Composition, cytotoxicity and antioxidant activities of polyphenols in the leaves of star anise (*Illicium verum* Hook. f.)

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ABSTRACT: The polyphenols in the leaves of star anise (*Illicium verum* Hook. f.) were qualitatively and quantitatively determined by LC-Q-TOF-MS and LC-MS/MS. Twelve flavones and a phenolic acid were identified, mainly comprising vitexin-2"-O-rhamnoside (8780.12 µg/g) and vitexin-2"-O-xylopyranoside (575.46 µg/g), which have high free radical scavenging abilities and strong lipid peroxidation inhibitory activities. In addition, they could also reduce reactive oxygen species in HT-29 cells without cytotoxicity.

KEYWORDS: *Illicium verum* Hook. f., polyphenols, vitexin-2"-O-rhamnoside, antioxidant activities, intracellular antioxidation

INTRODUCTION

Star anise (*Illicium verum* Hook f.) is an economic tree species, belonging to the *Illicium* genus of the Illiciaceae family and is distributed mainly in Vietnam, Thailand and southern China[1]. Its fruit is a famous spice. Seven compounds comprising flavones, such as naringenin-4"-O-β-D-glucopyranoside, quercitin-3-OL-rhamnose, and benzeneethanol-4-O-β-D-glucopyranoside were isolated and were obtained from *I. verum* plants for the first time[2]. In addition, Yi et al separated three flavone glycosides from *I. verum* leaves, including quercetin, kaempferol, and isorhamnetin, by high-performance liquid chromatography[3]. Sy and Brown discovered novel phenylpropanoids and lignans from *I. verum*, including some phenolics[4]. These compounds had significant antioxidant and anti-inflammatory properties[5]. As a by-product, *I. verum* leaves are abundant, but poorly utilized, resulting in wasted resources.

To the best of our knowledge, there has been no systematic report on the polyphenols of *I. verum* leaves and their antioxidant activities. In the present study, polyphenols in *I. verum* leaves were identified and quantified via LC-Q-TOF-MS and LC-MS/MS. Antioxidant activities were determined by chemical and cellular modelling methods. The relationship between the structure of the polyphenol and the antioxidant activity was also evaluated.

MATERIALS AND METHODS

Plant Materials

The fresh *I. verum* leaves were collected from Hechi City, Guangxi Province, China on September 2014 and verified by Professor Xie Junbo (School of Chinese Materia Medica, Tianjin University of Traditional Chinese Medicine, Tianjin, China).

Extraction, Purification, and Analysis of polyphenols from *I. verum* leaves

The polyphenols were extracted according to the following process. 5 g of ground leaf powder and 130 ml of 67% ethanol (V:V) were mixed using microwave-assisted extraction. The conditions were as follows: extraction time 5 min, microwave power 500 W and extraction temperature 75 °C. The resulting extract was vacuum filtered and further extracted three times with isopycnic petroleum ether to remove the fat-soluble components. The extract was concentrated and freeze-dried. According to our previous study[6], this crude extract was purified and separated by macroporous adsorption resin D101 and preparative chromatography (Grace

Reveleris[TM] system, Alltech, US), then analysed qualitatively and quantitatively by LC-MS/MS.

Validation of assay

The limit of detection (LOD) was detected by measuring the signal-to-noise ratio (S/N ratio) 3 times, while the limit of quantification was detected by measuring the S/N ratio 10 times. The accuracy of the method was evaluated by analysing six replicates of mixed standard solutions or samples and expressed as the relative standard deviation (RSD). The accuracy of the method is assessed by a recovery test. An appropriate amount of the mixed standard solution was added to the purified polyphenol extract solution. Subsequently, the mixture was processed and analysed according to the method described by Yang et al[6].

Antioxidant activity analyses

Various aspects of antioxidant activities were evaluated by chemical methods[7–9], including superoxide anion radical scavenging activities, the lipid peroxidation inhibition activities, the hydroxyl radical scavenging activities, the total antioxidant activities and the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activities. Compared to chemical methods, the cell model method has a better biological correlation and could more accurately predict the antioxidant activity of antioxidants in the human body. Hence human colon cancer cells (HT-29 cells) were chosen to simulate an in vivo environment to study the intracellular antioxidant activities of polyphenols. HT-29 cells were obtained from the Institute of Biochemistry and Cell Biology (Shanghai, China) and there was no mycoplasma contamination according to the mycoplasma test method[10]. Cell viability and intracellular antioxidation were measured according to the previous method described by both Schantz et al[11] and Yang et al[6]. The intracellular antioxidant activity (CAA) was calculated according to the following equation: CAA(%) = 100-($\int$ SA/$\int$ CA) × 100, fluorescence intensities were measured every 5 minutes during the 1-h incubation time, $\int$ SA and $\int$ CA are the integral areas of the relative fluorescence intensity of the sample and control[11, 16].

Statistical Analysis

Student's *t*-test was performed to compare the differences between total polyphenols and vitexin-2"-O-rhamnoside at the same concentration in the antioxidation assay. Cell viability and intracellular antioxidation were compared by one-way ANOVA.
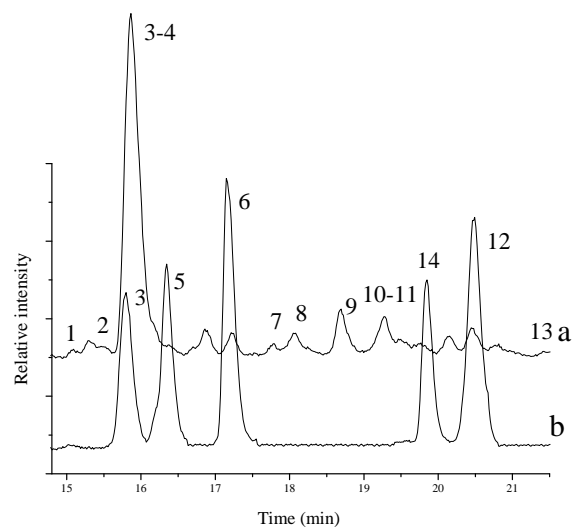

Fig. 1 Total ion chromatograms of (a) the purified polyphenols extracted from *I. verum* leaves and (b) the mixture of reference substances. Peak numbers refer to Table 1.

All statistical analyses were performed by SPSS (version 16.0). All results were based on three replicates indicated as mean values and standard deviations.

RESULTS AND DISCUSSION

Qualitative Analysis

Under the same analytical conditions, the retention time, molecular ion and MS[2] spectrum (peaks 3, 5, 6, 7, and 12) identified five components with standard substances (Fig. 1a and Fig. 1b). The other peaks in Fig. 1a were determined by MS/MS and comparison with Refs. 12–20. Twelve flavones and a phenolic acid have been identified and the results are presented in Table 1. The content and variety of the identified polyphenols were different from previously reported data[2–4], which could be explained by the different origins and sources of the collected plant. The collected plant can be age or tissue-specific. The other eight components that do not contain available standard substances could be distinguished by relevant pieces of literature, but they deserve further study once standards are available.

Quantitative Analysis

Five different concentrations of standard substances were analysed by LC-MS/MS for the quantification of polyphenols. Table 2 shows the calibration equations of the five components and the contents

Table 1 Retention times and fragment ions of components and standard substances in *I. verum* leaves.

Peak	Compound	[M−H]⁻ (m/z)	LC-Q-TOF-MS RT (min)	LC-Q-TOF-MS MS² (m/z)	LC-MS/MS RT (min)	LC-MS/MS MS² (m/z)
1	Vitexin-4″-O-glucoside	593	15.09	413, 293	10.80	413, 293
2	P-coumaroylquinic acids	337	15.41	191, 173	10.85	191, 173
3	Vitexin-2″-O-rhamnoside	577	15.58	413, 311, 293	11.86	413, 311, 293
4	Vitexin-2″-O-xylopyranoside	563	16.00	413, 311, 293	12.08	413, 311, 293
5	Rutin	609	16.46	301 300	13.69	301, 300
6	Vitexin	431	17.24	311, 283, 161	12.41	341, 311, 283
7	Hyperoside	463	17.83	300, 271, 255	13.96	300, 271, 255
8	Isorhamnetin-3-O-rutinoside	623	18.03	315, 314, 299	14.02	315, 314, 299
9	Kaempferol-7-glucoside	447	18.94	285, 255, 227	15.39	285, 255, 227
10	Quercetin-3-O-xylopyranoside	433	19.53	300, 271, 255	14.87	300, 271, 255
11	Isorhamnetin-3-O-glucoside	477	19.55	314, 300, 271	15.66	314, 271
12	Genistin	431	20.42	268	15.18	268
13	Kaempferol-3-O-arabinoside	417	21.46	285, 255, 227	16.33	285, 255, 227
14	Avicularin	433	20.08	300, 271, 255	15.04	300, 271, 255

Table 2 Quantification results of components in *I. verum* leaves.

Peak	Compound	Calibration curve	Linear range (µg/ml)	R^2	Content[a] (µg/g)	Recovery[a] (%)
1	vitexin-4″-O-glucoside	–	–	–	71.4 ± 2.0[b]	–
2	3-p-coumaroylquinic acids	–	–	–	112.6 ± 3.3[b]	–
3	vitexin-2″-O-rhamnoside	$Y = -42\,449.72 + 19\,565.48X$	0.1–10.0	0.998	8780 ± 185	105.2 ± 3.2
4	vitexin-2″-O-xylopyranoside	–	–	–	575 ± 13[b]	–
5	rutin	$Y = -3392.61 + 17\,337.44X$	0.05–6.0	0.998	38.8 ± 1.7	107.3 ± 2.6
6	vitexin	$Y = 108\,442.17 + 11\,324.55X$	0.1–4.0	0.995	20.7 ± 0.6	103.8 ± 3.4
7	hyperoside	$Y = 169\,852.67 + 15\,585.71X$	0.05–6.0	0.999	10.7 ± 0.1	98.7 ± 1.3
8	isorhamnetin-3-O-rutinoside	–	–	–	23.0 ± 0.3[b]	–
9	kaempferol-7-glucoside	–	–	–	7.3 ± 0.3[b]	–
10	quercetin-3-O-xylopyranoside	–	–	–	43.8 ± 1.3[b]	–
11	isorhamnetin-3-O-glucoside	–	–	–	10.5 ± 0.2[b]	–
12	genistin	$Y = 71\,095.55 + 18\,714.78X$	0.05–6.0	0.999	4.4 ± 0.1	93.8 ± 3.8
13	kaempferol-3-O-arabinoside	–	–	–	9.2 ± 0.2[b]	–

[a] Mean ± SD of three replicates;
[b] Expressed in the equivalents of vitexin.

of 13 components. Although vitexin has a structure similar to the eight non-standard components available and the eight-component content has been expressed as equivalents of the quantitative method of vitexin equivalence, they deserve further study once standards become available.

Validation of assay

LOD and the limit of quantitation (LOQ) were 0.10–14.80 ng/ml and 0.33–49.41 ng/ml, respectively. Correlation coefficients (R^2), recoveries and RSD values indicating satisfactory reliability and accuracy of the current method are presented in Table 2.

Separation of extract from *I. verum* leaves

The polyphenols were separated and collected by macroporous resin and preparative chromatography. The main components accounting for 96.3% of the total polyphenols were vitexin-2″-O-rhamnoside (90.4%) and vitexin-2″-O-xylopyranoside (5.9%). The structures of the two compounds were shown in Fig. 2, illustrating rings A and B.

Antioxidant activity analyses

All the antioxidant activities measured in this study were concentration-dependent (Fig. 3). In the present study, there was a linear relationship between absorption and antioxidant concentration of
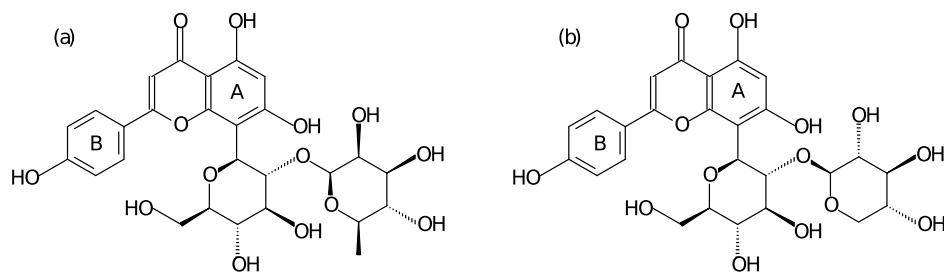
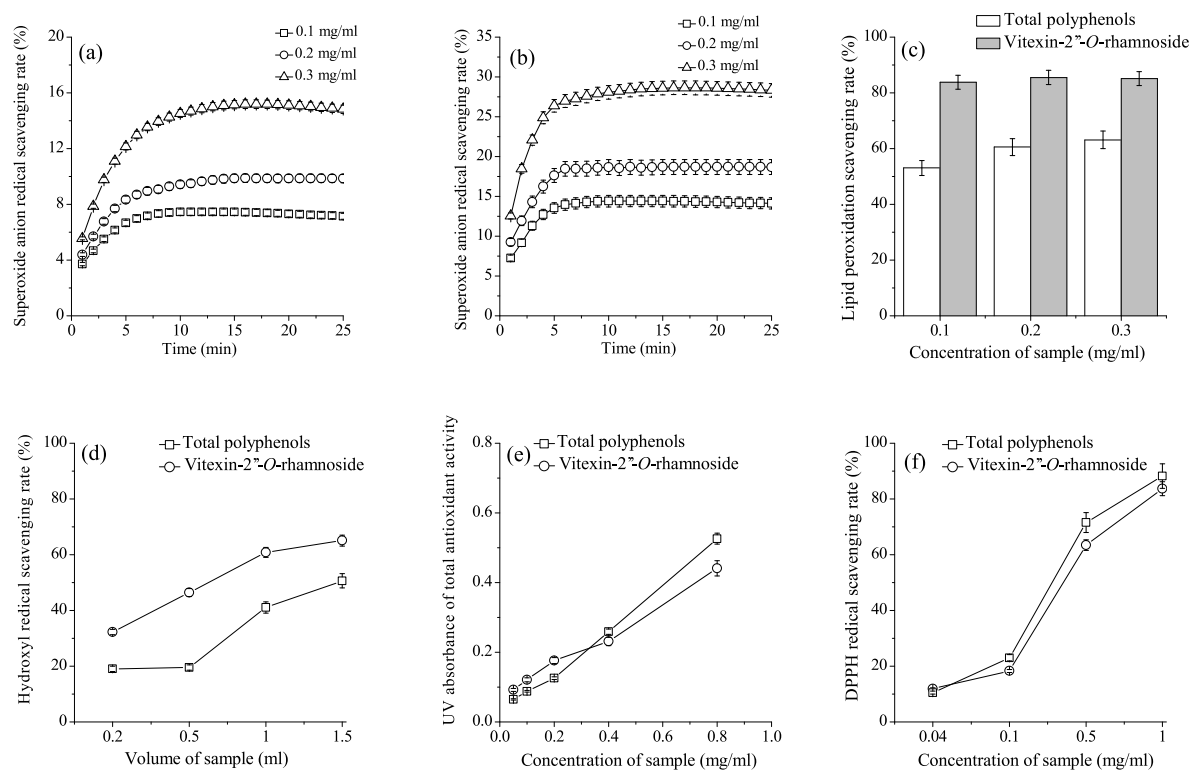**Fig. 2** The structures of (a) vitexin-2"-O-rhamnoside and (b) vitexin-2"-O-xylopyranoside.

Fig. 3 Superoxide anion scavenging activity of (a) total polyphenols and (b) vitexin-2"-O-rhamnoside; (c) lipid peroxidation inhibition activity, (d) hydroxyl radical scavenging activity, (e) total antioxidant activity, and (f) DPPH radical scavenging activity of both total polyphenols and vitexin-2"-O-rhamnoside. Data are reported as the mean $\pm$ SD of three replicates.

0–3 min for superoxide anion scavenging (Fig. 3a and Fig. 3b). The antioxidant capability of vitexin-2"-O-rhamnoside was higher than that of total polyphenols (t-test, $p < 0.05$), but the two capabilities of vitexin-2"-O-rhamnoside and polyphenols totals were low (Fig. 3a and Fig. 3b). This could be explained by the glycoside groups of vitexin-2"-O-rhamnoside which could introduce steric effects, thereby reducing the scavenging activity of superoxide anion radicals[21].

The effects of the lipid peroxidation inhibitory activity are shown in Fig. 3c the scavenging rate

of vitexin-2"-O-rhamnoside was significantly higher than that of total polyphenols (t-test, $p < 0.05$). This result indicated that vitexin-2"-O-rhamnoside had high scavenging activity even at low concentrations, possibly due to the 4-ketonic group and the 5-hydroxyl group structure in vitexin-2"-O-rhamnoside could chelate Fe^{2+}, while the 4-ketonic group also facilitates the formation of more stable free radical intermediates, which reduces the reaction strength[22].

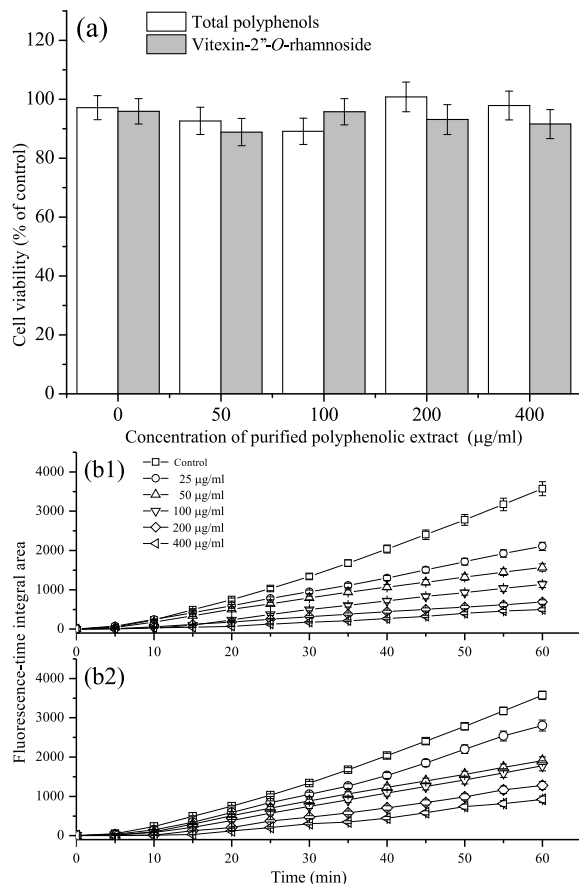For hydroxyl radical scavenging (Fig. 3d), vitexin-2"-O-rhamnoside had the highest scaveng-

Fig. 4 Effects of total polyphenols and vitexin-2"-O-rhamnoside on (a) cell viability and (b1 and b2) intracellular radical scavenging activities in HT-29 cells. Data are reported as the mean ± SD of three replicates.

ing activity (IC_{50} = 0.06 mg/ml) relative to total polyphenols (IC_{50} = 0.15 mg/ml). A 4-OH group on B-ring is important for antioxidants, while the 5-OH and 7-OH groups on A-ring could bind to transition metals to form complexes, such as Fe^{2+}, to block the reaction of Fenton[23]. Hence vitexin-2"-O-rhamnoside exhibited high scavenging activity of hydroxyl radicals.

The results showed that the total antioxidant activity increased rapidly with increasing sample concentration (Fig. 3e), and the polyphenol content was more pronounced than that of vitexin-2"-O-rhamnoside. This may be due in part to total polyphenols, which are a mixture of all antioxidant components such as rutin and quercetin-3-O-glucopyranoside. Their pyrocatechol structure on the B-ring increases the antioxidant activity of the total polyphenols[24].

The DPPH scavenging activity increased as the concentration of total polyphenols and vitexin-2"-O-rhamnoside increased from 0.04–1.0 mg/ml (Fig. 3f). The IC_{50} values were 0.32 mg/ml and 0.31 mg/ml, respectively. These results indicate that there is no significant difference in DPPH free radical scavenging capacity between total polyphenols and vitexin-2"-O-rhamnoside solution in the range of 0.04–1.0 mg/ml.

Cytotoxicity and anti-oxidative intracellular effects of polyphenolic compounds were evaluated in HT-29 cells. The results showed that there was no significant difference in HT-29 cell viability between total polyphenols and vitexin-2"-O-rhamnoside at different concentrations. Fig. 4a shows that they had no cytotoxic effect until the test concentration was 800 μg/ml.

For the intracellular free radical scavenging activities (Fig. 4b1 and Fig. 4b2), the average fluorescence intensity of the control group treated with 200 μmol/l of H_2O_2 was almost eight times that of the blank. Total polyphenols and vitexin-2"-O-rhamnoside significantly reduced average fluorescence intensity compared to the control group. The CAA value of total polyphenols increased from 39.47 to 86.39, while the vitexin-2"-O-rhamnoside increased from 21.50 to 74.12. At the same concentration, the value of total polyphenols was higher than vitexin-2"-O-rhamnoside (by t-test, $p < 0.05$). Vitexin-2"-O-rhamnoside had high activity for scavenging ROS in cells, attributed to the carbon 2, 3-double bond on the B-ring of the vitexin-2"-O-rhamnoside structure, which may extend the conjugated system and significantly protect cells against H_2O_2-induced damage[25].

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