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Chemical and physicochemical profiles of essential oil from *Piper betel (L.)* cultivated in different regions of Vietnam

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Abstract

This study aimed to investigate the variation of essential oils from Piper betel cultivated in three different geographic regions of Vietnam (Hoc Mon, Tien Giang, and Dak Lak). The extraction of essential oil was conducted by hydro-distillation for 3 h using a Clevenger-type apparatus. GC-MS was employed for the analysis of obtained essential oil. Physicochemical parameters were determined as per standard test methods. GC-MS revealed the highest amount of chavibetol present in all three essential oils of Piper betel cultivated in three different geographic regions of Vietnam: Hoc Mon (49.077%), Tien Giang (39.982%) and Dak Lak (34.888%). In contrast to the absence of Cadinene in both Hoc Mon and Tien Giang essential oils, there was remarkably an extremely high content of Cadinene present in Dak Lak essential oil, accounting for approximately 49% of the total constituents of essential oil. a-Cadinene was recorded to be the highest quantity (19.826%), followed by γ-Cadinene (15.470%) and δ-Cadinene (13.369%). Nature variation in different geographic regions of Vietnam (Hoc Mon, Tien Giang, and Dak Lak) plays a key role in the diversification of essential oil composition in Piper betel.

Keywords: Essential oil, geographic regions, GC-MS, Piper betel leaf, variations

Introduction

Piper betel (L.) (*P. betel*), commonly known as betel vine, belongs to the genus Piper of the Piperaceae family (Tutu et al., 2022). Betel vine is locally known as *Paan* (India), *Sulath* (Sri Lanka), *Ikmo* (Philippines), or *Phlu* (Thailand) (Azahar et al., 2020). Betel leaf is an evergreen, glossy, heart-shaped perennial climber. This species is widely cultivated for its leaves in tropical and sub-tropical regions such as India, Bangladesh, Sri Lanka, and other Asian countries. Betel leaves are commonly used for different purposes including culture, religion, and alternative and complementary medicine (Biswas et al., 2022). The practice of chewing betel leaves is truly a typical image of Vietnamese culture, penetrating the folk culture, folk songs, traditional festivals, and sacred rituals. Betel vine has long been traditionally used as herbal medicine mainly as an antiseptic in dental practice. In Malaysia, betel leaves have been traditionally used to treat joint pain, arthritis, and headaches. The juice of betel leaves has been applied to treat various skin ailments in Sri Lanka. Besides, its boiled betel leaves could be utilized as a tonic, astringent, or a cough remedy (Nayaka et al., 2021). However, betel vine nowadays is widely used to treat many diseases such as headaches, colds, coughs, sore throat, asthma, bad breath, rheumatic arthritis, conjunctivitis, constipation, wounds, cuts, and burns. Phytochemical analysis of betel leaf extract using different solvents revealed glycosides, sterols, saponins, flavonoids, alkaloids, tannins, and essential oils (Biswal, 2014). Notably, the distinct pungent aroma of betel leaves is attributed primarily to the essential oil containing a predominant quantity of terpenes and phenols (Biswas et al., 2022) existing in various proportions depending on the botanical origin of betel leaves.

Betel leaf essential oil (BLO) is a complex chemical mixture consisting of approximately 30 to 60 naturally

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occurring volatile compounds. In general, the quality and quantity of essential oil and its molecules might be extremely variable depending on various factors including plant species, genotypes, geographical origin, climatic conditions, cultivation practices, harvesting time, and experimental conditions (Madhumita et al., 2020). As indicated from a previous literature review, the most common molecules present in BLO are eugenol, chavicol, chavibetol, hydroxychavibetol, eugenol acetate, germacrene-D, isoeugenol, and safrole (Sahoo et al., 2023). Vietnam is best known as one of the leading growers, suppliers, and exporters of betel leaves to the global trade markets. Vietnamese betel leaves are widely consumed in overseas markets such as China, Taiwan, and Cambodia. In Vietnam, betel plants are grown in many regions across the nation ranging from the North to the South of Vietnam. Betel vine could be cultivated in small scales (home gardens) or large scales (farms). In this study, three different betel vine-growing regions, namely Hoc Mon, Dak Lak, and Tien Giang, were selected for the investigation (**Figure. 1**). Hoc Mon (HM) (*10°53'22'' N, 106°35'32'' E*) is a suburban district located in the Northwest of Ho Chi Minh City. Hoc Mon is famous for its 18 villages of betel gardens which have been established since the early 17th century. Dak Lak (*12°42'36" N, 108°14'15" E*) is a mountainous province located in the Central Highlands region of Central Vietnam. The province has a mild climate and approximately 300,000 hectares of fertile red basalt soil which would be ideal for growing perennial industrial plants. Tien Giang (*10°23'35'' N, 106°16'43'' E*) is a coastal province in the Mekong Delta of Southern Vietnam. The province's land is typically characterized by alluvial soil, accounting for approximately 55% of the natural area of the province (General Statistics Office of Vietnam, 2022).

Figure 1. Geographical map of three selected regions (highlight) for sampling.

Materials and methods

Plant Materials and Essential Oil Extraction

Fresh betel leaves were collected from various gardens and farms located in three different regions of Vietnam (HM, TG, and DL) (**Figure 2**) and then washed several times with running water to remove any impurities. This species was identified by Associate Professor Dr. Tran Van Minh of the Institute of Tropical Biology, Vietnam, and deposited in the herbarium of Applied Biochemistry Laboratory, Department of Applied Biochemistry, School of Biotechnology, International University, Vietnam National University–Ho Chi Minh City, Vietnam with voucher No. HB-BIO-10-09-03. The betel leaves were immediately air-dried, cut into small pieces, and then ground into fine powder using a mechanic grinder. The extraction of essential oil was carried out by hydro-distillation for 3 hours using a Clevenger-type apparatus. Once the distillation had been complete, the obtained essential oil was dehydrated using anhydrous sodium sulfate and then stored in sealed opaque brown bottles at 4℃ until GC-MS analysis.

GC-MS Analysis

Gas chromatography-mass spectroscopy (GC-MS) was employed for the analysis of obtained essential oil. GC-MS analyses were performed using SCION SQ 456-GC equipped with a Rxi-5ms RESTEX column (30 m x 0.25 mm x 0.25 µm). Helium was used as carrier gas at a constant flow rate of 1 mL/min. The oven temperature was initially programmed at 50℃ for 1 min and then increased to 80℃ at 30℃/min. Shortly afterward, it was increased to 230℃ at 5℃/min, and finally to 280℃ at 25℃/min where it was thermally held for 3 min. The injector temperature was set at 250℃ and the rate of Division was 1:30. Fragmentation was done by electron impact under a field of 70 eV. The mass spectra were recorded over the mass range of 50-500 amu with the full-scale mode at a rate of 1s/scan.

Physicochemical Analysis

The determination of the physicochemical parameters of essential oil was performed as per standard test methods. Physicochemical properties included in this research were refractive index, specific gravity, acid

Figure 2. *Piper betel* leaves from three different geographic regions of Vietnam- (A): Hoc Mon (Ho Chi Minh City); (B): Tien Giang and (C): Dak Lak

value, saponification value, ester value, and peroxide value. The obtained essential oils were also subjected to organoleptic evaluation including appearance, color, odor, and taste.

Determination of Refractive Index (RI)

The refractive index of essential oil was measured using a digital refractometer. The prism must be clean and dry prior to the test. Two drops of essential oil were applied to the prism using a disposable micropipette. Once the protective cover had been firmly closed, the power was turned on to take a measurement and the reading was recorded from the (ISO 489:2022, 2022).

Determination of Specific Gravity (SG)

The specific gravity of essential oil was determined using a 50-mL density bottle (pycnometer) according to the AOAC standard method (AOAC 920.212, 1920). The density bottle must be clean and dry prior to the assay. The empty dry bottle with a stopper was initially weighed on the analytical balance and documented as W_0. The bottle was then filled with distilled water and the stopper was inserted, followed by gently tapping the sides of the bottle to remove the air bubbles. Once the bottle had been carefully wiped off, it was weighed and recorded as W_1. The same process was repeated but using essential oil instead of distilled water. The bottle was then weighed and noted as W_2.

$$SG = \frac{W_2 - W_0}{W_1 - W_0}, \text{ where:}$$

W_0: Weight of empty specific gravity bottle
W_1: Weight of distilled water + specific gravity bottle
W_2: Weight of essential oil + specific gravity bottle

Determination of Acid Value (AV)

One gram of essential oil was accurately weighed into a conical flask containing 25 mL of ethanol mixed with 25 mL of diethyl ether. Two drops of phenolphthalein were added and then titrated with 0.1 N KOH solution until the color of the endpoint turned pale pink and must persist for at least 30 seconds (AOAC 969.17, 1995). The AV was then calculated using the following formula:

$$AV = \frac{V x N x 56{,}1}{W}, \text{ where:}$$

V: Volume of potassium hydroxide used
N: Normality of potassium hydroxide
W: Weight in g of the sample

Determination of Saponification Value (SV)

Two grams of essential oil were accurately weighed into a conical flask containing 30 mL of 0.2 N ethanolic KOH solution (AOAC 920.160, 1920). The mixture was then refluxed with continuous agitation for 2 h, followed by the addition of two drops of phenolphthalein. Shortly thereafter, the mixture was titrated with 0.5 N HCl until the pink color disappeared completely. Titration was simultaneously conducted for blank determination, and calculated by:

$SV = (T-B) \times N \times 56.1$, where:

B: Volume of HCl required by blank (mL)
T: Volume of HCl required by the oil sample (mL)
N: Normality of HCl
W: Weight of oil in gram

Determination of Ester Value (EV)

The ester value (ISO 709:2001, 2001) was determined based on the acid value and values using the following formula:

$EV = SV – AV$

Determination peroxide value (PV)

Two grams of essential oil were accurately weighed into the 250-mL glass-stoppered Erlenmeyer flask containing 30 mL of a mixture of three volumes of glacial acetic acid and two volumes of chloroform. The flask was promptly closed with the glass stopper and then swirled until completely dissolved. Shortly afterward,1mL of freshly prepared saturated KOH solution was added to the mixture. The flask was then closed and agitated for 60 sec in the dark, and subsequently, 30 mL of distilled water was added and titrated against a standard solution of sodium thiosulfate (0.01 N) till the yellow color was almost gone. Thereafter, 0.5 mL of starch solution (1%) was added to the mixture with continuous agitation, followed by titrating with sodium thiosulfate until the blue color disappeared. A blank titration was simultaneously carried out under the same condition (ISO 3960:2017, 2017). The volume of sodium thiosulfate was then recorded and the peroxide value (milliequivalent peroxide/ kg of sample) was calculated using the following formula:

$$PV = \frac{S \times N \times 100}{\text{Weight of essencial oil}}$$ where:

S: Volume of sodium thiosulfate (mL)
N: Normality of sodium thiosulfate

Statistical Analysis

All experiments were conducted in triplicate, and the results were expressed in terms of Mean ± Standard Error of Mean (SEM). Statistical analysis was performed by SPSS and analysis of variance (ANOVA) with the threshold of statistical significance at $p < 0.05$.

Results and Discussion

The obtained HMO and TGO were pale yellow oily liquids whilst DLO had a dark yellow (**Figure. 3**). All three essential oils were insoluble in water and soluble in non-polar organic solvents. There was generally no significant difference in odor and taste as they all had a creosote-like smell and a sharp bitter, pungent taste.

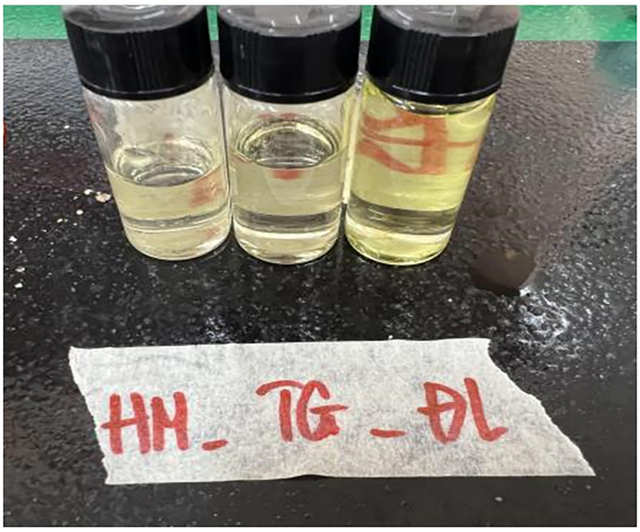

Figure 3. Essential oils of *Piper betel* leaves from three sampling regions Hoc Mon, Tien Giang, and Dak Lak, respectively (left-right)

The lowest yield of essential oil was recorded in HMO (0.152 ± 0.005%, w/w) whereas the highest yield was documented in TGO (0.404 ± 0.005%) which was approximately twice greater than that of DLO (0.208 ± 0.005%) (**Table 1**). There were no statistically significant differences in SV and EV between HMO and DLO; however, both these indices were found extremely high in TGO with the values of 101.327 ± 1.527 and 86.741 ± 0.012 mg KOH/g essential oil, respectively. Apart from SV and EV, there were no other significant differences in physicochemical parameters with regard to RI, SG, AV, and PV.

As expected, there were statistically significant differences in chemical constituents and their quantity among essential oils tested. HMO contains 18 compounds, representing approximately 97.460% of the total constituents of HMO. GC-MS analysis revealed the presence of chavibetol (49.077%), followed by other components: (1R,3aS,8aS)-7-Isopropyl-1,4-dimethyl-1,2,3,3a,6,8a-hexahydroazulene (11.811%), Naphthalene, 1,2,4a,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, [1S-(1α,4aβ,8aα)]- (7.547%), Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1S-cis)- (6.968%), Cyclohexene, 1-methyl-4-(1-methylethylidene)- (6.85%) and γ-Muurolene (4.066%). Meanwhile, TGO was composed of 16 compounds, accounting for approximately 98.636% of the total components of TGO. The major component was chavibetol (39.982%), followed by other components: (1R,3aS,8aS)-7-Isopropyl-1,4-dimethyl-1,2,3,3a,6,8a-hexahydroazulene (14.337%), Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1S-cis)- (13.057%), Naphthalene, 1,2,4a,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, [1S-(1α,4aβ,8aα)]- (10.096%), γ-Muurolene (4.542%) and Cyclohexene, 1-methyl-4-(1-methylethylidene)- (3.685%). DLO, on the other hand, consisted of 13 components, composing approximately 96.800% of the total components of DLO. The predominant constituent present in DLO was chavibetol (34.888%), followed by other components: α-Cadinene (19.826%), γ-Cadinene (15.470%), δ-Cadinene (13.369%) and γ-Muurolene (5.266%). Compounds that made contributions of less than 3% each to the total composition of essential oils are also presented in **Table 2**.

In this study, chavibetol (m-eugenol) was recorded to be the highest component present in all three essential oils tested, but at significantly different concentrations. As reported from previous literature, chavibetol, an isomer of eugenol, has been scientifically proven to possess

Table 1. Yields and physicochemical properties of essential oils from *Piper betel* leaves cultivated in three different geographic regions of Vietnam

Physicochemical properties	Description/Value		
	Hoc Mon	Tien Giang	Dak Lak
Appearance	Oily liquid		
Solubility	Immiscible in water, but soluble in non-polar organic solvents		
Color	Pale yellow	Pale yellow	Dark yellow
Odor	Creosote-like note		
Taste	Sharp bitter, pungent taste		
Yield of essential oil (%,w/w)	0.152 ± 0.005	0.404 ± 0.005	0.208 ± 0.005
Refractive index (27℃)	1.472 ± 0.001	1.471 ± 0.001	1.480 ± 0.001
Specific gravity	0.802 ± 0.001	0.927± 0.001	0.804 ± 0.001
Acid value (mg KOH/g)	12.342 ± 0.577	14.586 ± 0.001	10.659 ± 0.577
Saponification value (mg KOH/g)	25.176 ± 1.527	101.327 ± 1.527	27.396 ± 1.528
Ester value (mg KOH/g)	12.834 ± 0.015	86.741 ± 0.012	16.737 ± 0.040
Peroxide value (meq peroxide/kg)	0.046 ± 0.001	0.058 ± 0.006	0.044 ± 0.001

The data are presented as mean ± SEM and $p < 0.05$.

Table 2. Chemical composition of essential oils of *Piper betel* cultivated in three different geographic regions of Vietnam (Hoc Mon, Tien Giang, and Dak Lak)

No.	**Identified compound**	**Quantity (%,w/w)**		
		Hoc Mon	**Tien Giang**	**Dak Lak**
1	α-Phellandrene	0.947	0.504	0.579
2	Bicyclo[3.1.0]hexan-2-ol, 2-methyl-5- (1-methylethyl)-,(1α,2β,5α)-	0.627	-	-
3	Cyclohexene, 1-methyl-4-(1-methylethylidene)-	6.850	3.685	-
4	Chavicol	-	-	0.405
5	Chavibetol	49.077	39.982	34.888
6	Ylangene	0.595	0.750	-
7	β-Elemene	-	-	1.281
8	Cyclohexane, 1-ethenyl-1-methyl-2,4-bis(1-methylethenyl)-, [1S-(1α,2β,4β)]-	1.008	1.219	-
9	Caryophyllene	1.342	1.440	1.419
10	Humulene	0.72	0.737	0.788
11	γ-Muurolene	4.066	4.542	5.266
12	(1R,2S,6S,7S,8S)-8-Isopropyl-1-methyl-3-methylenetricyclo[4.4.0.02,7]decanerel-	2.146	2.427	-
13	Naphthalene, 1,2,4a,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1α,4aα,8aα)-	1.945	2.922	-
14	Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4-methylene-1-(1-methylethyl),(1α,4aβ,8aα)-	0.909	-	-
15	Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1S-cis)	6.968	13.057	-
16	δ-Cadinene	-	-	13.369
17	β-Longipinene	0.556	0.523	0.602
18	Naphthalene, 1,2,3,4,4a,7-hexahydro-1,6-dimethyl-4-(1-methylethyl)-	0.793	0.886	-
19	Eremophila-1(10),8,11-triene	-	-	1.032
20	Naphthalene, 1,2,4a,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, [1S-(1α,4aβ,8aα)]-	7.547	10.096	-
21	α-Cadinene	-	-	19.826
22	Copaene	1.453	1.629	1.885
23	(1R,3aS,8aS)-7-Isopropyl-1,4-dimethyl-1,2,3,3a,6,8a-hexahydroazulene	11.811	14.337	-
24	γ-Cadinene	-	-	15.470

significant pharmacological activities such as antiseptic, antimicrobial, and anti-inflammatory capacity. In view of the whole components present in three essential oils, remarkably, all molecules present in TGO could be found in HMO, but at different concentrations. Notably, GC-MS analysis revealed the presence of six other compounds in all three EOs (**Figure. 4-B**). However, two components were only detected in HMO (**Figure. 4-C1**) and six in DLO (**Figure. 4-C2**).

Perhaps the most remarkably significant variation in the composition of essential oils tested was the absence of cadinene in both HMO and TGO, but extremely high amounts in DLO, accounting for approximately 49% of total constituents of DLO. Cadinenes, chemically known as bicyclic sesquiterpenes, were isolated from various essential oil-bearing plants. Cadinenes are a group of isomeric compounds, including α-cadinene, β-cadinene, γ-cadinene and δ-cadinene. In this study, three cadinene isomers were detected, namely α-cadinene (19.826%), γ-cadinene 15.470% and δ-cadinene (13.369%). Cadinene has been previously reported to possess various pharmacological properties including antioxidant, anti-

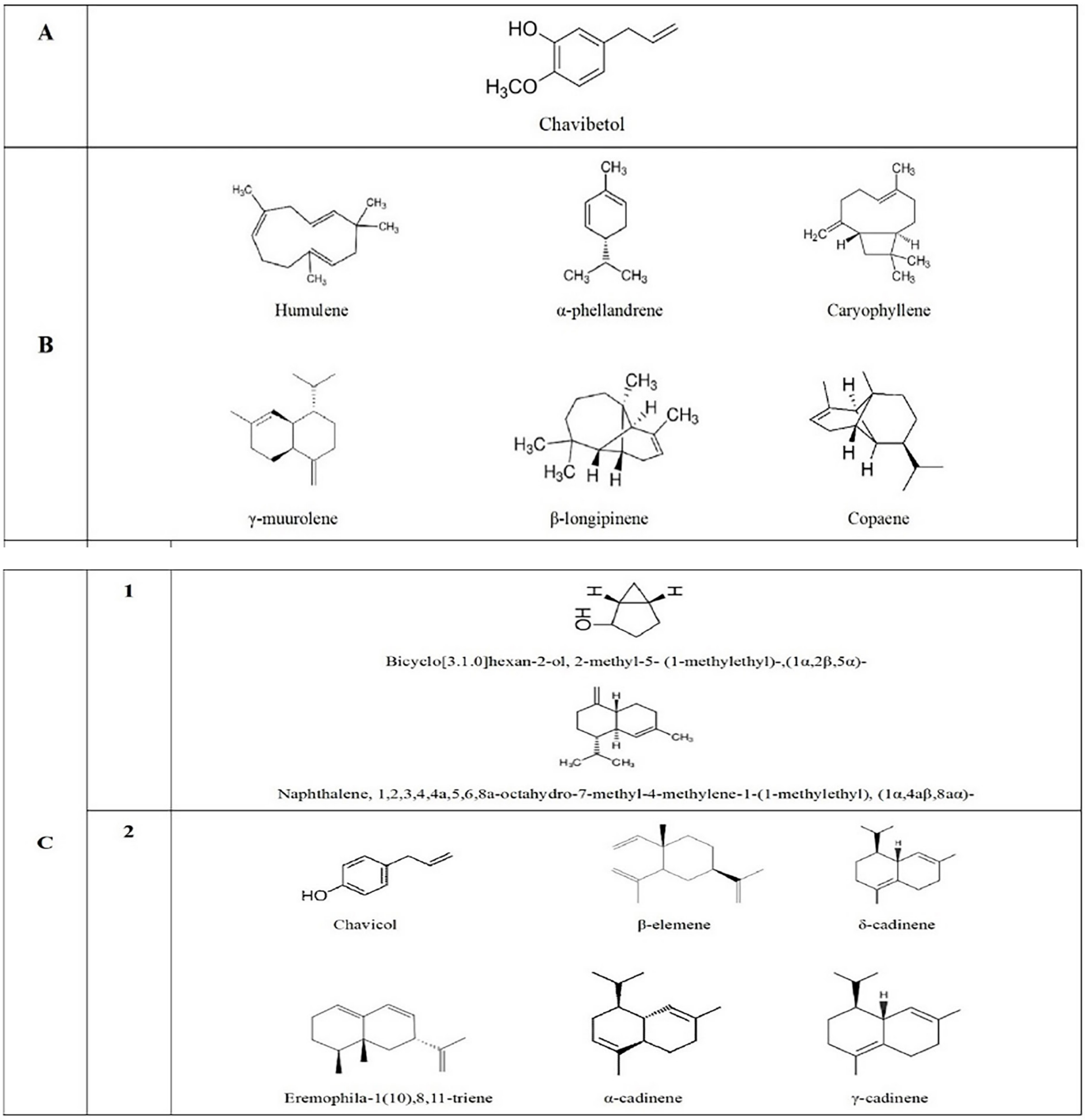

Figure 4. Major chemical compositions of essential oils from Piper betel cultivated in three different geographic regions. (A): Chavibetol; (B): Six components present in all samples; (C1): Two components in Hoc Mon essential oil only; (C2): Six components in Dak Lak essential oil only.

inflammatory, antimicrobial, antifungal, and insecticidal. In addition, cadinene is believed to be helpful in improving mood and has had potential as an antidepressant agent (Aditi Kundu et al., 2013)

It should be noted, nevertheless, that the biological activities of essential oil are not always correlated with the variation of essential oil composition. Indeed, essential oil is not composed of a single molecule. Instead, essential oil is a complex mixture of natural volatile organic compounds. There are thus no general principles relevant to postulate the correlation between the biological properties and essential oil composition. Practically, the interaction between molecules present in essential oil may likely result in antagonistic, indifferent, additive, or synergistic biological effects. Furthermore, nature is not always consistent. Natural conditions may be likely variable from time to time. Moreover, apart from natural variation, unpredictable natural disasters and human-induced factors may also dramatically influence the natural conditions leading to the significant variation of the secondary metabolites in general and essential oil in particular present in plants. Unpredictable natural disasters can be volcanic eruptions, bushfires, drought, floods, haze, cyclones, heat waves, and saline water intrusion. Greenhouse effects, on the other hand, are one of the typical examples of human-driven causes of climate change.

In view of the natural conditions of HM and TG, both regions have a tropical monsoon climate with an annual average temperature of around 27-28℃ and the average relative humidity is approximately 82-84% throughout the year. Annual average rainfall is approximately estimated to be 1467 mm in TG and 1398 mm in Ho Chi Minh City (Ministry of Transport of Vietnam, 2000). DL, on the other hand, features the "tropical wet and dry or savanna" climate (Köppen classification: Aw) with an annual average temperature of around 24-25℃ and the average relative humidity is approximately 78-80% throughout the year. Annual average rainfall is around 1552 mm (Dinh et al., 2022). Perhaps types of soil could be one of the key factors considerably contributing to the variation in essential oil composition. HM (as part of Ho Chi Minh City) and TG's soils are generally rich in both macro- and micronutrients, minerals, and organic matter, slightly acidic, and high basic saturation. In addition to nutrients, alluvial soil is porous and has a high level of aeration, owing to the abundant presence of microorganisms (Nguyen et al., 2019). Red basalt soil, on the other hand, is generally rich in iron and aluminum oxides and has poor organic matter content and low pH. This type of soil lacks potassium, calcium, magnesium, nitrogen, and phosphate (Thuy T. Nguyen & Anh The Luu, 2017).

In addition to the variation in essential oil composition, noticeably, there was also a typically remarkable variation in the phytomorphological features of betel leaves. Indeed, by visual examination, DL betel leaves are characteristically smaller in size and virtually thinner in texture than those cultivated in HM and TG (Figure 2). Overall, there is no doubt that different natural conditions from different geographic regions of Vietnam, namely Hoc Mon, Tien Giang, and Dak Lak, considerably attributed to the variation in essential oil composition from betel leaves. This present research, suffice it to say, scientifically contributes to the herbal medicine database system, particularly the roles of geographic regions in varying natural components of essential oil. However, further studies are necessary to extensively investigate the pharmacological potentials of isolated essential oils and their single molecules. Besides, their adverse reactions and toxicity may also need to be included in future research.

Conclusions

Chavibetol was recorded to be the highest value in all three essential oils of *Piper betel* cultivated in three different geographic regions of Vietnam, namely Hoc Mon, Tien Giang, and Dak Lak. α-Cadinene, γ-Cadinene, and δ-Cadinene were the major components present in Dak Lak essential oil, but not detected in both Hoc Mon and Tien Giang essential oils. Nature variation truly plays a crucial role in diversifying the composition of essential oil in *Piper betel* planted in different geographic regions of Vietnam. Future research should focus more on geographical scope, investigating molecular mechanisms, assessing health benefits, developing novel pharmaceutical products, and considering other varieties within this species based on geographical and environmental factors.

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Conflict of interest

The authors declare to have no conflicts of interest.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.