

Insecticidal Activities and Phenological Variations of Characteristic Component from *Michelia yunnanensis*

Yi-Xi Feng^{1,2}, Xin-Xin Lu¹, Jia-Wei Zhang¹, Yue-Shen Du¹, Yu Zheng¹, and Shu-Shan Du^{1*}

¹ Beijing Key Laboratory of Traditional Chinese Medicine Protection and Utilization, Faculty of Geographical Science, Beijing Normal University, Haidian District, Beijing 100875, People's Republic of CHINA

² Qujing No.1 Middle School, Qujing City, Yunnan Province, 650000, People's Republic of CHINA

Abstract: Volatile secondary metabolites of plants interact with environments heavily. In this work, characteristic components of *Michelia yunnanensis* essential oils (EOs) were isolated, purified and identified by column chromatography, GC-MS and NMR. Leaves of *M. yunnanensis* were collected monthly and extracted for EOs to investigate chemical and insecticidal activity variations as well as potential influencing environments. Different organs were employed to reveal distribution strategies of characteristic components. Results of insecticidal activities showed that all EOs samples exerted stronger contact activity to *Lasioderma serricorne*, but repellent effect was more efficient on *Tribolium castaneum*. One oxygenated sesquiterpene was isolated from EOs, basically it could be confirmed as (+)-cyclocolorenone (1). It exerted contact toxicity to *L. serricorne* ($LD_{50} = 28.8 \mu\text{g/adult}$). Chemical analysis showed that *M. yunnanensis* leaves in reproductive period would produce and accumulate more 1 than in vegetative period. Moreover, reproductive organs (flowers and fruits) contained more 1 than vegetative organs (leaves and twigs). Partial correlation analysis indicated that temperature-related elements positively correlated with the relative content of 1.

Key words: contact activity, repellent effect, *Tribolium castaneum* Herbst, *Lasioderma serricorne* Fabricius

1 Introduction

Storage products including grains, tea, tobaccos and medical herbs play great importance in modern society. They are not only consumed locally but also traded worldwide. During transportation, these products are easily contaminated by insect pests. Therefore, quality safety of storage products has long been a serious issue¹. Between countries, strict rules and regulations have been proposed and agreed for quality control, and more importantly for management of insects and bacteria. Even so, insect pests are still a big concern because of their adaptability and fecundity. *Tribolium castaneum* Herbst (Coleoptera: Tenebrionidae), *Lasioderma serricorne* Fabricius (Coleoptera: Anobiidae), *Liposcelis bostrychophila* Badonnel (Psocoptera: Liposcelididae) and *Sitophilus oryzae* L. (Coleoptera: Curculionidae) are reported as destructive stored-product insects, they usually cause extensive harms to these products^{2, 3}. Breakouts of insect pests are often followed by pathogens infesting, leading to quantity and quality reduction of storage productions.

In China, one kind of stored products particularly face great challenges for insect controlling, that is Chinese tra-

ditional medical materials. Chinese traditional medicines use plants' fruits, roots and rhizomes as medical herbs, such as the famous ginseng *Panax ginseng* C. A. Meyer. These plant parts are often rich in oils, fats and sugars, thus are popular to be consumed by insect pests. The infested medical materials will lose their therapeutic efficiency. To prevent valuable materials from being wasted, co-storage has been found in practical. This approach could ensure long-period and high-quality preservation of valuable materials. It is called co-storage in China and shares the same concept of biological management in western world⁴. One kind of biological management on insect species is botanical insecticides. It mainly relies on plants' toxic substances, which are known as secondary metabolites^{5, 6}.

Secondary metabolites are considered to take no direct participation in plant growth, development or reproduction, but play ecological roles such as defense against pests⁷. The practice use of plant secondary metabolites in agriculture could be dated back to two millennia in ancient China, Egypt, Greece and India, which is far predating the discovery of synthetic insecticides in mid-1930s to 1950s⁸.

*Correspondence to: Shu-Shan Du, Beijing Key Laboratory of Traditional Chinese Medicine Protection and Utilization, Faculty of Geographical Science, Beijing Normal University, Beijing, CHINA

E-mail: dushushan@bnu.edu.cn ORCID ID: <https://orcid.org/0000-0003-0037-2480>

Accepted January 30, 2024 (received for review July 22, 2023)

Journal of Oleo Science ISSN 1345-8957 print / ISSN 1347-3352 online

<https://www.jstage.jst.go.jp/browse/jos/> <https://mc.manuscriptcentral.com/jjocs>



With the rapid biodegradation, great selectivity, low resistant emergence and widespread bioactivities^{9,10}, plant secondary metabolites have been a focus for botanical insecticides. As interests and demands for botanical insecticides increase, researches turn from wide screen bioassays to mechanism of action investigation. A wide range of fragrant plant families have been performed on insecticidal activities, including contact and fumigant toxicities, antifeedant and repellent effects^{11–13}. Magnoliaceae plants are widely distributed in China¹⁴, thus have been used as Chinese traditional medical materials such as Xinyi (flower buds of several species from Magnoliaceae) and Houpu (barks of *Houpoa officinalis* N. H. Xia & C. Y. Wu)¹⁵. According to antique recordings and practices, insect management efficiency of Magnoliaceae plants have been investigated. Sima *et al.* have investigated the chemical constituents of several species from Magnoliaceae^{16–19}. Huang *et al.* have reported the chemical similarity of twigs, leaves and flowers from *Michelia alba* DC.^{20,21}. Apart from broad-range assessment of bioactive species, mechanism of action has been also considered. It has been reported that *H. officinalis* is particularly rich in neolignans. Among those constituents, honokiol and magnolol have been selected as lead compounds for structural modification^{22–24}.

In traditional Chinese medicine, collection timing and organ is of great importance, deciding the efficacy of medicine materials. For example, Xinyi should be collected during late winter to early spring, Houpu should be obtained from April to June¹⁵. Plants strongly interact with environment by producing secondary metabolites. It has been noticed widely and observed specifically. He *et al.* investigated the genotypic and environmental effects on volatile chemotype of *Valeriana jatamansi* Jones²⁵. Filomeno *et al.* reported significant differentiations on essential oils (EOs) yield, chemical types as well as insecticidal activities between *Corymbia citriodora* (Hook.) K.D. Hill & L.A.S. Johnson in dry and rainy season²⁶. It has reached broad agreement that environmental factors especially season and water availability are vital for secondary metabolites.

According to literature research, we performed a pilot test on four species from Magnoliaceae and focused on *M. yunnanensis* Franch. ex Finet et Gagnep., shrubs commonly seen on hills in southwestern China²⁷. In the pilot test, *M. yunnanensis* was rich in fragrant EOs and showed impressive insect-management potential. Based on above-mentioned results, further experiments have been conducted to reveal three questions: 1) Chemical constituents of *M. yunnanensis* EOs and isolation of bioactive component(s). 2) Phenological variations and influence environmental factors of bioactive constituent(s). 3) The variation of bioactive constituent content and insecticidal activities among different organs.

Leading by three questions, accumulation regularity of bioactive compounds would be discussed. To answer three

questions, firstly we collected *M. yunnanensis* leaves monthly and produced EOs to perform insecticidal assays individually. Secondly we collected different organs (leaves, twigs, flowers and fruits) of *M. yunnanensis* at the same time, EOs of each sample were performed for insecticidal activity. Finally, all EO samples were analyzed and used for bioactive compounds isolation.

2 Materials and Methods

2.1 General experimental procedures

GC-MS and GC-FID analyses were performed on an Agilent 6890N gas chromatograph coupled with an Agilent 5973N mass selective detector. A DB-5MS capillary column (30 m × 0.25 mm × 0.25 μm) was used. EI-MS spectrum was obtained on a GCT CA127 Micronass UK mass spectrometer. One- and two-dimensional NMR experiments were carried out on Bruker Advance III spectrometer (500 MHz for ¹H and 125 MHz for ¹³C) with TMS as internal standard. Column chromatography was performed on silica gel (SiO₂ · nH₂O, 100–200 mesh and 200–300 mesh, Qingdao Marine Chemical Factory, China). Thin-layer chromatography (TLC) was performed on silica gel G plates (Qingdao Marine Chemical Factory, China). Fractions were monitored by TLC and spots were visualized by UV light (365 nm) and spraying with 10 H₂SO₄ in ethanol, followed by heating.

2.2 Plant materials

The plant identification was performed by Dr. Quan-Ru Liu (College of Life Science, Beijing Normal University, Beijing, China). Voucher specimens were deposited in the Herbarium (BNU) of the College of Resources Science and Technology, Faculty of Geographical Science, Beijing Normal University.

Leaf samples of *M. yunnanensis* were collected monthly from September 2019 to August 2020 on the same day at Yuxi City, Yunnan Province, China (Site 1: 24° 3' 34" N, 102° 45' 45" E, altitude 1980 meters). Samples in February 2020 were collected twice because of COVID-19 breakout, one was on 4th February 2020 (Site 2: 24° 6' 21" N, 102° 43' 52" E, altitude 2020 meters), the other was on 14th February 2020 (collected at Site 1). The other two were collected in October 2018 and in February 2019 (both at Site 1). Totally 15 samples were collected.

A total of 4 organs (leaves, twigs, flowers and fruits) were collected at Site 1 and same day. The flowering period of *M. yunnanensis* is from January to April, the fruiting period is from August to September. While flowers and fruits were collected, leaves were collected as well for comparison. Mature fruits could be separated into seedcases and seeds easily, obtaining a total of 17 samples. Detailed information about plant materials could be seen in Table S1.

2.3 EOs and isolation

Except leaf sample collected on 4th February 2020, each sample of *M. yunnanensis* was powered freshly by mini-type super high-speed pulverizer (Xinzhen Enterprise Co., Ltd., Taiwan, China) and subjected to hydrodistillation on a Clevenger-type apparatus for 6 h to produce EOs. Raw EOs were dried over anhydrous sodium sulfate and stored in airtight glass bottles. The volume of individual EOs was determined for yield calculation.

M. yunnanensis EOs from leaves, flowers and fruits (11 mL) were subjected to silica gel column chromatography (100–200 mesh) with a gradient elution of petroleum ether-ethyl acetate (*v/v*, 100–0, 50–1, 20–1 and 10–1). According to thin-layer chromatography monitoring, a total of 29 fractions were obtained. Fr. 21–29 (5.40 g) were further purified by silica gel column chromatography (200–300 mesh) to give **1** (5.01 g).

2.4 Chemicals

Pyrethrin (pyrethrins 1: 24%; pyrethrins 2: 13%; cinnerin 1: 2%; cinnerin 2: 2%; jasmolin 1: 1%; jasmolin 2: 1%) and DEET (*N,N*-diethyl-3-methylbenzamide) were purchased from Dr. Ehrenstorfer, Germany. Acetone, petroleum ether and ethyl acetate were purchased from Beijing Chemical Works (Beijing, China). The deuterated solvent (DMSO-*d*₆, deuterated ratio, 99.8%) with TMS as the internal referent was purchased from Cambridge Isotope Laboratories, Inc (Andover, USA). Fluon was purchased from Beijing Sino-Rich Material Science (Beijing, China).

2.5 Bioassays

Insecticidal assays were conducted with two stored-product insect species *T. castaneum* and *L. serricorne*. Both insect species were reared on mixtures of wheat flour and active yeast (10:1, *w/w*), and kept in a dark incubator at 28–30°C and 70–80% relative humidity. Insects used in all bioassays were 1-week old adults.

2.5.1 Contact activity

Topical method used in this work was described by Liu and Ho²⁸⁾. To find proper ranges for formal experiments, pre-experiments with 3 concentrations (50, 10 and 2%) were performed in advance. Testing samples were diluted in acetone and 0.5 µL of solutions were treated on the dorsal thorax of tested insects. Every 10 treated insects were reared on a glass vial (2.5 cm diameter, 5.5 cm height). For each concentration, 5 replications were performed. Acetone served as solvent and negative control. Glass vial with treated insects were kept under the same condition as insects rearing. Mortality was recorded after 24 h and used for LD₅₀ values estimation by Probit analysis²⁹⁾.

2.5.2 Repellent effect

Area preference method was adopted from Liu and Ho²⁸⁾. Five concentrations (78.63, 15.73, 3.15, 0.63, and 0.13 nL/

cm²) were prepared in acetone. A qualitative filter paper disc (9 cm in diameter) was cut into 2 halves. One was loaded with 500 µL of diluted samples, and the other was with 500 µL of acetone. After solvent evaporation, two halves were fixed on the bottom of a Petri dish (9 cm in diameter) lengthwise. With releasing 20 insects at the center, a cover was put on the dish to prevent insect escaping. For each concentration, 5 replications were performed. Processed units were kept under the same condition as insects rearing. After 2 and 4 hours' exposure, the number of insects on each side were recorded for calculating Percent Repellency (PR) values, following eq. (1).

$$PR(\%) = \frac{N_c - N_t}{N_c + N_t} \times 100\% \quad (1)$$

Where N_c is the number on the negative control side, N_t is the number of the treated side. PR values were transferred into arcsine values, and subjected to Analysis of Variance (ANOVA) followed by Tukey's HSD test at $p < 0.05$ (SPSS V 22.0).

2.6 Relationship with environmental factors

2.6.1 Meteorological data

Meteorological data in this work can be obtained in China Meteorological Data Service Centre (<http://data.cma.cn/>). Dataset named Monthly data from surface meteorological stations in China was available and station Yuxi Hongta with designator 56875 was selected. Totally 9 weather elements related to temperature, water available and sun exposure were used for partial correlation analysis. Nine elements were minimum air temperature (°C, T_{min}), maximum air temperature (°C, T_{max}), mean air temperature (°C, T_{ave}), mean minimum temperature (°C, T_{min}), mean maximum temperature (°C, T_{max}), precipitation amount in 24 hours (mm, Pre_{24}), mean relative humidity (% , RH_m), the days with daily precipitation > 0.1 mm (days, $DPre$) and hours of sun exposure (hours, Sh).

2.6.2 Partial correlation analysis

Secondary metabolites are influenced by environmental factors heavily. To investigate potential relationship between the content of **1** and weather elements, partial correlation analysis was conducted on SPSS V 22.0. Correlation parameter R was provided and considered significant at $p < 0.01$. Partial correlation was analyzed between content of **1** and temperature-related elements, when water-related and sunlight-related elements were controlled. Water-related and sunlight-related elements were performed as well when the other two group elements were controlled.

3 Results

3.1 Chemical profiles of EOs

The yield of *M. yunnanensis* EOs (leaf samples collect-

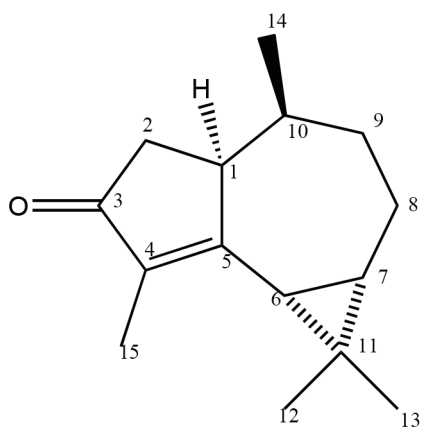


Fig. 1 The structure of (+)-cyclocolorenone (1).

ed monthly as well as different organ samples) was provided in Table S1. EOs yield of leaf samples ranged from 0.11 to 0.91%. The lowest was collected in January 2020, the highest was in September 2019. No significant fluctuation over season can be observed. Different organs of *M. yunnanensis* provided various capacity for EOs production, with yield ranging from 0.03 to 0.91%. Two samples Leaves-1909 and Seedcases-1909 showed the highest EOs yield (0.91% for each). Two stem samples Stems-1810 and Stems-1902 provided the lowest yield, with 0.03% and 0.05%, respectively. Within the same collection period, the yield of stems was always the lowest; leaves showed re-

markable yield, which was comparable or even superior to flowers and fruits.

Among 15 leaf EOs obtained monthly, 159 compositions were detected totally, representing from 94.78 to 100.00% (full data could be found in Table S2.1). Fifteen leaf EOs samples shared 9 compositions, which were eucalyptol, linalool, camphor, 4-terpineol, bornyl acetate, β -elemene, α -gurjunene, caryophyllene and (+)-cyclocolorenone (1). Within common compounds, 1 was detected in all EOs samples with remarkable relative content (8.22 to 78.75%). Moreover, the relative content of 1 showed phenological pattern (Fig. 2): from flowering period to vegetative period, the relative content increased from the least to steady; entering into fruiting period, the content grown to peak; with the ending of fruit period and starting of the flower period, the content fell monthly. Shortly, *M. yunnanensis* in reproductive stage would produce more 1 than in vegetative stage.

In 17 EOs samples from different organs, a total of 157 compounds were identified, representing from 91.40 to 100.00% (Full data was provided in Table S2.2). Among those compounds, eight (eucalyptol, linalool, camphor, borneol, 4-terpineol, bornyl acetate, caryophyllene oxide and (+)-cyclocolorenone (1)) were common ones, which was identified in more than 11 EOs samples. Notably, Compound 1 showed relative content ranging from 17.67 to 91.56%. Similarly, certain pattern could be found in different organs (Fig. 3). Stems and leaves collected at the same

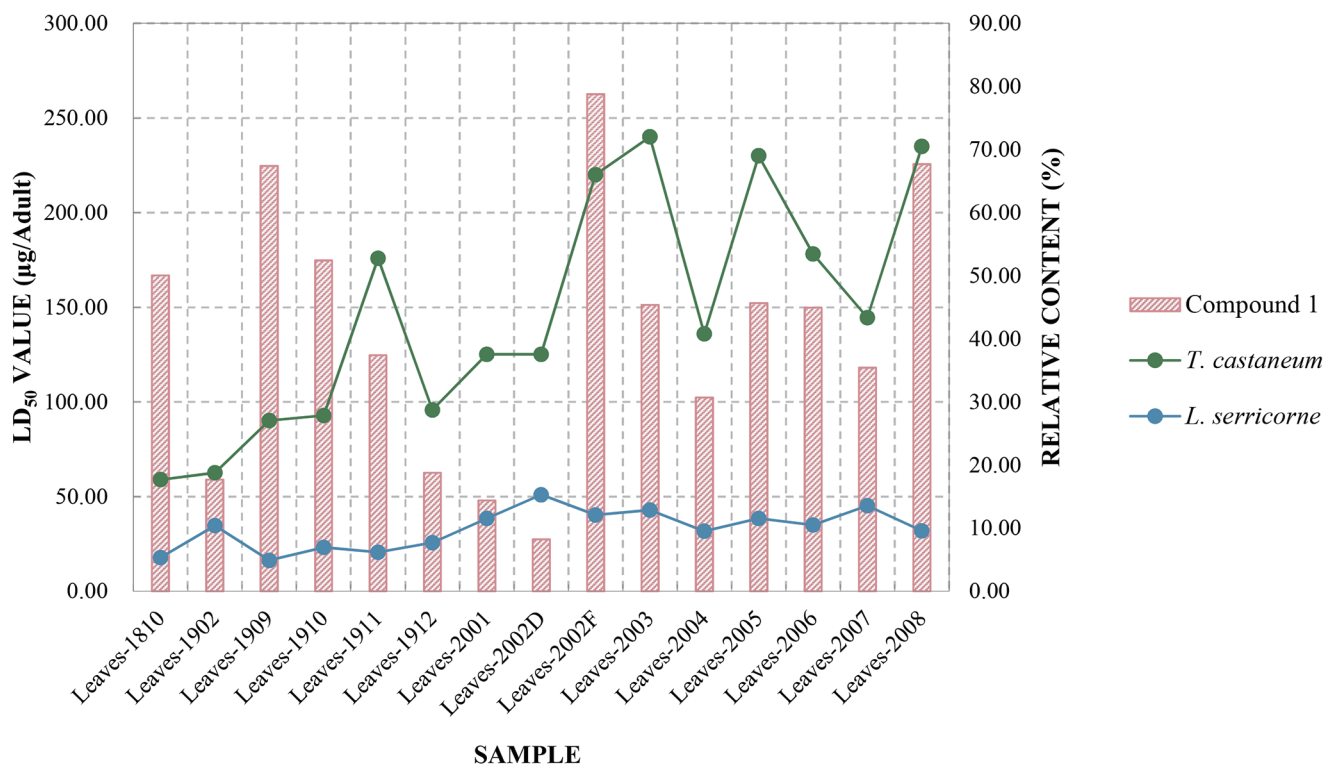


Fig. 2 The contact toxicity and (+)-cyclocolorenone (1) content of *M. yunnanensis* leaves EOs collected monthly.

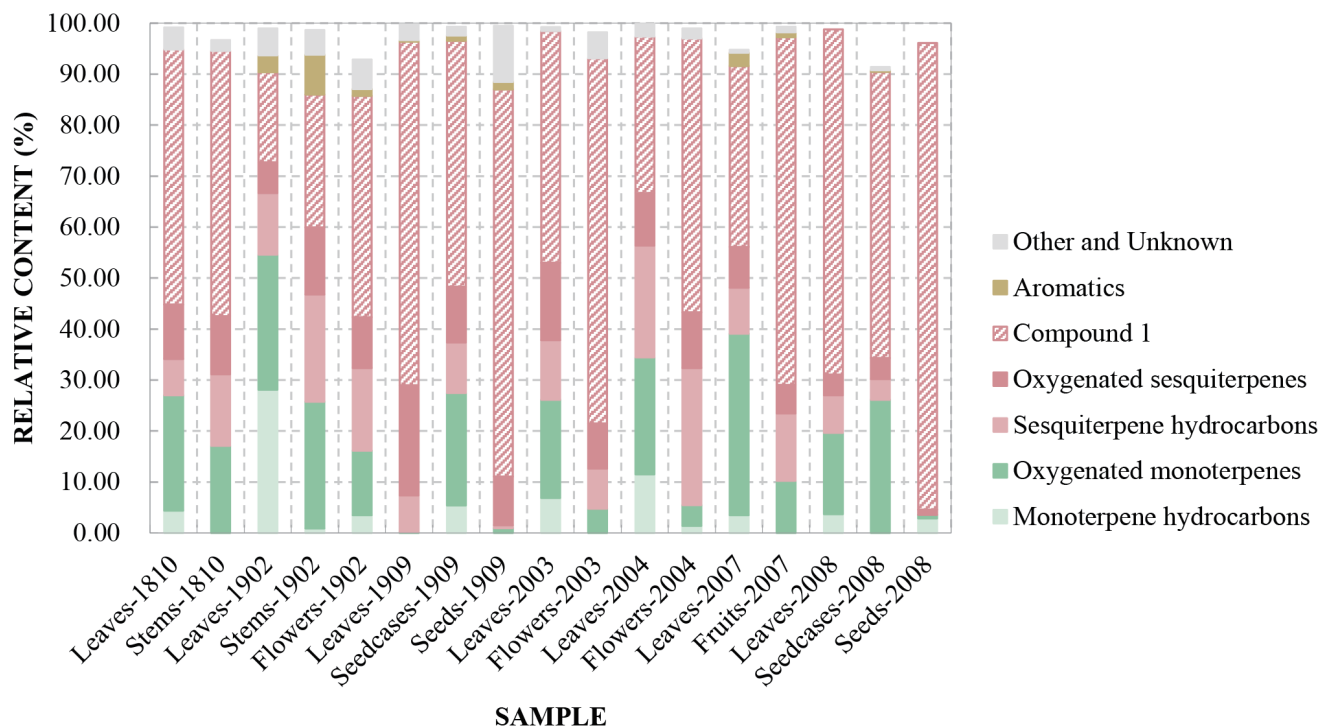


Fig. 3 The relative content of chemical groups in *M. yunnanensis* EOs from different organs.

time showed relatively close content; during flowering period, the content found in flowers was much higher than that in leaves; during fruiting period, the content of seeds was higher than that of leaves than that of seedcases. Generally, Compound **1** content of reproductive organs was always higher than that of vegetative organs collected at the same time.

3.2 Structure elucidation of **1**

Eos from *M. yunnanensis* afforded a known oxygenated sesquiterpene, namely (+)-cyclocolorenone (**1**). **1** was isolated as yellowish oil; $C_{15}H_{22}O$. EI-MS m/z 219.4 $[M+H]^+$. ^1H and ^{13}C NMR data (DMSO- d_6): see Table 1. One- (^1H , ^{13}C and APT) and two-dimensional (COSY, HMBC and HSQC) NMR spectrometry could be seen in Supplementary Materials (Figs. S1–S6). The identification was done by comparing spectrometry data in this work with those reported in literatures. According to literature, (+)-cyclocolorenone (**1**) was the known liverwort metabolite³⁰. Its structure could be seen in Fig. 1.

3.3 Influence of weather elements

Two leaf samples collected in February 2020 were used for partial correlation analysis individually. Results were presented in Table 2 (with sample of leaf 2002 dry) and Table 3 (with sample of leaf 2002 fresh). Results with Leaves-2002D sample showed that the content of **1** was positively correlated with temperature-related elements. Correlation was significant, as Sig. value was lower than

Table 1 The ^1H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectra data for (+)-cyclocolorenone (**1**) in DMSO- d_6 .

No.	H (δ [ppm], J [Hz])	C (δ [ppm])
1	—	25.9
2	1.35 m 2.05 m	32.6
3	1.60 m 1.90 m	21.1
4	1.27 m	32.1
5	1.96 m	31.5
6	3.02 br s	42.1
7	1.96 br d (18.5) 2.41 dd (6.6, 18.5)	40.1
8	—	206.9
9	—	139.5
10	—	176.1
11	1.52 d (8.1)	28.3
12	1.62 d (1.7)	8.5
13	0.74 d (7.0)	17.8
14	1.22 s	29.5
15	1.01 s	16.9

Table 2 Partial correlation analysis between **1** and weather elements (with Leaves-2002D).

	Weather Elements	R	df	Sig. ^a
Temperature-related (water and sunlight were controlled)	Tmax	0.810	8	0.005
	Tmin	0.750	8	0.013
	Tave	0.789	8	0.007
	Tmmin	0.813	8	0.004
	Tmmx	0.777	8	0.008
Water-related (temperature and sunlight were controlled)	Pre24	0.014	6	0.974
	DPre	0.399	6	0.328
	RHm	0.305	6	0.463
Sunlight-related (temperature and water were controlled)	Sh	−0.144	4	0.786

^a Sig.: R was significant when Sig. < 0.01**Table 3** Partial correlation analysis between **1** and weather elements (with Leaves-2002F).

	Weather Elements	R	df	Sig. ^a
Temperature-related (water and sunlight were controlled)	Tmax	0.382	8	0.276
	Tmin	0.403	8	0.249
	Tave	0.373	8	0.289
	Tmmin	0.370	8	0.292
	Tmmx	0.375	8	0.286
Water-related (temperature and sunlight were controlled)	Pre24	−0.272	6	0.515
	DPre	−0.135	6	0.749
	RHm	0.012	6	0.978
Sunlight-related (temperature and water were controlled)	Sh	0.139	4	0.793

^a Sig.: R was significant when Sig. < 0.01

0.01. It indicated that the content of **1** would be influenced strongly by environmental temperature. The content of **1** and water-related as well as sunlight-related elements presented positive and negative correlations, individually. Though the partial correlation was not significant, with Sig. value much higher than 0.01. Partial correlation analysis using sample Leaves-2002F displayed interesting results. Temperature-related elements and sun exposure hours were positively correlated with the content of **1**. Precipitation amount in 24 hours and days with daily precipitation >0.1 mm were negatively correlated with the content of **1**, relative humidity correlated positively. But all correlation relationships were not significantly. Generally, temperature-related elements showed positive correlation with the

content of **1**.

3.4 Biological activities of EOs and **1**

3.4.1 Contact activity

The contact activities of **1** and 25 *M. yunnanensis* EOs samples to *T. castaneum* and *L. serricorne* were presented in **Table 4**. **1** exerted weak contact toxicity to *T. castaneum* (LD₅₀ value was higher than 225 µg/adult) but considerable toxicity to *L. serricorne* (LD₅₀ value was 28.8 µg/adult). Generally, *L. serricorne* was much sensitive to testing samples (**1** and EOs) than *T. castaneum*. For *L. serricorne*, all EOs samples showed stronger contact activities than **1** did. Even Seeds-2008 EOs displayed more promising contact toxicity (LD₅₀ = 48.5 µg/adult), which

Table 4 Contact activities of **1** and *M. yunnanensis* EOs against *T. castaneum* and *L. serricorne*.

Insects ^a	Sample	LD ₅₀ Value (µg/adult)	95% Confidence Limit	Slope ± SE	χ ²	P value
TC	1	>225	—	—	—	—
	Leaves-1810	58.9	46.8 – 76.9	0.9 ± 0.2	8.0	0.841
	Stems-1810	43.6	33.9 – 54.8	2.2 ± 0.5	3.9	0.992
	Leaves-1902	62.6	47.6 – 103.9	2.3 ± 0.5	9.6	0.477
	Stems-1902	22.0	17.3 – 26.4	1.3 ± 0.2	3.2	0.997
	Flowers-1902	53.3	27.8 – 111.1	0.5 ± 0.1	5.6	0.590
	Leaves-1909	90.1	44.6 – 287.9	0.4 ± 0.1	8.8	0.269
	Seedcases-1909	32.6	5.9 – 130.5	0.3 ± 0.1	10.3	0.172
	Seeds-1909	>195	—	—	—	—
	Leaves-1910	92.8	64.2 – 137.7	2.2 ± 0.4	2.8	0.901
	Leaves-1911	175.9	116.9 – 337.2	2.0 ± 0.5	3.9	0.791
	Leaves-1912	95.7	85.8 – 106.7	6.0 ± 0.8	9.4	0.744
	Leaves-2001	125.2	112.1 – 140.7	5.9 ± 0.9	8.6	0.572
	Leaves-2002D	125.2	106.6 – 149.9	3.3 ± 0.5	7.2	0.889
	Leaves-2002F	>220	—	—	—	—
	Leaves-2003	>240	—	—	—	—
	Flowers-2003	>225	—	—	—	—
	Leaves-2004	136.0	98.0 – 196.2	2.8 ± 0.5	4.7	0.698
	Flowers-2004	>225	—	—	—	—
	Leaves-2005	>230	—	—	—	—
	Leaves-2006	178.1	111.2 – 382.7	1.6 ± 0.4	4.8	0.685
	Leaves-2007	144.5	121.2 – 182.5	3.7 ± 0.6	12.8	0.238
	Fruits-2007	>240	—	—	—	—
	Leaves-2008	>235	—	—	—	—
	Seedcases-2008	>245	—	—	—	—
	Seeds-2008	>225	—	—	—	—
	Pyrethrin ^b	0.3	0.2 – 0.3	3.3 ± 0.3	13.1	0.950

was comprised by 91.56% **1**. Within all EOs samples, Seedcases-1909 showed the firmest toxicity (LD₅₀ = 11.1 µg/adult). For *T. castaneum*, stems-1902 exhibited the strongest contact toxicity (LD₅₀ = 22.0 µg/adult).

Among leaf EOs obtained monthly, the contact toxicity to *L. serricorne* and the relative content of **1** showed a weak but noticeable pattern (Fig. 2): the LD₅₀ value increased with the relative content of **1** decreasing. Especially for leaf EOs collected from September 2019 (Leaves-1909) to February 2020 (Leaves-2002D). *M. yunnanensis* EOs from different organs presented a pattern as well: the contact toxicity of reproductive organs (flowers, fruits, seeds and seedcases) was slightly stronger than that of vegetative organs (leaves and stems). However, dose-dependent effect was not significant to *T. castaneum*. Besides, 10 out of 25 EOs showed LD₅₀ values to *T. casta-*

neum higher than 190 µg/adult, indicating less than 50% mortality caused at the highest concentration.

3.4.2 Repellent effect

Detailed PR values of *M. yunnanensis* EOs could be found in Table S3 for *T. castaneum* and Table S4 for *L. serricorne*. Generally, EOs samples as well as **1** showed better repellent effect on *T. castaneum* than on *L. serricorne*. The repellent effect on *L. serricorne* decreased rapidly with the decreasing of concentration and the increasing of exposure time. For *T. castaneum*, Flowers-1902, Leaves-1910, Leaves-1911, Leaves-1911, Leaves-2001, Leaves-2002F, Leaves-2003, Leaves-2005, Leaves-2006, Leaves-2008, Fruits-2007 and Seeds-2008 exhibited great repellent effect at the concentration of 78.63, 15.73 and 3.15 nL/cm² after 2- and 4-h exposure, which was comparable to that of positive control DEET. EOs of Leaves-

Table 4 Continued.

Insects ^a	Sample	LD ₅₀ Value (µg/adult)	95% Confidence Limit	Slope ± SE	χ ²	P value
LS	1	28.8	25.7 – 32.2	5.7 ± 0.8	8.3	0.827
	Leaves-1810	17.7	15.4 – 20.2	1.9 ± 0.3	8.7	0.798
	Stems-1810	17.5	13.6 – 21.0	0.3 ± 0.0	6.0	0.947
	Leaves-1902	34.7	31.4 – 38.5	3.0 ± 0.4	3.2	0.997
	Stems-1902	16.1	13.8 – 18.4	1.8 ± 0.3	4.7	0.981
	Flowers-1902	19.8	16.6 – 23.9	1.3 ± 0.2	4.8	0.980
	Leaves-1909	16.3	13.6 – 19.1	1.5 ± 0.2	12.1	0.518
	Seedcases-1909	11.1	8.8 – 13.3	1.3 ± 0.2	6.6	0.920
	Seeds-1909	13.7	11.2 – 16.3	1.3 ± 0.2	9.9	0.703
	Leaves-1910	23.1	18.5 – 29.1	2.3 ± 0.5	2.6	0.999
	Leaves-1911	20.5	18.6 – 22.4	5.4 ± 0.6	25.3	0.337
	Leaves-1912	25.7	23.5 – 28.1	9.5 ± 1.5	2.5	0.991
	Leaves-2001	38.4	35.3 – 41.8	10.2 ± 1.7	6.0	0.814
	Leaves-2002D	51.0	48.2 – 54.0	10.9 ± 1.5	13.4	0.416
	Leaves-2002F	40.2	37.4 – 43.3	8.8 ± 1.4	13.3	0.428
	Leaves-2003	42.9	40.6 – 45.9	10.9 ± 1.5	5.9	0.998
	Flowers-2003	21.1	19.1 – 23.3	7.4 ± 1.1	7.6	0.867
	Leaves-2004	31.7	28.4 – 35.7	6.3 ± 1.0	7.9	0.642
	Flowers-2004	32.9	30.2 – 35.1	8.2 ± 1.3	4.5	0.985
	Leaves-2005	38.4	36.5 – 40.3	15.5 ± 2.6	2.9	0.983
	Leaves-2006	35.0	33.5 – 36.5	15.4 ± 2.6	4.8	0.979
	Leaves-2007	45.1	42.8 – 47.8	11.9 ± 1.8	12.6	0.245
	Fruits-2007	32.0	28.6 – 36.4	5.8 ± 0.9	8.5	0.581
	Leaves-2008	31.8	28.7 – 35.4	7.0 ± 1.1	7.1	0.717
	Seedcases-2008	30.1	27.0 – 34.0	6.1 ± 0.9	14.0	0.175
	Seeds-2008	48.5	45.7 – 51.4	9.9 ± 1.4	9.2	0.758
	Pyrethrin ^c	0.2	0.2 – 0.4	1.3 ± 0.2	17.4	0.791

^a TC for *T. castaneum*; LS for *L. serricorne*^b data from You *et al.*⁴⁴⁾ ^c data from Yang *et al.*⁴⁵⁾

1810, Stemes-1810, Leaves-1912, Leaves-2002F, Leaves-2003, Flowers-2004, Seedcases-2008 and Seeds-2008 showed remarkable repellent effect at 0.63 and 0.13 nL/cm². For *L. serricorne*, Leaves-1902, Leaves-1911, Leaves-1912, Leaves-2003, Flowers-2004, Fruits-2007 and Seedcases-2008 showed great repellent effect, which was comparable to DEET.

4 Discussion

EOs samples Leaves-2002D and Leaves-2002F were collected both in February 2020. However, the EOs yield of 2 samples (0.72% for the former and 0.48% for the latter)

showed a difference which cannot be ignored. It could be the results of different pre-processing (shaded-dry or fresh), collecting sites (at a distance of about 10 kilometers) and time (about 11 days). More research data should be obtained for exact reasons. The difference between Leaves-2002D and Leaves-2002F also appeared on chemical compositions. Leaves-2002D was mainly composed of monoterpenes (82.51%), leaves-2002F was dominated by sesquiterpenes (98.27%). The relative content of 1 was 8.22% for Leaves-2002D and 78.75% for Leaves-2002F. Leaves-2002D was shaded-dried before hydrodistillation, oxygenated sesquiterpenes would decompose into monoterpenes eucalyptol, camphor, and bornyl acetate.

There was a great fluctuation among leaves collected

monthly and different organs, it was worthwhile to figure out the potential fluctuation pattern. *M. yunnanensis* samples collected monthly and from different organs showed a pattern: plants in reproductive period/organs would produce more **1** than plants in vegetative period/organs. The fluctuation pattern synchronized with the phenology of *M. yunnanensis*. Chemical composition variations over seasons can be observed massively (the effects of season and water availability on chemical composition, secondary metabolites and biological activity in plants)³¹⁾. According to the insecticidal activity of **1**, it exerted contact toxicity to *L. serricornis* and repellent effect on *T. castaneum*. Biological assays suggested that **1** could play a defense role in *M. yunnanensis*. As interacted frequently with living environments, secondary metabolites played diverse ecological roles, including defending insect diseases, attracting pollinators, and allelopathy with other plants³²⁾. During growing and developing, secondary metabolites were distributed to different organs selectively. Optimal defense theory proposed that plants tend to distribute defense secondary metabolites to vulnerable and crucial organs, such as flowers and fruits^{33, 34)}. In this work, **1** showed insecticidal activities and were accumulated heavily in reproductive organs and period. The result would support the proposition of optimal defense theory. Moreover, dose-dependent effect of **1** was not significant, indicating other ecological roles it may play. Figueiredo *et al.* reviewed that several species showed a chemical composition fluctuation within phenology, such as *Crithmum maritimum*, *Achillea millefolium* L., and *Mentha × piperita* L. The flowers of *Lonicera japonica* Thunb. presented a daily rise and fall of EOs chemical profiles because of pollinators. The fragrant substances could be a leader for pollinators³⁵⁾. As entomophilous plants, *M. yunnanensis* distributed and accumulated more **1** to flowers than to leaves and stems during florescence. It may protect flowers from phytophagous insects and help pollination.

The EOs yield of *M. yunnanensis* samples collected in September 2019 (0.91% for leaves, 0.91% for seedcases and 0.63% for seeds) was much higher than that in August 2020 (0.42, 0.34 and 0.22%, respectively). Meteorological data recorded precipitation in August 2019 (one month earlier before collection) was 42.9 mm, that in August 2020 was 229.2 mm. It suggested that chemical fluctuation was the result of various factors, including weather elements (precipitation, temperature and light) and seasonal change. Partial correlation analysis indicated that temperature-related elements were positively correlated to the relative content of **1**. Temperature affected volatile secondary metabolites in many ways. It influenced terpenoids from liquid to gaseity, determined the stomatal conductance, and affected the activity of enzyme to regulate the synthesis of secondary metabolites³⁶⁾. In the lowest temperature and shortest sunlight months, the content of volatile substances

decreased significantly. It has been proposed that the emission rate of *Pinus elliottii* Engelm. monoterpenoids was logarithmic related to temperature³⁷⁾.

Water-available elements also determined the composition of volatile components by controlling physiological activity. Thus drought stress for secondary metabolites production was studied widely. It has been agreed that slight and moderate drought stress cannot restrain the emission of secondary metabolites. Under severe drought condition, plants would reduce transpiration by stomatal closure, decreasing the amount of CO₂ fixed in cells^{38, 39)}. The content of secondary metabolites fell sharply because of lacking carbon sources⁴⁰⁾. Filomeno *et al.* reported that drought affected *Corymbia citriodora* not only chemical compositions but also insecticidal activities. *C. citriodora* EOs in dry season showed a EOs yield of 2.72%, dominated by citronellal (86.8%). *C. citriodora* from rainy season had a lower EOs yield (1.53%) and lower citronellal content (31.4%), associated with an increasing of citronellol (43.6%)²⁶⁾. Citronellol can be directly oxidized to citronellal with alcohol dehydrogenase. Hydric stress could regulate the biosynthesis pathway, affecting biological activity indirectly. Filomeno *et al.* also reported that dry-season EOs exerted stronger insecticidal activity to *Plutella xylostella* (L.) than rainy-season EOs did²⁶⁾. Riahi *et al.* collected *Artemisia absinthium* from four bioclimatic areas ranging from humid to arid stage, EOs yield decreased gradually and antioxidant capacities increased significantly⁴¹⁾. However, dose-dependent effect of **1** and insecticidal activity was not significant in this work. Apart from attracting pollinators, **1** would promote seed germination, for *M. yunnanensis* seeds were abundant in **1** but less toxic than seedcases and leaves. Absciscic acid, which is a sesquiterpenoid, and its analogue xanthoxin possessed biological activity to regulate plant growth and development. Xia *et al.* investigated the ecological role of *Michelia macclurei* Dandy, and reported that the performance of an autotoxic conifer *Cunninghamia lanceolata* could be facilitated by underground chemical interactions with *M. macclurei*⁴²⁾. Moreover, ecosystem carbon storage and soil organic carbon stability of *C. lanceolata* could be enhanced by introducing *M. macclurei*, resulting in carbon sequestration and carbon cycle improvement (ecosystem carbon storage and soil organic carbon)⁴³⁾. *M. yunnanensis* was often found in Pinaceae forests (*Pinus yunnanensis* Franch., *Pinus massoniana* Lamb. and *Keteleeria evelyniana* Mast.). Divers ecological roles of *M. yunnanensis* in mixed-species ecosystems shall be studied widely.

It was widely known that EOs were strongly affected by seasons, environmental factors (light, temperature, water and wind) and even disease. In this work, we collected *M. yunnanensis* samples in the field without controlling variables in experiments. It resulted in strong dependence of season, temperature and water. Statistic methods such as

partial correlation analysis were employed to minimize the effect and reveal the potential mechanism. However, due to COVID-19 the samples were not collected in succession, leading to unexpected results. Longer sequence and more parallel samples could reveal the accumulating patterns. Moreover, experiments with strict controlling of variables shall be done in laboratory if it was allowed.

5 Conclusion

In this work, *M. yunnanensis* samples were collected and subjected to hydrodistillation for EOs. One oxygenated sesquiterpene was isolated from EOs and identified as (+)-cyclocolorenone (**1**). It exerted great contact toxicity to *L. serricorne* ($LD_{50} = 28.8 \mu\text{g}/\text{adult}$) and promising repellent effect on *T. castaneum*. GC-MS analysis showed a potential phenological variation over the relative content of **1**: *M. yunnanensis* in reproductive period/organs would produce more **1** than plants in vegetative period/organs. Volatile secondary metabolites were results of environmental factors. Partial correlation analysis suggested that the relative content of **1** positively correlated with temperature-related elements. Variation on chemical compositions resulted in insecticidal activity variation, but dose-dependent effect was not significant.

Acknowledgement

Michelia yunnanensis samples were collected by Yong-He Feng and Li-Zhi Qian, and were identified by Dr. Quan-Ru Liu (College of Life Science, Beijing Normal University, Beijing, China).

Supporting Information

This material is available free of charge via the Internet at doi: 10.5650/jos.ess23136

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