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GC-MS Analysis and Bioactivity Screening of Leaves and Fruits of *Zanthoxylum armatum* DC.

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Abstract: *Zanthoxylum armatum* DC. is a plant that has been homologated for medicine and food by the Chinese for three thousand years. In this study, the essential oils of fresh leaves and fruits were extracted by hydrodistillation, the aromas of fresh leaves and fruits were extracted by headspace solid-phase microextraction and their chemical compositions were analyzed by gas chromatography mass spectrometry. The main components of the leaf essential oils were linalool (62.01%), 2-undecanone (9.83%) and 2-tridecanone (5.47%); the fruit essential oils were linalool (72.17%), limonene (8.05%) and sabinene (6.77%); the leaf aromas were limonene (39.15%), β-myrcene (15.8%), sabinene (8.17%) and linalool (5.25%); the fruit aromas were limonene (28.43%), sabinene (13.56%), linalool (11.47%) and β-myrcene (8.64%). By comparison, it was found that the composition of leaf essential oils and fruit essential oils were dominated by oxygenated monoterpenes, while the composition of their aromas were both dominated by monoterpenes; the relative content of non-terpene components in leaf essential oil and leaf aroma is second only to oxygenated monoterpenes, while their content in fruits is low; the chemical composition of leaf aromas and fruit aromas were richer than those of essential oils. In this study, we reported for the first time that the antitumor, tyrosinase inhibition, HMGR inhibition and nitric oxide production inhibition activity of leaf essential oils were stronger than those of fruit essential oils in in vitro tests. The results of the study can provide a reference for the recycling and green low-carbon transformation of the leaves, and also help to deepen the understanding of the value of the volatile chemical constituents of this plant in "forest medicine" or "aromatherapy", and provide new ideas for the transformation of the value of the plant in the secondary and tertiary industry chain.

Keywords: *Zanthoxylum armatum* DC.; essential oil; aroma; chemical composition; biological activity

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1. Introduction

Zanthoxylum armatum DC. (*Z. armatum*) is a small tree or shrub belonging to the genus *Zanthoxylum* in the family Rutaceae. It is also known as "green Sichuan pepper" because the peel of the fruit remains dark green until it is about nine minutes ripe, and is grayish green when dried [1]. It has been found from India in South Asia to the countries of Sout–East Asia, to the Korean Peninsula and Japan in East Asia. It is known as "Tejphal" "Tumru" "Trimal", etc. in India and "Timu" in Nepal [2]. The extract of *Z. armatum* has various medicinal functions, such as hepatoprotective effect [3], antidiabetic activity [3], insecticidal activity [4], antibacterial and antioxidant activity [5], antispasmodic effect [6], anticancer activity [7], memory enhancing property [8], antidepressant activity [9], neurological efficiency and other effects [10]. Based on its wide applicability and high commercial value, *Z. armatum* has been considered as a socio economically potential species by scholars in Nepal and India [11,12].

The aromas and essential oils of plants are secondary metabolites produced by the isoprene pathway, and are mainly a mixture of terpenoids and their oxygenated derivatives, stored in specialized epidermal oil glands [13]. The aromas come from the natural emanation of the plant, such as friction between leaves, physical damage to the plant body, etc., in a gaseous state. Essential oils are natural products extracted by artificial means, such as distillation or solvent extraction, and are in liquid form. "Forest medicine" research has found that plant aroma enters the body through the respiratory tract and human epidermis, and is absorbed by the body to improve its own regulation and immunity, such as increasing anti-cancer immune function, reducing stress hormone levels, reducing sympathetic nerve activity and increasing parasympathetic nerve activity [14]. Therefore, "forest therapy" or "forest bathing" using plant aromas has been recognized by the governments of Japan, Germany, Korea and China among health management or preventive medicine therapies. Plant essential oils have diverse biological activities and therefore have a wide range of uses in the fields of daily chemicals, healthcare, pest control and the food industry. Especially in the field of "aromatherapy", they are often used to improve human mental or physical health. The *Z. armatum* leaf essential oil (ZLO) has antimicrobial and antioxidant activity [15,16], antinociceptive and anticonvulsant activity [17], sedative–hypnotic, anxiolytic and muscle relaxant properties [18], anti-inflammatory and analgesic activity [19], and antispasmodic effects [20]. The *Z. armatum* fruit essential oil (ZFO) has antifungal, antibacterial and antioxidant activities [21–24].

Humans have been using the essential oils and aromas of plants for thousands of years for disinfection, antisepsis and curing diseases, and *Z. armatum* has been a famous homology of medicine and food plant of the Chinese people for more than 3000 years. The earliest Chinese poetry collection "Shi jing" praised the fragrance of *Z. armatum* for its ability to bring peace to many elderly people. "Lady Dais" buried in the ancient tomb of Mawangdui in Hunan Province, China (around 186 B.C.), held in her hands spice sachets filled with *Z. armatum*. In addition, several herbal bags containing *Z. armatum* fruits were also found in the tomb. Researchers believed that it was because the tomb owner believed in the medicinal value of the spice and its effects on the human body; she stored *Z. armatum* in sachets when she was not sick in order to tonify her body while enjoying it by smelling its slowly releasing fragrance [25]. To date, China is the world's largest cultivator of "green Sichuan pepper" (nearly 6667 km^3 planted) and the largest producer of the fruit (about 270,000 tons per year), but its purpose is to produce dried fruit pellets or extract fruit oil. These primary agricultural products are generally used as a spicy food seasoning in Chinese cuisine or as a traditional Chinese medicine for rheumatoid arthritis, vomiting and diarrhea, and to repel insects and relieve itching, etc. There are still many research gaps in their high-value utilization.

Based on this, this study focused on the extraction of the essential oils from freshly collected leaves and fruits of *Z. armatum* using hydrodistillation, sampling their aroma using headspace solid-phase microextraction, and analyzing the chemical composition of both samples by gas chromatography mass spectrometry (GC-MS). Meanwhile, the leaf and fruit essential oils were tested for the first time for their in vitro antitumor activity, tyrosinase inhibition activity, HMGR inhibition activity and NO production inhibition activity to explore the potential of their aromatic volatiles for healthcare development, with a view to break through the existing utilization and provide a reference for the high-value transformation of this plant resource.

2. Materials and Methods

2.1. Plant Materials

Plant materials were obtained from the forestry trial base of Sichuan Agricultural University, Chongzhou City, Sichuan Province, China (103°38′23″ E, 30°35′40″ N). Samples were collected from 3-year-old *Zanthoxylum armatum* 'Hanyuanputaoqing' (No.: Chuan S-SV-ZA-002-2018), an improved variety of *Z. armatum* (Figure 1). The samples were stored in the herbarium of the College of Forestry, Sichuan Agricultural University (China). When

Z. armatum was fully ripe, i.e., early August in summer, healthy and undamaged leaves and fruits from the upper, middle and lower parts of the sunny side of the plant were picked on a sunny day and put into sample bottles, and brought back to the laboratory immediately to extract the chemical constituents.



Figure 1. *Z. armatum* cultivated by Sichuan Agricultural University (photo Jie Ma).

2.2. Chemicals and Reagents

The mouse mononuclear macrophage cell line RAW264.7 was purchased from Shanghai Cell Bank of Chinese Academy of Sciences (Shanghai, China). DMEM medium and fetal bovine serum were purchased from Biological Industries, Kibbutz Beit Haemek, Israel. Griess reagent, lipopolysaccharide (LPS), nitric oxide synthase inhibitor (L-NMMA), mushroom tyrosinase, levodopa (L-Dopa), kojic acid and HMG-CoA reductase assay kits were purchased from Sigma-Aldrich Co. (St Louis, MO, USA). Leukemia cell line HL-60, colon cancer cell line SW-480, lung adenocarcinoma cell line A-549, liver cancer SMMC-7721 cell line, breast cancer MCF-7, cisplatin (DDP) and paclitaxel (Taxol) were purchased from Kunming Institute of Botany, Chinese Academy of Sciences (Kunming, China).

2.3. Experiment Apparatus

SPME fiber (100 μm) divinylbenzene-carboxen-polydimethylsiloxane (DVB-CAR-PDMS) from Supelco, Bellefonte, PA, USA, was used in this study. The Clevenger apparatus produced by Sichuan Shubo (Group) Co., Ltd., Chongzhou, China was adopted. The Agilent Model 7890A-5975C GC-MS was from Agilent, Santa Clara, CA, USA. The Multiskan FC microplate photometer was from Thermo Fisher Scientific, Waltham, MA, USA. The Flex Station 3 multifunctional enzyme labeler was from Molecular Devices, San Jose, CA, USA. The XS105 electronic balance was from Mettler Toledo, Zurich, Switzerland.

2.4. Extraction of Essential Oil

Fresh leaves of *Z. armatum* were crushed (1000 ± 0.1 g), fresh fruits were taken whole (1000 ± 0.1 g), added to distilled water at a solid-liquid ratio of approximately 1:4 respectively and distilled in a Clevenger-type distillation unit for 3 h. After the device was cooled to room temperature, the essential oil was dried with anhydrous sodium sulfate and stored in a 4 °C environment away from light.

2.5. Extraction of Aroma

Fresh leaves (3 ± 0.1 g) and fresh fruits (3 ± 0.1 g) of *Z. armatum* were cut and sealed in the extraction vials, respectively. The SPME handle fiber head was inserted into the bottle and placed about 0.5 cm directly above the sample, and the aroma was extracted at 50 °C for 40 min. Then the fiber head was withdrawn and inserted into the GC inlet of GC-MS and desorbed for 10 min.

2.6. GC-MS Detection

Essential oil gas chromatographic conditions: The column used for the essential oil analysis was an Agilent HP-5ms column (30 m × 0.25 mm × 0.25 μm). The initial temperature of 40 °C was maintained for 2 min, then ramped up to 180 °C at 5 °C/min and held for 2 min, followed by a ramp up to 240 °C at 5 °C/min and held for 8 min. The injection port temperature was 250 °C, the injection volume was 1 μL, and the splitting ratio was 50:1. The carrier gas was high-purity helium at a flow rate of 1 mL/min.

Aroma gas chromatography conditions: The column used for aroma analysis was an Agilent HP-5ms column (30 m × 0.25 mm × 0.25 μm). The initial temperature was 50 °C for 4 min, followed by a ramp-up to 150 °C at 6 °C/min for 2 min, and then to 250 °C at 7 °C/min for 8 min. The injection port temperature was 250 °C, the injection volume was 1 μL without splitting. The carrier gas was high purity helium at a flow rate of 1 mL/min.

Mass spectrometry conditions: Electron ionization was used with an ionization energy of 70 eV, transmission line temperature of 250 °C, ion source temperature of 230 °C, and scan range of 35–550 m/z. The plots of the mass spectra were obtained by scanning each mass spectrum of the total ion flow. The chemical composition was finally determined by software search of the NIST14.L library (Version 2014) and review of relevant literature. The relative content of each component was measured by the peak area normalization method [26].

2.7. Preliminary Screening of Antitumor Activity In Vitro

The leukemia HL-60 cell line, colon cancer SW-480 cell line, lung cancer A-549 cell line, hepatocellular carcinoma SMMC-7721 cell line and breast cancer MCF-7 cell line were prepared 12~24 h in advance as single cell suspensions in DMEM culture containing 10% fetal bovine serum. Then, the cells were inoculated into 96-well plates at 3000~15,000 cells per well in a volume of 100 μL per well. The essential oil was dissolved with DMSO to 100 μg/mL in a final volume of 200 μL per well, and three replicate wells were set up for each treatment.

After incubation at 37 °C for 48 h, the culture supernate was removed from the wells with adherent cells, followed by the addition of 20 μL of MTS solution and 100 μL of DMEM solution to each well; the culture supernate was removed from the wells with suspension cells, followed by the addition of 20 μL of MTS solution to each well. The incubation was continued for 2~4 h to allow the reaction to proceed adequately. Subsequently, the light absorption values of each well at 492 nm were measured by a microplate photometer and the results were recorded. DDP and Taxol were set as positive controls. The detection concentration of DDP was 40 μM, the detection concentration of Taxol was 5 μM, and the detection concentration of essential oil samples were all 100 μg/mL. After data processing, the inhibition rate of the five cell lines was plotted with essential oil as the horizontal coordinate and cell inhibition rate as the vertical coordinate.

2.8. Primary Screening of Tyrosinase Inhibition Activity In Vitro

The samples were mixed with L-Dopa and tyrosinase was added (final concentration 25 U/mL). Three replicate wells were set up and the reaction was carried out at room temperature for 5 min. Then, the optical density (OD) value was measured by the instrument with a detection wavelength of 490 nm. A blank control without samples was set up, as well as Kojic Acid as a positive control. Kojic Acid was detected at a concentration of

10 μg/mL and the essential oil samples were detected at a concentration of 100 μg/mL. The formula of inhibition rate was calculated as follows:

$$\text{Inhibition rate (\%)} = (1 - \text{sample OD}_{490\text{ nm}}/\text{blank OD}_{490\text{ nm}}) \times 100\% \quad (1)$$

2.9. Primary Screening of HMGR Inhibition Activity In Vitro

The samples were added to a 96-well ELISA plate with NADPH (final concentration 0.33 μM), substrate HMG-CoA and HMGR (final concentration 0.05 mg/mL–0.07 mg/mL). Mix thoroughly and set three replicate wells, along with a blank control without sample, and Pravastatin as a positive control. Pravastatin was detected at a concentration of 0.25 μM and the essential oil samples were all detected at 200 μg/mL. After incubation at 37 °C for 10 min, the OD value was measured by the instrument at 515 nm and the results were recorded. The formula was calculated as follows:

$$\text{Inhibition rate (\%)} = (1 - \text{sample OD}_{515\text{ nm}}/\text{blank OD}_{515\text{ nm}}) \times 100\% \quad (2)$$

2.10. Primary Screening of Nitric Oxide (NO) Production Inhibition Activity In Vitro

RAW264.7 cells were inoculated into 96-well plates and stimulated with 1 μg/mL LPS for induction, while samples were added (final concentration 50 μg/mL). A blank control without samples was set, as well as L-NMMA as a positive control. After the cells were cultured overnight, the medium was taken to detect NO production and the OD value at 570 nm was measured by the instrument. Cell viability assay was performed by adding MTS to the remaining medium to exclude the toxic effects of the compounds on the cells. L-NMMA was detected at a concentration of 50 μM and the essential oil samples were all detected at a concentration of 50 μg/mL. The formula was calculated as follows:

$$\text{Inhibition rate (\%)} = (1 - \text{sample OD}_{570\text{ nm}}/\text{blank OD}_{570\text{ nm}}) \times 100\% \quad (3)$$

3. Results

3.1. The Main Chemical Composition of Essential Oils and Aromas from Leaves and Fruits

As in Table 1, the main chemical components of the essential oils and aromas with a relative content greater than 0.1% were collated, and the compounds are listed in the order of elution. The ZLO and ZFO identified 29 and 24 components, respectively, with relative contents of 97.32% and 98.84% of the total essential oils. The components of the ZLO with relatively high content included linalool (62.01%), 2-undecanone (9.83%), 2-tridecanone (5.47%), terpinen-4-ol (3.61%), limonene (3.75%), α-terpineol (2.27%) and β-caryophyllene (2.02%). The components with higher relative content in ZFO included linalool (72.17%), limonene (8.05%), sabinene (6.77%), terpinen-4-ol (3.41%) and β-myrcene (1.25%). Leaf aroma of *Z. armatum* (ZLA) and fruit aroma of *Z. armatum* (ZFA) identified 48 and 45 components with relative contents of 98.91% and 97.7% of the total aroma, respectively. The components with relatively high content in ZLA included limonene (39.15%), β-myrcene (15.8%), sabinene (8.17%), linalool (5.25%), cis-hex-3-en-1-ol (2.98%), α-thujene (2.94%), trans-β-ocimene (2.63%), γ-terpinene (2.45%) and leaf acetate (2.11%). The components with relatively high content in ZFA included limonene (28.43%), sabinene (13.56%), linalool (11.47%), β-myrcene (8.64%), β-caryophyllene (4.91%), γ-selinene (3.72%), (-)- beta-elemene (3.46%), trans-β-ocimene (3.29%), germacrene D (3.11%), α-thujene (2.17%) and γ-terpinene (1.42%). Of these, the chemical structures of the major chemical components with relative contents greater than 5% are shown in Figure 2.

Table 1. Chemical Composition of essential oil and Aroma from leaves and fruits of *Z. armatum*.

No.	Compound Name	Chemical Formula	Essential Oil			Aroma		
			Retention Time (min)	Fruits Area (%)	Leaves Area (%)	Retention Time (min)	Fruits Area (%)	Leaves Area (%)
1	1,3-Hexadiene	C_6H_{10}	ND	ND	ND	2.523	ND	0.14
2	2-Ethylfuran	C_6H_8O	ND	ND	ND	3.205	ND	0.37
3	3-Hexenal	$C_6H_{10}O$	ND	ND	ND	5.096	0.64	0.97
4	2-Hexenal	$C_6H_{10}O$	ND	ND	ND	6.363	0.33	ND
5	3-Hexen-1-ol	$C_6H_{12}O$	ND	ND	ND	6.63	0.43	ND
6	cis-Hex-3-en-1-ol	$C_6H_{12}O$	ND	ND	ND	6.667	ND	2.98
7	1,2-Xylene	C_8H_{10}	ND	ND	ND	7.047	ND	0.33
8	Phenylethylene	C_8H_8	ND	ND	ND	7.047	ND	0.8
9	α-Thujene	$C_{10}H_{16}$	5.282	0.08	ND	8.817	2.17	2.94
10	α-Pinene	$C_{10}H_{16}$	6.292	0.2	ND	9.019	1.01	0.17
11	Sabinene	$C_{10}H_{16}$	7.253	6.77	0.55	10.313	13.56	8.17
12	β-Pinene	$C_{10}H_{16}$	7.347	0.28	ND	ND	ND	ND
13	β-Myrcene	$C_{10}H_{16}$	7.651	1.25	0.4	10.944	8.64	15.8
14	α-Phellandrene	$C_{10}H_{16}$	ND	ND	ND	11.284	0.43	1.11
15	Leaf acetate	$C_8H_{14}O_2$	ND	ND	ND	11.389	0.19	2.11
16	Octanal	$C_8H_{16}O$	7.948	0.13	ND	ND	ND	ND
17	α-Terpinene	$C_{10}H_{16}$	8.352	0.53	ND	11.683	0.99	1.94
18	Limonene	$C_{10}H_{16}$	8.685	8.05	3.75	12.225	28.43	39.15
19	trans-Ocimene	$C_{10}H_{16}$	ND	ND	ND	12.362	0.27	0.3
20	trans-β-Ocimene	$C_{10}H_{16}$	9.17	0.18	ND	12.691	3.29	2.63
21	γ-Terpinene	$C_{10}H_{16}$	9.487	0.83	0.21	13.016	1.42	2.45
22	trans-4-Thujanol	$C_{10}H_{18}O$	9.724	0.82	ND	13.262	0.14	ND
23	Terpinolene	$C_{10}H_{16}$	10.309	0.25	ND	13.921	0.98	1.51
24	2,4-Dimethylstyrene	$C_{10}H_{12}$	ND	ND	ND	14.027	ND	0.1
25	Linalool	$C_{10}H_{18}O$	10.8	72.17	62.01	14.027	11.47	5.25
26	β-Thujone	$C_{10}H_{16}O$	10.898	0.96	ND	14.842	0.07	ND
27	neo-Alloocimene	$C_{10}H_{16}$	ND	ND	ND	15.165	0.18	0.38
28	β-Ocimene	$C_{10}H_{16}$	ND	ND	ND	15.527	0.19	ND
29	α-Thujone	$C_{10}H_{16}O$	11.168	0.6	ND	ND	ND	ND
30	trans-p-Menth-2-en-1-ol	$C_{10}H_{18}O$	11.279	0.22	0.18	ND	ND	ND
31	cis-p-Menth-2-en-1-ol	$C_{10}H_{18}O$	11.749	0.15	0.18	ND	ND	ND
32	Citronellal	$C_{10}H_{18}O$	12.09	0.12	0.26	ND	ND	ND
33	Terpinen-4-ol	$C_{10}H_{18}O$	12.82	3.41	3.61	16.608	0.06	0.12
34	cis-3-Hexenyl butyrate	$C_{10}H_{18}O_2$	ND	ND	ND	16.849	0.04	0.14
35	α-Terpineol	$C_{10}H_{18}O$	13.17	0.97	2.27	16.996	0.14	0.07
36	(-)-Myrtenal	$C_{10}H_{14}O$	13.354	0.41	ND	ND	ND	ND
37	Piperitone	$C_{10}H_{16}O$	ND	ND	ND	18.827	0.12	ND
38	Nerol	$C_{10}H_{18}O$	14.159	0.05	0.4	ND	ND	ND
39	Citronellol	$C_{10}H_{20}O$	ND	ND	ND	18.057	ND	0.21
40	cis-3-Hexenyl 2-methylbutanoate	$C_{11}H_{20}O_2$	ND	ND	ND	18.171	ND	0.13
41	Linalyl acetate	$C_{12}H_{20}O_2$	14.93	ND	0.19	18.833	ND	0.29
42	Geraniol	$C_{10}H_{18}O$	14.86	0.07	0.46	ND	ND	ND
43	Phenethyl acetate	$C_{10}H_{12}O_2$	14.952	0.34	ND	ND	ND	ND
44	2-Undecanone	$C_{11}H_{22}O$	15.951	ND	9.83	19.876	0.07	0.51
45	γ-Pyrone	$C_5H_4O_2$	ND	ND	ND	21.13	0.36	0.1
46	2,6-Dimethyl-2,6-octadiene	$C_{10}H_{18}$	ND	ND	ND	21.464	ND	0.11
47	(-)-α-Cubebene	$C_{15}H_{24}$	ND	ND	ND	21.455	0.13	ND
48	(-)-α-Copaene	$C_{15}H_{24}$	ND	ND	ND	22.172	0.15	0.06
49	(-)-β-Elemene	$C_{15}H_{24}$	22.99	ND	0.72	22.584	3.46	0.92
50	Tetradecane	$C_{14}H_{30}$	ND	ND	ND	22.675	0.19	0.21
51	β-Caryophyllene	$C_{15}H_{24}$	23.81	ND	2.02	23.335	4.91	1.79
52	β-Cubebene	$C_{15}H_{24}$	ND	ND	ND	22.695	0.19	ND
53	(-)-γ-Elemene	$C_{15}H_{24}$	ND	ND	ND	23.635	0.1	0.05
54	(+)-Aromandendrene	$C_{15}H_{24}$	ND	ND	ND	23.819	0.29	0.11
55	α-Caryophyllene	$C_{15}H_{24}$	24.72	ND	0.57	24.187	1.47	0.49
56	Decanohydrazide	$C_{10}H_{22}N_2O$	25.1	ND	0.34	ND	ND	ND
57	β-Ionone	$C_{13}H_{20}O$	25.25	ND	0.57	ND	ND	ND
58	(-)-Isoledene	$C_{15}H_{24}$	ND	ND	ND	24.359	0.16	0.08
59	Bicyclosesquiphellandrene	$C_{15}H_{24}$	ND	ND	ND	24.413	0.19	0.06
60	γ-Muurolene	$C_{15}H_{24}$	ND	ND	ND	24.726	0.68	0.3
61	Germacrene D	$C_{15}H_{24}$	25.36	ND	0.51	24.867	3.11	0.37
62	β-Eudesmene	$C_{15}H_{24}$	ND	ND	ND	25.006	1.59	0.55
63	2-Tridecanone	$C_{13}H_{26}O$	25.63	ND	5.47	25.09	ND	0.29
64	γ-Selinene	$C_{15}H_{24}$	ND	ND	ND	25.219	3.72	1.34
65	α-Farnesene	$C_{15}H_{24}$	25.84	ND	0.25	ND	ND	ND
66	Elemicin	$C_{12}H_{16}O_3$	26.88	ND	0.12	ND	ND	ND
67	cis-Nerolidol	$C_{15}H_{26}O$	27.23	ND	0.93	ND	ND	ND
68	Caryophyllene Oxide	$C_{15}H_{24}O$	27.84	ND	0.36	ND	ND	ND
69	(-)-α-Amorphene	$C_{15}H_{24}$	ND	ND	ND	25.655	0.52	0.23
70	δ-Cadinene	$C_{15}H_{24}$	ND	ND	ND	25.871	0.92	ND
71	cis -Calamenene	$C_{15}H_{22}$	ND	ND	ND	25.862	ND	0.54
72	α-Cadinene	$C_{15}H_{24}$	ND	ND	ND	26.208	0.23	0.1

Table 1. *Cont.*

No.	Compound Name	Chemical Formula	Essential Oil			Aroma		
			Retention Time (min)	Fruits Area (%)	Leaves Area (%)	Retention Time (min)	Fruits Area (%)	Leaves Area (%)
73	Hexadecanal	$C_{16}H_{32}O$	ND	ND	ND	27.525	0.07	0.14
74	cis-9-Octadecenal	$C_{18}H_{34}O$	37.87	ND	0.16	ND	ND	ND
75	9,17-Octadecadienal, (Z)-	$C_{18}H_{32}O$	ND	ND	0.14	ND	ND	ND
76	Oleic alcohol	$C_{18}H_{36}O$	39.24	ND	0.18	ND	ND	ND
77	Phytol	$C_{20}H_{40}O$	40.13	ND	0.68	ND	ND	ND
Grouped compounds								
Monoterpene hydrocarbons				18.42	4.91		61.56	76.55
Oxygenated monoterpenes				79.95	69.37		12.04	5.79
Sesquiterpene hydrocarbons					4.07		21.82	6.99
Oxygenated sesquiterpenes					1.29			
Others				0.47	17.68		2.28	9.58
Total percentage of identified components				98.84	97.32		97.7	98.91

ND: not detected.

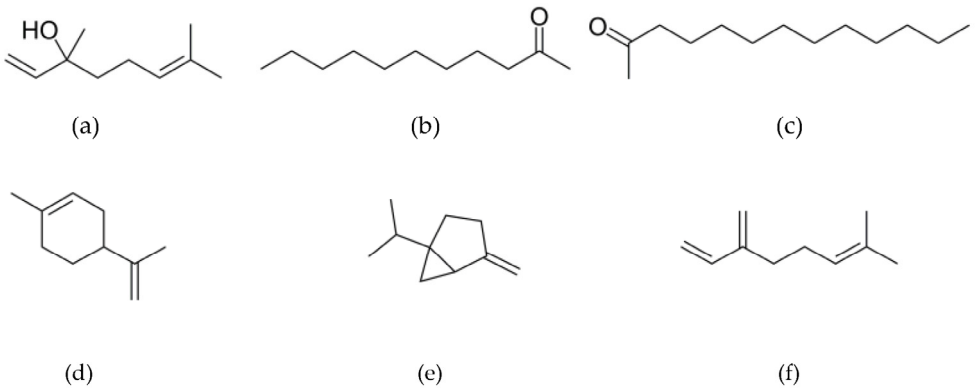


Figure 2. Chemical structures of the main chemical components in ZLO, ZFO, ZLA and ZFA. (**a**) Linalool. (**b**) 2-Undecanone. (**c**) 2-Tridecanone. (**d**) Limonene. (**e**) Sabinene. (**f**) β-Myrcene.

The chemical compositions of ZLA and ZFA were more complex than ZLO and ZFO in terms of the number of major compounds measured. This may be due to the fact that the essential oil extraction technique was hydrodistillation and some water-soluble compounds were not detected. The aroma extraction was performed by headspace solid-phase microextraction, which better presents the plant in a more natural state at a lower extraction temperature, thus detecting a more diverse range of trace components. In addition, leaves and fruits, as different organs, have different physiological and biochemical functions, and their effects on secondary metabolites are expressed in terms of compound species. Both ZLO (17.68%) and ZLA (9.58%) were detected in high amounts of non-terpenoid components, which were present in very small amounts in ZFO (0.47%) and ZFA (2.28%) Even for the same organ, there are many differences in the representative volatile components presented in gaseous and liquid forms. The ZLO (69.37%) and ZFO (79.95%) components were dominated by oxygenated monoterpenes, with linalool accounting for the absolute majority. The ZLA (76.55%) and ZFA (61.56%) components were dominated by monoterpenes, represented by limonene, β-myrcene and sabinene.

The main component, limonene, has a fresh lemon fragrance and has neuroprotective [27], anti-anxiety [28], anticancer [29] and immunomodulatory activities [30], as well as anti-inflammatory activity to prevent and control respiratory system damage [31]. The fragrance of β-myrcene is balsamic, with anti-tumor activity [32], anti-asthma [33] and whitening effects [34]. Sabinene has a woody and creamy fragrance, and can prevent skeletal muscle atrophy in rats [35]. Linalool has a sweet and fresh floral fragrance with anticancer activity [36], hepatoprotective activity [37], anti-inflammatory, analgesic [38] and hypocholesterolemic activities [34]. They are all edible spices, so there is great potential for the development of health and wellness products from *Z. armatum* essential oils and aromas. In particular, the health and service values in "forest therapy" and "aromatherapy" have

not been translated, and the effects of *Z. armatum* on human respiratory system diseases, immune function and neurological activity can be further investigated subsequently by forest medicine experiments. Based on the biological activities exhibited by the main components of the essential oils, the following is a preliminary screening of the in vitro antitumor, whitening, hypocholesterolemic and anti-inflammatory activities of ZLO and ZFO, with a view to providing a reference for the high-value utilization of *Z. armatum*.

3.2. In Vitro Bioactivity of ZLO and ZFO

From Figure 3, it can be seen that the two essential oils had different effects on cancer cell proliferation inhibition at 100 μg/mL concentration. ZLO inhibited the leukemia HL-60 cell line, colon cancer SW-480 cell line, lung cancer A-549 cell line and liver cancer SMMC-7721 cell line better than ZFO. Both essential oils showed no inhibitory effect on the MCF-7 cell line of breast cancer. Among them, ZLO inhibited leukemia HL-60 cell line up to 93.43% and its extremely strong antitumor activity deserves to be focused on. Several studies had shown that the in vitro cytotoxicity of essential oils on leukemic HL-60 cell lines is mediated by a mitochondria-dependent apoptosis. That is, by inducing the release of mitochondrial cytochrome c protein into the cytoplasm, which activates the caspase cascade and ultimately leads to apoptosis [39–41]. The apoptotic activities of the essential oils of *Citrus aurantium* var. *dulcis* (sweet orange), *Citrus paradisi* (grapefruit) and *Citrus limon* (lemon) on leukemia HL-60 cell lines were closely related to the limonene in the essential oils [42]. This is due to the ability of limonene to increase the expression of the apoptosis-promoting gene Bax in cancer cells, release cytochrome c, and activate the caspase pathway [29]. In addition, linalool also showed inhibitory effects on cell proliferation and apoptosis induction in the leukemia HL-60 cell line [43].

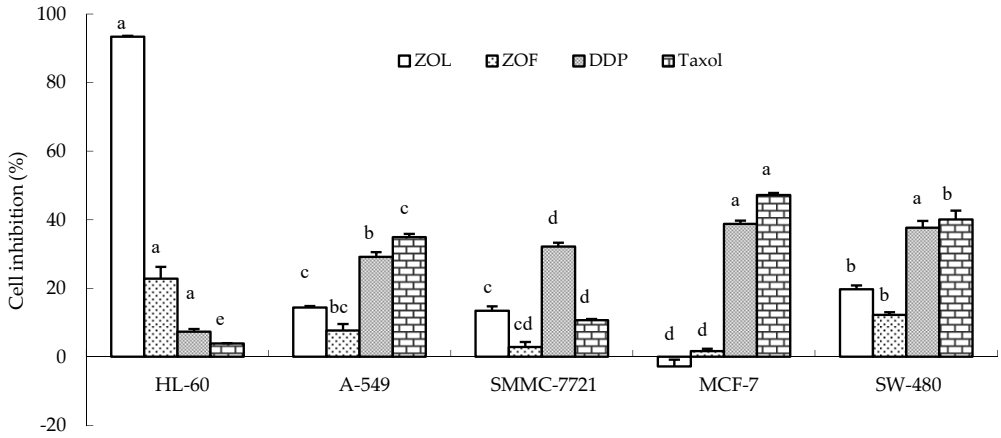


Figure 3. Different effects on cancer cell proliferation inhibition from ZOL and ZOF at 100 μg/mL concentration. Differences between them are not significant if they have a letter with the same label. Differences are significant if they have different letters ($p \leq 0.05$).

Melanin biosynthesis is regulated to a large extent by tyrosinase. This enzyme converts tyrosine into dopa pigment, which then undergoes a series of steps to become melanin. If the tyrosinase enzyme is more active in the body, it will lead to the production of more melanin, which will manifest as dull skin and visible pigmentation. Therefore, one of the main ways that skin whitening products on the market work is by inhibiting tyrosinase activity to solve the problem of hyperpigmentation [44]. Plant essential oils can effectively inhibit the oxidation of L-Dopa catalyzed by tyrosinase by disrupting the tertiary structure of the enzyme [45,46]. Significant differences in the in vitro studies have been identified in Figure 4. From Figure 4(1), it can be seen that ZLO and ZFO showed mild in vitro tyrosinase inhibitory activity at 100 μg/mL concentration, with 30.66 ± 1.26% and 15.15 ± 0.91% inhibition, respectively. This may be related to the presence of β-myrcene in the essential oils. A study of the activity of 13 citrus essential oil components revealed that myrcene is a competitive inhibitor of tyrosinase [34]. The presence of β-myrcene in the

essential oils of *Litsea cubeba* (Lour.) Pers and *Verbena officinalis* L. contributes to the overall activity of tyrosinase inhibition [47].

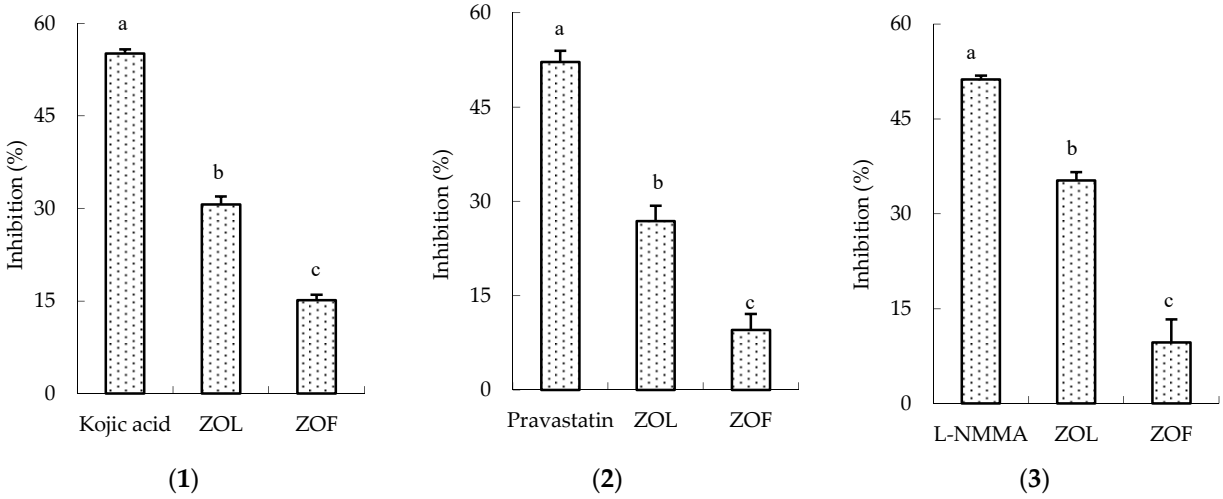


Figure 4. In vitro tyrosinase inhibition (**1**), HMGR inhibition in vitro (**2**) and inhibition of NO production in vitro (**3**) by ZLO and ZFO. If the letters are the same between groups, it means they are not significantly different. If the letters are different, it means that the differences are significant ($p \leq 0.05$).

HMGR, a transmembrane glycoprotein located in the endoplasmic reticulum, is a rate-limiting enzyme in the synthesis of cholesterol in hepatocytes. Inhibition of HMGR prevents cholesterol synthesis and is therefore the main target of cholesterol-lowering drugs [48]. As shown in Figure 4(2), ZLO showed stronger HMGR inhibition than ZFO at a concentration of 200 μg/mL, with an inhibition rate of 26.89 ± 2.42% for ZLO and 9.54 ± 2.56% for ZFO. This is attributed to the natural monoterpenes and sesquiterpenes in the essential oil, such as β-caryophyllene, geraniol, limonene and linalool. They all lead to reduced transcription and accelerated degradation of HMGR, thus improving blood cholesterol levels or sterol metabolic pathways [49].

NO has a wide range of biological regulatory functions. When immune cells are stimulated by microbial endotoxins and inflammatory mediators, they generate large amounts of inducible nitric oxide synthase (iNOS) and produce NO for immune response. Therefore, inhibition of NO production is a direct indicator of the anti-inflammatory activity of the compound [50,51]. From Figure 4(3), it can be seen that at 50 μg/mL concentration, ZLO inhibited the production of NO more than ZFO, 35.24 ± 1.32% for ZLO and 9.66 ± 3.65% for ZFO. Studies on the anti-inflammatory mechanism of *Zanthoxylum schinifolium* essential oil revealed that it inhibited the gene transcription of the iNOS and cyclooxygenase-2 (COX-2) from LPS-induced RAW 264.7 cells [52]. In addition, ZLO showed significant anti-inflammatory and analgesic activity against paw edema in rats [19]. The main component, linalool, played a notable role in this regard, as it not only inhibited NO formation in the in vitro assay, but also inhibited COX-2 overexpression in the mouse assay [38,53].

4. Conclusions

In this study, the essential oil and aroma components of fresh leaves and fruits of *Z. armatum* from China were analyzed by GC-MS. It was found that there were large numbers of non-terpenoid components in the leaves, but their content in the fruits was very small; the essential oil components were dominated by oxygenated monoterpenes, and the aroma components were dominated by monoterpenes; the aroma chemical components were more abundant than the essential oil. The main volatile chemical components represented by linalool, limonene, β-myrcene and sabinene, are not only used as flavor

additives in food, but also have a wide range of biological activities. Therefore, *Z. armatum* has great potential for development in "forest medicine" and "aromatherapy", etc.

Furthermore, ZLO showed better in vitro antitumor, whitening, hypocholesterolemic and anti-inflammatory activities than ZFO in screening tests, which is the first report in the study of this species. In particular, at a concentration of 100 μg/mL, ZLO exhibited extremely strong antitumor activity against the leukemia HL-60 cell line, with an inhibition of 93.43%. Whether the ZLO can be utilized in adjuvant therapy for leukemia, requires in-depth studies in combination with concentration gradients and toxicological animal tests to better evaluate the observations. In production practice, only *Z. armatum* fruits are currently used as agricultural products for primary use, while the leaves are disposed of as waste by incineration. This study may provide a reference for leaf recycling and green low-carbon conversion [54–56].

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Conflicts of Interest: The authors declare no conflict of interest.

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