Metabolome analysis of genus *Forsythia* related constituents in *Forsythia suspensa* leaves and fruits using UPLC-ESI-QQQ-MS/MS technique

Lingdi Liu, Yu Sun, Chunxiu Wen, Tao Jiang, Wei Tian, Xiaoliang Xie, Xusheng Cui, Ruike Lu, Jiaxing Feng, Aihong Jin, Saiquen Wen, Wei Wei

1 Institute of Cash Crops, Hebei Academy of Agricultural and Forestry Sciences, Shijiazhuang, Hebei, China, 2 Hebei Medicinal Plant Engineering Technology Research Center, Shijiazhuang, Hebei, China, 3 Shijiazhuang Institute of Pomology, Hebei Academy of Agricultural and Forestry Sciences, Shijiazhuang, Hebei, China, 4 Shijiazhuang Yiling Pharmaceutical Co, Ltd, Shijiazhuang, Hebei, China

These authors contributed equally to this work.
‡ TJ, WT, XX, XC, RL, JF, AJ, SW and WW also contributed equally to this work.
* chunxiuwenny@163.com

Abstract

*Forsythia suspensa* is a traditional Chinese herb. Its numerous metabolites have important roles, as they possessed a wide range of biological activities. This study explored the accumulations of *F. suspensa* metabolites by performing widely targeted metabolomic analysis. The metabolites were studied at four stages of fruit development. Metabolites in the fruits and leaves of *F. suspensa* during fruit development included phenolic acids, flavonoids, lipids, lignans and coumarins, amino acids and their derivatives, terpenes, organic acids, nucleotides and their derivatives, alkaloids, quinones, steroids, and tannins. Fourteen *Forsythia* related metabolites were detected. Their contents varied among the developmental stages. Statistically significant correlations were found between the levels of forsythoside B and 11-methyl-forsythide, and forsythialan B and phillygenin, in both leaves and fruits. According to the correlation analysis between metabolites, *Forsythia* related metabolites were divided into two classes and five subclasses. In total, 33 compounds presented significant correlations in both fruits and leaves, which indicated the potential relationship in the synthesis of *Forsythia* related metabolites. Forsythialan B and phillygenin were both negatively correlated with L-valine, while Z-6,7-epoxyligustilid was positively correlated with both compounds. The quality control compounds forsythiaside A and phillyrin were positively and negatively correlated with uracil, respectively. These metabolomics results may facilitate the biosynthesis of *Forsythia* related metabolites.

Introduction

*Forsythia suspensa* (Tunb.) Vahl is widely used in medical applications [1–4]. Previous studies indicated that the compounds present in *F. suspensa* include phenylethanoid glycosides,
Gerplasm Resources in Technical System of Traditional Chinese Medicine Industry in Hebei Province to LL (HBCT201806201), China Agriculture Research System to CW (CARS21). Shijiazhuang Yiling Pharmaceutical Co, Ltd provided support for this study in the form of salary for XC. The specific roles of these authors are articulated in the ‘author contributions’ section. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: X.C. and Shijiazhuang Yiling Yiling Yiling Pharmaceutical Co.,Ltd did not develop products and apply patents. X.C. does not hold a dissenting opinion as being a co-author in this study. Each co-author has no potentially competing interests occurred within 5 years of conducting the research under consideration, or preparing the article for publication. This does not alter our adherence to Plos One policies on sharing data and materials.

The medicinal values vary in different plant organs of the same species due to the different metabolic processes and accumulation of metabolites in various plant tissues, and it is widely believed that the medicinal compounds in F. suspensa are present mainly in the fruit. However, previous studies have indicated that the medicinal compound, forsythiaside A, exists in leaves, fruits, and even stems. Thus, the leaves of F. suspensa may have potential medicinal value. Moreover, forsythiaside A has been reported to be even more abundant in leaves than in fruits. Differences in the levels of the chemical components of F. suspensa among plant organs have been confirmed. It also appears that all F. suspensa tissues likely contain many other medicinal compounds, although differences in abundance of these compounds among plant organs are also likely [8]. Besides, the amounts of the metabolites are varying depending on the stage of development, which greatly effects the medicinal metabolites production [9].

Studying the chemical composition and contents of the active compounds of herbal plants is complicated, as many species-distinctive metabolites are derived from secondary metabolic processes [9]. Secondary metabolites have important regulatory roles in resistance to abiotic and biotic stresses and adaptation to the environment. Thus, unique metabolites of different species may be formed during adaptation to growing conditions. Previous studies have isolated and identified various medically active compounds in the fruits of Forsythia suspensa, including the structures of rengyosides A, B and C [11]. Numerous fruit-derived metabolites have been isolated from previous studies. Phillyrin, which exists in family Oleaceae, was identified as the main component of F. suspensa [12]. Forsythiaside and forsythiaside A were isolated from air-dried fruits of F. suspensa. Previous studies demonstrated their anti-inflammatory and antioxidant effects [13, 14]. Knowledge of metabolites in herbal plants is very important. Metabolome studies [15–21] have shown positive roles in identifying the functions of multiple compounds in plants, including abiotic and biotic stress and medicinal aspects. With the wide applications of metabolomes in plant science studies, various mechanisms of environment adaptation and target compound biosynthesis in plants have been identified. In addition, the biosynthesis and metabolism of a large range of medicinal compounds have been elucidated [19].

Previous studies on F. suspensa have investigated medicinal compounds in different plant organs in terms of their effects on diseases and their metabolism in vivo [19–21]. The biosynthesis and metabolic pathways of Forsythia related metabolites have received little attention and remain poorly understood [20–22]. Although, the use of enzyme assays has revealed the biosynthesis of lignans and iridoids in F. suspensa compounds [23, 24].

In this study, we conducted a targeted metabolomic analysis of metabolites in the leaves and fruits of F. suspensa at four fruit developing stages to investigate the vast Forsythia related metabolites simultaneously to analyze their potential associations and the correlations with other metabolites to provide basic theoretical supports on their biosynthesis studies. We also
analyzed the dynamics of *Forsythia* related medicinal metabolites in fruits and leaves to determine the optimum harvesting time and plant organ.

**Materials and methods**

**Plant material**

The *Forsythia suspensa* plants were located in Jingxing, Shijiazhuang, Hebei, China. The leaves and fruits were sampled at four fruit developing stages in 2019. About twenty uniform asexual propagation seedlings of *Forsythia suspensa* were used for this study. We sampled fifty fruits which have relatively uniform growth and physiological state each sampling time as one biological replicate, and the fourth leaves were sampled with the same method. Each sampling was repeated three times to reduce the error and ensure measurement data validation and accuracy. The sample were collected around ten a.m. each time. After sampling, the samples were transported to the laboratory and cleaned in distilled water, and then put into liquid nitrogen and stored at −80°C. We sampled the fruits and leaves every 50 days since May 30th. The sampling dates in 2019 were as follows, May 30th, July 20th, September 10th and October 30th. The four stages were as follows: early-stage (T1), mid-stage (T2), mid-late-stage (T3) and late-stage (T4).

**Sample preparation and extraction**

The leaf and fruit samples were freeze-dried to extract the metabolites in the tissues. The freeze-dried samples were crushed using a mixer mill (MM 400, Retsch) with a zirconia bead for 1.5 min at 30 Hz. We weighted 100 mg powder of each sample and extracted overnight at 4°C with 0.6 ml 70% aqueous methanol (HPLC grade, Merck, www.merckgroup.com/). Following centrifugation at 10,000 g for 10 min, the extracts were absorbed (CNWBOND Carbon-GCB SPE Cartridge, 250 mg, 3 ml; ANPEL, Shanghai, China, www.anpel.com.cn/cnw) and filtrated (SCAA-104, 0.22 μm pore size; ANPEL, Shanghai, China, http://www.anpel.com.cn/) before ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) analysis.

**UPLC and electrospray ionization triple quadrupole linear ion trap (ESI-Q TRAP-MS/MS) conditions**

The sample extracts were analyzed using an UPLC-ESI-MS/MS system (UPLC, Shim-pack UFLC SHIMADZU CBM30A system, www.shimadzu.com.cn/; MS, Applied Biosystems 4500 Q TRAP, www.appliedbiosystems.com.cn/). The analytical conditions were as follows, UPLC: column, Agilent SB-C18 (1.8 μm, 2.1 mm 100 mm); The mobile phase was consisted of solvent A, pure water with 0.1% formic acid (HPLC grade, Merck, www.merckgroup.com/), and solvent B, acetonitrile (HPLC grade, Merck, www.merckgroup.com/). Sample measurements were performed with a gradient program that employed the starting conditions of 95% A, 5% B. Within 9min, a linear gradient to 5% A, 95% B was programmed, and a composition of 5% A, 95% B was kept for 1min. Subsequently, a composition of 95% A,5.0% B was adjusted within 1.10 min and kept for 2.9 min. The column oven was set to 40°C; The injection volume was 4μl. The effluent was alternatively connected to an ESI-triple quadrupole-linear ion trap (QTRAP)-MS.

LIT and triple quadrupole (QQQ) scans were acquired on a triple quadrupole-linear ion trap mass spectrometer (Q TRAP), API 4500 Q TRAP UPLC/MS/MS System, equipped with an ESI Turbo Ion-Spray interface, operating in positive and negative ion mode and controlled by Analyst 1.6.3 software (AB Sciex). The ESI source operation parameters were as follows: ion
source, turbo spray; source temperature 550˚C; ion spray voltage (IS) 5500 V (positive ion mode)/-4500 V (negative ion mode); ion source gas I (GSI), gas II(GSII), curtain gas (CUR) were set at 50, 60, and 30.0 psi, respectively; the collision activated dissociation (CAD) was set at high level. Instrument tuning and mass calibration were performed with 10 and 100 μmol/L polypropylene glycol solutions in QQQ and LIT modes, respectively. QQQ scans were acquired as MRM experiments with collision gas (nitrogen) set to 5 psi. DP and CE for individual MRM transitions was done with further DP and CE optimization. A distinctive set of MRM transitions were monitored for each period according to the metabolites eluted within this period.

**Correlation analysis of metabolites in leaves and fruits**

The correlation analysis was performed with *Forsythia* related metabolites and other metabolites detected in this experiment from four fruit developing stages. The correlation values between *Forsythia* related metabolites and other metabolites were based on data of their ion intensities. All data were analyzed by bivariate analysis followed by Pearson’s correlation and Two-tailed test of significance among different groups.

Experimental data of *Forsythia* related metabolites contents were analyzed by one-way analysis of variance followed by Tukey’s multiple range test to detect differences among the groups. A $p$-value < 0.05 was considered significant. All statistical analyses were performed using IBM SPSS Statistics 22 software (IBM Corp., Armonk, NY, United States).

**Results**

**Analysis of levels of *Forsythia* related metabolites in leaves and fruits**

In this study, ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) was used to determine the fold-change values of *Forsythia* related metabolites in leaves and fruits. Quantifications utilized a QqQ mass analyzer. The total ion current (TIC) maps of the mixed sample (quality control sample, QC) were used to verify the reliability and repeatability of the experiment (S1 and S2 Figs). The peak shapes of the TIC maps were consistent, indicating that the results of the experimental results were reliable and repeatable. The extracted ion chromatograms (XIC) exhibited the chromatographic peaks corresponding to the mass metabolites (S3 and S4 Figs). The representative TICs an XICs of fruits and leaves at each harvesting time are shown in S5–S36 Figs. The mass spectrum information of *Forsythia* related constituents is shown in S1 Table.

Forsythiaside C, forsythoside B, rengyoside B, rengyoside, forsythiaside B, forsythiaside J, forsythiaside A, isoforsythoside A, forsythianal B, forsythianal A, phillygenin, phillyrin, 11-methyl-forsythide and forsythide were all detected in fruits and leaves. The *Forsythia* related metabolites were classified as phenolic acids, lignans, and terpenoids, respectively. The fold-change values in the relative levels of metabolites in the fruits and leaves at different fruit developing stages varied markedly (Fig 1). The relative contents of forsythide, phillyrin, forsythiaside A, and isoforsythoside A were higher in leaves than in fruits, with the fold-change values < 1 at the four stages, which indicated that these constituents may be rich in leaves. In contrast, 11-methyl-forsythide, forsythinside B, forsythiaside J, forsythiaside C, forsythoside B, forsythianal A and forsythianal B might be the compounds rich in fruit. Forsythiaside J, forsythiaside A and forsythiaside C represented similar variation among the four fruit developing stages. Forsythiaside A in fruit exhibited lower levels than that in leaves, which differed from forsythiaside J and forsythiaside C. Phillygenin, forsythianal A and forsythianal B showed similar variations in fold-change values. However, phillygenin in fruits did not show much higher levels than that in leaves during the fruit development process.
Fig 1. Fold-changes of *Forsythia* related metabolites in leaves and fruits in four harvesting stages.

https://doi.org/10.1371/journal.pone.0269915.g001
Correlation analysis of Forsythia related metabolites

Fourteen metabolites were detected in the fruits and leaves of Forsythia. Correlation analyses revealed that forsythiaside C was positively correlated with forsythiaside J, and forsythoside B was positively correlated with 11-methyl-forsythide (Tables 1 and 2). Phillygenin was negatively correlated with rengyoside B, but was positively correlated with forsythialan A. In leaves, forsythiaside A, isoforsythoside A, and rengyoside A were not significantly correlated with any other Forsythia related metabolites.

Correlations were evident among metabolites in fruits. Forsythiaside C had significant positive correlations with forsythialan B, forsythialan, and phillygenin. Forsythoside B was positively correlated with 11-methyl-forsythide and forsythide. Forsythenside B was negatively correlated with both forsythiaside and isoforsythoside A, and positively correlated with phillyrin. Forsythiaside A was positively correlated with isoforsythoside A and negatively correlated with forsythoside B and phillyrin. Forsythialan A was positively correlated with forsythiaside C and forsythialan B; forsythoside B, 11-methyl-forsythide, and forsythialan B were all positively correlated with phillygin in both leaves and fruits. However, forsythiaside J and rengyoside B were not significantly correlated with the other Forsythia related metabolites.

Despite the inconsistent correlations between Forsythia related metabolites in fruit and leaves. Forsythialan B, phillygenin and 11-methyl-forsythide and forsythoside B showed significant and corresponding positive correlations.

Correlations between Forsythia related metabolites and other metabolites

To reduce errors caused by inconsistent amounts of metabolites in fruits and leaves, among the Forsythia-related metabolites, we screened for metabolites that exhibited similar correlations in both fruits and leaves. Of the 489 metabolites identified, 225 were selected and classified into 10 types (S2–S4 Tables), comprising phenolic acids (n = 47), lignans and coumarins (n = 21), terpenoids (n = 19), amino acids and their derivatives (n = 11), quinones (n = 2), flavonoids (n = 41), alkaloids (n = 6), nucleotides and their derivatives (n = 13), tannins (n = 1), lipids (n = 18), organic acids (n = 14) and others (n = 31).

Based on the correlations shown in Figs 2 and 3, the Forsythia related metabolites were divided into two groups. Forsythiaside C, forsythenside B, forsythiaside J, forsythialan B, phillygenin, and phillyrin were all negatively or positively correlated with the other metabolites. However, the correlations between forsythoside B, rengyoside B, rengyoside A, forsythiaside A, isoforsythoside A, 11-methyl-forsythide, and forsythide and the other metabolites were inconsistent.

The Forsythia related metabolites and their substance types did not present any correlations. Phenolic acids were distributed in both classes, whereas only monoterpenoids and lignans were present in two classes. The correlations between metabolites of the same substance type and Forsythia related metabolites were inconsistent.

When ranked by forsythiaside, heatmaps of correlation values of Forsythia related metabolites showed a similarity of correlations between metabolites in fruits and leaves (Fig 3). Correlation values between other metabolites and Forsythia related metabolites in different plant organs exhibited similar results. These findings indicated the reproducibility of the potential metabolic pathways and the differentiation of metabolites. The results suggested that the metabolic pathways of Forsythia related metabolites could be divided into different groups.

Molecular structures and cluster analysis of Forsythia related metabolites

To further explore the relationships between the Forsythia related metabolites and other metabolites, a cluster analysis was performed (Fig 3). The Forsythia related metabolites could be
divided into two classes, which were each further divided into two subclasses. *Forsythia* related metabolites in the first subclass of class 1 were forsythialan B, phillygenin, forsythiaside C, phillyrin, forsythiaside J, and forsythenside B. Only forsythialan A was clustered in the second class. The *Forsythia* related metabolites in the first subclass of the class 2 included rengyoside B, forsythiaside A, forsythoside B, 11-methyl-forsythide, rengyoside A, and forsythide. Only isoforsythiaside A was clustered in the second subclass. The *Forsythia* related metabolites in classes 1 and 2 were lignans, phenolic acids, monoterpenoids, and phenolic acids. (Table 3, Fig 4).

The *Forsythia* related metabolites in the first class displayed two structures: 5-(4-(3,4-dimethoxyphenyl) tetrahydro-1H,3H-furo[3,4-c]furan-1-yl)-2-methoxyphenol (phillygenin) and (2R,3R,4S,5S,6S)-tetrahydro-2H-pyran-2,3,4,5,6-pentaol (Fig 5). The differentiation of phillyrin and phillygenin was caused by the insertion of (2R,3R,4S,5S,6S)-tetrahydro-2H-pyran-2,3,4,5,6-pentaol. The insertion may also occur during the biosynthesis processes of forsythialan A and forsythenside B. Forsythialan A, rengyoside B, phillygenin, and forsythialan B have similar molecular structures, and their differentiations may be due to hydroxylation.

### Table 1. Correlation analysis of *Forsythia* related metabolites in leaves of *Forsythia suspense*.

| Forsythiaside C | Forsythoside B | Rengyoside B | Rengyoside A | Forsythene B | Forsythiadiene B | Forsythiadiene J | Forsythiadiene A | Isoforsythiadiene A | Forsythiadiene B | Forsythiadiene A | Phillyrin | 11-Methyl-forsythide | Forsythide |
|-----------------|----------------|-------------|-------------|-------------|-----------------|---------------|----------------|------------------|----------------|----------------|------------|-----------------|-------------|
| Forsythiaside C | 1              |             |             |             |                 |               |               |                   |                 |               |            |                 |             |
| Forsythoside B  | -0.73          | 1           |             |             |                 |               |               |                   |                 |               |            |                 |             |
| Rengyoside B    | -0.78          | 0.214       | 1           |             |                 |               |               |                   |                 |               |            |                 |             |
| Rengyoside A    | -0.87          | 0.914       | 0.420       | 1             |                 |               |               |                   |                 |               |            |                 |             |
| Forsythene B    | 0.645          | -0.802      | -0.114      | -0.936       | 1               |               |               |                   |                 |               |            |                 |             |
| Forsythiadiene J| 0.93           | -0.555      | -0.915      | -0.75        | 0.582           | 1             |               |                   |                 |               |            |                 |             |
| Forsythiadiene A| -0.093         | 0.149       | 0.091       | 0.489        | -0.3            | -0.876        | 1             |                   |                 |               |            |                 |             |
| Isoforsythiadiene A | 0.283 | 0.379       | -0.795      | 0.064        | -0.267          | 0.561         | -0.836       | 1                 |                 |               |            |                 |             |
| Forsythiadiene B | 0.853          | -0.487      | -0.874      | -0.501       | 0.364           | 0.840         | -0.955       | 0.457             | 1               |               |            |                 |             |
| Forsythiadiene A | 0.532          | 0.26        | -0.806      | 0.366        | -0.467          | 0.31          | -0.559       | -0.8              | 0.736           | 1             |            |                 |             |
| Phillyrin       | 0.85           | -0.575      | -0.364      | -0.485       | 0.153           | 0.915         | -0.761       | 0.624             | 0.971           | -0.779         | 1           |                 |             |
| Phillyrin       | 0.935          | -0.621      | -0.806      | -0.851       | 0.675           | 0.360         | -0.868       | 0.468             | 0.704           | -0.299         | 0.782       | 1                 |             |
| 11-Methylforsythide | -0.577      | 0.861       | -0.047      | 0.785        | -0.784          | -0.504        | -0.13        | 0.618             | 0.437           | -0.148         | -0.38       | 1                 |             |
| Forsythide      | -0.279         | 0.519       | 0.705       | 0.064        | 0.056           | -0.557        | 0.884        | -0.949            | -0.272          | -0.576         | -0.492      | -0.543          | -0.564      |

Note:

* "p < 0.05 was considered to demonstrate statistically significant differences.*

** "p < 0.01 was considered to demonstrate statistically significant differences, the same below.*

https://doi.org/10.1371/journal.pone.0269915.t001

### Table 2. Correlation analysis of *Forsythia* related metabolites in fruits of *Forsythia*.

| Forsythiaside C | Forsythoside B | Rengyoside B | Rengyoside A | Forsythene B | Forsythiadiene B | Forsythiadiene J | Forsythiadiene A | Isoforsythiadiene A | Forsythiadiene B | Forsythiadiene A | Phillyrin | 11-Methylforsythide | Forsythide |
|-----------------|----------------|-------------|-------------|-------------|-----------------|---------------|----------------|------------------|----------------|----------------|------------|-----------------|-------------|
| Forsythiaside C | 1              |             |             |             |                 |               |               |                   |                 |               |            |                 |             |
| Forsythoside B  | -0.6           | 1           |             |             |                 |               |               |                   |                 |               |            |                 |             |
| Rengyoside B    | -0.6           | 0.9         | 1           |             |                 |               |               |                   |                 |               |            |                 |             |
| Rengyoside A    | -0.3           | 0.08        | 0.597       | 1           |                 |               |               |                   |                 |               |            |                 |             |
| Forsythene B    | 0.78           | -0.1        | -0.451      | 0.31         | 1               |               |               |                   |                 |               |            |                 |             |
| Forsythiadiene J| 0.66           | 0.14        | 0.175       | 0.26         | 0.559           | 1             |               |                   |                 |               |            |                 |             |
| Forsythiadiene A| -0.7           | 0.12        | 0.484       | -0.3         | -0.99           | -0.422        | 1             |                   |                 |               |            |                 |             |
| Isoforsythiadiene A | -0.8       | 0.19        | 0.526       | -0.3         | -0.99           | -0.455        | 0.996         | 1                 |                 |               |            |                 |             |
| Forsythiadiene B | 0.99          | 0.6        | -0.441      | -0.4         | 0.68            | 0.627         | 0.586         | 0.955             | 1               |               |            |                 |             |
| Forsythiadiene A | 0.96          | -0.7        | -0.622      | -0.5         | 0.562           | 0.662         | -0.458        | -0.932            | 0.989           | 1             |            |                 |             |
| Phillyrin       | 0.99          | -0.6        | -0.724      | -0.3         | 0.806           | 0.35          | -0.744        | -0.798            | 0.972           | -0.35          | 1           |                 |             |
| Phillyrin       | 0.75           | -0.2        | -0.5        | 0.3           | 0.996           | 0.478         | -0.966        | -0.999            | 0.651           | 0.529          | 0.793       | 1                 |             |
| 11-Methylforsythide | -0.4          | 0.98        | 0.919       | 0.85         | -0.08           | 0.339         | 0.104         | 0.157             | -0.508          | -0.547         | -0.512     | -0.126          | 1           |
| Forsythide      | -0.5           | 0.90        | 0.769       | 0.97         | 0.163           | 0.15          | -0.186       | -0.105            | -0.423          | -0.503         | -0.173     | 0.15            | 0.957       |

https://doi.org/10.1371/journal.pone.0269915.t002
Forsythialan A and forsythenside B both have a hydroxyl group inserted, whereas forsythialan A also undergoes hydrogenation.

In the second subclass of *Forsythia* related metabolites, isoforsythoside A, forsythoside B, rengyoside A, forsythiaside A, rengyoside B, forsythide and 11-methyl-forsythide all displayed the same structure as (2R,3R,4S,5S,6S)-tetrahydro-2H-pyran-2,3,4,5,6-pentaol (Figs 4 and 5). Although 11-methyl-forsythide and forsythoside B were in the same cluster, but 11-methyl-forsythide was more likely to be a derivative of forsythide. In addition, methylation was observed in the molecular structures of 11-methyl-forsythide and forsythiaside A as well. Although forsythiaside

---

**Fig 2. Correlation heatmap of the *Forsythia* related metabolites in leaves and fruits in each harvesting period.** The two heatmaps represent the correlations between the metabolites and the *Forsythia* related metabolites in fruits and leaves, respectively. Heatmap of fruits was listed on the left and that of leaves was listed on the right. The left column of the heatmap shows the classification of different types of metabolites. Each *Forsythia* related metabolite is represented by a single column, as follows: A: forsythiaside C, B: forsythoside B, C: rengyoside B, D: rengyoside A, E: forsythiaside B, F: forsythiaside J, G: forsythiaside A, H: isoforsythoside A, I: forsythialan B, J: forsythialan A, K: phillygenin, L: phillyrin, M: 11-methyl-forsythide, and N: forsythide. Red and blue color represent positive and negative correlations. https://doi.org/10.1371/journal.pone.0269915.g002

Forsythialan A and forsythenside B both have a hydroxyl group inserted, whereas forsythialan A also undergoes hydrogenation.

In the second subclass of *Forsythia* related metabolites, isoforsythoside A, forsythoside B, rengyoside A, forsythiaside A, rengyoside B, forsythide and 11-methyl-forsythide all displayed the same structure as (2R,3R,4S,5S,6S)-tetrahydro-2H-pyran-2,3,4,5,6-pentaol (Figs 4 and 5). Although 11-methyl-forsythide and forsythoside B were in the same cluster, but 11-methyl-forsythide was more likely to be a derivative of forsythide. In addition, methylation was observed in the molecular structures of 11-methyl-forsythide and forsythoside B. Methylation also occurred in the cluster of rengyoside B and forsythiaside A as well. Although forsythiaside
A and isoforsythoside A have the same groups, cluster analysis revealed a distant relationship. However, isoforsythoside A also undergoes methylation.

**Screening of compounds significantly correlated with Forsythia related metabolites**

A total of 33 compounds displayed significant correlations with nine *Forsythia* related metabolites in leaves and fruits (*S5 Table*). Nine metabolites had significant or highly significant
positive correlations with forsythiaside C in the fruits and leaves during the fruit development process. Guanosine, cynarin, D-pinitol, (S,S,8R,9Z,12Z)-5,8-dihydroxyoctadeca-9,12-dienoate, hispidulin, glucose-1-phosphate, 9-hydroxy-12-oxo-15(Z)-octadecenoic acid, D-glucose 6-phosphate, and (S)-2-phenyloxirane were all positively correlated with forsythiaside C. The metabolites were classified as nucleotides and their derivatives, phenolic acids, lipids, flavonoids, and others. Galactinol was positively correlated with forsythoside B in both fruits and leaves. Both of the metabolites were classified as phenolic acids. Rengyoside B was significantly or highly significantly positively correlated with hesperetin-6-C-glucoside-7-O-glucoside and D-panose. D-Galactaric acid was significantly negatively correlated with forsythenside B in fruits and had a highly significantly negative correlation in leaves. 2-α-Linolenoyl-glycerol-1,3-di-O-glucoside was positively correlated with forsythiaside J in both fruits and leaves. LysoPC 18:3 and 2-α-linolenoyl-glycerol-1,3-di-O-glucoside, which are both lipids, were negatively correlated with forsythiaside J, and 2-α-linolenoyl-glycerol-1,3-di-O-glucoside had a highly significant negative correlation (correlation coefficient = -0.999) in leaves. The Forsythia suspensa quality control compound forsythiaside A was positively correlated with uracil in both plant organs. The recently identified compound forsythialan B, was positively correlated with Z-6,7-epoxyligustilide, and esculetin, whereas it was negatively correlated with L-valine. Phillygenin and forsythialan B were classified as lignans and had similar patterns of correlation with the other metabolites. L-Valine had very similar correlations with phillygenin and forsythialan B. Forsythialan B and Z-6,7-epoxyligustilide were both positively correlated with phillygenin. Phillyrin was significantly negatively or positively correlated with 10 metabolites. Only chestnutlignansoide and calycosin were positively correlated with phillyrin, β-amyrenone, D-glucurono-6,3-lactone, sedoheptulose, citric acid, D-galacturonic acid, uracil and δ-amyrenone, and euscaphic acid had significant or extremely significant negative correlations with phillyrin.

**Discussion**

In this study, Forsythia related metabolites in leaves and fruits during different fruit development stages were analyzed. The goal was to clarify the optimal harvesting time necessary to achieve a high content of medicinal compounds, and to establish the plant organs with the highest metabolite contents.
Forsythia suspensa has multiple medical applications because of its complex mixture of metabolites in leaves and fruits [25–27]. Significant differences in plant metabolic processes among plant organs have been described. Studies on F. suspensa have challenged the belief that the fruit is the main source of medicinal compounds, with leaves reportedly being a better source [26]. In the present study, the levels of phillyrin, forsythiaside A, isoforsythoside A, and forsythide in leaves were higher than those in fruits, consistent with the results of some previous studies, whereas the levels of forsythenside B, forsythoside B, forsythialan A, forsythialan B, and forsythiaside J were higher in fruits than in leaves. These compounds derived from leaves may be less suitable for medical use. The levels of the Forsythia related metabolites differed among development stages and plant organs, which is an important consideration for harvesting.

Studies on Forsythia related compounds have indicated that their main effects are antibacterial, antifungal, antiviral, antioxidant, anti-inflammatory, antitumor, and hypolipidemic activities [2, 21, 27–30]. Different compounds have different medicinal effects.

Isoforsythoside A and forsythiaside A have been reported to have inhibitory effects on Staphylococcus aureus and other pathogenic bacteria [31]. One study indicated that these molecules also have potent activities against fungi and influenza A virus [32]. Forsythia suspensa metabolites also have antiseptic properties. Forsythin has been reported to exhibits anti-oxidation activity, lowers blood lipid levels and inhibits the oxidation of low-density lipoprotein [33]. Another study demonstrated that phillyrin can enhance the scavenging of 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 3-ethylbenzothiazoline-6-sulfonic acid (ABTS) free radicals [34]. The present study confirmed differences in the content of Forsythia related metabolites.
Fig 5. Molecular structures of *Forsythia* related metabolites.

https://doi.org/10.1371/journal.pone.0269915.g005
between leaves and fruits. Different therapeutic effects can be expected between compounds derived from the leaves and fruits of *F. suspensa*. The plant organs from which compounds are extracted and the harvesting stage should be considered to ensure that the desired medicinal effects are achieved. We suggest that the leaves of *Forsythia suspensa* harvested at an intermediate stage, would likely provide the greatest medicinal effects because of their high levels of forsythiaside A, isoforsythoside A, phillygenin, and phillyrin.

Significant progress has been made in determining the components of medicinal herbs with medical applications, and the biosynthetic pathways of podophyllotoxin and colchicine have been identified [35, 36]. Studies on colchicine and podophyllotoxin have indicated that a combined metabolomic and transcriptomic analysis approach is suitable for studying biosynthetic pathways. Metabolomics is the major tool for the identification and screening of target substances, and determination of their levels in different plants. However, the biosynthetic pathways in *F. suspensa* are still not fully understood. Although previous studies have involved only enzyme assays, metabolic research offers much promise as a means of understanding the basis of biosynthetic pathways. A previous transcriptome study of the biosynthesis pathways of lignans performed in *Forsythia koreana* revealed that pinoresinol-lariciresinol reductase (PLR) and matairesinol O-methyltransferase (MOMT) from this plant catalyzed the biosynthesis of lariciresinol from pinoresinol and biosynthesis of larctigenin from matairesinol [37]. Phillygenin, forsythialan A and forsythialan B were all classified into one cluster by analyzing their correlations. PLR and MOMT regulate the transformations of pinoresinol to lariciresinol and of matairesinol to arctigenin, which are similar to the transformation of phillygenin to forsythialan A and from forsythialan A to forsythialan B. Thus, PLR and MOMT may be involved in the biosynthesis of distinctive metabolites of *Forsythia*. Because PLR and MOMT of *F. koreana* participate in the biosynthesis processes of forsythialan A and forsythialan B, we assumed that forsythialan A and forsythialan B are the downstream metabolites of phillygenin. We suggest that the medicinal functions of phillygenin, forsythialan A and forsythialan B should be further studied to verify the functions of PLR and MOMT in *Forsythia suspensa*. Ying et al. [38] reported the different catalytic efficiencies and specificities of pinoresinol-lariciresinol reductases and pinoresinol reductases (PrRs) in *Arabidopsis thaliana* and *Isat...
metabolism. The chemical structures of the metabolites in each subclass further indicated the differentiation of the biosynthetic pathways. Although the chemical structures of many metabolites have already been identified, their biosynthetic pathways remain poorly understood. Phenolic acids presented in both classes, and their biosynthetic processes may play important roles in their upstream metabolism. Terpenoids and lignans were clustered into different subclasses separately. The biosynthetic pathways of the lignans and terpenoids may have significant effects on metabolites differentiation. The biosynthetic pathways of the *Forsythia* related metabolites were significantly different, and there might be different upstream biosynthetic processes in the two subclasses. Differences in synthesis and differentiation of downstream products may underlie the different metabolite compositions of the two classes. In the present study, forsythialan B and phillygenin, 11-methyl-forsythide and forsythoside B showed negative or positive correlations, respectively. The cluster analysis also showed that forsythialan B and phillygenin 11-methyl-forsythide and forsythoside B may have similar processes in their biosynthesis pathways, which also confirmed the validity of the correlation and cluster analysis. Forsythialan B, phillygenin, forsythiaside C, forsythiaside J, forsythiaside B, phillyrin and forsthalan A were clustered in one subclass. However, forsythialan B, phillygenin, phillyrin and forsythialan A were identified as lignans. Although the other metabolites were phenolic acids, and phenolic acids present in both subclasses. Thus, we suggest that the studies on metabolites of these lignans should focus on the transformations from phenolic acid to lignans and the differentiations of their chemical groups, such as methoxy and hydroxyl. Forsythide and 11-methylforsythide were monoterpenoids, and clustered into one subclass. Their chemical structures and cluster results showed similarities in biosynthesis processes. Enzymes that catalyze the insertion of the ether bond may facilitate the biosynthesis of 11-methylforsythide. In addition, phillygenin was identified to play an important role in the biosynthesis pathways of the *Forsythia* related metabolites, and hydroxylation-related genes and enzymes may be involved in the processes of forsythialan A and forsythialan B. Tetrahydro-1H,3H-furo[3,4-c]furan was a common group of the *Forsythia* related metabolites in the first subclass. *Forsythia* related metabolites of the second subclass did not have similar furan groups. Thus, we assume that the biosynthesis and insertion of tetrahydro-1H,3H-furo[3,4-c]furan may occur upstream. (2R,3R,4s,5S,6S)-tetrahydro-2H-pyran-2,3,4,5,6-pentaol, was found in most of the *Forsythia* related metabolites as a common group. Its biosynthesis may occur upstream, as there was no indication of its formation in the differentiation process of the *Forsythia* related metabolites. As only ether bond of the common group, (2R,3R,4s,5S,6S)-tetrahydro-2H-pyran-2,3,4,5,6-pentaol, of 11-methylforsythide was replaced with a methylene. We assume that compared with other *Forsythia* related metabolites, 11-methylforsythide was a downstream metabolite. As phillyrin had both of two common groups, its biosynthesis may be a cross procedure among the biosyntheses of the two subclasses of *Forsythia* related metabolites. Cluster analysis and molecular structures indicated that there are the potential biosynthesis orders of the *Forsythia* related metabolites.

In this study, we analyzed the *Forsythia* related constituents in leaves and fruits at different harvesting stages. The findings support the suggestion that harvesting should be timed with the metabolites contents. The stability and differences in metabolism of forsythiaside A and phillyrin levels during fruit development could facilitate the identification of the two classes of *Forsythia* related metabolites reported in this study. This confirms the rationality of the quality evaluation method for *F. suspensa* in the Chinese Pharmacopeia. This study provides a theoretical basis for further study of the *Forsythia* related metabolites in *F. suspensa*, including in terms of biosynthesis and metabolic pathways.
Conclusions

Different metabolites levels and proportions of metabolites may influence the medicinal properties of *F. suspensa*. We found that the metabolites levels in both fruits and leaves varied during different developmental periods. Thus, the harvest timing and plant organs should be carefully considered. Correlation and cluster analyses indicated two major types of potential biosynthesis pathways of the *Forsythia* related metabolites in *F. suspensa*. (2R,3R,4s,5S,6S)-Tetrahydro-2H-pyrano-2,3,4,5,6-pentaol and phillygenin are two typical molecular structures of *Forsythia* related metabolites. The related enzymes and genes involved in the insertion of (2R,3R,4s,5S,6S)-tetrahydro-2H-pyrano-2,3,4,5,6-pentaol may play important roles in the formation of both types of *Forsythia* related metabolites. Related hydroxylation enzymes and hydrogenases may be involved in the differentiation of metabolites into the first type.

Supporting information

S1 Fig. Total ion chromatogram of quality control samples in positive ion mode. (DOCX)
S2 Fig. Total ion chromatogram of quality control samples in negative ion mode. (DOCX)
S3 Fig. Multi-peak detection plot of metabolites acquired in positive ion multiple reaction monitoring mode. (DOCX)
S4 Fig. Multi-peak detection plot of metabolites acquired in negative ion multiple reaction monitoring mode. (DOCX)
S5 Fig. T1 of fruits_XIC_detection_of_multimodal_maps-N. (PDF)
S6 Fig. T1 of fruits_XIC_detection_of_multimodal_maps-P. (PDF)
S7 Fig. T1 of fruits_QC_MS_TIC-N. (PDF)
S8 Fig. T1 of fruits_QC_MS_TIC-P. (PDF)
S9 Fig. T1 of leaves_XIC_detection_of_multimodal_maps-P. (PDF)
S10 Fig. T1 of leaves_XIC_detection_of_multimodal_maps-N. (PDF)
S11 Fig. T1 of leaves_QC_MS_TIC-N. (PDF)
S12 Fig. T1 of leaves_QC_MS_TIC-P. (PDF)
S13 Fig. T2 of fruits_XIC_detection_of_multimodal_maps-N. (PDF)
Acknowledgments

We gratefully thank the project funds, leaders in our team, co-authors and other colleagues of Institute of Cash Crops of Hebei Academy of Agricultural and Forestry Sciences, Hebei Medicinal Plant Engineering Technology Research Center, Shijiazhuang Fruit Research Institute of Hebei Academy of Agriculture and Forestry Sciences, Shijiazhuang 050061, China and Shijiazhuang Yiling Pharmaceutical Co, Ltd for the supports they provided.

Author Contributions

Conceptualization: Lingdi Liu, Chunxiu Wen.
Data curation: Yu Sun.
Formal analysis: Yu Sun.
Funding acquisition: Lingdi Liu, Chunxiu Wen, Xiaoliang Xie.
Investigation: Ruike Lu, Jiaxing Feng, Aihong Jin, Saiqun Wen, Wei Wei.
Methodology: Tao Jiang, Wei Tian.
Project administration: Xiaoliang Xie.
Resources: Lingdi Liu, Chunxiu Wen, Xiaoliang Xie, Xusheng Cui.
Supervision: Chunxiu Wen.
Visualization: Yu Sun.
Writing – original draft: Yu Sun.
Writing – review & editing: Lingdi Liu.
References
1. Guo H, Liu AH, Ye M, Yang M, Guo DA. Characterization of phenolic compounds in the fruits of Forsythia suspensa by high-performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry. Rapid Commun Mass Spectrom. 2007; 21(5): 715–29. https://doi.org/10.1002/rcm.2875 PMID: 17279595.
2. Qu H, Zhang Y, Wang Y, Li B, Sun W. Antioxidant and antibacterial activity of two compounds (forsythiaside and forsythin) isolated from Forsythia suspensa. J Pharm Pharmacol. 2008; 60(2): 261–6. https://doi.org/10.1211/jpp.60.2.0016 PMID: 18237475.
3. Chenwei P, Guangyao Z, Weilai Chen, et al. Protective effect of forsythiaside A on lipopolysaccharide/d-galactosamine-induced liver injury, International Immunopharmacology, 2015, 26(1): 80–85. https://doi.org/10.1016/j.intimp.2015.07.011 PMID: 25797347.
4. Cheng L, Li F, Ma R, Hu X. Forsythiaside inhibits cigarette smoke-induced lung inflammation by activation of Nrf2 and inhibition of NF-κB. Int Immunopharmacol. 2015; 28(1): 494–9. https://doi.org/10.1016/j.intimp.2015.03.009 PMID: 26209933.
5. Huang C, Lin Y, Su H, Ye D. Forsythiaside protects against hydrogen peroxide-induced oxidative stress and apoptosis in PC12 cell. Neurochem Res. 2015; 40(1): 27–35. https://doi.org/10.1007/s11064-014-1461-5 PMID: 25344274.
6. Iizuka T, Nagai M. Vasorelaxant effects of forsythiaside from the fruits of Forsythia suspensa. Yakugaku Zasshi Journal of the Pharmaceutical Society of Japan, 2005, 125(2):219–24. https://doi.org/10.1248/yakushi.125.219 PMID: 15684577.
7. Jia J, Zhang F, Li Z, Qin X, Zhang L. Comparison of Fruits of Forsythia suspensa at Two Different Maturation Stages by NMR-Based Metabolomics. Molecules. 2015; 20(6): 10065–81. https://doi.org/10.3390/molecules200610065 PMID: 26035103.
8. Guo H, Liu AH, Li L, Guo DA. Simultaneous determination of 12 major constituents in Forsythia suspensa by high performance liquid chromatography—DAD method. J Pharm Biomed Anal. 2007; 43 (3):1000–6. https://doi.org/10.1016/j.jpba.2006.09.033 PMID: 17156960.
9. Jensen PR, Williams PG, Oh DC, Zeigler L, Fenical W. Species-specific secondary metabolite production in marine actinomycetes of the genus Salinispora. Appl Environ Microbiol. 2007 Feb; 73(4):1146–52. https://doi.org/10.1128/AEM.01891-06 PMID: 17158611.
10. Kazuhiko S, Katsuya E, Hiroshi H. Structures of rengyosides A, B and C, three glucosides of Forsythia suspensa fruits. Phytochemistry, 1989; 28(5):1495–1498. https://doi.org/10.1016/S0031-9422(00)97772-0.
11. Ma B, Li J, Lou T, et al. Comprehensive Screening and Identification of Phyllirin Metabolites in Rats Based on UHPLC-Q-Exactive Mass Spectrometry Combined with Multi-Channel Data Mining. International Journal of Analytical Chemistry, 2020; 2: 1–20. https://doi.org/10.1155/2020/8274193 PMID: 32670374.
12. Wang Y, et al. Forsythiaside A Exhibits Anti-inflammatory Effects in LPS-Stimulated BV2 Microglia Cells Through Activation of Nrf2/HO-1 Signaling Pathway. Neurochem Res. 2016; 41(4):659–665. https://doi.org/10.1007/s11064-015-1731-x PMID: 26498935.
13. Guangdong C, Yulian Z, He L, Yue W, Xianxian L, Qiang H, et al. Forsythiaside attenuates lipopolysaccharide-induced inflammatory responses in the bursa of Fabricius of chickens by downregulating the NF-κB signaling pathway. Experimental & Therapeutic Medicine, 2014; 7: 170–184 https://doi.org/10.3892/etm.2013.1378.
14. Kong F, Jiang X, Wang R, Zhai S, Zhang Y, Wang D. Forsythoside B attenuates memory impairment and neuroinflammation via inhibition on NF-κB signaling in Alzheimer’s disease. J Neuroinflammation. 2020; 17(1): 305. https://doi.org/10.1186/s12974-020-01967-2 PMID: 33059746.
15. de Jager CM, Buto RP, van der Meijden E, Verpoorte R. The role of primary and secondary metabolites in chrysanthemum resistance to Frankliniella occidentalis. J Chem Ecol. 1996; 22(11): 1987–99. https://doi.org/10.1007/BF02040090 PMID: 24227211.
16. Ashry N A, Mohamed H I. Impact of secondary metabolites and related enzymes in flax resistance and/or susceptibility to powdery mildew. African Journal of Biotechnology, 2011, 11(5). https://10.5897/ AJB11.1023.
17. Niemeyer H M, Copaja S V, Barria B N. The Triticeae as sources of hydroxamic acids, secondary metabolites in wheat conferring resistance against aphids. Hereditas, 2010, 116(3):295–299. https://doi.org/10.1111/j.1601-5223.1992.tb00158.x
18. Chen Y, Guo Q, Liu L, et al. Influence of fertilization and drought stress on the growth and production of secondary metabolites in Prunella vulgaris L. Journal of Medicinal Plant Research, 2011, 5(9):1749–1755. https://10.2147/DDDT.S10498.
19. Lu Q, Jiang J G. Chemical metabolism of medicinal compounds from natural botanicals. Current Medicinal Chemistry, 2012; 19(11): 1682–705. https://doi.org/10.2174/092986712799945076 PMID: 22414089.

20. Nett RS, Lau W, Sattely ES. Discovery and engineering of colchicine alkaloid biosynthesis. Nature. 2020; 584(7819): 148–153. https://doi.org/10.1038/s41586-020-2546-8 PMID: 32699417.

21. Shizuka K, Hiroki T, Sue H, et al. Studies on the Chinese crude drug "forsythia fructus." VII. A new caffeoyl glycoside from forsythia viridissima. CHEMICAL & PHARMACEUTICAL BULLETIN, 1984, 32 (3):1209–1213. https://doi.org/10.1248/cpb.32.1209

22. Kim HJ, Ono E, Morimoto K, Yamagaki T, Okazawa A, Kobayashi A, et al. Metabolic engineering of lignan biosynthesis in Forsythia cell culture. Plant Cell Physiol. 2009; 50(12):2200–9. https://doi.org/10.1093/pcp/pcp156 PMID: 19887541.

23. Kim H J, Ono E, Morimoto K, et al. Metabolic Engineering of Lignan Biosynthesis in Forsythia Cell Culture. Plant and Cell Physiology, 2009, 50: 2200–9. https://doi.org/10.1093/pcp/pcp156 PMID: 19887541.

24. Damtoft S, Franzyk H, Jensen S R. Biosynthesis of iridoids in Forsythia spp. Phytochemistry, 1994, 37 (1):173–178. https://10.1016/0031-9422(94)85019-4.

25. Nishibe S, Kawamura T, Tanaka T, et al. On the contents of lignans in Forsythia Fruit and in Forsythia leaf. Natural Medicines, 2001, 55:300–303.

26. Sung, Yoon-Young, Kim, et al. Forsythia suspensa fruit extracts and the constituent matairesinol confer anti-allergic effects in an allergic dermatitis mouse model. Journal of Ethnopharmacology An Interdisciplinary Journal Devoted to Bioscientific Research on Indigenous Drugs, 2016. 187:49–56, https://doi.org/10.1016/j.jep.2016.04.015.

27. Guang-Ming L, Jun-Dong Z, Rui L, et al. Antibacterial effect of eucommia, forsythia, garlic and ginger. Journal of Chengdu Medical College, 2011.

28. Law AHY, Yang CLH, Lau ASY. Antiviral effect of forsythoside A from Forsythia suspensa (Thunb.) Vahl fruit against influenza A virus through reduction of viral M1 protein. Journal of ethnopharmacology, 2017, 14; 209:236–247. https://doi.org/10.1016/j.jep.2017.07.015. Epub 2017 Jul 14. https://doi.org/10.1016/j.jep.2016.04.015.

29. Qu X Y, Li Q J, Zhang H M, et al. Protective effects of phillyrin against influenza A virus in vivo. Archives of Pharmacal Research, 2016, 39(7):998–1005. https://doi.org/10.1007/s12272-016-0775-z PMID: 27323762

30. Dong-Bei G, Xin L I, Ying-Ai P, et al. Research on forsythia anti-tumor component (LQ-4) effect on apoptosis of SGC-7901 cells in vitro. Chinese Journal of Clinicians (Electronic Edition), 2011.

31. Kang W, Wang J. In vitro antioxidant properties and in vivo lowering blood lipid of Forsythia suspendea leaves. Medicinal Chemistry Research, 2010, 19(7):617–628. https://10.1007/s00044-009-9217-5.

32. Guo Y, Lin L & Wang Y. Chemistry and pharmacology of the herb pair Flos Lonicerae japonicae-Forsythia fructus. Chin Med 10, 16 (2015). https://doi.org/10.1186/s13020-015-0044-y PMID: 26163698

33. Ying Xiao, Qian, et al. Combined transcriptome and metabolite profiling reveals that IiPLR1 plays an important role in lariciresinol accumulation in Isatis indigotica. Journal of Experimental Botany, 2015. https://doi.org/10.1038/jxb.2015.662 PMID: 26163698

34. Xiao Y, Shao K, Zhou J, Wang L, Ma X, Wu D, et al. Structure-based engineering of substrate specificity for pinoresinol-lariciresinol reductases. Nat Commun. 2021; 12(1):2828. https://doi.org/10.1038/s41467-021-23095-y PMID: 33990581; PMCID: PMC8121951.