In Vitro Antimicotic Activity and Structural Damage Against Canine Malassezia pachydermatis Strains Caused by Mexican Stinglees Bee Propolis

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Article

In Vitro Antimicotic Activity and Structural Damage Against Canine *Malassezia pachydermatis* Strains Caused by Mexican Stinglees Bee Propolis

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Abstract: This work demonstrates the antimycotic activity and structural damage of stingless bee propolis against *Malassezia pachydermatis*,causative agent of canine otitis.Two propolis ethanolic extracts(EEP) derived from the stingless bees, *Scaptotrigona mexicana* and *Tetragonisca angustula* were tested against three clinical strains of *Malassezia pachydermatis* and one of reference (ATCC 14522). Each ethanolic extract of propolis was analyzed by Gas Chromatography – Mass Spectrometry(GC-MS). Antimycotic activity was evaluated by plate microdilution. To evaluate the induced changes in yeasts, by fluorescence microscopy, stains were performed with chalcofluor white and propidium iodide. For both propolis, sesquiterpenes were the main components determined by GC-MS. Minimum inhibitory concentration (MIC) of 32 mg/mL and a minimum fungicidal concentration of 64 mg/Ml were determined in both extracts. EPP of *Scaptotrigona mexicana* and *Tetragonisca angustula* caused significant damage to yeast morphology. Propidium iodide penetration was observed, indicating damage to the yeast and with calcofluor-white stain, only morphology deformation was observed..The antimycotic activity and structural damage of propolis from *Scaptotrigona mexicana* and *Tetragonisca angustula* against *Malassezia pachydermtais* was demonstrated .This probably being the first scientific report that demonstrates structural damage in *Malassezia pachydermatis* of Mexican stingless bee propolis

Keywords: Mexican stingless bee; propolis; antimicotic activity; structural damage; *Malassezia pachydermatis*

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1. Introduction

Propolis is a natural resinous substance produced by bees from substances collected from the vegetation in which antifungal, antibacterial, antiviral and antiparasitic activities have been recognized, showing variation in their biological activity depending on their geographical origin [1–3].

In Latin America, there is a great variety of ecosystems each with a very diverse vegetation from which native bees' extract propolis, which in turn results in great medicinal richness. In general, to be able to identify the origin from which bees extracted the material with which they elaborate their propolis, is not as simple as it is to do so with honey or pollen, and for this reason it has been proposed that chemotaxonomic studies should be undertaken or the behavior of each bee species in each region to be observed [4–7]. Since ancient times, products elaborated by stingless bees from the species *Scaptotrigona Mexicana* and *Tetragonisca angustula* have been used, both species being highly employed in central American beekeeping, however, very little information exists in the currently available literature concerning the scientific evidence demonstrating the medicinal efficacy of their products. Also, propolis from the *Apis mellifera* species presents a fungicidal effect against several fungi species [8–10]. Moreover, its applications can prove beneficial in veterinary medicine, as in the case of canines. Propolis coming from *Apis mellifera* has been used as a prophylactic agent against gastrointestinal and respiratory diseases, mycoses, as well as a wound healing agent, and its therapeutic use has spread to many areas [11,12], one example being that of canine external otitis, which is the inflammation of the external auditory canal and represents between 5 to 20 % of consultation reasons. The main causative agent of canine external otitis is the yeast *Malasezzia pachydermatis*, which is part of the normal microbiota of the external auditory canal in dogs [13–16]. Propolis possess an alternative for the treatment of canine otitis, instead of common antifungals, for patients with a high incidence of relapse, because of its antifungal, anti-inflammatory and wound healing properties; however, up to this time the only propolis that has been evaluated is that obtained from the bee *Apis mellifera* [17]. To undertake studies regarding the potential antimycotic properties of propolis from native bees can provide the scientific ground for its use as an alternative treatment against canine otitis.

2. Materials and Methods

2.1. Ethanolic Extracts Propolis (EEP)

Two propolis samples from stingless bees were obtained. One from, found in the municipality of Yecuatla, Veracruz, geolocation at 19°51 north and 96°46 west, at an altitude of 432 m.a.s.l. The other one from *Tetragonisca angustula*, found in the municipality of Chalchihuitán, Chiapas, latitude of 16°57 north, longitude of 92°37 west and an altitude of 1461 m.a.s.l. Collected material was evaluated for its physical properties according to Mexican regulation regarding color, odor, taste, and consistency [18]. 30 g of propolis from *Sm* and 12 g from *Ta* were weighted and any present impurities were eliminated. Thereafter 100 mL of 70 % ethanol were added to each sample and the obtained mixture was subjected to ultrasonic extraction (Branson, CPX1800H, Danbury, EE. UU.). Each sample was then vacuum filtered and, finally, the obtained filtrates were concentrated by rotovapor (Science MED, SM100-PRO, Finlandia) and left to dry by vacuum pump. Then, both dried extracts were placed in light resistant containers and refrigerated at 4 °C until they were needed [19].

2.2. Gas Chromatographic-Mass Spectrometry (GC-MS)

Chromatographic analysis of ethanolic extracts was performed using a gas chromatograph (6850) coupled to a mass spectrometer (7890 model, JEOL MC-GC-Mate II, Japan). A HP-5MS (30 m × 0.32 mm) capillary column and a film thickness of 0.25µm were used. Helium gas was used as the carrier gas. Elected injection method was split mode with an injection volume of 1 µL. Separation conditions were as follows: 70°C at the beginning for two minutes followed by two ramp increments. The first one of 20°C per minute until a temperature of 230°C was reached; the second one of 8°C per minute

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until a temperature of 290°C was reached, keeping this temperature for a period of 5 minutes. Total analysis time was of 21.25 minutes. The detected mass range was of 35 m/z to 750 m/z, the sample was subjected to electron impact ionization at 70 eV, with the ionization source reaching a temperature of 230 °C. Compound identification was carried out by comparison with the library database from the equipment [20].

2.3. Evaluation of antimycotic activity:

2.3.1. Inoculum preparation

Three different *M. pachydermatis* strains were used, one of reference ATCC 14522 and two obtained from clinical isolates from dogs. All three strains were identified by proper biochemical testing [21]. Microorganisms were provided by the Laboratorio del Servicio de Análisis de Propóleos (LASAP), a branch of the Multidisciplinary Research Unit of FES Cuautitlán, UNAM. To activate *M. pachydermatis* strains, each yeast was seeded in modified Dixon Agar (mDA). Each was seeded in a different petri dish and then incubated for 72 hours at 28 °C. Thereafter, samples were reseeded in other mDA-containing plates and were incubated for 48 hours at 28°C in order to rule out strain contamination [22,23]-

2.3.2. Determination of Minimum Inhibitory Concentration and minimum fungicidal concentration

Broth microdilution was carried out by determination of the minimum inhibitory concentration (MIC) and the minimum fungicidal concentration (MFC). For this purpose, the EEP were subjected to serial dilutions from 32 to 0.0312 mg/mL in Sabouraud Dextrose broth (SDA) supplemented with glucose (Bioxon, México). SDA broth containing the microorganism was used as the positive witness, while SDA broth without the microorganism was used as the negative witness and were incubated for 48 hours at 28°C. To detect the respiratory activity of the microorganisms, a solution of 0.08 % oxidated tetrazolium salt (TTC) was used, which generates a red pigment (formazan) in the presence of the microorganism. The afore mentioned procedure was performed in the following manner: 50 µL of TCC were added to each inoculated well, plates were mixed by plate agitator and incubated at 33 °C for 30 minutes. By the end of the 30 minutes, it was observed that an insoluble, red-colored precipitate had formed, representing the MIC. When there was no growth, the solution stayed clear-colored, indicating the MIC or MFC. To confirm these results, it was determined whether the observed effect was fungistatic or fungicidal by taking a sample of the culture with an inoculation loop and seeding the material in a petri dish containing SDA agar supplemented with glucose, followed by an inoculation period of 72 hours at 33 °C. It was considered that plates exhibiting growth was indicative of a fungistatic effect, while plates lacking growth of the microorganisms was indicative of a fungicidal effect [24].

2.4. Structural damage

To evaluate the structural changes induced by the EEP on *Malazassia pachidermatis* fluorescence microscopy was used. In this experiment, the reference strain and one clinical strain were used. A concentration of 64 mg/mL of each EEP of *Scaptotrigona mexicana* and *Tetragonisca angustula* were added to each strain. Incubated at 28 ° C for 48 hrs. When the incubation ended, the yeast was stained with calcofluor-white (M2R 1g/L,Sigma) and propidium iodide (2.4 mmol, Sigma), as a negative control, a culture without EEP was used. Preparations were viewed on a microscopy Zeiss Axioscop 40, coupled to an Evolution VF Cooled Color camera from Media Cibernetics. All experiments were performed in triplicate [25,26].

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3. Results

3.1. Gas Chromatographic-Mass Spectrometry (GC-MS)

Results from each propolis ample analyzed by coupled gas chromatography- mass spectrometry (GC-MS) are shown in Tables 2 and 3, as well as Figures 1 and 2, these show the main identified compounds with an accurate identification (> 90%) as related to the equipment's database. For both propolis simples, the main constituent compounds were sesquiterpenes

Table 1. Constituents of Mexican *Scaptotrigona mexicana* propolis characterized by CG-EM.

Bak	Time Retention (TR)	Composite proposed by the database	Chemical classification	Biological activity	Reference
~~2~~	30.6	1,4-Methanecycloocta[d]piri-dazine, 1,4,4a,5,6,9,10,10a-octahydro-11,11-dimethyl-, (1.alpha.,4.alpha.,4a.alfa. ,10a.alfa.) -	Pyridazine (heterocyclic compound)	Antioxidant	[27]
5	31.58	Fanersol Isomer a	Sesquiterpene	Antimicrobian	[28]
6	32.48	Ethanone, 1-(1,3a,4,5,6,7-hexahydro-4-hydroxy-3,8-dimethyl-5-azulenyl)-	Ketone Sesquiterpene	Antimicrobian	[29]
8	33.68	2H-1-Benzoxacyclohexadecin-16(18aH)-one,3,4,5,6,7,8,9,10,11,12,13,14-dodecahydro-18,18a-dihdroxy-2-methyl	Macrocycle	Activity Not reported	[30,31]
9	33.7	Furan-2,5-dicarbaldhyde 2,5-Furandicarboxaldehydo	Heterocyclic compound with aldehyde groups	Antioxidant Antimicrobian	[32,33]

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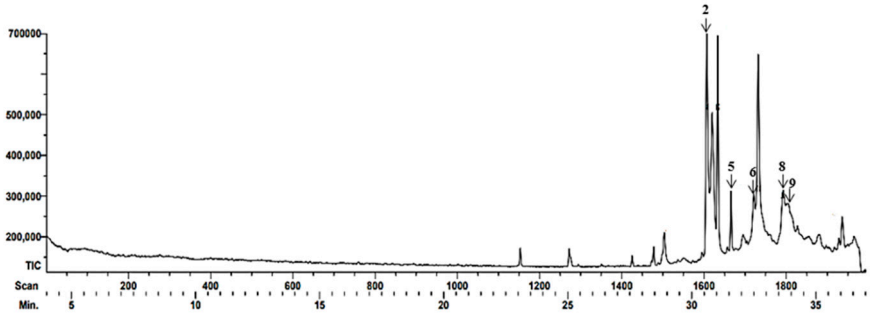

Figure 1. Gas chromatogram corresponding to the propolis of *Scaptotrigona mexicana* and the main compounds detected.

Table 2. Main constituents of propolis of *Tetragonisca angustula* characterized by CG-EM.

Beak	Time Retention (TR)	Composite proposed by the database	Chemical classification	Biological activity	Reference
7	30.68	Solavetivona	Sesquiterpenoid and a cyclic ketone	Antifungal anti-inflammatory	[34–36]
8	31.65	2-Methyl-3-(3-methyl-but-2-enyl)-2-(4-methyl-pent-3-enyl)-oxethane	Heterocycle compound	Not found Information	
9	32.75	(1S,6R,9S)-5,5,9,10-Tetramethyltricycle [7.3.0.0(1,6)]dodec-10(11)-ene	Sesquiterpene	Antibacterian antifúngal	[37]

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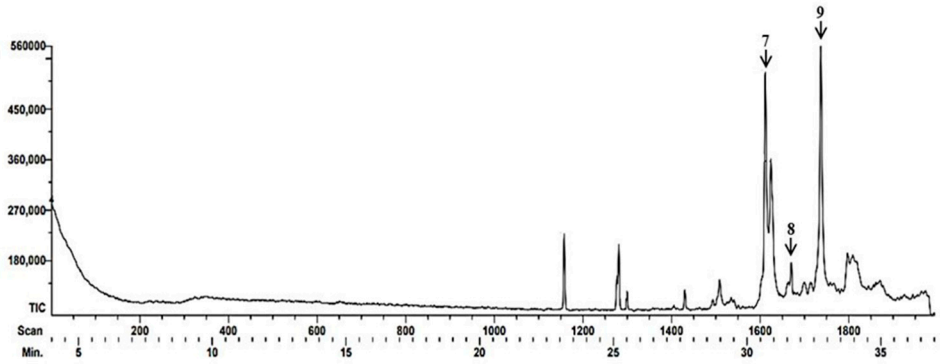

Figure 2. Gas chromatogram corresponding to the propolis of *Tetragonisca angustula* and the main compounds detected.

3.2. Evaluation of the antimycotic activity

It was found that all tested *Malassezia pachydematis* strains were susceptible to all evaluated propolis extracts in the present study, the value of the Minimum Inhibitory Concentration was of 32 mg/mL and that of the Minimum Fungicidal Concentration was of 64 mg/mL (Table 4).

Table 3. Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (FMC) values of stingless bee propolis extracts from two regions of the Mexican Republic on the reference strain *M. pachydermatis* ATCC 14522 and strains isolated from clinical samples.

	Origen	MIC (mg/ml) *M. pachydermatis* ATCC 14522	MFC(mg/ml) *M. pachydermatis* ATCC 14522	Media CMI (mg/ml) in isolation Clinical (n = 3)	Media CMF (mg/ml) in isolation Clinical (n = 3)	Number of Isolates Clinical Inhibited (n = 3)
Scaptotrigona mexicana	Municipio de Yecuatla Veracruz	32	64	32	64	3
Tetragonisca angustula	Municipio de Chalchihuitan Chiapas	32	64	32	64	3

3.3. Structural damage

Figures 3 and 4 show the effect of EEP on the structure of *M. pachydermatis* (ATCC 14522) and clinical strain. Both EEPs penetrated the yeasts.

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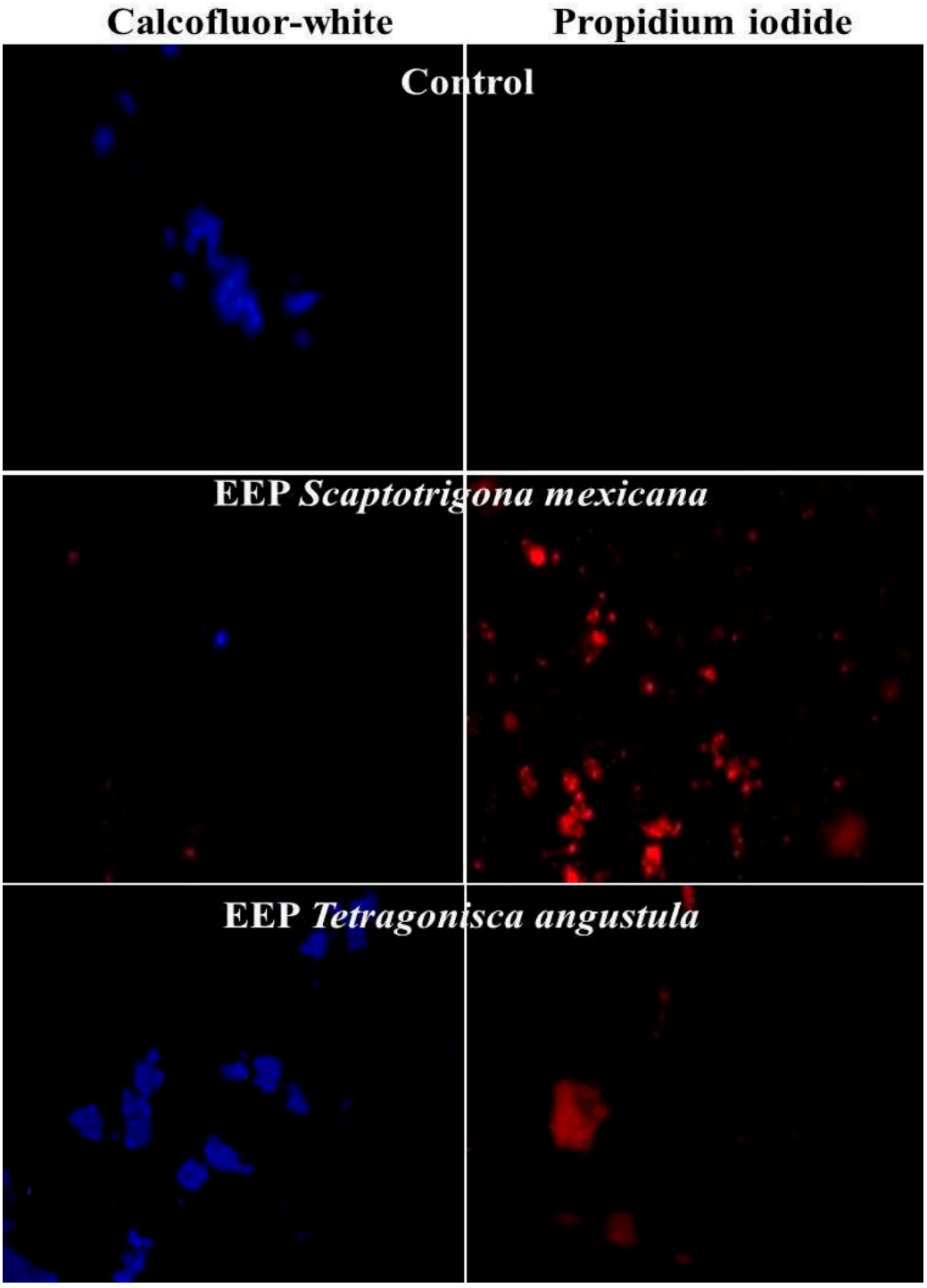

Figure 3. Effect of EEP of *Scaptotrigona mexican* and *Tetragonisca angustula* on reference strain *Malassezia pachydermatis* ATCC 14522 were obtained by fluorescence microscopy and dyed with calcofluor-white and propidium iodide. Cultures were exposed to EEP at a concentration of 64 mg/ml for 48 h at 28 °C. Propidium iodide penetration was observed, indicating damage to the yeast. With calcofluor-white stain, only morphology deformation. (40x magnification).

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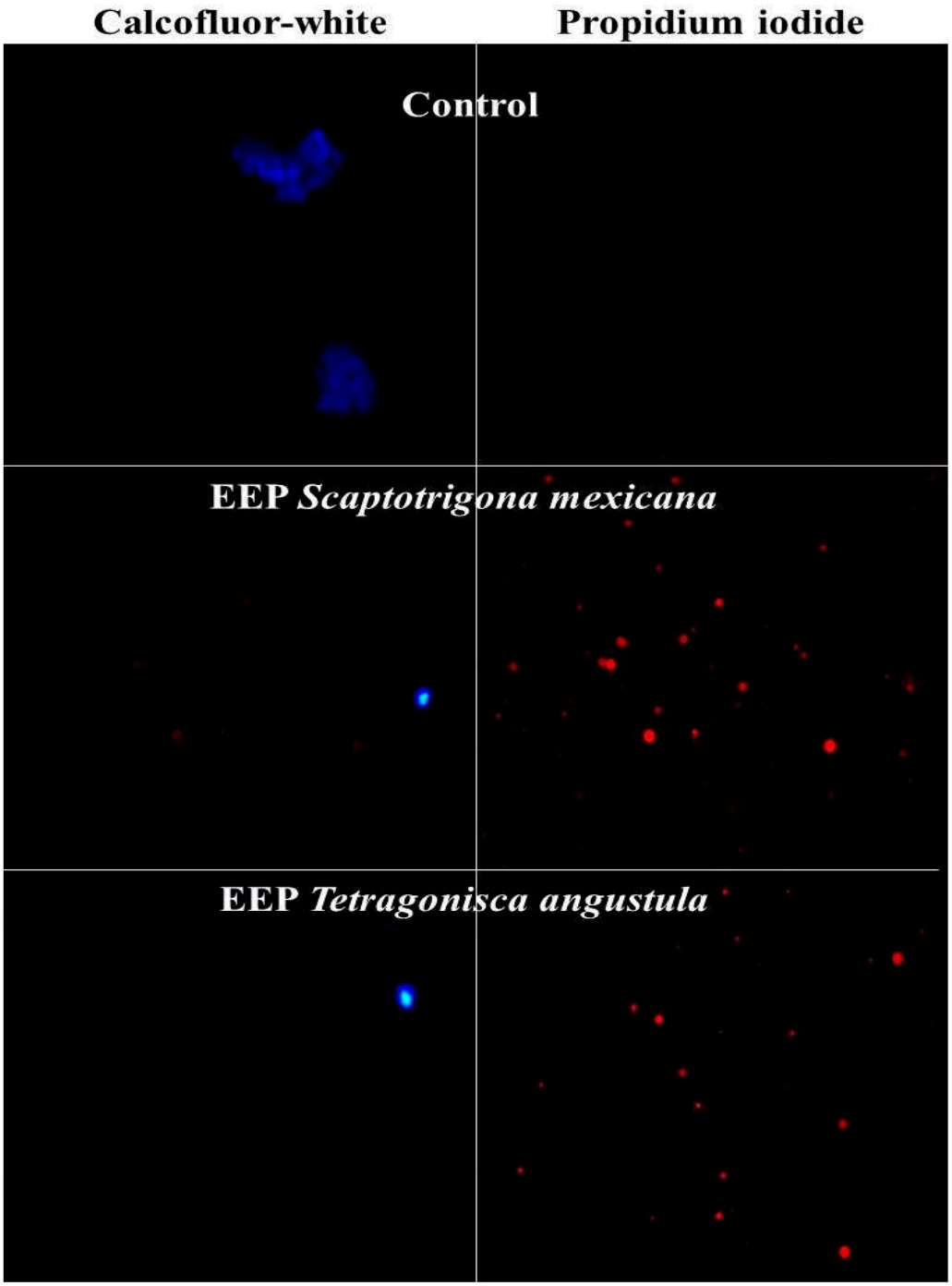

Figure 4. Effect of EEP of *Scaptotrigona mexicana* and *Tetragonisca angustula* on the clinical strain oof *Malassezia pachydermatis* were obtained by fluorescence microscopy and dyed with calcofluor-white and propidium iodide. Cultures were exposed to EEP at a concentration of 64 mg/ml for 48 hr at 28 °C. Propidium iodide penetration was observed, indicating damage to the yeast. With calcofluor-white stain, alteration of morphology is observed. A greater damage was observed since it was not detected the presence with the calcofluor- white of the yeast and only the red of the propidium iodide was detected with Ethanolic Extract of *Tetragonisca angustula* Propolis.(40x magnification).

The lack of fluorescence in the stain of calcofluor-white in some preparations indicates a probable destruction of the yeast.Propidium iodide was detected in all treated samples, which indicates alteration of the wall and membrane, observing a greater amount of altered yeasts in the reference strain treated with the EEP of *Scaptotrigona mexicana.*

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4. Discussion

Many of the identified metabolites of propolis stingless bees have been reported to exhibit a myriad of biological activities, including antimicrobial, anti-inflammatory, cytotoxic, antioxidant, hepatoprotective, antiulcer, among others [39–42]. Analyzed propolis extracts exhibit chemical compositions with significant differences among them, this being consistent with other studies which have found that the quantity and diversity of compounds found in propolis is high [43,44], in comparison with the obtained results from the present study for *Scaptotrigona mexicana* and *Tetragonisca angustula* as well as other species such as *Melipona beecheii* and others worldwide [2].It is important to consider that floral diversity, collection time of the year and bee species are all determinant factors for the final composition of each propolis. Compounds belonging to the sesquiterpenes were primarily detected, these have been attributed with antimicrobial activity [34,45–47]

Regarding *Scaptotrigona mexicana*, it is important to mention that a bibliographic research of furan-2,5-dicarbaldehyde, which is a heterocyclic compound with aldehyde groups, was unsuccessful using that exact denomination, however we did find mention of a similar compound, 2-acetil-5-metilfurane, reported to exhibit antimicrobial activity against *Escherichia coli, Candida albicans* and *Staphylococcus aureus* [33].

For *Tetragonisca angustula*, the antibacterial and antifungal activity of solavetivone has been reported [36,37]; on the other hand, concerning l-(1S,6R,9S)-5,5,9,10-tetramethyltricyclo [7.3.0.0(1,6)]dodec-10(11)-ene, there in no specific information regarding its activity; nonetheless, antifungal and antibacterial activities have been reported for a similar compound, namely 3,3,7,7-tetramethyl-5-(2-methyl-1-propenyl)-tricyclo [4.1.0.0(2,4)]heptane [37].

Antimycotic potential of propolis extracts, mainly from *Apis mellifera*, has been demonstrated against *Candida albicans*. Also, fungicidal and fungistatic properties of green and red propolis extracts from Brazil against other fungi genera such as *Saccharomyces* [48] have been observed; inhibition and morphologic alterations of *Cyptococcus neoformans* [49].

None the less, information regarding the evaluation of the antimycotic activity of propolis coming from stingless bees mainly against *Candida albicans* has been studied for the following species: *Lestrimellata spp., Melipona favora orlinge, Melipona marginata, Melipona quadrifasciata, Melipona scutellaris, Nannotrigona testaceicornis, Plebeia droriana, Plebeia remota, Scaptotrigona bipunctata, Tetragona clavipes* [7] and *Tetragonisca angustula* and that of propolis from *Tetragonisca fiebrigi* against *Candida glabrata* [50] .In the case of propolis coming from the Malaysia-stingless bee *Trigona thoracica,* it has been demonstrated that it acts against *Cryptococcus neoformans* [51]. Furthermore, an Indonesian propolis from the species *Tetragonula sp.* has been evaluated as a possible therapeutic agent for the treatment of vaginal candidiasis [52].

Regarding *Malassezia pachydermatis*, there are studies evaluating the activity of propolis from *Apis mellifera,* but not from native bees, against this yeast. In a recent study a correlation was established between the antifungal activity of EPP from Brazilian green and red propolis against *M. pachydermatis,* with a MIC between 4 y 8 mg/mL and a MFC of 8 and 16 mg/mL. It was determined that as the total content of phenols and flavonoids increases, propolis exhibits a better biological activity, proposing that the action mechanism of EEP is due to the rupture of the cellular wall, given the fact that during the investigation it was observed that some azole- resistant *Malassezia pachydermatis* strains were inhibited by the EEP. However, they also extern concern because it is still unclear if high EPP concentrations could induce cytotoxicity. Therefore, there remains work to be done in order to accurately identify the active principles of propolis, as well as their action mechanisms. Currently the are two theories aiming to explain the antifungal activity of propolis, the first one proposing that it elicits cellular wall lysis and the other proposing that it damages the cellular membrane by inhibiting ergosterol synthesis [53]. Likewise, the efficacy of an Argentinian propolis against *M. pachydermatis* was evaluated by different in vitro techniques, which concluded that the yeast was vulnerable to all employed propolis concentrations, with a MIC of 0.30 mg/mL, however, they were not able to determine the MFC [17]. The efficacy of a 2.5 % EEP against 48 clinical strains of *Malazessia pachydermatis* isolated from dogs diagnosed with external otitis was also proven, as it

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was found that all of them were susceptible to it [54]. Antimycotic activity against clinical isolates from dogs with external otitis has also been observed in a EEP from Rio Grande do Sul, Brazil, with a MIC of 2.6 mg/mL and a MFC of 5.3 mg/mL [55]. In contrast, our results for both propolis of the evaluated bee species regarding MIC and MFC was of 32 mg/mL and 64 mg/ml respectively, both concentrations being significantly higher than those reported in studies of propolis coming from *Apis mellifera.*

According to the images obtained, the EEP proved to be able to penetrate the membrane cellular, causing severe damage and eventually death of yeasts with structural and functional damage by membrane disruption. Calcofluor white has a high affinity for fungal wall components [56,57]. The alteration observed with this stain was mainly deformity of the morphology and in some cases, we have the hypothesis that the complete destruction of the cell wall caused the yeasts not to be observed, which would be a possible effect by the sesquipertenes present in the EEP as previously described [58]. It would be advisable to perform computational chemistry studies to specifically establish the damage of these compounds in the yeast wall.

On the other hand, propidium iodide binds to nucleic acids and increases its red color when there is damage to the cell membranes. which indicates severe cell damage and death, which was seen in the reference strains and the clinic treated with both EEP, which demonstrates the effectiveness of this type of propolis. This effect with propidium iodide has been observed in other fungi such as *Fusarium* species and *Colletotrichum fructicola*, [59] when naturally occurring compounds have been evaluated against their growth [60].

These stains have been used to detect cellular damage using *Apis mellifera* propolis against other yeast of medical importance such as *Candida albicans* [61–63] as well as the bacterium *Staphylococcus aureus* [64].

Therefore, it is shown that propolis of the Mexican stinglees bee *Scaptrigona mexicana* and *Tetragonisca angustula* have antimycotic effect and cause structural damage on *Malazessia pachidermatis*, which supports its possible use for therapeutic purposes in the treatment.. The findings and their implications should be discussed in the broadest context possible. Future research directions may also be highlighted.

5. Conclusions

In conclusion, the present study has shown the antifungal properties and damage structural of two propolis ethanolic extracts from two stingless bee species (*Scaptotrigona mexicana* and *Tetragonisca angustula)* proceeding from two Mexican municipalities (Yecuatla, Veracruz and Chalchihuitán, Chiapas) against different *Malazessia pachydermatis* strains, one of reference (ATCC14522) and three clinical isolates, being highly likely that for both propolis. Sesquiterpenes, along with other compounds, are possibly the reason the reason for the antimycotic activity of both extracts. It is important to mention that, to our knowledge, the present work is the first to demonstrate the damage structural and antifungal effect of Mexican stingless bee propolis against this *Malazessia pachydermatis*. None the less, further research must be undertaken in order to provide more solid scientific basis for the future employment of propolis as an alternative treatment for canine external otitis.

Supplementary Materials: Table 1. Constituents of *Scaptotrigona mexicana* propolis characterized by CG-EM, Figure 1. Gas chromatogram corresponding to the propolis of *Scaptotrigona mexicana* and the main compounds detected. **Figure 2.** Gas chromatogram corresponding to the propolis of *Tetragonisca angustula* and the main compounds detected. **Table 2.** Main constituents of propolis of *Tetragonisca angustula* characterized by CG-EM. **Figure 2.** Gas chromatogram corresponding to the propolis of *Tetragonisca angustula* and the main compounds detected**. Table 3**. Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (FMC) values of stingless bee propolis extracts from two regions of the Mexican Republic on the reference strain *M. pachydermatis* ATCC 14522 and strains isolated from clinical samples. **Figure 3**. Effect of EEP of *Scaptotrigona mexican* and *Tetragonisca angustula* on reference strain *Malassezia pachidermatis* were obtained by fluorescence microscopy and dyed with calcofluor-white and propidium iodide. Cultures were exposed to EEP at a concentration of 64 mg/ml for 48 hr at 28 °C. Propidium iodide penetration was observed, indicating damage to the yeast. With calcofluor-white stain, only morphology deformation.(40x magnification).**Figure 4.** Effect of EEP of *Scaptotrigona mexican* and *Tetragonisca angustula* on clinical strain of *Malassezia pachidermatis* were obtained by

fluorescence microscopy and dyed with calcofluor-white and propidium iodide . Cultures were exposed to EEP at a concentration of 64 mg/ml for 48 hr at 28 °C. Propidium iodide penetration was observed, indicating damage to the yeast; with calcofluor-white stain, alteration of morphology was observed. A greater damage was observed since it was not detected the presence with the calcofluor- white of the yeast and only the red of the propidium iodide was detected with Ethanolic Extract of *Tetragonisca angustla* Propolis. .(40x magnification).

Author Contributions: Evaluation antimicotic activity, D.B.F E.; Analysis of propolis samples ,B.R.P; Collection of propolis in the meliponaries,FHG ; Clinicals and reference strains, and conservation of microorganisms, NTB; Gas Chromatographic-Mass Spectrometry,J.P.F..; Performance of chemical determinations , J.G.P.C. ; Fluorescence microscopy and revision manuscript, C.G.G.T.; Funding acquisition and revision manuscript TACS .All authors have read and agreed to the published version of the manuscript

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