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*Article*

# Comparison of Volatile Oil between the Fruits of *Amomum villosum* Lour. and *Amomum villosum* Lour. var. *xanthioides* T.L.Wu et Senjen Based on GC-MS and Chemometric Techniques

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**Abstract:** Fructus Amomi (FA) is usually regarded as the dried ripe fruits of *Amomum villosum* Lour. (FAL) or *Amomum villosum* Lour. var. *xanthioides* T. L.Wu et Senjenis (FALX). However, FAL, which always has a much higher price because of its better quality, is confused with FALX in the market. As volatile oil is the main constituent of FA, a strategy of chromatography-mass spectrometry (GC-MS) and chemometric approches was applied to compare the chemical composition of FAL and FALX. The results showed that the oil yield of FAL was significantly higher than that of FALX. Total ion chromatograph (TIC) showed that cis-nerolidol existed only in FALX. Bornyl acetate and camphor, could be considered as the most important ones in FAL and FALX respectively. Moreover, hierarchical cluster analysis (HCA) and principal component analysis (PCA) successfully distinguished the chemical constitutes of the volatile oils in FAL and FALX. Additionally, bornyl acetate, $\alpha$-cadinol, linalool, $\beta$-myrcene, camphor, d-limonene, terpinolene and endo-borneol were selected as the potential markers for discriminating FAL and FALX by partial least squares discrimination analysis (PLS-DA). In conclusion, this present study first developed a scientific approach to separate FAL and FALX based on volatile oils by GC-MS combined with chemometric techniques.

**Graphical abstract**

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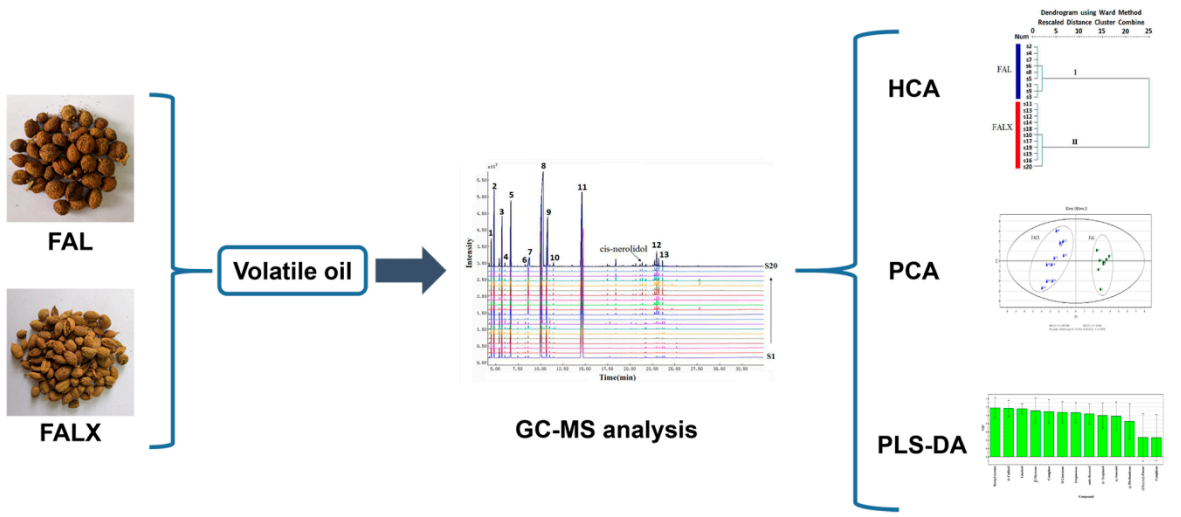


**Keywords:** *Amomum villosum* Lour.; *Amomum villosum* Lour. var. *xanthioides* T. L.Wu et Senjenis; GC-MS; Chemometric Techniques; volatile oil.

## 1. Introduction

Fructus amomi (FA), also called Sharen, is a famous traditional Chinese medicine (TCM). It was firstly recorded as a medicinal resource in Yao Xing Lun (Tang Dynasty), has a long medical history of more than one thousand years for treating gastrointestinal diseases and pregnancy diseases in China. In Chinese Pharmacopeia (2015 version), FA is defined as the dried ripe fruit of three ginger plants—*Amomum villosum* Lour., *A. villosum* Lour. var. *xanthioides* T. L. Wu et Senjen, and *A. longiligulare* T. L. Wu [1], which has the effect of eliminating dampness and appetizing the stomach, warming spleen and stopping diarrhea. The first two are the main varieties in China market [2]. The dried ripen fruits of *A. villosum* Lour. (FAL) are called Yang Chun Sha, while those of *A. villosum* Lour. var. *xanthioides* T. L.Wu et Senjenis (FALX) are called Lv Ke Sha. Although the two species are used in the same way according to Pharmacopeia, it is generally acknowledged that FAL is of the better public praise, and considered one of the four most famous TCMs in the South of China with a high price. In China market, FAL costs five-ten times higher than FALX. Therefore, FALX is often used to be a counterfeit commodity of FAL because of their similar appearance and close genetic relationship. Precisely, they both originate from genus Amomum in the Gingeraceae family, having the indistinguishable shape, color, surface characteristics, odor and other characteristics. Therefore, it is imperative to clarify the differences between the two species and obtain valuable additional evidences for identification.

So far, there are relatively few studies on the identification of FAL and FALX. Fourier Transform Infrared Spectroscopy (FTIR) had been reported to differ from FA (including FALX and FALX) from other confusable varieties, but could not distinguish FAL from FALX [3]. A previous study had proved ITS-1 sequence could effectively identify FAL and FALX [4], which indicated that the two herbs were biologically differ from each other. However, because FA is the dried processed products of their fruits, most of DNA is degraded and destroyed during the processing. High quality DNA is difficult to be extracted. So DNA barcode is not suitable for identification of dried FA.

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FA is rich in volatile oil. Several studies have reported the volatile oil of FA and its major compounds possessed anti-inflammatory and analgesic activities, and could be regarded as potential drugs for digestive diseases such as nonalcoholic fatty liver disease, 5-fluorouracil-induced intestinal mucositis and inflammatory bowel disease [5-9]. Therefore, volatile oil is regarded as the main active ingredient of FA. Steam distillation combined with gas chromatography-mass spectrometry (GC-MS) is used as the routine methods for the analysis of the volatile oils of FA [10-12].But till now, no study could tell us the chemical difference between volatile oil of FAL and FALX.

Conventional mutual chemical comparison cannot find out elements which result in quality variance. Chemometrics, based on computer and modern computing technology, is a new interdisciplinary subject. In recent years, chemical analysis combined with chemometrics methods such as cluster hierarchical cluster analysis (HCA), principal component analysis (PCA) and partial least squares discrimination analysis (PLS-DA) have been widely used in the identification, qualitative character, quality control and efficacy relationship of herbs [13-18], seems to be an ideal tool for this problem.

Therefore, in this study, a comprehensive strategy combining GC-MS analysis and chemometrics methods was firstly proposed to compare the OFAL with OFALX. Precisely, GC-MS combined with chemometrics methods including HCA and PCA were employed for identification of the volatile oils in the two confused species while unpaired T-test and PLS-DA were exploited to discover the potential chemical markers for discriminating these two herbal medicines. The aim of the present study is to investigate the chemical differences of the volatile oils between the two confusing species of FA by GC-MS in general and in detail.

## 2. Results

### *2.1. Fingerprints of FAL and FALX*

According to TIC, the chemical composition of the two species were similar in general but still had a little difference. The number of peaks of FALX from 17 min to 25 min was more than those of FAL. The peak (RT 21.345min) identified as cis-nerolidol, appeared only in the chromatogram of FALX, but its relative percentages were too low, only 0.23% to 0.48%. The chromatograms of FAL possessed 16 common peaks while those of FALX had 17 common peaks. Among the above peaks, 13 common peaks, whose total areas accounted above 90% of the volatile constituents and represented the chemical characteristic of the samples well, could be found in the chromatograms of FAL and FALX. The results were shown in Table 1 and Figure 1, 2.

In order to find the marker compounds for chemical comparison of FAL and FALX, unpaired T-test was employed for analyzing the relative contents of the common peaks of FA. The p-value was set as the filtering standard to maintain the contents. The relative contents of two compounds, camphor and bornyl acetate, regarded as the main chemical components in the essential oils of FA, were significantly different in FAL and FALX. In FAL samples, the relative contents of bornyl acetate were the highest ranging from 41.32% to 60.20%, with an average of 49.16±5.13% while that of camphor was the second-highest ranging from 16.84% to 28.90% with an average of 22.81±3.79%, and the relative contents of both was above 70% with an average of 70.97±2.14%. Meanwhile, in

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FALX samples except S16, the relative contents of camphor was the highest ranging from 29.07% to 44.76% with an average of 36.13 ± 4.18% and that of bornyl acetate was the second-highest from 15.85% to 31.63% with an average of 24.51±4.10%. The sum of the relative contents of the two was above 56% with an average of 60.64 ± 2.54%. In general, the relative contents of bornyl acetate in FAL were significantly higher than that in FALX, but those of camphor in FAL were significantly lower than in FALX. In addition, the relative contents of other common peaks in the two species of FA were also obviously different. The relative contents of β-myrcene, α-phellandrene, d-limonene, terpinolene, linalool, camphor, α-terpineol, α-cadinol and α-santalol in FAL were 2.49~3.38%, 0.29~0.39%, 6.70~8.31%, 0.23~0.28%, 0.25~0.85%, 1.89~4.66%, 0.26~0.36%, 0.16~0.37% and 0.16~0.34% successively. The relative contents of those in FALX were 3.89~6.07%, 0.21~0.30%, 8.10~11.35%, 0.18~0.23%, 1.76~3.62%, 3.79~8.09%, 0.31~0.57%, 0.80~1.79% and 0.40~0.87% in order. Among them, the relative contents of α-phellandrene and terpinolene accounted for significantly higher in FAL than in FALX, while β-myrcene, d-limonene, linalool, camphor, endo-borneol, α-terpineol, α-cadinol and α-santalol were significantly lower in FAL than in FALX. Furthermore, whether in FAL or in FALX, bornyl acetate and camphor, were the first two highest percentage compounds and accounted for more than half in all oil samples. Bornyl acetate, the quality control component of FA in the Chinese pharmacopeia, always accounted more percentage in FAL than in FALX. Comparatively, the percentage of camphor, was significantly higher in FALX than in FAL（$P<0.05$）. Moreover, the ratio of the percentages of bornyl acetate to camphor was significantly higher in FLA than in FLAX. Therefore, it was concluded that FAL was a plant represented by bornyl acetate while FALX seemed to be a plant, the oil of which was mainly dependent on camphor. As was known, bornyl acetate exhibited anti-inflammatory[19-21], analgesic, anti-tumor[22], whitening, anti-oxidative[23] and immune-regulatory[24] effects while camphor showed various pharmacological effects including while camphor showed various pharmacological effects including anti-tussive[25], anti-oxidative[26], anti-fungal[27], anti-wrinkle[28] and wound healing[29] activities. However, no report regarding the pharmacological differences of FAL and FALX had been seen, which would hinder the development and rational utilization of FA. Further study is needed to compare the bioactivities of the two species.

The results were shown in Figure 3.

**Table 1.** The relative contents of the common peaks of FA from two species.

| No. | Retent time | Molecular weight | Component | Molecular Formula | S1 | S2 | S3 | S4 | S5 | S6 | S7 | S8 | S9 | S10 | S11 | S12 | S13 | S14 | S15 | S16 | S17 | S18 | S19 | S20 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 4.487 | 136.2 | (1S)-(-)-α-Pinene | $C_{10}H_{16}$ | 1.92 | 1.65 | 0.72 | 1.63 | 1.98 | 1.86 | 1.65 | 1.56 | 1.49 | 1.69 | 1.86 | 0.84 | 1.89 | 0.66 | 1.5 | 0.91 | 1.18 | 1.12 | 1.19 | 1.79 |
| 2 | 4.810 | 136.2 | Camphene | $C_{10}H_{16}$ | 7.92 | 6.84 | 4.80 | 7.33 | 7.98 | 7.64 | 5.72 | 7.25 | 7.67 | 8.34 | 8.26 | 4.38 | 7.80 | 3.17 | 6.38 | 5.03 | 5.63 | 5.51 | 5.62 | 7.01 |
| 3 | 5.681 | 136.2 | β-Myrcene | $C_{10}H_{16}$ | 3.25 | 2.54 | 2.65 | 2.49 | 3.34 | 2.88 | 2.62 | 3.38 | 3.33 | 4.97 | 6.07 | 4.13 | 5.37 | 4.42 | 4.90 | 3.89 | 5.6 | 4.85 | 4.82 | 4.35 |
| 4 | 6.022 | 136.2 | α-Phellandrene | $C_{10}H_{16}$ | 0.35 | 0.31 | 0.29 | 0.29 | 0.31 | 0.29 | 0.31 | 0.39 | 0.32 | 0.29 | 0.29 | 0.21 | 0.30 | 0.23 | 0.29 | 0.23 | 0.28 | 0.23 | 0.27 | 0.26 |
| 5 | 6.663 | 136.2 | D-limonene | $C_{10}H_{16}$ | 7.96 | 6.87 | 7.02 | 6.70 | 7.98 | 7.24 | 7.25 | 7.76 | 8.31 | 10.72 | 11.35 | 9.12 | 11.17 | 9.75 | 9.96 | 9.06 | 9.61 | 9.75 | 10.74 | 8.10 |
| 6 | 7.439 | 136.2 | Terpinolene | $C_{10}H_{16}$ | 0.28 | 0.26 | 0.25 | 0.25 | 0.24 | 0.26 | 0.27 | 0.28 | 0.23 | 0.21 | 0.19 | 0.21 | 0.23 | 0.23 | 0.22 | 0.21 | 0.19 | 0.19 | 0.21 | 0.18 |
| 7 | 8.610 | 154.1 | Linalool | $C_{10}H_{18}O$ | 0.38 | 0.56 | 0.31 | 0.51 | 0.79 | 0.48 | 0.85 | 0.25 | 0.46 | 2.49 | 2.11 | 3.62 | 2.87 | 3.51 | 2.45 | 2.42 | 2.08 | 2.55 | 3.28 | 1.76 |
| 8 | 10.069 | 152.2 | Camphor | $C_{10}H_{16}O$ | 18.65 | 25.68 | 16.84 | 25.95 | 28.90 | 22.29 | 23.82 | 22.7 | 20.46 | 32.97 | 36.08 | 38.28 | 38.35 | 37.88 | 34.16 | 29.07 | 33.02 | 38.14 | 34.73 | 44.76 |
| 9 | 10.651 | 154.2 | endo-Borneol | $C_{10}H_{18}O$ | 1.98 | 3.80 | 3.12 | 3.80 | 2.87 | 1.89 | 4.66 | 2.37 | 2.41 | 6.29 | 7.15 | 5.24 | 3.79 | 5.58 | 4.94 | 6.25 | 6.35 | 5.27 | 6.61 | 8.09 |
| 10 | 11.433 | 154.2 | α-Terpineol | $C_{10}H_{18}O$ | 0.29 | 0.26 | 0.26 | 0.24 | 0.32 | 0.36 | 0.34 | 0.30 | 0.27 | 0.44 | 0.31 | 0.55 | 0.48 | 0.57 | 0.39 | 0.53 | 0.42 | 0.45 | 0.55 | 0.37 |
| 11 | 14.692 | 196 | Bornyl acetate | $C_{10}H_{20}O_2$ | 51.68 | 47.15 | 60.20 | 46.83 | 41.32 | 49.07 | 47.02 | 47.81 | 51.46 | 25.31 | 20.11 | 26.52 | 22.49 | 24.87 | 27.95 | 31.64 | 25.75 | 25.21 | 23.89 | 15.85 |
| 12 | 22.956 | 222.2 | α-Cadinol | $C_{15}H_{26}O$ | 0.29 | 0.18 | 0.37 | 0.16 | 0.33 | 0.16 | 0.22 | 0.36 | 0.37 | 1.19 | 1.57 | 1.39 | 0.80 | 1.52 | 1.10 | 1.31 | 1.79 | 1.48 | 1.32 | 1.04 |
| 13 | 23.609 | 220.2 | α-Santalol | $C_{15}H_{24}O$ | 0.31 | 0.21 | 0.34 | 0.16 | 0.24 | 0.27 | 0.26 | 0.34 | 0.34 | 0.52 | 0.43 | 0.66 | 0.49 | 0.87 | 0.58 | 0.87 | 0.46 | 0.51 | 0.77 | 0.41 |
| | | | Oil yield (%) | | 3.2 | 3.4 | 2.9 | 2.6 | 3.3 | 2.5 | 3.0 | 3.2 | 2.4 | 1.7 | 1.5 | 1.1 | 0.9 | 1.2 | 1.4 | 1.5 | 1.6 | 1.3 | 1.3 | 1.6 |
| | | | Others | | 4.74 | 3.71 | 2.85 | 3.66 | 3.41 | 5.37 | 5 | 5.25 | 2.87 | 4.59 | 4.22 | 4.88 | 3.95 | 6.74 | 5.18 | 8.61 | 7.65 | 4.73 | 6.01 | 6.03 |
| | | | Number of total peaks | | 30 | 27 | 26 | 27 | 24 | 27 | 31 | 36 | 21 | 27 | 27 | 25 | 25 | 31 | 31 | 40 | 38 | 25 | 33 | 42 |

| | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Number of identified peaks | 28 | 27 | 25 | 26 | 24 | 27 | 29 | 34 | 20 | 27 | 26 | 25 | 24 | 29 | 27 | 38 | 35 | 25 | 32 | 38 |

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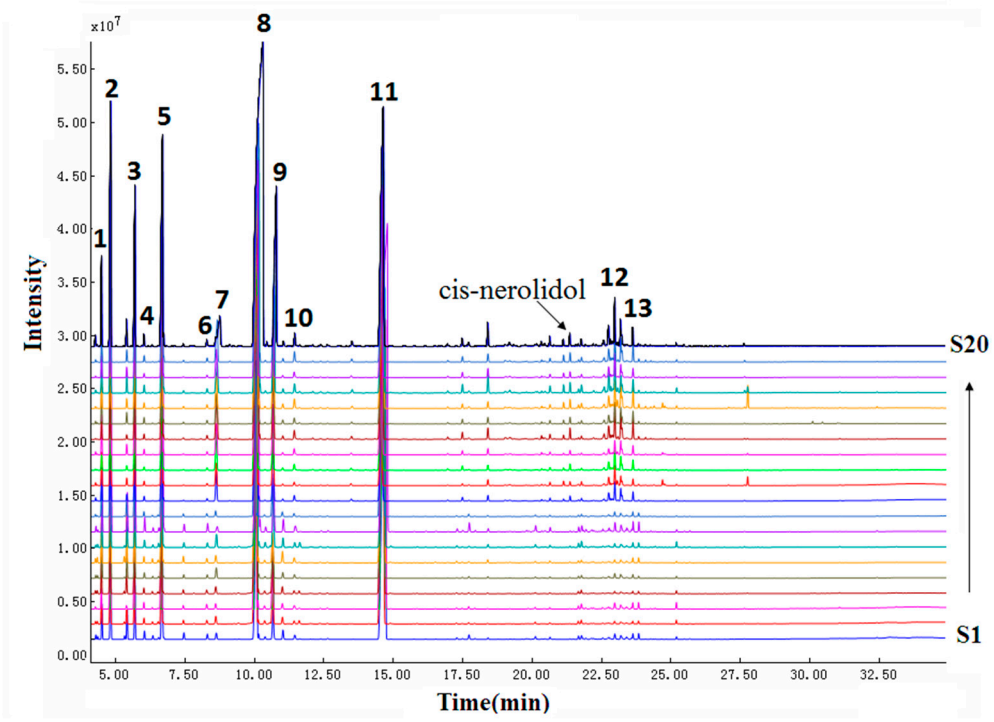


**Figure 1.** GC-MS chromatogram of 20 batches of FA from two species.

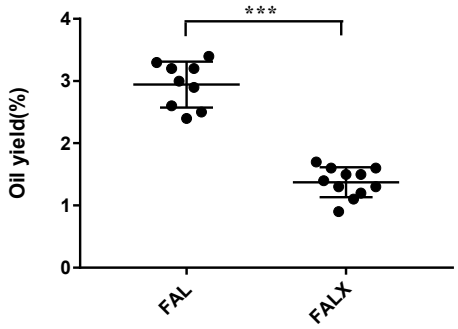


**Figure 2.** Oil yields of 20 batches of FA from two species. Data was presented as mean ± SEM. ***$P<0.001$, **$P<0.01$, *$P<0.05$ compared with FALX.

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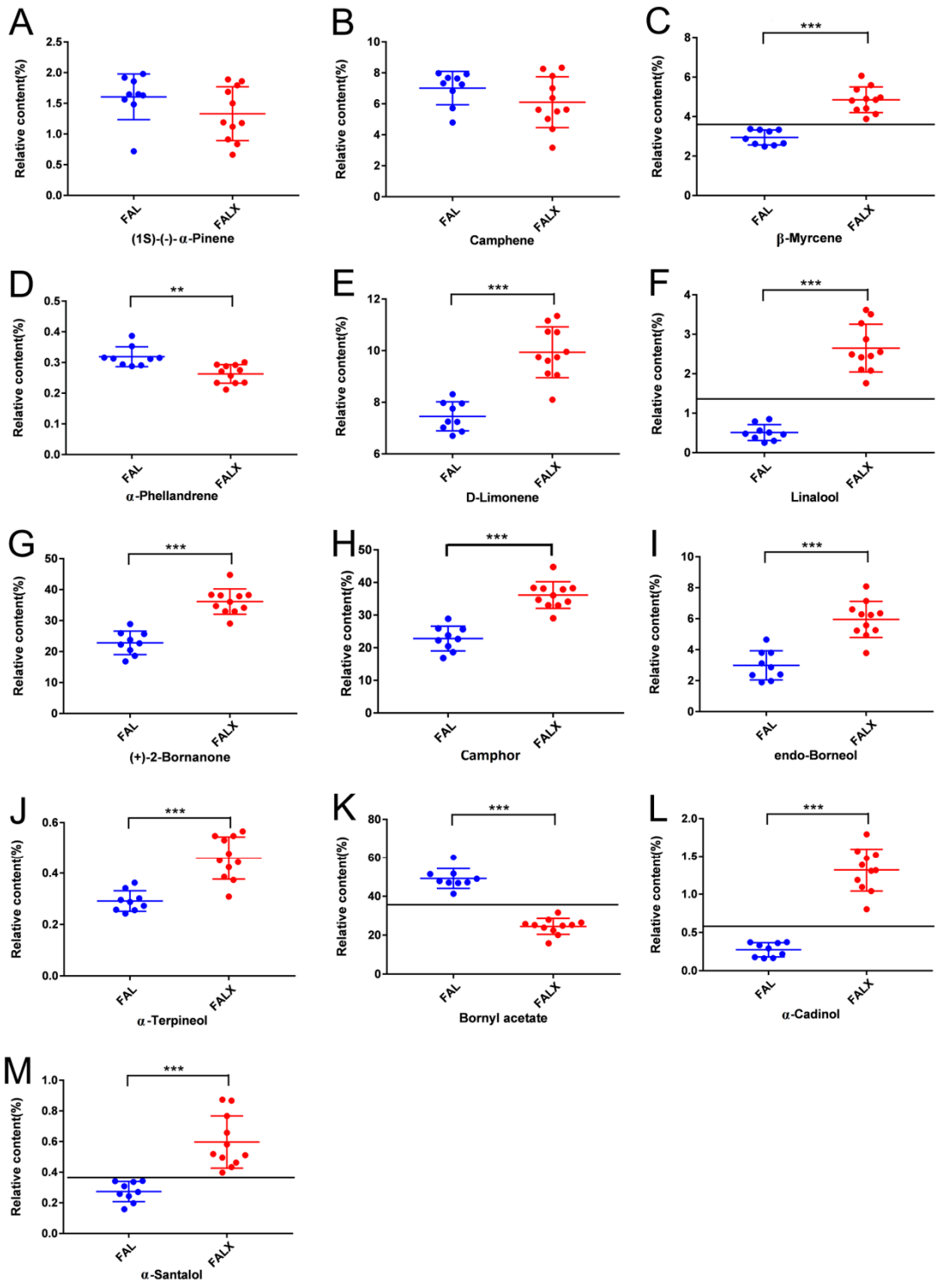


**Figure 3.** Relative contents of the common peaks in TIC of FA from two species (A) (1S)-(-)-$\alpha$-pinene, (B) Camphene, (C) $\beta$-Myrcene, (D) $\alpha$-Phellandrene, (E) D-Limonene, (F) Linalool, (G) (+)-2-Bornanone, (H) Camphor, (I) endo-Borneol, (J) $\alpha$-Terpineol, (K) Bornyl acetate, (L) $\alpha$-Cadinol and (M) $\alpha$-Santalol. Data was presented as mean ± SEM. $^{***}P<0.001$, $^{**}P<0.01$, $^{*}P<0.05$ compared with FALX.

*2.2 HCA*

HCA is a clustering technique that measures either the distance or the similarity between the objects to be clustered. Based on the relative contents of each component in essential oil, the samples

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of FA with close similarities will be crudely classified into the same cluster by HCA. All the test samples were performed using a Ward method to visualize the differences and/or similarities among samples through Euclidean distance. The result showed that the samples were clustered into 2 groups. The first group included S1~S9 (FAL) and the second group covered S10~S20 (FALX). In other words, FAL and FALX could be distinguished based on the composition of the oils. The results were shown in Figure 4.

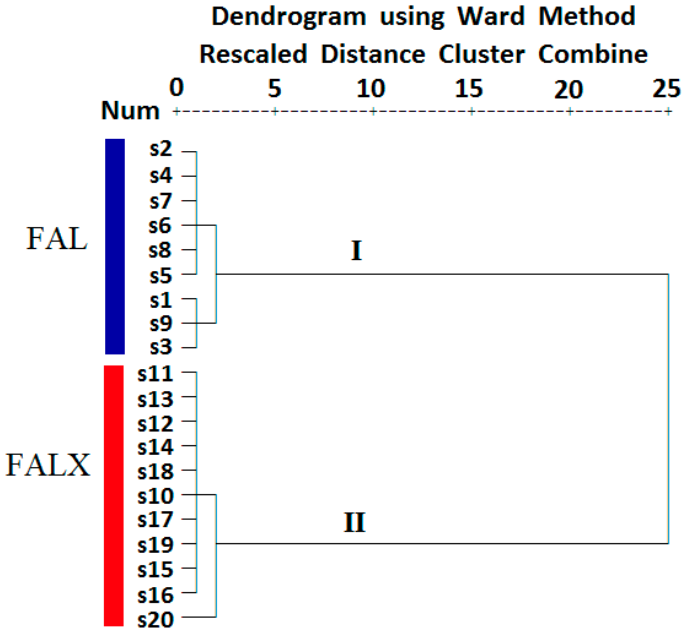


**Figure 4.** HCA of 20 FA samples from two species using Ward's method based on the Euclidean distance.

*2.3. PCA*

To provide more information about differentiation of the origins of FA samples, PCA was performed based on the 13 common peak areas. It was applied for reducing the number of variables (13 variables corresponding to the components in essential oil from FAL and FALX) to a smaller number of new derived variables (PCs) that adequately summarize the original information. The first two PCs explained approximately 83.9% of the original data variability (Figure 5). The score scatter plot was displayed in Figure 6. From the PCA scatter plot, FAL (S1~S9) and FALX (S10~S20) were divided into two areas respectively, which were similar to the result of cluster analysis results. Moreover, dots which presented S1~S9 were relatively nearer to each other, suggesting a closer relationship among the 9 batches of FAL. Dots of S10~S20 were relatively scattered, indicating diversification of the 11 batches of samples. It was indicated that the chemical composition of FALX were less stable compared with FAL. This was probably because FALX were collected from throughout Asia, for instance, Vietnam, Thailand and Myanmar and Yunnan, compared with FAL, which were cultivated only in the south and southeast of China. The results were shown in Figure 5.

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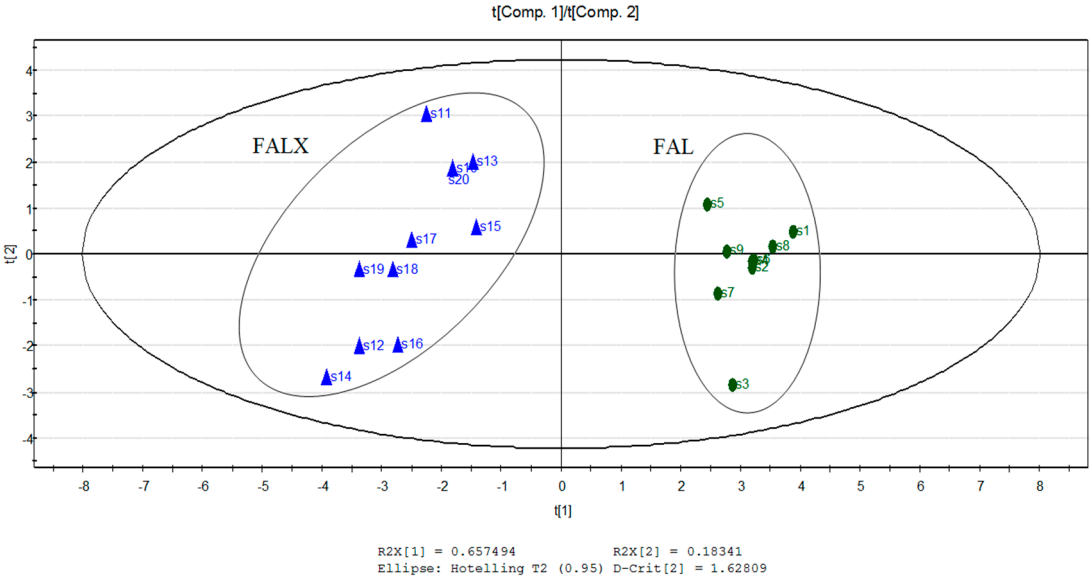


**Figure 5.** Score plot of Principal component analysis of 20 samples of FA from two species.

*2.4. PLS-DA*

Both of HCA and PCA could clearly clarify the two species, but failed to find out the variables for sample classification. Therefore, a supervised PLS-DA technique was used to visualize the variations among these samples. R2X, R2Y and Q2Y of the PLS-DA mode were 0.840, 0.972 and 0.962 respectively, which were suitable for fitness and prediction. Based on the PLS-DA, a loadings plot was drown to exhibit the contribution of each variable to the discrimination of FAL and FALX. As shown in Fig. 7, the 13 common peaks were listed in order according to their contribution value. 8 components with VIP values greater than or equal to 1.00, were selected as the potential markers, including bornyl acetate, $\alpha$-cadinol, linalool, β-myrcene, camphor, d-limonene, terpinolene and endo-borneol. Notably, unpaired T-test had proved that all of the selected constituents could distinguish FAL from FALX. Therefore, these markers could be used for specie identification and quality control of FA. The results were shown in Figure.6.

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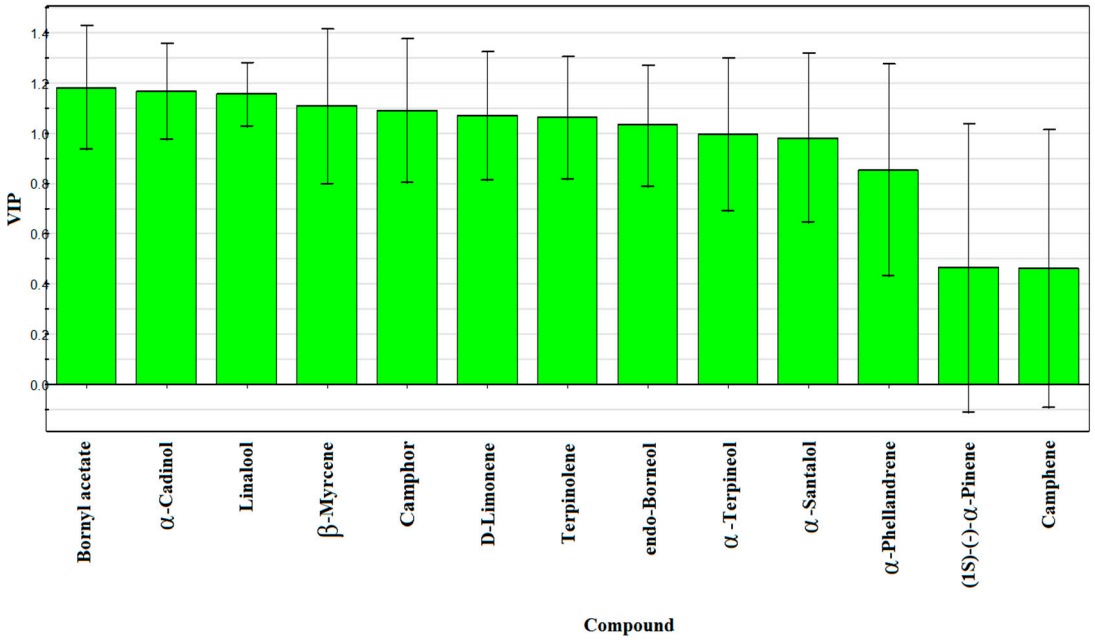


**Figure 6.** VIP of identified compounds of 20 samples of FA from two species based on PLS-DA.

## 3. Methods

### *3.1. Plant Materials*

A total of 20 batches of FA including 9 batches of FAL and 11batches of FALX were collected from Chengdu Lotus Pond Chinese Herbal Medicine Market. FAL were cultivated in Yangchun and Gaozhou in Guangdong Province, Baise in Guangxi, and Ruili, Menghai and Mengla in Yunnan. The origins of FALX were Vietnam, Thailand and Myanmar in addition to Yunnan. 20 batches of samples, authenticated by Lu Chen (Associate Professor of Chengdu University of Traditional Chinese Medicine) were deposited in the chemical laboratory.

### *3.2. Solvents and Chemicals*

Analytical grade n-hexane was purchased from Beijing Chemical works (Beijing, China). Anhydrous sodium sulfate was provided by Chemical Reagent Co. Ltd. of SINOPHARM.

### *3.3. Steam Distillation for Volatile Oil*

About 30 g FAL or 50 g FALX was put into a 1000 ml distillation flask. 10 times of water were added and volatile oil distillation apparatus was set according to the Chinese Pharmacopoeia. The oil was distilled for 6 h, obtained from the condenser, and dried over anhydrous sodium sulfate. The oil yields were calculated in milliliter of oil per 100 g of FA. 20 μl of the obtained essential oil was introduced in 1.5 ml autosampler vials after filtered through 0.22 μm filter and the final volume of the extract was adjusted to1.0 ml with *n*-hexane.

### *3.4. GC-MS Analysis*

GC-MS analysis was performed on an Agilent Technologies apparatus 7890A-5975C with HP-5 MS capillary column (30 m×0.25 mm, 0.25 μm film thickness). Helium was applied as the carrier gas at a constant flow rate of 1 mL•min-1. The injector temperature was 250°C and interface temperature was 280 °C. The initial oven temperature was kept at 60ºC. Then it was gradually raised to124℃ at

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4ºC•min-1, to196℃ at 8ºC•min-1, to 260℃ at 10ºC•min-1 and finally kept for 2 min. The spectrometer operated at 70 ev with the full scan style. The injection mode was split with a 60:1 ratio. Then the components of the volatile oil were positively identified using National Institute of Standards and Technology (NIST) 14.0 Mass Spectra Database. The semi-quantitative analysis of volatile compounds was performed by comparing their peak areas in the GC-MS total ion chromatogram. The percentage compositions of compounds were calculated by area normalization method.

### *3.5. Data Analysis*

Statistical analysis was carried out using unpaired-t test by GraphPad Prism 7. Moreover, these data were also analyzed were processed by HCA, PCA and PLS-DA using SPSS13.0(SPSS Inc., Chicago, IL) or SIMCA P11.0 (Umetrics, Umea, Sweden). Results were expressed in means ± SEM, and the level of $P < 0.05$ was considered as statistically significant.

## 4. Conclusions

In the present study, an efficient strategy for species identification of FA was developed by GC-MS analysis and chemometrics methods. GC-MS fingerprints showed that FAL and FALX were similar and had 13 common peaks. But at the same time, the difference was also very significant. First of all, the main component of the volatile oils was different. Bornyl acetate had the largest relative peak area in FA ranging from 41.32% to 60.20%, while in FALX camphor accounted for the highest percentages ranging from 29.07% to 44.76%. Moreover, some markers with important identification value were found. Cis-nerolidol was only found in PAL, but not in FALX, and the other 8 components, bornyl acetate, α-cadinol, linalool, camphor, β-myrcene, d-limonene, terpinolene and endo-borneol, could also be used as the distinguishing components by unpaired T-test. Moreover, chemometric analysis based on the GC-MS spectrum including HCA, PCA and PLS-DA showed that there were significant differences in the volatile components of FAL and FALX, and the samples from the same variety were clustered together.

Conclusively, the authors believe that chemical composition lays the foundation for the material basis of efficacy. Among Chinese herbs, some species looks similar in extrinsic feature and used in the same way, but they perhaps have different ingredients and effects to some extends. GC-MS analysis combined with chemometrics technologies can provide an accurate method to distinguish these similar Chinese herbs containing volatile oils.

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