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Research Article

Comparative Analysis of Volatile Oils from Fresh and Grilled *Citrus hystrix* Peels: Phytochemicals and Antifungal Activity

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ABSTRACT

In Thai traditional medicine, grilled fruits of *Citrus hystrix* DC. are halved and applied to the scalp for anti-dandruff, anti-itching, and hair nourishment purposes. However, no studies have reported phytochemical profile and antifungal activity of volatile oil extracted from peels of grilled *C. hystrix* fruits. This study aimed to analyze phytochemical profile and evaluate antifungal activities against cutaneous pathogenic fungi as *Candida albicans*, *Candida tropicalis*, *Trichophyton rubrum*, *Trichophyton mentagrophytes*, *Microsporum canis*, and *Nannizzia gypsea* of volatile oil extracted from peels of grilled *C. hystrix* fruits (G-ChVO) in comparison with those extracted from fresh peels (F-ChVO). Chemical composition of the volatile oils was determined using GC-MS. The analysis revealed that F-ChVO contained β-pinene (22.02%), D-limonene (18.22%), and terpinen-4-ol (11.54%), whereas G-ChVO contained β-pinene (7.66%), D-limonene (10.86%), and terpinen-4-ol (23.93%). The grilling process reduced content of β-pinene and D-limonene while content of terpinen-4-ol was increased. Antifungal activities were assessed using disk diffusion method with different concentrations of volatile oils and incubation periods. G-ChVO exhibited significantly the growth inhibitory activity against all six pathogenic fungi greater than F-ChVO. At a concentration of 10 µL/disc and incubation period of 5 days, G-ChVO demonstrated the highest antifungal activity, particularly against *C. albicans*. Thus, the grilling process altered chemical composition in volatile oils, leading to enhanced antifungal activity. The volatile oils extracted from the peels of grilled *C. hystrix* fruits have potential as a natural antifungal agent against cutaneous pathogenic fungi.

Keywords:
Grilled *Citrus hystrix, Citrus hystrix,* volatile oil, antifungal activity

1. INTRODUCTION

Fungal infections affect approximately 13 million people worldwide, with the prevalence continuing to rise[1]. Cutaneous mycoses, caused by dermatophytes and non-dermatophytes, commonly affect the skin, nails, and hair. Symptoms such as irritation, lesions, and inflammation are frequently observed[2]. In severe cases, these infections may progress to fungal wounds, disfigurement, and reduced quality of life, with complications such as sepsis occurring in advanced stages[3,4]. A fungal infection of the skin, hair, or nails caused by dermatophytes is called dermatophytosis. Dermatophytosis occurs commonly in both animals and humans. The primary fungal pathogens responsible for these infections include *Trichophyton rubrum*, *T. mentagrophytes*, *Microsporum canis*, and *Nannizzia gypsea*[5]. These species are considered significant zoophilic dermatophytes capable of causing zoonotic transmission to humans.

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A retrospective study in Thailand reported that infections with *T. rubrum*, the *T. mentagrophytes* complex, and *M. canis* were associated with cases of tinea faciei, particularly in women and individuals who had close contact with pets[6]. Among them, *M. canis* is recognized as the most prevalent zoophilic dermatophyte[7]. It is known to cause tinea corporis and tinea capitis in individuals exposed to infected animals[8]. Tinea capitis is observed among children in nursery care, where *M. canis* is often the causative agent[9]. Outbreaks of *M. canis* infections have been reported in both household environments [8] and military facilities[10]. It has been typically transmitted through direct contact, either animal-to-human or person-to-person. Additionally, the geophilic fungus *N. gypsea* has been isolated from domestic animals such as dogs and cats. It is capable of causing tinea capitis in humans. Consequently, *T. rubrum*, *T. mentagrophytes*, *M. canis*, and *N. gypsea* are of clinical significance as zoophilic dermatophytes contributing to human fungal infections. With the increasing prevalence of pet ownership and the growing trend of pet humanization, the prevention of zoonotic fungal transmission is becoming increasingly important for both human and animal health.

This study also focuses on *Candida albicans* and *C. tropicalis*, which are well-known opportunistic fungal pathogens capable of causing a wide spectrum of diseases, ranging from superficial mucosal infections to life-threatening systemic mycoses. *C. albicans* is typically a commensal organism in healthy individuals and remains non-pathogenic under normal conditions. However, it can become pathogenic in immunocompromised individuals or those with disrupted microbial flora, potentially resulting in candidemia and other invasive infections[11]. A key virulence factor of *Candida* species is their ability to form biofilms, which enable adherence to surfaces and confer protection against host immune defenses and antifungal agents. These biofilms significantly contribute to antifungal resistance and treatment failure. Furthermore, at present, systemic fungal infections are responsible for approximately 1.6 million deaths annually, with about 90% of these fatalities attributed to *Candida* species. *C. albicans* is the predominant species, accounting for approximately 80% of clinical isolates[12]. Although *C. tropicalis* is considered the second most virulent species, it has been shown to produce denser and more resistant biofilms than *C. albicans*[13]. Infections caused by *C. tropicalis*, particularly candidemia, are associated with high morbidity and mortality rates[14].

Current antifungal agents, including amphotericin B, itraconazole, terbinafine, and voriconazole, are chemically synthesized and often associated with adverse effects, particularly nephrotoxicity or hepatotoxicity[4,15]. Furthermore, the emergence of antifungal resistance among dermatophytes and *Candida* spp. poses a significant therapeutic challenge[16-18].

In conclusion, this study focuses on the clinically significant fungal pathogens *T. rubrum*, *T. mentagrophytes*, *M. canis*, and *N. gypsea*, which are important zoophilic dermatophytes, as well as the opportunistic yeasts *C. albicans* and *C. tropicalis*, due to their relevance in both human and veterinary medicine, including their association with antifungal drug resistance.

In Thailand, several medicinal plants with antifungal properties are used in traditional medicine. *Citrus hystrix* DC. (Rutaceae), commonly known as Ma Krut, is one such plant, widely available and traditionally used for hair and scalp care. Grilled *C. hystrix* fruit is commonly applied in folk remedies for anti-dandruff and anti-pruritic properties[19, 20]. Phytochemical analyses of *C. hystrix* volatile oil have identified major constituents such as limonene, β-pinene, terpinen-4-ol, citronellal, and linalool, which have demonstrated antifungal activity against *C. albicans* and *T. mentagrophytes*[21,22]. However, the phytochemical composition and antifungal potential of volatile oil derived from grilled *C. hystrix* peels (G-ChVO) remain uninvestigated. This study aimed to compare the physical and chemical properties of fresh and grilled *C. hystrix* fruits. Microscopic examinations were performed on fruit tissues (exocarp, mesocarp, and endocarp) to assess structural differences. Volatile oils from both fresh and grilled fruits were extracted using hydrodistillation, following [23]. Chemical profiling of the oils was conducted via gas chromatography–mass spectrometry (GC-MS). Antifungal activity was evaluated against six pathogenic fungi—*T. mentagrophytes*, *T. rubrum*, *M. canis*, *N. gypsea*, *C. tropicalis*, and *C. albicans*—using the disk diffusion method. Difference between the fresh and grilled *C. hystrix* fruit including chemical profiles of the volatile oils was revealed in this research.

2. MATERIALS AND METHODS

2.1. Chemicals and Reagents

All chemicals and reagents were analytical grade. Reference standards as (-)-*β*-pinene (99% purity), (±)-citronellal, (≥ 95% Purity), D-limonene (97% purity), sabinene (75% purity) and terpinen-4-ol (≥ 99% purity) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Voriconazole 1 µg/disc was purchased from Himedia, India. Hexane was purchased from QRëC, New Zealand. Water type I and II, produced by Milli-Q®, Germany, were used in this experiment.

For antifungal activity test, sabouraud dextrose agar (SDA) was purchased from Difco, France. Potato dextrose agar (PDA) was purchased form Oxoid, United Kingdom. Mueller-Hinton agar plates supplemented with 2% glucose and 0.5 µg/mL methylene blue (Himedia, India) was used in this experiment.

2.2. *Preparation of plant materials*

Citrus hystrix fruits were harvested on August, 2022 from Pathum Thani Province, Thailand. The plant material, including fruit-bearing branches with leaves, was identified by the Plant Varieties Protection Division Department of Agriculture, Bangkok, Thailand. A voucher specimen (registration number TTM No. 0005468) was deposited at the Department of Thai Traditional and Alternative Medicine, Nonthaburi, Thailand. Fruits measuring 25–30 mm in diameter were washed 4–5 times with deionized water and air-dried using an electric fan for 48 hours at ambient temperature[24]. Each fruit was individually weighed prior to grilling. The fruits were then grilled on a hot charcoal stove for 30 minutes at 300–350 °C until the peel color changed from green to yellow. After grilling, the fruits were allowed to cool to room temperature. The weight of each grilled fruit was subsequently recorded[19,25,26].

2.3. Morphological studies of fresh and grilled C. hystrix fruits using microscopic method

External appearance of each fruit was studied based on sensory observations, including colour, size, shape, and texture. Fruit tissues from the exocarp, mesocarp, and endocarp of fresh and grilled *C. hystrix* fruits were separately dissected. Oil gland was studied under a SNZ745T stereomicroscope (10x) (Nikon, China) equipped with an MDX503 microscope camera. The images were magnified, captured and recorded with iWork software (Lanoptik Technologies Ltd., China).

2.4. Titratable Acidity of C. hystrix juice squeezing from fresh and grilled C. hystrix fruits[27]

Two milliliters of *C. hystrix* juice was mixed with 0.3 mL of 1% phenolphthalein. The solution was titrated with 0.1M sodium hydroxide. The titration was stopped when pink color was appeared. The acidity was calculated.

2.5. Extraction of volatile oil from peels of fresh and grilled C. hystrix fruits

Volatile oil was extracted using the hydrodistillation method with a Clevenger-type apparatus (NK Laboratory, Thailand), following [23]. The peels (exocarp and mesocarp) of *C. hystrix* were separated from the fruits and cut into uniform pieces. A total of 25 g of the cut peels was weighed and transferred to a round-bottom flask, followed by the addition of 250 mL of distilled water. The distillation was carried out at 130 °C for 5 hours. The resulting volatile oil, appearing as a light-yellow liquid, was collected and stored in amber glass bottles. The percentage yield of the oil was calculated. The oil was stored at 4 °C until further analysis[28].

2.6. Phytochemical analysis of volatile oil extracted from peels of fresh and grilled C. hystrix fruits (modified from [29])

Qualitative analysis of phytochemical constituents in the volatile oil extracted from fresh and grilled *C. hystrix* peels using the gas chromatography-mass spectrometry (GC-MS) method.

2.6.1 Preparation of test samples

Volatile oils extracted from fresh *C. hystrix* peels (F-ChVO) and grilled *C. hystrix* peels (G-ChVO) were dissolved in hexane at a concentration of 4 µL/mL. All samples were then filtered through a 0.45 µm nylon membrane filter. In this study, reference standards including *β*-pinene, sabinene, D-limonene, citronellal, and terpinen-4-ol were analyzed using gas chromatography–mass spectrometry (GC-MS) to determine their respective retention times.

2.6.2 GC-MS conditions

GC-MS analytical techniques were employed be GC-MS instruments (Agilent technologies, USA) for the analysis and identification of the phytochemicals in F-ChVO and G-ChVO. The analysis utilized a HP-Innowax column with dimensions of 30 m × 0.25 mm and a film thickness of 0.25 µm (Agilent J&W GC, USA). Helium was used as the carrier gas at a flow rate of 1.0 mL/min. The injector volume was set to 1.0 µL with a split ratio of 1:100, and the temperature was maintained at 200°C. The column oven temperature was gradually increased by 5°C per minute. An initial temperature was set at 50°C and it was maintained for 5 minutes. Then, the temperature was increased until reaching 250°C and it was held for an additional 5 minutes. The mass spectrum of phytochemicals in F-ChVO and G-ChVO were obtained using electron impact energy of 70 eV. The scan time and mass range were set to 1 second and 40-1000 m/z, respectively. Instrument parameters included a quadrupole mass filter, an interface temperature of 210°C, an ion source temperature of 230°C, and a solvent cut time of 4 minutes. The scan speed was set to 1,562 u/s. The obtained spectra were compared to the National Institute of Standards and Technology (NIST) 2014 mass spectral library for compound identification.

2.7. Anti-fungal activity test using disc diffusion assay

2.7.1 Fungal strain and growth condition

The fungal strains used in this study included *Candida tropicalis* (unpublished data), *Microsporum canis*[7], and *Nannizzia gypsea*[30], which were clinical or environmental isolates previously collected. Additionally,

Candida albicans (ATCC 90028), *Trichophyton mentagrophytes* (ATCC MYA-4439), and *Trichophyton rubrum* (ATCC MYA-4438) were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). *Candida* species was maintained on sabouraud dextrose agar at 37°C for 24 hours and dermatophytes were maintained on potato dextrose agar at 25°C for 4-15 days[31,32]. All fungal strains used in this study were kindly provided by Professor Dr. Passanesh Sukphopetch (Faculty of Tropical Medicine, Mahidol University, Thailand).

2.7.2 Determination of antifungal activity

The antifungal activity of F-ChVO, G-ChVO, and voriconazole was evaluated using the disc diffusion assay by measuring the diameter of the inhibition zones. A fungal inoculum suspension (1×10^6 CFU/mL) was prepared and evenly spread onto Mueller-Hinton agar plates supplemented with 2% glucose and 0.5 µg/mL methylene blue using sterile cotton swabs. Sterile paper discs (6 mm diameter) were placed on the agar surface, and 2 µL, 4 µL, 6 µL, 8 µL, and 10 µL of F-ChVO or G-ChVO were applied to each disc. Voriconazole (1 µg/disc) was used as the positive control. The plates were incubated at 37 °C, and the diameters of the inhibition zones were measured on days 1, 3, 5, 7, and 9 using a vernier caliper[32, 33].

2.8. Statistical analyses

Each assay was performed in triplicate. Titratable acidity and extract yield were analyzed using GraphPad Prism (GraphPad Software, San Diego, CA, USA). Results were expressed as mean ± standard deviation. Statistical significance ($p < 0.05$) was determined using independent Student's t-tests. For the disc diffusion assay, results were expressed as mean ± standard deviation (SD). Statistical significance was assessed using independent Student's t-tests at $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

3. RESULTS AND DISCUSSION

3.1. Morphological studies of fresh and grilled *C. hystrix* fruits using microscopic method

The physical characteristics of fresh and grilled *C. hystrix* fruits were analyzed. Fresh fruits exhibited a green color, hard exocarp, and an oval to elliptic shape (**Figure 1A**). The average diameter and weight of the fresh fruits were 30.2 ± 0.14 mm and 67.13 ± 0.001 g, respectively. Upon longitudinal sectioning, the exocarp and mesocarp appeared green and white, respectively (**Figure 1B; white and red arrows**). The endocarp contained green juice sacs (**Figure 1B; blue arrow**). Oil glands containing volatile oil were visible in the exocarp layer (**Figure 1C; black arrow**). In contrast, grilled *C. hystrix* fruits exhibited yellow pericarps (**Figures 2A and 2B**). The average weight of the grilled fruits was 63.08 ± 0.09 g, showing a reduction of average weight approximately 4.05 ± 0.09 g compared to the fresh fruits. This weight loss was attributed to the evaporation of water, volatile compounds, and essential oils due to heating[34]. Longitudinal cross-sections of the grilled fruits revealed a yellow exocarp (**Figures 2B; white arrow**), pale yellow mesocarp (**Figure 2B; red arrow**), and yellow endocarp (**Figure 2B; blue arrow**). The endocarp of the grilled fruits appeared drier than that of fresh fruits. Heat exposure also damaged the oil glands, resulting in ruptured structures and devoid of oil (**Figure 2C; black arrow**). Upon texture analysis, the grilled fruit's pericarp left more oil residue on the hands compared to the fresh fruit. Additionally, the grilled fruit emitted a noticeably stronger odor, distinct from the fresh fruit. The texture of the grilled fruit was softer and smoother, making it easier to squeeze. These changes suggested that heat altered morphological characteristics, including pericarp color[35], texture, odor profile, and a reduction or absence of volatile oil in oil gland[36].

3.2. Titratable acidity of *C. hystrix* juice squeezing from fresh and grilled *C. hystrix* fruits

Total acidity values of juice extracted from fresh and grilled *C. hystrix* fruits were determined using a titration assay. Juice from fresh fruits exhibited the highest total acidity at 8.15 ± 0.35% w/v, calculated as citric acid, followed by juice from grilled fruits, which showed a significantly lower value of 7.18 ± 0.03% w/v. The reduction of total acidity in the grilled fruit juice was attributed to the thermal degradation of citric acid. Citric acid, a key organic acid responsible for the sour taste of *C. hystrix* fruit, peel, and juice, was known to degrade at temperatures between 177–501 °C[37,38]. Therefore, heating likely led to the decomposition of citric acid and other organic acids, resulting in a decrease in total acidity. Interestingly, recent studies have reported that citric acid exhibits inhibitory effects on hair growth[39]. Consequently, the reduction of citric acid and overall acidity in grilled *C. hystrix* juice may contribute to hair growth promotion. However, further experimental studies are necessary to evaluate the hair growth–promoting potential of juice extracted from grilled fruits.

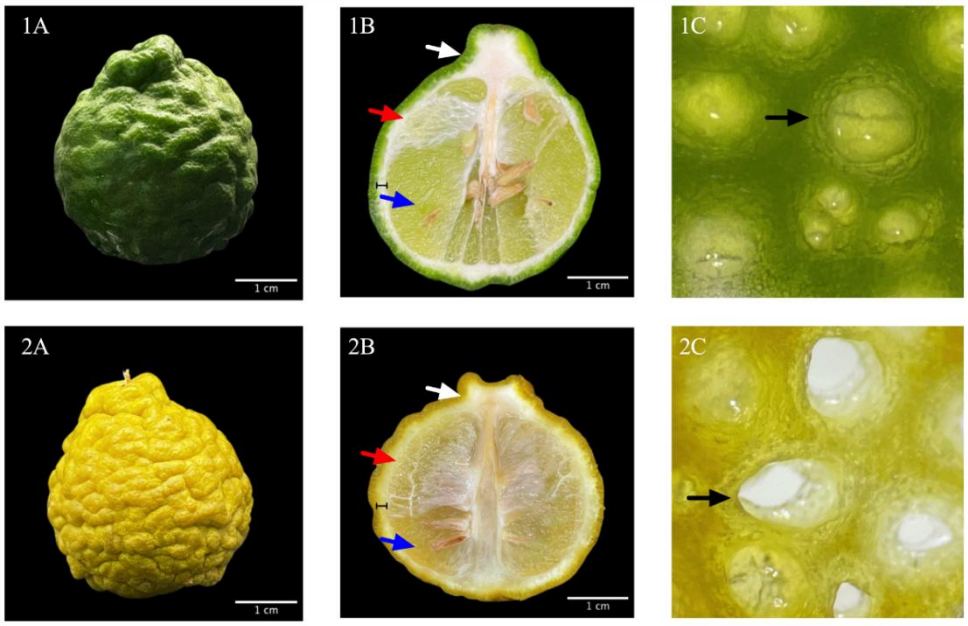

Figure 1 Comparison of morphological characteristics between fresh *C. hystrix* fruits (1A) and grilled *C. hystrix* fruits (2A). Longitudinal cross-sections of fresh (1B) and grilled (2B) fruits were differences in tissue layers: exocarp (white arrows), mesocarp (red arrows), and endocarp (blue arrows). Oil glands containing volatile oil was visible in the peel of the fresh fruit (1C), while heat-damaged and ruptured oil glands were observed in the peel of the grilled fruit (2C; black arrow).

3.3. Extraction of volatile oil from peels of fresh and grilled *C. hystrix* fruits

Volatile oils extracted from the peels of both fresh and grilled *C. hystrix* fruits were clear, pale-yellow liquid The yield of volatile oil from the peels of grilled fruits (1.2% v/w) was significantly higher than that from fresh fruit peels (1.0% v/w), suggesting that the grilling or heating process enhanced volatile oil extraction. This finding was consistent with previous research by [35], which reported that elevated temperatures increased the yield of volatile oil extracted from the peel of *Citrus aurantium* L.

3.4. Phytochemical analysis of volatile oil extracted from peels of fresh and grilled C. hystrix fruits using GC-MS analysis

Thirty-three compounds were identified in the volatile oil extracted from the peels of fresh *C. hystrix* fruit (F-ChVO), while ninety-one compounds were detected in the volatile oil extracted from grilled fruit peels (G-ChVO). The GC-MS analysis results were presented in the Supplementary Information. Previous studies reported that β-pinene, sabinene, D-limonene, terpinen-4-ol, and citronellal were the major constituents of volatile oil containing in *C. hystrix* peels[40,41]. In our study, GC-MS chromatograms (**Figure 2**) confirmed the presence of four major compounds as *β*-pinene, sabinene, D-limonene, and terpinen-4-ol in both F-ChVO and G-ChVO. However, the phytochemical profiles of F-ChVO and G-ChVO markedly differed. *β*-Pinene was the predominant compound in F-ChVO, whereas G-ChVO was characterized by a higher content of terpinen-4-ol, indicating that thermal processing influenced the chemical composition of the volatile oil.

In F-ChVO, the major compound was *β*-pinene (22.02%), followed by D-limonene (18.22%), terpinen-4-ol (11.54%), sabinene (10.31%), and citronellal (9.21%). In contrast, G-ChVO contained terpinen-4-ol as the dominant component (23.93%), followed by D-limonene (10.86%), β-pinene (7.66%), and sabinene (1.90%). Notably, citronellal was present in high amounts in F-ChVO but was undetectable in G-ChVO (**Table 1 and Figure 2B**).

Heating or grilling influenced the chemical composition of volatile oils by inducing degradation and transformation of terpenes. In G-ChVO, the content of terpinen-4-ol increased, while the levels of β-pinene, sabinene, and D-limonene decreased, and citronellal was undetectable, compared to F-ChVO. A previous study reported that terpinen-4-ol could undergo thermal degradation [42]. This suggested that heating may enhance terpinen-4-ol content due to chemical transformation processes.

The grilling process was an important factor which caused chemical transformations and led to significant alterations in the phytochemical profile. Subsequently, it potentially affected to the biological activity of the volatile oil extracted from heat-treated plant materials [43]. In this study, antifungal activity was of particular interest. Based on the GC-MS qualitative analysis, the phytochemical profiles of F-ChVO and G-ChVO differed significantly. Therefore, their antifungal activities were comparatively evaluated.

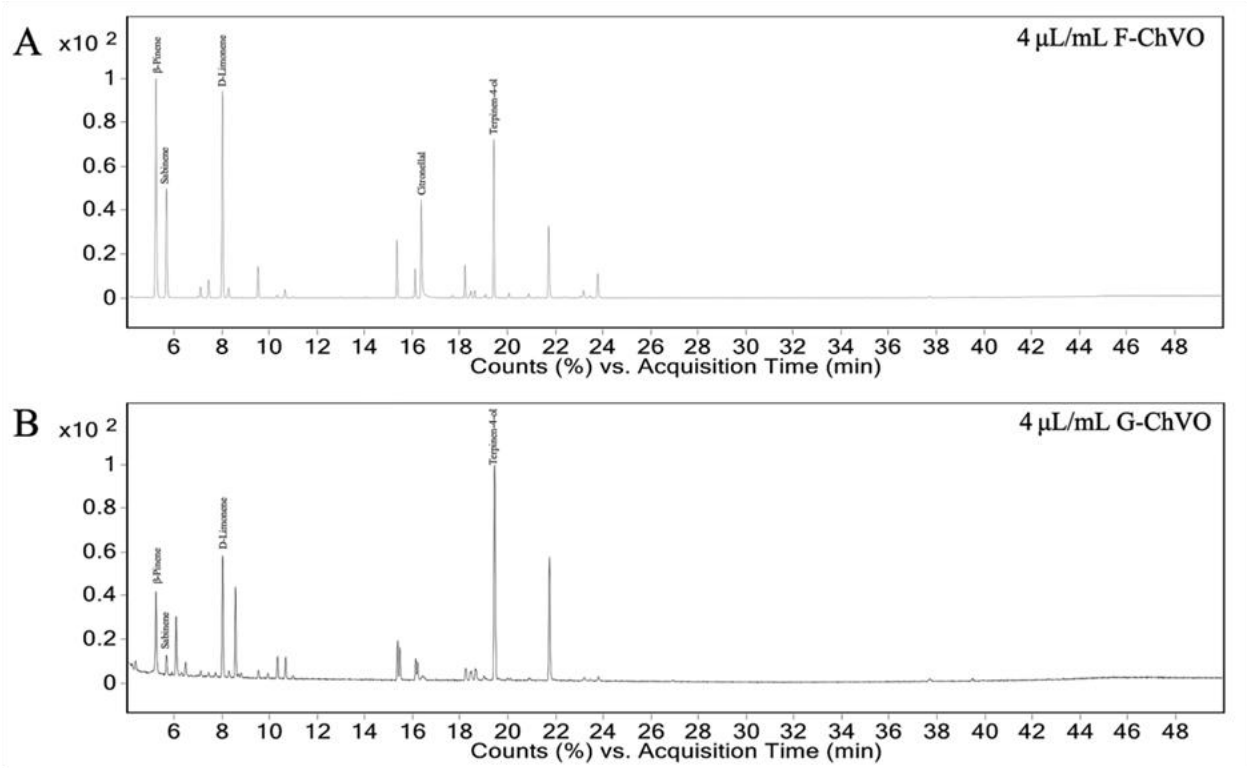

Figure 2. GC-MS chromatograms of F-ChVO (A) and G-ChVO (B) detected at a concentration of 4 µL/mL

Table 1. Phytochemical composition of F-ChVO and G-ChVO

F-ChVO				G-ChVO			
SI. no	RT*	%Area	Compound name	SI. no	RT[a]	%Area	Compound name
1.	5.246	22.02	*β-pinene*	1.	5.235	7.66	*β-pinene*
2.	5.681	10.31	Sabinene	2.	5.681	1.90	Sabinene
3.	8.03	18.22	D-limonene	3.	8.03	10.86	D-limonene
4.	16.384	9.21	Citronellal	4.	19.445	23.93	Terpinen-4-ol
5.	19.424	11.54	Terpinen-4-ol				

* RT was retention time (min)

3.5. Anti-fungal activity test using disc diffusion assay[32,33,44-47]

The disc diffusion assay is a simple, cost-effective, and time-efficient method for the preliminary screening of antimicrobial activity. It enables visual detection and measurement of inhibition zones, thereby providing a qualitative assessment of the efficacy of test compounds. Currently, this assay has utilized to evaluate the antifungal activity of selected volatile oils. Previous studies have demonstrated the potential of plant-derived essential oils against various *Candida* species (*C. albicans*, *C. glabrata*, *C. tropicalis*, and *C. parapsilosis*)[44,45], as well as dermatophytes such as *T. rubrum*, *T. mentagrophytes*, *M. canis*, and *N. gypsea*[46,47], using the disc diffusion method.

Due to the high volatility of essential oils, there is a significant risk of cross-contamination between samples when multiple discs are placed on the same Petri dish. Additionally, volatilization may lead to inaccurate or inconsistent measurements of inhibition zones. To mitigate these limitations and ensure experimental reliability, each Petri dish in the present study was prepared with a single disc impregnated with one concentration of volatile oil.

In this research, the *in vitro* antifungal activity of F-ChVO and G-ChVO was evaluated using the disc diffusion assay against *C. albicans*, *C. tropicalis*,*T. rubrum*, *T. mentagrophytes*, *M. canis*, and *N. gypsea*. Both volatile oils, at concentrations of 2, 4, 6, 8, and 10 µL/disc, exhibited dose-dependent growth inhibitory activity against all tested fungal strains, as indicated by the formation of inhibition zones (**Figure 3**). Notably, at the same concentration of 10 µL/disc, the inhibition zones were observed on the fifth day of incubation. G-ChVO produced larger inhibition zones compared to F-ChVO. G-ChVO generated inhibition zones of 24.34 ± 0.59 mm against *T. rubrum* and 24.24 ± 0.78 mm against *M. canis*, whereas F-ChVO produced inhibition zones of 15.31 ± 0.56 mm and 18.07 ± 1.04 mm,

respectively. The antifungal activity of G-ChVO was significantly greater than that of F-ChVO, with p-values of 0.002 and 0.0087, respectively. At a concentration of 10 µL/disc, both volatile oils demonstrated the highest antifungal activity across all tested fungi. Inhibition of *C. albicans* and *C. tropicalis* was observed on the first day of incubation, G-ChVO exhibited stronger inhibition against *C. albicans* (**Figure 3B**) compared to *C. tropicalis* (**Figure 3E**). By day 3, both volatile oils inhibited the growth of *T. rubrum* and *T. mentagrophytes*. Although *T. mentagrophytes* was initially more sensitive to G-ChVO, by days 5 to 9, G-ChVO exhibited greater inhibitory activity against *T. rubrum* (**Figure 3H**) than against *T. mentagrophytes* (**Figure 3K**).

On day 5, the both volatile oils showed the growth inhibition of *M. canis* (**Figures 3M and 3N**) and *N. gypsea* (**Figures 3P and 3Q**). G-ChVO demonstrated stronger activity than F-ChVO. Notably, the growth inhibition of G-ChVO against *N. gypsea* was greater than that of *M. canis*.

Voriconazole, used as a positive control at concentration 1 µg/disc, exhibited antifungal activity against all tested fungi over the 9-day incubation period. It possessed the strongest antifungal activity against *T. rubrum* (**Figure 3I**) and *M. canis* (**Figure 3O**).

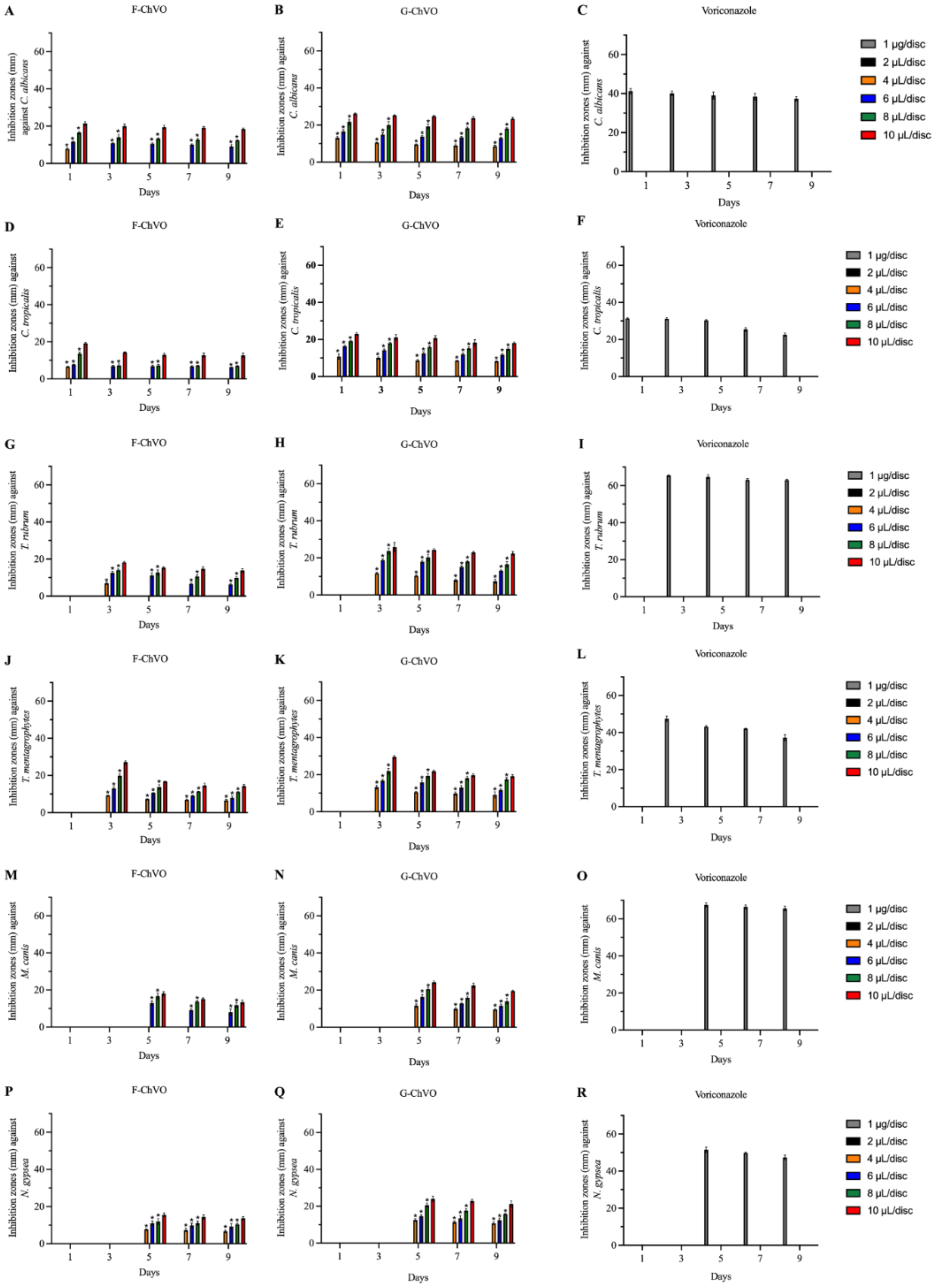

Figure 3. At concentration 10 µL/disc and in the same incubation period (1, 3, 5, 7, 9 day), growth inhibitory activity of cutaneous pathogenic fungi treated with F-ChVO and G-ChVO was observed within 9 days and compared. The results were presented as inhibition zone(mm). Asterisks indicated to statistically significant differences between the concentration at 10 µL/disc and at the same incubated period. Voriconazole was used as positive control (C, F, I, L, O, R).

At a highest concentration of 10 µL/disc on the fifth day of the incubation period, G-ChVO exhibited the highest antifungal activity. The potency of antifungal activity followed the order: *C. albicans* > *T. rubrum* > *M. canis* > *N. gypsea* > *T. mentagrophytes* > *C. tropicalis* (**Figure 4**).

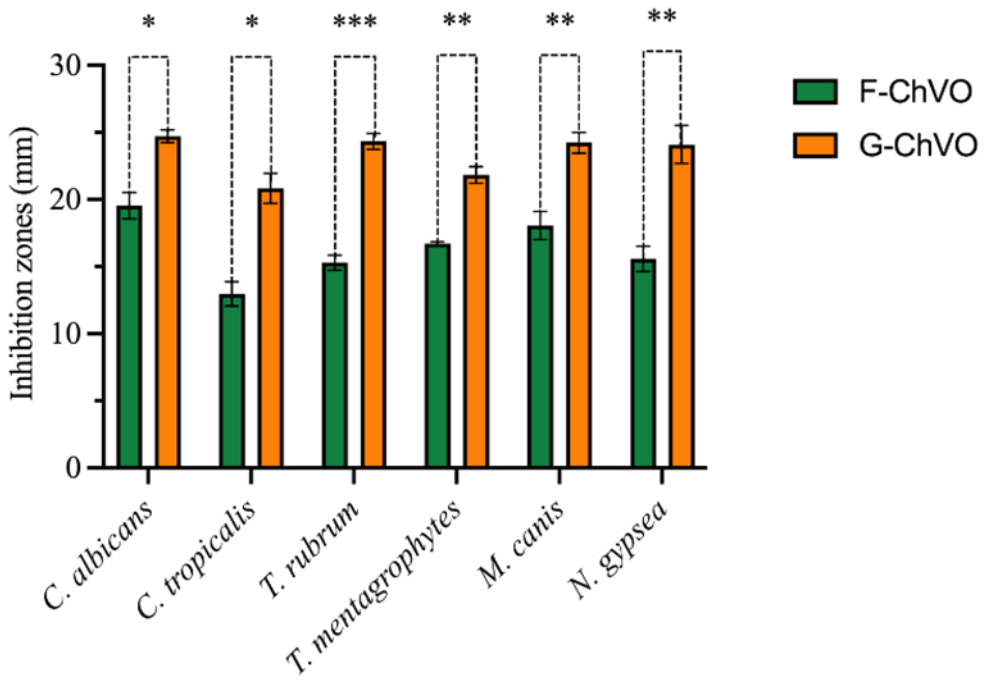

Figure 4. Inhibition zones of F-ChVO and G-ChVO at a concentration of 10 µL/disc against cutaneous pathogenic fungi after five days of incubation at 37 °C. Asterisks (*) indicate statistically significant differences between F-ChVO and G-ChVO for the same fungal strain.

In this study, volatile oils extracted from the peels of both fresh and grilled *C. hystrix* fruits demonstrated antifungal activity against cutaneous pathogenic fungi, including *C. albicans*, *C. tropicalis*, *T. rubrum*, *T. mentagrophytes*, *M. canis*, and *N. gypsea*. G-ChVO exhibited the strongest antifungal activity, likely due to heat-induced degradation of terpenes and an increased concentration of terpinen-4-ol, a compound known for its potent antifungal properties[21,48-50]. Additionally, other compounds such as *β*-pinene, D-limonene, and citronellal may contribute synergistically to the observed activity.

These findings suggested that volatile oil extracted from the peels of grilled *C. hystrix* fruit has potential for use as an antifungal ingredient in future health products. However, further studies are necessary to evaluate its toxicity and ensure its safety for topical or therapeutic applications.

4. CONCLUSIONS

According to Thai traditional knowledge, fresh *C. hystrix* fruits are typically grilled until the green exocarp turns yellow. The pericarp of the grilled fruit became softer and smoother, compared to the fresh fruit. The oil glands were visibly damaged. The grilled fruit also emitted a stronger odor than the fresh fruit. The total acidity of juice extracted from the grilled fruit was lower due to the thermal degradation of citric acid. Phytochemical profiling of volatile oils extracted from the peels of fresh (F-ChVO) and grilled fruits (G-ChVO), as analyzed by GC-MS, was differences. G-ChVO was predominantly composed of terpinen-4-ol, while F-ChVO contained higher levels of β-pinene. G-ChVO exhibited greater antifungal activity than F-ChVO against *C. albicans*, *C. tropicalis*, *T. rubrum*, *T. mentagrophytes*, *M. canis*, and *N. gypsea*. Among these, *C. albicans* showed the highest sensitivity to G-ChVO, followed by *T. rubrum* and *T. mentagrophytes*. These findings indicated that heating played a critical role in enhancing the antifungal activity of *C. hystrix* volatile oil by promoting the transformation of active compounds into more active compounds. For example, the conversion of *β*-pinene to terpinen-4-ol. This study revealed the potential antifungal activity of volatile oil extracted from the peels of the grilled *C. hystrix* fruits. The volatile oil extracted from the peels of the grilled *C. hystrix* fruits could be used as an active ingredient in health products for the treatment or prevention of fungal infections.

5. ACKNOWLEDGMENTS

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Author contribution

FL: Methodology, Investigation, Formal Analysis, Visualization, Writing – original draft.
PS: Methodology, Visualization, Resources, Funding acquisition, Writing – review and editing.
TK: Methodology, Investigation, Visualization, Writing – review and editing.
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Conflict of interest

The authors declare that they have no conflict of interest.

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