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Antioxidants screening in *Limonium aureum* by optimized on-line HPLC–DPPH assay

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ABSTRACT

Limonium aureum (Linn.) is a traditional Chinese medicine with strong antioxidant activity. To search for antioxidants in *L. aureum*, an optimized, on-line high performance liquid chromatography (HPLC) method with DPPH (1,1-diphenyl-2-picrylhydrazyl) for the detection of radical scavenging ability was developed. Response surface methodology (RSM) was used to determine the best combination of experimental conditions, such as DPPH concentration, DPPH velocity and the reaction tank length. The optimized method uses a DPPH concentration of 25.0 μg/mL, DPPH velocity of 0.45 mL/min and a 15.0 m long reaction tank. The highly sensitive, optimized method can not only be used for the detection of antioxidants in plants, but can also be coupled with mass spectrometry (MS) to obtain the mass-to-charge ratios of chemical species corresponding to the different peaks in the HPLC profiles. Seven antioxidants were identified in *L. aureum* using the optimized method, including myricetin-3-*O*-β-D-(6"-*O*-galloyl)-glucopyranoside, myricetin-3-*O*-glucoside, myricitrin, eriodictyol-7-*O*-glucoside, myricetin, eriodictyol and homoeridictyol.

1. Introduction

Oxidative stress is necessary for aerobic life, but, under certain conditions, can also be toxic and responsible for causing a variety of diseases including Alzheimer's disease, Parkinson's disease, aging, cancer, neuronal disorders, and cardiovascular disease (Ames, 1983; Aniya et al., 2002; Leong and Shui, 2002; Lim et al., 2007; Lopes et al., 1998; Chapple, 1997). Therefore, antioxidants may be important tools for the prevention or postponement of these diseases. In the past decade, numerous studies aimed to identify safe and effective antioxidants (Pyrzynska and Pękal, 2013; Sánchez-Moreno, 2002).

In the search for new natural antioxidants, complex mixtures are frequently encountered and often times there is a loss of antioxidant activity during the isolation and purification processes due to the loss of the synergism between the molecules that are present in the crude extract (Skaper et al., 1996; Liu et al., 1998). Bioassay-guided fractionation of plant samples is time consuming with low efficiency (Sudha and Srinivasan, 2014); fortunately, on-line high performance liquid chromatography (HPLC) methods can be used for more efficient analyses. In this method, stable free radical, such as 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS) or 1,1-diphenyl-2-picrylhydrazyl (DPPH), is added to the HPLC flow post-column and the antioxidants are detected by absorbance reduction at certain visible wavelengths. This method has been previously used for the detection of antioxidants in many plants and foods and has resulted in the identification of several new active compounds (Damašius et al., 2014; Murkovic, 2002; Zhang et al., 2014).

Limonium aureum (Linn.) (hereafter referred to as *L. aureum*), a perennial herb from the *Plumbaginaceae* family, is distributed mainly in the Qinghai, Gansu and Inner Mongolia regions of China (Liu, 1999). *L. aureum* is an important traditional herbal medicine and has been shown to relieve fever, nourish blood, eliminate inflammation and have analgesic and detoxification effects (Yang, 1991). Several compounds, especially flavonoids, have been isolated from *L. aureum* (Liu et al., 2013; Ye and Huang, 2006). Currently there are few papers reporting the molecular components of this plant and here we report the first on-line HPLC analysis of antioxidants found in *L. aureum*.

In this work, a highly sensitive on-line HPLC–DPPH assay was performed. Response surface methodology (RSM) was used to optimize the experimental conditions and a non-return valve was added to the system to reduce the noise of the assay, thus,

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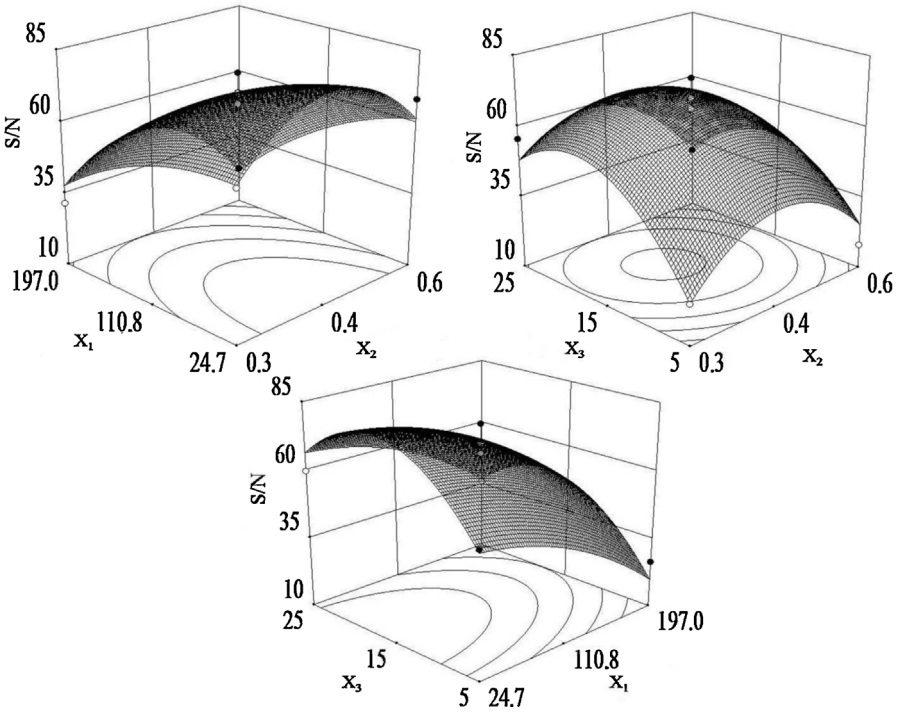

Fig. 1. Response surface plots.

increasing the sensitivity. The optimized method was used for the detection of the antioxidants in *L. aureum*. Antioxidant activity of each peak in the HPLC profile was determined and mass spectrometry (MS) was used to elucidate the structures of the detected antioxidants.

2. Experimental

2.1. Chemicals

DPPH was purchased from Sigma–Aldrich (Steinheim, Germany). Vitamin C and rutin were obtained from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Methanol (HPLC grade) was purchased from Yu Wang Chemical Factory (Shandong, China), all other chemicals and solvents (analytical grade) used were acquired from Beijing Chemical Co. (Beijing, China).

2.2. Plant sample preparation and extraction

The aerial parts of *L. aureum* were harvested in August 2013, from a lower mountain slope in Dulan, Qinghai province, China. The sample was identified by Shizhen Ma (Northwest Institute of Plateau Biology, Qinghai, China) and the voucher specimen (NWIPB-BHD-2013-08-2) was deposited in the herbarium of the Northwest Institute of Plateau Biology, Xining, Qinghai, China. Fresh samples were air-dried in shade at room temperature, ground to a fine powder and passed through sieves, to provide homogeneous powder.

The air-dried and powdered *L. aureum* (5.0 g) was extracted in a 34 mL stainless steel extraction pool in accelerated solvent extraction (ASE-350, Dionex, USA). First, petroleum ether was used as the degreasing solvent and the extraction was performed one time for 10 min. After purging for 100 s, the extract was discarded. Second, the residue was extracted by methanol two times for 10 min at 70 °C. The pressure was 1500 psi. After purging for 100 s, the crude extraction solution was filtered and concentrated by vacuum evaporator (IKA, Germany) at 45 °C. The evaporated supernatant was re-dissolved in methanol and stored at 4 °C for use.

2.3. Instrumentation

On-line HPLC–DPPH assay was performed using an Agilent 1100 series HPLC with a diode array detector (DAD) and an Agilent 1260 series HPLC with a visual web detector (VWD). The separation of the sample extract was carried out using an Agilent Eclipse XDB-C_{18} column (250 mm × 4.6 mm, 5 μm). The reaction coils used were made of polyetheretherketone (PEEK) tubing (15.0 m × 0.25 mm i.d.). A HPLC–MS (Agilent 1100 Series LC/MSD Trap, Agilent Technologies, USA) fitted with an electrospray ionization (ESI) source

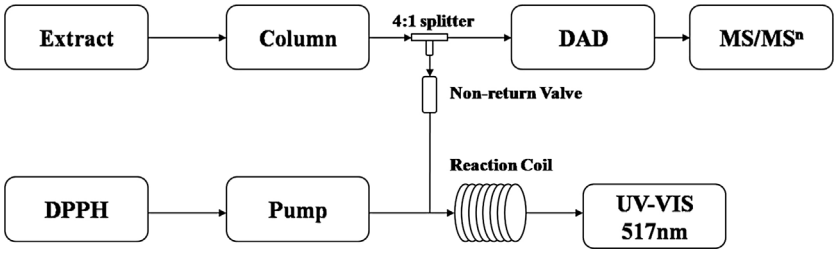

Fig. 2. Instrument set-up of on-line system.

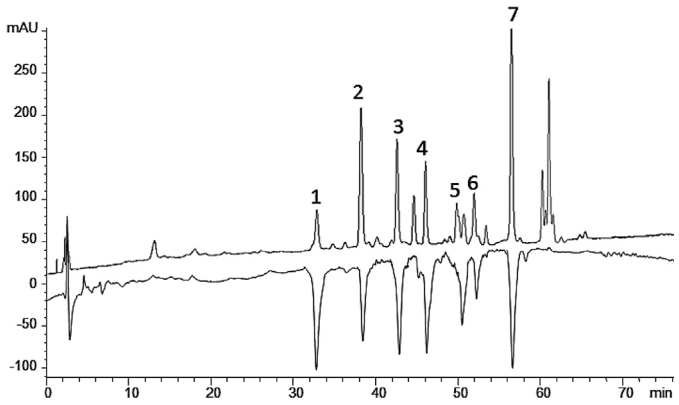

Fig. 3. The HPLC profiles (330 nm) and the DPPH radical scavenging profiles (517 nm) of the *Limonium aureum*.

was used to identify the antioxidant compounds presented in the extract.

2.4. Off line DPPH radical scavenging activity assays

Total flavonoid content (TFC) was measured at 510 nm with a UV/vis spectrophotometer (Cary 300, Agilent Technologies, USA) according to the method described by Han and Liu (2007) The assays of off line DPPH radical scavenging activity was monitored by UV following the method described by Sun et al. (2012) with slight modification. DPPH was dissolved in methanol at a concentration of 0.1 mM/L. 2 mL of diluted extract was added to 2 mL of DPPH solution. The absorbance of the mixture was measured at 517 nm with a UV/vis spectrophotometer (Cary 300, Agilent Technologies, USA) after reacting for 30 min in the dark. The scavenging activity was calculated according to the following equation:

$$\text{Scavengingactivity}(\%) = \left(1 - \frac{A_{\text{sample}-}A_{\text{blank}}}{A_{\text{control}}}\right) \times 100\%$$

A_{sample} refers to the absorbance of DPPH with sample added at 517 nm; A_{blank} is the absorbance of the sample at 517 nm; A_{control} refers to the absorbance of DPPH without sample added at 517 nm.

The concentrations of samples scavenging 50% DPPH free radicals (IC50 values) were calculated by GraphPad Prism 5.0 software. Vitamin C and rutin were used as reference compounds for the evaluation of free radical scavenging activity.

2.5. HPLC–ESI-MS/MS analysis

Eluents for *L. aureum* samples were water (solvent A) and methanol (solvent B), with a flow rate of 1.0 mL/min. The elution gradient was carried out as follows: solvent B was 15–35% from 0 to 30 min, 35–65% from 30 to 70 min, and 65–100% from 70 to 80 min. The temperature of the column was 30 °C and the detection wavelength was 330 nm as determined by UV scanning.

The HPLC–MS analysis was performed under the same gradient program as mentioned above. Nitrogen was used as the nebulizing gas with a flow rate of 9.0 L/min at a pressure of 30 psi and drying gas temperature of 300 °C. The capillary voltage was maintained at 4000 V. The scanning range of the mass spectrometer was 100–1500 *m/z*. The MS data were acquired in the negative ion mode and tandem mass spectrometer (MS/MS) was acquired in automatic mode.

Table 1
Antioxidant activities of the extract.

Anti-oxidant	TFC	IC50
Limonium aureum extract	9.69	6.15 ± 0.01[a]
Rutin	ND	5.46 ± 0.01[a]
Vitamin C	ND	5.08 ± 0.02[a]

Each value in the table is the mean ± standard deviation ($n = 3$).
[a] The IC50 value was calculated by GraphPad Prism 5.0 software; TFC: total flavonoid content (%); IC50 (μg/mL); ND: no determination.

2.6. Optimization of the system

DPPH concentration, DPPH velocity and reaction tank length are all factors that influence the estimated radical scavenging capacity of the sample (Damašius et al., 2014; Yan et al., 2014; Zhang et al., 2014). Rutin, a model antioxidant, was used to optimize the DPPH concentration, DPPH velocity and reaction tank length; the signal to noise patio (S/N) of the radical was used as the evaluation standard (Esin Çelik et al., 2014; Zhang et al., 2014). Response surface methodology (RSM) was used to determine the best combination of experimental conditions for the on-line HPLC–DPPH assay. A Box-Behnken design (BBD, software Design-Expert 7.0.1.0, Stat-Ease, Inc., Minneapolis, U.S.) (Box and Behnken, 1960) with three independent variables (X_1, DPPH concentration; X_2, DPPH velocity; X_3, reaction tank length) at three levels was performed and among the three factors, the DPPH concentration ranged from 12.3 to 197.5 μg/mL; the DPPH velocity ranged from 0.2 to1.0 mL/min, and the reaction tank length ranged from 5 to 25 m. A total of seventeen experiments was carried out randomly in this design. In order to estimate the sum of squares for pure error, five replicates at the center of the design were applied. An empirical second-order polynomial model was used to fit the regression analysis which performed on the experimental data. The model is shown in the following equation:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3$$

here, Y is the response variable; β_0 is an intercept; and β_i, β_{ii}, and β_{ij} are the regression coefficients of variables for the linear, quadratic and interaction terms, respectively. X_i and X_j are independent variables ($i \neq j$). The relationship between the responses of each experiment and independent variables was analyzed by Design-Expert software. The correlation coefficients (R^2) and variance analysis (ANOVA) were used to evaluate the fitness of the model, and its statistical significance and significances of the regression coefficients were confirmed by F-test at a probabilities (P) of 0.001, 0.01, or 0.05.

3. Results and discussion

3.1. DPPH radical scavenging activity

The radical scavenging activity of the extract was determined in order to evaluate the antioxidant activity of the *L. aureum*. The TFC and radical scavenging activities are shown in Table 1. The high level of TFC was found in *L. aureum* extract (9.69%). As it is known, the lower the IC50 value the higher the antioxidant activity of the antioxidants. The *L. aureum* extract exhibited strong antioxidant activity, which is lower than that of rutin and vitamin C.

3.2. Optimization of on-line experimental set-up

On-line HPLC–DPPH assay parameters were optimized using RSM according to the BBD. Table 2 lists the parameters used in the BBD and the obtained experimental yield. A multiple regression

Table 2
BBD and experimental result of RSM.

Run	Parameters			S/N
	Factor 1 (X_1)[a]	Factor 2 (X_2)[a]	Factor 3 (X_3)[a]	
1	−1 (24.7)	−1 (0.3)	0 (15)	60.6 ± 0.6
2	−1 (24.7)	1 (0.6)	0 (15)	68.6 ± 0.9
3	1 (197.0)	−1 (0.3)	0 (15)	32.1 ± 1.2
4	1 (197.0)	1 (0.6)	0 (15)	22.8 ± 0.8
5	0 (110.8)	−1 (0.3)	−1 (5)	24.2 ± 0.9
6	0 (110.8)	1 (0.6)	−1 (5)	18.2 ± 1.3
7	0 (110.8)	−1 (0.3)	1 (25)	56.3 ± 0.6
8	0 (110.8)	1 (0.6)	1 (25)	31.9 ± 0.8
9	−1 (24.7)	0 (0.45)	−1 (5)	55.1 ± 0.8
10	1 (197.0)	0 (0.45)	−1 (5)	26.9 ± 1.2
11	−1 (24.7)	0 (0.45)	1 (25)	60.4 ± 0.5
12	1 (197.0)	0 (0.45)	1 (25)	36.7 ± 0.9
13	0 (110.8)	0 (0.45)	0 (15)	70.8 ± 0.7
14	0 (110.8)	0 (0.45)	0 (15)	66.9 ± 0.7
15	0 (110.8)	0 (0.45)	0 (15)	77.4 ± 0.7
16	0 (110.8)	0 (0.45)	0 (15)	70.2 ± 0.6
17	0 (110.8)	0 (0.45)	0 (15)	70.6 ± 0.8

Each value in the table is the mean ± standard deviation (n = 3).
[a] BBD: Box-Behnken design (software Design-Expert 7.0.1.0); RSM: Response surface methodology; X_1: DPPH concentration; X_2: DPPH velocity; X_3: reaction tank length; S/N: signal to noise patio.

equation was developed and evaluated by analyzing the regression coefficient, ANOVA, P-value and T-test. Predicted values were calculated by applying a multiple regression analysis. The polynomial equation was as following:

$$Y\left(\frac{S}{N}\right) = 71.18 - 3.96X_1 - 15.78X_2 + 7.61X_3 - 4.32X_1X_2 - 4.60X_1X_3 + 1.13X_2X_3 - 18.64X_1^2 - 6.51X_2^2 - 19.89X_3^2, R^2 = 0.9486$$

The results of ANOVA are shown in Table 3. The regression model was highly significant (P = 0.0010 < 0.01) and the lack of fit was not significant (P = 0.0507 > 0.05). The quality of fit of this model was expressed based on the R^2 value. The quadratic regression model showed that the R^2 was 0.9486 (Yolmeh et al., 2014). All of linear coefficients were significant except the DPPH concentration (P < 0.05). Based on the significance of the regression coefficients, the sequence of influential significance was as follows: DPPH velocity > reaction tank length > DPPH concentration. All the mutual interactions between the test variables were found to be insignificant (P > 0.05). Among quadratic coefficients only the DPPH velocity was insignificant (P > 0.05).

The three-dimensional (3D) response surface and two dimensional (2D) contour plots are shown in Fig. 1. The plots give a better visualization. The shapes of the contour plots indicate whether the interactions between the corresponding variables are significant or not. The elliptical contour plots indicate that the mutual interactions between the corresponding variables are significant.

The predicted S/N was 81.0 with the following examined variables: DPPH concentration of 24.70 μg/mL, DPPH velocity of 0.45 mL/min, reaction tank length of 16.65 m. The optimum parameters were DPPH concentration of 25.0 μg/mL, DPPH velocity of 0.45 mL/min, reaction take length of 15.0 m.

In order to verify the applicability of the model equations, a verification test was carried out under the optimal conditions mentioned above. Under the optimal conditions the experimental yield was 76.3 (n = 3), which is consistent with the predicted value of 81.0. The difference between 76.3 and 81.0 (n = 3) were not significant (P > 0.05) which confirmed that the response model was accurate and adequate to reflect the experimental data.

Table 4
Comparison the S/N with the non-return valve.

Anti-oxidant	S/N (without non-return valve)	S/N (with non-return valve)
Rutin	76 ± 4	542 ± 17
Vitamin C	81 ± 6	619 ± 31

Each value in the table is the mean ± standard deviation (n = 3).
The concentration of the antioxidant was 0.5 Mm/L.
S/N: signal to noise patio.

3.3. Application of non-return valve

Noise greatly influences the sensitivity of the on-line HPLC–DPPH assays. According to the previous studies, the fluctuation of the pump pressure and flow rate was the primary source of noise in the assay (Damašius et al., 2014; Esin Çelik et al., 2014; Murkovic, 2002; Sun et al., 2012; Yan et al., 2014; Zhang et al., 2014). To reduce the noise, a non-return valve was added to the system which decreases the fluctuations in pump pressure and prevents the solution reverse. S/N for rutin and vtamin C (0.5 mM) in reaction with DPPH are shown in Table 4 . As expected, the S/N increased significantly in the system with the non-return valve. Once the system was optimized, the influence of the radical concentration was explored once more and found to be in accord with the system without the non-return valve.

Table 3
Results of the ANOVA.

	Sum ofsquares	df	Meansquare	F-value	P-valueprob > F
Model	6363.99	9	707.11	14.35	0.0010***
DPPH concentration	125.61	1	125.61	2.55	0.1543
DPPH velocity	1990.80	1	1990.80	40.41	0.0004***
Reaction tank length	463.60	1	463.60	9.41	0.0181*
DPPH concentration × DPPH velocity	74.82	1	74.82	1.52	0.2576
DPPH concentration × reaction tank length	84.64	1	84.64	1.72	0.2313
DPPH velocity × reaction tank length	5.06	1	5.06	0.10	0.7579
DPPH concentration × DPPH concentration	1462.95	1	1462.95	29.70	0.0010***
DPPH velocity × DPPH velocity	178.72	1	178.72	3.63	0.0985
Reaction tank length × reaction tank length	1665.74	1	1665.74	33.81	0.0007***
Residual	344.83	7	49.26		
Lack of fit	286.38	3	95.46	6.53	0.0507
Pure error	58.45	4	14.61		
Cor total	6708.82	16			
R^2	0.9486				

ANOVA: analysis of variance.
* P-value < 0.05, significant interaction.
*** P-value < 0.001, extremely significant interaction.

Table 5
Proposed compounds detected in *Limonium aureum* extract obtained by HPLC–ESI-MS/MS.

Peak[a]	UV–vis max (nm)	*m/z*Experimental	MS/MS fragments	Identification
1	265,360	631	479,316	Myricetin-3-*O*-β-D-(6"-*O*-galloyl)-glucopyranoside
2	255,360	479	316,271	Myricetin-3-*O*-glucoside
3	260,355	463	316,271	Myricitrin
4	255,355	449	287,151	Eriodictyol-7-*O*-glucoside
5	255,355	316	271,179	Myricetin
6	240,380	287	287,151	Eriodictyol
7	260,375	301	151,107	Homoeridictyol

[a] Peak number assigned according to the overall elution order.

3.4. Identification of the antioxidants by MS

To identify the bioactive compounds in the *L. aureum* extract, the extracted solution was analyzed by online HPLC coupled with ESI-MS/MS. The instrument set-up of on-line system is shown in Fig. 2. A 4:1 splitter was linked up with the DAD; which means one part of HPLC eluent flowed to the mass spectrometer and four parts to the reaction coil. In this way, UV, MS and radical scavenging activity data of every peak could be acquired in only one sample injection. The chromatograms of *L. aureum* extract are displayed in Fig. 3. Detailed information of the MS and ultraviolet (UV) characteristics for the main antioxidant peaks is given in Table 5.

Peak 1 showed low DPPH radical scavenging activity and had an $[M-H]^-$ of *m/z* 631.2, and its MS/MS produced ions were *m/z* 479.0 and 315.9. By comparison with the published data (Ye et al., 2005; Ye and Huang, 2006) compound 1 was putatively identified asmyricetin-3-*O*-β-D-(6"-O-galloyl)-glucopyranoside.

The MS ions of peak 2 were observed at *m/z* 479. An MS/MS fragment at *m/z* 316 is a characteristic fragmentation pattern of myricetin derivatives, and corresponds to the loss of glucoside (179 units). By comparing the MS/MS ions and the UV data with published literature, (Amico et al., 2004; De Brito et al., 2007; Downey and Rochfort, 2008) compound 2 was tentatively identified as myricetin-3-*O*-glucoside.

The ESI-MS/MS spectrum of peak 3 showed the $[M-H]^-$ ion at *m/z* 463 with the fragments at *m/z* 316 $[M-H-146]^-$ and *m/z* 271 $[M-H-192]^-$. The fragment ion *m/z* 316 was attributed to a loss of a rhamnoside from the parent ion and also matched with myricetin derivatives. By comparison with the literature data, (Paul et al., 1974; Ye et al., 2005; Ye and Huang, 2006) compound 3 was identified as myricitrin.

The molecular ions of peak 4 were observed at *m/z* 449. The MS/MS spectrum showed ion fragment at *m/z* 287, corresponding again to a loss of glucoside. Published literature reports that this fragmentation pattern was a characteristic of eriodictyol. Therefore, compound 4 was tentatively identified as eriodictyol-7-*O*-glucoside (Areias et al., 2001; Hvattum and Ekeberg, 2003).

The MS ions of peak 5 were detected at *m/z* 316. The ESI-MS/MS spectrum showed fragments at *m/z* 271 and 179. By comparing the MS and UV data with the literature data (De Brito et al., 2007; Downey and Rochfort, 2008; Hvattum and Ekeberg, 2003), compound 5 was tentatively identified as myricetin.

The MS of peak 6 displayed an intense parent ion at *m/z* 287 and an MS/MS fragment at *m/z* 151. By comparison with the UV–vis data reported in literature and retention time of the standard compound (Krause and Galensa, 1991; Ye et al., 2005; Ye and Huang, 2006) compound 6 was confidently identified as eriodictyol.

The MS ions of peak 7 were observed at *m/z* 301, with fragments at *m/z* 151 and 107. By comparing the MS/MS ions and the UV data with literature data, compound 7 was tentatively identified as homoeridictyol (Ye et al., 2005; Ye and Huang, 2006).

4. Conclusion

In this study, the potential antioxidant activity of flavonoids from *L. aureum* extracts was evaluated using an on-line HPLC assay. RSM was used to optimize the on-line HPLC–DPPH assay and seven compounds in the extract, exhibiting strong antioxidant activity, were identified.

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