

Effect of different growth stages of *Ziziphora clinopodioides* Lam. on its chemical composition

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ABSTRACT

Aims: The aim of this study is to monitor the changes in the chemical composition of *Ziziphora clinopodioides* Lam. throughout nine different growth stages. **Materials and Methods:** Volatile components such as essential oils were analyzed using the gas chromatography (GC) and GC-mass spectrometry, and the contents of non-volatile components were determined by a visible spectrophotometer. **Results:** Hydro-distilled essential oil content ranged from a minimum of 1.1% (in the post-flowering stage) to a maximum of 1.8% (in the flowering stage). The essential oils included pulegone, which was the most abundant component (77.48-87.3%), p-menthanone (2.79-12.39%), trans-isopulegone (1.04-2.06%), d-limonene (0.51-3.03%) and eucarvone (1.5-4.48%). The contents of non-volatile components, such as that of total phenolics (TPC), total flavonoids (TFC), total triterpenoids content (TTC) and total free amino acids content (TFAAC) were measured using visible spectrophotometry. In the growing stage, TPC, TFC, TTC and TFAAC were 9.91-12.80 mg/g, 29.84-50.63 mg/g, 0.57-1.41 mg/g and 13.33-28.56 mg/g, respectively. **Conclusion:** These data can be used as a basis to determine the optimal harvest time of *Z. clinopodioides* Lam.

Key words: Chemical composition, dynamic changes, *Ziziphora clinopodioides* Lam.

INTRODUCTION

Ziziphora clinopodioides Lam. is a traditional Uygur medicinal plant widely distributed in China, Mongolia, Turkey, Kazakhstan and Kyrgyzstan Liu 1985.^[1] In Iran, this plant is mostly used in traditional medicine as a sedative, carminative, anti-emetic, anti-inflammatory and antiseptic substance in food Maya 2011.^[2] The Uygur use *Z. clinopodioides* Lam. as an edible medicinal plant and its leaves, flowers and stems as a wild vegetable or an additive in food for richer aroma and flavor. This plant is also used to treat different diseases as an antiseptic, cold, cough and wound-healing medicine. Studies on *Z. clinopodioides* Lam. have mainly focused on their chemical constituents and biological activity Senejoux *et al.* 2012; Ji *et al.* 2012.^[3,4] Much of the biological activity of the plant has been reported, such as its antibacterial, antimicrobial and antioxidant properties, its relaxing effect on the vascular

system and its immunity-boosting effect on laying hens Ali *et al.* 2012 and Soltani 2012.^[5,6]

Our research team has previously studied the stability of the essential oils of the plant Shi *et al.* 2009,^[7] described the differences in the essential oil content of four *Z. clinopodioides* Lam. types growing in northern Xinjiang, China Zhou *et al.* 2011,^[8] investigated the total polyphenolic and flavonoid content and antioxidant activity of plant extracts of different polarities Tian *et al.* 2011,^[9] determined the oleanolic and ursolic acid content in the plant by using the high-performance liquid chromatography Tian *et al.* 2010,^[10] identified diosmin, linarin and pulegone content in the plant from different places in Xinjiang Halmuart *et al.* 2012^[11] as well as its caffeic and rosmarinic acid content Zhou *et al.* 2011^[12] established the fingerprint of the plant Yu *et al.* 2012.^[13] and established the quality standard of *Z. clinopodioides* Lam. initially Yu *et al.* 2012,^[14] However, the changes in the chemical composition of *Z. clinopodioides* Lam. throughout different growth stages have not been reported and the harvest date affects the chemical composition of the plant. Thus, this study was undertaken to determine the dynamic changes of chemical composition of *Z. clinopodioides* Lam. in different growth stages as well as its optimum harvest time.

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EXPERIMENTAL

Standards and solvents

Standards of rutin, uralic acid were purchased from National Institute for The Control of Pharmaceutical and Biological Products (China), arginine and gallic acid were from Wuxi Jinghai Amino Acid Co. (Wuxi, China).

Solvents and reagents were all of analytical grade from Tianjin Fu-Yu Chemical Ltd., Co. (Tianjin, China).

Plant material

Fresh plants (i.e., the entire plant) were collected from Tuoli, a township in Urumq (N 43°26' 51.2", E 87°39' 18.9") from May 1 to August 31, 2012, once every 2 weeks of harvest, authenticated by Yonghe Li, the chief apothecary of Chinese medicine at the Hospital of Xinjiang and dried naturally in the shade. Voucher specimens were deposited in the Department of Traditional Chinese Medicine Ethnical Herbs Specimen Museum of Xinjiang Medical University.

Analysis of essential oil composition

The whole plants from nine different growth stages (50 g) were extracted through hydro-distillation by using a Clevenger-type apparatus for 6 h. The essential oils were dried over anhydrous Na₂SO₄ and stored in sealed vials under refrigeration prior to analysis. Gas chromatography (GC) analyses were carried out on essential oil samples by using a Shimadzu QP-2010 GC-MS system equipped with DB-5 ms (30 m, 0.25 mm, film thickness of 0.25 μm) capillary column and connected to a flame ionization detector. The temperatures of the injector and detector were both 250°C. Helium was used as the carrier and the flow rate was 1.0 mL/min. The temperature program was 40°C to 250°C at a rate of 5°C/min. The split ratio was 1:100. GC-MS analyses were performed in the electron-impact ionization mode with energy of 70 eV. The inlet temperature was set at 200°C and the transfer line temperature was 250°C. The temperature program used was similar to that adopted for GC analysis. The injected volume was 0.2 μL. Finally, the components were identified by comparing the retention time of each component with the n-alkane series (C6-C22) internal standards in identical experimental conditions. The mass spectra and relative retention index of the components were compared with those of commercial samples National Institute of Standards and Technology (NIST 05 and NIST 05 s). The relative amounts of individual components were calculated based on GC integrator peak areas without the use of correction factors.

Ethanol extract

Samples from nine different growth stages of *Z. clinopodioides* Lam. were pulverized into a fine powder by using a stainless steel blender. The dried and powdered plant materials (1 g)

were extracted with 25 mL of 70% ethanol with 100 W ultrasonic bath (KQ2200E, Kunshan Ultrasonic Instrument Co., Jiangsu, China) for 30 min and were finally filtered.

Water extract

The powders from the nine stages of *Z. clinopodioides* Lam. were accurately weighed (2 g) and placed in a 250 mL round bottom flask. Boiling water was added three times. The volume of the first addition of water was 100 mL. The solution underwent reflux extraction for 1 h and was filtered while hot. The volume of the second addition of water was 50 mL. The solution was boiled for 0.5 h and filtered while hot. The volume of the third addition of water was 50 mL. The solution was boiled for 0.5 h and filtered while hot. The filtrate that was merged three times was concentrated to 100 mL as the test solution.

Total phenolic content

TPC was determined using the Folin-Ciocalteu method. First, 1 mL of ethanol was extracted and diluted to 10 mL with 70% ethanol. About 0.5 mL of the diluents was then mixed with 1 mL of the Folin-Ciocalteu reagent and was left to stand for 1 min at room temperature. About 2 mL of sodium carbonate (20% Na₂CO₃) solution was then added and the volume of distilled water was increased to 10 mL. The solution was left to stand at room temperature for 10 min. Supernatant absorbance was measured at 760 nm with a visible spectrophotometer (Vis)-722S, Shanghai Jinghua, China and the quantification was done on the basis of the standard curve ($Y = 14.31X + 0.1173$) of gallic acid concentration ranging between 0.01 and 0.05 mg/mL ($r^2 = 0.9949$). The results were different from the standards prepared similarly with known gallic acid concentrations. All samples were analyzed three times.

Total flavonoid content

TFC was determined by the method of Lv Lin and Ningping Tao Lv *et al.* 2009^[15] at 510 nm with a Vis-722S spectrophotometer. The quantification was performed on the basis of the standard curve ($Y = 10.7007X + 0.0136$) of rutin concentration ranging between 0.02 and 0.06 mg/mL ($r^2 = 0.9949$).

Total triterpenoid content

In this assay, the ethanol extracts needed to be purified because the other impurities in the extract would affect the measurement results. The ethanol extracts were concentrated and filtrated with a rotary evaporator, dissolved with 30 mL of chloroform and then moved to a separatory funnel. The saturated sodium bicarbonate extraction of chloroform was performed four times (15 mL × 4) and was combined with a sodium bicarbonate solution. The chloroform extraction was performed four times (15 mL × 4) with the pH regulated between 2 and 3 by using 6 mol/mL hydrochloric acid.

The chloroform liquid was merged and the chloroform solution was washed with water. The water was then left to stand. The chloroform solution with anhydrous sodium sulfate was dried and filtered. The anhydrous sodium sulfate was washed with chloroform three times (15 mL × 3). The washing liquid and filtrate were merged. The solution was decompressed and evaporated until dry. The residue with the anhydrous ethanol solution was set at a constant volume of 5 mL. The solution was shaken and the product solution was obtained. TTC was determined through colorimetry. An accurate quantity of the purification extract was taken (0.4 mL in a test tube). Anhydrous ethanol was placed in a boiling water bath. The solution was then cooled. About 5 mL of 5% vanillin-glacial acetic acid solution and 1 mL of concentrated sulfuric acid solution were prepared. The test tube was heated to 60°C in the water bath for 10 min and the solution was cooled to room temperature. A constant volume of glacial acetic acid was added to the solution, which was then agitated. Each sample solution's absorbance was measured at 553 nm by using a Vis-722S spectrophotometer. Uralic acid (1.2 mg/mL to 7.2 mg/mL) was used as a standard. All samples were analyzed three times.

Total free amino acids content

The TFAAC of the extracts was determined based on the method of Rosen (1957). First, 0.5 mL of water extractions was added to 1 mL of sodium carbonate buffer salts and 2 mL of 2% ninhydrin solution in a tube. The tubes were heated in a boiling water bath for 15 min and allowed to cool at room temperature for 15 min. A constant volume of water was added to the solution, which was then agitated. Each sample solution's absorbance was measured at 566 nm by using a Vis-722S spectrophotometer. Arginine (4 µg/mL to 16 µg/mL) was used as a standard. All samples were analyzed three times.

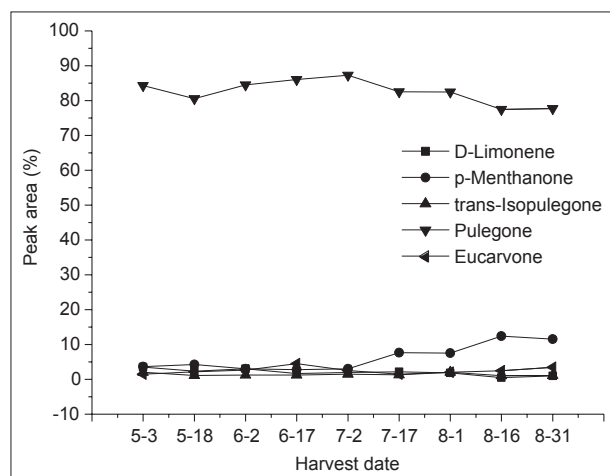


Figure 1: The dynamic changes of main chemical compositions of essential oils in the *Ziziphora clinopodioides* Lam.

RESULTS AND DISCUSSIONS

Volatile components

The growth stages were as follows: May 3 and May 17 were the seedling stage, June 2 and June 17 were the pre-flowering stage, July 2 was the initial flowering stage, July 2 to August 1 was the flowering stage, August 16 was the blossom stage and August 31 was the post-flowering stage. The essential oil compositions in different growth stages are presented in Table 1 and the dynamic changes are shown in Figure 1. The nine essential oils from different growth stages were mainly composed of oxygenated monoterpenes and had no significant differences in contents. The main component was pulegone in each stage, especially in the pre-flowering stage (June 17), where it was as high as 86.05%. p-Menthanone was as high as 12.39% on August 16, the blossoming stage. However, the content of d-limonene was lower during the flowering stage, but was the highest in the seeding stage, May 3 (3.5%). However, the trans-isopulegone content was higher in both the seeding and flowering stages at 2.06% and 2.01%, respectively. Overall, both the content of essential oils and the main chemical compositions in *Z. clinopodioides* Lam. were higher in the flowering stage than in other stages. Thus, *Z. clinopodioides* Lam. should be harvested during the flowering stage if its essential oils need to be studied.

Non-volatile components

Z. clinopodioides Lam. is rich in total flavonoids, total polyphenols and total free amino acids [Table 2]. The harvest date had a significant effect on its content [Figure 2]. However, TTC in the plant was much lower and had a little change during the different growth stages. Overall, TPC was higher on May 3 and in August and reached values as

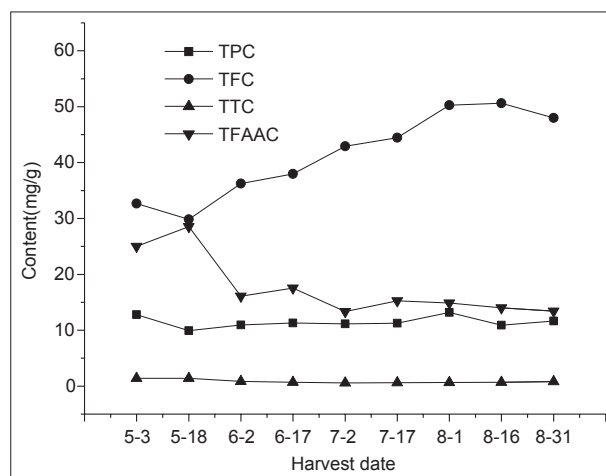


Figure 2: Content of chemical composition in *Ziziphora clinopodioides* Lam. at different growth stage

Table 1: Chemical compositions of the essential oil of *Ziziphora clinopodioides* Lam in different growth stages

RI	Name	Peak area (%)								
		May 3	May 18	June 2	June 17	July 2	July 17	August 1	August 16	August 31
939	α -Pinene	0.26	0.43	0.47	0.15	0.4	0.49	0.62	*	*
978	2-Thujene	0.15	0.2	0.2	0.14	0.15	0.2	0.23	*	0.1
984	β -Pinene	0.38	0.48	0.52	0.28	0.45	0.56	0.69	*	0.2
996	β -Myrcene	0.29	0.32	0.32	0.23	0.27	0.35	0.35	*	0.16
1037	D-limonene	3.5	2.33	3.03	1.64	1.95	2.14	1.84	0.51	1
1040	Eucalyptol	0.37	0.53	0.39	0.49	0.24	0.48	0.35	0.19	0.43
1167	P-Menthanone	3.66	4.27	3.02	2.79	3.05	7.66	7.52	12.39	11.52
1184	Trans-isopulegone	2.06	1.14	1.22	1.23	1.47	1.29	2.01	1.04	1.13
1193	Terpinen-4-ol	*	*	*	*	0.36	0.5	0.61	1.23	*
1203	Neoisomenthol	0.15	*	0.12	*	*	0.22	0.24	0.25	*
1233	Cumaldehyde	*	*	0.19	*	*	0.12	0.15	0.24	0.2
1257	Pulegone	84.34	80.54	84.48	86.05	87.3	82.51	82.49	77.48	77.72
1268	1-tert-Butoxy-6-methylcyclohexene	0.26	0.15	0.14	0.15	0.19	0.28	0.29	0.4	0.62
1312	Thymol	0.17	0.23	0.2	0.24	0.21	*	*	*	*
1328	2-methoxy-4-vinylphenol	0.33	0.27	0.25	0.19	*	*	*	*	*
1355	Eucarvone	1.5	2.16	2.66	4.48	2.54	1.49	2.05	2.49	3.48
1499	Germacrene D	0.45	0.52	0.46	*	0.45	0.39	0.24	0.45	0.43
1701	Patchouli alcohol	*	1.04	*	0.1	*	*	*	*	*
	Total identified	97.87	94.61	97.67	98.16	99.03	98.68	99.68	96.67	96.99
	Oxygenated	92.25	90.78	92.28	95.38	95.17	94.27	95.42	95.31	94.48
	Monoterpenes	4.58	3.76	4.54	2.44	3.22	3.74	3.73	0.51	1.46
	Sesquiterpenes	0.45	0.52	0.46	*	0.45	0.39	0.24	0.45	0.43
	Fatty acids and aliphatic esters	0.59	0.42	0.39	0.34	0.19	0.28	0.29	0.4	0.62
	Essential oils content (%)	1.6	1.5	1.5	1.4	1.6	1.7	1.8	1.7	1.1

*Not detected. RI: Retention indices relative

Table 2: The dynamic changes of chemical components of *Ziziphora clinopodioides* Lam

Harvest time	TPC (mg/g)	TFC (mg/g)	TTC (mg/g)	TFAAC (mg/g)
May 3	12.80±0.04	32.66±0.22	1.41±0.19	25.01±0.35
May 18	9.91±0.18	29.84±0.18	1.39±0.08	28.56±0.34
June 2	10.93±0.21	36.23±0.38	0.85±0.02	16.11±0.48
June 17	11.30±0.11	37.96±0.61	0.69±0.04	17.54±0.31
July 2	11.13±0.13	42.91±0.71	0.57±0.01	13.33±0.21
July 17	11.25±0.19	44.47±0.35	0.61±0.02	15.25±0.19
August 1	13.18±0.19	50.28±0.81	0.66±0.01	14.86±0.21
August 16	10.89±0.35	50.63±0.59	0.71±0.02	14.00±0.25
August 31	11.66±0.31	48.01±0.33	0.82±0.02	13.43±0.19

Each value is expressed as mean±standard deviation (n=3). TFC: Total flavonoid content, TPC: Total phenolic content, TTC: Total triterpenoid content, TFAAC: Total free amino acids content

high as 12.80 and 13.18 mg/g, respectively. TFC increased from May 18 to August 16 from 29.84 mg/g to 50.63 mg/g and then decreased to 48.01 mg/g on August 31. TFAAC was rich in the seeding stage, followed by a rapid decrease on June 2 and became relatively stable in the subsequent growth stage. TTC was always maintained less than the others we had determined, a trend similar to that of TFAAC.

CONCLUSIONS

Based on the investigation of the essential oils in *Z. clinopodioides* Lam., the contents during the growth stage is different. However, in all stages, the content accounts for 1% or above. The common dates were very rich in pulegone, especially at the flowering stage. Thus, the best harvesting time for the essential oils of *Z. clinopodioides* is during the flowering stage.

Z. clinopodioides Lam. is rich in total phenolics, total flavonoids and total free amino acids. Both TFC and TFAAC significantly varied in the growth process, whereas TPC was relatively stable. TTC was low in these varieties and similar in all stages.

The differences in the content of chemical components may be attributed to the different harvest dates. Growth time also affects the change in chemical constituents in the plants. The influence of growth phase on the essential oil composition of *Z. clinopodioides* Lam. in Iran has been previously investigated Amiri 2009.^[16] However, the present study is the first to determine simultaneously the volatile and non-volatile components of *Z. clinopodioides* Lam. in

Xingjiang, China at different growth stages. This method can also be used to monitor and rapidly predict the quality of *Z. clinopodioides* Lam. to decide the optimal harvest date.

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