Antioxidant and xanthine oxidase inhibitory activity of *Eucommia ulmoides* Oliver leaf extracts

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Abstract: The total phenolic content, flavonoid content, *in vitro* xanthine oxidase (XOD) inhibitory activity and antioxidant activity (AA) of *Eucommia ulmoides* Oliver leaf extracts were investigated. The AA investigations included 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, *β*-carotene/linoleic acid bleaching assay and oxygen radical absorbance capacity (ORAC) test. The ethyl acetate fraction (EE) showed the highest AA and xanthine oxidase inhibitory activity. Whilst the lowest 50% inhibition (IC_{50}) value of this fraction for DPPH free radical scavenging was 0.045mg/mL, its highest ORAC value was 10.57 μmol TE/mg. The highest inhibition rate against linoleic acid oxidation observed was 69.41%, and the lowest IC_{50} value for xanthine oxidase activity inhibition was 2.47mg/mL. These results show that *E. ulmoides* leaf extract is a promising source of natural antioxidants because it contains high contents of bioactive compounds, including chlorogenic acid, rutin, hyperin and astragalin, as detected by high-performance liquid chromatography coupled to HPLC-DAD-ESI-MS.

Keywords: *E. ulmoides* leaf extracts, total phenolics, total flavonoids, antioxidant activity, xanthine oxidase inhibitory activity.

INTRODUCTION

Interest in restraining the generation of reactive oxygen species (ROS), involving the super oxide anion ($O_2^{-}\cdot$) and hydrogen peroxide (H_2O_2) and hydroxyl ($HO\cdot$) radicals, has steadily grown through the years. These ROS are highly reactive and harmful when present in excessive amounts (Gill and Tuteja, 2010), and can cause biological aggression and various diseases, including cardiovascular disease, diabetes mellitus, cancer and neurological disorders in the human body (Valko *et al.,* 2007). Many antioxidants can either capture free radicals or reduce oxidative stress by activating or inhibiting the key regulating enzymes, such as SOD or xanthine oxidase. Artificial antioxidants are commonly used in the food industry, although their safety is often doubted (Sokmen *et al.,* 2004). Therefore, efforts to determine natural antioxidants that can potentially protect humans from oxidative stress-induced damages have gradually intensified (Scalbert *et al.,* 2005).

Eucommia ulmoides Oliver (Du-zhong) is one of the earliest tonic herbs described in traditional Chinese Medicine (Hung *et al.,* 2006). Its roasted cortex could be used as a folk medicine to reinforce the muscle and lung, lower blood pressure, prevent miscarriages, improve the liver and kidney condition and promote longevity (Huang, 1998). The leaves of *E. ulmoides* contain bioactive components similar to those found in its bark (Zhang, 1994), including polyphenolics [e.g. pyrogallol, coumaric acid, chlorogenic acid (CGA) and protocatechuic acid] and flavonoids (e.g. quercetin and kaempherol); these compounds exhibit excellent antioxidant activities (AA) as revealed by various antioxidant assays (Park *et al.*, 2006; Wong *et al.*, 2006; Yen and Hsieh, 2000). The hot water extract of *E. ulmoides* leaf is a potential ROS scavenger, and its effects are much stronger than those of protocatechuic acid in inhibiting oxidation of linoleic acid at a similar concentration of 0.1mg/mL (Yen and Hsieh, 2000). Furthermore, the flavonoid extract of *E. ulmoides* leaf exhibits significant AA (Huang *et al.*, 2009). These results demonstrate that *E. ulmoides* possesses strong AA for free radical removal.

Consistent with the reported findings, our previous results have confirmed the presence of several flavonoids in the leaves of *E. ulmoides* (Fu *et al.*, 2007). In addition, the AAs of flavonoids purified by AB-8 macroporous resin are stronger than those of the crude flavonoid extracts of *E. ulmoides* leaf at similar concentrations (Luo *et al.*,

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2011). However, whether the flavonoids in *E. ulmoides* are the sole antioxidant constituent of its leaf extract is unclear and requires further investigation.

This study aimed to prepare different solvent fractions of *E. ulmoides* Oliver leaf material and investigate the relationship between the contents of flavonoids or phenolics and their AAs to evaluate the contribution of flavonoids to the health benefits of *E. ulmoides* leaf.

MATERIALS AND METHODS

Raw material

Eucommia ulmoides Oliver leaf were gathered from Pingxiang area, Jiangxi province, China, and identified by Professor Guiming Fu of Nanchang University. The specimen of leaf material was conserved in State Key Laboratory of Food Science and Technology, Nanchang University, Jiangxi province, China.

Reagents

Standards of rutin, quercetin and *β*-Carotene, were purchased from Merck (*Merck* & Co Inc., Germany). Chlorogenic acid, hyperin and astragalin were bought from National Institutes for Food and Drug Control (NIFDC, Beijing, China). DPPH, linoleic acid, AAPH (2,2′-azobis (2-methylpropionamidine) dihydrochloride), Trolox, fluorescein sodium salt, xanthine, xanthine oxidase (XOD), allopurinol, 2,6-Di-tert-butyl-4-methylphenol (butylated hydroxytoluene, BHT) and gallic acid were purchased from Sigma–Aldrich (St. Louis, MO, USA). All other reagents of analytical pure grade were purchased from China National Medicine Group Shanghai Corporation (Shanghai, China).

Preparation of the extracts

Air-dried leaves (100g) were ground and extracted using a Soxhlet extractor with ether for 8h to remove the lipophilic fraction. The remaining residue was mixed with 60% ethanol at a material-to-liquid ratio of 1:15 and extracted at 80°C for 2h. This extraction step was repeated thrice. After extraction, the solvent was removed by a rotary evaporator to obtain a concentrated water suspension, which was equally divided into two parts: one part was labelled ‘ethanol fraction (EL)’, whereas the other part was further dried and then extracted successively using equal volume of chloroform, ethyl acetate and water-saturated *n*-butanol. Each fraction was then concentrated under vacuum condition, resulting in six fractions, namely, ether fraction (ER), EL, chloroform fraction (CM), ethyl acetate fraction (EE), water-saturated *n*-butanol fraction (WL) and residual fraction (RL). These fractions were freeze-dried or dried under moderate heating in a vacuum.

Determination of total flavonoids content (TFC)

TFC was determined using a colorimetric method described by Chai *et al.* (2013). Briefly, 1mL of diluted sample and 0.3mL of $NaNO_2$ solution (5%, m/v) were mixed vigorously. This mixture was allowed to stand for 4 min, after which 0.3mL of $Al(NO_3)_3$ solution (10%, m/v) was added to it. Mixing followed. Four minutes later, 0.3mL of NaOH solution (1 mol/L) was added to the mixture and the final volume was adjusted to 10mL using 50% ethanol solution. The mixture was allowed to stand for 10 min, and its absorbance was measured at 510nm. Using rutin as the standard substance, we determined a TFC standard curve. The regression equation of this curve was y=11.172x-0.007 (R^2=0.9995), where y is the absorbance value of sample and x is the sample concentration.

Determination of total phenolics content (TPC)

TPC was determined using Folin Ciocalteu’s phenol reagent according to the method described by Gálvez *et al.* (2005). Using gallic acid as the standard substance, we obtained a TPC standard curve. The regression equation of this curve was y=0.0926x-0.0281 (R^2 = 0.9918), where y is the absorbance value of the sample and x is the sample concentration.

Antioxidant tests

2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay

DPPH is one highly stable and colour-free radical that could sequester labile hydrogen atoms from phenolic antioxidants and simultaneously form a colourless hydrazine (Diouf *et al.*, 2009). The radical scavenging activity of the extracts was evaluated according to the method described by Manzocco *et al.* (1998) with some modifications. Exactly 0.5mL of the fractions, vitamin C (V_C) or BHT in methanol was added respectively to 5mL of DPPH solution (6×10^{-5} mol/L) in methanol. The mixture was left in the dark for 30 min at room temperature. The decrease of absorbance was measured at the wavelength 517 nm, and the percentage of the reacted DPPH was calculated by the following equation:

$$1\% = \left[\frac{(A_O - A_S)}{A_O}\right] \times 100\% \qquad (1)$$

where A_0 is the initial absorbance (no antioxidant) of the solution and As is the absorbance in the presence of the different concentrations of the extract, V_C or BHT. All experiments were performed in triplicate.

β-Carotene/linoleic acid bleaching assay

The AA of the leaf extracts to restrain linoleic acid oxidation was evaluated with measuring the inhibition of volatile organic compounds and conjugated diene hydroperoxides producing in the system. The method described by Jayaprakasha *et al.* (2001) was used with minor modification. *β*-Carotene (0.2mg), linoleic acid (20 mg) and Tween 40 (200 mg) were mixed in chloroform (0.5mL) to obtain stock solutions of *β*-carotene and linoleic acid. The chloroform was evaporated under reduced pressure at 40°C, and the resulting mixture was immediately blended with 10mL of triple-distilled water and then mixed for 1-2 min. The emulsion was diluted with oxygenated water to 50mL. BHT was used as the positive control. Methanol (0.2mL) and the above emulsion (4 mL) also served as controls. The samples were dissolved in methanol to get the sample solution (2 mg/mL) and 0.2 mL of each sample solution was added to 4 mL of the above emulsion in test tubes, which were then incubated in a water bath at 50°C for 4h. The absorbances of the solutions were measured at 470nm. All absorbances were measured at 30-minute intervals from zero time (t=0) until the control reaction became colourless (t=240 min). A mixture without *β*-carotene was prepared following above step and served as a blank. The AAs of the fractions were calculated through the equation below:

$$\mathrm{AA}\% = \left[1 - \frac{(\mathrm{A_O} - \mathrm{At})}{\mathrm{A_{CO}} - \mathrm{A_{ct}}}\right] \times 100\% \qquad (2)$$

Where A_0 and A_{c0} are the absorbances of the sample and control at 0min, respectively and A_t and A_{ct} are the absorbances of the sample and control at 240 min, respectively. All experiments were performed in triplicate.

ORAC assay

The fluorescent probe fluorescein was oxidised by AAPH, resulting in quenching of its fluorescence and generation of hydrophilic peroxyl radicals (ROO·). Hence, the ability to reduce ROO· can be used as a measure of inhibition of antioxidant quenching (Gomes *et al.*, 2009). The ORAC method employed was adapted from a method described by Zill-e-Huma *et al.* (2011). All steps followed were similar to the original method except that the fluorescence intensity was measured every minute within 30 min and that Trolox solutions (0, 6.25, 12.5, 25, 50 and 100 μmol/L) were used as the standard.

In vitro xanthine oxidase inhibitory activity

XOD activity was assayed spectrophotometrically under aerobic conditions using xanthine as the substrate as described by Umamaheswari *et al.* (2007) with some changes. The enzyme concentration was 0.1 unit/mL, and different concentrations of the fractions were dissolved in dimethyl sulphoxide (DMSO), resulting in a final DMSO concentration of 5%, which does not affect the enzymatic assay. Allopurinol and quercetin, which are XOD inhibitors, were used as positive controls. All other steps followed were similar to those previously described. All of the fractions were assayed for their xanthine oxidase inhibitory activity *in vitro*.

Liquid chromatography mass spectrometry (LC-MS) analysis

The EE fraction was dried, dissolved in 50% (v/v) methanol water solution up to a final concentration of 2 mg/mL and then analysed by LC-MS. Separation by HPLC was performed using Waters dual gradient chromatographic system (Waters Corporation, Boston, MA, USA) with Symmetry C_{18} column (5μm, 4.6mm × 250 mm; Waters Corporation, USA) interfaced to a Millennium-32 workstation and equipped with a PCM pump controller, two 515 HPLC pumps, a Waters 2996 photodiode array detector, a thermostatic column compartment and a sample injector with a 20μL sampling loop. Mass spectra were obtained on Waters ZQ4000 mass spectrometer (Waters Corporation, USA) equipped with a nano-electrospray ionisation (ESI) source in both positive and negative ion modes.

The mobile phases included 0.1% (v/v) formic acid in water (solvent A) and methanol (solvent B). A gradient elution program with a flow rate of 0.8mL/min was performed as follows: 15%-30% B (0-3min), 30% B (3-10 min), 30%-45% B (10-20 min), 45% B (20-35min), 45%-80% B (35-40min), 80% B (40-45min) and 80%-15% B (45-50min). Full-scan mass spectra were obtained in positive and negative ion modes at m/z 100-1000. Data obtained from MS analysis were compared to standard values and references.

STATISTICAL ANALYSIS

The experimental results presented are the mean ± standard deviation (SD) of three replicates. The results were analysed using Excel 2010 (Microsoft Corporation, Redmond, WA, United States) and SPSS 17.0 (SPSS Inc., Chicago, IL, United States).

RESULTS

TFC

Amongst the six fractions prepared, the fraction EE showed the highest amount of flavonoids; but no flavonoids were detected in ER (table 1). When TFC in every fraction was divided by the highest TFC, the relative values were obtained (table 2). Except the relative TFC value of EE was 1, values of other fractions were lower far from 1. The TFC of these fractions could be arranged in decreasing order as follows: EE > WL > EL > RL > CM > ER.

TPC

Same to the results of the TFC assay, EE showed the highest amount of TPC (table 1). When TPC of every fraction was divided by the highest TPC, the relative TPC values were obtained (table 2). Similarly, except the relative TPC value of EE was 1, values of other fractions were all lower than 0.5. The TPC of the six fractions could be arranged in decreasing order as follows: EE >WL >EL >RL >CM >ER; this trend is basically consistent to the TFC trend.

Antioxidant results

DPPH assay

The radical-scavenging activity of the six *E. ulmoides* leaf extract fractions was evaluated using the DPPH assay. Table 1 shows the extract concentrations resulting in 50% inhibition (IC_{50}). The fraction EE, with an IC_{50} value of

0.045 mg/mL between the IC_{50} values of two positive controls V_C (0.010mg/mL) and BHT (0.074 mg/mL), showed the highest scavenging activity, followed by WL, with an IC_{50} value of 0.105 mg/mL. EL and RL, with IC_{50} values of 0.181 and 0.186 mg/mL, respectively, showed moderate AA against DPPH radicals; this disparity was not significant. CM, with an IC_{50} value of 0.374 mg/mL, showed relatively weak activity, whilst ER with an IC_{50} value of 30.12 mg/mL, showed the weakest scavenging potency.

β-Carotene/linoleic acid bleaching assay

The inhibitory potential of the extract fractions against lipid peroxidation was evaluated using the *β*-carotene/linoleic acid bleaching test, which measures an antioxidant's potential in inhibiting the formation of conjugated diene hydroperoxide from linoleic acid oxidation (fig. 1). Table 1 presents the AA of the six fractions, which exhibited varying degrees of AA. The EE fraction showed the highest AA of 69.4%, whilst RL showed the lowest AA of 12.2%; the latter AA is significantly lower than those of the five other fractions.

In this system, one hydrogen atom located on C-11 between the two double bond of linoleic acid is abstracted from the bis-allylic methylene group. The formed pentadienyl free radical then attacks the *β*-carotene molecules with highly unsaturation degree to obtain another hydrogen atom. Given that the *β*-carotene molecules lose their conjugation after losing a hydrogen atom, the orange colour of the carotenoids faded. This process can be monitored by spectrophotometry. The phenolic antioxidants in the plant extracts could impede the extent of *β*-carotene destruction through 'neutralising' the linoleate free radical and any other free radical formed within the system. Hence, the antioxidant potential of the plant extracts can be revealed by this assay.

ORAC test

The ORAC test revealed that the antioxidants inhibit the AAPH-induced oxidation of fluorescein by reducing the AAPH-derived ROO·. Table 1 shows the results obtained, and the AA of the fractions may be arranged in decreasing order as follows: EE (10.57μmol TE/mg) >BL (6.40μmol TE/mg) >ER (4.85μmol TE/mg) >CM (4.50μmol TE/mg) >EL (2.12 μmol TE/mg) >RL (0.78 μmol TE/mg). EE showed the highest AA, which may be attributed to the high amounts of natural antioxidants in this fraction.

In vitro xanthine oxidase inhibitory activity

The fractions can dose-dependently restrain xanthine oxidase from catalysing oxidation of xanthine into uric acid (fig. 2). The IC_{50} of allopurinol and quercetin to XOD were 0.21 μg/mL and 92.49 μg/mL, respectively. The EE fraction exhibited the highest potency (IC_{50} = 2.47 mg/mL), followed by WL (IC_{50} =3.62 mg/mL) and ER (IC_{50} =3.86 mg/mL) (table 1). EL, CM and RL showed weak XOD inhibitory activities. XOD is an important enzyme in nucleic acid metabolism; it catalyses oxidation of xanthine and hypoxanthine into uric acid and generates oxidative free radicals. Therefore, inhibiting XOD activity reduces uric acid formation, effectively halts or delays gout and its complications and weakens tissue damage caused by free radicals, all of which are critical to human health.

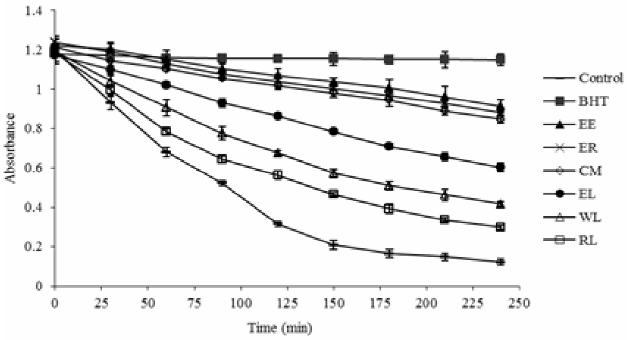
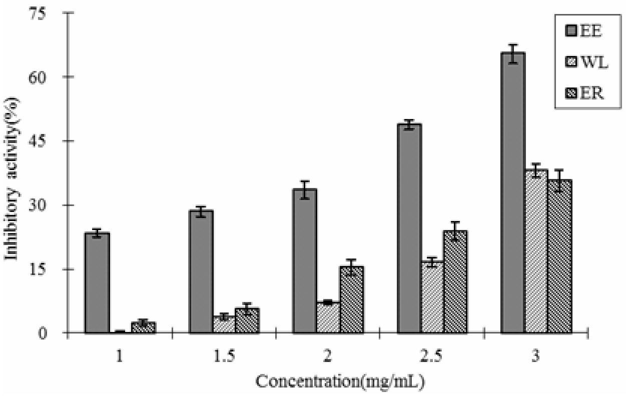


Fig. 1: Antioxidant activities of the different fractions *of E. ulmoides leaf extract* and BHT in *β*-Carotene/linoleic acid bleaching assay (ER: ether fraction, EL: ethanol fraction, CM: Chloroform fraction, EE: ethyl acetate fraction, WL: water-saturated *n*-butanol fraction, RL: residual fraction)



(ER: ether fraction, EE: ethyl acetate fraction, WL: water-saturated *n*-butanol fraction)

Fig. 2: Inhibitory effects of three fractions EE, WL and ER on XOD activity

Correlation of AA with TFC and TPC

We investigated the relationship of AA (expressed as the reciprocal of the calculated IC_{50} for both assays) to TFC and TPC (table 3). A strong tendency was detected between scavenging activity and TPC (R^2=0.9799) or TFC (R^2=0.9783) and a weak tendency was detected between ORAC and TFC (R^2=0.8019). These correlations suggest that TFC is partly responsible for the significant antioxidant effect of the *E. ulmoides* leaf fractions. However, this relationship was not obvious in the *β*-carotene/linoleic acid bleaching assay.

Table 1: Extraction yield, TFC, TPC and antioxidant test results of different fractions of *Eucommia ulmoides* leaf extract (n = 3) (ER: ether fraction, EL: ethanol fraction, CM: chloroform fraction, EE: ethyl acetate fraction, WL: water-saturated *n*-butanol fraction, RL: residual fraction, TFC: total flavonoids content, TPC: total phenolics content)

Fraction	Extraction yield (%)	TFC (mg/g)	TPC (mg/g)	IC_{50} of scavenging the DPPH free radical (mg/mL)	Linoleic acid oxidation inhibiting rate (%)	ORAC (μmol TE/mg)	IC_{50} of in vitro XOD inhibitory (mg/mL)
ER	4.63±0.35	-	62.51±5.24	30.12±0.850	64.25±2.27	4.85±0.35	3.86±0.42
CM	0.75±0.05	65.36±3.43	99.89±9.15	0.374±0.008	63.85±5.24	4.50±0.35	-
RL	21.98±1.88	69.78±5.85	103.4±3.22	0.186±0.006	12.21±0.55	0.78±0.03	-
EL	29.06±2.07	121.9±8.32	156.1±7.04	0.181±0.004	43.59±2.97	2.12±0.18	-
WL	3.86±0.32	175.2±11.40	229.4±7.20	0.105±0.005	23.24±2.26	6.40±0.34	3.62±0.29
EE	1.38±0.14	376.6±20.05	461.2±25.66	0.045±0.011	69.41±8.71	10.57±0.37	2.47±0.20
Vc				0.010±0.000	-	-	-
BHT				0.074±0.002	97.12 ± 4.08	-	-
Allopurinol				-	-	-	$(2.1±0.20) \times 10^{-4}$
Quercetin				-	-	-	$(9.25±0.78) \times 10^{-2}$

- Not investigated

Table 2: Relative TFC and TPC values of fractions of *E. ulmoides* leaf extract (ER: ether fraction, EL: ethanol fraction, CM: chloroform fraction, EE: ethyl acetate fraction, WL: water-saturated *n*-butanol fraction, RL: residual fraction, TFC: total flavonoids content, TPC: total phenolics content)

	ER	EL	CM	EE	WL	RL
Relative TFC value	-	0.3237	0.1735	1	0.4652	0.1854
Relative TPC value	0.1356	0.3384	0.2166	1	0.4973	0.2241

Table 3: Correlation of TFC and TPC with the antioxidant activity (AA) (TFC: total flavonoids content, TPC: total phenolics content)

	Correlation factors R^2		
	DPPH scavenging test	*β*-Carotene/linoleic acid bleaching assay	ORAC assay
TFC	0.9783	0.2375	0.8019
TPC	0.9799	0.0662	0.6822

Table 4: Chromatographic and spectroscopic properties of the main phenolic compounds of the ethyl acetate fraction (EE)

Peak	t_R (min)	λ_{max} (nm)	MS^+/MS^- (m/z)	M_W	Compound
1	10.953	219.1, 242.6, 296.7, 329.1	353.3$[M-H]^-$, 191.5$[M- Caffeoyl]^-$, 707.4 $[2M-H]^-$; 355.3$[M+H]^+$, 163.4$[M-Quinic acid]^+$	354	Chlorogenic acid
2	22.394	256.8, 355.3	609.4$[M-H]^-$, 463.3$[M-Rha]^-$	610	Rutin
3	26.036	255.6, 359.6	463.3$[M-H]^-$, 927.5$[2M-H]^-$	464	Hyperin
4	30.851	266.2, 347.0	447.3$[M-H]^-$, 895.4$[2M-H]^-$	448	Astragalin

Identification of main phenolic chemicals in EE by LC-MS

The main flavonoid and phenolic compounds in the EE fraction were analysed by the HPLC-DAD-ESI/MS method. Fig. 3 shows the HPLC spectrum of EE under 330 nm, and table 4 lists the relevant retention times (t_R), maximum absorption wavelength (λ_{max}) and MS fragmentation ions. The four components observed were identified as CGA, rutin, hyperin and astragalin by comparing their characteristic UV spectra and mass spectra with those of the standard compounds.

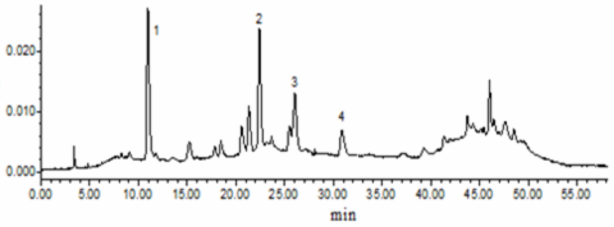


Fig. 3: The HPLC Chromatogram of the ethyl acetate fraction (EE). 1: chlorogenic acid, 2: rutin, 3: hyperin, 4: astragalin

DISCUSSION

By comparing the content of total flavonoids with the total phenolics of the six fractions, we concluded that EE contains the highest amounts of total flavonoids and phenolics amongst the fractions studied, which made important contribute to the strongest antioxidant activity of EE. Though ER and CM had low contents of total flavonoids and phenolics, they still showed certain antioxidant activity. Safaei-Ghomi *et al.* (2009), who reported similar results, found that the volatile oil and nonpolar components of *Thymus caramanicus* exhibit high AA in the β-carotene/linoleic acid system, achieving 79.03% and 84.55% inhibition, respectively; these inhibition rates are significantly higher than those of other components. The researchers thus suggested that the high AA of *T. caramanicus* may be attributed to the presence of compounds containing hydrogen atoms in the allylic and/or benzylic positions. We speculate that this phenomenon is the same reason causing the high AAs of ER and CM.

CGA, rutin, hyperin and astragalin are four ingredients of EE, and also widespread natural antioxidants in plants. A number of studies have investigated the free radical-scavenging capacities and structure–activity relationships of flavonoids (Trouillas *et al.,* 2006), especially quercetin and its glucosides, which are the most commonly observed compounds in related studies. Cai *et al.* (2014) showed that quercetin, rutin and hyperin exhibit high inhibitory activity against ·OH and $O_2^{-}\cdot$ radicals, low bond dissociation enthalpies and high AAs. Parejo *et al.* (2004) also reported that CGA, hyperin and astragalin from fennel (*Foeniculum vulgare* Mill.) waste exhibit strong radical scavenging activities. An investigation of the relationship of CGA content to caffeine content and AAs in commercial whole coffee fruit extracts/powder samples showed that the total AA of the samples displays strong correlations with CGA content (Mullen *et al.,* 2011). Therefore, the AA of EE may be related to the aforementioned phenolic components.

CONCLUSIONS

The *in vitro* xanthine oxidase inhibitory activities of various *E. ulmoides* leaf fractions are reported in this study. This study showed that the EE fraction of *E. ulmoides* leaf extract exhibits the highest *in vitro* AA and xanthine oxidase inhibitory activity amongst the other fractions of *E. ulmoides* leaf extract studied. The correlation between AA and TFC/TPC suggested that TFC/TPC is partly responsible for the significant antioxidant effects of these fractions. HPLC-DAD-ESI-MS analysis revealed that CGA, rutin, hyperin and astraganin are the main antioxidant components of EE. Additional studies are currently being conducted to better understand the inhibitory mechanism of EE on XOD and the relationship between AA and the inherent phenolic components of *E. ulmoides*.

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