Research Article

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Caprylic acid in *Vitex mollis* fruit and its inhibitory activity against a thiabendazole-resistant *Colletotrichum gloeosporioides* strain

Jordi G López-Velázquez,[a†] Lidia E Ayón-Reyna,[b†] Misael O Vega-García,[a,b] Gabriela López-Angulo,[b] Martha E López-López,[b] Blanca A López-Zazueta[b] and Francisco Delgado-Vargas[a,b*]

Abstract

BACKGROUND: *Colletotrichum gloeosporioides* causes anthracnose in a large number of crops. Synthetic fungicides are employed to prevent this disease, even though their effectiveness and safety is questionable. Thus, effective and innocuous antifungal compounds are proposed as natural alternatives against anthracnose. The hexane fraction of *Vitex mollis* pulp (HF-VM) reduces anthracnose incidence in papaya fruit; however, the active compounds and antifungal mechanism of HF-VM are unknown. The aims of this study were to characterize the activity of HF-VM sub-fractions (sHF_1–sHF_7) against a thiabendazole-resistant *Colletotrichum gloeosporioides* strain, identify the chemical components and investigate the mechanism of the most active sub-fraction.

RESULTS: The sHF_3 showed the highest inhibitory activity against *Colletotrichum gloeosporioides* with a minimal inhibitory concentration (MIC) of 0.5 mg mL^{-1}, whereas thiabendazole (TBZ) had a MIC value higher than 2 mg mL^{-1}. The gas chromatography–mass spectrometry (GC–MS) analysis showed that the compounds in sHF_3 were methyl 4-decenoate, caprylic acid, and 24-methylencycloartanol. These compounds are rarely found in fruits and are reported for the first time on *Vitex* species. The purified 24-methylencycloartanol was inactive (MIC > 0.5 mg mL^{-1}). In contrast, the commercial standard of caprylic acid presented an elevated activity (MIC = 0.125 mg mL^{-1}), indicating that this compound is the main one responsible for the antifungal properties of sHF_3. Furthermore, the sHF_3 inhibited the spore germination and induced membrane disruption in both the spore and mycelium of *Colletotrichum gloeosporioides*.

CONCLUSION: *Vitex mollis* fruit is a novel source of antifungal caprylic acid that could be employed as a marker to prepare standardized extracts with antifungal properties.

Keywords: anthracnose; natural fungicides; *Vitex mollis*; GC–MS

1 INTRODUCTION

Plant diseases compromise the production and quality of the most important crops in the world, hindering the efforts of the Food and Agriculture Organization (FAO) to reduce hunger.[1] Different phytopathogen microorganisms cause diseases, and fungi are the most harmful crop pathogens because they produce spores that resist extreme abiotic conditions. Some of the most relevant pathogenic fungi genera in agriculture are *Botrytis*, *Fusarium*, and *Colletotrichum*.[2]

Within the *Colletotrichum* spp., *Colletotrichum gloeosporioides* is one of the most harmful phytopathogens. It infects many crops and induces anthracnose, a disease that deteriorates the visual and nutritional quality of fruit. Papaya is highly susceptible to anthracnose, being the main one responsible for its postharvest spoilage.[3]

Farmers struggle with anthracnose worldwide, given that most fungicides are not 100% effective against *Colletotrichum gloeosporioides*, resulting in poor disease management.[4] Moreover, synthetic antifungals affect natural ecosystems and human health.[5] Benomyl and thiabendazole (TBZ) are benzimidazoles commonly used in postharvest disease prevention, whose mechanism and effectiveness are well documented.[6,7] However, *Colletotrichum* species in Mexico have developed resistance,[8,9] and new economic sources of natural and non-toxic antifungals are needed in agriculture to substitute the synthetic ones.

* *Correspondence to: F Delgado-Vargas, Doctorado Regional en Biotecnología, Facultad de Ciencias Químico Biológicas, Universidad Autónoma de Sinaloa, Cd. Universitaria, Av. de las Américas y Josefa Ortiz S/N, Culiacán, Sinaloa, 80010, Mexico, E-mail: fdelgado@uas.edu.mx, fcodelgadovargas@gmail.com*

† *These authors should be considered joint first authors.*

a *Doctorado Regional en Biotecnología, Facultad de Ciencias Químico Biológicas, Universidad Autónoma de Sinaloa, Culiacán, Mexico*

b *Posgrado en Ciencia y Tecnología de Alimentos, Facultad de Ciencias Químico Biológicas, Universidad Autónoma de Sinaloa, Culiacán, Mexico*

Many plant metabolites possess antifungal activity. The spore germination inhibition is one of the most desirable antifungal mechanisms, affecting the most infectious and resistant fungal structure.[2] Many plant compounds (e.g. alkaloids, fatty acids, terpenes and phenolics) interact with ergosterol or fatty acids on the fungal membrane, increasing its permeability and disturbing cell compartmentalization and energy production.[10–13] Such membrane interactions have been observed in spores or mycelium, causing germination inhibition or mycelium deformation and fungus death.

Discovering novel sources of innocuous antimicrobials is complex. Essential oils (EOs) extracted from aromatic plants have led this field for years. EOs inhibit an extensive spectrum of phytopathogens and have a distinguished recognition as GRAS (generally recognized as safe) additives by the Food and Drug Administration (FDA).[14] Besides EOs, other examples of natural antimicrobials recognized as GRAS and their respective sources are the following: benzoic acid (*Styrax* spp.), curcumin (*Curcuma longa*), and caprylic acid (*Cocos nucifera*).[15] It is important to mention that the investigation of a little-studied plant genus included in ancient popular medicine could result in new sources of GRAS antimicrobials.

In this regard, the chemical composition and biological activities of most *Vitex* spp. are poorly characterized. The *Vitex* genus comprises more than 250 species distributed worldwide and produces 0.2–2 cm diameter spherical drupes. The fruits of several *Vitex* species have medicinal properties, and some are included in folk medicine. For instance, an extract of *Vitex agnus-castus* is employed to treat human hormonal imbalance.[16] Furthermore, examples of non-clinical applications are the following: *Vitex trifolia* exhibits anti-feeding activity against the insect *Spodoptera frugiperda*, and *V. agnus-castus* is effective against harmful phytopathogenic fungi (e.g. *Alternaria alternata*, *Fusarium tricinctum*, and *Penicillium funiculosum*).[17] Therefore, *Vitex* species are potential sources of safe antifungals to prevent or reduce the negative impact of postharvest diseases.

Vitex mollis fruit is native to Mexico, but it is scarcely consumed or employed for any other purpose and within the least studied *Vitex* spp. Previously, we reported that a hexane fraction of *V. mollis* (HF-VM) inhibited the mycelial growth of *Colletotrichum gloeosporioides* and reduced the anthracnose incidence and severity on papaya.[18] However, the HF-VM is poorly characterized; therefore, this article aims to identify compounds in the HF-VM with activity against *Colletotrichum gloeosporioides* and advance in establishing its antifungal mechanism.

2 MATERIAL AND METHODS

2.1 Plant material

Mature *V. mollis* fruits were collected in the municipality of Culiacan, Sinaloa, Mexico, between June and August 2020. Samples were stored at −70 °C until freeze-drying, the seeds were discarded, and the recovered pulp was pulverized and sieved with a 40 mesh (425 μm of diameter).

2.2 Pathogen

Colletotrichum gloeosporioides was isolated from papaya and characterized by molecular analysis (18S rDNA, accession number HM222960.1).[3] Inoculated Petri dishes were incubated for 2 weeks at 27 °C. To obtain the spore suspension, sterilized water was added to a Petri dish, then the Petri dish was scraped with a microscope slide and deposited on a sterilized glass container. Finally, the suspension was filtered through organza fabric to remove mycelium, and the spore concentration was adjusted using a hemocytometer. For mycelium, a *Colletotrichum gloeosporioides* spore suspension (1×10^6 spores mL^{-1}) was added to potato dextrose broth (PDB) (1:10), and the mixture was incubated at 27 °C for 3 days. Then, the mycelium was collected with sterilized tweezers and washed with sterile water.

2.3 Fraction preparation

The HF-VM was prepared as described by López-Velázquez *et al.*[18] The pulverized pulp (560 g) was mixed with methanol (1:10 *w/v*), sonicated (30 min, 35 °C), filtered (Whatman 1), and the residue obtained was re-extracted two additional times under the same conditions. The filtrates were mixed, and the solvent was removed in a rotary evaporator (BÜCHI Labortechnick AG, Flawil, Switzerland) at 40 °C. The extract was re-suspended in 90% methanol (1:2 *w/v*) and partitioned three times with hexane. The polar fraction was discarded, and the hexane fraction was concentrated in a rotary evaporator to obtain the HF-VM. The HF-VM was stored under a nitrogen (N_2) atmosphere at −20 °C.

2.4 Chemical analysis of the components in the HF-VM with antifungal activity

2.4.1 *Silica gel chromatography*

The HF-VM (9 g in dry weight) was separated by column chromatography (55 cm × 9 cm) using silica gel 60 (35–70 μm of diameter). Compounds were eluted using the following solvent mixtures and proportions (*v/v*): hexane + ethyl acetate [9.75 + 0.25 by volume (400 mL), 9.5 + 0.5 by volume (200 mL), 9.25 + 0.75 by volume (200 mL), 9 + 1 by volume (300 mL), 8.5 + 1.5 by volume (100 mL), 8 + 2 by volume (300 mL), 7.5 + 2.5 by volume (100 mL), 7 + 3 by volume (100 mL), 6 + 4 by volume (100 mL), and 1 + 1 by volume (100 mL)], ethyl acetate (100 mL), and ethyl acetate + methanol [9 + 1 by volume (300 mL)]. Aliquots of 10 mL were collected and analyzed by thin-layer chromatography (TLC), using hexane + ethyl acetate (8 + 2 by volume) as the mobile phase. The TLC spots were visualized with the Lieberman–Burchard reagent (solution of sulfuric acid and acetic anhydride in chloroform) for triterpene detection (purple coloration).[19] Aliquots were pooled into seven sub-fractions (sHF_1–sHF_7) based on their TLC retention factor (R_f). The R_f was calculated as follows:

$$R_f = \frac{\text{Distance traveled by the solute}}{\text{Distance traveled by the mobile phase}}$$

2.4.2 *Antifungal activity by microdilution assay*

The microdilution assays were carried out in microplates as described by Miron *et al.*[20] with some modifications. On each microplate well was added potato dextrose agar (PDA), and the microplate was incubated at 27 °C to ensure the absence of microorganisms. The sub-fractions were dissolved in hexane at different concentrations (0.062, 0.125, 0.25, 0.5, 1, 1.5, 2, 2.5, 5, and 10 mg mL^{-1}), sterilized by filtration (Nylon, 0.22 μmol L^{-1} of aperture), and added to each well with solidified PDA. The solvent was evaporated in a laminar flow cabinet. For inoculation, a spore suspension of *Colletotrichum gloeosporioides* (1×10^6 spores mL^{-1}) was added to each well, and the microplate was incubated for 2 days at 27 °C. The antifungal activity of the sub-fractions was determined based on minimal inhibitory concentration (MIC), which is the lowest sub-fraction concentration with the absence

of any visible mycelial growth. Hexane and pure TBZ (Sigma-Aldrich, St Louis, MO, USA) (0.5–2 mg mL^{-1}) were used as negative and positive controls, respectively.[21] The sub-fraction with the best antifungal properties was selected for further analysis.

2.4.3 Gas chromatography–mass spectrometry (GC–MS) analysis

The sub-fraction sHF_3 was dissolved in hexane (4 mg mL^{-1}). The sample was analyzed by gas chromatography–mass spectrometry (GC–MS) with the HP 6890 GC Instrument, 5973 Network (Agilent Technologies, Santa Clara, CA, USA). The separation was carried out on a capillary column QUADREX 007 CARBOWAX 20 M [30 m × 0.25 mm inner diameter (i.d.), film thickness 0.25 µm) (Quadrex Corporation, Woodbridge, CT, USA) using helium as carrier gas (0.9 mL min^{-1}). The operating temperatures were as follows: injector, 250 °C; oven, 60 °C for 1 min, 5 °C min^{-1} to 200 °C, 10 °C min^{-1} to 275 °C, and held at 275 °C to the end of the analysis; ion source, 245 °C; and quadrupole, 150 °C. The samples were injected (5 µL) without flow division. MS detection was performed in electron impact mode at 70 eV ionization energy and full-scan mode in the 50–800 amu range.[19] The sample components were identified by comparison (match factor) with the National Institute of Standards and Technology mass spectra library (NIST08.LIB) (USA). The match factor for compound identification was assigned as follows: excellent (> 900), good (800–900), fair (700–800), and poor (≤ 699).[22] The relative percentage amount of each compound was calculated by comparing its average peak area to the total area.

2.4.4 Triterpene isolation and antifungal inhibitory evaluation

The sub-fraction sHF_3 (1.5 g) was separated again by column chromatography (45 cm × 4 cm). Compounds were eluted with hexane (100 mL), hexane + ethyl acetate [9.75 + 0.25 by volume (150 mL), 9.5 + 0.5 by volume (150 mL), 9.25 + 0.75 by volume (100 mL), 9 + 1 by volume (100 mL), 8.5 + 1.5 by volume (150 mL), 8 + 2 by volume (100 mL), and 1 + 1 by volume (100 mL)], and ethyl acetate (100 mL). Aliquots of 5 mL were collected and analyzed by TLC as described in Section 2.4.1. Aliquots with the highest triterpene presence were pooled and evaporated on a rotary evaporator. The residue was re-suspended in cold hexane and crystallized under refrigeration. The crystals ($sHF_{3\text{-}c}$) were washed three times with hexane and dried at 50 °C for 1 h. The crystal melting point was determined with a melting point apparatus (Fisher Scientific Inc., Waltham, MA, USA) and used as purity criterion; besides, the sample was dissolved in hexane (1 mg mL^{-1}), analyzed by TLC employing hexane + ethyl acetate (8 + 2, 6 + 4, and 1 + 1 by volume) as mobile phase, and visualized with the Lieberman–Burchard reagent. The antifungal activity of the compound (0.062, 0.125, 0.25, and 0.5 mg mL^{-1}) was evaluated as described in Section 2.4.2.

2.4.5 Caprylic acid identification, quantification, and antifungal inhibitory evaluation

A solution of caprylic acid (Sigma-Aldrich) was prepared (4 mg mL^{-1}) to confirm the presence of caprylic acid in sHF_3, and it was added to the sub-fraction (4 mg mL^{-1}) at a final concentration of 1 mg mL^{-1}. Samples were analyzed by GC–MS as previously described. The caprylic acid content was determined using a calibration curve of this compound (0–4 mg mL^{-1}) and expressed as micrograms of caprylic acid per milligram of sHF_3. Regarding the antifungal inhibitory evaluation, the pure caprylic acid (Sigma-Aldrich) was prepared at different concentrations (0.015, 0.031, 0.062, 0.125, 0.25, and 0.5 mg mL^{-1}) in hexane and evaluated accordingly.

2.5 Antifungal activity and potential mechanism

2.5.1 Spore germination

The sub-fraction sHF_3 (0.25 and 0.50 mg mL^{-1}) and pure TBZ (0.5 mg mL^{-1}) were dissolved in hexane and methanol, respectively. Solutions were sterilized by filtration (Nylon, 0.22 µmol L^{-1} of aperture), added on Petri dishes containing solidified PDA, air-dried on a laminar flow cabinet, and inoculated with a spore suspension (1×10^6 spores mL^{-1}). Hexane and methanol were employed as negative controls. Petri dishes were incubated for 8, 16, 24, and 32 h at 27 °C. Spore germination was observed with a light microscope at 10× magnification (Carl Zeiss, Oberkochen, Germany) followed by image capture using a digital camera (Nikon Inc., Tokyo, Japan). One hundred spores were observed. A spore was considered germinated when its germ tube length exceeded half the spore length. Results were expressed as the percentage of germinated spores.[3]

2.5.2 Mycelial growth

Sterile sHF_3 (0.25 and 0.50 mg mL^{-1}) and TBZ (0.5 mg mL^{-1}) were prepared as previously described and added on Petri dishes containing PDA medium. Each Petri dish was inoculated into the center with a *Colletotrichum gloeosporioides* suspension (1×10^6 spores mL^{-1}) and incubated at 27 °C for 4 days. Results were daily reported as colony diameter in centimeters.

2.5.3 Protection assays

The interaction of the sHF_3 with ergosterol, sorbitol, palmitic acid, and linoleic acid was carried out as described by Haraguchi *et al.*[10] and Miron *et al.*[20] with some modifications. The sHF_3 was prepared at different concentrations (0.5–3.0 mg mL^{-1}) and pipetted on a microplate with PDA. Each well was added with ergosterol (2.5 mg mL^{-1}), sorbitol (145 mg mL^{-1}), palmitic acid (100 µg mL^{-1}), or linoleic acid (100 µg mL^{-1}); and then inoculated with a spore suspension (1×10^6 spores mL^{-1}). These compounds are expected to protect the spores, increasing the MIC of the sHF_3. Sorbitol assay revealed if the antifungal agent was disrupting the plasmatic membrane. However, the fatty acid and ergosterol assays were used to establish a possible interaction of the antifungal with lipids and ergosterol in the plasmatic membrane. The MIC values were determined after 24 and 48 h (27 °C) of incubation.

2.5.4 Intracellular leakage

Fresh mycelia samples (30 mg each) were suspended in Tween-80 solutions (1 mL) containing the sHF_3 at 0.25 or 0.50 mg mL^{-1}. For spores, a spore suspension was prepared (2×10^6 spores mL^{-1}) and mixed with Tween-80 solutions containing the sHF_3 at 0.5 or 1 mg mL^{-1} (1:1) to obtain the final concentrations. A Tween-80 solution (0.5% *v/v*) was used as control. The samples were incubated on a rotary shaker at 25 °C for 4 h. Spores and mycelia were filtered (Nylon, 0.22 µm of aperture), and the filtrates were used for protein and DNA analysis: the leaked proteins (in µg mL^{-1}) were determined by the Bradford assay,[23] and leaked DNA by measuring the absorbance at 260 nm.[24] The microplate reader (Bio-Tek Instruments, Winooski, VT, USA) was adjusted to zero with a solution of sHF_3 (0.25 or 0.50 mg mL^{-1}).

Electrolyte leakage from spores and mycelia was determined with a manual conductivity meter (HI98312; Hanna Instruments, Woonsocket, RI, USA). Total electrolyte leakage was determined by measuring the conductivity of the unfiltered samples before

(initial conductivity) and after (final conductivity) boiling the mixture for 10 min.[13] Results were expressed as the percentage of leaked electrolytes as follows:

$$\text{Electrolyte leakage}\,(\%) = \left(\frac{\text{final conductivity} - \text{initial conductivity}}{\text{final conductivity}}\right) \times 100$$

2.6 Statistical analysis

A completely randomized experimental design with three replicates was carried out. A one-way analysis of variance was performed for leakage of intracellular content, and a multivariate analysis of variance for spore germination and mycelial growth. Means were compared using the Fisher's least significant difference (LSD) test ($P < 0.05$). The Statgraphics Centurion XVI software (Statpoint Technologies, Warrenton, VA, USA) was employed for all the analyses.

3 RESULTS

3.1 Chemical analysis of the components in the HF-VM with antifungal activity

3.1.1 *Silica gel chromatography*

After silica gel chromatography, 92 eluates of 10 mL were obtained and pooled into seven sub-fractions (sHF_1–sHF_7) based on their TLC profile (Table 1). All sub-fractions were positive for triterpenes (purple coloration) but sHF_5 (yellowish coloration). The sub-fraction yields ranged from 0.03% w/w (sHF_5) to 1.88% w/w (sHF_1) (Table 1).

Regarding the antifungal activity against *Colletotrichum gloeosporioides*, lower MIC values were obtained for the sub-fractions sHF_3 (0.5 mg mL^{-1}) and sHF_2 (2 mg mL^{-1}), whereas the values for the remaining sub-fractions were ≥ 10 mg mL^{-1}. Both sHF_2 and sHF_3 had lower MIC values than TBZ; in fact, TBZ prepared up to 2 mg mL^{-1} did not inhibit the mycelial growth of *Colletotrichum gloeosporioides*.

Table 1. Yield, retention factor, and inhibitory activity against *Colletotrichum gloeosporioides* of the sub-fractions (sHF_1–sHF_7), obtained by silica gel chromatography of the hexane fraction of *Vitex mollis* (HF-VM)

Sub-fractions	Yield[†]	R_f[‡]	MIC[§]
HF-VM	2.65	—	20
sHF_1	1.88	0.94	>10
sHF_2	0.10	0.51	2
sHF_3	0.20	0.45	0.5
$sHF_{3\text{-}c}$	0.001	0.45	>0.5
sHF_4	0.22	0.36	10
sHF_5	0.03	0.22	10
sHF_6	0.06	0.064	10
sHF_7	0.14	0	>10
Caprylic acid	—	—	0.125
Thiabendazole (TBZ)	—	—	>2

[†] Yield was expressed as % w/w.
[‡] R_f stands for retention factor, spots were revealed with the Lieberman–Burchard test.
[§] Antifungal activity was expressed as minimal inhibitory concentration (MIC, mg mL^{-1}), evaluation was repeated three times.

3.1.2 *Gas chromatography–mass spectrometry (GC–MS) analysis*

The GC–MS analysis showed ten main components in the sHF_3 (Fig. 1). The most abundant compounds were 4-decenoic acid, methyl ester (39.43%), fatty acids (33.86%), being caprylic acid (27.53%) the most abundant, the pentacyclic triterpene 24-methylencycloartanol (14.29%), and other compound families (9.76%) (Fig. 1 and Table 2).

3.1.3 *Triterpene isolation and antifungal activity evaluation*

The chromatographic separation of sHF_3 resulted in 50 eluates of 5 mL, and the triterpene was mainly found in eight of them. Then, 10 mg of purified triterpene was crystallized, and the crystal melting point was 80–81 °C. The sharpness in melting point and a single spot in TLC developed with different mobile phases suggested the compound purity. However, the pure triterpene ($sHF_{3\text{-}c}$) was inactive against *Colletotrichum gloeosporioides* up to 0.5 mg mL^{-1} (Table 1).

3.1.4 *Caprylic acid identification, quantification, and antifungal activity evaluation*

The caprylic acid standard exhibited a potent antifungal effect against *Colletotrichum gloeosporioides* with a MIC value of 0.125 mg mL^{-1} (Table 1). The caprylic acid standard (purity > 99%) presented a retention time of 21.2 min in GC–MS analysis, coinciding with compound **2** (Fig. 2(a,b)). When sHF_3 and the standard were mixed, compound **2** in sHF_3 and caprylic acid coeluted in the GC–MS analysis (Fig. 2(c)), intensifying the signal and confirming the compound identity as caprylic acid. Regarding the quantification, caprylic acid content was 464.19 ± 19.89 μg mg^{-1} sHF_3.

3.2 Antifungal activity mechanism

3.2.1 *Spore germination and mycelial growth*

Spore germination in control and TBZ treatments reached about 80% in the first 8 h, whereas germination was inhibited by the sHF_3 treatment (0.25 and 0.50 mg mL^{-1}), reaching approximately 5% (Fig. 3(a)). After 16 h, all treatments reached 100% germination but the sHF_3 treatment at 0.50 mg mL^{-1} with a value lower than 10%. After 24 and 32 h, the spore germination on treatment sHF_3 at 0.50 mg mL^{-1} remained lower than 10%, and there was a significant difference with the other treatments ($P < 0.05$). As observed by optical microscopy, the spores in the control treatment presented visible germination after 8 h and development of mycelium after 32 h. However, only a small number of spores treated with sHF_3 (0.50 mg mL^{-1}) were germinated (Fig. 4).

Analyzing the mycelial growth speed, the antifungal treatments had the following order: control (≅ 1 cm d^{-1}) > sHF_3 (0.25 mg mL^{-1}) and TBZ (≅ 0.5 cm d^{-1}) > sHF_3 (0.50 mg mL^{-1}) (0 cm d^{-1}) (Fig. 3(b)).

3.2.2 *Protection assays*

Ergosterol, sorbitol, linoleic acid, and palmitic acid protected the *Colletotrichum gloeosporioides* spores from the sHF_3 antifungal activity (Table 3). The best results were obtained with sorbitol and linoleic acid, which increased the sHF_3 MIC (2.5 mg mL^{-1}) after 48 h. The sHF_3 MIC value for the negative control (hexane or water) was the same (0.5 mg mL^{-1}).

3.2.3 *Intracellular leakage*

Intracellular leakage of *Colletotrichum gloeosporioides* spores and mycelium was affected by the addition of the sHF_3 ($P < 0.05$) (Table 4). On spores, the treatment at 0.25 mg mL^{-1} increased

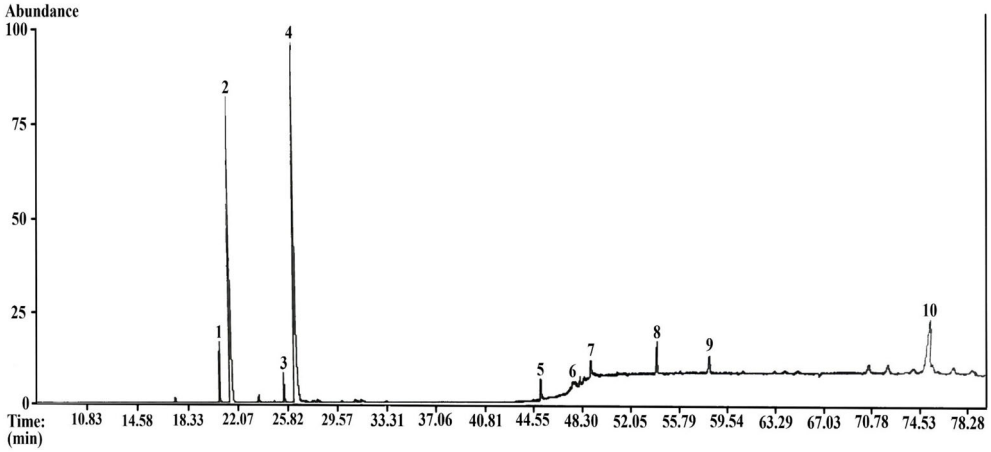

Figure 1. GC–MS chromatogram of the most active sub-fraction (sHF_3) of the hexane fraction of *Vitex mollis* (HF-VM). The names of numbered peaks are shown in Table 2.

Table 2. Gas chromatography–mass spectrometry (GC–MS) analysis of the most active sub-fraction (sHF_3) from *Vitex mollis* pulp

Chemical compound					
Number	RT[†]	Name[‡]	MW[§]	Match factor[¶]	Area (%)
1	20.43	Cinnamaldehyde	132	937	3.77
2	21.22	Caprylic acid	144	940	27.53
3	25.29	Capric acid	172	930	2.62
4	26.11	4-Decenoic acid, methyl ester	184	759	39.43
5	45.20	Palmitic acid	256	932	1.91
6	48.28	Stearic acid	284	851	0.63
7	49.12	Linoleic acid	280	862	1.17
8	54.29	Hexacosane	366	883	3.36
9	58.42	Eicosanol	298	886	2.63
10	75.45	24-Methylencycloartanol	440	913	14.29

Detected compounds by family: esters (39.43%), fatty acids (33.86%), triterpenes (14.29%), aldehydes (3.77%), n-alkanes (3.36%), and fatty alcohols (2.63%).
[†] RT stands for retention time and was expressed in minutes.
[‡] Common name was employed when possible.
[§] MW stands for molecular weight and was expressed in g mol^{-1}.
[¶] The match factor is the similarity degree between the mass spectra of the sample and that stored in the National Institute of Standards and Technology mass spectra library. The match factor takes values in the range of 0 and 1000.

protein and electrolyte leakage, whereas all evaluated intracellular components leaked at 0.50 mg mL^{-1}. On mycelium, sHF_3 treatment (0.25 and 0.5 mg mL^{-1}) only induced electrolyte leakage.

4 DISCUSSION

The identified fatty acids, n-alkanes, aldehydes, and fatty alcohols in the sHF_3 are commonly found in plants (Table 2). The saturated (caprylic, capric, palmitic, and stearic acids) and unsaturated (linoleic acid) fatty acids found in the sHF_3 are synthesized in the cell plastids; then, they are transported into the endoplasmic reticulum for lipid assembly.[25] The occurrence of medium-chain fatty acids such as caprylic acid is rare in fruits. This potent antimicrobial is GRAS by the FDA,[12,15] and is commonly found in coconut, milk of some mammals, and palm kernel oil.[26] Therefore, *V. mollis* pulp is a novel source of this valuable GRAS-type compound.

The identified n-alkane (hexacosane) and fatty alcohol (eicosanol) have been isolated from fruit waxes and are incorporated on the outer layer of the epidermal cells, helping to decrease the surface wetting and moisture loss.[27] For the detected aldehyde, cinnamaldehyde is a phenylpropanoid with potent antifungal properties associated to cell wall disruption.[28]

The methyl ester of 4-decenoic acid is uncommonly found in nature. However, Keeney and Patton[29] and Blum *et al.*[30] identified decenoic acid derivatives on milk and royal jelly, respectively. Regarding the presence of the compound 24-methylencycloartanol, plants of the *Vitex* genus contain a wide variety of pentacyclic triterpenes (oleanane, ursane, norursane, lupane, and friedelane type). However, this is the first study to report this compound on a *Vitex* species.[16,31]

Compared with the antifungal activity of the HF-VM (MIC = 20 mg mL^{-1}), the activity was concentrated on sHF_3 (MIC = 0.5 mg mL^{-1}), indicating that sHF_3 contains the main compounds responsible for the antifungal activity. Moreover, the antifungal activity of sHF_3 was also assessed in liquid medium (PDB) and showed a MIC value of 0.20 mg mL^{-1}. Treatments in the range of 0.05 to 0.15 mg mL^{-1} presented small mycelium

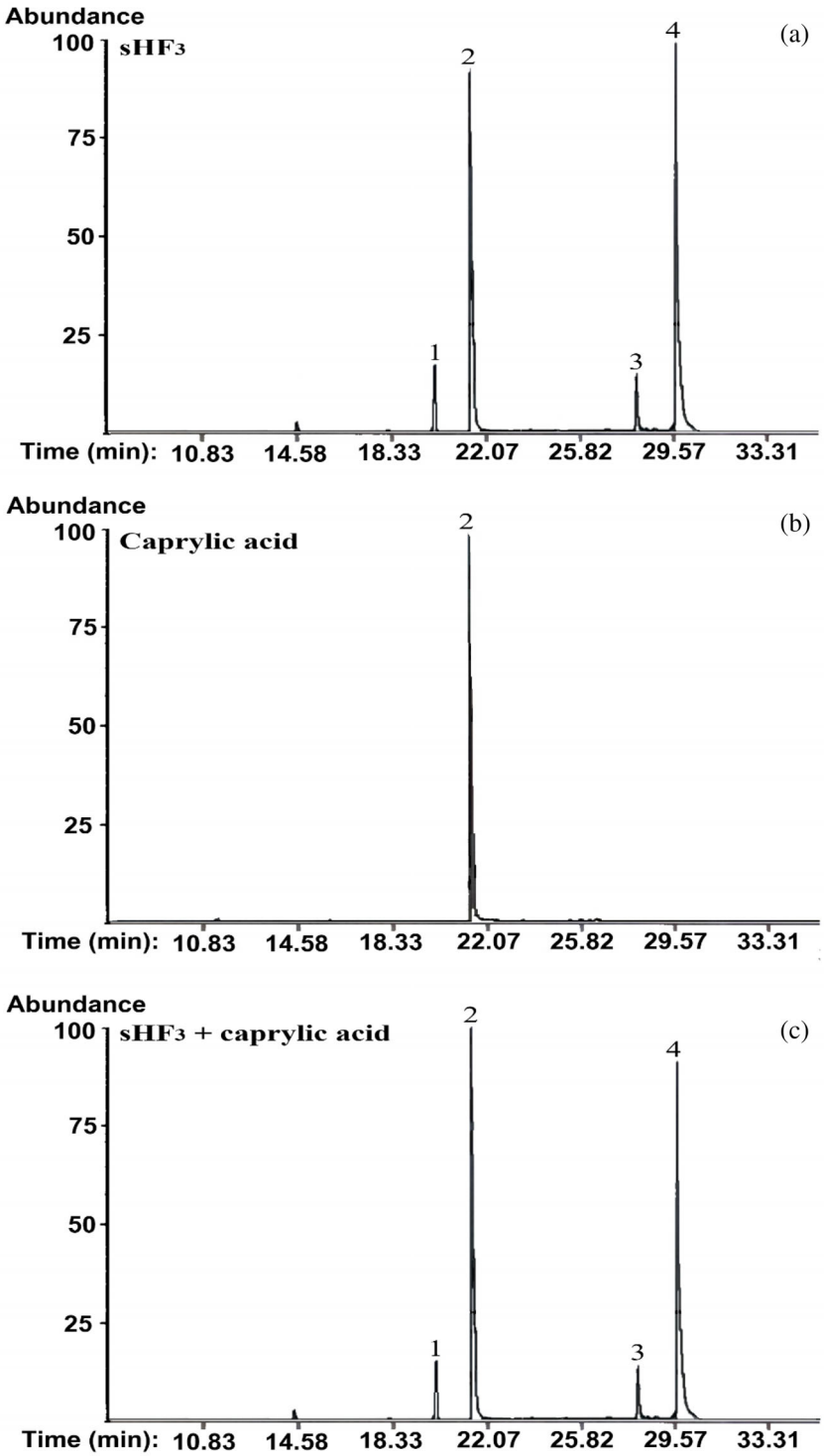

Figure 2. GC–MS chromatogram of sHF_3 (a), caprylic acid (b), and sHF_3 + caprylic acid (c). 1, Cinnamaldehyde; 2, caprylic acid; 3, capric acid; 4, 4-decenoic acid, methyl ester.

particles and a large proportion of ungerminated spores (data not shown). These data indicate that MIC values depended on the experimental conditions.

The main components (> 5%) in sHF_3 were 4-decenoic acid, methyl ester, caprylic acid, and 24-methylencycloartanol, all of which have antimicrobial properties.[12,30,32] However, pure 24-methylencycloartanol was inactive against *Colletotrichum gloeosporioides*, discarding its role in the antifungal properties of sHF_3.

The elevated antifungal activity of caprylic acid (MIC = 0.125 mg mL^{-1}) indicated that the antifungal properties of sHF_3 are mainly related to the presence of this compound and not to a synergistic effect among its major components. Liu *et al.*[12] reported that a mixture of medium-chain fatty acids (caprylic acid, pelargonic acid, and

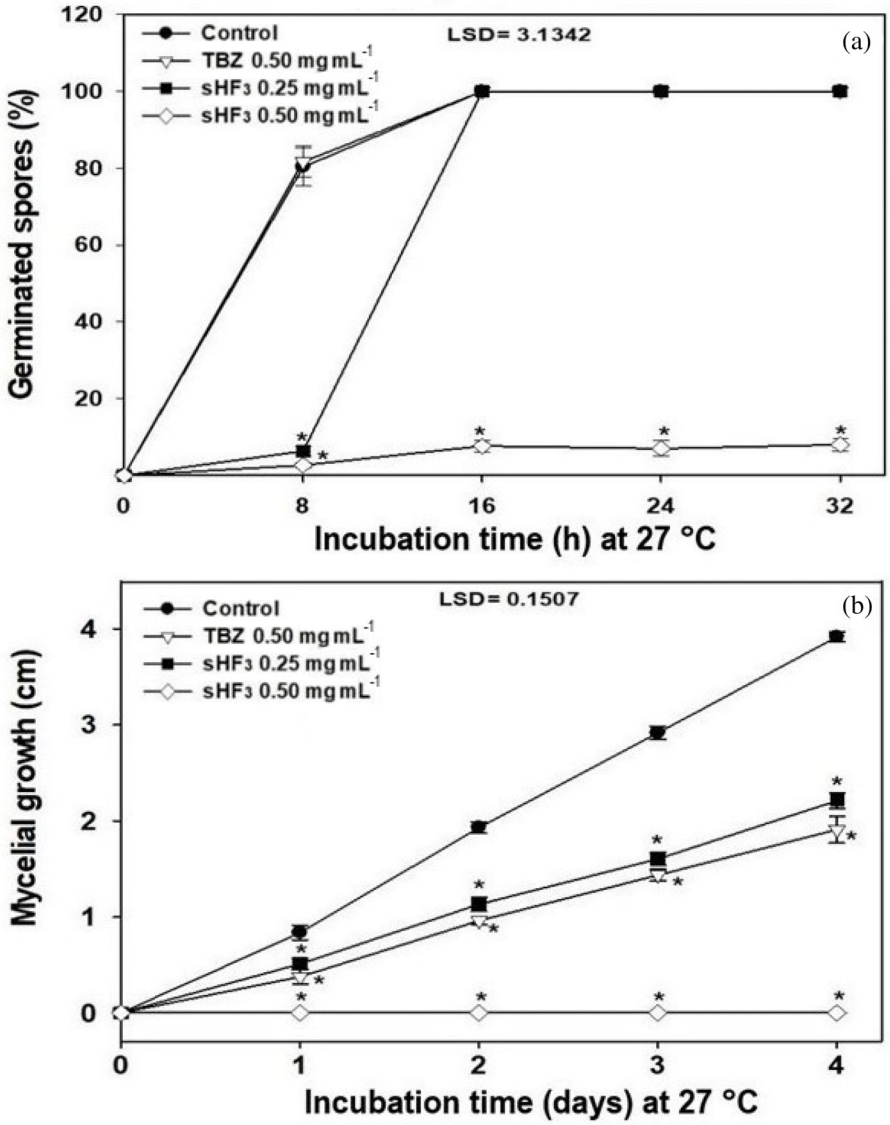

Figure 3. Effect of the most active sub-fraction sHF_3 (0.25 and 0.50 mg mL^{-1}) of the hexane fraction of *Vitex mollis* (HF-VM) on spore germination (a) and mycelial growth (b) of *Colletotrichum gloeosporioides*. Hexane and thiabendazole (TBZ) on potato dextrose agar (PDA) were employed as the negative and positive controls, respectively. Values are the means of three replicates. Vertical bars indicate standard deviation (SD). Asterisk (*) denotes statistical difference ($P > 0.05$) with control on the same evaluation time and according to the Fisher's least significant difference (LSD) test.

capric acid) inhibited the mycelial growth of phytopathogenic fungi. Similar to our results, they reported a MIC value of 0.125 mg mL^{-1} for *Colletotrichum gloeosporioides*, whereas for *Phytophthora infestans* and *Botrytis cinerea* the MIC values were 0.1 and 0.2 mg mL^{-1}, respectively. It must be mentioned that this article was focused on mycelial growth inhibition, and aspects regarding the mechanism were omitted.

Studies on the antifungal effect of medium-chain fatty acids such as caprylic acid against phytopathogenic fungi are scarce. However, there are multiple antifungal clinical studies. Bae[33] and Bhattacharyya *et al.*[34] reported the effect of these compounds on *Candida albicans and Trichophyton rubrum*, showing that they damaged the plasmatic membrane and inhibited different efflux pumps.

The effect of sHF_3 upon *Colletotrichum gloeosporioides* germination suggested that it compromised the spore functionality and could stop the fruit infection at early stages. The MIC for TBZ against *Colletotrichum gloeosporioides* strain (accession number HM222960.1) from Sinaloa, Mexico, is higher than values for reference strains,[8] suggesting its TBZ resistance. This TBZ resistance has been registered for other *Colletotrichum* spp. from Mexico.[9]

The protection assays provided relevant information about the antifungal mechanism of the sHF_3 against *Colletotrichum gloeosporioides*. The assay with sorbitol revealed that caprylic acid damaged the membrane as previously described with *Candida albicans*,[33] being inferred because sorbitol is an osmoprotectant used to stabilize cellular membranes. The assays with palmitic and linoleic acids revealed that they interacted with caprylic acid; thus, the spore membrane was less exposed to caprylic acid, suggesting that the antifungal mechanism of caprylic acid was partially mediated by interaction with membranal fatty acids. This mechanism has been previously described by Bhattacharyya *et al.*[34] On an *in silico* assay, molecules of caprylic (eight carbon atoms) and lauric acid (12 carbon atoms) were placed next to a

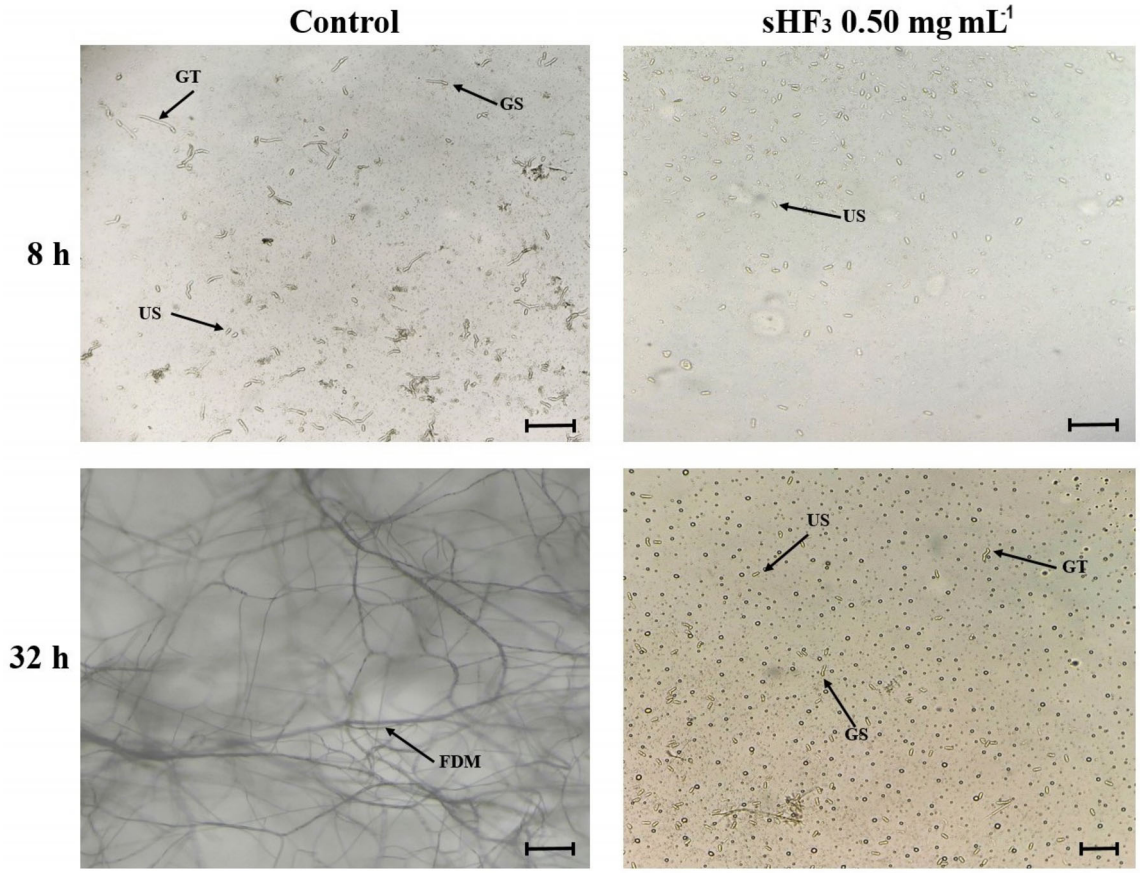

Figure 4. Images of the effect of the most active sub-fraction sHF_3 (0.50 mg mL^{-1}) on spore germination of *Colletotrichum gloeosporioides*. Hexane was employed in the negative control. US, ungerminated spore; GS, germinated spore; GT, germ tube; FDM, full developed mycelium. Images were taken on a light microscope at 10× magnification. Bars = 50 µm.

Table 3. Protective effect of ergosterol, sorbitol, and fatty acids (palmitic and linoleic acids) against the inhibitory effect of sHF_3 on development of *Colletotrichum gloeosporioides* mycelia[†]

	MIC value for sHF_3 (mg mL^{-1})	
Treatment	24 h	48 h
Control	0.5	0.5
Ergosterol	0.5	1.0
Sorbitol	1.5	2.5
Palmitic acid	1.0	2.0
Linoleic acid	1.5	2.5

[†] The experiment was repeated three times. MIC stands for minimal inhibitory concentration.

phospholipid bilayer until equilibrium. In the simulation with caprylic acid, the molecules tend to self-aggregate; however, they started to interact with the bilayer and penetrated the membrane within 1000 ns. However, lauric acid remained as an aggregate until equilibrium (1500 ns). The authors concluded that the proportion of hydrophilic and lipophilic characteristics of caprylic acid was adequate to interact with the membrane, and the rate of lipophilic properties in lauric acid was too high and tended to form micelles in an aqueous medium.

The protective effect of palmitic acid was lower than that exhibited by linoleic acid, indicating a better interaction between caprylic acid and unsaturated fatty acids. Thus, sHF_3 would be a more effective antifungal on phytopathogen spore membranes composed of a higher proportion of unsaturated fatty acids. In concordance with our results, Liu *et al.*[35] reported that developed resistance of *Saccharomyces cerevisiae* against caprylic acid was due to a reduced presence of unsaturated fatty acid on the membrane.

Ergosterol had the lowest protective effect on spores and was detected only after 48 h (Table 4). Thus, data suggested that the membrane disrupting effect of sHF_3 mediated by ergosterol binding was not predominant. This result was expected as ergosterol binding is associated with volatile compounds such as geraniol, nerol, and citral. Such volatiles interact with ergosterol, resulting in channel formation, cell membrane disruption, and mitochondria malfunction.[24]

Leaked protein, DNA, and electrolytes observed in the control treatment are caused by exposure to a hypotonic medium, which causes the release of some intracellular components. The increase in leaked components on spore and mycelium treated with sHF_3 confirmed the destabilization of the plasmatic membrane, being more effective on spores.

Previous reports have shown that selenium, caprylic acid combined with synthetic fungicides, and antifungal compounds from natural sources lead to intracellular leakage.[10,13,24] Besides the

Table 4. Leakage of protein, DNA, and electrolytes from spores and mycelium of *Colletotrichum gloeosporioides* treated with sHF_3[†]

Treatment	Leaked components		
	Protein (μg mL^{-1})	DNA ($Abs_{260\ nm}$)	Electrolytes (%)
Spores			
Control	2.41 ± 0.010 c	0.10 ± 0.01 b	68.49 ± 3.190 b
sHF_3 (0.25 mg mL^{-1})	2.60 ± 0.061 b	0.11 ± 0.004 b	78.93 ± 3.434 a
sHF_3 (0.50 mg mL^{-1})	2.70 ± 0.085 a	0.14 ± 0.005 a	81.33 ± 3.949 a
Mycelium			
Control	5.16 ± 0.203 a	0.04 ± 0.005 a	60.48 ± 4.530 b
sHF_3 (0.25 mg mL^{-1})	5.07 ± 0.257 a	0.04 ± 0.008 a	76.10 ± 4.740 a
sHF_3 (0.50 mg mL^{-1})	5.32 ± 0.164 a	0.04 ± 0.006 a	76.52 ± 2.175 a

[†] For spores and mycelium, different letters on the same column indicate statistical difference ($P < 0.05$) according to the Fisher's least significant difference (LSD) test. Values are means ± standard deviation (SD) of three replicates.

mechanism previously described (fatty acid interaction), these authors suggested that fungal cell leakage was due to oxidative stress.

In conclusion, the HF-VM activity against spores and mycelium of *Colletotrichum gloeosporioides* was mainly due to caprylic acid. *Vitex mollis* represents a novel source of this rare and innocuous compound and contains other unidentified antifungal compounds. Data suggest that caprylic acid interacts with lipids in fungal membranes inducing its disruption, and this effect was higher on spores. Furthermore, caprylic acid could be employed as a marker to develop standardized antifungal HF-VM preparations.

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CONFLICT OF INTEREST

The authors declared that they have no conflict of interest that are relevant to this article.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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