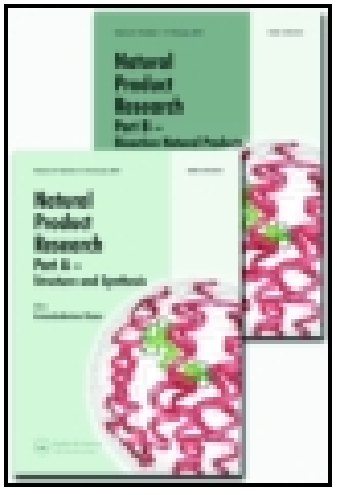

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Efficient quantification of the phenolic profiles of Zanthoxylum bungeanum leaves and correlation between chromatographic fingerprint and antioxidant activity

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SHORT COMMUNICATION

Efficient quantification of the phenolic profiles of *Zanthoxylum bungeanum* leaves and correlation between chromatographic fingerprint and antioxidant activity

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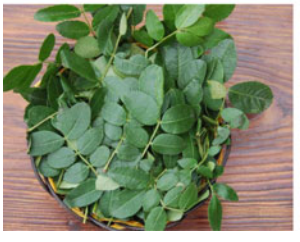

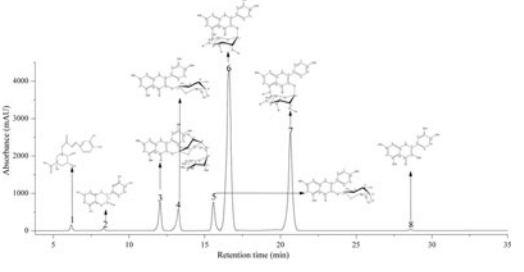

Sixteen subsequent fractions were prepared from the ethyl acetate fraction of *Zanthoxylum bungeanum* leaves after bio-guided chromatographic separation. The HPLC profiles and antioxidant activity of the various fractions indicated that the content of eight phenolic compounds (chlorogenic acid, epicatechin, rutin, hyperoside, trifolin, quercitrin, afzelin and quercetin) and antioxidant activity vary significantly, and high concentrations of a combination of eight phenolic compounds would result in an increase of the antioxidant activity. These results suggested that the eight compounds could be used as chemical markers for quality assessment of *Z. bungeanum* leaves. Correlation between chromatographic fingerprint and antioxidant activity of the fractions showed that quercitrin and hyperoside play crucial roles in the antioxidant activity, and they can be seen as the milestone for quality control. The findings also suggested that five obtained fractions (E-3-3, E-2-4, E-7, E-5 and E-4) could become useful supplements for functional food ingredients and health-related products.

Keywords: *Zanthoxylum bungeanum* leaves; phenolic profiles; antioxidant activity; chromatographic fingerprint; HPLC-UV

1. Introduction

Zanthoxylum bungeanum is an aromatic tree and shrub plant of the family Rutaceae. The leaves of *Z. bungeanum* are commonly consumed as condiments and vegetables in China because of their special flavour properties (Yang 2008). Recent literatures indicated that *Z. bungeanum* leaves possessed rich polyphenolics (Xu & Fan 2010) and 11 phenolic compounds were identified from it by HPLC-ESI-MS/MS (Yang et al. 2013). In our previous studies, the ethyl acetate fraction (EAF) from the leaves of *Z. bungeanum* was selected as the most active fraction with the highest level of total phenolics and significant antioxidant activities (Zhang,

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Luo, et al. 2014); based on this result, we bio-guided isolated and characterised nine flavonoids, including quercetin, kaempferol, isorhamnetin glycosides and C-glycoside flavonol from the leaves of *Z. bungeanum* (Zhang, Wang, et al. 2014). In addition, simultaneous quantification of 12 major bioactive chemicals in the leaves was established. In this study, a bio-guided chromatographic fractionation strategy was used to obtain 16 fractions from EAF. A qualitative HPLC method was developed to compare the phytochemical profiles of various fractions of *Z. bungeanum* leaves, and the correlation between chromatographic fingerprints and antioxidant activity of the fractions was also investigated, aiming to find out which phenolic compounds would contribute most to the antioxidant activity. The HPLC fingerprint as a characteristic distinguishing method combining antioxidant activities and quantification analysis can be successfully used to assess the quality of *Z. bungeanum* leaves and related products.

2. Results and discussion

2.1. *Antioxidant activities of various fractions*

Previous research in our laboratory proved that EAF was the most active fraction with powerful antioxidant activities (Zhang, Luo, et al. 2014). Thus, a bioassay-guided chromatographic fractionation was conducted on EAF to produce seven sub-fractions (E-1–E-7, Figure S1). The antioxidant activities of the seven sub-fractions were summarised in Table 1. Among these fractions, the IC_{50} value of E-7 ($4.85 \pm 0.27\ \mu g\ ml^{-1}$) was the lowest, indicating the highest level of DPPH radical scavenging ability, followed by E-4 and E-5 (14.08 ± 0.61 and $16.22 \pm 0.21\ \mu g\ ml^{-1}$, respectively, $p < 0.05$). The seven sub-fractions from EAF also exhibited varying abilities to neutralise the radical cation $ABTS^{+}$. E-4, E-5 and E-7

Table 1. Antioxidant activity of various fractions from *Z. bungeanum* leaves.

Samples		Yield (%)	DPPH IC_{50} ($\mu g\ ml^{-1}$)	FRAP ($\mu mol\ equiv\ trolox\ g^{-1}$)	ABTS ($\mu mol\ equiv\ trolox\ g^{-1}$)
EAF	E-1	15.38	400.57 ± 9.25^{g}	600.37 ± 23.13^{f}	2393.83 ± 39.88^{g}
	E-2	30.69	36.73 ± 4.33^{e}	1080.62 ± 41.30^{e}	8442.20 ± 60.92^{ef}
	E-3	37.94	23.53 ± 0.07^{d}	1413.95 ± 31.50^{de}	16364.91 ± 121.83^{b}
	E-4	3.64	14.08 ± 0.61^{c}	1526.30 ± 28.93^{d}	18917.19 ± 219.64^{ab}
	E-5	2.68	$16.22 \pm 0.\ 21^{c}$	1644.81 ± 16.97^{cd}	19661.61 ± 143.79^{a}
	E-6	6.10	$16.34 \pm 0.\ 44^{c}$	1347.28 ± 66.70^{de}	11725.61 ± 398.79^{d}
	E-7	0.71	4.85 ± 0.27^{b}	1731.23 ± 18.27^{c}	18172.78 ± 179.83^{ab}
E-2	E-2-1	40.93	33.15 ± 1.05^{de}	1343.58 ± 63.54^{de}	6961.02 ± 194.83^{ef}
	E-2-2	22.21	29.15 ± 0.63^{d}	1749.75 ± 134.68^{c}	13757.18 ± 222.47^{c}
	E-2-3	3.37	28.84 ± 3.00^{d}	937.41 ± 38.67^{e}	3961.60 ± 215.89^{g}
	E-2-4	11.74	3.86 ± 1.09^{ab}	2676.91 ± 170.63^{b}	9871.85 ± 79.04^{e}
	E-2-5	7.91	7.97 ± 0.64^{bc}	1832.47 ± 63.11^{c}	7062.27 ± 133.34^{f}
E-3	E-3-1	5.37	75.51 ± 1.55^{f}	760.86 ± 16.70^{f}	2658.05 ± 79.04^{g}
	E-3-2	38.10	41.01 ± 1.20^{e}	774.44 ± 41.24^{f}	6771.18 ± 95.55^{f}
	E-3-3	44.68	2.01 ± 0.04^{a}	7901.60 ± 233.67^{a}	20173.67 ± 21.92^{a}
	E-3-4	6.87	7.03 ± 0.26^{b}	2171.98 ± 51.90^{bc}	9454.21 ± 133.34^{c}
Standard compounds	Curcumin	–	2.53 ± 0.04^{a}	1747.28 ± 28.77^{c}	18983.66 ± 39.88^{ab}
	Quercetin	–	2.63 ± 0.02^{a}	1865.80 ± 33.40^{c}	20113.58 ± 23.20^{a}
	Rutin	–	9.53 ± 0.11^{bc}	1722.59 ± 6.42^{c}	20153.46 ± 46.05^{a}
	Ascorbicacid	–	2.70 ± 0.01^{a}	1855.93 ± 13.35^{c}	20166.75 ± 23.02^{a}

Notes: Values are the mean of three replicates ± SD ($n = 3$). Means with different letters within a column were significantly different ($p < 0.05$).

(18917.19 ± 219.64, 19661.61 ± 143.79, 18172.78 ± 179.83 μmol equiv. Trolox g^{-1}, $p < 0.05$) showed the most effective $ABTS^{+}$ scavenging abilities, which were not significantly different with the reference compounds. In the FRAP assay, the same trends were found in the reduction of Fe^{+3}/ferric cyanide complex to the ferrous form, that is, E-4, E-5 and E-7 (1526.30 ± 28.93, 1644.81 ± 16.97 and 1731.23 ± 18.27 μmol equiv. Trolox g^{-1}, $p < 0.05$) exhibited good reducing abilities and were not significantly different with the references. In summary, E-4, E-5 and E-7 were the most active fractions obtained from EAF, thus further separation should be conducted on these fractions. However, for the low yields of E-4, E-5 and E-7 (3.64, 2.68 and 0.71%), they were not further separated in the next work. Due to the better antioxidant activity and higher yields (30.69% and 37.94%), E-2 and E-3 were further fractioned to produce five (E-2-1–E-2-5) and four sub-fractions (E-3-1–E-3-4), respectively (Figure S1). E-3-3 showed the highest antioxidant abilities, with the highest ABTS (20173.67 ± -21.92 μmol equiv. Trolox g^{-1}), DPPH radical scavenging (2.01 ± 0.04 μg ml^{-1}) and reducing ability (7901.60 ± 233.67 μmol equiv. Trolox g^{-1}), which were even better than the references. E-2-4 also exhibited stronger antioxidant activities, with significant ABTS (9871.85 ± -79.04 μmol equiv. Trolox g^{-1}), DPPH radical scavenging (3.86 ± 1.09 μg ml^{-1}) and reducing ability (2676.91 ± 170.63 μmol equiv. Trolox g^{-1}).

2.2. *Total phenolic and flavonoids content of various fractions*

In the seven sub-fractions of EAF, the total flavonoid and phenolic content of E-5 (137.60 ± 13.56 mmol QE 100 g^{-1}, 230.02 ± 5.86 mmol GAE 100 g^{-1}), E-4 (132.42 ± 5.74 mmol QE 100 g^{-1}, 236.62 ± 4.11 mmol GAE 100 g^{-1}) and E-7 (128.64 ± 14.57 mmol QE 100 g^{-1}, 184.44 ± 6.53 mmol GAE 100 g^{-1}) were high and not significantly different from each other. In the five sub-fractions of E-2, E-2-2 had the highest total flavonoid (164.93 ± 7.22 mmol QE 100 g^{-1}) and phenolic (270.06 ± 12.80 mmol GAE 100 g^{-1}) content combined, not significantly different from E-2-4 (178.71 ± 13.15 mmol QE 100 g^{-1}, 207.06 ± 12.80 mmol GAE 100 g^{-1}). In the four sub-fractions of E-3, total flavonoid (247.56 ± 13.15 mmol QE 100 g^{-1}) and phenolic content (668.75 ± 19.22 mmol GAE 100 g^{-1}) of E-3-3 were the highest of all (Figure S1, Table S1).

2.3. *Content of eight phenolic compounds of various fractions*

In this study, the proposed HPLC method was successfully applied to the simultaneous determination of the eight phenolic compounds in the fractions of *Z. bungeanum* leaves. The identity of the marker compounds peaks in the chromatogram was confirmed by their retention times and their UV profiles (Figure S2). Also, a previous study in our laboratory proved the existence of these marker compounds in *Z. bungeanum* leaves (Zhang, Wang, et al. 2014). The contents of the eight chemical markers in various fractions of *Z. bungeanum* leaves are summarised in Table 2. In the seven sub-fractions of EAF, E-7 had the highest content of chlorogenic acid and epicatechin (36.75 ± 1.10, 729.30 ± 0.22 mg g^{-1}). E-4 had the highest content of rutin (51.73 ± 0.14 mg g^{-1}), E-5 had the highest hyperoside (396.08 ± 1.23 mg g^{-1}), E-3 had the highest content of trifolin and quercitrin (70.46 ± 6.24, 421.28 ± 0.90 mg g^{-1}) and E-2 had the highest content of afzelin and quercetin (112.12 ± 0.41, 54.58 ± 1.28 mg g^{-1}). From the five sub-fractions of E-2, E-2-4 had the highest content of quercetin (363.36 ± 1.83 mg g^{-1}), while E-2-2 had the highest content of afzelin (758.34 ± 1.13 mg g^{-1}). From the four sub-fractions of E-3, E-3-3 had the highest content of quercitrin (871.13 ± 7.64 mg g^{-1}), while E-3-4 had the highest content of trifolin (206.76 ± 2.82 mg g^{-1}). These results suggested that some compounds were gathered in different fractions due to the enrichment effects during chromatographic fractionation.

Table 2. Content of eight phenolic compounds of various fractions from *Z. bungeanum* leaves.

Samples		Content (mg g^{-1})							
		Chlorogenic acid	Epicatechin	Rutin	Hyperoside	Trifolin	Quercitrin	Afzelin	Quercetin
EAF	E-1	ND	ND	ND	ND	ND	ND	5.38 ± 0.56^{l}	20.74 ± 0.07^{f}
	E-2	ND	66.80 ± 8.36^{d}	ND	22.49 ± 0.01^{e}	ND	13.78 ± 0.43^{kl}	112.12 ± 0.41^{c}	54.58 ± 1.28^{c}
	E-3	ND	ND	ND	ND	70.46 ± 6.24^{c}	421.28 ± 0.90^{c}	93.66 ± 0.23^{d}	ND
	E-4	ND	ND	51.73 ± 0.14^{a}	249.51 ± 3.06^{b}	61.18 ± 0.47^{d}	65.41 ± 0.13^{f}	9.80 ± 0.70^{j}	ND
	E-5	ND	ND	39.66 ± 0.16^{b}	396.08 ± 1.23^{a}	36.06 ± 0.28^{e}	42.42 ± 0.92^{h}	8.13 ± 0.06^{k}	ND
	E-6	ND	ND	9.28 ± 0.48^{g}	173.15 ± 1.87^{c}	14.25 ± 1.00^{f}	25.71 ± 0.10^{j}	3.69 ± 0.07^{m}	24.20 ± 1.42^{e}
	E-7	37.65 ± 1.10^{a}	729.30 ± 0.22^{a}	22.25 ± 0.20^{d}	99.53 ± 0.20^{d}	6.37 ± 0.24^{g}	29.55 ± 0.54^{j}	5.78 ± 0.23^{l}	40.70 ± 2.49^{d}
E-2	E-2-1	ND	ND	ND	ND	2.14 ± 0.13^{g}	12.60 ± 0.35^{klm}	ND	26.03 ± 0.34^{e}
	E-2-2	ND	ND	ND	ND	2.45 ± 0.02^{g}	35.47 ± 0.46^{i}	758.34 ± 1.13^{a}	9.24 ± 0.24^{g}
	E-2-3	ND	ND	ND	ND	ND	6.81 ± 0.02^{mn}	28.32 ± 0.30^{f}	5.88 ± 0.21^{h}
	E-2-4	ND	311.97 ± 5.86^{b}	ND	30.07 ± 0.08^{e}	3.68 ± 0.05^{g}	39.87 ± 0.52^{hi}	11.73 ± 0.91^{i}	363.36 ± 1.8^{a}
	E-2-5	ND	277.19 ± 5.38^{c}	ND	28.68 ± 0.19^{e}	2.97 ± 0.23^{g}	26.95 ± 0.38^{j}	2.05 ± 0.15^{n}	141.32 ± 3.9^{b}
E-3	E-3-1	ND	ND	ND	ND	5.16 ± 0.04^{g}	9.08 ± 0.11^{lm}	ND	ND
	E-3-2	ND	ND	ND	ND	56.22 ± 0.22^{d}	58.46 ± 0.93^{g}	463.27 ± 0.94^{b}	9.52 ± 0.11^{g}
	E-3-3	ND	ND	ND	ND	114.48 ± 0.40^{b}	871.13 ± 7.64^{a}	6.61 ± 0.21^{l}	5.95 ± 0.13^{h}
	E-3-4	ND	ND	ND	ND	206.76 ± 2.82^{a}	717.76 ± 4.92^{b}	18.33 ± 2.07^{g}	4.00 ± 0.14^{h}

Notes: Values are the mean of three replicates ± SD ($n = 3$). Means with different letters within a column were significantly different ($p < 0.05$).

2.4. *Correlation between chromatographic fingerprint and antioxidant activity of various fractions*

Combination of fingerprints with quantitative analysis of several marker compounds for quality control of herbal plant is definitely an improvement over the old methodology (Yudthavorasit et al. 2014; Zhang, Chen, et al. 2014). The chromatographic fingerprint has predominance in showing the authenticity, quality consistency and stability of herbal plants, while the quantification of several marker compounds can better reflect the quality (Fukahori et al. 2014; He et al. 2015). Previous work proved that there exist quercetin glycosides, kaempferol glycosides, isorhamnetin glycosides and C-glycoside flavonals in *Z. bungeanum* leaves, which evidenced that a wide range of polyphenolic concentrations are responsible for its significant antioxidant activity (Zhang, Wang, et al. 2014). Chromatograms of the ECE and its five sub-fractions revealed that there were significant differences among the five fractions (Figure S3a). Peaks 1–8 were common peaks in EAF and AF. The higher contents of these phenolic compounds in EAF and AF were inferred to be responsible for their more potent antioxidant activity. Mocan and others found that the antioxidant potential of plant and fruit extracts is strongly correlated with their phytochemicals (Mocan, Crisan, et al. 2014a; Mocan, Vlase, et al. 2014b). This sustains that in our current study, a combination of the eight phenolic compounds, in different levels, would increase the antioxidant activity in the different fractions. In detail, in sub-fractions of EAF (Figure S3b), E-4, E-5 and E-7 were selected as the fractions with significant antioxidant activity. HPLC chromatograms of E-7 suggested that it was composed of different levels of peaks 1–8. Meanwhile, peaks 3–6 (rutin, hyperoside, trifolin and quercitrin) were commonly seen in chromatograms of E-4 and E-5 at high levels, whereas hyperoside (peak 4) was the highest. As for chromatograms of E-6, a decrease of peaks 2–6 and peak 8 would result in a corresponding decrease in the antioxidant activity. In sub-fractions of E-2 (Figure S3c), E-2-4 was screened as the most significant fraction with potent antioxidant activity. As compared to HPLC chromatogram of E-2-5, an increase of peak 2 and peaks 4–8 in E-2-4 would definitely result in an increase of its antioxidant activity. In chromatograms of E-2-2, a significant increase of peak 7 (afzelin) alone would not guarantee an increase on the antioxidant activity. In sub-fractions of E-3 (Figure S3d), E-3-3 was screened as the most significant. As compared to chromatograms of E-3-4 and E-3-2, an increase of peak 5 (trifolin) and peak 7 alone would not guarantee an increase in the antioxidant activity, yet a significant increase of peak 6 (quercitrin) would definitely guarantee a significant increase in the antioxidant activity. It is indicated that quercitrin was truly the phytochemical with profound antioxidant activity.

3. Conclusion

Hyperoside and quercitrin were the dominant compounds in the leaves of *Z. bungeanum*, and they were found to be the most significant radical scavengers (Zhang, Luo, et al. 2014; Zhang, Wang, et al. 2014). The higher concentrations of them in the fractions indicate that they play major roles in radical scavenging and reducing power, while other compounds, such as chlorogenic acid, epicatechin, rutin, trifolin, afzelin and quercetin, play partial roles in the antioxidant activity. The eight phenolic compounds could be used as chemical markers for the quality assessment of the *Z. bungeanum* leaves and related products, yet hyperoside and quercitrin can be seen as the milestone for the quality control. The five selected fractions (E-4, E-5, E-7, E-3-3 and E-2-4) could be used as functional food additives to promote human health and prevent oxidation-related diseases.

Supplementary material

Experimental details relating to this paper are available online, alongside Table S1 and Figures S1–S3.

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