DOI: 10.31276/VJSTE.2023.0074

Study on chemical composition and biological activities of essential oil of *Limnophila aromatica* (Lamk.) Merr.

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Received 17 August 2023; revised 13 December 2023; accepted 8 April 2024

Abstract:

***Limnophila aromatica* (Lamk.) Merr. is a widely used spice and medicinal herb in many countries and Vietnam is one of them. However, there are relatively few studies on this plant, especially within Vietnam. This research focuses on the chemical composition analysed via Gas Chromatography-Mass Spectrometry (GC-MS), antioxidant activity (measured by the DPPH method), and antimicrobial properties (determined using the paper plate method) of the essential oil derived from *Limnophila aromatica* (Lamk.) Merr. The results show that the primary component of the essential oil is limonene (46.86%), followed by β-cis-ocimene (24.1%), α-pinene (5.69%), and 1,2-epoxy-5,9-cyclododecadiene (4.57%). The antioxidant activity was quantified with an IC50 value of 266.6±0.56 µg/ml. The antimicrobial assays demonstrated that the essential oil was effective against six of the eight tested microorganisms, including *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633, *Listeria innocua* ATCC 33090, *Bacillus cereus* ATCC 14579, and *Aspergillus niger* ATCC 6275. These findings suggest that *Limnophila aromatica* has potential applications in functional foods, cosmetics, and antibiotics.**

Keywords: **essential oil, hydrodistillation, *Limnophila aromatica*, steam distillation**.

Classification numbers: **2.2, 3.4**

1. Introduction

Limnophila aromatica (Lamk.) Merr., commonly known as rice paddy herb, is a tropical plant from the *Scrophulariaceae* family, belonging to the genus *Limnophila*, and can be taxonomically classified as follows [1]:

Kingdom	Plantae
Sub-kingdom	Tracheobionta
Division	Magnoliophyta
Class	Magnoliopsida
Sub-class	Asteridae
Order	Scrophulariales
Family	Scrophulariaceae
Genus	*Limnophila*
Species	*L. aromatica*
Binomial name	*Limnophila aromatica* (Lamk.) Merr. [1]

This plant originates from Southeast Asia and was introduced to North America in the 1970s [1]. It thrives in aquatic or semi-aquatic environments such as rivers, lakes, ponds, and marshy areas [2, 3]. The plant can be harvested approximately 40 days after planting [3]. *Limnophila aromatica* (Lamk.) Merr. is a staple vegetable in Laotian, Thai, Cambodian, and Vietnamese cuisines, valued for the fragrance it adds to sour soups, noodle soups, and sauces [1-3].

In traditional medicine, *Limnophila aromatica* (Lamk.) Merr. is used for its antispasmodic, antiseptic, anthelmintic, anti-inflammatory, and diuretic properties. Its leaves are also used in treatments for fever and kidney stones [1-3]. The plant's beneficial effects are thought to be due to its essential oil (EO), flavonoids, and phenolic compounds [3]. There have been several studies on the EO of *Limnophila aromatica* cultivated in Thailand, Malaysia, and Bangladesh [2-5]. However, limited research has been conducted on the EO of *Limnophila aromatica* (Lamk.) Merr. in Vietnam.

This study aims to investigate the chemical composition, antioxidant activity, and antimicrobial properties of the EO of *Limnophila aromatica* (Lamk.) Merr. in Vietnam, contributing to the scientific understanding of the plant's potential applications in the economic, pharmaceutical, and culinary fields.

2. Materials and methods

2.1. Distillation of essential oil

The *Limnophila aromatica* (Lamk.) Merr. plant was harvested in the urban area of Can Tho city, Vietnam. After

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harvesting, the plant (without roots) was washed, drained, and stored at 5°C. The fresh samples (approximately 90% moisture) underwent EO extraction via hydrodistillation using a Clevenger apparatus. Several parameters influencing EO yield were investigated, including material size (5 and 0.5 mm), the ratio of material to water (1:2 to 1:4, g/ml), and distillation time (1.5-3.5 hours). After the distillation process, the essential oil was dehydrated using anhydrous sodium sulphate and subsequently analysed for physicochemical properties, chemical composition, and biological activities.

The EO yield was calculated as shown below:

$$Essential\ oil\ yield\ (‰) = \frac{weight\ of\ essential\ oil\ (g)}{weight\ of\ material\ (g)} x1000 \quad (1)$$

2.2. Physicochemical index analysis

Physicochemical indices were analysed according to the International Organization for Standardization (ISO) methods: ISO 279:1998: density at 26°C of EO [6], ISO 1242:1999: acid index analysis of EO [7].

2.3. Analysis of essential oil

The chemical composition of *Limnophila aromatica* (Lamk.) Merr. EO was determined using GC-MS with a Thermo Fisher Scientific system equipped with a TG SQC column (15 m x 0.25 mm x 0.25 µm). The following conditions were applied: injection volume (1 µl), carrier gas (helium at 1 ml/min), oven temperature programme (50 to 80°C at 5°C/min, 80 to 200°C at 10°C/min, and 200 to 250°C at 10°C/min), ion source temperature (200°C), and transfer line temperature (275°C). The mass spectrometer was operated in EI mode at 70 eV with a mass scan range of 35-450 amu. Compounds were identified using the National Institute of Standards and Technology (NIST MS) spectral library [8, 9].

2.4. Biological activities

2.4.1. Antioxidant activity: The antioxidant activity was evaluated using the DPPH (1,1-diphenyl-2-picrylhydrazyl) method. A solution of 180 µl DPPH (78 µg/ml in methanol) was mixed with 20 µl of essential oil (1% v/v in ethanol). The reaction mixture was left at room temperature for 30 minutes, after which the absorbance was measured at 515 nm to determine the percentage of inhibition (Q%) [10].

$$Q\ (\%) = \frac{A_0 - A_c}{A_0} x100 \quad (2)$$

where A_0 is the absorbance of DPPH (control) and A_c is the absorbance of the sample.

2.4.2. Antimicrobial activity: The antimicrobial activity was assessed using the paper plate method against 7 microorganisms: *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923TM, *Escherichia coli* ATCC 25922TM, *Bacillus cereus* ATCC 10876TM, *Listeria innocua* ATCC 33090TM, *Enterococcus faecalis* ATCC 29212TM, and *Aspergillus niger* ATCC 6275.

A clean tube containing 4 ml of Luria-Bertani (LB) liquid medium was inoculated with the tested microorganisms and shaken at 200 rpm overnight. Subsequently, 200 µl of the inoculum (concentration of approximately 4-5x10^8 CFU/ml) was spread onto the surface of petri dishes containing LB solid medium. A sterile paper plate impregnated with 10 µl of diluted EO (dimethyl sulfoxide, concentration of original EO-10^{-2}) was then placed on the petri dish, and the plate was incubated at 37°C for 18-20 hours. After incubation, the diameter of the inhibition zone was measured. A sterile ring with a diameter (D)>6 mm indicated antimicrobial activity; D=6 mm indicated no inhibition [11].

2.5. Statistical analysis

Data were expressed as the average of duplicate experiments. A two-sample T-test assuming equal variance was performed using Excel software (2019) to evaluate significant and insignificant differences in the investigated parameters.

3. Results and discussion

3.1. Investigation of influencing parameters affecting essential oil yield

3.1.1. Effects of material size on essential oil yield: A 2000 ml flask containing 200 g of *Limnophila aromatica* leaves (either 5 mm or 0.5 mm in size) and 400 ml of deionised water (DIW) was used for distillation. The yield of EO for the two material sizes is shown in Fig. 1. The highest yield was obtained with the larger material size (5 mm), as smaller materials (0.5 mm) tended to float on the surface of the distillation flask, limiting the evaporation of water. This resulted in a lower EO yield compared to the larger material size. Hence, a material size of 5 mm was chosen for subsequent experiments.

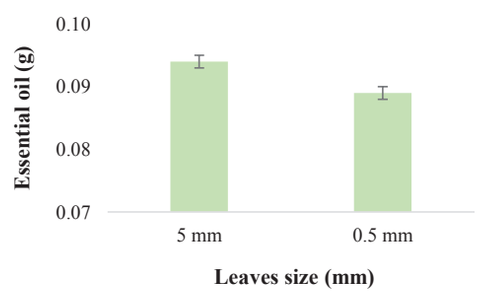

Fig. 1. Effect of material sizes on essential oil yield. Conditions: 200 g of sample, 400 ml deionised water and 2 hours of distillation.

3.1.2. Effect of time on EO yield: A 2000 ml flask containing 200 g of *Limnophila aromatica* leaves (5 mm) and 400 ml of DIW was used to study the effect of distillation time on EO yield. Distillation times of 1.5, 2.0, 2.5, 3.0, and 3.5 hours were investigated, as shown in Fig. 2.

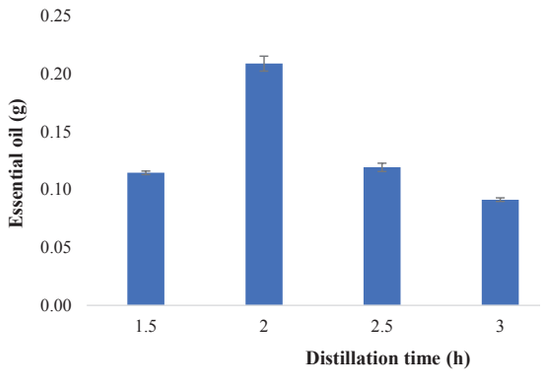

Fig. 2. Effect of distillation time on essential oil yield. Conditions: 200 g of sample, 400 ml deionised water, and material size of 5 mm.

The EO yield as seen in Fig. 2 increased from 0.11 to 0.21 g as the distillation time increased from 1.5 to 2 hours. However, EO yield decreased after 2 hours, similar to the observations of V.D. Zheljazkov, et al. (2013) [12], who reported that EO yields for lavender peaked at 60 minutes and declined thereafter. A shorter distillation time (<2 hours) does not fully extract the EO, whereas a distillation time of 2 hours allows the essential oil sacs to rupture and release the maximum amount of oil. Prolonged distillation times, however, lead to a decrease in EO yield due to the dissolution of polar components in water. Thus, a distillation time of 2 hours was considered optimal.

3.1.3. Effect of water volume on essential oil yield: A total of 200 g of *Limnophila aromatica* leaves was placed in a 2000 ml flask and distilled with leaf-to-water ratios of 1:1, 1:2, 1:3, and 1:4 (g/ml) for 2 hours. The effect of the water amount on essential oil yield is illustrated in Fig. 3.

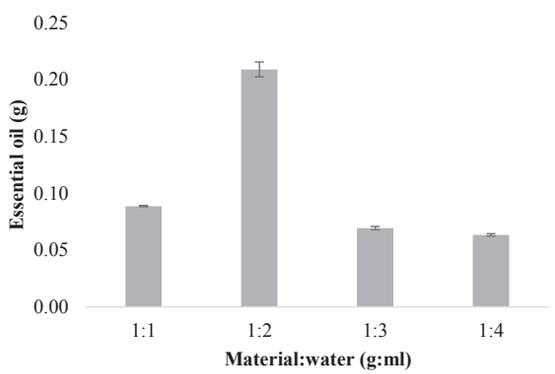

Fig. 3. The effect of water volume on essential oil yield. Conditions: material size of 5 mm and 2 hours of distillation time.

As shown in Fig. 3, a low water amount (1:1) is insufficient to allow water to penetrate the material, resulting in a lower EO yield. Conversely, an excessive amount of water (e.g., 1:4) reduces the amount of EO obtained, as the distillation process requires more time with additional water. Therefore, a leaf-to-water ratio of 1:2 was selected as optimal for the experiments.

In summary, the optimal parameters for the distillation process of *Limnophila aromatica* EO were found to be: material size (5 mm), distillation time (2 hours), and a material-to-water ratio of 1:2 (g/ml). Under these conditions, the highest oil yield was 0.21 g per 200 g of material, corresponding to a distillation yield of 1.05‰ (*w/w)*.

3.2. Physicochemical index analysis

The obtained essential oil from *Limnophila aromatica* had a characteristic fragrance, a light yellow colour, and was transparent.

Table 1. Physicochemical indices.

Index	This study	References
Density (26°C) (g/cm^3)	0.88±0.04	0.87 [13]
Acid	2.79±0.17	5.61 [14]

As shown in Table 1, the density of the obtained essential oil is less than 1, which is consistent with the reference [13]. However, there is a noticeable difference in the acid value compared with the reference [14], which could be attributed to geographical variation. A lower acid value indicates a high-quality essential oil with less degradation or rancidity [15].

3.3. Chemical composition

The essential oil of *Limnophila aromatica* contains 18 components, with limonene (46.86%) being the most abundant. Limonene is widely used in the cosmetics, food, and industrial cleaning industries [16]. According to J.U. Chowdhurya, et al. (2011) [5], limonene is also combustible and has potential applications as a biofuel. Interestingly, the limonene content in *Limnophila aromatica* cultivated in Bangladesh was reported to be significantly lower (3.75%), while *Z*-ocimene content was higher (39.21%) [5]. These variations suggest that the chemical composition of the EO is influenced by geographical origin [17, 18].

The second most prevalent compound is β-cis-ocimene (24.1%), one of the most common monoterpenes found in nature. In medicinal applications, β-cis-ocimene exhibits anticonvulsant, antifungal, antitumour, and pest-resistant properties [19]. Other notable constituents of *Limnophila*

Table 2. Chemical composition of the essential oil.

No	Compounds	(%)
1	***α-Pinene***	**5.69**
2	*β*-Myrcene	2.39
3	**Limonene**	**46.86**
4	***β*-cis-Ocimene**	**24.10**
5	Carane, 4,5-epoxy-, trans	2.16
6	1,2-Epoxy-5,9-cyclododecadiene	4.57
7	Perilla aldehyde	0.81
8	*β*-Elemen	0.51
9	*Cis*-Myrtanyl acetate	1.05
10	Germacrene	0.86
11	*α*-Caryophyllene	1.57
12	Germacrene D	2.60
13	*α*-Amorrphene	0.44
14	*γ*-Muurolene	0.63
15	*δ*-Cadinene	1.17
16	Ledol	0.44
17	*δ*-Cadinol	2.02
18	*α*-Cadinol	2.13

aromatica EO include α-pinene (5.69%), β-myrcene (2.39%), and germacrene D (2.6%) (Table 2).

3.4. Biological activities

3.4.1. Antioxidant activity: The antioxidant activity of the EO is shown in Fig. 4. The free radical scavenging efficiency (Q%) of the oil increases in direct proportion to its concentration. As the concentration of the EOincreases from 50 to 300 µg/ml, the scavenging efficiency increases from 26.64±1.00% to 54.65±0.08%. The IC_{50} value of the EO was determined to be 266.6±0.56 µg/ml, as shown in Fig. 4.

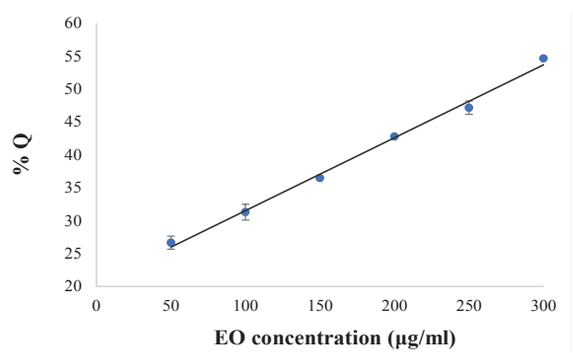

Fig. 4. Antioxidant capacity of *Limnophila aromatica* essential oil.

In comparison, the IC_{50} value of ascorbic acid (used as a positive control) was found to be 9.54±1.22 µg/ml, based on concentration ranges from 5.0 to 25 µg/ml. Thus, the antioxidant activity of the EO is approximately 27.9 times weaker than that of ascorbic acid. No published data on the IC_{50} of *Limnophila aromatica* EO could be found for comparison; however, relative to other plants [20, 21], the IC_{50} value of *Limnophila aromatica* EO (266.6±0.56 µg/ml) is lower than that of *Ammodaucus leucotrichus* (IC_{50} =3326.7 µg/ml) [20], *Thymus vulgaris* (IC_{50}=861.5 µg/ml), *Cymbopogon citratus* (IC_{50}=601.60 µg/ml), *Origanum marjorana* (IC_{50}=524.00 µg/ml), and *Rosmarinus officinalis* (IC_{50}=444.30 µg/ml), but higher than that of *Zingiber* officinalis (IC_{50}=129.4 µg/ml), *Salvia officinalis* (IC_{50}=59.07 µg/ml), and *Petroselinum crispum* (IC_{50}=15.51 µg/ml) [21]. This indicates that the antioxidant activity of *Limnophila aromatica* EO is weaker than that of ascorbic acid, *Zingiber officinalis, Salvia officinalis*, and *Petroselinum crispum*, but stronger than that of *Ammodaucus leucotrichus*, *Thymus vulgaris*, *Cymbopogon citratus*, *Origanum marjorana*, and *Rosmarinus officinalis*.

3.4.2. Antimicrobial activity: The antimicrobial activity of the EO was tested against eight microorganisms, as reported in Table 3.

Table 3. Antimicrobial activity of *Limnophila aromatica* essential oil based on sterile ring diameter (D).

Strains	Dilution concentration (C): µl/sterile ring diameter (D): mm			
	C_0 *(Original EO)*	$C_1=5^{-1}$	$C_2=10^{-1}$	$C_3=10^{-2}$
Escherichia coli ATCC 25922	D=13±0.20	D=10±0.04	D=6±0.00	D=6±0.00
Pseudomonas aeruginosa ATCC 27853	D=6±0.00	D=6±0.00	D=6±0.00	D=6±0.00
Enterococcus faecalis ATCC 29212™	D=6±0.00	D=6±0.00	D=6±0.00	D=6±0.00
Staphylococcus aureus ATCC 25923	D=12±0.08	D=6±0.00	D=6±0.00	D=6±0.00
Bacillus subtilis ATCC 6633	D=16±0.10	D=10±0.05	D=6±0.00	D=6±0.00
Listeria innocua ATCC 33090	D=14±0.09	D=8±0.09	D=7±0.10	D=6±0.00
Bacillus cereus ATCC 14579	D=10±0.10	D=6±0.00	D=6±0.00	D=6±0.00
Aspergillus niger ATCC 6275	D=25±1.20	D=6±0.00	D=6±0.00	D=6±0.00

D>6 mm: antimicrobial activity; D=6 mm: no-inhibition.

Table 3 illustrates that the pure EO (C_0) exhibited antimicrobial activity against six bacterial strains, including *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633, *Listeria innocua* ATCC 33090, *Bacillus cereus* ATCC 14579, and the fungus *Aspergillus niger* ATCC 6275. This activity may be attributed to the bactericidal properties of limonene, which is known to significantly inhibit both Gram-negative and Gram-positive bacteria as well as fungi [22].

Research by A. Gupta, et al. (2021) [23] demonstrated that limonene effectively disrupts cell membranes, causing leakage and cell death in *Escherichia coli*. Similarly, Y. Han, et al. (2021) [24] observed that limonene inhibited bacterial growth by damaging the cell morphology and wall integrity of *Staphylococcus aureus*, as confirmed through scanning electron microscopy (SEM) and fluorescence microscopy. As shown in Table 2, limonene constitutes 46.86% of the EO composition, likely contributing to its antimicrobial properties. Additionally, the presence of other compounds, such as β-cis-Ocimene, α-Pinene, and others, which are known for their antibacterial effects, may further enhance the oil's activity [25, 26].

At a 5^{-1} dilution (C_1), the EO remained effective against three bacterial strains: *Escherichia coli* ATCC 25922, *Bacillus subtilis* ATCC 6633, and *Listeria innocua* ATCC 33090. However, the fungus *Aspergillus niger* ATCC 6275 did not show any inhibition at this concentration. When the EO was diluted 10 times (C_2), inhibition was only observed for *Listeria innocua* ATCC 33090. No antimicrobial activity was detected when the oil was diluted 100 times (C_3).

Comparatively, the EO of *Mentha arvensis* L. var. *javanica* (Blume) Hook. f. [27] exhibited antimicrobial activity against *Listeria innocua* ATCC 33090 (D=9.0±0.2 mm), *Bacillus cereus* ATCC 10876 (D=8.0±0.03 mm), and *Bacillus subtilis* ATCC 6633 (D=7.0±0.05 mm) at a 10^{-1} dilution. These results demonstrate the strong antimicrobial potential of herbs like Limnophila aromatica EO on the tested bacterial strains.

4. Conclusions

The optimal parameters for the distillation of *Limnophila aromatica* EO were identified as follows: material size (5 mm), distillation time (2 hours), and a material-to-water ratio of 1:2 (g/ml). Under these conditions, the highest oil yield was 0.21 g per 200 g of material, corresponding to a distillation yield of 1.05‰ (*w/w*). Limonene, the primary component of the EO (46.86%), played a significant role in its biological activities. The EO exhibited antioxidant capacity with an IC_{50} value of 266.6 µg/ml and demonstrated antimicrobial activity against six bacterial strains: *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633, *Listeria innocua* ATCC 33090, *Bacillus cereus* ATCC 14579, and *Aspergillus niger* ATCC 6275. These results highlight the potential applications of *Limnophila aromatica* in pharmaceutical and cosmetic industries.

CRediT author statement

Nguyen Thi Bich Thuyen: Methodology, Data analysis, Writing - Reviewing and Editing; Tran Thanh Men, Ho Quoc Phong, Huynh Lien Huong: Methodology, Data analysis.

COMPETING INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this article.

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