Homogenate-Ultrasound-Assisted Ionic Liquid Extraction of Total Flavonoids from Selaginella involvens: Process Optimization, Composition Identification, and Antioxidant Activity

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ABSTRACT: In this paper, an efficient approach to extract total flavonoids (TFs) from Selaginella involvens (Sw.) Spring using homogenate-ultrasound-assisted ionic liquid (IL) extraction (HUA-ILE) was first developed. The results indicated that EPyBF₄ was selected as the suitable extractant. According to the single factor experiment and response surface methodology, the IL concentration of 0.10 mol/L, the extraction time of 160 s, the liquid/solid ratio of 13:1 mL/g, and the extraction power of 300 W were concluded as the best conditions. A yield of 8.48 ± 0.27 mg/g TF content was obtained. Compared with HUA ethanol extraction, ultrasound-assisted IL extraction, and percolation extraction, the TF content obtained by the HUA-ILE method could be increased by 2 to 4 times, and the extraction time could be reduced by 100 times. Furthermore, 16 compounds of the TF extract were finally identified through ultra-performance liquid chromatography quadrupole time-of-flight tandem mass spectrometry, among which 11 compounds were first discovered in S. involvens. The contents of six biflavonoids in S. involvens were determined simultaneously adopting high-performance liquid chromatography, including amentoflavone, hinokiflavone, bilobetin, ginkgetin, isoginkgetin, and heveaflavone. The TF extract in S. involvens was proved to have potent antioxidant activity through the four antioxidant experiments. In conclusion, HUA-ILE was applied for the first time to exploit a green, efficient, and novel approach to extract TFs, and the research also provided promising prospects for applications of S. involvens.

1. INTRODUCTION
The research on the application and processing of natural products has attached great attention all over the world, contributing to the globalization development of Chinese medicine. Selaginella involvens (Sw.) Spring, a traditional herb, belongs to the family of Selaginellaceae and is abundantly distributed in south China as well as the other Asian countries including North Korea and Japan. Selaginella involvens has the effect of treating hemoptysis, asthma, jaundice, traumatic bleeding, scald, and scrofula.¹

Flavonoids were investigated widely as the active ingredients of S. involves. Among them, biflavonoids are the main effective components of S. involvens, which consist of two mono flavonoids.² So far, scholars all over the world have isolated more than 30 kinds of biflavonoids, including amentoflavone (AME), hinokiflavone (HIN), and bilobetin (BIL). Most biflavonoids not only have significant antioxidant activity but also have other vital pharmacological activities, including anticancer, antiviral, antimyocardial ischemia, and antibacterial effects.³ Kim et al. discovered that AME could be a potential antitumor agent because of the inhibition of epithelial mesenchymal transition (EMT), which was a...
significant step in tumor metastasis.\(^4\) Cai et al. found that AME could inhibit complement component 3 (C3) and regulate the B-cell receptor (BCR)/NF-kappa B signaling pathways as well as high mobility group box 1 (HMGB1) negatively.\(^5\) Mu et al. found that HIN could activate the mtROS/JNK/caspase pathway and inhibit NF-kappa B signaling, which could be a potential therapeutic agent for treating hepatocellular carcinoma.\(^6\) Huang et al. found that HIN could effectively treat breast cancer, and the migration and invasion of breast cancer cells were inhibited through the EMT and signaling pathway.\(^7\) Zhang and Wang found that BIL could inhibit the influenza A virus polymerase acidic endonuclease which was a significant target to develop the anti-influenza new drug. Li et al. found that ginkgetin (GIN) could markedly reduce the hemolytic activity of sulfoxin which was a pivotal virulence-related factor and had various cytotoxicities.\(^9\)

Usually, the most common traditional approaches for selecting total flavonoids (TFs) are reflux extraction\(^10\) and maceration extraction,\(^11\) which have inevitable disadvantages, including high energy consumption, a long extraction time, and environment destruction. The ionic liquid (IL), as a kind of green solvent, is a potential alternative for the conventional organic solvents owing to special characteristics, such as high polarity, strong solvent compatibility, low viscosity, and wonderful hydrophobicity.\(^12\) In recent times, ILs have been widely applied in the extraction of natural products, including flavonoids, phenolic acids, and terpenoids.\(^13\) The physical and chemical properties of ILs can be adjusted through the design of anions and cations so that ILs are able to infiltrate into the plant cell rapidly and dissolve the target compounds.\(^14\) However, ILs also have some disadvantages, such as the long time to synthesize ILs.\(^15\) In this study, homogenate-ultrasound-assisted IL extraction (HUA-ILE) was applied to extract TF.

HUA-ILE is a novel technique for efficient development of natural products. The homogenization process can promote the product release from the raw materials into the solvent by high-speed mechanical shearing, fluid cutting action, stirring, and pulverization.\(^16\) In addition, the ultrasonic waves can cause cavitation, resulting in bubble rupture and local high temperature and pressure, which is conducive to the fragmentation of the cell wall.\(^17\) Based on the above advantages, HUA-ILE is an efficient extraction method with extensive application prospect. For instance, Chen et al. extracted orientin and vitexin from Trollius chinensis employing IL-based homogenate extraction (ILHE), and the contents of orientin and vitexin were 5.66 and 0.85 mg/g, respectively. Compared with the traditional methods, ILHE enhanced the orientin and vitexin yields by 1.05 to 2 times and shortened the extraction time by 4 to 180 times. In conclusion, the HE as an appropriate approach could obtain more target components.\(^18\) Tong et al. extracted crocins from saffron using HE. Under the optimal conditions, 22.96% of crocin yield was obtained. Compared with the ultrasonic extraction, the crocin yield of HE was increased by 4.45%. The result included that HE could be an efficient approach with higher yields.\(^19\)

In this paper, HUA-ILE, a rapid, efficient, and stable method, was established to extract TFs from S. involvens. The effects of ILs with diverse anions and cations were investigated. Single-factor experiments and response surface methodology (RSM) design were used to optimize the process parameters. Different extraction methods, including HUA ethanol extraction (HUA-EE), ultrasound-assisted IL extraction (UA-ILE), and percolation extraction (PE), were compared with the novel approach. Ultra-performance liquid chromatography quadrupole time-of-flight tandem mass spectrometry (UPLC-Q-TOF-MS) was deeply employed to analyze the components of the TF extract. In addition, antioxidant activity of the TF extract was investigated by four antioxidant experiments.

2. RESULTS AND DISCUSSION

2.1. Screen ILs for TF Extraction. 2.1.1. Effects of Anions. The solubility of ILs could be distinctly impacted by their anion structure.\(^20\) Therefore, the N-ethyl pyridinium ILs with four classes of anions (\(\text{Br}^−, \text{BF}_4^−, \text{HSO}_4^−, \text{and Cl}^−\)) were evaluated. In Figure 1A, EPyBF\(_4\) was found to be the most appropriate IL for the extraction of TF from S. involvens, which also verified that BF\(_4^−\) interacts with ethanol molecules for promoting IL solution into cells, resulting in the better solvation of the TFs. Furthermore, the hydrogen bonding between IL and BF\(_4^−\) may generate high solubility of the targets in the IL solution. Thus, BF\(_4^−\) was selected as the optimal anion for further research.

2.1.2. Effects of Cations. The length of the alkyl chain is also vital in the extraction effect. With anions fixed as BF\(_4^−\), four N-alkylpyridinium ILs, including EPyBF\(_4\), [BPy]BF\(_4\), [OPy]BF\(_4\), and [HPy]BF\(_4\) were evaluated. As presented in Figure 1B, EPyBF\(_4\) had a prominent extract effect toward TF content among the four ILs. When the alkyl chain lengths of cations increased, the content of TF declined significantly, which was due to the increase of solution viscosity, leading to
the enhancement of the space block effect and the weakening of the hydrophobic effect.21 Based on this, EPyBF₄ was a kind of appropriate IL and selected for further research.

2.2. Single-Factor Experiments. 2.2.1. Effect of IL Concentration. To obtain the appropriate IL concentration for the further experiment, the effect of EPyBF₄ concentration on TF extraction was analyzed. Under the established extraction conditions (280 W of extraction power, 150 s of extraction time, and 12:1 of liquid/solid ratio), the influences of different IL concentrations (0.05, 0.1, 0.15, 0.2, 0.25, and 0.30 mol/L) on TF yields are as presented in Figure 2A. As the EPyBF₄ concentration was increased to 0.1 mol/L, the TF content enhanced gradually. This phenomenon indicated that when the IL concentration gradually enhanced, their extraction efficiency and the extract solubility might improve. However, with the further increase of EPyBF₄ concentration, the TF content decreased. In previous research of Wang,22 the increase of IL concentration will trigger the increase of solvent viscosity. The solvent with high viscosity is likely to be unfavorable to penetrate into the raw material and to dissolve the target compound, resulting in a low extraction rate of the targets. Therefore, the concentrations of 0.05, 0.1, and 0.15 mol/L EPyBF₄ were selected for the further RSM optimization.

2.2.2. Effect of Liquid/Solid Ratio. Liquid/solid ratio is one of the important factors for investigation. Under the established extraction conditions (0.1 mol/L of IL solution, 280 W of extraction power, and 150 s of extraction time), the influences of six liquid/solid ratios (6:1, 8:1, 10:1, 12:1, 14:1, and 16:1 mL/g) on TF yields are as presented in Figure 2B. As it was increased to 12:1 mL/g, the TF content enhanced gradually, which may be due to the infiltration of a large amount of solvents into the cells, so as to dissolve the TFs rapidly. However, with the further increase of the ratio, the TF content decreased, which may be due to the excessive solvent contacting with the medicinal materials, resulting in the appearance of a large number of impurities.23,24 During the extraction of natural products, the appropriate solid–liquid ratio could maximize the extraction content, yet the excess solid–liquid ratio would waste the solvents. The phenomenon indicated that the best extraction yields were achieved under the appropriate liquid/solid ratio. Therefore, the ratios of 10:1, 12:1, and 14:1 mL/g were selected for the further RSM optimization.

2.2.3. Effect of Extraction Power. Under the established extraction conditions (0.1 mol/L of IL solution, 12:1 mL/g of liquid/solid ratio, and 150 s of extraction time), the effects of different extraction powers (120, 160, 200, 240, 280, and 320 W) on TF yields are as presented in Figure 2C. As the extraction power was increased to 280 W, the TF content enhanced gradually. The extraction efficiency of natural medicines can be clearly increased by enhancing the speed of molecular motion and the solvent penetration because of the mechanical effect, acoustic cavitation, and thermal effect characteristics.25,26 Nonetheless, when the power further increased, the extraction yields of TF declined. During the extraction, the increase in power will cause a lot of impurities, which led to a decrease in TF yields. Therefore, the powers of 240, 280, and 320 W were selected for the further RSM optimization.

2.2.4. Effect of Extraction Time. Figure 2D presents the effects of different extraction times (30, 60, 90, 120, 150, and 180 s) on TF yields, with the conditions of 0.1 mol/L of IL solution, 12:1 mL/g of liquid/solid ratio, and 280 W of...
As the extraction time was increased to 150 s, the TF content enhanced gradually. Generally, the extraction time was a vital factor in extraction efficiency. Less extracting time leads to less TF content.

Nonetheless, when the time further increased, the extraction yields of TF dropped slightly. After reaching complete extraction, excess time would lead to energy consumption, increasing the cost and producing impurities. Therefore, the times of 120, 150, and 180 s were selected for the further optimization.

2.3. Response Surface Analysis. 2.3.1. Fitting the Model. The four variables (liquid/solid ratio, IL concentration, extraction power, and extraction time) and three levels (10:1, 12:1, 14:1 mL/g; 0.05, 0.1, 0.15 mol/L; 240, 280, 320 W; 120, 150, 180 s) were investigated by Box–Behnken design (BBD) of RSM. Table 1 exhibited the ANOVA prediction quadratic model of RSM. It can be seen from the table that the models were significant with $p < 0.05$, the determination coefficient ($R^2$) of TF content is 0.9607, and the adjusted coefficient of determination (adj. $R^2$) is 0.9215. The lack of fit was not significant ($p > 0.05$). The model items of EPyBF$_4$ concentration, extraction power, and extraction time had a negative correlation action, and the other three modes had a positive relationship action on TF yields. Figure 3 indicates a significant correlation between predicted and actual values. The equation from the BBD was eventually obtained for the TF content by HUA-ILE

\[
Y = -95.07 + 164.59X_1 + 0.35X_2 + 4.38X_3 + 0.25X_4 \\
- 0.31X_1X_2 - 3.27X_1X_3 - 0.13X_1X_4 \\
- 1.13 \times 10^{-3}X_2X_3 \\
- 3.53 \times 10^{-5}X_2X_4 - 3.98 \times 10^{-5}X_2X_3X_4 - 142.77X_1^2 \\
- 6.07 \times 10^{-4}X_2^2 - 0.15X_3X_4 + 3.10 \times 10^{-2}X_4^2 \\
(1)
\]

2.3.2. Effect of Extraction Parameters. For TF content, Figure 4 illustrates the 3D surface plots of the virtual interaction terms of $X_1X_3$, $X_1X_4$, $X_2X_3$, $X_2X_4$, and $X_3X_4$, with the other two variables fixed. Based on these results, a 0.10 mol/L EPyBF$_4$ concentration, a 156 s extraction time, a 13:1 mL/g liquid/solid ratio, and a 296 W extraction power were selected as the optimal conditions. Furthermore, the maximized TF content of 8.39

![Normal Plot of Residuals](image)

![Internally Studentized Residuals](image)

![3D Surface Plots](image)
mg/g was predicted. The above conditions were slightly modified at the optimum factors of a 0.10 mol/L EPyBF$_4$ concentration, a 160 s extraction time, a 13:1 mL/g liquid/solid ratio, and a 300 W extraction power with the TF content of 8.48 ± 0.27 mg/g.

2.4. Scanning Electron Microscopy. The microcharacterizations of *S. involvien* before and after various extractions were investigated, including PE, UA-ILE, HUA-EE, and HUA-ILE. Scanning electron microscopy (SEM) (8100 Hitachi, Japan) was adopted to observe the characterization of the raw material and the residues after different extractions. As illustrated in Figure 5, the cell wall structure of the raw material (Figure 5A, A1) obviously illustrated a thick cell wall, a complete cellular structure, and a distinct boundary between various tissues before extraction. After PE, UA-ILE, HUA-EE, and HUA-ILE, the cell wall and boundary of *S. involvien* were damaged in varying degrees. Among them, ultrasonic homogenization treatment exhibited the surface and the cell wall structures were extremely destroyed, resulting in the rough cell wall, an incomplete cellular structure, and an indistinct boundary. It was illustrated that ultrasound and homogenization produced violent vibration and destroyed the cell tissue of *S. involvien* completely and accelerated the solvent into the cell wall during the extraction process, so more TFs were obtained after HUA-ILE. To sum up, the extraction effect of HUA-ILE was better than those of other three methods.

2.5. Comparison of Four Extraction Methods. Four methods, including HUA-ILE, HUA-EE, IL-UAE, and PE were adopted and compared for the TF content from *S. involvien*. As illustrated in Figure 6, compared with PE, the TF content of HUA-ILE was enhanced about 3 times. Moreover, the extraction time was significantly shortened, and the consumption of the solvent was apparently reduced by HUA-ILE. In addition, the extraction times of HUA-EE, IL-UAE, and HUA-ILE were the same, but the TF content of HUA-ILE was enhanced distinctly compared to the other three methods. Through the contrast effect with the above methods, we could conclude that HUA-ILE had a vital potential application value. It is expected to be an efficient, environment-friendly, fast, and green extract method for TF content from *S. involvien*.

2.6. Recovery of EPyBF$_4$ in Subsequent Extractions. After extraction, EPyBF$_4$ was recovered and adopted to extract TFs in the further extraction of *S. involvien*. First, the extract liquids of *S. involvien* were concentrated to remove ethanol. Then, the samples were extracted three times with ethyl acetate ($V_{\text{ethyl acetate}}:V_{\text{extraction}} = 3:1$). After that, the extracted IL aqueous solution was collected and concentrated under reduced pressure and dried under vacuum at 60 °C to obtain EPyBF$_4$.

Under the optimal conditions, the effects of recycled EPyBF$_4$ on the extraction efficiency of TF from *S. involvien* were investigated in detail. As shown in Figure 7, with the increase of repeated operations, the extraction yields of TF decreased slightly, and the extraction yields in the fifth cycle still reached about 90% of the excellent extraction rate. The result indicated that the recovered EPyBF$_4$ could be repeated five times to extract TF from *S. involvien*. In conclusion, EPyBF$_4$ was an ideal solvent for extracting TF from *S. involvien*, which had the advantages of high extraction yields and great recyclability.

2.7. Identification of Main Flavonoids. As a traditional Chinese medicine, *S. involvien* has remarkable biological activity. In order to analyze the chemical composition of the *S. involvien* extract comprehensively, the UPLC-Q-TOF-MS method was employed, which could afford information pertaining to the targeted compounds, such as elemental composition, molecular structures, and molecular weight. So far, UPLC-Q-TOF-MS has been widely employed in the confirmation of bioactive components in natural products. Therefore, the flavonoids in *S. involvien* were identified by UPLC-Q-TOF-MS with the negative-ion mode. Ion chromatograms of the compounds are shown in Figure 8, and the constituents are listed in Table 2.
naringenin. The 6th to 12th compounds had a bimolecular flavonoid skeleton and are connected by C−O−C bonds. According to a large number of experimental data and MS references, they are judged to be biflavonoids. Among them, vicenin, genistin, luteolin, naringenin, 2,3-dihydroameto flavone, BIL, HIN, isoginkgetin (IGIN), isochamaejasmin, cryptomerin B, and kaya flavone were discovered for the first time in *S. involvent.*

### 2.8. Method Validation

AME, HIN, BIL, GIN, IGIN, and HEV belonged to biflavonoids and were the main active ingredients of *S. involvent.* A quantitative analysis of the main six biflavonoids under optimal conditions was explored to accurately reflect the quality of the *S. involvent* extract. The biflavonoid standard solutions were applied as an index to investigate the linearity of calibration curves. The correlation between Y (peak area) and X (standard concentration, μg/mL) indicated that the data were suitable for linear regression.

The standard curves of AME, HIN, BIL, GIN, IGIN, and HEV were $Y_{AME} = 6825.7X - 53472$, $Y_{HIN} = 3156.4X + 37.7438$, $Y_{BIL} = 4593.2X - 76.1582$, $Y_{GIN} = 4177.3X + 8.2955$, $Y_{IGIN} = 2438.1X + 25.7536$, and $Y_{HEV} = 8246.5X + 43.6721$, respectively. As shown in Table 3, the linearity of six kinds of biflavonoids was favorable in 2.00−200.00 μg/mL and the $R^2$ was higher than 0.9993. Meanwhile, the limits of detection (LODs) and limits of quantification (LOQs) of the six biflavonoids were in the range of 0.326−0.663 and 0.859−1.962 μg/mL, respectively.

Under the optimal conditions, the intraday precision, interday precision, stability, repeatability, and recovery rate of the IL extracts were evaluated. Under the above-mentioned "2.5" chromatographic conditions, the intraday precision was used to determine the biflavonoids 6 times in the same day, and the interday precision was adopted to determine the sample on 6 consecutive days. As shown in Table 3, the relative standard deviation (RSD) values were less than 2%, which indicated that the approach had great intraday precision and interday precision. The stability of each biflavonoid was detected for 0, 3, 6, 9, and 12 h at room temperature (25 °C). The results showed that the RSD values of the six biflavonoids were in the range of 1.13−1.72%, indicating that the six biflavonoids in the IL extract were stable for 12 h.
determine the repeatability, the same sample was weighed six times to prepare the extract and then detected by high-performance liquid chromatography (HPLC). The RSD values of the extract were in the range of 1.02−1.85%, which indicated that the extraction yields of the six biflavonoids had good repeatability. To evaluate the recovery for the six biflavonoids, the same targets with a known content were added into 4.0 g powders of S. involven. As shown in Table 3, the established methods had great recoveries for the six biflavonoids. Thus, HUA-ILE was credible to determine the six biflavonoids simultaneously.

2.9. Antioxidant of the Total Flavonoid Extract. In order to explore novel natural antioxidants, the antioxidant activities of TF extracts (TFEs) and other antioxidants were compared by four antioxidant approaches, including DPPH radical scavenging activity, chelation of ferrous ion assay, ferric ion reducing power assay, and lipid peroxidation. As shown in Figure 9A, the TFE had good radical scavenging capacity. Within the experimental concentration (20−120 μg/mL), the scavenging ability of TFE on DPPH free radicals increased gradually, which could reach 47.69% in the concentration of 120 μg/mL. Furthermore, quercetin and Vc had a strong scavenging effect on DPPH free radicals in the entire concentration range, which maintained above 90%.

According to the method adopted by Li,14 the principle of ferrous chelation was as follows: ferrous ions had a catalytic effect in the oxidation of free radicals, which could damage the process to organisms caused by the oxidation of other oxidants. Figure 9B indicates that as the concentration of two samples increased, the inhibition rate also increased. Ethylenediaminetetraacetic acid (EDTA) and TFE showed good antioxidative activity. Figure 9C indicates that as the concentration of quercetin, Vc, and TFE increased, the reducing power increased obviously. In addition, at the same concentration, the antioxidative activity of quercetin and Vc was higher than that of TFE.

The result of lipid peroxidation is illustrated in Figure 9D. As shown in Figure 9C, when the concentration of quercetin, Vc, and TFE increased, the reducing power increased obviously. In addition, at the same concentration, the antioxidative activity of quercetin and Vc was higher than that of TFE.

The result of lipid peroxidation is illustrated in Figure 9D, and TFE, quercetin, and Vc showed a significant inhibition effect. With the concentration of the three samples increased, the inhibition rates increased significantly, which indicated that there existed a dose dependence in concentration range from 20 to 120 μg/mL. Furthermore, at the concentration of 120 μg/mL, the inhibition rates of TFE, quercetin, and Vc were increased to 40.69, 60.82, and 58.61%, respectively. The results indicated that TFE had an inhibition effect on lipid peroxidation.

2.10. Mechanism of HUA-ILE. As an emerging technology, HUA-ILE has been successfully applied in the extraction of essential oil, flavonoids, proanthocyanidins, and phenolic compounds.54,55 The HUA-ILE process has two inherent advantages, which can obtain the high content of flavonoids.

Above all, according to the previous study, there are π−π interactions and hydrogen bonds in pyridine ILs, which enhance their ability to dissolve cellulose in the cell wall.56 Therefore, EPyBF4, the suitable pyridine IL, can destroy the cell wall distinctly, and the solvent can penetrate into the cell more easily so as to extract more target components. At the same time, the optimum concentration of EPyBF4 can achieve the best TF content. When the IL concentration is too high, the TF content will reduce because of the enhancement of the solvent viscosity and the decrease of diffusion ability.

Second, the homogenate treatment can carry out high-speed mechanical shearing, fluid cutting action, and stirring of the material, which can promote the raw material contact with the solvent adequately, and accelerate the flavonoids dissolved in the solvent. Compared with the physical milling method, homogenate treatment is able to extract target compounds without extra heating and pressure as well as the lack of environmental pollution. The ultrasonic extraction technology is an efficient extraction technology. When ultrasonic waves are applied to the liquid, cavitation will occur, causing bubbles to collapse and produce local high temperature and high pressure. The medium absorbs the ultrasonic waves and the friction inside the medium, which causes the molecules to vibrate violently, resulting in the augment of the medium temperature and generating a thermal effect. Ultrasonic waves can also form effective agitation and flow in the liquid, producing a mechanical effect.

Hence, HUA-ILE has been found for target compound extraction and has many superiorities, such as easy operation, time saving, and environmental protection (Figure 10).

3. CONCLUSIONS

In this study, an efficient and novel HUA-ILE method was successfully established to extract the TFs from S. involven, and the compositions in S. involven were detected by UPLC-Q-TOF-MS. In addition, the antioxidant activity of TFs was explored by DPPH, radical scavenging activity, chelation of ferrous ion assay, ferric iron reducing power assay, and lipid peroxidation.

Among all the ILs, EPyBF4 was finally identified as the optimal IL for extraction of TFs. According to the single-
Table 2. Chemical Constituents from *S. involven*

| No. | Retention time (min) | Analyte          | Chemical structure | Neutral mass (Da) | Determined mass [M+H]+ (Da) | Product mass (m/z) | Mass error (ppm) | Reference |
|-----|----------------------|-------------------|--------------------|------------------|--------------------------|------------------|----------------|-----------|
| 1   | 3.15                 | Vincamine         | ![Vincamine](image1) | 594 1585         | 595 1500                 | 430 04 267 04 152 03 124 02 118 12 | ±0.2 | 29       |
| 2   | 4.95                 | Genistein         | ![Genistein](image2) | 432 1056         | 431 0981                 | 268 33 132 04 124 04 118 35 | ±0.2 | 36       |
| 3   | 5.52                 | Luteolin          | ![Luteolin](image3) | 286 6477         | 285 6425                 | 137 24 109 96 76 03 41 57 | ±0.4 | 36±3     |
| 4   | 5.96                 | Apigenin          | ![Apigenin](image4) | 270 5828         | 269 5472                 | 121 1395 14 76 13 41 21 | ±0.3 | 31       |
| 5   | 6.57                 | Naringenin        | ![Naringenin](image5) | 272 2983         | 271 2489                 | 152 03 120 03 124 04 | 0 | 33±6     |
| 6   | 7.99                 | Amelogenin        | ![Amelogenin](image6) | 538 0913         | 537 0829                 | 419 17 401 05 375 22 157 12 121 06 | 0.2 | 35       |
| 7   | 8.21                 | 2,3-Oxyhydrooxyflavone | ![2,3-Oxyhydrooxyflavone](image7) | 540 1054         | 539 0962                 | 421 11 403 07 377 09 157 03 121 06 | ±0.2 | 36±5     |
| 8   | 8.69                 | Rubriflavone      | ![Rubriflavone](image8) | 538 0914         | 537 0826                 | 411 06 385 05 268 13 | 0 | 36±5     |
| 9   | 9.42                 | Stilbenes        | ![Stilbenes](image9) | 552 1056         | 551 0864                 | 433 06 291 06 322 20 295 06 139 60 121 06 | 0 | 36±4     |
| 10  | 9.86                 | Hesperiflavone    | ![Hesperiflavone](image10) | 558 0915         | 557 0827                 | 284 14 264 29 255 55 246 21 | 0 | 4±3      |
| 11  | 11.84                | Gbekgetin        | ![Gbekgetin](image11) | 366 1213         | 565 1144                 | 447 03 415 26 405 11 166 20 121 15 | 0.4 | 4±4      |
| 12  | 12.23                | Leskgetin        | ![Leskgetin](image12) | 366 1213         | 565 1142                 | 433 04 381 07 325 20 295 06 153 13 355 24 | 0.1 | 4±4      |
| 13  | 13.34                | Inschuankeojen    | ![Inschuankeojen](image13) | 542 1213         | 541 1146                 | 415 08 389 10 265 07 237 03 15 00 | 0 | 4±6      |
| 14  | 14.22                | Cryptomeria II   | ![Cryptomeria II](image14) | 566 1213         | 565 1139                 | 433 24 262 19 254 34 135 15 | ±0.1 | 40       |
| 15  | 15.34                | Kayafavone        | ![Kayafavone](image15) | 580 1369         | 579 1295                 | 447 04 405 05 377 05 295 06 153 00 335 15 | ±0.4 | 30       |
| 16  | 16.49                | Huafluavone       | ![Huafluavone](image16) | 583 1369         | 579 1299                 | 447 33 415 31 403 11 166 23 135 15 | 0 | 31       |
The 0.10 mol/L IL concentration, the 160 s extraction time, the 13:1 mL/g liquid/solid ratio, and the 300 W extraction power were obtained as the optimal conditions. Under the above conditions, the TF content was 8.48 ± 0.27 mg/g. Based on the comparison of different extraction methods, the TF content of HUA-ILE was significantly better than that of HUA-EE, IL-UAE, and PE. Furthermore, an approach for the simultaneous detection of six biflavonoids was established by HPLC, and their effectiveness and feasibility were verified by methodological review. Additionally, 16 flavonoids of the IL extract were identified by UPLC-Q-TOF-MS, in which 11 compounds were first discovered. The results of four antioxidant experiments indicated that TFs had potent antioxidant activity.

Table 3. Method Validation for Six Biflavonoids

| analyte | calibration curve | R² | LOD (μg/mL) | LOQ (μg/mL) | intraday precision (n = 6) | interday precision (n = 6) | stability (n = 5) | repeatability (n = 6) | recovery (n = 6) |
|---------|-------------------|----|-------------|-------------|---------------------------|---------------------------|-----------------|---------------------|-----------------|
| AME     | Y = 6825.7X − 5.3472 | 0.9996 | 0.663 | 1.962 | 1.04 | 1.81 | 1.46 | 1.34 | 97.61 |
| HIN     | Y = 3.156.4X + 37.7438 | 0.9995 | 0.392 | 1.018 | 0.63 | 1.47 | 1.58 | 1.02 | 100.20 |
| BIL     | Y = 4.593.2X − 76.1582 | 0.9996 | 0.326 | 0.859 | 0.98 | 1.65 | 1.72 | 1.17 | 97.18 |
| GIN     | Y = 4.177.3X + 8.2955 | 0.9994 | 0.574 | 1.677 | 0.77 | 1.26 | 1.66 | 1.85 | 96.34 |
| IGIN    | Y = 2.438.1X + 25.7536 | 0.9997 | 0.345 | 1.184 | 1.12 | 1.37 | 1.38 | 1.22 | 97.68 |
| HEV     | Y = 8.246.5X + 43.6721 | 0.9993 | 0.451 | 1.208 | 0.84 | 1.72 | 1.13 | 1.63 | 98.33 |

LOD, limit of detection (S/N = 3); LOQ, limit of quantification (S/N = 10). Intraday precision, interday precision, stability, repeatability, and recovery are expressed as the RSD (%) of peak area.

Figure 9. Antioxidant effects of TFE. (A) DPPH radical scavenging activity; (B) ferric chelation ability; (C) reducing ability; (D) lipid peroxidation.

Figure 10. Contribution of HUA-ILE to improve the extraction efficiency of TF.
antioxidant activity. In brief, HUA-I LE is confirmed an
effective approach for extraction of flavonoids from natural
products.

4. MATERIALS AND METHODS

4.1. Materials and Chemicals.  *S. involven* was collected
at Simianshan (Zunyi, China) on 08/28/2019, washed out,
and dried naturally. Seven kinds of ILs, including EPyBF₄,
[BPy]BF₄, [OPy]BF₄, [HPy]BF₄, EPyBr, EPyHSO₄, and
EPyCl (Table S1 in the Supporting Information) were bought
from Kaite Co., Ltd. (Lanzhou, China). Six biflavonoids,
including AME, HIN, BIL, GIN, IGIN, and hevea
flavone (HEV) (Figure 11, ≥98% purity), were obtained from
Ruifeng Co., Ltd. (Chengdu, China). Other reagents,
including 2,2-di(4-tert-octylphenyl)-1-picrylhydrazyl, 3-(2-pyr-
idyl)-5,6-bis (4-sulfoph-enyl) -1,2,4-triazine disodium salt,
ascorbic acid, quercetin, methanol, and acetonitrile (chroma-
tographic grade), were obtained from Bailingwei Co., Ltd.
(Beijing, China).

4.2. HUA-I LE Apparatus. HE was accomplished on a UP-
400 homogenate extractor (Luoshang Intelligent Technology,
Ltd., Ningbo, China).

4.3. HUA-I LE Extraction.  *S. involven* herbs were crushed
through a 100 mesh sieve (137.67–162.42 μm). 15 g of raw
materials was mixed with a certain volume of IL solution.
After that, TFs were extracted in the ultrasonic homoge-
nization device under the predetermined extraction power, IL
concentration, liquid/solid ratio, and extraction time. After
filtering the reaction liquid, the MINI-10K high-speed
centrifuge (Young, Hangzhou, China) was applied to
centrifuge the liquid. After concentration and vacuum drying
of the liquid, TFs were obtained and stored at 5 °C until the
next analysis. As ILs are relatively sticky, according to a
previous study, it is found that EpyBF₄ is best soluble in a
series of ethyl ethanol compounds at the same temperature, so
we use ethanol to dissolve ILs.

4.4. Optimization of Extraction. 4.4.1. Screening of ILs.
The optimal anions and cations were regarded as the vital
factors. The anions, including Br⁻, BF₄⁻, HSO₄⁻, and Cl⁻, and
the cations, including [EPy]+, [BPy]+, [OPy]+, and [HPy]+
were investigated. The extraction parameters were as follows:
a 0.1 mol/L IL concentration, 280 W of extraction power,
12:1 mL/g of liquid/solid ratio, and 150 s of extraction time.
The results indicated that [BPy]BF₄ was selected for further
investigation.

4.4.2. Screening of IL Concentration. The [BPy]BF₄
concentrations with 0.05, 0.1, 0.15, 0.2, 0.25, and 0.30 mol/
L were investigated under the other parameters
fixed: 280 W of extraction power, 12:1 mL/g of liquid/solid ratio,
and 150 s of extraction time. The result indicated that at the
concentration of 0.1 mol/L, the TF content was the highest.

4.4.3. Screening of Liquid/Solid Ratio. In order to save
solvents and extract completely, the liquid/solid ratio was
necessary to be determined. The different liquid/solid ratios
(6:1, 8:1, 10:1, 12:1, 14:1, and 16:1 mL/g) were investigated,
with the other parameters fixed: a 0.1 mol/L IL concentration,
280 W of extraction power, and 150 s of extraction time. The
results indicated that the liquid/solid ratio of 12:1 mL/g was
selected for further investigation.

4.4.4. Screening of Extraction Power. The same
parameters were used: a 0.1 mol/L IL concentration, 12:1
mL/g of liquid/solid ratio, and 150 s of extraction time. The
extraction power (120, 160, 200, 240, 280, and 320 W) was
investigated. To ensure that the extraction was complete, the
280 W of extraction power was selected for further
investigation.

4.4.5. Screening of Extraction Time. To ensure efficient
extraction, different extraction times of 30, 60, 90, 120, 150,
and 180 s were investigated with the other parameters fixed: a

![Figure 11. Chemical structures of six biflavonoids from *S. involven*.](https://doi.org/10.1021/acsomega.1c01087)
0.1 mol/L IL concentration, 280 W of extraction power, and 12.1 mL/g of liquid/solid ratio. The result indicated that at the extraction time of 150 s, the TF content was the highest.

4.4.6. RSM Experiments. A BBD was adopted to optimize the HUA-ILE process with respect to four variables (X_1: EPyBF_4 concentration, mol/L; X_2: extraction time, min; X_3: extraction power, W; X_4: liquid/solid ratio, mL/g) as presented in Table S2 in the Supporting Information. A total of 29 runs were implemented as presented in Table S3 in the Supporting Information. The extraction yields of TFS were established as responses to combine with the independent variables. Furthermore, the following equation illustrated the relationships between the variables and the responses

\[ Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ij} X_i X_j \]

where Y is the determined response of TFS, (mg/g); \( \beta_0, \beta_i, \beta_{ij} \) represent the parameters of regression, namely, the intercept, linearity, square, and interaction values, respectively; and \( X_i, X_j \) represent the four variables.

4.5. Conventional Reference Extraction Methods. 4.5.1. HUA Ethanol Extraction. With reference to the previous method of Jin with some modifications, the powders of \( S. \ involv \) (15.0 g) and 95% ethanol (180 mL) were put into the flask with 250 mL. Then, the flask was immers in the ultrasonic homogenization device. The mixture was reacted with the ultrasonic power of 250 W for 180 s. After the end of extraction, the supernatant was collected by filtering and centrifuging the mixture. Then, it was concentrated and stored at 4 °C.

4.5.2. Ultrasound-Assisted IL Extraction. According to the approach of Jiang with some modifications, the powders of \( S. \ involv \) (15.0 g) and IL (180 mL) were put into the flask with 250 mL. Then, the flask was exposed to the ultrasound device. The mixture was reacted with the ultrasonic power of 250 W for 180 s. After the end of extraction, the supernatant was collected by filtering and centrifuging the mixture. Then, it was concentrated and stored at 4 °C.

4.5.3. Percolation Extraction. According to the report of Cao-Ngoc with some modifications, the raw materials (15.0 g) were moistened with 180 mL of 95% ethanol and swelled in a closed container for 3 h. Then, the swollen material was transferred into the percolator, and the appropriate amount of 95% ethanol was added to extract for 24 h. After that, the first concentrated extract was collected. Extraction was continued until the constituents were completely exhausted. The second extraction was concentrated and combined with the first extraction. After the end of extraction, the supernatant was collected by filtering and centrifuging the mixture. Then, it was concentrated and stored at 4 °C.

4.6. Analytical Methods. 4.6.1. Determination of TF Content. For single-factor experiments and RSM experiments, the TF content was determined by ultraviolet spectrophotometry. According to the report of Sun with some modifications, rutin was chosen as the standard material. In brief, the 10.0 mg of rutin was dissolved in methanol (50 mL, 0.2 mg/mL) as a mother liquor. The diluted standard (1 mL: 0.02, 0.04, 0.06, 0.08, 0.1, and 0.12 mg/mL) was transferred to a 10 mL test tube and then mixed with 0.3 mL of sodium nitrite (5%, w/v), 0.3 mL of aluminium nitrate (10%, w/v), and 4 mL of sodium hydroxide (4%, w/v). The solution was diluted to 10 mL with methanol. After 15 min, the absorbance was detected at 510 nm with the UV spectrophotometer (TU-1900, Puxi, Beijing, China). The solution without rutin was used as the blank reference, and the liner response was obtained (\( Y = 1.0057X + 0.0109, R^2 = 0.9993 \)). 1 mL of the TF extract was taken and operated according to the above method. The TF content was expressed as the rutin equivalents applying the standard calibration curve.

4.6.2. HPLC Determination of Main Flavonoids. An HPLC approach was established to explore the main flavonoids in \( S. \ involv \). According to Li with some modifications, an analytical HPLC system (1260, Agilent, USA) was applied to form a set of HPLC analysis systems to determine the flavonoids. An ACE Excel C18 reverse-phase column was used to separate the sample (5 μm, 250×4.6 mm, Phenomenex Technologies, Torrance, California, United States). Acetonitrile and 0.1% formic acid solution were applied as the mobile phase using gradient elution (see Table S4 in the Supporting Information). The flow rate of 1.0 mL/min, an injection volume of 20 μL, and a column temperature of 25 °C were set as chromatographic conditions. As shown in Figure 12, the retention times of the flavonoids, including AME, HIN, BIL, GIN, IGIN, and HEV, were 7.47, 9.94, 12.09, 14.83, 15.62, and 21.14 min, respectively.

![Figure 12. Comparison of diverse extraction methods for extraction of TF from S. involv.](https://doi.org/10.1021/acsomega.1c01087)
electrospray ionization was set to +3.0 kV. The sample cone voltage (33 V) and the collision energy (2.5 eV) were set. The mass scan was in the range of 100−4000 m/z, and the scanning time was 1 s.

4.7. Research on Antioxidant Activity. 4.7.1. DPPH Radical Scavenging Assay. According to the former approach with some modifications, 2 mL of TFE solution of six concentrations (20−120 μg/mL) was prepared and mixed with 2 mL of DPPH solution (0.2 mmol/L). The reaction was carried out in the dark for 30 min. After reaction, the absorbance of the reactant was detected at 517 nm. Absolute ethanol was used as a blank sample. In addition, quercetin and ascorbic acid with the same concentration were adopted as positive controls. The clearance rate was calculated as follows:

\[ I (%) = \left[\frac{(A_c - A_f)}{A_c}\right] \times 100\% \quad (3) \]

where \( A_c \) was the absorbance of the control (without samples), \( A_f \) represented the absorbance of the sample, and I represented the inhibition rate of the sample.

4.7.2. Chelation of Ferrous Ion Assay. With reference to the previous method for a slight modification, 1 mL of TFE solution of six concentrations (20−120 μg/mL) was added to 1.7 mL of distilled water and then mixed with 0.5 mL of FeCl₃ (2 mmol/L) and 1 mL of Ferrozine (0.1 mmol/L). The purple complex was generated after 10 min of reaction. The absorbance of the complex was measured at 562 nm. At the same time, the same concentration of EDTA was adopted as the positive control.

4.7.3. Ferric Ion Reducing Assay. According to the report of Li with some adjustment, the reducing effect of TFE was detected. 1.2 mL of TFE solution of six concentrations (20−120 μg/mL) was added to 1.2 mL of sodium phosphate buffer (pH = 6.6, 0.2 mol/L) and 1 mL of potassium ferricyanide solution (1% w/v). After 20 min of reaction in the 50 °C water bath, the solution was cooled rapidly, and 1.0 mL of trichloroacetic acid (10% w/v) was added into it. The volume was adjusted to 10 mL with distilled water. Then, the solution was centrifuged for 10 min at 3000 rpm. After that, the supernatant (2.5 mL) was taken and 0.5 mL of ferric chloride solution (0.1% w/v) was added into it. After mixing, the solution was made up to 5 mL with distilled water. After 10 min, the absorbance of the solution was detected at 700 nm. The same concentration of quercetin and ascorbic acid were selected as the positive controls.

4.7.4. Lipid Peroxidation Assay. The inhibition activity of TFE on lipid peroxidation was detected according to the method of Li with some modifications. 1 mL of TFE solution (20, 40, 60, 80, 100, and 120 μg/mL) was mixed with 0.2 mL of the yolk homogenate (4%, v/v) and 0.2 mL of FeCl₃ solution (25 mmol/L). Then, the sodium phosphate buffer was adopted to fix the volume of the extract into 2 mL. The mixture was shaken vigorously and put into a water bath at 37 °C for 0.5 h. At the end of reaction, 2 mL of trichloroacetic acid (10%, w/v) was added. The mixture was allowed to stand for 10 min to precipitate the protein. Then, 2 mL of thiobarbituric acid (0.67%, w/v) was mixed into the sample. At the same time, the sample was placed in a boiling water bath for 15 min, cooled naturally, and centrifuged at 3000 rpm. After 10 min, the absorbance of the supernatant was detected at 532 nm. The same concentration of quercetin and ascorbic acid were selected as the positive controls.

4.8. Statistical Analysis. The Design Expert 8.0 software was used to design and optimize the procedure, and the RSM technology was employed to obtain the response model. The ANOVA test was adopted to analyze the RSM results as well as predict the TF content. Each group of experiments was performed three times in parallel. The mean and standard deviation were calculated after each sample was determined.

## ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.1c01087.

Chemical structures of the ILs, levels, and factors selected for the experiment, results of BBD for four variables in coded and real values; and HPLC gradient elution program (PDF)

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**Notes**

The authors declare no competing financial interest.

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