

Original Research Article

Comparative studies on flavor substances of leaves and pericarps of *Zanthoxylum bungeanum* Maxim. at different harvest periods

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Abstract

Purpose: To study the transformation of the aroma components and pungent constituents of *Zanthoxylum bungeanum* Maxim. (ZBM) leaves and pericarps at different periods, and to provide a basis for selecting an appropriate harvest time for the pericarps and leaves.

Methods: Quantitative analysis of the pungent components of ZBM leaves and pericarps was performed by high performance liquid chromatography (HPLC) while their aroma constituents were analyzed by headspace solid phase micro-extraction combined with gas chromatography-mass spectrometry (HS-SPME-GC-MS).

Results: HPLC analysis revealed that hydroxy- α -sanshool was the predominant pungent component in both the leaves and pericarps of different parts of ZBM at different harvest periods, followed by hydroxy- γ -sanshool and hydroxy- β -sanshool. During the growth of ZBM, the contents of pungent substances in the leaves declined gradually, while pungent substances in the pericarps increased. The results of HS-SPME-GC-MS analysis showed that linalyl acetate, linalool and limonene were the major aroma components of the leaves and pericarps of ZBM at different harvest periods. During the growth of ZBM, the contents of monoterpenes in the leaves decreased gradually, whereas monoterpenes in the pericarps increased.

Conclusion: These results suggest that the pungent and aroma components produced in ZBM at early developmental stages are stored in the leaves, and are gradually transferred to the pericarps at the final developmental stages. Thus, the leaves of ZBM can be used as a new source of food and medicine.

Keywords: *Zanthoxylum bungeanum* Maxim., Pericarp, Pungent components, Aroma components

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INTRODUCTION

The pericarp of *Zanthoxylum* is commonly named Huajiao or Szechuan pepper, and the most popular commercially goods are

"honghuajiao" and "qinghuajiao" in China. The "qinghuajiao" is derived from the green pericarps of *Zanthoxylum armatum* DC. and *Zanthoxylum schinifolium* Sieb. et Zucc., which are used for food seasoning. Another type of "honghuajiao" is

derived from the bright red pericarp of the *Zanthoxylum bungeanum* Maxim. (ZBM), which is the most popular commercial product in the genus *Zanthoxylum*, with a long history of application as a popular condiment in cooking and medicine in China [1].

The pericarps of ZBM are widely used as a flavor condiment, and also as traditional Chinese medical herbs for their therapeutic properties such as a warming action, relieving pain, dispelling dampness, stopping diarrhea, and preventing itch [1]. Flavor substances (aroma constituents and pungent components), are mainly responsible for these pharmacological activities. Essential oils are the principle source of the special fragrant odor in *huajiao*. It has a variety of effects such as antibacterial, antifungal, antiviral, insecticidal, antithrombotic, anti-atherosclerosis, etc [2,3].

The tingling sensation of *huajiao* is a unique pungent taste, known as “ma”. Furthermore, constituents responding for the special taste of “ma” are unsaturated fatty acid amides of sanshool, especially the hydroxy- α -sanshool, hydroxy- β -sanshool, and hydroxy- γ -sanshool *etc*, and possess various pharmacological activities such as antitumor, insecticidal, anti-inflammatory, anesthetic and analgesic properties, etc. [4-7]. In addition, the leaves of ZBM is widely utilized in food seasonings because of its unique aroma and taste which are similar to the flavor of the pericarps of ZBM [1].

The flavor substances in ZBM are not only the basic ingredients of the seasonings, but also the active ingredients responding for its pharmacological activities. Although the flavor substances, including the aroma components and the pungent components, have been identified in many previous studies, little information is available about changes in the potent flavor substances of laves and pericarps of ZBM as a function of t harvest periods. The scarcity of available data prompted us to reinvestigate the aroma constituents and numb-taste components of ZBM pericarps and leaves.

In the present study, comparison of the aroma components and the pungent components of ZBM leaves and pericarps at different harvest periods by high performance liquid chromatography (HPLC) and headspace solid phase micro-extraction combined with gas chromatography-mass spectrometry (HS-SPME-GC-MS) analyses. In addition, the possibility of translocation of the flavor substances between the leaves and the pericarps was studied.

EXPERIMENTAL

Plant materials

In order to study the flavor substances of leaves and pericarps of ZBM at different harvest periods, samples were collected samples at three different time points on the same tree (Figure 1). The fruits and leaves of ZBM were collected from the same tree in Hanyuan, Sichuan province, China in 2017. The young fruits and leaves were harvested on May 20, and the half-mature fruits and leaves were obtained on June 27, while the mature fruits and leaves were gathered on August 8. The fresh leaves and fruits were dried at 40 °C for 6 h. The pericarp of the dried fruits was separated from the seeds and used for further analyses. The dried pericarps and leaves were vacuum sealed and refrigerated.



Figure 1: Plant materials used. L1: young leaves; L2: half-mature leaves; L3: mature leaves; F1: young fruits; F2: half-mature fruits; F3: mature fruits

Chemicals and reagents

Hydroxy- α -sanshool, hydroxy- β -sanshool, and hydroxy- γ -sanshool were all made in-house. They were identified by NMR and consistent with the literature (Mizutani *et al.*, 1988). The purity of the sample was determined using HPLC peak area normalization method greater than 98.5 %. Methanol and acetonitrile HPLC grade were bought from Sigma (St. Louis, MO, USA). Water was distilled using a UPT water purification system (Ulupure Corporation, Chengdu, China).

Apparatus

The quantitative analysis of the pungent components was performed with Shimadzu liquid chromatographic system (Shimadzu LC-2010A, Japan) consisting of SIL-20A autosampler, SPD-20A detector, CTO-20A Column Thermostat, LC solution Chromatography Workstation Software. The analytical column was Phenomenex C18 (250mmx4.6mm, 4 μ m particle size). Aroma components analysis was accomplished by HS-SPME-GC-MS system comprising AOC-6000 headspace sampler (Shimadzu, Japan), HP-

1510 gas chromatography headspace heater (Jicheng Analytical Instrument Company, Shanghai, China.), solid phase microextraction device (Supelco, USA), 100 μ m PDMS solid phase microextraction fiber (Supelco, USA), Shimadzu gas chromatography system (Shimadzu, GC-2010, Japan), Shimadzu mass spectrometer (Shimadzu, TQ-8040, Japan), and GC-MS solution Chromatography Workstation Software. The components were separated on an Agilent DB-17MS column (30m x 0.25mm x 0.25 μ m film thickness) (Agilent Technologies, USA).

Preparation of samples and standards

Mixed standard solutions

Reference stock solutions of hydroxy- α -sanshool, hydroxy- β -sanshool and hydroxy- γ -sanshool (400, 10, and 25 μ g/mL) in methanol were prepared in a 10-mL volumetric flask. Then, 2 mL of each reference stock was diluted to 10 mL with methanol in a 10-mL volumetric flask to yield mixed standard solutions. The mixed standard solution were then serially diluted in a series of concentrations [9]. The lowest concentration of the reference solution was serially diluted, with signal-to-noise ratios of 3 and 10 as detection and quantitation limits, respectively.

Pungent substance samples

The dried pericarps and leaves of ZBM were ground to powder, passed through a sieve of 355 μ m pore size, and used as sample powders. Half a gram sample (accurate to 0.0001 g) was accurately weighed into a sealed Erlenmeyer flask and ultrasonically extracted with 50 mL of methanol for 30 min. Each sample extract was analyzed in triplicate. In the preliminary experiments, the contents of pungent substances in the half-mature and mature pericarps of ZBM were found to be quite different from those of other samples. In order to ensure the accuracy of experimental data, the total volume of each extract was adjusted to 500 mL in the case of half-mature pericarps, and to 1000 mL for mature pericarps, while 50 mL was used for other samples. The solvent used was methanol. The solutions were filtered through a 0.45- μ m microporous membrane into the injection bottle prior to use.

High performance liquid chromatography

Chromatographic separation was performed in a column (Phenomenex C18) with a detection wavelength of 270 nm. The column temperature

was maintained at 30 °C. The mobile phase used for analyzing the three hydroxyl sanshools by HPLC was acetonitrile: water at a volume ratio of =40 : 60 for 55 min, at a flow rate of 1.0 mL/min and injection volume of 10 μ L.

Construction of reference standard curve

For the HPLC analysis, a series concentration reference solution (10 μ l) was injected into the HPLC. Taking the peak area as the ordinate and the solution mass concentration as the abscissa for linear regression, the regression equations for hydroxy- α -sanshool, hydroxy- β -sanshool, and hydroxy- γ -sanshool were obtained.

HS-SPME-GC-MS

In this procedure, 0.3 g of the sample powder was accurately weighed into a headspace vial, sealed and incubated for 30 minutes at a temperature of 50 °C in the headspace heater. The SPME fiber was activated prior to use each time by inserting it into the GC injector at 230 °C for 30 min. For sampling, the fiber was inserted in the headspace vial and extracted for 10 min at 50 °C. Immediately after extraction, the SPME fiber was exposed to the hot GC injection port at 230 °C for 3 min for desorption of aroma components, where it remained in the inlet during the run, thus preparing it for the next collection of volatiles.

In GC-MS procedure, the injector temperature was set at 230 °C, with desorption time of 3 min. Helium was used as the carrier gas at a flow rate of 1 mL/min. The components were separated on an Agilent DB-17MS column. The oven temperature program was 50 °C for 2 min, increased at 4 °C/min to 200 °C, and held isothermally for 5min, followed by increase at the rate of 15 °C/min to 240 °C, and held isothermally for 5 min. Helium was used as carrier gas. The gas flow rate was set at 1.2 mL/min with a splitting ration of 1:35. The GC-MS interface and ion source temperatures were 240 and 200 °C, respectively. GC-MS detection was carried out in the complete scanning mode (SCAN) in the range m/z 50–550. The MS was operated in EI mode with standard electron energy of 70 eV.

RESULTS

Pungent components

The HPLC chromatograms of reference substances are shown in Figure 2, and the regression equation results are shown in Table 1.

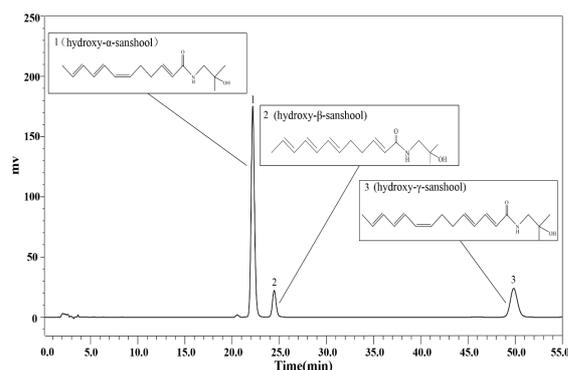


Figure 2: HPLC chromatograms of reference substances. 1: hydroxy- α -sanshool; 2: hydroxy- β -sanshool; 3: hydroxy- γ -sanshool

The peak assignment of each hydroxy sanshool in the sample was based on its retention time in the reference product. The HPLC-DAD chromatograms of samples detected at 270 nm are shown in Figure 3. The amount of each hydroxy sanshool was quantified with HPLC by comparing the measured peak area with calibration curves obtained from hydroxy- α -sanshool, hydroxy- β -sanshool, and hydroxy- γ -sanshool as standards, and the results are summarized in Table 2.

Table 1: Linear regression and sensitivity data

Analyte	Regression equation(n) ^a	Linear range ($\mu\text{g/ml}$)	R-squared	LOQ(ng/mL) ^b	LOD(ng/mL) ^b
Hydroxy- α -sanshool	$y=82952.5903x+149089.5000(5)$	5.00-80.00	0.9996	29.95	7.14
Hydroxy- β -sanshool	$y=132103.8925x+4226.0833(5)$	0.13-2.00	0.9998	3.28	0.96
Hydroxy- γ -sanshool	$y=147605.4968x+1944.7500(5)$	0.31-5.00	0.9998	21.95	6.12

^a y = peak area; x = concentration of the standard in ($\mu\text{g/ml}$); n = number of data points (concentration levels); limits of detection (LOD) and quantitation (LOQ) in ($\mu\text{g/ml}$ L) and in (ng)

Table 2: Content of hydroxy sanshool in the leaves and pericarps of ZBM at different harvest periods

Analyte	Leaf			Pericarp		
	Young (May 20)	Half-mature (June 27)	Mature (August 8)	Young (May 20)	Half-mature (June 27)	Mature (August 8)
Hydroxy- α -sanshool (mg/g) [*]	1.93 \pm 0.15	1.18 \pm 0.10	0.89 \pm 0.12	6.78 \pm 0.45	8.99 \pm 0.64	52.70 \pm 2.42
Hydroxy- β -sanshool (mg/g)	0.02 \pm 0	0.03 \pm 0.01	0.05 \pm 0.02	0.08 \pm 0.02	0.18 \pm 0.01	0.96 \pm 0.05
Hydroxy- γ -sanshool (mg/g) [*]	0.03 \pm 0.01	0.05 \pm 0	0.06 \pm 0.02	0.45 \pm 0.10	1.53 \pm 0.12	11.28 \pm 1.21
Total (mg/g) [*]	1.98 \pm 0.16	1.26 \pm 0.11	1.00 \pm 0.16	7.31 \pm 0.57	10.70 \pm 0.77	64.94 \pm 3.68

^{*}Milligram of hydroxy sanshool per gram of dry sample powder; each value is expressed as mean \pm SD (n = 3, mg/g dry weight)

Aroma components

Extract (1.0 μL) was used for the GC-MS analysis. The total ion chromatogram of the GC-MS analysis for each sample is shown in Figure 4. Individual constituent were identified by comparison with the internal spectral library of the instruments (Wiley library ver. 9, NIST library ver. 14 and NIST library ver. 14s) and retention times based on references. Statistically, volatile components with a degree of match greater than 90% were used, and quantitative analysis was based on the relative percentage of normalized peak areas.

A total of 38 components were identified from the volatile components by HS-SPME-GC-MS. The chemical composition and relative contents are shown in Table 3. Linalyl acetate, limonene, linalool, germacrene D, β -myrcene, cineole, caryophyllene and β -phellandrene were the main components, and they comprised more than 81 % of the total aroma components. In particular, the linalyl acetate constituted 23.53 to 44.95 % (Table 4).

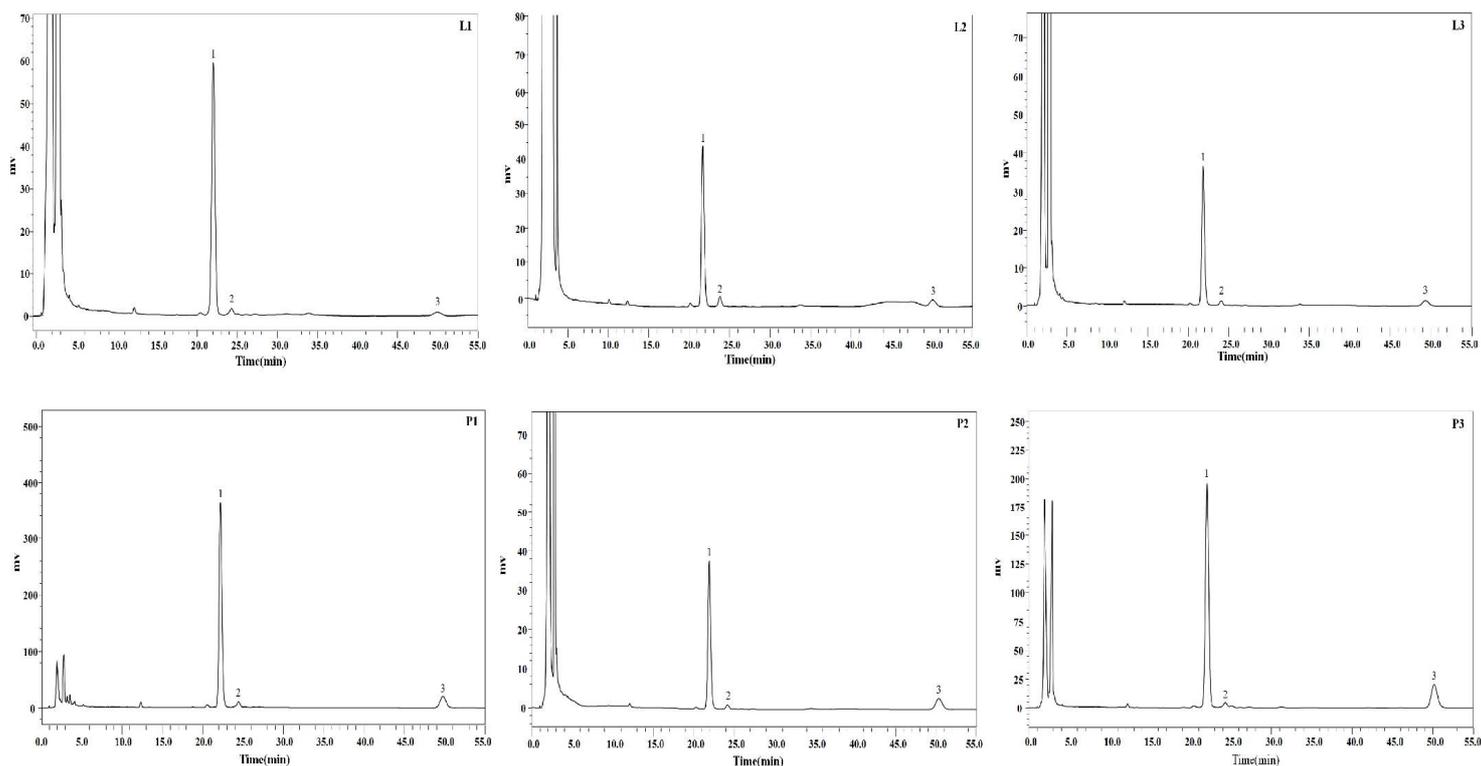


Figure 3: HPLC chromatograms of the samples (L1= young leaves; L2= half-mature leaves; L3=mature leaves;P1=young pericarps; P2= half-mature pericarps; P3, mature pericarps). 1: hydroxy- α -sanshool; 2:hydroxy- β -sanshool; 3:hydroxy- γ -sanshool

The aroma constituents comprised mainly monoterpenes and sesquiterpenes, and belong to a structurally diverse group of natural products known as isoprenes. Regardless of the growth stage of ZBM leaves and pericarps, its

monoterpenes content is higher than sesquiterpenes content, and the content is more than 70 % (Table 5).

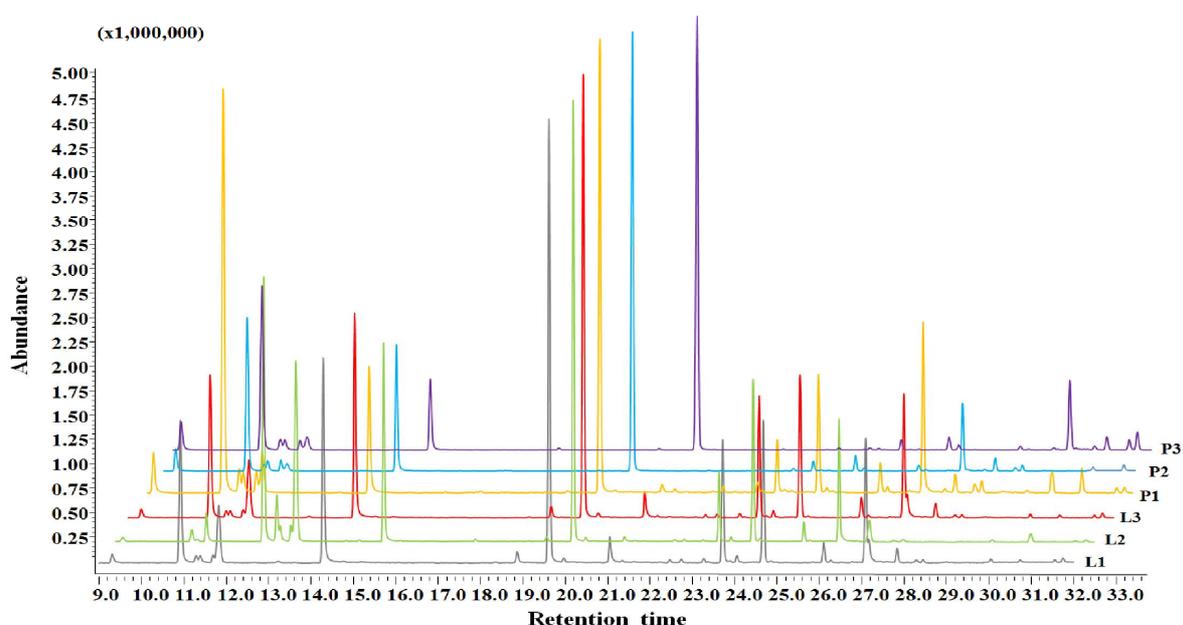


Figure 4: Total ion chromatogram of volatile components in the leaves and pericarps of ZBM at different harvest times: L1 = young leaves; L2 = half-mature leaves; L3 = mature leaves; P1 = young pericarps; P2 = half-mature pericarps; P3 = mature pericarps

Table 3: Volatile constituents of the leaves and pericarps of ZBM at different harvest periods

No.	Retention time (min)	Compound	Relative content (%)					
			L1	L2	L3	P1	P2	P3
1	9.15	α -Pinene	—	0.37	—	—	—	—
2	11.05	Sabinene	—	0.89	—	—	—	—
3	11.20	β -Pinene	—	0.18	—	—	—	—
4	11.74	β -myrcene	3.03	1.88	0.73	2.72	2.73	3.68
5	13.14	Limonene	26.42	17.41	14.40	27.25	20.35	19.02
6	13.47	β -phellandrene	3.12	3.09	0.51	1.67	1.31	0.78
7	13.89	Trans- β -Ocimene	1.03	0.94	0.58	1.31	1.20	1.19
8	14.79	β -Ocimene	0.90	0.93	0.54	1.33	1.26	1.09
9	15.01	Cineole	1.97	15.18	7.95	3.57	2.21	1.23
10	16.33	Linalool	9.58	11.29	14.84	7.54	8.01	10.73
11	18.86	Citronellal	1.12	0.13	—	—	—	0.20
12	20.82	α -Terpineol	0.26	0.27	0.82	—	—	0.11
13	21.57	Linalyl acetate	36.58	24.75	23.53	24.06	44.61	44.95
14	21.90	γ -Terpinene	—	0.18	0.36	—	—	—
15	22.97	2-Hendecanone	—	0.24	2.09	0.46	—	—
16	23.26	α -Cubebene	0.15	—	—	0.17	—	—
17	24.35	α -Copaene	0.33	0.08	0.20	0.38	0.23	0.15
18	24.62	4-Terpinenyl acetate	—	0.11	0.22	—	—	—
19	24.84	Citronellyl acetate	0.26	—	—	—	—	0.05
20	25.14	β -Cubebene	0.12	0.09	0.29	0.62	0.25	0.14
21	25.24	β -Elemene	0.08	—	—	0.15	—	—
22	25.58	Terpinyl acetate	0.08	0.11	8.26	3.01	1.09	0.98
23	25.74	α -Gurjunene	—	0.23	0.23	0.23	—	—
24	26.51	Caryophyllene	3.56	9.40	10.90	7.10	1.67	1.40
25	26.70	Geranyl acetate	1.35	0.23	0.13	0.36	0.51	0.33
26	27.91	α -Humulene	0.62	1.10	1.37	1.98	0.63	0.41
27	28.07	Aromadendrene	—	—	0.15	0.35	0.14	0.09
28	28.56	γ -Muurolene	0.14	—	—	—	0.14	0.24
29	28.88	Germacrene D	4.07	7.04	8.25	9.91	7.56	7.29
30	29.00	α -Farnesene	0.13	—	—	—	0.16	0.13
31	29.37	α -Muurolene	0.25	—	—	0.18	0.20	0.24
32	29.61	Bicyclogermacrene	0.51	1.12	1.00	1.06	1.38	1.43
33	30.05	γ -cadinene	0.78	—	0.27	0.62	0.41	1.19
34	30.21	δ -cadinene	1.18	0.11	0.27	0.70	0.57	1.94
35	31.79	Germacrene B	0.10	0.11	0.19	1.19	0.35	0.20
36	34.27	Spathulenol	—	—	0.16	0.28	—	—
37	32.47	Cubebol	—	0.42	0.16	1.42	0.66	—
38	33.45	Caryophyllene oxide	0.11	0.10	0.28	0.29	—	—

L1, L2, and L3 represent samples of young leaves, half-leaves, and mature leaves, respectively; P1, P2, and P3 represent young pericarps, half-mature pericarps, and mature pericarps samples, respectively; “—” represents not detected

Table 4: Major compounds of the leaves and pericarps of ZBM collected during different seasons

Compound	Relative content (%)					
	L1	L2	L3	P1	P2	P3
Linalyl acetate	36.58	24.75	23.53	24.06	44.61	44.95
Limonene	26.42	17.41	14.40	27.25	20.35	19.02
Linalool	9.58	11.29	14.84	7.54	8.01	10.73
Germacrene D	4.07	7.04	8.25	9.91	7.56	7.29
β -myrcene	3.03	1.88	0.73	2.72	2.73	3.68
Cineole	1.97	15.18	7.95	3.57	2.12	1.23
Caryophyllene	3.56	9.4	10.9	7.1	1.67	1.4
β -phellandrene	3.12	3.09	0.51	1.67	1.31	0.78
Total	88.33	90.04	81.11	83.82	88.36	89.08

L1, L2, and L3 represent samples of young leaves, half-leaves, and mature leaves, respectively; P1, P2, and P3 represent young pericarps, half-mature pericarps, and mature pericarps samples, respectively

Table 5: Relative contents of monoterpenes and sesquiterpenes in leaves and pericarps of ZBM at different harvest periods

Monoterpenoids	Relative content (%)					
	L1	L2	L3	P1	P2	P3
Monoterpenes	85.	76.	76.	73.	83.	83.
	70	46	18	28	19	34
Sesquiterpenes	12.	19.	23.	26.	15.	13.
	13	80	72	63	54	95

L1, L2, and L3 represent samples young leaves, half-leaves, and mature leaves, respectively; P1, P2, and P3 represent young pericarps, half-mature pericarps, and mature pericarps samples, respectively

DISCUSSION

The results of this study suggest that the pungent components in the leaves and pericarps of ZBM were same basically. However, the content of pungent components in the leaves was less than that of pericarps. At different periods, hydroxy- α -sanshool was the most abundant pungent component in the leaves and pericarps of ZBM, followed by hydroxy- γ -sanshool and hydroxy- β -sanshool. However, in the previous studies regarding pungent substances in other genus *Zanthoxylum* (Rutaceae), such as *Z. schinifolium* and *Xanthoxylum piperitum*, hydroxy- α -sanshool and hydroxy- β -sanshool were considered to be the most important pungent components, and the hydroxy- γ -sanshool was not a major pungent component [10,11].

Therefore, hydroxy- γ -sanshool may be a characteristic pungent component of ZBM, unlike what was reported in other *Zanthoxylum* varieties. In addition, it was found that during the growth of ZBM, the pungent substances in the leaves were reduced gradually, while those in the pericarps were increased continuously, indicating that these substances were gradually translocated from the leaves to the pericarps during this process.

The HS-SPME-GC-MS has advantages of simplicity, rapidity and high-efficiency in the analysis of volatile constituents from leaves and pericarps of ZBM [12]. Linalyl acetate, linalool, and limonene were the major components of the leaves and pericarps of ZBM. This is consistent with the results of previous studies [13]. In addition, the aroma components of the leaves and pericarps were generally similar. However seasonal changes could affect the contents of main constituents in the leaves and pericarps of ZBM.

The main aroma components in the pericarps and leaves of ZBM are monoterpenes. Similar to the pungent substances, the monoterpene

contents decreased gradually in the leaves, but increased in the pericarps, suggesting that the monoterpenes in leaves could be transferred to the pericarps continuously following the growth and development of ZBM. However, in a different study, it was reported that the essential oil of *Z. piperitum* was rich in monoterpenes at the early stages, but changed to being enriched with sesquiterpenes at the maturation stages [14]. These differences in results may be related to differences in the varieties of *Zanthoxylum* used.

CONCLUSION

The results obtained in this study indicate that the pungent and aroma components produced from ZBM leaves at the early developmental stages are stored in leaves, and are transferred to the pericarps at the final developmental stages. The hydroxyl sanshool level in the pericarps of ZBM gradually increases, and the content and distribution of the aroma components also change during maturation of the pericarps. These changes in pungency and fragrance during maturation appear to support the use of both species as sources of huajiao in traditional Chinese medicine. These results provide a basis for selecting an appropriate harvest time for the pericarps and leaves, as well as afford a reference for their quality control. The results also provide a useful basis for the development and utilization of leaves and pericarps of ZBM as value-added food and medicinal resource.

DECLARATIONS

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. C.-J. Wu conceived the research. L. Zhu., L. Wang., X. Chen., and F. Liang performed the detail experiments. L. Zhu collected test data and

drafted the manuscript. L. Wang and X. Chen. analyzed the data. W. Peng and Y.-J. Liu contributed reagents/materials/analysis tools. W. Peng and L.-Y. Yu revised the manuscript.

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