The American Journal of Chinese Medicine, Vol. 45, No. 7, 1513–1536

Institute for Advanced Research in Asian Science and Medicine
DOI: 10.1142/S0192415X17500823

Differences in Chemical Component and Anticancer Activity of Green and Ripe Forsythiae Fructus

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Published 26 September 2017

Abstract: Forsythiae Fructus, Lianqiao in Chinese, is one of the most fundamental herbs in Traditional Chinese Medicine. Both green Forsythia (GF) and ripe Forsythia (RF) are referred to Forsythiae Fructus in medicinal applications. In most cases, they are used without distinction. In this study, a metabolomics approach was performed to compare componential differences of two Forsythiae Fructus aqueous extracts subtypes. Principal component analysis (PCA) score plots from the UPLC-MS data showed clear separation between the two subtypes, indicating there are significant differences in the chemical components between GF and RF. Meanwhile, the anticancer activity of them was also compared. GF exhibited much stronger antitumor activity than RF against B16-F10 murine melanoma both *in vitro* and *in vivo*. 15 chemical compounds were identified as specific markers for distinguishing GF and RF. Among these marker compounds, forsythoside I, forsythoside A, forsythoside E and pinoresinol were demonstrated to be key important active compounds that account for the different anticancer efficacies of GF and RF. Our data suggest that GF and RF should be distinctively used in clinical applications, particularly in the anticancer formulas, in which GF should be preferentially prescribed.

Keywords: *Forsythia suspensa*; Metabolomics; Forsythoside; Anticancer; B16 Melanoma.

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Introduction

Forsythiae Fructus (FF), the dry fruit of *Forsythia suspensa* (Thunb.) Vahl in the family Oleaceae, is considered to be one of the most fundamental herbs and has been widely used in traditional Chinese Medicine (TCM) for thousands of years. FF is classified as a typical heat-clearing and detoxifying herb according to TCM theory with characteristics of bitterness, coolness, and slight acridity (China, 2010). The main active ingredients are lignans and flavonoids (Bai *et al.*, 2015). FF is commonly used for treating many diseases in China, such as allergic rashes, boils, carbuncles, cancers, colds or flu-like symptoms (e.g. fever, chills, and headache), mumps, tonsillitis, urinary tract infections, etc. (Kang *et al.*, 2008; Cheng *et al.*, 2015; Sung *et al.*, 2016). FF is also the main ingredient of many Chinese brand-name OTC drugs (Fang *et al.*, 2015; Zhang *et al.*, 2015; He *et al.*, 2016), such as the fructus forsythiae tablet for detoxification (Yin-Qiao-Jie-Du tablet), heat-clearing and detoxificating oral liquid (Qing-Re-Jie-Du oral liquid), Shuang-Huang-Lian oral liquid. Among all these applications, the anticancer property of FF makes it one of the most popularly applied and studied herbs due to its heat-clearing and detoxifying effects. It is estimated that the market demand of FF is continuously growing at 25% annually in recent years, which shows the great market potential of FF.

In general, FF includes two subtypes, green Forsythia (GF) and ripe Forsythia (RF), in reference to the different maturity stages. GF is usually harvested in early September, when the fruit is almost mature, but still green. Most of GF have the seeds in the fruit with no crack. Differently, RF is usually harvested in October, and its fruit is fully ripe, yellow, cracked and dry, with some seeds falling off. According to the *Chinese Pharmacopoeia*, both GF and RF are referred to as FF in medicinal applications (China, 2010).

In present clinical applications, there is no strict distinction on the preference for either GF or RF. In most cases, they are interchangeably used. It is believed that the chemical components might be different between GF and RF due to the different harvest time. Thus, their pharmacological activity could also be different. Our hypothesis is supported by the records in one ancient Chinese medical book named *BenCaoYuanShi* (A.D. 1612) (Xie, 1992) which described that "Forsythia Fructus without crack is a good product, while the one with crack is not," indicating that the medicinal activity of GF was higher than RF. More recent studies also reported that GF had higher antioxidant activity than RF (Jia *et al.*, 2015). However, the majority of other ancient Chinese medical books did not distinguish the use of GF and RF, and most TCM prescription used today also did not distinguish GF and RF. In this study, we globally investigate the differences in the chemical components between GF and RF using metabolomics approaches and their antitumor activity.

Metabolomics is a system biology-based newly developed chemical composition analysis approach, which could provide a global unbiased analysis of metabolites or small molecular chemicals compounds in biological samples (Hall *et al.*, 2002; Wang *et al.*, 2015). Through employing high-resolution Mass Spectroscopy or NMR and bioinformatics tools, metabolomics could quickly identify the discrepant compounds (Hall *et al.*, 2002; De Vos *et al.*, 2007). The TCM herbs, like FF, usually contain hundreds of compounds, and many of the compounds have not been identified yet. It is of great advantage to apply a

metabolomics approach to perform a non-targeted qualitative and quantitative analysis to determine the chemical composition differences in GF and RF. Previous studies have compared the contents of a small proportion of components derived from GF and RF. Their results showed that forsythoside A, phillyrin and rutin were significantly higher in GF than those in RF (Guo *et al.*, 2007a,b; Xia *et al.*, 2009). However, these studies only examined either a single compound or only the main constituents in FF by conventional thin-layer chromatography (TLC), capillary electrophoresis (CE) or high performance liquid chromatography (HPLC) methods (Guo *et al.*, 2007). Thus, a systematic global comparison on the chemical composition between GF and RF is in high demand.

In the present study, we examined the chemical differences between GF and RF using non-targeted metabolomics approaches. Ultra-performance liquid-chromatography/quadrupole time of flight mass spectrometry (UPLC/Q-TOF MS) method was employed to obtain the comprehensive metabolic signatures of the aqueous extracts, and pattern recognition approaches, including principal component analysis (PCA), orthogonal projections to latent structures discriminant analysis (OPLS-DA) and other bioinformatics methods were used to identify the componential differences. Because of the application of non-targeted metabolomics approach, a wide range of chemical components in both GF and RF could be covered with further analysis on their content differences. From our analysis results, 15 compounds were identified as specific markers for distinguishing GF and RF. Meanwhile, we also evaluated their pharmacological differences in anticancer activity using melanoma cell lines and tumor allograft model. We found that GF exhibited stronger antitumor activity against B16-F10 melanoma than RF did both *in vitro* and *in vivo*.

Materials and Methods

Chemicals and Reagents

Forsythoside A, phillyrin, (+)-pinoresinol-4-O-β-D-glucoside, forsythoside E and rutin were purchased from Sichuan Weikeqi Biological Technology Co., Ltd. (Chengdu, Sichuan, China). Forsythoside I and pinoresinol were purchased from Winherb Medical Technology Co., Ltd. (Shanghai, China) and Tauto Biotech Co., Ltd. (Shanghai, China), respectively. HPLC-grade acetonitrile (CH3CN) and formic acid were obtained from Merck KGaA (Darmstadt, Germany). HPLC-grade methanol was purchased from Sigma-Aldrich (St. Louis, MO, USA). Water was purified by the Milli-Q water purification system (Millipore, Bedford, MA, USA). All other reagents were of analytical grade.

Preparation of FAE Aqueous Extract

A total of 41 batches of FF, including 26 batches of GF and 15 batches of RF were collected from different retails and hospital pharmacies in China. The original producing areas were Shanxi, Henan and Anhui Provinces of China. The voucher specimens of those FF were deposited at the Institute of Chinese Medical Sciences, University of Macau,

Macao, China. Forsythiae Fructus aqueous extract (FAE) was prepared by adding 10 g dried herbal powder into 100 ml distilled water to form slurry, then the mixture was simmered at 80°C for 60 min on the boiler. After cooling down, the upper aqueous layer was carefully transferred to another fresh container for further use. The concentrations of FAE were normalized to 100 mg/ml, which were relative to the quantity of the dried herbal powder. The extract was stored at −80°C until use. Generally, the extracts were tested within a month after preparation.

LC-MS Analysis

Liquid chromatography was performed using a Waters ACQUITYTM ultra performance liquid chromatography (UPLC, Waters Corp., Milford, MA, USA). Five μL aliquot of each sample was injected into an ACQUITY UPLC BEH C18 column (50 mm × 2.1 mm, 1.7 μm) maintained at 45°C. The mobile phase consisted of a linear gradient system of 0.1% formic acid in water (solution A) and 0.1% formic acid in acetonitrile (solution B), 0–3 min, 10% B; 3–15 min, 10–20% B; 15–18 min, 20–100% B; 18–20 min, 100% B. The flow-rate was 0.4 mL/min.

Mass spectrometry was performed using a SYNAPT G2-Si high-definition mass spectrometer (Waters Corp., Milford, MA, USA) operated using the negative (ESI-) ion mode. Source temperature was set at 120°C with a cone gas flow of 10 L/h. Meanwhile, the desolvation gas temperature was 450°C with gas flow of 900 L/h. The capillary voltage was set to 2.4 kV (ESI-), sampling cone voltage was set to 40 V. The extraction cone voltage was 4.0 V; the TOF acquisition rate was 0.1 s/scan. MS/MS data were collected for all the ions observed in the preceding MS scan. In order to ensure the accuracy and reproducibility of Q-TOF MS, the leucine enkephalin calibrant solution at the concentration of 200 ng/mL was used as the lock mass in negative ion mode (m/z 554.2615). A full scan mass range from m/z 50 to m/z 1000 was scanned.

Data Processing and Analysis

The raw data were imported to Progenesis QI Software (Waters Corporation, MA, USA) for peak detection and alignment to obtain a peak list containing the retention time, m/z, and peak area of each sample. The peak area was normalized to an internal standard for further statistical analysis. Then, the resultant data matrices were introduced into the SIMCA-P Software (Umetrics AB, Umea, Sweden) for multivariate pattern recognition analysis, including PCA and OPLS analysis. From the S-plot of OPLS, the clustering information and potential markers of GF and RF were acquired. Significantly changed potential markers between the two groups were chosen according to the VIP values based on their contribution to the variation and correlation within the data set. To support the potential chemical marker identification and further understanding of this study, PubChem (http://ncbi.nim.nih.gov), MassBank (http://www.massbank.jp), METLIN (http://metlin.scripps.edu), and Respect for Phytochemicals (http://spectra.psc.riken.jp/) were queried.

Cell Culture and Cell Proliferation Assay

Murine melanoma B16-F10 cell line and Lewis lung carcinoma (LLC) cells were purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2. The media were changed every other day. Cell proliferation was determined by MTT colorimetric assay as described in our previous paper (Bao *et al.*, 2016).

Animals and Treatments

All the animal procedures were approved by Animal Ethics Committee of Institute of Chinese Medical Science, University of Macau, and every effort was made to minimize animal suffering. Female C57BL/6 mice, weighting 22 ± 2 g at 10 weeks age, were purchased from Laboratory Animal Services Center, the Chinese University of Hong Kong (Hong Kong, China). The mice were housed in individually cage (IVC) system in a temperature (23 ± 2°C) and humidity (50 ± 5%) controlled environment under a 12 h light/dark cycle. For tumor allograft experiment, B16-F10 cells (5×10^4 cells in 0.1 ml phosphate-buffered saline, PBS) were transplanted subcutaneously (s.c.) into mice on day 0. Tumor-bearing mice in treatment group ($n = 8$) received FAE treatment at 10 g/kg by oral gavage every two days since day 0. The concentrations of FAE were normalized by the quantity of the dried herbal powder. Mice in control group received water by oral gavage. Body weight and mortality of the mice were monitored every two days until the end point. Mice were sacrificed when the tumor size reached 10% of the body weight.

Statistical Analysis

Data were expressed as the mean ± standard error of the mean (SEM) or standard deviation (SD). Student's *t*-test was used for between-group comparison and one-way ANOVA analysis with Tukey post hoc was performed to compare differences in variables of different treatment groups using GraphPad Prism 5.0 Software (La Jolla, CA, USA). The comparison of survival curves were evaluated by Log-rank (Mantel-Cox) Test. Statistical significance was accepted at the level of $p < 0.05$.

Results

Collection of GF and RF

According to the *Chinese Pharmacopoeia*, both GF and RF are referred to as FF. Even though their harvest time and herbal processing method are different, GF and RF are commonly interchangeably used, and both of them are applied as FF in present clinical application. We randomly purchased FF from 31 retail and hospital pharmacies in different

areas of China to see the preferential application of GF and RF. 26 GF samples and 15 RF samples in total were collected from 31 pharmacies (10 pharmacies were able to provide both GF and RF), which implied that generally there is no obvious distinction in usage preference for either GF or RF.

Analysis of Chemical Profiles in FAE by UPLC/Q-TOF MS

Decoction is the main usage method of FF in traditional Chinese medicine application. Therefore, the aqueous extract of FF was used for chemical component analysis and further pharmacological activity evaluation in this study. The chemical profiles of FAE samples from GF ($n = 26$) and RF ($n = 15$) groups were characterized by UPLC/Q-TOF MS in negative ion mode. The typical UPLC-Q/TOF MS base peak intensity (BPI) chromatograms of representative samples collected from each group were presented in Fig. 1. There were obvious differences between the GF and RF groups via visual inspection of the typical chromatograms. Multivariate statistical analysis was applied to test the differences of the chemical components and identify potential chemical markers in FF at two different maturation stages. Data matrix was generated after pre-processing consisting of the retention time (RT), m/z value and the normalized peak intensity in Progenesis QI software. 1032 pairs of RT-m/z in negative ion mode in peak list were observed.

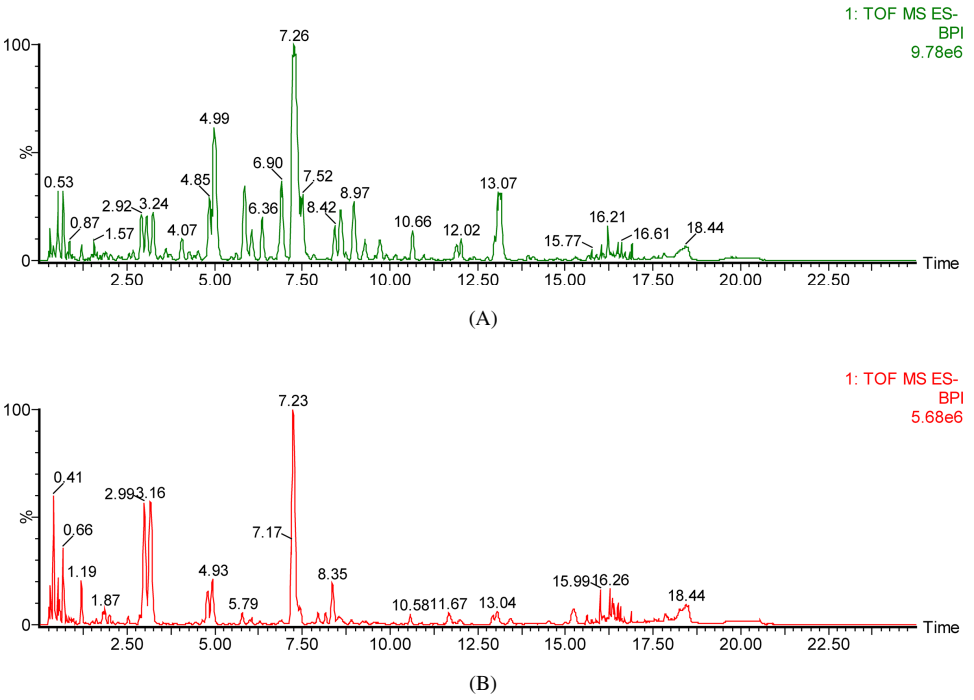


Figure 1. Typical UPLC-Q/TOF MS BPI chromatograms of *Forsythia suspensa* aqueous extracts. The aqueous extracts of GF (A) and RF (B) were analyzed in negative ion mode.

Validation of UPLC/Q-TOF MS Method

In a metabolomics study, the reproducibility and stability of the UPLC/Q-TOF MS method should be evaluated to ensure that the significant difference was originated from inherent differences between groups instead of the instrumental drift of chromatography and MS. The QC sample of FAE was prepared by mixing the aliquots of each batch of FAE in a single sample vial, which provided a representative sample of all components in a relatively average concentration. The QC sample served as technical replicates throughout the data set to validate system stability and reproducibility of the UPLC/Q-TOF MS method. Prior to each run, the QC sample was analyzed three times to ensure that the system was properly equilibrated. The QC sample was also analyzed once every five tested samples to further assess the method stability.

The system reproducibility was evaluated using relative standard deviation (RSD) of the remaining ions in 10 QC samples. As shown in Fig. 2, 95.06% of the variables had RSD less than 20% among the 1032 ions acquired from the QC samples in ESI negative ion mode, suggesting that the analytical platform was robust with excellent repeatability and stability. In addition, the reproducibility and stability of the metabolomics analysis were also examined by the overall amount of the QC samples variability using PCA analysis. If there is instability in the instrumental system, it could be reflected by the shifts in retention time, peak intensity, and mass accuracy from one run to the next, and produce a characteristic pattern for the QC samples under PCA analysis. As shown in Fig. 3A, the cluster of the QC samples showed in a tight manner in the PCA score plots of all samples analyzed. These results demonstrated that the present UPLC/Q-TOF MS method had excellent stability and reproducibility.

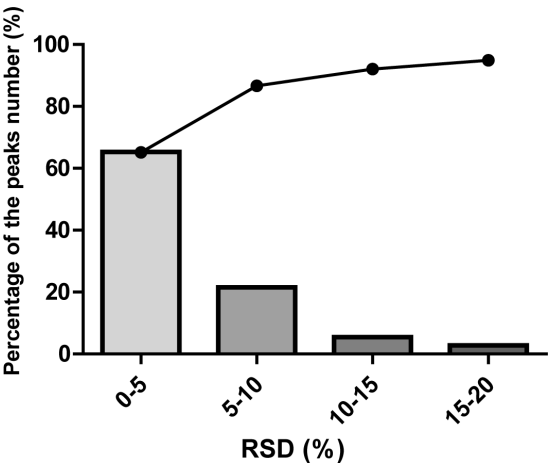
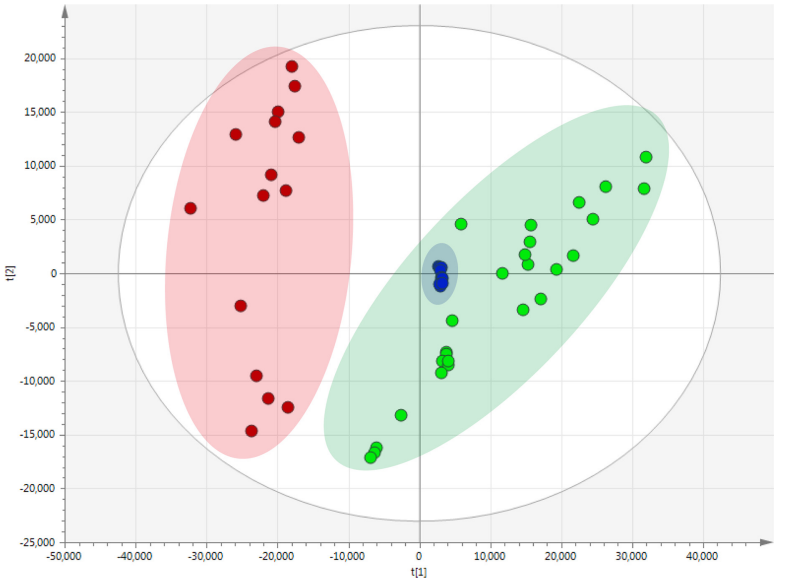
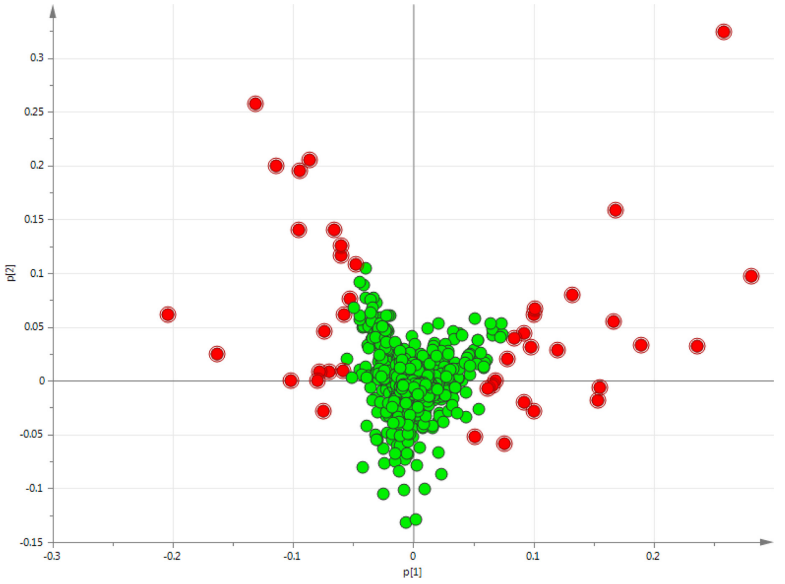


Figure 2. RSD (%) distribution of all metabolites in the pooled quality control (QC) samples. The columns represent the percentage of the peak number within the specified RSD range. The line show the cumulative percentages of the peak number within the specified RSD range.



(A)



(B)

Figure 3. PCA score plot and its corresponding loading plot based on global chemical profiling of *Forsythia suspensa* aqueous extracts. (A) PCA score plot in GF group (green), RF group (red) and QC group detected in negative ion mode, with fitting and predictive performance (2 latent variables, $R^2X = 0.843, Q^2 = 0.594$). (B) Loading plot in GF group and RF group detected in negative ion mode. The variables contributing most to be differences (VIP $> 2.0, p < 0.05$) were highlighted as red filled circles.

Multivariate Statistical Analysis

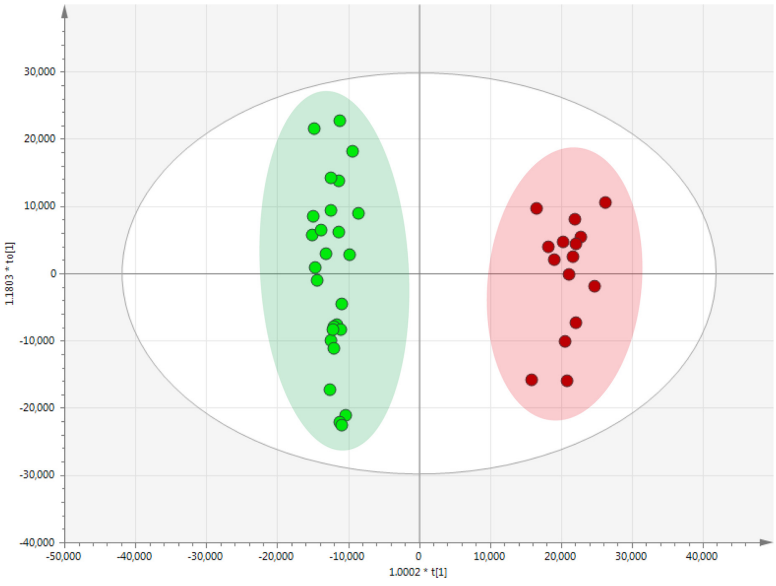
The peak list containing retention time, m/z, and ion intensity of each sample were imported into the SIMCA-P Software for multivariate pattern recognition analysis, which were processed by PCA for discriminating between the two groups (including QC group) based on their chemical compositions differences. After Pareto scaling and mean-centering, these data were displayed as scores and loadings in a coordinate system of principal components resulting from data dimensionality reduction. The two-component PCA score plots (Fig. 3A) showed that 41 FF samples could be clearly separated into two different clusters, reflecting their corresponding grouping of GF and RF, based on their global chemical profiles differences. Notably, all of the observations in the current study fell within the Hotelling T2 (0.95) ellipse. Moreover, the R2X (cum) and Q2 (cum) values were 0.843 and 0.594, respectively, highlighting the good quality of present PCA model. The loading plot of PCA (Fig. 3B) was used to show the differences between GF and RF based on the global chemical profiling of FAE. The variables highlighted as a red filled circle contributed most to the differences (VIP $> 2.0, p < 0.05$).

In order to obtain better discrimination between the GF and RF group, OPLS-DA approach was applied. As shown in Fig. 4A, each point represented a tested FAE sample. The loading plot of OPLS-DA clearly separated tested samples into two blocks according to their chemical profiles of different groups in negative ion mode. In the corresponding S-plot, each point represented an ion RT-m/z pair. The variables with higher p and p (corr) values were more important to discriminate two groups as shown in the upper-right and lower-left quadrants of the S-plot. The variables with importance in projection (VIP) values exceed 2.0 were highlighted with red filled circle (Fig. 4B). These ion RT-m/z pairs were selected as potential chemical markers for the discrimination of GF and RF.

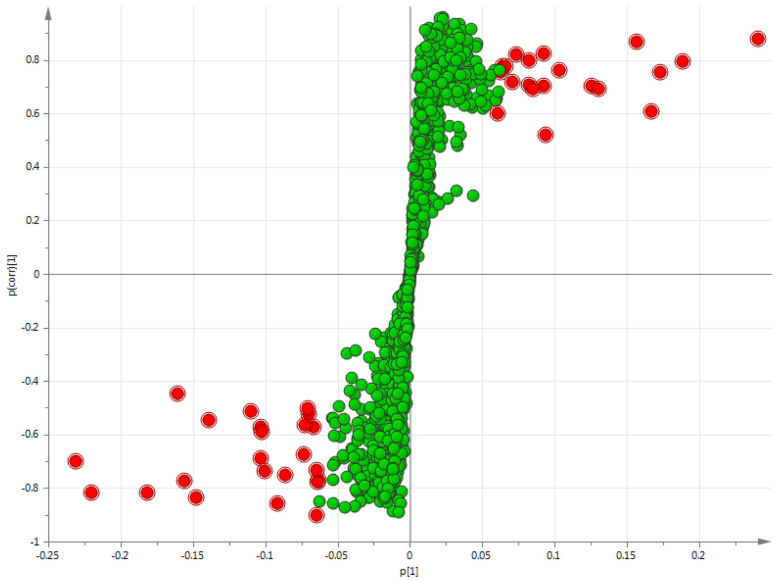
Identification of Potential Chemical Markers

The variables with high VIP value (> 2.0) were considered as potential chemical markers which were highlighted in the PCA loading plots (Fig. 3B) and the S-plots of OPLS-DA (Fig. 4B). Molecular mass was determined within a reasonable degree of measurement error (< 2 ppm) using Q-TOF MS, and the potential element composition, and degree of saturation of the compounds were obtained. To identify these metabolites, these variables were predicted by comparing the accurate MS and MS/MS fragments with the metabolites found in on-line databases. These structures were only tentatively assigned based on a comparison of their molecular weights, molecular formulas and MS/MS fragment ions with that of compounds isolated from *Forsythia* species published in the literature or databases as listed in methods.

The representative MS/MS spectra, chemical structures and proposed fragmentation pathway details for the chemical markers (Var 164 and 50) are shown in Fig. 5. MS analysis of chemical marker Var 164 revealed an m/z value of 579.2070 for $[M+FA-H]^-$ in the negative ion mode, suggested that its empirical molecular formula was $C_{27}H_{34}O_{11}$. The difference in the mass of this parent ion and its main fragment ion (m/z 371.1499) were



(A)



(B)

Figure 4. OPLS-DA score plot and its corresponding S-plot based on UPLC-MS profiling data of *Forsythia suspensa* aqueous extracts. (A) OPLS-DA score plot in GF group (green) and RF group (red) detected in negative ion mode, with fitting and predictive performance (3 latent variables, $R^2X = 0.712, R^2Y = 0.983, Q^2 = 0.929$). (B) S-plot in GF group and RF group detected in negative ion mode. The variables with high VIP value (VIP > 2.0) were highlighted with red filled circles.

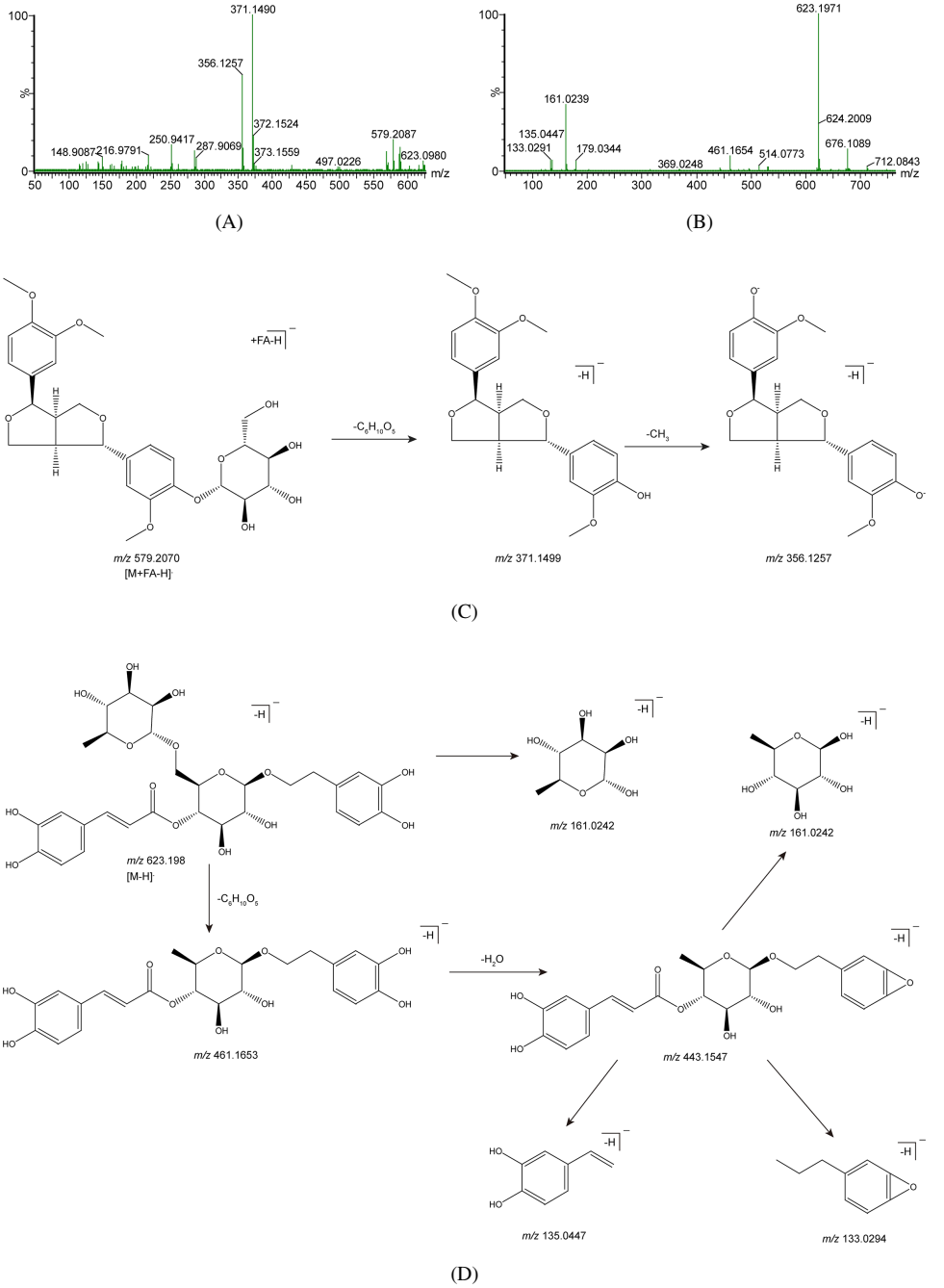


Figure 5. Mass spectra for the proposed fragmentation pathways in the negative ionization mode. MS/MS spectrum of phillyrin (A) and forsythoside A (B). The cleavage pathways of phillyrin (C) and forsythoside A (D).

162, which corresponded to the loss of $C_6H_{10}O_5$. This daughter ion (m/z 371.1499) afforded main fragment ion with an m/z value of 356.1257, representing mass differences of 15 Da, which corresponded to the successive loss of CH_3. Combining the information of comparison with data from published literature, our data suggested that this compound was phillyrin, and the most plausible interpretation of its fragmentation pathway is shown in Fig. 5C, which is primarily based on the information from the MS/MS data (Fig. 5A).

Similarly, MS analysis of Var 50 (Fig. 5B) revealed an m/z value of 623.1983 for $[M-H]^-$ in the negative ion mode, which indicated that this compound had an empirical molecular formula of $C_{29}H_{36}O_{15}$. Var 50 gave a fragment ion with an m/z value of 461.1652, representing a 162 Da mass difference compared with the parent ion, indicating the loss of $C_6H_{10}O_5$. Followed, this daughter ion (m/z 461.1652) afforded the main fragment ion with m/z values of 443.1547, representing mass differences of 18 Da, which corresponded to the successive loss of an H_2O. Then, the ion (m/z 443.1547) afforded three main fragment ions with m/z values of 161.0242, 135.0447 and 133.0294, respectively. These data therefore indicated that this compound was forsythoside A and its fragmentation pathway is shown in Fig. 5D.

According to the protocol described above, a total of 15 chemical markers (Table 1) among difference variables were identified through comparing their MS data with those from the literature, and searching those online databases listed above as well. Among them, one of the 15 chemical markers identified showed an increased pattern and 14 of them were decreased in the RF group shown in Fig. 6.

Comparison of Signal Intensities between GF and RF

The signal intensities of the chemical markers identified in the GF and RF were analyzed by *t*-test to provide a further comparison of the differences between GF and RF at different maturation. Differences with $p < 0.001$ were considered significant. As shown in Fig. 6, the marker levels varied considerably between GF and RF samples. The intensity of 14 chemical markers were found to be significantly ($p < 0.001$) higher in GF than that in RF. GF group contained a higher signal level of forsythoside I, phillyrin, (+)-pinoresinol-4-O-β-D-glucoside, forsythoside A, adoxosidic acid, forsythenside A, forsythoside H, forsythoside J, rutin, forsythoside E, forsythenside B, pinoresinol, forsythenside L and cornoside, while a lower signal level of β-hydroxyacteoside compared to the RF group, suggesting that these chemical markers could be used as a specific marker for the discrimination of GF and RF.

Discrimination between GF and RF Based on Identified Chemical Markers

In order to evaluate the capability of 15 identified chemical markers in the discrimination between GF and RF, we applied PCA analysis for these identified markers. The dataset included 41 observations of all samples and 15 variables of identified chemical markers was constructed and subjected to PCA. As shown in Fig. 7, where the observations and variables of the multivariate data were presented in the same resulting PCA/bi-plot,

Table 1. Identification of 15 Metabolites that Contribute to the Discrimination of GF and RF

Var ID	Ret. Time	m/z Determined	m/z Calculated	MS Error (ppm)	Ion Form	Molecular Weight	Molecular Formula	Metabolite Identification	VIP Value	GF/RF Fold Change
24	3.17	639.1938	639.1925	1.3	[M−H]	640.59	$C_{29}H_{36}O_{16}$	β-hydroxyacteoside	7.75283	0.47
79	4.93	623.1982	623.1976	0.6	[M−H]	624.59	$C_{29}H_{36}O_{15}$	Forsythoside I*	7.40425	3.18
164	13.06	579.2086	579.2083	0.3	[M+FA−H]	534.55	$C_{27}H_{34}O_{11}$	Phillyrin*	7.08365	3.01
58	7.43	519.1878	519.1867	1.1	[M−H]	520.53	$C_{26}H_{32}O_{11}$	(+)-pinoresinol-4-O-β-D-glucoside*	5.85586	2.66
50	7.22	623.1983	623.1976	0.7	[M−H]	624.58	$C_{29}H_{36}O_{15}$	Forsythoside A*	5.06428	1.20
6	0.53	375.1303	375.1291	1.2	[M−H]	376.36	$C_{16}H_{24}O_{10}$	Adoxosidic acid	5.01559	7.43
16	2.86	449.1454	449.1448	0.6	[M−H]	450.44	$C_{22}H_{26}O_{10}$	Forsythenside A	4.71356	6.28
216	6.85	623.1971	623.1976	−0.5	[M−H]	624.59	$C_{29}H_{36}O_{15}$	Forsythoside H	4.40248	13.25
64	5.77	609.1826	609.1820	0.6	[M−H]	610.56	$C_{28}H_{34}O_{15}$	Forsythoside J	3.60243	2.00
875	4.80	609.1466	609.1456	1.0	[M−H]	610.52	$C_{27}H_{30}O_{16}$	Rutin*	3.27576	1.48
25	0.68	461.1672	461.1659	1.3	[M−H]	462.45	$C_{20}H_{30}O_{12}$	Forsythoside E*	3.22963	2.49
1	0.85	511.1457	511.1457	0.0	[M+FA−H]	466.44	$C_{22}H_{26}O_{11}$	Forsythenside B	2.93279	2.86
298	7.43	357.1343	357.1338	0.5	[M−H]	358.39	$C_{20}H_{22}O_6$	Pinoresinol*	2.78476	3.01
202	6.91	443.1553	443.1554	−0.1	[M−H]	444.43	$C_{20}H_{28}O_{11}$	Forsythenside L	2.12526	20.17
4	0.39	315.1097	315.1080	1.7	[M−H]	316.30	$C_{14}H_{20}O_8$	Cornoside	2.01929	4.23

FA, Formic acid. * Metabolites identified by comparison with authentic standards.

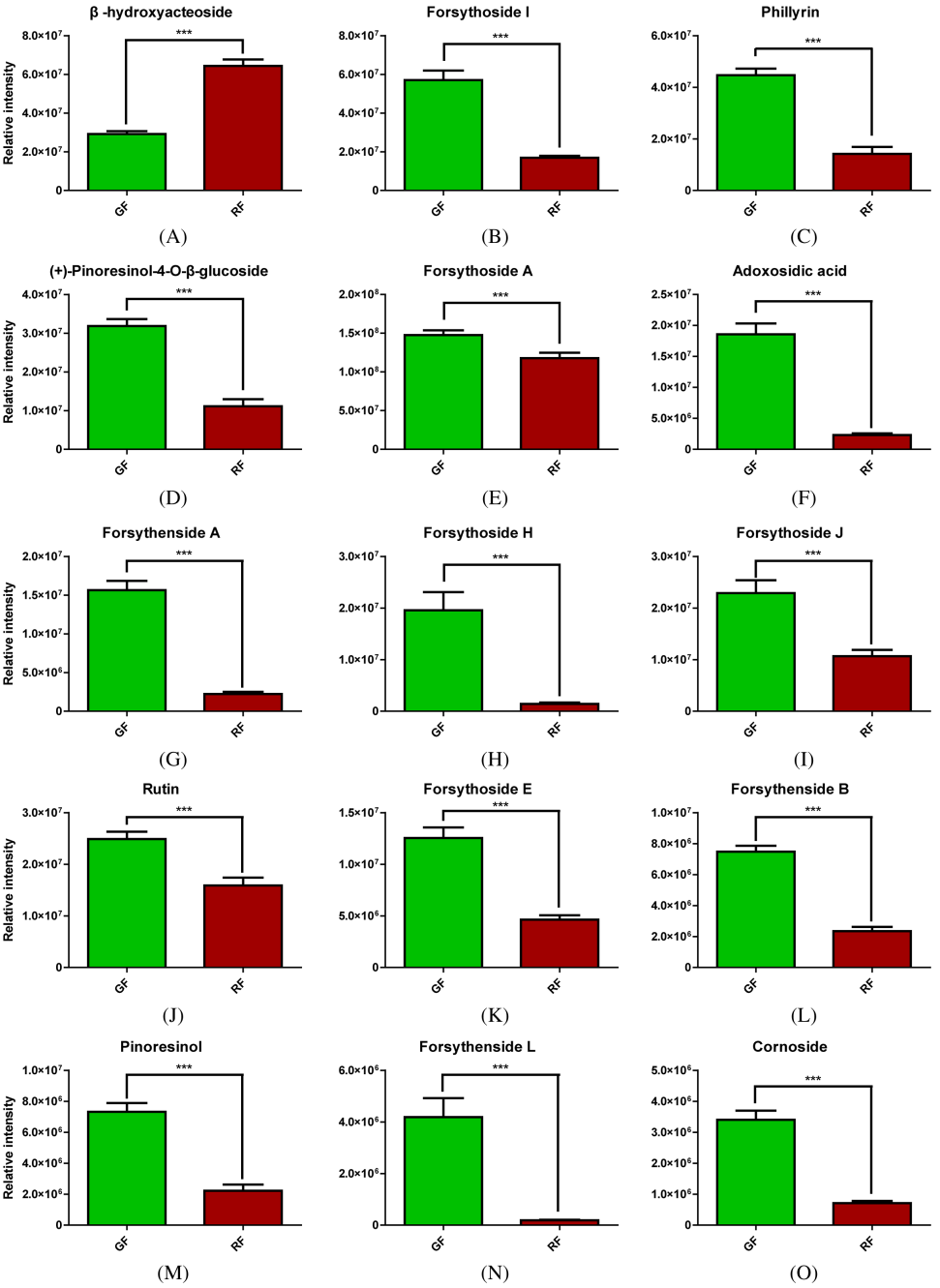


Figure 6. Relative signal intensities of the chemical markers in GF and RF identified by UPLC/MS. Data represent the mean ± SD. ***$p < 0.001$.

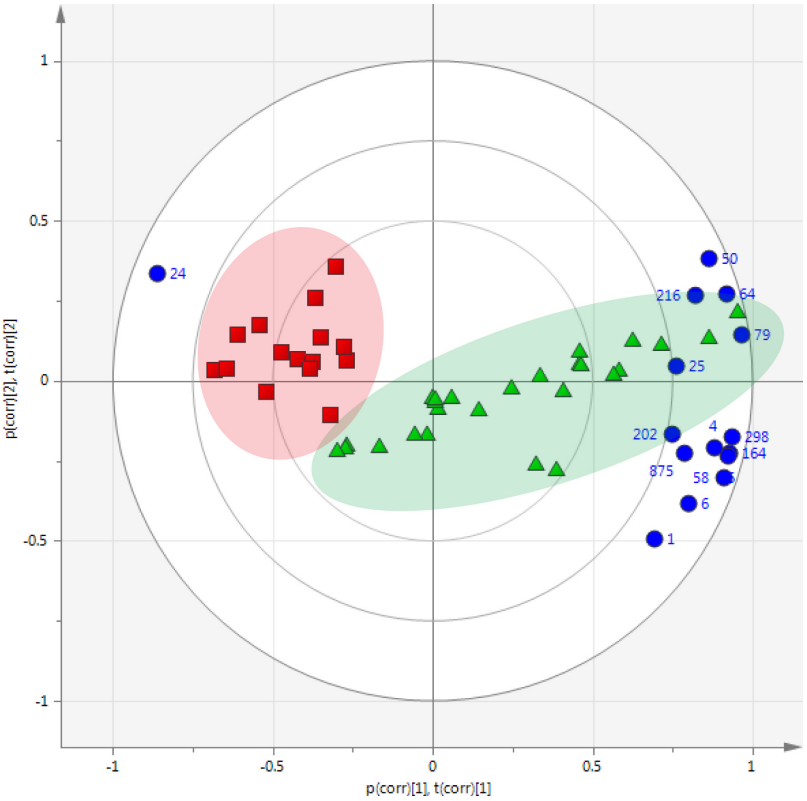


Figure 7. PCA/bi-plots of the 41 *Forsythia suspensa* aqueous extracts samples constructed using 15 chemical markers.

variables with similar loadings appeared to be strongly correlated with the observations, and all GF and RF samples were successfully classified into their corresponding groups in a similar manner as shown in Fig. 3A. The PCA/bi-plot results revealed that applying the 15 identified chemical markers provided a good solution to discriminating GF and RF successfully.

Different Anticancer Activity of GF and RF on B16-F10 Melanoma and LLC Lung Cancer Cells In Vitro

In order to investigate the *in vitro* anticancer activity of GF and RF, an MTT assay was applied to examine the proliferation of murine melanoma B16-F10 and LLC lung cancer cells treated with series concentrations of GF and RF. As shown in Fig. 8, treatment with GF exhibited a strong cytotoxicity against both B16-F10 cells and LLC cells in a dose-dependent manner. Significant inhibition of cell proliferation was exhibited after 24 h treatment in the GF treatment group, but not in the RF treatment group (Figs. 8A and 8C). The IC_{50} of GF was about 5.0 mg/ml in B16-F10 cells and 1.87 mg/ml in LLC cells, while

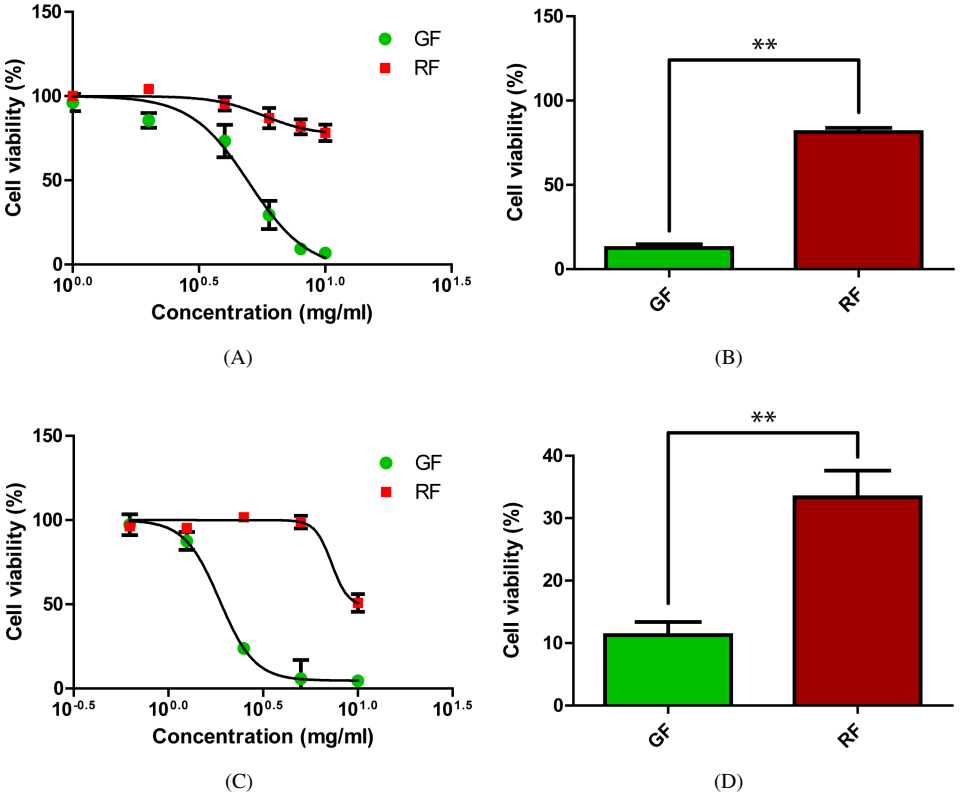


Figure 8. The anticancer activity of GF and RF measured by MTT assay. The cell viability curve of B16-F10 cells (A) and LLC cells (C) treated with a series of concentrations for 24 h. The cell viability of B16-F10 cells (B) and LLC cells (D) treated with 10 mg/ml GF and RF for 24 h, respectively. Results are based on independent-sample *t*-test and presented as the mean ± SEM (GF, $n = 26$; RF, $n = 15$). $^{**}p < 0.01$.

the IC_{50} of RF was more than 10 mg/ml in both cell lines. GF and RF (10 mg/ml) treatment had 87.41 ± 2.23% and 18.74 ± 2.54% inhibitory rate on B16-F10 melanoma cell proliferation comparing with the control group, respectively (Fig. 8B). Similar results were also achieved in LLC cells, which had 88.72 ± 2.12% and 66.66 ± 4.28% inhibitory rate in GF and RF treated groups, respectively (Fig. 8D). These results suggested that GF exhibited much stronger anticancer activity against B16-F10 melanoma and LLC cells *in vitro* than RF did.

Different Antitumor Activities of GF and RF Against Melanoma In Vivo

We applied a tumor allograft mouse model to compare the anticancer tumor activity of GF and RF *in vivo*. Murine melanoma B16-F10 cells were transplanted subcutaneously into mice on day 0. GF and RF were given at concentration of 10 g/kg by oral gavage on the same day and every two days after that. Comparing the survival curve of GF and RF treatment group with the control group, the survival curve of RF group was not

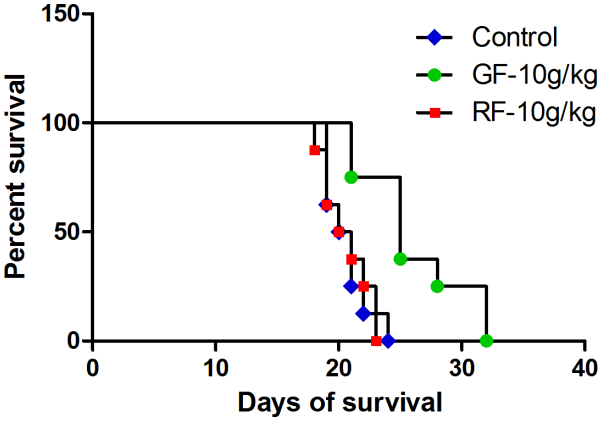


Figure 9. The antitumor activity of GF and RF on B16-F10 melanoma *in vivo*. Survival rate was monitored along with oral administration of FAE or distilled water to C57BL/6 mice ($n = 8$). When the tumor volume is greater than 2500 mm^3, the mice were sacrificed and marked death. The animal survival rate was analyzed by Log-rank (Mantel-Cox) Test. The survival rate of mice in GF group was significantly higher than that in control group ($p = 0.009$).

significantly different from the one of control group. However, the average survival time of GF treatment group was significantly extended compared to the control group (Fig. 9). Mice began to die in the control group since day 18 after initiating drug administration, and the number of surviving animals in the control group decreased dramatically. On experimental day 24, all mice in the control group and RF group had died from the heavy tumor burden, whereas 75% of the GF-treated group survived. The last mouse in the GF treatment groups survived until day 32. Thus, the longest survival time in GF treatment group was prolonged by 33% compared to the control group. The median survival time of both control and RF groups was 20.5 days, while the median survival time of GF group was 25 days, which was prolonged by 22% compared with the control group. These results demonstrated that GF and RF exhibited different antitumor activities against B16-F10 melanoma in C57BL/6 mice, and GF had better antitumor activity than RF *in vivo*.

The Anticancer Activity of the Chemical Markers

We have identified 15 compounds that could be used as chemical markers to distinguish GF and RF. We further explored their anticancer activity to determine whether they are responsible for the different anticancer activities of GF and RF. As shown in Fig. 10, forsythoside I, forsythoside A, forsythoside E and pinoresinol had significant inhibitory effect on cancer cell proliferation, while phillyrin, (+)-pinoresinol-4-O-β-D-glucoside and rutin had no significant inhibitory effect. Since forsythoside I and forsythoside A are structural isomers, they had similar growth inhibitory activity on B16-F10 cells. The 72 h IC_{50} of both forsythoside I and forsythoside A was 32 μg/ml. These results shown that forsythoside I, forsythoside A, forsythoside E and pinoresinol may have important roles in the anticancer activity of GF.

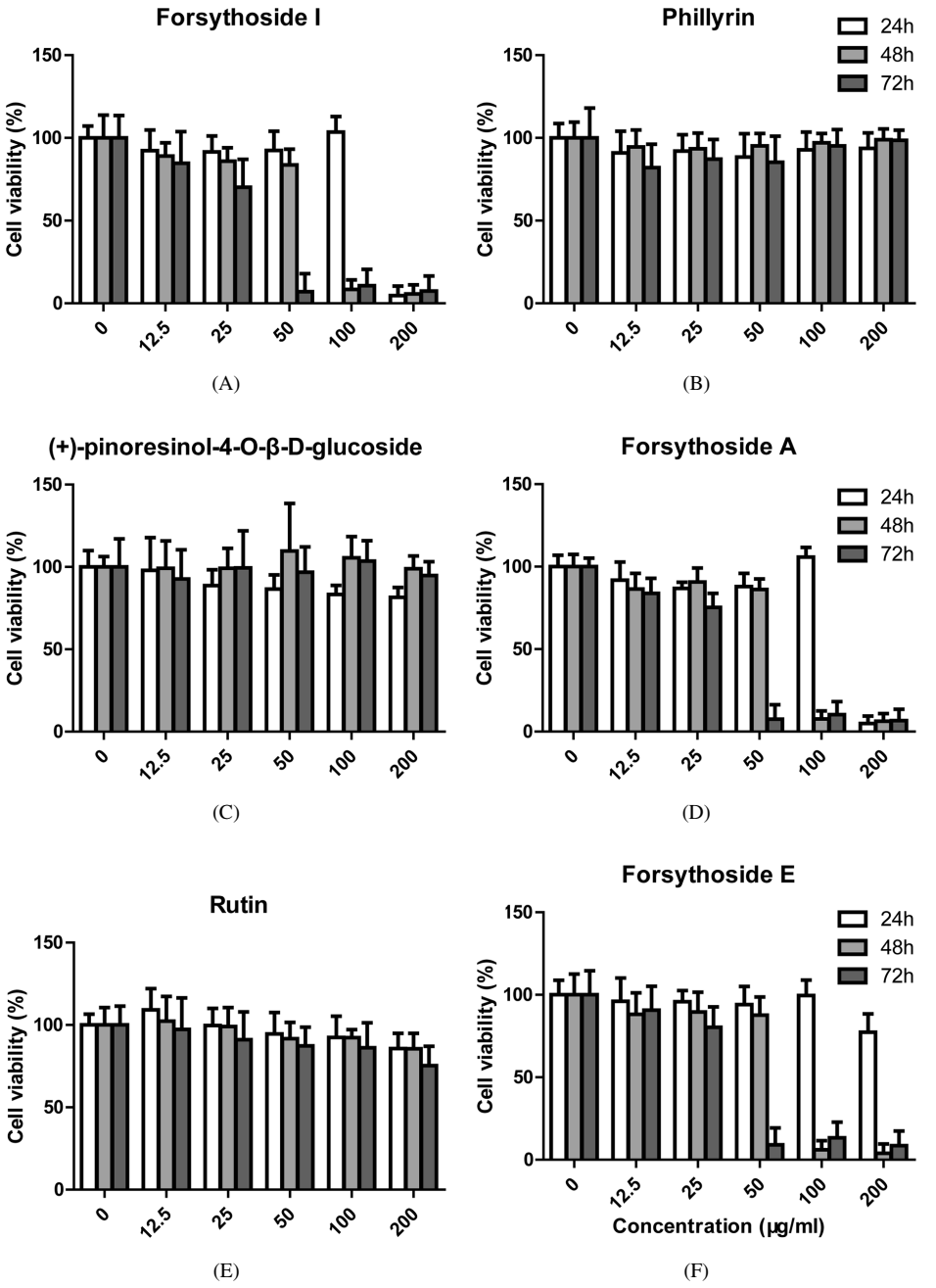
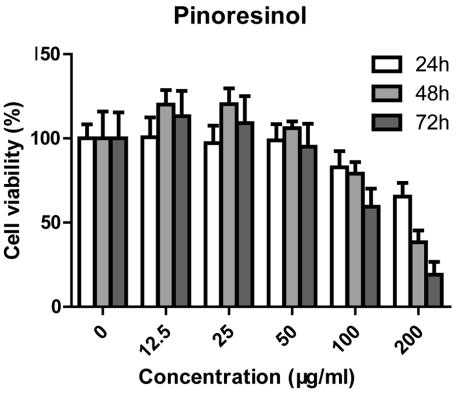


Figure 10. The anticancer activity of the chemical markers on B16-F10 cells. The cell viability was determined by MTT assay. B16-F10 cells were treated with the seven chemical markers at a series of concentrations for 24 h, 48 h and 72 h, respectively. Data represent the mean ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with control group.



(G)

Figure 10. (*Continued*)

To more conclusively determine the contribution of the four constituents of the anticancer effect of GF, we examined the contents of each constituent in the aqueous extract and then combined the constituents based on the contents to determine the anticancer effect for comparison with the aqueous extract. The contents of the four compounds in aqueous extract based on MS intensity of m/z were shown in Table S1.

As shown in Fig. S1, the mixture (GF) exhibited a stronger cytotoxicity than mixture (RF). The mixture (GF) and mixture (RF) treatment group had $52.33 \pm 1.88\%$ and $16.53 \pm 2.74\%$ inhibitory rate on B16-F10 melanoma cell proliferation compared with the control group, respectively. Compared to the inhibitory rate ($87.41 \pm 2.23\%$) of GF aqueous extract, mixture (GF) had a lower anticancer activity. This indicates that there might be other unknown anticancer constituents in the aqueous extract of GF. However, the mixture (RF) had a similar anticancer activity compared with the aqueous extract of RF.

Discussion

Both GF and RF are referred to as Forsythiae Fructus (FF) according to the *Chinese Pharmacopoeia*, even though their harvest time and herbal processing method are different. They are commonly interchangeably used without distinction in present clinical application. In order to see the preferential application of GF and RF, we randomly collected FF from different retail and hospital pharmacies from different areas of China. Totally 26 GF samples and 15 RF samples were collected from 31 pharmacies (10 pharmacies were able to provide both GF and RF), which indicated that generally there is no obvious distinction in usage preference for either GF or RF. It brings concerns on the drug efficacy and safety in clinical application, as their chemical composition and pharmacological activity might be markedly different between GF and RF. Thus, it is necessary to systematically investigate the differences in chemical composition of GF and RF through global characterization and their pharmacological activities like anticancer effect.

Metabolomics provides a robust approach for the analyses of complex metabolites mixtures, such as TCM herbs or formulas, which is comprehensive, unbiased, and high-throughput (Hall *et al.*, 2002; Buriani *et al.*, 2012; Hu *et al.*, 2014). Consistent with the holistic concept of TCM, the integrated strategy of metabolomics has been well accepted in TCM studies (Liu *et al.*, 2015; Chen *et al.*, 2016; Kim *et al.*, 2016). Recently, more and more studies applied metabolomics approaches for chemical characterization, bioactive component screening, efficacy evaluation of TCM herbs or formulas (Hu *et al.*, 2014; Wang *et al.*, 2014; Shi *et al.*, 2015). For example, UPLC-QTOF-MS/MS-based metabolomics approach had been used for evaluating the holistic qualities and exploring the characteristic chemical components of commercial white ginseng (WG) and red ginseng (RG). The characteristic components of RG and WG were found through the multivariate statistical analysis, including PCA and OPLS-DA pattern recognition analysis. The inconsistencies in the quality of commercial WG and RG and the possible reasons were also revealed (Zhang *et al.*, 2012). In the present study, similar non-targeted metabolomics approach was employed to investigate the major chemical differences in aqueous extracts from 41 batches of FF (26 GF and 15 RF). The results of UPLC-MS showed that there were significant differences between GF and RF in chemical constituents, and further PCA and OPLS-DA pattern recognition analysis identified 15 chemical markers which could successfully discriminate GF and RF into corresponding groups very well. Among 15 identified markers, forsythoside A and phillyrin were previously believed to be the major bioactive components in Forsythia suspense, and forsythoside I and (+)-pinoresinol-4-O-β-D-glucoside were newly found to be characteristic components of GF, while β-hydroxyacteoside was determined to be a featured compound in RF. Importantly, 14 out of 15 identified markers were significant higher in GF than those in RF, while only β-hydroxyacteoside was higher in RF. These results provided a chemical basis for the discrimination of GF and RF, and for their potential discrepant pharmacological activities.

FF has been traditionally used as a main herb in TCM formulas to treat multiple cancers in clinical cases, including esophageal cancer, stomach cancer, breast cancer, liver cancer, pancreatic cancer, cervical cancer and other cancers (Ma, 2003; Zhang, 2006; He, 2007; Men *et al.*, 2010; Li, 2011; Zhu, 2011). In recent years, several studies also proved that FF exhibited good *in vitro* anticancer activity on various cancer cell lines through inducing apoptosis and other mechanisms, as well as enhanced the sensitivity of cancer cells to chemotherapy (Hu *et al.*, 2007; Liu *et al.*, 2009; Zhong *et al.*, 2009; Sun *et al.*, 2010; Yan *et al.*, 2012; Cai *et al.*, 2013). Our recent published results also demonstrated FF aqueous extract possessed potent anti-tumor activity against melanoma both *in vitro* and *in vivo* through MAPKs/Nrf2/HO-1 mediated anti-oxidation and anti-inflammation pathways. In the present study, the anticancer activities of GF and RF were separately re-evaluated on B16-F10 melanoma and LLC lung cancer cell lines as well as tumor allografts. To our surprise, GF exhibited significantly higher growth inhibitory efficacy on B16-F10 melanoma and LLC lung cancer cells *in vitro* than RF. Moreover, RF almost has no effect on the survival of B16-F10 melanoma bearing mice compared to control mice, whereas GF treatment significantly prolonged the survival of tumor bearing mice. These results demonstrated that GF showed much better anticancer activity than RF.

We identified 15 chemical markers allowing us to successfully distinguish GF and RF, which were believed to be the potential main active constituents that account for the pharmacological activity differences. Next, several marker compounds were applied for further *in vitro* anticancer evaluation. Four compounds with significant higher contents in GF, including forsythoside I, forsythoside A, forsythoside E and pinoresinol, were demonstrated to have relatively strong anticancer activity, indicating that at least these four compounds accounted for the higher anticancer effect of GF than RF. FF is a commonly used Chinese medicinal herb in TCM prescriptions and an active pharmaceutical ingredient (API) for herbal products. In TCM prescription, there is no obvious preference in the usage of GF and RF. However, GF is more frequently applied as an API based on the market sales data, owing in part to its higher content of some effective constituents. It is consistent with our results that 14 out of 15 identified chemical markers might account for the higher anticancer activity of GF. Therefore, our results strongly suggest that GF and RF should be distinctively applied in TCM prescriptions or herbal products.

Taken together, the present study analyzed the global chemical constitutes in GF and RF through non-targeted metabolomics approach, and demonstrated that there are significant componential differences between GF and RF. Moreover, the anticancer activities of these two subtypes of FF were also proved to be very different. Through pattern recognition analysis, 15 chemical compounds were identified as specific markers to distinguishing GF and RF. Among these marker compounds, forsythoside I, forsythoside A, forsythoside E and pinoresinol were demonstrated to be key important active compounds which may account for the differences in anticancer activity between GF and RF. Considering the efficacy and safety concerns on the clinical application of FF, we strongly suggest that the usage of GF and RF should be distinguished, particularly in the anticancer formulas, in which GF should be preferentially prescribed. In addition, evaluation of the differences in pharmacological activities other than anticancer between GF and RF is also highly demanded.

Acknowledgments

This study was supported by the Macao Science and Technology Development Fund (041/2016/A), the Research Fund of the University of Macau (MYRG107(Y1-L3)-ICMS13-HCW, MYRG2015-00081-ICMS-QRCM).

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Supplementary Table

Table S1. Contents of the Major Constituents in GF and RF Aqueous Extracts

(% of Dry Herb, w/w)	Forsythoside I	Forsythoside A	Forsythoside E	Pinoresinol
GF	1.10	2.12	0.32	0.56
RF	0.32	2.00	0.14	0.22

Supplementary Figure

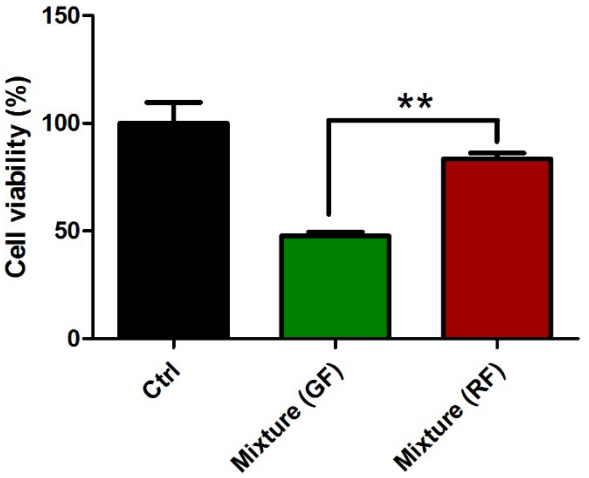

Figure S1. The anticancer activity of Mixture (GF) and Mixture (RF) measured by MTT assay. The cell viability of B16-F10 cells treated with Mixture (GF) and Mixture (RF) for 24 h. Results are based on independent-sample *t*-test and presented as the mean ± SD ($n = 4$). $^{**}p < 0.01$.