

Comparative Analysis of Phytoncides Released from *Liquidambar formosana* Hance Trees and Seedlings

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Abstract

This study compared the phytoncides released from *Liquidambar formosana* Hance forest trees and its seedlings with volatile compounds present in the essential oil. Thirteen volatile compounds were identified in the essential oil of *L. formosana*. The main components were: α -pinene (40.9%), β -pinene (24.8%), D-limonene (18.3%), α -phellandrene (8.9%), and terpinen-4-ol (8.1%). Using solid-phase microextraction (SPME), we found that D-limonene (29.5%) was the dominant compound emitted from seedling leaves, followed by α -pinene (21.4%), β -pinene (19.2%), myrcene (10.7%), and α -terpinene (6.1%). Temperature influenced the emission of phytoncides from seedlings. The emission of volatiles increased as the temperature increased from 25°C to 35°C, with maximum emission of volatiles at 35°C. In the *L. formosana* forest, regardless of whether sampling was conducted close to or far from waterfall sites; α -pinene was

the most abundant compound in the atmosphere. In addition to α -pinene, α -thujene, β -pinene, myrcene, and D-limonene were also detected at the forest sites both close to and far from waterfalls. We conclude from the data that walking in the *L. formosana* forest breathing the phytoncides emitted from the trees may worthy of investigation for stress management, relaxation, and general regulation of health.

Key words: *Liquidambar formosana*, α -pinene, β -pinene, phytoncides, solid-phase microextraction

Introduction

Empirical evidence of the benefits of the forest environment to human health is slowly emerging^(1, 2). Sensory input such as that from volatiles emitted by trees in the forest is processed in the corresponding sensory areas of the brain, to control emotions and physiological functions, and effect physiological changes⁽³⁾. Olfaction-

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related elements in the forest are one of the factors that are thought to bring about physiological and psychological changes. Plants produce large quantities of diverse metabolites, including “phytoncides”, antimicrobial volatile compounds that are emitted from plants.⁽⁴⁾ The investigation of the specific volatile compounds released by trees is important to gain scientific understanding of the effect of “Shinrin-yogu” (forest-air bathing and walking). “Aowanda National Recreation Area” is a popular forest recreation area located in central Taiwan and is one of 18 such national forest recreation areas set up by Forest Bureau of the government of Taiwan. Formosan sweet gum (*Liquidambar formosana* Hance) is the main plantation tree in the area.

L. formosana is a deciduous tree that is native to Taiwan. *L. formosana* has three-lobed leaves that are downy and violet-red when young and turn to delicate shades of rose in the autumn.⁽⁵⁾ *L. formosana* is also an economically important heartwood species, the wood of which is used for construction, furniture, and mushroom cultivation. The aromatic exudates of *L. formosana* have traditionally been used in folk medicines in Asian countries as anti-inflammatory agents, analgesics, and for promotion of blood circulation, removing blood stasis, and wound healing. Triterpenoids isolated from the fruits of *L. formosana* exhibited strong inhibitory activity against nuclear factor of activated T cells (NFAT).⁽⁶⁾ Our recent investigation of the composition of exudates

emitted from *L. formosana* revealed a total of 26 volatile and non-volatile compounds: 13 triterpenoids, 2 sesquiterpenoids, 9 monoterpenoids, and two cinnamyl derivatives. Three of these compounds, 2 α , 3 α -dihydroxyolean-12-en-28-al, 3 α -hydroxyolean-12-en-30-ol, and 3 α -hydroxyolean-2-oxo-12-en-28-al were new compounds⁽⁷⁾. Okuda and colleagues described the structure of hydrolyzed tannin in the leaves of *L. formosana*.^(8, 9) The antioxidant activities of leaf extracts were further studied.⁽¹⁰⁾ In the current study, we analyzed the composition of phytoncides (volatile compounds) from *L. formosana*. Tenax TA resin tubes coupled with automatic portable air pumps were used to collect the phytoncides emitted at different forest sites in an *L. formosana* forest. The phytoncides emitted from *L. formosana* were characterized and analyzed by GC/MS.

Materials and methods

Plant Materials

Four-year-old seedlings of *L. formosana* were provided by the Nantou Forest District Office of the Forestry Bureau of Taiwan. After the seedlings were brought to the laboratory, they were cultured in a growth chamber under controlled light (12 h/12 h day/night cycle) and temperature (25°C/20°C day/night cycle) conditions. The humidity was set at 80%.

Essential oil preparation

Leaves collected from the seedlings of *L.*

formosana (150 g) were cut into small pieces (2-3 cm), then subjected to water distillation for 6 h in a Clevenger type apparatus, followed by determination of oil content (mL/kg) based on leaf dry weight. After deoxygenation with N₂, the essential oil was stored in sample vials prior to analysis by gas chromatography (GC) and GC-mass spectrometry (GC/MS).

Collection of phytoncides from leaves of seedlings

To obtain phytoncides from leaves for analysis, the solid-phase microextraction (SPME) technique was used to collect the volatile compounds from seedlings. A SPME holder and carboxen-polydimethylsiloxane (75 µm) were purchased from Supelco (Bellefonte, USA). The SPME fibers were conditioned by heating in a hot injection port of a GC device at 250°C for 20 min in order to remove contaminants before use. The leaves of *L. formosana* were put into a Ziplock bag (S. C. Johnson, Racine, WI, USA) and then a SPME fiber was introduced into the bag and exposed to the headspace of the bag for 30 min. The conditions selected in this study were based on a previous experiment where complete optimization of the extraction conditions was carried out⁽⁴⁾. After extraction for 20 min, the SPME fiber was removed from the Ziplock bag, flash frozen and stored in an ice box at -20°C until analysis. Finally, the SPME was inserted into the injection port of a GC device using SPME-liner where the terminal description occurs at 250°C for 30 seconds. Next, to investigate the effect of

temperature on the amounts of phytoncides emitted from *L. formosana*, the same seedlings were placed into growth chambers at 25°C, 30°C, 35°C, and 40°C. The same SPME method was used to extract the volatile compounds and semi-quantification of amounts of phytoncides were calculated according to the total area of the peak in the GC/MS chromatograms.

Collection of phytoncides in the *L. formosana* forest

To collect the phytoncides emitted from *L. formosana* at different sites in the “Aowanda National Recreation Area” sampling was conducted with automatic portable air pumps (Gilian LFS 113D C Low Flow Personal Air Sampling Pump; Sensidyne, USA) coupled with glass PE ATD tube (Perkin Elmer, Rodgau-Juegesheim) filled with Tenax TA 60/80 mesh resin (Supelco, Bellefonte, USA). The flow rate of the pump was set at 250 ml/min, and the collection time was from 8:00 am to 4:00 am. After phytoncide collection, sample tubes were immediately stored in an ice pail until they could be returned to the laboratory. The phytoncides were then eluted with ethyl ether and analyzed by GC/MS.

GC-MS analyses of essential oil composition and phytoncides

The composition of the essential oils were analyzed by a trace GC-polaris Q mass spectrometer (Finnigan-spectronex, Thermo, MA, USA), equipped with a DB-5 column (30 m×0.25

mm i.d., 0.25 μm film thickness; J & W Scientific). The temperature program was as follows: 40°C for 1 min, then increased by 4°C min^{-1} to 260°C and held for 4 min. The other parameters were as follows: injection temperature, 270°C; ion source temperature, 280°C; EI, 70 eV; carrier gas, He at 1 ml/min; injection volume, 1 ml; split ratio, 1:50; and mass range, m/z 45-425. Quantification was from percentage peak areas from the gas chromatogram. Identification of individual compounds was carried out using a Wiley/NBS Registry of Mass Spectral Data, National Institute of Standards and Technology (NIST) search and

authentic reference compounds. Chromatography results expressed as area percentages were calculated with a response factor of 1.0.

Results

Analysis of composition of *L. formosana* essential oil and phytoncides emitted from seedlings

The yield of essential oil, which was prepared by water distillation from the leaves of 4-year-old *L. formosana* seedlings, was 2.1 mL/kg. Table 1 shows the results of GC-MS analyses of leaf essential oil of *L. formosana*. The main components were α -pinene (40.9%), β -pinene

Table 1. Analysis of composition of essential oil and phytoncides from leaves of *Liquidambar formosana*

Compound	KI ^a	Content in essential oil (%)	Phytoncide (%)	Identification ^b
α -Thujene	921	0.5	1.5	MS, KI
α -Pinene	929	40.9	21.4	MS, KI, ST
Camphene	941	-	1.4	MS, KI, ST
Sabinene	967	5.6	-	MS, KI
β -Pinene	969	24.8	19.2	MS, KI, ST
Myrcene	983	2.4	10.7	MS, KI, ST
α -Phellandrene	1005	8.9	5.1	MS, KI, ST
α -Terpinene	1010	3.9	6.1	MS, KI, ST
p-Cymene	1016	0.7	-	MS, KI, ST
D-Limonene	1023	18.3	29.5	MS, KI, ST
γ -Terpinene	1052	4.7	1.3	MS, KI, ST
α -Terpinolene	1082	1.4	3.9	MS, KI, ST
Terpinen-4-ol	1174	8.1	2.5	MS, KI, ST
α -Terpineol	1183	1.2	-	MS, KI, ST

^a Kovats index on a DB-5ms column in reference to *n*-alkanes

^b MS, NIST and Wiley libraries and literature; KI, Kovats index; ST, authentic standard compounds

(24.8%), D-limonene (18.3%), α -phellandrene (8.9%), and terpinen-4-ol (8.1%). The SPME method was used to collect the phytoncides emitted from *L. formosana* seedlings. As shown in Table 1, the variety and relative quantities of phytoncides emitted from the seedlings differed from the compounds found in the essential oil. D-Limonene (29.5%) was the dominant compound emitted from the seedlings, followed by α -pinene (21.4%), β -pinene (19.2%), myrcene (10.7%), and α -terpinene (6.1%). Sabinen, *p*-cymene, and α -terpineol were only found in essential oil and not found emitted from seedlings. Effect of temperature on the emission of phytoncides emitted from seedlings

To investigate the influence of temperature

on the emission of phytoncides, *L. formosana* seedlings were placed in a growth chamber. Then phytoncide emission was measured at different temperatures, 25°C, 30°C, 35°C, and 40°C (Figure 1). Emission of volatiles increased as the temperature increased from 25°C to 35°C with maximum emission at 35°C. When the temperature was increased to 40°C the emission of volatiles dramatically decreased.

Analysis of phytoncides emitted from *L. formosana* forest trees

Automatic portable air pumps coupled with glass PE ATD tubes, filled with Tenax TA 60/80 mesh resin were used to collect the phytoncides at different sites in the *L. formosana* forest. Sites both near to and far away from waterfalls were

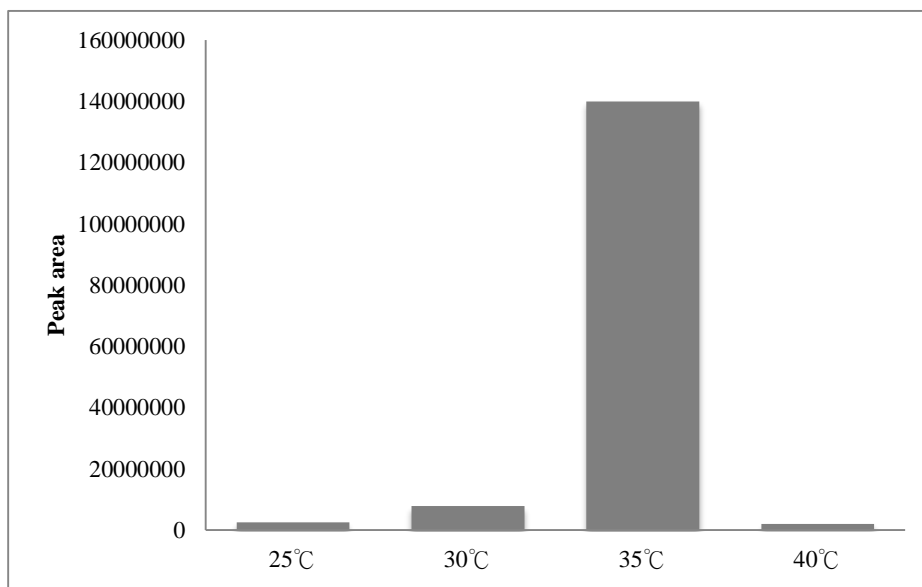


Fig. 1. Semi-quantification of phytoncides emitted from *Liquidambar formosana* seedlings at different temperatures.

Table 2. Analysis of phytoncides emitted by *Liquidambar formosana* forest trees

Compound	KI ^a	Far from waterfalls (%)	Near to waterfalls (%)	Identification ^b
α -Thujene	921	0.7	0.9	MS, KI
α -Pinene	929	90.2	81.4	MS, KI, ST
β -Pinene	969	1.2	0.8	MS, KI, ST
Myrcene	983	2.8	3.8	MS, KI, ST
D-Limonene	1023	3.8	5.5	MS, KI, ST
γ -Terpinene	1052	- ^c	0.8	MS, KI, ST
α -Caryophyllene	1450	-	1.9	MS, KI, ST

^a Kovats index on a DB-5ms column in reference to *n*-alkanes

^b MS, NIST and Wiley libraries and literature; KI, Kovats index; ST, authentic standard compounds

^c Trace

chosen. The results are shown in Table 2. In general, fewer types of volatiles were collected in the forest than from seedlings by SPME in the laboratory. α -Pinene was the most abundant compound in the atmosphere both close to and far away from waterfalls. In addition to α -pinene, we also detected α -thujene, β -pinene, myrcene, and D-limonene in the forest at sites both close to and distant from waterfalls. Two further compounds, γ -terpinene and α -caryophyllene were identified at sites near to waterfalls that were not identified at sites far away from waterfalls.

Discussion

It is suspected that taking in the forest atmosphere or “forest bathing” may provide relaxation and reduce stress, and empirical evidence of the therapeutic effect of the forest environment is beginning to emerge. However,

scientific evidence is still needed to understand the mechanisms. The volatile compounds (phytoncides) emitted from trees vary according to species. In this study, the composition of the essential oil from the leaves of seedlings of *L. formosana*, and the phytoncides emitted from the seedlings were analyzed. The most abundant compound in the essential oil was α -pinene, followed by β -pinene, D-limonene, α -phellandrene, and terpinen-4-ol. In contrast, the most abundant compound emitted from the seedlings was D-limonene, followed by α -pinene, β -pinene, myrcene, and α -terpinene. In our previous study, we found that D-limonene, an abundant compound in *Cryptomeria japonica*, exhibited anti-anxiety and anti-nociceptive activities in mice.⁽⁷⁾ Guzmán-Gutiérrez and colleagues reported that β -pinene is one of main active principles of *Litsea glaucescens* essential

oil, and showed antidepressant-like and sedative-like activity; however, α -pinene did not show antidepressant-like activity in their study.⁽¹¹⁾ Kusuhara and his colleagues suggested that the provision of a fragrant environment maybe a useful cancer therapy.⁽¹²⁾ Their investigation indicated the mice kept in a fragrant environment enriched with α -pinene had reduced melanoma growth. Tumor volume in mice in an environment including α -pinene was about 40% smaller than that in control mice. According to cytotoxicity assay, α -pinene had no inhibitory activity on melanoma cell proliferation *in vitro*, they, therefore, predicted that reduction in cancer growth due to enrichment of α -pinene in the environment might be *via* the hypothalamic-pituitary-adrenal axis and leptin.⁽¹²⁾ Another report suggested that α -pinene might be of potential interest for the management of inflammatory and neuropathic pain.⁽¹³⁾ Our results indicate that α -pinene and other aromatic compounds, such as α -thujene, β -pinene, myrcene, D-limonene γ -terpinene and α -caryophyllene are emitted from the *L. formosana* forest. We further found that temperature also affected the emission of volatile compounds. Emission of volatiles from *L. formosana* seedlings increased along with temperature increases from 25°C to 35°C. The maximum emission of volatiles from *L. formosana* seedlings was at around 35°C. At temperature over 35°C, the emission rate decreased dramatically.

Collectively our data suggest that walking in the *L. formosana* forest and breathing the phytoncides emitted from the trees may be of interest for stress management, relaxation, and general regulation of health.

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