Chemical composition and antioxidant activity of phenolic compounds from *Dioscorea* (Yam) leaves

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Abstract: This study was aimed to assess the potential of *Dioscorea* (yam) leaves as a source of antioxidants. Microwave-assisted extraction (MAE) process was used to prepare the extracts. The phenolic compounds in *Dioscorea* leaves extracts were analyzed by HPLC-DAD-ESI-MS/MS method and the contents of major compounds were determined. Results indicated that a total of 17 phenolic compounds were separated identified by means of UV and mass spectra compared with authentic reference substances and/or reported values in the literature. The main phenolic compound was rosmarinic acid and its highest amount was found in *Dioscorea glabra Roxb.* leaves (22.31±1.33 mg/g DW). Rutin was the dominant flavonoid followed by quercetin which highest amount was found in *Dioscorea alata* leaves (8.66±0.29 mg/g DW). Antioxidant activity of the extracts was estimated by the use of DPPH and ABTS assays. Both kinds of leaves exhibited satisfied antioxidant capacity which was correlated with phenolic contents. In the cytoprotective effect on HUVECs viability assay, *Dioscorea glabra Roxb.* leaves extract was found to be more active than that of *Dioscorea alata* against H_2O_2-induced oxidative stress. Our findings support the promising role of *Dioscorea* leaves that can be used as an interesting source of phenolic antioxidants.

Keywords: Microwave-assisted extraction, HPLC-DAD-ESI-MS/MS, phenolic compounds, antioxidant activity, cytoprotective

INTRODUCTION

Phytochemicals such as phenolic compounds are major bioactive compounds known to be generally antioxidants and beneficial against many chronic diseases such as inflammation, cardiovascular, cancer, and neurodegenerative disorders. Although their precise mechanism of action is largely unknown, increasing health consciousness has attracted the attention of scientists towards phenolics widely found in vegetables, fruits, leaves, nuts, and seeds (Veitch and Grayer, 2011; Dahmoune *et al.*, 2015). Because of the complexity of structures and chemical characteristics of phenolic compounds mostly represented by phenolic acids and flavonoids occur in free and bound forms, it is still an analytical challenge to identify these compounds (Stalikas, 2007). With the development of separation protocols, high-performance liquid chromatography coupled with diode array detection (HPLC-DAD) represents the most widely used analytical technique for the characterization of phenolic compounds (Mertz *et al.*, 2009; Andrade *et al.*, 2010; Vichapong *et al.*, 2010). The use of HPLC-DAD coupled to mass spectrometry (MS) and newer methodologies based on tandem mass spectrometry experiments (MS/MS) have been gained attention due to the capacity of characterizing structurally similar compounds in complex matrices (Bataglion *et al.*, 2015).

Yams, belonging to the *Dioscorea* genus, are cultivated throughout the subtropical and tropical regions of the world. It is not only a common food in China, but also has been frequently used to cure diabetes, diarrhea, cough, spermatorrhea, leukorrhea and frequency of urination and arthritis (Zhong *et al.*, 2002; Ju *et al.*, 2014). *Dioscorea* leaves, the main byproduct of yam, are usually burned or discarded as waste, resulting in resource loss and environmental pollution. Recently, phenolics have been broadly regarded as great antioxidants and proved to show higher activity than common antioxidants including vitamins C, E and the carotenoids (Dai and Mumper, 2010). However, little is known about antioxidant action and chemical compositions of *Dioscorea* leaves of *Dioscorea alata* and *Dioscorea glabra Roxb.* (known as purple and red yam) are common two varieties of yam. Therefore, the objective of this study was to analyze the chemical composition of *Dioscorea alata* and *Dioscorea glabra Roxb.* leaves and to evaluate their antioxidant activity and cytoprotective properties against oxidative stress.

MATERIALS AND METHODS

Plant materials and extraction

Two species of the *Dioscorea* genus (*Dioscorea alata* L. and *Dioscorea glabra* Roxb.) leaves were collected during the autumn season from a local farm in Jiangsu Province, China and authenticated by Xuzhou Sweetpotato Research Institute, Chinese Academy of Agricultural Sciences. The fresh leaves were washed

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thoroughly with running water and drain overnight at room temperature (25^{o}C), then lyophilized in a ModulyoD-230 vacuum freeze drier (Thermo Savant, USA) and ground to a fine powder using an electric grinder. The ground powders with particle size under 40 meshes were collected and stored at -20^{o}C in airtight bags until further use.

Phenolic compounds in *Dioscorea* leaves were extracted using an automated microwave synthesizer (Initiator EXP, Sweden). In a 10 ml glass tube (Biotage Pressurized Reaction Vials), 0.1 g of *Dioscorea* leaves powder was dispersed in 4.0 ml 80% ethanol. After sealing the vessel by a cap, it was put into the microwave cavity and the temperature was set at 100^{o}C for 2 minutes. After cooled to room temperature online, the extract was filtered through the 0.22 µm PVDF and stored at 4^{o}C for future use. Extracts for cell experiment were prepared with 1.0 g leaves powder, then condensed and freeze-dried.

HPLC-DAD-ESI-MS/MS analysis

Phenolic compounds in the *Dioscorea alata* and *Dioscorea glabra Roxb.* leaves extracts were identified using a HPLC-MS system consisting of a Surveyor MS pump, an electrospray ionization source (ESI) and 6460 triple quad mass spectrometer (Agilent Technologies, Palo Alto, CA, USA), with Chem Station Agilent Technologies Revision B.04.00 software for data acquisition and analysis. The HPLC series 1290 equipped with a binary pump, autosampler and diode array detection (DAD) was used. Mass spectra were acquired using a Turbo Ionspray source in both negative and positive mode (NI and PI) with the operating parameters: ion spray voltage, 3.5 kV; auxiliary gas (N_2), 20 psi; tube lens voltage 135 V; desolvation gas temperature, 350^{o}C; sheath gas (N_2) flow 11 l/min. Compounds were detected by full-scan mass analysis from *m/z* 100 to 1000. The MS/MS fragment-targeted experiments were performed using a collision energy ranging from 5 to 20 eV.

Total phenolic content (TPC)

Total phenolic content of the *Dioscorea* leaves extract was determined by Folin-Ciocalteu colorimetry (Mocan *et al.*, 2014). 100 µl from each ethanol extract was added to 2 ml of 80% ethanol and 1.0 ml of 1 mol/l Folin-Ciocalteu reagent and diluted to 10.0 ml with saturated sodium carbonate solution (290 g/l). The absorbance was read at 765 nm with a T6 UV-Visible spectrophotometer (Persee, China) after incubation for 30 min at room temperature and compared with a gallic acid calibration curve. TPC of the extract was expressed as mg gallic acid equivalent/g dried weight plant materials (mg GAE /g DW).

Total flavonoid content (TFC)

Total flavonoid content of the extract was measured by the $NaNO_2$-$AlCl_3$-NaOH system with rutin as a standard (Andrade *et al.*, 2014). A 0.2 ml aliquot of ethanol extract was mixed with 0.2 ml of 15% $NaNO_2$. After 6 min, 0.2 ml of 10% $AlCl_3$ was added and diluted with 1 M NaOH to 10.0 ml. The resulting mixture was thoroughly mixed and allowed to stand for another 15 min and the absorbance was read at 515 nm. Total flavonoid content was expressed as mg rutin equivalent/g dried weight plant materials (mg RE /g DW).

Quantification of rosmarinic acid, rutin and quercetin

Quantitative analysis of the three major compounds was carried out by Agilent 1260 HPLC series equipped with a diode array detector (DAD). A reversed phase C18 column (Hypersil ODS-2; 250 mm×4.0 mm, 5 µm; Thermo Scientific) was used with 0.15% acetic acid (solvent A) and methanol (solvent B) as mobile phase at a flow rate of 1.0 ml/min at 30^{o}C. The gradient was varied linearly 15-25% B in 10 min, 25-35% B at 25 min, 35-65% at 40 min and held at 65% to 45 min, followed by reconditioning the column. Detecting wave-lengths were set at 320 nm for phenolic acids and 360 nm for flavonoids. Calibration curves for rosmarinic acid (RA), rutin (RT) and quercetin (QT) were constructed for each standard by plotting the concentration of compounds versus peak area response using the least-squares linear regression method and coefficient of determination. The curves showed good linearity for RA at 320 nm (R^2=0.9982), and for rutin and quercetin at 360 nm (R^2>0.9991), in the range of 5-500 µg/ml.

DPPH radical scavenging assay

Antioxidant activity of the samples was assessed by the stable 2, 2-diphenyl-1-(DPPH) radical according to the method outlined by Geresh *et al.* (2002) with some modifications. A 50 µl aliquot of appreciated diluted extract were added to 100 µl DPPH (0.3 mM). The mixture was left for 30 min in the dark after gently shaking, the absorbance was then measured at 517 nm using a microplate reader (Epoch, Bio Tek Instruments, USA). The percentage of DPPH consumption was converted to ascorbic acid (V_C) equivalent (mg V_CE/g dried plant material) using a calibration curve (R^2=0.9997) with ascorbic acid standard solutions of 0-50 µg/ml.

ABTS radical scavenging assay

ABTS radical scavenging ability of the samples was assessed using commercial assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the instructions. In brief, 10 µl of appreciated diluted extract were mixed with 150 µl ABTS working solution. The reaction mixture was incubated at ambient temperature for 20 min. The scavenging acitivity on the ABTS radical was determined by measuring the absorbance at 734 nm and expressed as µM trolox equivalent per gram dry weight leaves.

Cell culture and treatments

Human umbilical vein endothelial cells (HUVECs) were

cultured in RPMI medium 1640, supplemented with 10% fetal bovine serum (FBS). Cells were maintained at 37°C in a humidified atmosphere containing 5%CO_2 with media replenished every two days. The stock solution *of Dioscorea* leaves extract was prepared in DMSO and diluted with RPMI medium 1640 prior to the experiment (The final concentration of DMSO is less than 0.1%). Cells were incubated with increasing concentrations of extracts (0-200 μg/ml) from leaves of *Dioscorea glabra Roxb.* and *Dioscorea alata* for 24 h, and then cell viability was determined by MTT assay directly or after exposed for 2 h to 200 μM H_2O_2.

STATISTICAL ANALYSIS

All the experiments were conducted in triplicate. The results are shown as means ± standard deviations and statistically analyzed by one-way analysis of variance (ANOVA) test using SPSS software package (version 19.0) with $P<0.05$ as threshold for statistical significance.

RESULTS

Identification of phenolic compounds

The LC-ESI-MS (NI) total ion current (TIC) profiles of ethanol extract of *Dioscorea alata* leaves are shown in fig. 1. A total of 17 compounds were found in leaves of *Dioscorea alata*. Among them, five compounds (10,12, 15,16,17) were identified unequivocally by comparing retention times and MS data with those of reference compounds, and 12 other compounds (1-9,11,13-14) were tentatively identified by comparing MS and MS^2 spectra with those reported in the literature. Molecular weights of the compounds were confirmed based on protonated and deprotonated pseudo-molecular ions in the positive and negative ions mode, as well the sodium adduct ions ($[M+23]^+$) (table 1). Furthermore, these pseudo-molecular ions were then subjected to CID energies (5,15,20eV) in negative ion mode ESI-MS/MS to characterize the site of substitution in the aglycones for providing evidence for the proposed structure of phenolic compounds in *Dioscorea alata* leaves.

Compound 1 produced $[M-H]^-$ ion at *m/z* 353 with the maximum UV absorption at 240 nm and 325 nm and gave fragment ions $[QA-H]^-$ at *m/z* 191 and $[CA-H-H_2O]^-$ at *m/z* 161.1 (table 1). Based on the hierarchical key proposed by Gouveia-Figueira & Castilho (2015) and Narváez-Cuenca *et al.* (2012), this compound was identified as 5-*O*-caffeoylquinic acid. Compound 7 exhibited $[M-H]^-$ at *m/z* 367, gave fragment ion $[M+H-H_2O-CH_3OH]^+$ at *m/z* 319, and had a similar UV spectra with 5-*O*-caffeoylquinic acid. Its MS^2 fragment ions at *m/z* 179, 161 and 135, which were identical to diagnostic ions of caffeic acid. Thus, compound 7 could be proposed as 5-*O*-caffeoyl methyl quinate. Compound 5, small peak at 13.303 min, yielded $[M-H]^-$ ion at *m/z* 367, and its maximum absorption peaks and MS^2 specturm were similar to compound 7, hense, compound 5 might be caffeoyl methyl quinate isomer. Compound 3, UV_{max} at 230 and 285 nm with *m/z* 317 $[M-H]^-$ and its MS^2 base fragment ions at *m/z* 191 $[QA-H]^-$ and 163 $[QA-H-H_2O]^-$, was definitively identified as quinic acid derivative.

Compound 4 displayed a $[M-H]^-$ ion at *m/z* 337, and UV spectra showed the maximum absorption peaks at 235 nm and 315 nm. The MS^2 fragmentation gave a fragment ion $[QA-H]^-$ at *m/z* 191 as base peak and [M-H-quinine acyl-CO_2]$^-$ at *m/z* 119. This compound corresponds to 3-*p*-coumaroylquinic acid according to literature (Zhang *et al.*, 2015; Parveen *et al.*, 2011). Compounds 8 and 9 produced $[M-H]^-$ at *m/z* 351 and obtained the UV_{max} at 315 nm like compound 3. Their MS^2 fragmentation generated a base peak ion [M-H-quinine acyl-CO_2]$^-$ at *m/z* 119, two intense ions [M-H-quinine acyl]$^-$ and [M-H-quinine acyl-H_2O]$^-$ at *m/z* 179 and 145, respectively. This fragmentation pattern is consistent with 3-*p*-coumaroylquinic acid. Compounds 8 and 9 were tentatively identified as coumaroylquinic acid methyl ester isomers (Yu *et al.*, 2013).

Based on the UV spectrum (λ_{max} 240 nm and 326 nm, table 1) and its $[M-H]^-$ at *m/z* 179 yielded the fragments $[M-H-H_2O]^-$ 161 and [M-H-CO_2]$^-$ 135, Compound 2 was identified as caffeic acid according to Narváez-Cuenca *et al.* (2012). Compound 6 and 11 gave $[M-H]^-$ ion at *m/z* 335 and 349, respectively, and obtained UV_{max} at 315 nm and 320 nm. Their MS^2 in negative mode showed the base peak ions [M-shikimic acyl-H]$^-$/[M-shikimic acyl-CH_3]$^-$ at *m/z* 179, [M-H-shikimic acyl-H_2O]$^-$/[M-shikimic acyl-CH_3]$^-$ at *m/z* 161, and [M-H-shikimic acyl-CO_2]$^-$/[M-CH_3-shikimic acyl-CO_2]$^-$ at *m/z* 135. Compound 6 also exhibited $[M+H]^+$ at *m/z* 337, gave fragment ion [M+H-caffeoyl-CO]$^+$ at *m/z* 147. After comparing to published data (Parveen *et al.*, 2011), compound 6 and 11 could be 4-*O*-caffeoylshikimic acid and its methyl ester, respectively.

Compound 10 gained $[M-H]^-$ ion at *m/z* 359, and then generated product ion [M-H-caffeoyl]$^-$ at *m/z* 197, and UV spectra gave the maximum absorption peaks at 240 nm and 330 nm. Its MS^2 fragmentations showed $[CA-H]^-$ at *m/z* 179 and [CA-H-CO_2]$^-$ at *m/z* 135. Furthermore, by comparison with authentic standard, compound 10 was confirmed as rosmarinic acid. Compound 14, obtained the UV_{max} at 330 nm like compound 10, was identified as methyl rosmarinate by their ions at *m/z* 373 $[M-H]^-$, *m/z* 178.9 $[CA-H]^-$ and *m/z* 135 [CA-H-CO_2]$^-$ in NI-MS^2 mode and at *m/z* 397 $[M+Na]^+$, *m/z* 269 [M+H-catechol]$^+$ and *m/z* 163 $[CA+H-H_2O]^+$ in PI mode (Table 1).

Compounds 12 and 13 were identified to be disaccharides glycosides by examination of their MS data (table 1). These disaccarides glycosides were identified as quercetin and kaempferol derivatives on the basis of aglycone

Table 1: Identification of 17 constituents of *Dioscorea alata* leaves by HPLC-DAD-ESI-MS/MS in negative modes

Peak no.	Rt (min)	UV_{max} (nm)	$[M-H]^-$	NI-MS^2 ions *m/z* (%)	Adducts and Fragment ions of $[M+H]^+$	Proposed compounds
1	7.946	240, 325	353	191(100), 179(8)	377(39), 319(8), 194(10) 163(37)	5-*O*-caffeoylquinic acid[bc]
2	10.035	240, 322	179	135.1(100)	-	Caffeic acid[c]
3	10.446	230, 285	317	191(12), 163(52), 151(53), 109 (100)	341(38), 301(29), 137(19)	Quinic acid derivative
4	11.401	235, 315	337	191 (100), 119 (18), 93 (49)	361(52), 303(54), 163(26), 147(58)	3-*p*-Coumaroyl quinic acid[de]
5	13.303	245, 325	367	179(50), 160.9(25), 135(100)	391(26), 269(5), 163 (27)	Caffeoyl methyl quinateisomer[c]
6	13.721	220, 325	335	178.9 (50), 161 (27), 135 (100)	359(75),19(10), 163(100), 147(30)	4-*O*-Caffeoylshikimic acid[d]
7	16.392	240, 325	367	178.9(42), 160.9(25), 135(100)	391(100),319(11), 163 (55)	5-*O*-caffeoyl methyl uinate[c]
8	21.503	235, 315	351	163(58), 145(22), 119 (100)	375(100), 263(33), 147(85)	Coumaroyl quinic acid methyl ester isomer[e]
9	22.809	240, 310	351	163.1(52), 145(24), 118.9(100)	375(92), 269(5), 147(100)	Coumaroyl quinic acid methyl ester isomer[e]
11	26.002	245, 325	349	179(52), 160.7(18), 135 (100)	373(17), 298(19), 163(15)	Caffeoylshikimic acid methyl ester
10	25.005	240, 330	359	196.8(10), 179(38), 161(100), 134.9 (28)	383(22), 257(16), 163(44)	Rosmarinic acid[a]
12	26.771	255, 355	609	609 (100), 301 (12), 299.9 (17)	633(95), 465(6), 303(19)	Rutin[a]
13	29.629	235, 340	593	593(100), 284.9(45), 284 (66)	617(40), 454(9), 269(10), 187 (5)	Kaempferol-3-*O*-rut[f]
14	32.718	245, 330	373	178.9(100), 135 (81)	397(42), 269(9), 163 (96)	Methyl rosmarinate
15	33.859	255, 370	301	301(100), 273(8), 178.8(32), 151(88), 121.1 (22)	325(2)	Quercetin[a]
16	37.030	265, 365	285	284.9(100), 229(15), 187(12), 116.9(12)	255(5), 186.9(6), 158(14)	Kaempferol[a]
17	37.941	255, 370	315	314.9(66), 300(100), 151(7)	339(7), 269(8), 187(7)	Isorhamnatin[a]

The peak numbers correspond to those used in fig. 1. Rt: retention time.
[a]Compounds confirmed by comparison with authentic standards.
[b]Identification confirmed by comparison with UV and mass data reported by Gouveia-Figueira & Castilho (2015).
[c]Identification confirmed by comparison with UV and mass data reported by Narváez-Cuenca *et al*. (2012).
[d]Identification confirmed by comparison with UV and mass data reported by Zhang *et al*. (2015).
[e]Identification confirmed by comparison with UV and mass data by Parveen *et al*. (2011).
[f]Identification confirmed by comparison with UV and mass data reported by Stephen *et al*. (2010).

fragment ions $[M-308-2H]^-$ at *m/z* 299.9, 283.9 in NI-MS^2 and fragment ions at *m/z* 447 or 463 were not detected confirmed that these glycosyls were connected to the same phenolic hydroxyl (Zhang *et al.*, 2015; Cuyckens & Claeys, 2004). Furthermore, a high intensity MS^2 peak at *m/z* 299.9, 283.9 suggested that compounds 12 and 13 are quercetin-3-*O*-rutinaside and kaempferol-3-*O*-rutinaside instead of quercetin-7-*O*-rutinaside and kaempferol-7-*O*-rutinaside (Stephen *et al.*, 2010; Neugart *et al.*, 2015), which was confirmed by co-elution with rutin standard. Compounds 15, 16, and 17 were unambiguously identified as quercetin, kaempferol, and isorhamnetin, respectively. For these compounds, comparison of the retention time, UV data, and MS fragments was done against the authentic references.

Determination of total phenolic, flavonoid and major compounds content

As shown in table 2, the leaves of *Dioscorea glabra Roxb.* contain a considerable amount of phenolic compounds

Table 2: The content of phenolic compounds and antioxidant capacity of *Dioscorea alata* and *Dioscorea glabra Roxb.* leaves

Samples	TPC mg GAE/g DW	TFC mg RE/g DW	RA mg/g DW	RT mg/g DW	QT mg/g DW	DPPH mg VCE/g DW	ABTS μM Trolox/g DW
Dioscorea alata leaves	74.54±2.42	23.37±1.64	1.96±0.14	11.86±0.36	8.66±0.29	63.41±3.65	2292.98±75.41
Dioscorea glabra Roxb. leaves	85.58±2.51	31.95±1.38	22.31±1.33	18.05±2.03	5.17±0.72	134.71±5.20	2587.26±61.19

RA: rosmarinic acid; RT: rutin; QT: quercetin; DW: dry weight plant material.

(85.58±2.51 mg GAE/g DW) and flavonoids (31.95±1.38 mg RE/g DW). The *Dioscorea alata* leaves contain slightly lower amounts of phenolics (74.54±2.42 mg GAE/g DW) and flavonoids (23.37±1.64 mg RE/g DW). In contrast, *Dioscorea alata* roots have a significantly lower amount of phenolic compounds (4.78±0.12 mg GAE/g dried DW) according to the study by Fang *et al.* (2011). No previous data were found about TPC and TFC in *Dioscorea* leaves.

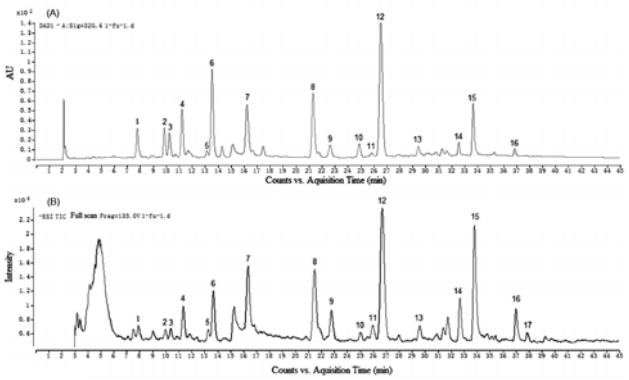

Fig. 1: Typical DAD chromatogram at 320 nm (A) and total ion chromatogram (TIC) in negative mode (B) of 17 phenolics from *Dioscorea alata* Leaves.

HPLC chromatogram of extract from *Dioscorea alata* leaves recorded at 360 nm is displayed in fig. 2A. Rutin (11.86±0.36 mg/g DW) was the most representative flavonoid found in the *Dioscorea alata* leaves extract followed by quercetin (8.66±0.29 mg/g DW). The content of rosmarinic acid (table 2) was significantly lower (1.96±0.14 mg/g DW). *Dioscorea glabra Roxb.* leaves showed different phenolic profile (fig. 2B). Interestingly, rosmarinic acid is found most notably in many Lamiaceae (Dimitrios *et al.*, 2013) such as hyssop (12.0±0.8 mg/g DW), or in plants of the genus Stachys.

However, it is the first time that rosmarinic acid has been identified in *Dioscorea* leaves and *Dioscorea glabra Roxb.* leaves presented the most significant amount of this compound (22.31±1.33mg/g DW). A higher concentration of rutin was observed in *Dioscorea glabra Roxb.* leaves (18.05±2.03mg/g DW) than that in *Dioscorea alata* leaves. Quercetin was also identified in the *Dioscorea glabra Roxb.* leaves extract but at a lower concentration (5.17±0.72 mg/g DW). The study revealed that twelve phenolic acids and five flavonoids were detected in yam leaves, especially the considerable amounts of rosmarinic acid, rutin and quercetin, indicating that yam leaves are a new and important source of phenolic acids and flavonoids.

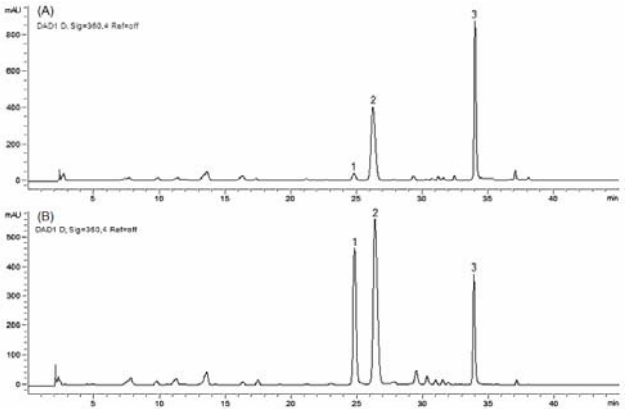

Fig. 2: HPLC-chromatograms of extracts from *Dioscorea alata* (A) and *Dioscorea glabra Roxb.* (B) *leaves* at 360 nm.1= rosmarinic acid, 2= rutin, and 3= quercetin.

Evaluation of antioxidant activity

DPPH radical scavenging assay is an easy and rapid method for screening of antioxidant in plant extracts. This free radical produces a violet solution in ethanol which is stable at room temperature. Antioxidant molecules are allowed to react with DPPH, the absorbance decrease of the radical could be measured at 517 nm. The DPPH method was conducted to evaluate the antioxidant capacity of extracts of *Dioscorea alata* and *Dioscorea glabra Roxb.* leaves and the results are presented in table 2. DPPH radical scavenging activity was presented as ascorbic acid equivalent by using a calibration curve (R^2=0.9997) with ascorbic acid (V_C) standard solutions of 0-50 μg/ml. The DPPH antioxidant activity varied considerably and *Dioscorea glabra Roxb.* sample (134.71±5.20mg V_CE/g DW) has a higher antioxidant capacity than the *Dioscorea alata* sample (63.41±3.65 mg V_CE/g DW). The results demonstrated that there is a

direct correlation between the level of phenolic compounds in *Dioscorea* leaves and its antioxidant activity.

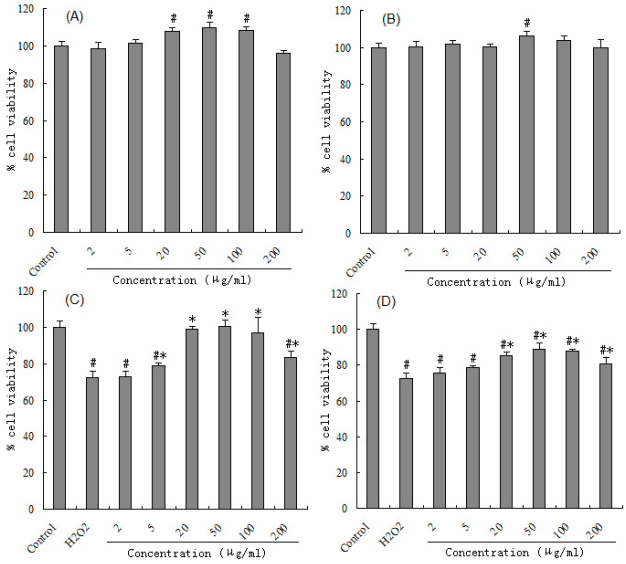

Fig. 3: Cell viability determined by MTT assay. Cells were incubated with increasing concentrations of extracts (0-200 µg/ml) from leaves of *Dioscorea glabra Roxb.* (A) and *Dioscorea alata* (B) for 24 h. HUVECs were pretreated with extracts (0-200 µg/ml) from leaves of *Dioscorea glabra Roxb.* (C) and *Dioscorea alata* (D) for 24 h, and then exposed for 2 h to 200 µM H_2O_2. Values are expressed as the mean ± SD, n=5. #*P*< 0.05 compared with untreated control HUVECs, **P*<0.05 compared with H_2O_2 exposed HUVECs.

ABTS radical also react directly with antioxidants to quantify the antioxidant capacity of the sample like the free DPPH radical (Dezsi *et al.*, 2015). Natural extracts were allowed to react with $ABTS^{+\cdot}$ and promoted the inhibition of absorbance at 734 nm. Total antioxidant activity of the extracts of *Dioscorea alata* and *Dioscorea glabra Roxb.* leaves was determined using the ABTS scavenging method. As shown in table 2, the ABTS radical scavenging assay results are in accordance with the DPPH values. *Dioscorea alata* and *Dioscorea glabra Roxb.* leaves extracts showed to have high antioxidant activity (2292.98±75.41 and 2587.2±61.19 µM Trolox/g DW). However, *Dioscorea alata* leaves showed the slightly lower ABTS scavenging activity, which could be explained by the lower levels of phenolic compounds.

Cytoprotective effect of dioscorea extracts on oxidative stress

To examine the toxicity of *Dioscorea alata* and *Dioscorea glabra Roxb.* leaves extracts (DALE and DGRLE), HUVECs were treated with DGRLE and DALE (2-200 µg/ml) for 24 h. Results showed that both extracts treatment did not cause toxic effects on HUVECs, however DGRLE caused proliferative effects at the concentration between of 20, 50, 100 µg/ml and DALE at 50 µg/ml. HUVECs were then exposed to 200 µM alone for 2 h or pretreated with the increasing concentrations of DALE and DGRLE for 24 h. Cell viability was assessed using MTT assay. As shown in fig. 3C and 3D, DGRLE and DALE attenuated the H_2O_2-induced decline in cell viability compared with H_2O_2 exposed HUVECs (*P*<0.05). By compared with the same untreated control, it can be deduced that the protective effect of DGRLE is higher than that of DALE, which is due to the higher phenolic contents in DGRLE.

DISCUSSIONS

In the present study, we determined the phenolic profile of *Dioscorea* (yam) leaves based on UV spectra, MS and MS/MS fragmentation patterns compared with those of available authentic reference compouds and/or reported values in the literature. Twelve phenolic acids and five flavonoids were identified in *Dioscorea alata* leaves, while two compounds (*p*-coumaroyl quinic acid nmethyl ester isomer and methyl rosmarinate) were not detected in *Dioscorea glabra Roxb.* leaves.

The TPC and TFC of the extracts obtained by MAE were analyzed through colorimetry. Contents of rosmarinic acid, rutin and quercetin were determined using HPLC-DAD. Results indicated that *Dioscorea* leaves are rich source of phenolic acids and flavonoids. Interestingly, the major phenolics found in leaves of *Dioscorea glabra Roxb.* were rosmarinic acid and rutin, however the most abundant compounds in leaves of *Dioscorea alata* were rutin and quercetin. Rosmarinic acid has been previously reported in medicinal herbs contributing to their antioxidant, anti-inflammatory activities (Del Rio *et al.*, 2013; Milica *et al.*, 2015). Rutin and quercetin are important secondary plant metabolites with potentially health-promoting properties like free radical scavenging properties, anti-lipid peroxidation, anti-inflammatory and cardioprotective properties. The activities of these compounds may be associated theoretically with *Dioscorea* leaves, which may be further tested through pharmacognosy in the laboratory.

The antioxidant potential of *Dioscorea alata* and *Dioscorea glabra Roxb.* leaves extracts was investigated using DPPH and ABTs assay. Results indicated that *Dioscorea glabra Roxb.* leaves were rich in rosmarinic acid and ruin, and exhibited strong antioxidant activity. In addition, there is a good correlation between the two radical assays, with a notable antioxidant activity for *Dioscorea glabra Roxb.* leaves. These found confirmed that high concentrations of rosmarinic acid and flavonoids are reflected in significant radical scavenging properties (Jordán *et al.*, 2012; Yang *et al.*, 2008).

Studies showed that oxidative stress is among the

prominent pathways of vascular endothelial dysfunction in many pathophysiological conditions (Fratantonio *et al.*, 2015). Therefore, treatments aimed at suppressing oxidative stress have potential therapeutic strategy for cardiovascular diseases (CVDs). Dietary intake of natural antioxidants including phenolic acids and flavonoids can act as potential candidates for preventing oxidative stress-related diseases (Halliwell, 2007). In this work, we used human umbilical vein endothelial cells as a model to determine the protective properties of *Dioscorea alata* and *Dioscorea glabra Roxb.* leaves extracts (DALE and DGRLE) against oxidative cell damage. Following treatment of HUVECs with various concentrations of DALE and DGRLE for 24 h, then incubation with H_2O_2 for 2 h, DALE and DGRLE significantly attenuates H_2O_2-induced decline in cell viability as demonstrated by MTT. By compared with the same untreated control, the DGRLE exhibited a higher antioxidant capacity than DALE. The results obtained from the cytoprotective properties suggest that *Dioscorea* leaves are a promising source of antioxidants inhibiting oxidative stress.

CONCLUSIONS

Microwave-assisted extraction (MAE) coupled to HPLC-DAD-ESI-MS/MS method was successfully used for fast determination of phenolic compounds in *Dioscorea* (yam) leaves. Twelve phenolic acids and five flavonoids were detected in yam leaves under the optimized analytical method. The phenolic profile was found to vary with species. The major phenolics found in leaves of *Dioscorea glabra Roxb.* were rosmarinic acid and rutin, however the most abundant compounds in leaves of *Dioscorea alata* were rutin and quercetin. Furthermore, *Dioscorea glabra Roxb.* leaves had higher antioxidant activity than that of *Dioscorea alata* due to the higher content of phenolic compounds. These results highlight the importance of *Dioscorea* leaves as a promising source of phenolic ingredients, with appreciable antioxidant activity and cytoprotective property against oxidative stress, to be used for different biological, medicinal and food applications.

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