Composition, antioxidants content and antioxidant activity of walnut residues, a by-product of oil extraction in China

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Summary
As by-products of walnut (*Juglans regia* L.) oil production, walnut residues are rich in polyphenols. The antioxidants content and antioxidant activities of walnut residues, which were obtained from three regions of China, were investigated in this study. Moreover, the correlation between polyphenols content and their antioxidant activities were analysed. The results showed that, for each walnut residue ethanolic extracts (WREE), there was a positive correlation between total polyphenols and antioxidant activity. Additionally, walnut residue ethanolic extracts from Xinjiang province (WREE-XJ) with the highest total polyphenols content possessed the highest antioxidant activity, which further demonstrated that polyphenols contributed to the antioxidant activity of the extracts. Furthermore, high resolution mass spectrometry was used to study the polyphenol composition of WREE-XJ. Results showed that the majority of WREE-XJ phenolics were procyanidins, gallic acids, ellagic acids, quercetin and their corresponding carbonyl compounds (glycosides).

Keywords
walnut; oil extraction; antioxidant activity; high resolution mass spectrometry; polyphenols

Walnut (*Juglans regia* L.), which belongs to the family Juglandaceae, is valuable for its wood and fruits. It is primarily distributed in temperate areas and cultivated commercially in Asia, western South America, USA as well as in central and southern Europe. China is the major world producer of walnuts [1]. In China, walnuts are generally used for oil extraction in food industry, owing to the high content of functional ingredients such as sphingolipids and phospholipids [2]. The processing produces large amounts of waste walnut residues, which are usually used as feed or fertilizers [1]. A better use of these wastes would be desirable.

China is a big country with diverse climate. It was demonstrated that cultivation region is the key factor of the phytochemical composition differences of walnuts [3]. In another study, the phenolics and flavonoids contents as well as antioxidant activity of the extracts of walnuts from 11 regions of Iran proved to be significantly different [4]. In addition, it was reported that the walnut flour is a rich source of polyphenols [3]. Polyphenols, known as the secondary metabolites of higher plants, are divided into four categories, that is phenolic acids, flavonoids, stilbenes and lignans. They are believed to provide health benefits, including anti-obesity, anti-diabetic, anti-cancer as well as anti-inflammatory activities [5]. All of these functional activities originate from a basic effect known as antioxidant activity [6]. Recent studies investigated antioxidant activity through the radical-scavenging activity of 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and ferric ion reducing antioxidant power (FRAP). For example, phenolics and flavonoid contents of six buckwheat varieties in free form were found to have higher antioxidant activities of these radicals than those of bound phenolics [7].

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Therefore, the aim of this research was to analyse the antioxidant components of walnut residues, a by-product of oil extraction, from different regions of China. We investigated the differences in contents of moisture, ash, protein, total carbohydrates, total phenolics and total flavonoids. In addition, the antioxidant activity of ethanolic extracts from walnut residues was evaluated through the radical-scavenging activity of DPPH, ABTS and reducing power. Furthermore, the correlation analysis between total phenolics and antioxidant activity and high resolution mass spectrometry (HRMS) analysis were performed on the walnuts residues with highest radical-scavenging activity. This research contributes to in-depth understanding of the neglected value of walnut residues and aims to highlight its potential benefits as a source of natural antioxidants.

MATERIALS AND METHODS

Chemicals

Petroleum ether, concentrated sulfuric acid, copper sulphate, sodium hydroxide, boric acid, methyl red, bromocresol green, ethanol, potassium sodium tartrate, concentrated hydrochloric acid, Folin-Ciocalteu reagent, sodium carbonate, sodium nitrite, aluminum nitrate, rutin, ABTS, DPPH, potassium persulfate, sodium dihydrogen phosphate, disodium hydrogen phosphate, methanol, potassium ferricyanide and trichloroacetic acid obtained from Sigma Aldrich (St. Louis, Missouri, USA). Gallic acid standards and rutin standards obtained from Sinopharm (Shanghai, China).

Sample collection and preparation

Walnuts, fruits of *Juglans regia* L., were collected from three regions with an N 23° to N 44° latitude span (Xinjiang province, XJ; Hubei province, HB; and Yunnan province, YN). Samples of approximately 2 kg of fresh walnuts were obtained from local retailers in each region in autumn 2020. After removing the shells and kernels (with pellicle, i. e. the brown skin), 10–20 nuts were diced into small pieces, subsequently milled in an FW80 high-speed grinder (Teste Instrument, Tianjin, China) and finally screened through a 841 μm sieve to obtain fine particles. In addition, walnut residues were prepared by Soxhlet extraction with petroleum ether (200 g·l^{-1}) according to AOAC 948.22 [8]. The walnut residues were placed in sealed plastic bags, kept at 2 °C and analysed within seven days.

Chemical composition analysis

Moisture content of the walnut residues was determined by heating the sample using a drying oven DHG-9075A (Yiheng, Shanghai, China) at 101 ± 2 °C overnight until constant weight. Ash content was determined by weighing the residues obtained after incineration using a Baidian muffle furnace at 525 ± 5 °C for 24 h according to AOAC 923.03 [8]. Crude protein content was determined using the Kjeldahl method according to AOAC 925.40 [8]. Total carbohydrate (sugar and starch combined) content was determined using the Lane-Eynon constant volumetric method [9].

Preparation of walnut residue ethanolic extracts

Walnut residue samples (10.0 g) were suspended in 600 ml of 70% (v/v) ethanol and stirred for 120 min at 30 °C in constant temperature shake bed (Yiheng Scientific Instruments Development, Shanghai, China). Samples were centrifuged at 3 800 ×*g* for 15 min, the upper layer was collected and extraction was repeated twice. Subsequently, the supernatants were combined and evaporated at 50 °C to remove ethanol by RE-5299 rotary evaporator (Aibote Science and Technology Development, Henan, China). Finally, the obtained solutions of walnut residue ethanol extracts (WREE) were vacuum freeze dried (LGJ-10; Beijing Songyuanhuaxing Technology Development, Beijing, China).

Total phenolics analysis of WREE was performed according to the methodology of DE CAMARGO et al. [10]. Under alkaline conditions, phenolic compounds reacted with Folin-Ciocalteu reagent to form blue compounds. The results were expressed in milligrams of gallic acid equivalents (GAE) per kilogram of dry weight (DW).

Total flavonoids analysis was carried out according to LIANG et al. [11]. Under alkaline conditions, flavonoid compounds reacted with the mixed reagent (0.51 mol·l^{-1} $NaNO_2$, 0.79 mol·l^{-1} $Al(NO_3)_3$, and 0.52 mol·l^{-1} NaOH) to form red complexes. The data were expressed as grams of rutin equivalents (RE) per kilogram DW.

ABTS radical-scavenging activity assay

ABTS radical ($ABTS^{\bullet+}$) scavenging activity was analysed according to GOWD et al. with slight modifications [12]. Specifically, $ABTS^{\bullet+}$ stock solution (0.007 mol·l^{-1}) and potassium persulfate (0.00245 mol·l^{-1}) were mixed and incubated in the dark for 12–16 h. Then, the mixed solution was diluted with phosphate buffered saline (PBS) buffer (0.01 mol·l^{-1}, pH 7.4), until the absorbance was 0.70 ± 0.02 as measured at 734 nm. A volume of 10 μl of sample was added to 1 ml of $ABTS^{\bullet+}$

diluting solution, then vigorously shaken for 30 s and incubated in the dark at room temperature for 6 min. The absorbance was measured at 734 nm using UV-1800PC spectrophotometer (AOE Instruments Development, Shanghai, China). ABTS radical-scavenging rate was calculated using Eq. 1 and expressed as percentage:

$$R = \frac{(A_0 - A_1)}{A_0} \times 100 \quad (1)$$

where A_0 is absorbance of $ABTS^{\bullet+}$ with ethanol and A_1 is absorbance of $ABTS^{\bullet+}$ with the sample solution.

ABTS radical-scavenging activity of samples was expressed as half-maximal inhibitory concentration (IC_{50}).

DPPH radical-scavenging activity assay

The DPPH radical-scavenging ability analysis was carried out as described previously with modifications [13]. Volumes of 1 ml each of various concentrations of WREE solutions were mixed with 4 ml of 6×10^{-5} mol·l^{-1} DPPH methanolic solution. The mixture was shaken vigorously, incubated in the dark for 60 min and absorbance was measured at 517 nm using UV-1800PC spectrophotometer. DPPH radical-scavenging rate was calculated by Eq. 1, where A_0 is absorbance of DPPH radical with ethanol and A_1 is absorbance of DPPH radical with the sample solution. DPPH radical-scavenging rate was expressed as percentage.

DPPH radical scavenging activity of samples was expressed as IC_{50}.

Reducing power assay

The reducing power of each sample solution was estimated according to the method described by Zhu et al. [14]. The reagent was prepared by mixing sample (2.5 ml), 1% potassium ferricyanide (2.5 ml) and 0.2 mol·l^{-1} PBS buffer (2.5 ml, pH 6.6), followed by incubation at 50 °C for 20 min. A volume of 20 ml of 10% trichloroacetic acid was added, the solution was mixed and then centrifuged at 1 000 ×g for 10 min. The supernatant (2.5 ml) was mixed with 2.5 ml of distilled water and 1 ml of 0.1% ferric chloride, followed by incubation at 50 °C for 10 min. Absorbance was measured spectrophotometrically at 700 nm (A_{700}) using UV-1800PC spectrophotometer. Increased absorbance of the reaction mixture indicated greater reducing power.

High resolution mass spectrometry analysis

The WREE with highest antioxidant activity was dissolved in methanol to be analysed in a high resolution mass spectrometer (HP 1100 Series; Agilent Technologies, Palo Alto, California, USA). All the analyses were carried out at room temperature. The following conditions of electrospray ionization (ESI) interface were used: drying gas flow 9.0 l·min^{-1}, nebulizer pressure 2.41×10^5 Pa, gas drying temperature 350 °C. Phenolic compounds were identified according to the mass-to-charge ratio in MS spectra [15].

Statistical analysis

All experiments were carried out in triplicate ($n = 3$) and each employed a new batch of ground walnut residues as an independent test. Data from all replications were analysed using one-way ANOVA test. Duncan's new multiple range test ($\alpha = 0.05$) was used to estimate the significance of values. Additionally, Fisher correlation analysis was carried out using the program Statistica 10.0 (StatSoft, Tulsa, Oklahoma, USA).

RESULTS AND DISCUSSION

Chemical composition of walnut residues

The chemical composition of walnut residues from the three regions is shown in Tab. 1. Means

Tab. 1. Chemical composition of walnut residues.

	Region		
	Xinjiang province	Hubei province	Yunnan province
Moisture [g·kg^{-1}]	0.59 ± 0.01 ^{b}	0.61 ± 0.01 ^{a}	0.57 ± 0.00 ^{c}
Ash [g·kg^{-1}]	0.55 ± 0.01 ^{a}	0.49 ± 0.01 ^{b}	0.52 ± 0.01 ^{ab}
Protein [g·kg^{-1}]	5.36 ± 0.18 ^{a}	5.32 ± 0.04 ^{a}	4.25 ± 0.14 ^{b}
Total carbohydrates [g·kg^{-1}]	2.43 ± 0.02 ^{b}	2.18 ± 0.01 ^{c}	2.96 ± 0.02 ^{a}
Reducing sugars [g·kg^{-1}]	0.06 ± 0.01 ^{b}	0.07 ± 0.00 ^{b}	0.19 ± 0.01 ^{a}
Total polyphenols [g·kg^{-1}]	0.78 ± 0.01 ^{a}	0.34 ± 0.00 ^{c}	0.45 ± 0.01 ^{b}
Flavonoids [g·kg^{-1}]	0.03 ± 0.00 ^{a}	0.02 ± 0.00 ^{b}	0.02 ± 0.00 ^{ab}

For moisture, the unit is on a wet weight basis; for all other components, the unit is on a dry weight basis.
Values represent mean ± standard deviation ($n = 3$). Different letters in superscript within a column indicate significant difference ($P < 0.05$).

and standard deviations of walnut residues composition were expressed on a dry weight basis except for moisture content. As an indicator for the shelf-life of walnut products, moisture content of walnut residues from Hubei province (HB) were significantly higher than the other two samples ($P < 0.05$). In addition, the average moisture content of the three walnut residues were between 5.7 % and 6.1 %, which was in concordance with New Zealand walnut residues (6.1 %) [16], while the differences in moisture content of three regions may be affected by the drying method of rough processing. The ash content of the three walnut residues was in the range of 4.9–5.5 %, which was obviously higher than that of six Portuguese walnut residues (1.8–2.1 %) [17]. Furthermore, protein content of the walnut residues ranged from 42.5 % (YN) to 53.6 % (XJ), which was significantly higher than that of fresh walnuts (15.7 %) owing to the degreasing process [18].

The results demonstrated that walnuts grown in the northern provinces tended to contain more protein than those grown in the southern region. As a by-product of edible kernel oil production, walnut residues (containing more than 42 % protein) were considered to have low economic value but are high-quality protein resources, which were used as fodder and fertilizers [19]. Carbohydrates, including both complex (starch) and simple (sugars) ones, were crucial for the processing and palatability characteristics. Interestingly, the walnut residues from the southern region (YN) contained the greatest total amount of carbohydrates, which might be due to both the ambient temperature and the length of daily sun exposure. Further, since carbohydrates were produced via photosynthesis, latitude could contribute to the effect, average autumn temperature being the highest in the south region (YN). Large temperature differences between morning and evening and high temperature during the day are known to be conducive to the growth of plant [20]. Except from these differences, what is more important is that walnut residues from all the three regions contained more than 20 % carbohydrates, which suggested that walnut residues are a very good carbon source for plants and animals.

Antioxidants content

Walnuts contain secondary metabolites with antioxidant activity, such as polyphenolics and flavonoids [21] and some evidence suggested that walnuts become more important in human nutrition due to the health protection effect of antioxidants [22]. In spite of the differences between production regions, all three groups of walnut residues were found to be rich in antioxidants, suggesting that they are excellent edible sources of bioactive compounds. Notably, the total phenolics contents of the three walnut residues were in a range of 0.34–0.78 $g \cdot kg^{-1}$ DW (Tab. 1), which was higher than in six Kermanshah walnut residues (0.01–0.02 $g \cdot kg^{-1}$ DW) in a previous study [23]. Total flavonoids contents were in the range of 0.02–0.03 $g \cdot kg^{-1}$ DW, which was higher than in six Kermanshah walnut residues (0.01 $g \cdot kg^{-1}$ DW) [23]. Walnut residues of XJ possessed the highest flavonoid content (0.03 $g \cdot kg^{-1}$ DW) and this was related to the strong sunlight in Xinjiang province, which is an environmental condition that might promote the synthesis of more flavonoids [24]. Our results suggested that walnut residues can be developed to a resource of natural antioxidants. However, it was not clear whether the soil quality also affected the synthesis of these compounds.

Total antioxidant activity

Total antioxidant activity was evaluated by ABTS radical-scavenging ability. The effects of WREE on $ABTS^{\bullet+}$ were determined and the results are shown in Fig. 1A and Tab. 2. All the tested WREE samples exhibited scavenging activities on $ABTS^{\bullet+}$ in a dose-dependent manner (0.005–0.035 $mg \cdot ml^{-1}$, the concentration range of WREE samples). The IC_{50} values were used to compare the antioxidant activity. Compared to chestnut (*Castanea sativa* Mill.) shells, were are by-products of chestnut industry process and showed IC_{50} of 0.065 $mg \cdot ml^{-1}$ [25], WREE-XJ showed an excellent scavenging activity (IC_{50} = 0.010 $mg \cdot ml^{-1}$), while the IC_{50} values of WREE-HB and WREE-YN were 0.023 $mg \cdot ml^{-1}$ and 0.013 $mg \cdot ml^{-1}$. These results clearly indicated that ethanol-soluble compounds in walnut residues were crucial for ABTS radical-scavenging activity.

DPPH radical-scavenging activity

In this assay, DPPH radical-scavenging activity was tested and the results revealed that the activities of all WREE increased with extracts concentration, in particular for WREE-XJ (Fig. 1B). The calculated IC_{50} values are listed in Tab. 2. Among the three groups of WREE, WREE-XJ exhibited the strongest effect, as its IC_{50} value was 0.019 $mg \cdot ml^{-1}$, followed by WREE-YN (0.029 $mg \cdot ml^{-1}$) and WREE-HB (0.041 $mg \cdot ml^{-1}$). Obviously, the DPPH radical-scavenging activity of all three WREE was stronger than that of chestnut shell extracts (IC_{50} = 0.044 $mg \cdot ml^{-1}$) [25]. The polyphenols in WREE may transform the DPPH radical into its reduced form (DPPH-H) through

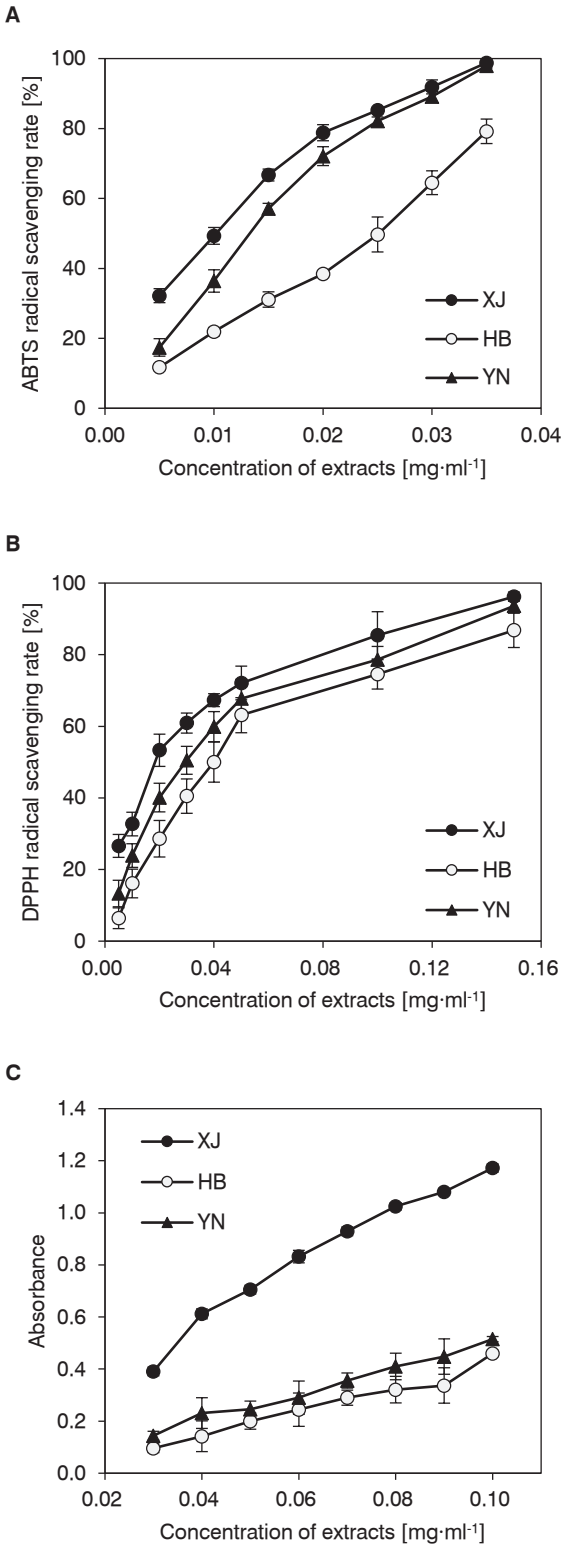

Fig. 1. Antioxidant ability of walnut residues.

A – ABTS radical-scavenging rate, B – DPPH radical-scavenging rate, C – reducing power.
Values represent mean ± standard deviation (n = 3).
ABTS – 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); DPPH – 2,2-diphenyl-1-picrylhydrazyl.
XJ – Xinjiang province, HB – Hubei province, YN – Yunnan province.

donating the electrons or hydrogen atoms [26]. In addition, high correlation coefficient was observed between total phenolics content and DPPH radical-scavenging activity in WREE (Tab. 2).

Reducing power

Reducing power is another significant indicator to estimate the antioxidant activity [27]. The results of analysis (Fig. 1C) suggested that the reducing power of the WREE increased with the concentration. However, compared to WREE-HB and WREE-YN, WREE-XJ possessed the highest reducing power. Reducing power of WREE may be attributed to phenolics, which are good electron donors and can terminate the radical chain reaction by converting free radicals to more stable products [28]. Indeed, the results demonstrated that WREE showed strong reducing power at concentrations of 0.10 mg·ml^{-1}, which was even more than that of vitamin E (A_{700} = 0.184 at 0.10 mg·ml^{-1}). Our results are in good agreement with those of Labuckas et al. [29], who showed that the antioxidant activity was concomitant with the development of reducing power.

Correlation analysis

It has been reported that total phenolics content and antioxidant activity in vitro were good indicators of the antioxidant properties of plant extracts [30]. Tab. 1. combined with Tab. 2 show correlations between the total phenolics and antioxidant activity of WREE. Overall, all the extracts from walnut residues showed excellent radical-scavenging capacity and the correlation coefficients established were good (R^2 = 0.964–0.997). In addition, significant differences were observed among the three WREE samples in antioxidant activity assays (P < 0.05). Positive correlations between total polyphenols and antioxidant activity values were shown in WREE sample from the same region, which was reported previously also for other nut species [31]. Another previous study showed that the numbers of hydroxyl groups in phenolic compounds might affect the radical-scavenging activity [32], which indicated that the chemical nature and content of phenolics present in each walnut extract may have a profound effect on their antiradical activity.

Identification of polyphenolic species

The composition of polyphenols of WREE-XJ with the highest polyphenol content and antioxidant activity was determined through high-resolution mass spectrometry analysis. Indeed, various polyphenolic and phenolic acid compounds were detected, for example, pyrogallic

Tab. 2. Antioxidant parameters of walnut residues.

Assay	Parameter	Walnut residue ethanolic extract		
		Xinjiang province	Hubei province	Yunnan province
ABTS radical-scavenging	IC_{50} [mg·ml^{-1}]	0.010 ± 0.000 ^{c}	0.023 ± 0.001 ^{a}	0.013 ± 0.000 ^{b}
	R^2	0.997	0.984	0.990
DPPH radical-scavenging	IC_{50} [mg·ml^{-1}]	0.019 ± 0.00 ^{b}	0.041 ± 0.01 ^{a}	0.029 ± 0.004 ^{ab}
	R^2	0.982	0.987	0.994
Total reducing power	Absorbance	1.17 ± 0.01 ^{a}	0.46 ± 0.02 ^{c}	0.52 ± 0.05 ^{b}
	R^2	0.971	0.967	0.988

Values represent mean ± standard deviation (n = 3). Different letters in superscript within a column indicate significant difference ($P < 0.05$). Absorbance values at polyphenol concentration 0.10 mg·ml^{-1}.
ABTS – 2,2'-azino-*bis*(3-ethylbenzothiazoline-6-sulfonic acid), DPPH – 2,2-diphenyl-1-picrylhydrazyl, IC_{50} – half maximal inhibitory concentration, R – correlation coefficient.

acid (*m*/*z* 125.0109) [33], gallic acid (*m*/*z* 169.1266) [33–36], caffeic acid (*m*/*z* 179.0535) [33, 34], syringic acid (*m*/*z* 197.0399) [33] and sinapic acid (*m*/*z* 224.9270) [37, 38]. In addition, a few gallic acid derivatives were identified, such as gallic acid derivative (*m*/*z* 186.9083) [33], monogalloyl-glucose (*m*/*z* 331.0591) [35, 41], gallotannin (*m*/*z* 633.0666) [35] and gallic acid derivative (*m*/*z* 740.1689) [38]. Digalloyl-hexahydroxydiphenoyl-glucose (digalloyl-HHDP-glucose) (*m*/*z* 785.0553) [35] was identified in WREE-XJ by accurate mass measurements, together with quercetin (*m*/*z* 301.2179) [36], quercetin pentoside (m/z 433.0329) [38–40], quercetin derivative (*m*/*z* 501.1672) [36] and quercetin-3-*O*-6-malonylglucoside (*m*/*z* 549.1522) [36]. Further, the WREE-XJ samples contained several polyphenol derivatives with pentose or hexose moieties attached to aglycone, for example, monogalloyl-glucose, quercetin pentoside, luteolin hexoside (*m*/*z* 447.1394) [40–42], HHDP-glucose (*m*/*z* 481.0534) [35], caffeoyl-hexose-rhamnose (*m*/*z* 487.1518) [40] and digalloyl-HHDP-glucose. Additionally, ellagic acid (*m*/*z* 301.2179) [36] and ellagic acid pentoside were identified in the WREE-XJ through the mass charge ratio. Monomer procyanidins (catechin or epicatechin, *m*/*z* 289.0746) [38, 40] and other flavonoids (luteolin, *m*/*z* 285.0480) [39] were identified. Du et al. [42] suggested that procyanidins possessed excellent antioxidant activity, which may be related to the antioxidant activity of WREE.

CONCLUSIONS

Walnut residues, which are traditionally considered as disposable by-products of walnut oil extraction, can be a good source of carbohydrates and antioxidants. The walnut residues from the southern region of China (YN) exhibited highest carbohydrate contents, which might be developed to fertilizers to providing carbon source for plants. In addition, XJ walnut residues showed the highest antioxidants content and their extract revealed the best antioxidant activity (significantly lowest IC_{50} values; $P < 0.05$). What's more, the XJ walnut residue ethanolic extracts were rich in various phenolic substances and their corresponding carbonyl compounds (glycosides), such as procyanidins, gallic acids, ellagic acids and quercetin. The extracts exhibited excellent antioxidant activity as well, which was realized by scavenging free radicals. From our results we can conclude that walnut residues, in particular from Xinjiang region, can be taken as natural antioxidants with potential benefits for human or animal health after further research on their bioavailability and metabolic conversion.

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