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FLAVONOIDS CONTENT AND ANTIOXIDANT ACTIVITY OF ETHANOL EXTRACTS OF *OSMANTHUS FRAGRANS* FLOWERS

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Abstract

Flavonoids content and antioxidant activity of ethanol extracts of *Osmanthus fragrans* flowers (EEOF) were investigated. Response surface methodology (RSM) and central composite design (CCD) were applied to examine the flavonoids extraction conditions, and high performance liquid chromatography (HPLC) with diode array detection (DAD) was used to determine flavonoids contents in *Osmanthus fragrans* flowers of different regions and various cultivar groups. Antioxidant activity of EFOF from various sources was evaluated in terms of IC50 values using DPPH and ABTS assays. The data showed that Albus group and Luteus group contained higher flavonoids content, although flavonoid contents in different samples had the significant difference. And there was a significant correlation between DPPH and ABTS scavenging activity and flavonoids contents of EEOF. It is indicated that flowers of *O. fragrans* have potential applications in biomedical science and Albus group and Luteus group have higher biomedical value.

Introduction

Flavonoids, as a family of antioxidants found in fruits and vegetables as well as in popular beverages such as red wine and tea, have great potential to inhibit the generation of reactive oxygen species (ROS), and may also protect cells from various insults (Agati *et al.* 2012, Demirkiran *et al.* 2013, Ishige *et al.* 2001). Besides antioxidation, flavonoids exhibit other important physiological functions such as antibacterial, anti-cancer and anti-inflammation (Huang *et al.* 2015).

Osmanthus fragrans is considered as one of the top traditional flowers of China and is widely cultivated in many places, among which five cities (Hangzhou, Suzhou, Guilin, Chengdu, and Xianning) become the production centre for *O. fragrans*, and the flower of *O. fragrans* is of economic importance because it is used as an additive for foods, tea, and other beverages (Wang *et al.* 2009). *O. fragrans* has the biomedical functions of anti-inflammation, antioxidation, anti-tussive, nitric oxide-scavenging and nitric oxide-suppressing, neuroprotection, aromatherapy properties and melanogenesis inhibitory effect (Wu *et al*. 2009). In China, flowers of *O. fragrans*, regarded as Chinese herbal medicines, have been applied to therapy of toothache, cough and asthma (1996). *O. fragrans* cultivar groups have been investigated and classified into Asiaticus and autumn division based on flowering season; the autumn division was further divided into three groups (Albus group, Luteus group, and Aurantiacus group) according to flower and leaf traits (Han *et al.* 2014, Yan *et al.* 2009). The concrete content of flavonoids in flowers of *O. fragrans* cultivar groups from different regions is not so clear, which will influence their biomedical value.

High performance liquid chromatography (HPLC) with diode array detection (DAD), more common and less costly, currently represents the most prevalent and reliable analytical method for the characterization of flavonoids, and also plays a key role in confirmation of the analyte identity, as it enables simultaneous acquisition of chromatograms at any wavelength accompanied by the absorption spectrum of each eluted band and verification of separation quality with peak purity

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analysis (Huang *et al.* 2015, Luo *et al.* 2011, Sriseadka *et al.* 2012, Zeraik and Yariwake 2010). Consequently, a normally manageable HPLC-DAD process was proposed in this research.

Among conventional extraction method of flavonoids, achieved by heating, boiling or refluxing, ethanol exhibited the highest extraction efficiency of flavonoids among various extraction solvents (Bae *et al.* 2012). Response surface methodology (RSM) is an effective collection of mathematical statistics method for establishing models to evaluate the relative significance of variables and determine optimal conditions of desirable responses, and then RSM and central composite design (CCD) have been applied for the optimization of multiple variables to predict the best operation conditions with a minimum number of experiments (Perales-Sánchez *et al.* 2014, Huang *et al.* 2009, Reis *et al.* 2015). Here, the extraction conditions of flavonoids from *O. fragrans* were optimized by RSM and CCD.

Antioxidants are vital substances which possess the ability to protect the body from damage caused by free radical induced oxidative stress, and there is increasing interest in natural antioxidants, e.g., total phenolics, total flavonoids, which present in medicinal and dietary plants and might help prevent oxidative damage (Ozsoy *et al.* 2008). Antioxidant activity of plants extracts were often determined to evaluate medical property of plants (Wu *et al.* 2009, Perales-Sánchez *et al.* 2014). Based on analysis of flavonoids content and antioxidant activity of ethanol extracts of O. fragrans flowers, the relationship of them will be defined and the biomedical value of flowers of *O. fragrans* cultivar groups from different regions will be assessed.

Materials and methods

Flowers of *O. fragrans* were obtained from different regions and ground into dry powder, followed by sieving through 200 mesh screen to control the particle size. 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulphonate (ABTS) and rutin were purchased from Sigma Chemical Co. Trolox was purchased from MP Biomedicals. Methanol was chromatographically reagent grade. All other chemicals used in this study were of analytical reagent grade.

Flavonoids were extracted from sample under the following methods: mixed with 50, 60, 70, 80 and 90% ethanol solution, respectively and reflux extraction 1hour at 70; 50, 60, 70 and 80°C respective reflux extraction 1 - 4 hrs, 70% ethanol solution reflux extraction at 70°C and vacuum refluxing extraction for 1 hr, respectively; solid to liquid ratios of 1 : 25, 1 : 50, 1 : 100, 1 : 200 respective reflux extraction 1hr two times by 70% ethanol solution at 70°C.

Flavonoids in samples were separated and quantified using a Waters HPLC (Milford, Massachusetts, USA) equipped with a Waters 1525 pump, a Waters 2707 autosampler, and Waters diode array detector 2998. Breeze software was used for the data processing. C18 Elite column (Dalian, Liaoning, China) (250 × 4.6 mm i.d., 5 μm particle size) was used to identify flavonoids. The chromatographic separation was operated with solvent A (MeOH) and B (water). The separated flavonoid peaks were identified by comparing the retention time of individual standards. The optimum procedure elution used in this study was as follows: a gradient of 70 - 20% A (0 - 15 min), 20 - 70% A (15 - 20 min). The samples were injected automatically (20.0 μl). The column and guard column were controled at 30°C and a 1.0 ml/min flow rate was applied. The sample injection volume was 20 μl, and the flavonoids were scanned from 220 to 400 nm in HPLC.

RSM and CCD were used to optimize the extraction condition. The software Design-Expert 7.0.0 Trial (Stat-Ease Inc., Minneapolis, MN, USA) was applied in the experimental design, data analysis, and quadratic equation construction (Lu *et al.* 2009).

$$x_i = \frac{X_i - X_0}{\Delta X_i} \qquad i = 1,\ 2,\ 3$$

X_i is the experimental value; ΔX_i is the step change in X_i ; X_0 is the midpoint of X_i; X_i represents the coded values for X_i. A second-order model was applied to find the optimal set of procedure condition, and the relationship between variables and response was described according to the following equation:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^{\,2} + \sum_{i=1}^{k} \sum_{j}^{k} \beta_{ij} x_i x_j$$

Y is the predicted response; x_i and x_j ($i < j$) are coded variables; β_0, β_i, β_{ii}, β_{ij} are regressive coefficients calculated from the experimental data by second-order multiple regression; and k is the number of factors. The experimental data were statistically analyzed by Fischer's statistical test for variance analysis. The significance of each coefficient was analyzed using variance analysis, and a p value (probability > F) less than 0.05 indicated that the model terms are significant (Ma *et al.* 2012).

Flavonoids contents in flowers of the same *O. fragrans* cultivar group from different regions and those of various cultivar groups in the same region were determined according to the optimal conditions.

Analytical method of antioxidant capacity: The DPPH radical scavenging ability and the ABTS radical cation decolorization assay of EEOF were evaluated by the spectrophotometric method (Hong *et al.* 2008, Du *et al.* 2009). The free radical scavenging activity was measured using the 2.2-diphenyl-1-picrylhydrazil (DPPH) assay. The absorbance values were measured at 518 nm and converted into the per cent antioxidant activity (AA) using the following formula: AA% = [(absorbance of the control - absorbance of the sample)/absorbance of the control] × 100 (Reis *et al.* 2015). The IC50 (50% activity lost) values were calculated from a linear regression of the AA of ascorbic acid (Vitamin C, VC).

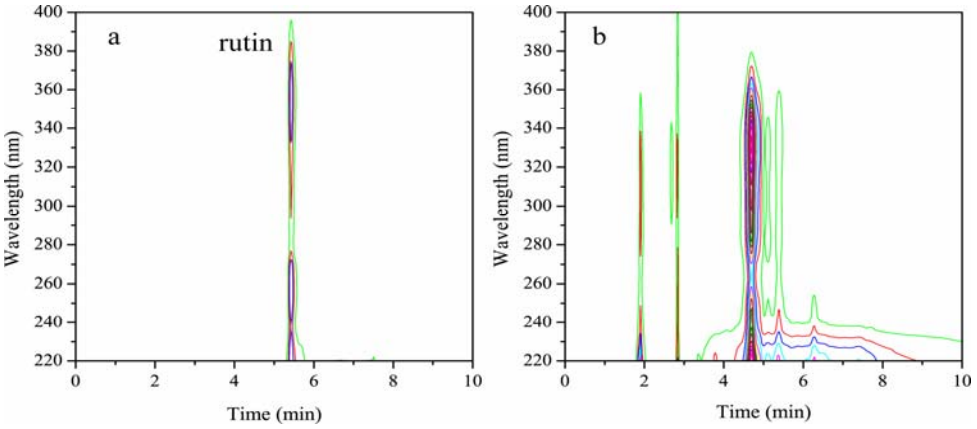
The scavenging activity aganist 2,2'-azino-bis(3-ethylbenzo thiazoline)-6-sulfonic acid (ABTS) radical cation was measured according to the method (Zengin *et al.* 2015): ABTS radical cation was produced directly by reacting ABTS solution with potassium persulfate and allowing the mixture to stand for 12-16 h in dark at the room temperature and prior to beginning the assay, and ABTS solution was diluted with methanol to an absorbance of at 734 nm, and then sample solution was added to ABTS solution and read at 734 nm after mixture and 30 min incubation at room temperature. The IC50 value of ABTS radical cation scavenging activity was expressed as trolox equivalent.

Results were showed as means ± standard deviation (Sd) of three measurements. Statistical analysis was performed using t-test and significant difference was statistically considered at the level of $p < 0.05$. Correlations analysis among data acquired was performed using the MS Excel software correlation coefficient statistical option. The correlations analysis between DPPH and ABTS scavenging activity of EEOF and flavonoids content was obtained by Origin 7.5.

Results and Discussion

The flavonoid peaks were identified by their UV/DAD spectra due to their characteristic UV spectral pattern (Band I, λ max around 300 - 350 nm and Band II, λ max around 230-280 nm) in Fig.1; and this UV pattern allows for the selection of flavonoids peaks for quantitative analysis; hence, UV/DAD is an important alternative in the absence of a mass detector (Zeraik and Yariwake 2012). Based on the technology, a few kinds of flavonoids were determined in flowers, because

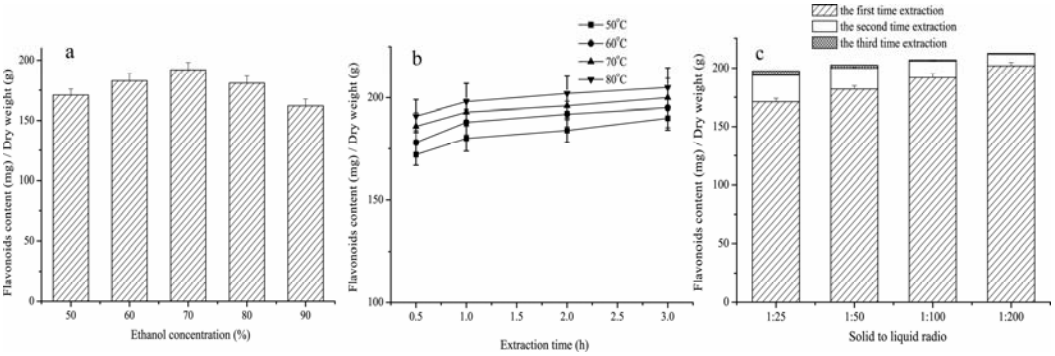
their characteristic UV spectral pattern and retention time is similar to rutin. Practically, UV/DAD scanning of herbal medicines without grinding makes flavonoids determination much more convenient. Perhaps, similar compounds were determned simultaneously.



(a) Chromatogram of rutin, (b) Chromatogram of flavonoids in *Osmanthus fragrans*.

Fig. 1. HPLC-UV/DAD chromatogram (λ from 220 to 400 nm) of flavonoids.

Extraction efficiency of flavonoids by various ethanol concentrations was determined. The result showed that the 70% ethanol concentration was the optimal flavonoids extraction condition (Fig. 2a). Fig. 2b described that longer heating time and higher temperature increased extraction efficiency. The extraction efficiency between 1 and 2 hrs, 70°C and 80°C was not significant. Then heating time of 1 hr at 70°C was selected for the further research. In addition, solid to liquid ratio is another factor effecting extraction efficiency. Fig. 2c showed that higher solid to liquid ratios enhanced extraction efficiency and flavonoids in EEOF, extracted for the third time (solid to liquid ratio of 1 : 100), were almost not detected. Then solid to liquid ratio of 1 : 100 extraction two times was selected for the further experiment.



(a) Ethanol concentration. (b) Extracting time and temperature. (c) Solid-liquid ratio

Fig. 2. Effect of various factors on the extraction efficiency of flavonoids.

CCD was applied to find the appropriate extraction condition and to predict the maximum flavonoids content. Based on the previous experiments, ethanol concentration and temperature are chosen as variables of response. The variables and responses of flavonoids content are listed in Table 1.

Analyses of variance for the quadratic model of flavonoids content are shown in Table 2. Values of "$p > F$" < 0.05 indicate, that the model terms were significant. In this case, X_1, X_2 , X_1X_2, X_1^2 and X_2^2 were significant model terms which indicated that changes of ethanol concentrations and temperature affected the flavonoids extraction directly. The F value of the X_1X_2 term was 0.0097, indicating that the interaction of ethanol concentration and temperature was significant. The "lack of fit F value" of 3.27 implies that the "lack of fit F value" was not significant relative to the error. There is a 14.14% chance that a "lack of fit F value" this large could be due to noise (Table 2). By ANOVA, the coefficient of determination (R^2) of the regression model was 0.9688 and the adjusted coefficient (Adj R^2) in the models was 0.9465, which means a good agreement between the experimental and predicted values of the flavonoids content.

The results of CCD to predict the flavonoids content were fitted with a second order polynomial function:

$$Y = -25.8 + 0.89X_1 + 0.35X_2 + 0.0035X_1X_2 - 0.0079X_1^2 - 0.0039X_2^2 \quad (1)$$

where, Y was response, i.e., flavonoids content, X_1 and X_2 were the ethanol concentration and temperature, respectively.

Table 1. Central composite design for flavonoids extraction.

Run	X_1	X_2	Y
1	80	60	190
2	70	70	206
3	70	70	206
4	70	70	204
5	70	70	208
6	70	70	206
7	60	80	192
8	70	84.14	206
9	70	55.86	190
10	55.86	70	188
11	60	60	188
12	84.14	70	192
13	80	80	208

X_1, X_2 and Y represent the ethanol concentration (%), temperature (^{o}C) and flavonoids content (mg/g), respectively.

The effects of ethanol concentration and temperature, and their combinations on the flavonoids content are shown in Fig. 3. The surface plots of yield indicated that the flavonoids content could not exceed 210 mg/g. In fact, the result showed that the experimental value (203 ± 5.1 mg/g) was consistent with the predictive value (206 mg/g). Therefore, the extraction conditions and result obtained by RSM and CCD were accurate and reliable.

Flavonoids contents were quantitated in flowers of *O. fragrans* groups from different regions under the optimal extraction conditions (Table 3). Flavonoids contents had the significant difference ($p < 0.05$) among *O. fragrans* groups. Albus group had the highest flavonoids content and Aurantiacus group had the lowest flavonoids content in the same region.

Table 2. Analysis of ANOVA for the fitted quadratic polynomial model of flavonoids extraction

Source	Sum of squares	df	Mean square	F value	p-value prob > F
Model	8.57	5	1.71	43.47	<0.0001
X_1	1.94	1	1.94	49.23	0.0002
X_2	0.31	1	0.31	7.81	0.0267
X_1X_2	0.49	1	0.49	12.43	0.0097
X_1^2	4.31	1	4.31	109.44	<0.0001
X_2^2	1.04	1	1.04	26.50	0.0013
Residual	0.28	7	0.039		
Lack of fit	0.2	3	0.065	3.27	0.1414
Pure error	0.08	4	0.02		
Cor total	8.84	12			
C.V. % = 1.0		R^2 = 0.9688 Adj R^2 = 0.9465			

X_1 and X_2 represent the ethanol concentration (%) and temperature (^oC), respectively.

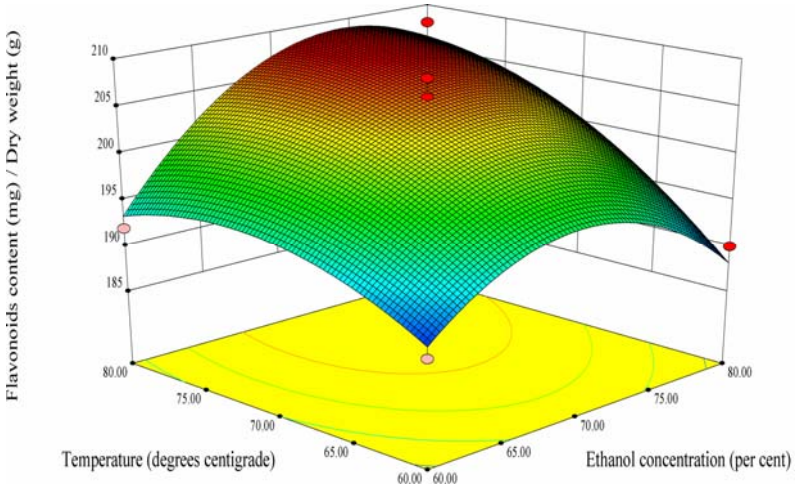


Fig. 3. Response surfaces for flavonoids extraction from flowers of *Osmanthus fragrans.*

Chinese herbal medicines (CHMs), under the guidance of traditional Chinese medicine theory, have been widely used in China and some Asian countries to treat and prevent diseases for thousands of years. Unlike western medicines, which usually contain a single chemical entity with clear efficacy, CHMs are rather complex samples containing a large number of compounds (Cao *et al.* 2014). In addition, even though CHMs are the same species, their qualities are different by geographical conditions such as climate and soil (Woo *et al.* 1999). In this study, flavonoid contents of the same *O. fragrans* group from different regions had more significant difference (Table 3).

Table 3. Flavonoids content in flowers of *Osmanthus fragrans*.

Regions	Groups	Flavonoids content (mg/g) *
Guilin	Luteus group	210 ± 4.1
	Aurantiacus group	172 ± 3.8
Hangzhou	Luteus group	206 ± 4.1
	Aurantiacus group	149 ± 3.6
Xianning	Albus group	193 ± 3.9
	Luteus group	182 ± 3.8
Kaifeng	Albus group	223 ± 4.5
	Luteus group	213 ± 3.9
	Aurantiacus group	133 ± 3.0

*Flavonoids contents had the significant difference ($p < 0.05$) among *O. fragrans* groups.

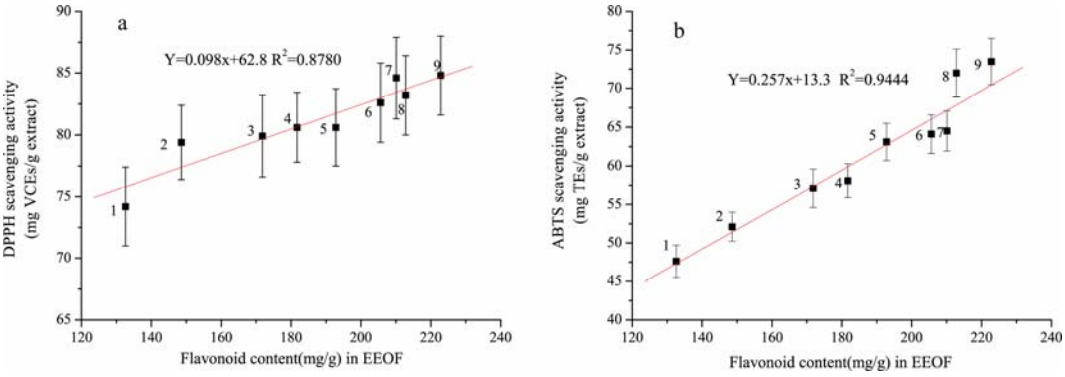


Fig. 4. The relationship between flavonoids content and antioxidant activity of ethanol extracts of *Osmanthus fragrans* flowers. (a) The relationship between flavonoids content and DPPH scavenging activity of EEOF. (b) The relationship between flavonoids content and ABTS scavenging activity of EEOF VCEs: vitamin C equivalents. TEs: trolox equivalents.

1: Aurantiacus group (Kaifeng). 2. Aurantiacus group (Hangzhou). 3. Aurantiacus group (Guilin). 4. Luteus group (Xianning). 5. Albus group (Xianning). 6. Luteus group (Luteus group). 7: Luteus group (Guilin). 8. Luteus group (Kaifeng). 9. Albus group (Kaifeng).

Moreover, there was a positive correlation between DPPH and ABTS scavenging activity of EEOF and flavonoids content (mg/g dry weight), based on VCEs (vitamin C equivalents) and TEs (trolox equivalents) of EEOF showed in Fig. 4.

$$Y_{DPPH} = 0.098x + 62.8 \; R^2 = 0.8788 \quad (2)$$

$$Y_{ABTS} = 0.257x + 13.3 \; R^2 = 0.9444 \quad (3)$$

where, Y_{DPPH} and Y_{ABTS} were DPPH scavenging activity (mg VCEs/g extract) and ABTS scavenging activity (mg TEs/g extract) respectively, and x was flavonoids content (mg/g), and R was the correlation coefficient. Both intercepts in equations were greater than zero. This showed that besides flavonoids, other ingredients in EEOF had the antioxidant activity as well.

Among different groups of *O. fragrans*, the contents of flavonoids in Albus group were the highest from various regions. Moreover, significant correlations existed between the contents of flavonoids and the DPPH and ABTS radical scavenging ability. This study indicates that flowers of *O. fragrans* have a high biomedical value based on their high contents of total flavonoids as well as strong antioxidant activity.

More than 6000 flavonoids have been characterized in nature, and many kinds of compounds similar to flavonoids exist in *O. fragrans*. In the future work, the compounds determined in flowers of *O. fragrans* will be identified and characterized and the antioxidant activity of the single compound will be studied.

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