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Terpenoid Profiling of Thai Strain Cannabis Leaves (*Cannabis sativa* L. subsp. *sativa*) by Headspace (HS) Couple with GC/MS

Phurin Watanakul[1], Jessada Phattaralerphong[2], Kitsada Pitija[3], and Pattarawadee Sumthong Nakmee[1*]

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ABSTRACT

Introduction: Cannabis terpenoids, especially volatile terpenes, were used for the classification of cannabis strains. The leaves of *Cannabis sativa* L. subsp. *sativa* Thai strain 'Hang Krarok' are used legally in traditional Thai medicines, cosmetics, and food ingredients in Thailand under the control of the tetrahydrocannabinol (if lower than 0.2% dry weight). One of the specific characteristics of this plant is the volatile oil which consists of mono-and the sesqui-terpenoids.

Materials and methods: Fresh cannabis leaves were ground and 1 g samples were kept in gas chromatography/mass spectrometry glass vials at 4 °C prior to measurement using headspace.

Results: More than 50 terpenoids were identified from the fresh leaves in the cannabis samples. The major compounds were *β*–ocimene, L–limonene, terpinolene, p–cymenene, *β*–(E)–caryophyllene, (Z,E)–*α*–farnesene, *β*–bisabolene, and (E)–*α*–bisabolene.

Conclusion: The variation in the unique terpenoids in the Thai strain could be used in novel medicines and food and cosmetic products.

KEYWORDS:

Cannabis sativa, terpenoids, Headspace-gas chromatography/mass spectrometry (HS-GC/MS)

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***Corresponding author:**
Asst. Prof. Dr. Pattarawadee Sumthong Nakmee
Email: pattarawadee.sum@ku.th
Phone: +66 (0) 38 354581
Fax:+66 (0) 38 354 587

Authors' Affiliation:

[1] *Faculty of Science at Sriracha, Kasetsart University Sriracha Campus, 199 Moo 6, Sukhumvit Rd., Sriracha, Chonburi, 20230, Thailand.*
[2] *Faculty of Natural Resource and Agricultural Industry, Kasetsart University, Chalermprakiat Campus, Chiang Khruea, Muang, Sakon Nakhon, 47000, Thailand.*
[3] *PerkinElmer Scientific (Thailand) Co., Ltd. (Head Office) No. 290 Soi Soonvijai 4, Bangkapi, Huay Kwang, Bangkok, 10310, Thailand.*

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Introduction

A Thai strain of cannabis (*Cannabis sativa* L. Cannabaceae) known as 'Hang Krarok' (squirrel tail) is commonly grown in Sakon Nakhon province, northeast Thailand. The leaves have been used as a folk medicine for the treatment of epilepsy, paralysis, stomachache, nausea, lack of appetite, and as a sleeping aid. In traditional Thai medicines, leaves with a size equal to or bigger than a human hand are recommended for the recipes, such as for a sleeping aid, lower abdominal pain, and paralysis (Duangdamrong et al., 2022; Pussapaphan & Busabong, 2022).

Cannabis is an dioecious annual plant with an erect stem 0.2−6 m height (UNODC, 2019). Leaves are palmately compound with 5−11 pointed, serrate leaflets 5−15 cm long and 1−2 cm wide. The female inflorescence is 10−30 cm long and 4−8 cm in diameter. The nearly spherical achene seeds with a color range from light brown to dark gray, are about 2.5−4 mm in diameter and are 3−6 mm long (Ehrensing, 1998).

Terpenoids in *Cannabis sativa* L. play an important role in the biosynthesis of the cannabinoids that contribute to the much-appreciated aroma and flavor of cannabis seed oil (Booth and Buhlmann, 2019; Zager et al., 2019). More than 200 terpenoids have been identified in cannabis, with the main constituents being mono-and sesqui-terpenes (Gallily et al., 2018). Flower buds contain about 0.5−3.5% essential oil (Fischedick et al., 2017). However, the ratios differ between the mono-and sesqui-terpenes in cannabis inflorescence and leaves (Gaggiotti et al., 2020; Casano et al., 2010). In addition, the cannabinoid-terpenoid ratio from the herbal extract supports the synergistic action of cannabinoids and terpenes in pain management, analgesia, cancer, and severe epilepsy (Brousseau, et al., 2021). Dziok et al (2021) reported the positive effect of *Cannabis sativa* L. extract on skin care and there are cannabis-based products used for skin inflammatory disease (Martin et al, 2022).

Volatile cannabis products are usually analyzed using gas chromatography coupled with flame ionization (GC-FID) or a mass spectrometric detector (GC/MS) (Casano et al., 2010; Fischedick et al., 2010; Giese et al., 2015; Ibrahim et al., 2019; Jin et al., 2017; Choi & Verpoorte, 2019). In addition, headspace (HS) coupled with GC-FID, HS-GC/MS, and solid phase headspace micro-extraction GC/MS have been used to determine the volatile constituents in *Cannabis sativa* L. inflorescence (Omar et al., 2014; Porto et al., 2014). The advantages of using headspace GC/MS are fast and simple by directly injected samples without extraction plant materials as for GC/MS or GC-FID. But the fresh samples needed to keep in GC/MS vials under cold temperature before directly injection. Thus, the aim of the present study was to characterize the terpenoids in the fresh leaves of the Thai medicinal strain of cannabis based on HS-GC/MS analysis.

The biochemical profiles of terpenoids in cannabis are more influenced by genetic factors than by the environment (Casano et al., 2014; Gillily et al., 2018). Cannabis terpenoids, especially volatile terpenes, could be used for the classification of cannabis strains. Thus, the identification of terpenoids in Thai cannabis leaves can be used to compare these results with other cannabis varieties and to learn more about the characteristic compounds in the Thai strain that are related pharmaceutical, food, and cosmetic industrial use.

Materials and methods

Cannabis leaf collection

Cannabis sativa L. Thai strain ('Hang Krarok') plants were grown in a greenhouse at Kasetsart University, Chalermprakiat Campus, Chiang Khruea, Muang, Sakon Nakhon, Thailand under a growth license (License number 6/2562) issued by the Narcotics Control Division, Food and Drug Administration, Ministry of Public Health, Thailand. The cannabis leaves were collected during 5−6 AM after growing for 50 days. The leaves were 15–20 cm long, which was consistent with the recommendations for traditional Thai medicine use. The fresh leaves were ground and 1 g samples were stored in HS-GC/MS glass tubes (size 22 ml) and kept at 4 °C in darkness prior to HS-GC/MS analysis.

Turbomatrix headspace extraction

The cannabis leaf samples were analysed using HS-GC/MS (Perkin Elmer; GC type Clarus 680; and MS type Clarus SQ8C; USA) at the Faculty of Science, Kasetsart University Sriracha Campus, Thailand under production license number 21/2563 and occupancy license number 43/2563 issued by the Narcotics Control Division, Food and Drug Administration, Ministry of Public Health, Thailand. Static HS analysis was carried out using a model TurboMatrix 40 Headspace Sampler (PerkinElmer Ltd; USA) as shown in Fig.1. Each sample of cannabis leaves (1 g) was placed in a 20 ml HS bottle. The temperatures for extraction, the needle, and transfer line were 125, 130, and 135 °C, respectively. The times for extraction, pressurization, and injection were 25, 1.5, and 0.03 min, respectively.

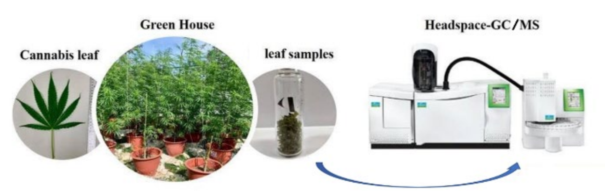

Figure 1: Cannabis leaf samples for Headspace-GC/MS analysis.

GC/MS analysis for separation and identification of cannabis volatiles

GC/MS analysis was performed using an Clarus model 690 gas chromatography (PerkinElmer, MA, USA) coupled to a model SQ8 mass-selective detector. The specifications for the PerkinElmer Elite-5M capillary column (USA) were 5% phenylmethylpolysiloxane, 30 m × 320 µm ID × 0.25 µm film thickness. Initially the oven temperature was held at 60 ºC, then increased at a rate of 7 ºC/min to a final temperature of 200 ºC. The injector temperature was 200 ºC. Purified helium was used as the carrier gas at a flow rate of 1 ml/min. Electron Ionization mass spectra were collected at a 70 eV ionization voltage over the m/z range 45–550. The electron multiplier voltage was 1,400 V. The ion source and quadrupole temperatures were both set at 200 ºC. The identification of terpenoids was based on Kováts retention indices (Adams 2001), relative to C_8–C_{22} n-paraffin hydrocarbon mixtures and mass spectral data comparison with database libraries (NIST, 2019), supported by the linear temperature program retention indices data (LTPRI), which were calculated from retention times on the first column. Retention times of known standard compounds were also used to confirm identities.

Results and Discussion

This is the first reported of terpenoid profile from *Cannabis sativa* L. subsp. *sativa* Thai strain 'Hang Krarok' leaves. The identification of terpenoid compounds in the leaves of *C. sativa* Thai strain 'Hang Krarok' was performed using HS-GC/MS (Fig. 1), which showed the separation of 53 terpenoids components (Table 1 and Fig. 2). The 53 signals were classified as 16 monoterpenes, 19 oxygenated monoterpenes, 11 sesquiterpenes, and 7 oxygenated sesquiterpenes. The major monoterpenes were β−ocimene and L−limonene, with terpinolene and *p*−cymenene as the major oxygenated monoterpenes, β−(E)−caryophyllene and β−bisabolene as the major sesquiterpenes, and (Z,E)−α−farnesene and (E)−α−bisabolene as the major oxygenated sesquiterpenes. Gaggiotti et al. (2020) and Porto et al. (2014) found monoterpenes and sesquiterpenes in different amounts and caryophyllene was the most abundant in *Cannabis sativa* L. inflorescence based on their GC/MS analysis. However, Porto et al. (2014) found the major compound caryophyllene in both fresh inflorescence and inflorescence extracted with supercritical carbon dioxide using HS-SPME combined with GC/MS analysis, as well as detecting major terpenoids, such as α–pinene, myrcene, limonene, terpinolene, caryophyllene, and farnesene from *Cannabis sativa* (hemp) inflorescence that had been grown and produced in northern Italy (Carnia region). In the present experiment, the *Cannabis sativa* L. Thai variety, which was grown in northeastern Thailand had cymenene as the most abundance terpenoid, followed by terpinolene, caryophyllene, bisabolene, and limonene, respectively. Thus, volatile terpenoid profiling could be used for the classification of *Cannabis* strains. Not only was the strain different (genetic factors), but in addition, environmental factors (such as soil, temperature, and moisture) influenced the profile and the ratios of plant natural compounds (Li et al., 2020; Sommano et al., 2020; Borges et al., 2017).

The most abundant mono-and sesqui-terpenoids identified in the cannabis Thai strain ('Hang Krarok') were *p*–cymenene, followed by terpinolene, β–(E) – caryophyllene, β–bisabolene, L–limonene, and β–ocimene (Fig.3). *p*–Cymenene was only found in the leaves of this Thai cannabis strain. This compound, possibly in combination with other cannabis terpenoids, caused the specific odor of cannabis Thai strain ('Hang Krarok') leaves. Some terpenoids found in cannabis, such as bisabolene, are used as a food additive and have pharmaceutical properties (Jou et al., 2015; Jou et al., 2016; Yeo et al., 2016), which is consistent with some traditional uses of cannabis leaves in Thailand. Nevertheless, some other compounds (or the synergistic effect of many compounds) in cannabis can be the source of other therapeutic effects. Cymenene, the most abundance terpenoid in the cannabis Thai strain leaves, is an oxygenated monoterpene present in the essential oil of *Ageratina pentlandiana* leaves with antibacterial activity (Quispe et al., 2019) and is also found in the essential oil of *Limbarda crithmoides* with antioxidant activity (Andreani et al., 2013). Another oxygenated monoterpene, terpinolene, is not only found in cannabis, but also in various plant sources, such as pine and fir trees, sage, apple, tea tree, and lemon and is widely used as a flavoring agent in the industry (Menezes et al., 2021). A sesquiterpene, caryophyllene, has been reported as a prominent constituent in many cannabis varieties (including the Thai variety in the present experiment) and caryophyllene oxide has been reported as a main component for cannabis identification by drug-sniffing dogs (Booth & Buhlmann, 2019; Gaggiotti et al., 2020). Bisabolene is in a group sesquiterpenes that has been used as food additives, with both β–and γ–bisabolene having anti-cancer properties (Jou et al., 2015; Jou et al., 2016; Yeo et al., 2016). In addition, bisabolene was identified in hop, lemon, and oregano. Limonene, a monoterpene, has been identified in the inflorescence of *Cannabis sativa* cultivars in Canada and the Netherlands (Jin et al., 2017; Fischedick et al., 2010). However, normally, it is found in lemons and other *Citrus* sp. (Nuutinen, 2018). Limonene has reported to have significant antimicrobial activity (Thielmann and Muranyi, 2019). Limonene, ocemene, terpinolene, caryophyllene, and bisabolene have been identified in the non-psychoactive chemotypes of *Cannabis sativa* harvested in Slovenia (Gallily et al., 2018).

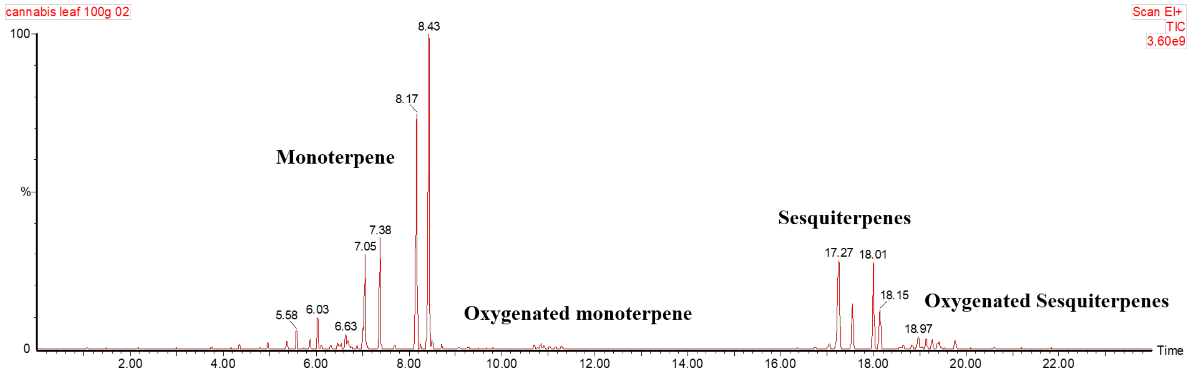

Figure 2: Headspace GC/MS chromatogram of terpenoid compounds in leaves of Cannabis sativa L. Thai strain ('Hang Krarok').

Table 1: Percentages of identified terpenoid compounds in leaves of Cannabis sativa L. Thai strain ('Hang Krarok') using HS-GC/MS.

Retention Time (min)	Assigned compound	Ri	Percentage relative abundance (%)±SD
	Monoterpenes		
4.34	Sabinene	897	0.3453 ± 0.03
4.96	Camphene	943	0.4253 ± 0.03
5.36	*β*–Pinene	979	0.5981 ± 0.03
5.58	*α*–Pinene	937	1.3889 ± 0.06
5.87	(+)–Camphene	952	0.7545 ± 0.03
6.03	*β*–Myrcene	958	2.1678 ± 0.07
6.11	*α*–Phellandrene	969	0.2918 ± 0.01
6.31	Ocimene	976	0.2513 ± 0.01
6.63	3,7,7–Trimethyl–1,3,5–Cycloheptatriene	971	1.1975 ± 0.01
6.69	*γ*–Terpinene	1060	0.9139 ± 0.02
6.74	*p*–Cymene	1022	0.1541 ± 0.01
6.87	*o*–Cymene	1025	0.2643 ± 0.01
7.01	Limonene	1030	1.2034 ± 0.06
7.07	*β*–Ocimene	1037	6.4091 ± 0.30
7.38	L–Limonene	1031	7.3742 ± 0.11
7.69	*γ*–Terpinene	1060	0.2671 ± 0.01
	Oxygenated monoterpenes		
8.19	Terpinolene	1088	17.3488 ± 0.82
8.25	*p*–Cymenene	1090	21.7511 ± 1.03
8.44	Isoterpinolene	1089	0.5967 ± 0.03
8.50	Cineole	1089	0.3073 ± 0.30
8.69	Linalool	1099	0.3217 ± 0.01
8.86	Fenchone	1121	0.0238 ± 0.01
9.05	Camphor	1121	0.1071 ± 0.01

Retention Time (min)	Assigned compound	Ri	Percentage relative abundance (%)±SD
9.25	exo–Fenchol	1116	0.1128 ± 0.01
9.48	Isoborneol	1138	0.0463 ± 0.01
9.61	Borneol	1138	0.0188 ± 0.01
9.67	*α*–Terpineol	1143	0.0331 ± 0.01
9.78	*γ*–Terpineol	1143	0.0391 ± 0.01
9.89	Isopulegol	1196	0.0203 ± 0.01
10.76	Carveol	1219	0.2578 ± 0.03
10.76	Pulegone	1212	0.0295 ± 0.01
10.82	Carveol	1219	0.3083 ± 0.03
10.89	Nerol	1228	0.1981 ± 0.01
11.02	Geraniol	1228	0.1986 ± 0.01
11.14	Geranyl acetate	1352	0.1559 ± 0.01
Sesquiterpenes			
16.05	*α*–Cubebene	1351	0.0611 ± 0.01
16.71	7–epi–Sesquithujene	1391	0.1294 ± 0.01
16.81	(Z) –*β*–Caryophyllene	1405	0.0159 ± 0.01
16.99	(E) –*α*–Bergamotene	1435	0.5373 ± 0.03
17.21	*β*–(E)–Caryophyllene	1419	11.2503 ± 0.81
17.31	(Z)–*β*–Farnesene	1444	0.0054 ± 0.01
17.68	Z,E)–*α*–Farnesene	1491	4.3015 ± 0.25
17.96	*β*–Bisabolene	1509	7.5583 ± 0.44
18.09	(E)–*α*–Bisabolene	1512	4.1021 ± 0.23
18.78	Germacrene B	1527	0.3473 ± 0.01
18.92	Aromandendrene	1530	1.1464 ± 0.06
Oxygenated sesquiterpenes			
19.09	Caryophyllene Oxide	1537	0.9545 ± 0.05
19.22	Cedrol	1543	0.7272 ± 0.06
19.34	Cis–Nerolidol	1564	1.0488 ± 0.04
19.37	(E)–Nerolidol	1564	1.0488 ± 0.04
19.49	*α*–Humulene	1579	0.0531 ± 0.01
19.72	Guaiol	1614	0.7604 ± 0.03
20.12	*α*–Bisabolol	1625	0.0682 ± 0.03

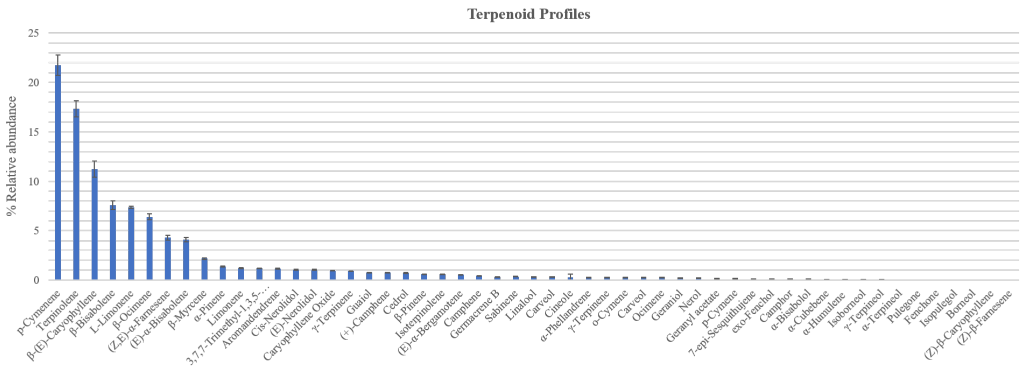

Figure 3:Profile of terpenoid compounds in leaves of Cannabis sativa L. Thai strain ('Hang Krarok') based on HS-GC/MS analysis

However, the absolute quantitative analysis for identification of terpenoids in the Thai strain of *Cannabis sativa* L. using internal standard could be further studied to identify precious major bioactive terpenoids.

Cannabis extracts can be a valuable source of biologically active substances that reduce oxidative stress, inhibit skin aging processes, and positively affect the viability of skin cells. Hydrogels based on cannabis extracts have a positive effect on skin hydration (Dziok et al., 2021). *Abies sibirica* terpenes had potential anti-aging and anti-cancer effects on senescent and cancer cell lines in human cells (Kydryavtseva et al., 2016). Thus, further study could focus on cannabis terpenes for skin treatment and therapy.

The green material left over from the production of medicinal cannabis could be an interesting source for various novel products. For pharmaceutical applications, the traditional uses could provide good indicators for producing interesting medicines that might also have a positive connection to cosmetic usage. In addition, the essential oil could be tested for various applications, such as an insect repellent, for food conservation, or as a taste enhancer. Further studies on these activities are needed as well as a proper analysis of the variability of the metabolome of the Thai strain and a comparison with other preparations on the market. The level of cannabinoids in the essential oil will be an important factor in obtaining licensed approval for the sale and use of such cannabis products.

Conclusion

Monoterpenes and sesquiterpenes were the main groups of volatile compounds from *Cannabis sativa* L. subsp. *sativa* Thai strain 'Hang Krarok' fresh leaves using Headspace-Gas Chromatography/Mass Spectroscopy. The major compounds were *β*–ocimene, L–limonene, terpinolene, *p*–cymenene, *β*–(E)–caryophyllene, (Z,E)–*α*–farnesene, *β*–bisabolene, and (E)–*α*–bisabolene. This terpenoid profile was different from other strains which has been reported in other country (Gaggiotti et al., 2020 and Porto et al., 2014). Thus, the volatile terpenes could be used for the classification of *Cannabis* strains in all over the world. Moreover, the variation in the unique terpenoids of cannabis Thai strain could be used to produce novel medicines, food ingredients and cosmetic products.

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Conflict of Interest

The authors declare no conflicts of interest.

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