightened light-protected containers in cold. Dried seeds (50 g) were powdered and extracted with methanol, acetone and hexane for 6 h at 30C using an orbital shaker (Gulcin 2005). After filtration through a filter paper (Whatman No.1), the solvent was evaporated under vacuum to 10 mL and then dried at –50C by a lyophilizer. Yields of the extracts of methanol, acetone and hexane were 17, 8 and 6 (w/w %), respectively. Extracts were kept at 4C for 1 week. All experiments were done within 1 week after extraction.

DPPH Radical Scavenging Method

The scavenging activity was estimated by the method described by Sanchez-moreno *et al*. (1998). According to the method, 0.5 mL of various concentrations of the extracts (0.1–0.5 mg/mL) were added to 3 mL of DPPH· (2,2-diphenyl-2-picrylhydrazyl) solution in methanol. After 30 min of incubation time at room temperature in darkness, the absorbance was recorded at 517 nm. DPPH· (I%) was calculated as follows:

$$I\% = \left[\left(A_{\text{blank}} - A_{\text{sample}}\right)/A_{\text{blank}}\right] \times 100$$

where A_{blank} was the absorbance of the control reaction and A_{sample} is the absorbance of the extracts or standards.

IC_{50} values of each extract and standard were determined from the plot of scavenging activity against the extract concentrations, which were defined as the total antioxidant necessary to decrease the initial DPPH· radical concentration by 50%. Experiments were carried out in triplicate, and BHT and BHA were used as standard antioxidants.

β-Carotene–Linoleic Acid Assay

In β-carotene–linoleic acid assay, the antioxidant capacity was determined by measuring the inhibition of the volatile organic compounds and the conjugated diene hydroperoxides arising from linoleic acid oxidation (Kartal *et al*. 2007).

The antioxidant activity of *A. dioscoridis* extracts was determined according to the method of Velioglu *et al*. (1998). For this purpose, a stock solution of β-carotene–linoleic acid mixture was prepared as follows: 0.2 mg of β-carotene in chloroform was added to the mixture of 200 mg of Tween 20 and 0.02 mL of linoleic acid. After the chloroform was evaporated using a vacuum evaporator (Heidolph, Kelheim, Germany), 100 mL of distilled water saturated with oxygen was added vigorously. Approximately 0.2 mL of this reaction mixture was transferred to the test tubes including 5 mL of the extracts (2 mg/mL), and the emulsion system was incubated at 40C. The same procedure was repeated with synthetic antioxidants, BHT and BHA, as positive control, and a blank. Absorbance was measured at 470 nm during 120 min. The antioxidant capacity of the extracts was compared with those of BHT, BHA and blank. Degradation rate (DR) was calculated using the following equation based on Al-saikhan *et al*. (1995):

$$DR = \ln(a/b)$$

where a is the absorbance at initial time, and b is the absorbance at 120 min. Antioxidant activity (AA) was expressed as percent of inhibition relative to the control, using the following equation:

$$AA = \left[\left(DR_{\text{control}} - DR_{\text{sample}}\right)/DR_{\text{control}}\right] \times 100$$

where DR_{control} is degradation rate of control, DR_{sample} is degradation rate of the extract or the standard antioxidant. The samples were run in triplicate.

Total Phenolic Content

The amount of total phenolic content was measured by the method described by Singleton and Rossi (1965). In this method, an aliquot of diluted extracts and standard solutions of gallic acid at different concentrations between 0.01 and 0.5 mg/mL were added to a 25-mL volumetric flask containing 9 mL of ddH_2O. Approximately 2.5 mL of 10% (v/v) Folinand Ciocalteu's phenol reagent and 7.5 mL of 20% (w/v) Na_2CO_3 were added to the mixture and shaken vigorously. After incubation of 120 min at room temperature, the absorbance was recorded at 750 nm. Total phenolic contents of *A. dioscoridis* extracts were expressed as milligram gallic acid equivalents per gram (mg GAE/g) dry extract. Total phenolic content was calculated using the following equation:

$$\text{Absorbance} = 5.343x + 0.047 \quad (r = 0.998)$$

All tests were carried out in triplicate.

Total Flavonoid Content

Total flavonoid content was determined based on the formation of flavonoid–aluminum complex by a spectrophotometric assay described by Ebrahimzadeh *et al.* 2008. One milliliter of extract (0.1–1 mg/mL) was mixed with 0.1 mL of 1 M sodium acetate and 0.1 mL of 10% (w/v) aluminum chloride solution. After incubation of 30 min at room temperature, the absorbance of the aluminum complex was measured at 415 nm. Total flavonoid content of the extract was expressed as milligram equivalents to quercetin per gram (mg QE/g) dry weight of the extract. The total flavonoid content was calculated using the following equation:

$$\text{Absorbance} = 6.299x + 0.088 \quad (r = 0.995)$$

All tests were carried out in triplicate.

Phytochemical Analysis

A Shimadzu 1100 series HPLC (Shimadzu Corporation, Kyoto, Japan) equipped with a SIL-10AD vp autosampler (Shimadzu Corporation) and LC-10Advp pump system (Shimadzu Corporation), diode array detector and an Inertsil Agilent Eclipse XDB column (240 mm × 4.60 mm, 5 μm particle size) (Agilent Technologies, Palo Alto, CA) was used to analyze several phenolic acid and flavonoids. The method described by Maltas *et al.* 2010 was used for HPLC analysis. The mobile phase composition was the following: (A) 100% methanol; (B) 3% (v/v) aqueous acetic acid. Samples were analyzed using the linear gradient flow program from 5% to 66.5% solvent A over 75 min at 0.8 mL/min and absorbance was detected at 278 nm of the standard and the extract solutions was injected. Amounts of each antioxidant compounds were calculated on the basis of the calibration curve for each one. These were gallic acid, catechin hydrate, caffeic acid, epicatechin, p-coumaric acid, ferulic acid, vitexin, rutin, naringin, hesperidin, rosmarinic acid, eriodictyol, quercetin and naringenin as standards provided from Sigma Aldrich (St. Louis, MO). Carvacrol was also analyzed in this method. Samples were filtered through a 0.22-μm syringe filter and run in triplicate.

Statistical Analysis

The statistical analysis was carried out by using OriginPro 7.5 software (OriginLab Corp., Northampton, MA). One-way ANOVA was applied to data and results were compared by using Tukey test. A difference was considered to be statistically significant when the P value is lower than 0.05 ($P < 0.05$).

RESULTS AND DISCUSSION

Antioxidant Activity

DPPH· is a stable free radical that accepts an electron or a hydrogen radical to become a stable molecule. DPPH· radical scavenging method, one of the methods widely used in determining antioxidant activity, is an easy, rapid and sensitive way to survey the antioxidant activity of phenolic compound or the plant extracts (Koleva *et al.* 2002). Many plant extracts exhibit efficient antioxidant properties due to their phytoconstituents, including phenolic acids and flavonoids (Chu *et al.* 2000; Pourmorad *et al.* 2006). In the present study, reduction of DPPH· radicals can be significantly observed at 517 nm by the extracts of *A. dioscoridis* (Fig. 1). Measured by DPPH· method (Table 1), the free radical scavenging activity of the methanolic extract of the plant with an IC_{50} value of 0.50 ± 0.19 mg/mL was significantly higher than those of the acetone and the hexane extracts ($P < 0.05$). IC_{50} values of the standards, BHT and BHA, were lower than those of all extracts. The methanolic extract with 81.2 ± 1.7% exhibited the highest scavenging activity in all extracts at the same concentration.

In β-carotene–linoleic acid system, free radical form of linoleic acid attacks β-carotene. The antioxidant compounds in the plant extract prevent oxidation of β-carotene inhibiting the linoleate radicals in the system. In this system, oxidation of linoleic acid was effectively inhibited by different extractsof *A. dioscoridis* (Fig. 2). Antioxidant activity of the extracts and standards measured by degradation rate (*DR*) of β-carotene in the presence and absence of the extract and standards were also given in Table 1. The methanolic extract of *Arum* showed high antioxidant activity with 78.3 ± 1.7% by inhibiting the formation of conjugated dienes, followed by the acetone extract with 52.8 ± 1.4% and the hexane extract with

Extracts	DPPH· scavenging activity† (%)	IC_{50}‡ (mg/mL)	*AA* (β-carotene system)§ (%)
Hexane extract	22.7 ± 0.9^a	3.03 ± 0.5^b	41.5 ± 1.0^a
Acetone extract	27.4 ± 1.4^b	2.85 ± 0.21^b	52.8 ± 1.4^a
Methanol extract	88.2 ± 1.7^c	0.50 ± 0.19^c	78.3 ± 1.7^c
BHT	91.5 ± 0.6^d	0.020 ± 0.001^d	84.2 ± 1.1^e
BHA	88.8 ± 1.0^e	0.035 ± 0.007^d	81.4 ± 1.7^e

Data are expressed as mean ± standard deviation (n = 3). Means within each column with different letters (*a*–*e*) differ significantly ($P < 0.05$).

† DPPH· scavering activity (%) at 1 mg/mL concentration of mg dry weight.

‡ Antioxidant activity by β-caroten system (%) at concentration of 2 mg/mL.

§ Extract concentration inhibited 50% of DPPH·.

AA, antioxidant activity; BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; DPPH·, 2,2-diphenyl-1-picrylhydrazyl.

TABLE 1. FREE RADICAL SCAVENGING ACTIVITY MEASURED BY DPPH· ASSAY AND ANTIOXIDANT ACTIVITY BY B-CAROTENE–LINOLEIC ACID ASSAY AND IC_{50} OF *ARUM DIOSCORIDIS* EXTRACTS AND STANDARDS

41.5 ± 1.0%. The highest inhibition was provided by BHT and BHA, followed by *A. dioscoridis* extracts at the same extract and standard concentration. It can be concluded that the methanolic extract of *A. dioscoridis* had weaker activity than those of standards, BHA with 81.4 ± 1.7% and BHT with 84.2 ± 1.1% ($P < 0.05$). However, the presence of polar phenolics in the methanolic extract might promote the inhibiting effect on linoleic acid oxidation.

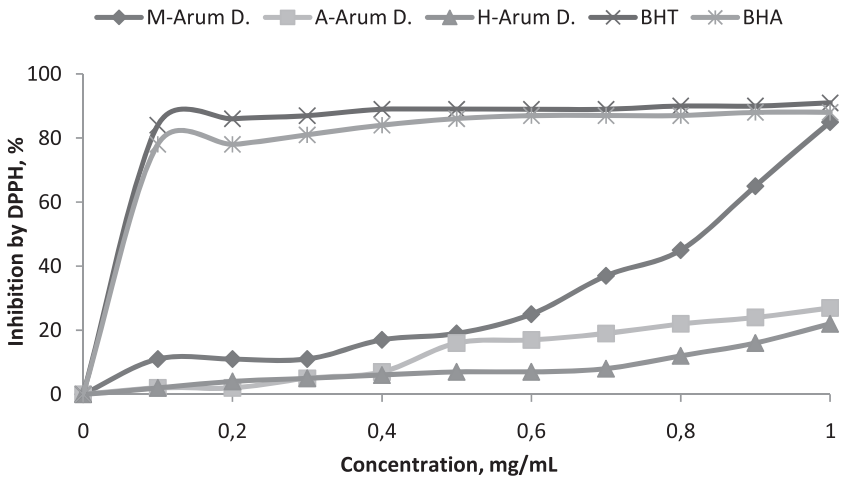

FIG. 1. INHIBITION (%) BY DPPH· AGAINST INCREASING CONCENTRATION OF THREE EXTRACTS OF *ARUM DIOSCORIDIS*

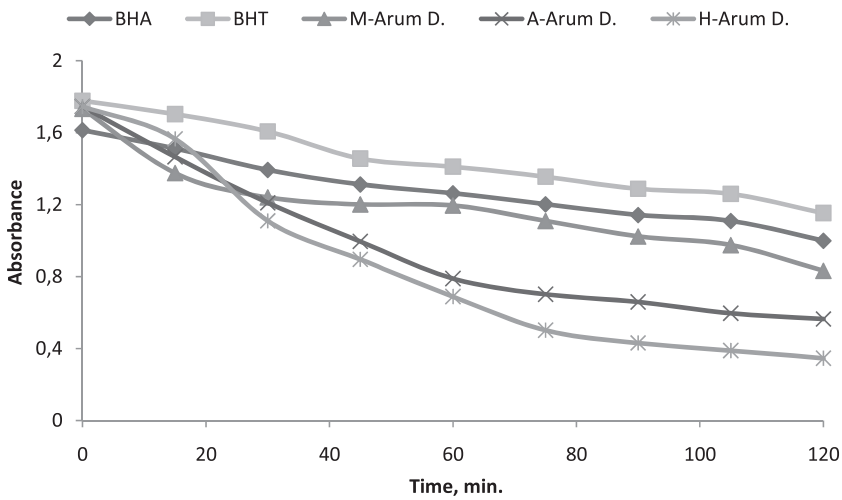

FIG. 2. ABSORBANCE CHANGE OF β-CAROTENE AT 490 nm IN THE PRESENCE OF THREE EXTRACTS OF *ARUM DIOSCORIDIS*

TABLE 2. TOTAL PHENOLIC AND FLAVONOID CONTENTS OF THREE EXTRACTS FROM *ARUM DIOSCORIDIS*

Extracts	Total phenolics† (mg GAE/g)	Total flavonoids‡ (mg QE/g)
Hexane extract	33.5 ± 0.6[a]	19.5 ± 0.2[a]
Acetone extract	40.1 ± 1.2[b]	21.5 ± 0.8[b]
Methanol extract	56.8 ± 1.8[a]	45.6 ± 1.4[b]

Data are expressed as mean ± standard deviation ($n = 3$). Means within each column with different letters (*a*, *b*) differ significantly ($P < 0.05$).

† Total phenolic content was expressed as gallic acid equivalents (GAE).

‡ Total flavonoid content was expressed as quercetin equivalents (QE).

The total phenolic and flavonoid contents of the *A. dioscoridis* extracts were expressed as gallic acid and quercetin equivalent per dry extract, respectively. The amount of total phenolic and flavonoid contents of the methanolic, acetone and hexane extracts of *A. dioscoridis* was presented in Table 2. The methanolic extract of *A. dioscoridis* showed higher phenolic and flavonoid contents compared with the acetone and hexane extracts. These observations clearly indicated a close linkage between phenolic compounds and antioxidant activity due to their phytochemical diversity related to the solvent.

Phytochemical Analysis

Typical phenolics that possess antioxidant activity have been characterized as phenolic acids and flavonoids. Phenolic acids have repeatedly been implicated as natural antioxidants in fruits, vegetables and other plants. For example, caffeic acid, ferulic acid and vanillic acid are widely distributed in the plants (Al-Saikhan *et al*. 1995; Yu *et al*. 2005; Hajdú *et al*., 2009; Wu *et al*. 2009; Kumazawa *et al*. 2010). This is the first time several selected phenolic acids and flavonoids of the *A. dioscoridis* extracts have been eluted and identified by

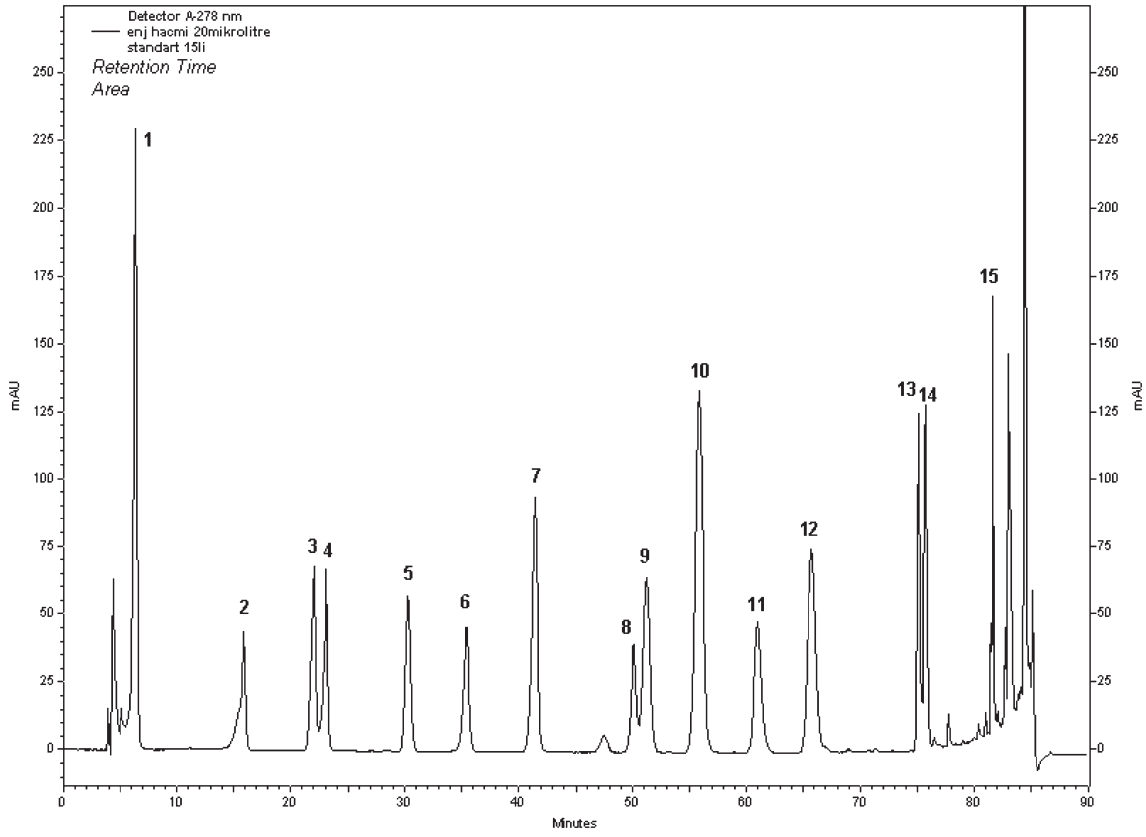

FIG. 3. STANDARD HPLC CHROMATOGRAM OF 15 ANTIOXIDANTS, 1: GALLIC ACID, 2: CATECHIN HYDRATE, 3: CAFFEIC ACID, 4: EPICATECHIN, 5: P-COUMARIC ACID, 6: FERULIC ACID, 7: VITEXIN, 8: RUTIN, 9: NARINGIN, 10: HESPERIDIN, 11: ROSMARINIC ACID, 12: ERIODICTYOL, 13: QUERCETIN, 14: NARINGENIN, 15: CARVACROL

TABLE 3. CONTENT OF SEVERAL PHENOLIC ACIDS AND FLAVONOID COMPOUNDS FROM *ARUM DIOSCORIDIS* EXTRACTS (μg/g)

Compound	Methanolic extract	Acetone extract	Hexane extract
Gallic acid	–	–	–
Catechin hydrate	–	–	–
Caffeic acid	–	–	–
Epicatechin	–	–	–
p-coumaric acid	5.4 ± 0.8	5.6 ± 0.4	–
Ferulic acid	325.5 ± 2.5	255.9 ± 1.9	
Vitexin	1,125.0 ± 13.4	–	23.4 ± 2.4
Rutin	–	–	–
Naringin	98.4 ± 5.5	19.4 ± 3.2	–
Hesperidin	–	–	–
Rosmarinic acid	–	–	–
Eriodictyol	43.7 ± 3.1	25.0 ± 4.2	5.2 ± 0.7
Quercetin	–	–	–
Naringenin	–	–	–
Carvacrol	–	–	–
Σ Total	1,598.0	305.9	34.2

Data are expressed as mean ± standard deviation ($n = 3$, $P < 0.05$).

comparison with authentic standards using reversed-phase HPLC (Fig. 3). The main compounds of the *A. dioscoridis* extracts were predominantly found to be as vitexin and ferulic acid. Sum of them in the methanolic, acetone and hexane extracts were 1,598.0 μg/g, 305.9 μg/g and 34.2 μg/g, respectively. Hovewer, several compounds were not observed in any extract of *A. dioscoridis* (Table 3). As a result of chromatographic analysis of the phytochemicals, naringin and eriodictyol also contributed to the antioxidant activity of *A. dioscoridis* extracts. Many studies on health benefits have been linked to the effective phenolic acids and flavonoid components in most of medicinal plants (Yang *et al*. 2001). The pharmaceutical effects can be attributed to strong antioxidative and free radical scavenging activities of flavonoids that have been reported to have clinically relevant functions including antihypertensive, anti-inflammatory, antihemorrhagic activity, and strengthen of the capillary permeability and stabilization of platelets (Yang *et al*. 2001; Guo and Wei 2008). They are known to inhibit free radical processes in cells (Afanas *et al*. 1989). Therefore, the potent activity of these compounds isolated from *A. dioscoridis*, which has been attributed to the most active phenolic fractions, has been reported for the first time in this study.

CONCLUSION

Our results are to show total phenolic and flavonoid contents and the antioxidant activity of *A. dioscoridis* extracts, and to search relationship between antioxidant activity and phenolic content. Results indicated that the antioxidant activity of *A. dioscoridis* can be explained by its higher content of antioxidant phenolic compounds. By HPLC analysis, vitexin, ferulic acid, naringin and eriodictyol appeared to be responsible for the antioxidant activity of *A. dioscoridis*, together with other unidentified compounds. Also data showed that the methanolic extract of *A. dioscoridis* exhibited stronger antioxidant activity related to its polar phenolic constituents such as vitexin and ferulic acid. As conclusion, *A. dioscoridis* is a new naturally potential antioxidant source.

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