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Antifungal Activities of the Essential Oils in *Syzygium aromaticum* (L.) Merr. Et Perry and *Leptospermum petersonii* Bailey and their Constituents against Various Dermatophytes

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This study was carried out in order to investigate the potential of using plant oils derived from *Leptospermum petersonii* Bailey and *Syzygium aromaticum* L. Merr. Et Perry as natural antifungal agents. The antifungal effects of essential oils at concentrations of 0.05, 0.1, 0.15, and 0.2 mg/ml on the dermatophytes *Microsporum canis* (KCTC 6591), *Trichophyton mentagrophytes* (KCTC 6077), *Trichophyton rubrum* (KCCM 60443), *Epidermophyton floccosum* (KCCM 11667), and *Microsporum gypseum* were evaluated using the agar diffusion method. The major constituents of the active fraction against the dermatophytes were identified by gas chromatography-mass spectrometry and high-performance liquid chromatography analysis. The antifungal activities of *S. aromaticum* oil (clove oil) against the dermatophytes tested were highest at a concentration of 0.2 mg/ml, with an effectiveness of more than 60%. Hyphal growth was completely inhibited in *T. mentagrophytes*, *T. rubrum*, and *M. gypseum* by treatment with clove oil at a concentration of 0.2 mg/ml. Eugenol was the most effective antifungal constituent of clove oil against the dermatophytes *T. mentagrophytes* and *M. canis*. Morphological changes in the hyphae of *T. mentagrophytes*, such as damage to the cell wall and cell membrane and the expansion of the endoplasmic reticulum, after treatment with 0.11 mg/ml eugenol were observed by transmission electron microscopy (TEM). At a concentration of 0.2 mg/ml, *L. petersonii* oil (LPO) was more than 90% effective against all of the dermatophytes tested, with the exception of *T. rubrum*. Geranial was determined to be the most active antifungal constituent of *L. petersonii* oil. Taken together, the results of this study demonstrate that clove and tea tree oils exhibited significant antifungal activities against the dermatophytes tested in this study.

Keywords: *Leptospermum petersonii, Syzygium aromaticum,* essential oil, antifungal activity, dermatophytes, transmission electron microscopy

Essential oils have a long history of use as natural microbial agents. Essential oils have recently been used in a number of pharmaceutical, food, and cosmetic products because these oils effectively inhibit the growth of a wide range of microorganisms, and they cause fewer side effects than synthetic antimicrobial agents in humans. Despite the widespread use of essential oils by humans, little is known about the exact mechanism of their antimicrobial action. Thus, many researchers have recently attempted to identify the antimicrobial properties of essential oils. Tea tree and clove oils have been widely investigated due to their popularity, availability, and high essential oil content.

Clove oil (*Syzygium aromaticum*) is widely used as a perfume and food flavoring (Zheng *et al.*, 1992), as a medicine for the treatment of asthma and various allergic disorders in Korea (Kim *et al.*, 1998), and as a general antiseptic in medical dental practices (Cai and Wu, 1996). Importantly, Lee and Shibamoto (2001) reported that clove oil might also be used as an anti-carcinogenic agent due to its antioxidant properties. Their results also suggested that clove oil might be of use as a potential chemopreventative agent.

The term "tea tree" generally refers to species in the *Leptospermum*, *Melaleuca*, and *Kunzea* genera. Tea tree oil, generally called melaleuca oil (TTO), is an essential oil produced through the steam distillation of leaves of *Melaleuca alternifolia*, a shrub-like tree native to northern New South Wales and southern Queensland in Australia. Because TTO possesses both antibacterial and antifungal activities, it has been used as an antiseptic for various skin diseases for many years (Bassett *et al.*, 1990; Benger *et al.*, 2004). TTO is also widely used as a principal antimicrobial agent and as a natural preservative in various types of cosmetic and medicinal products. While the majority of studies investigating the bioactivity of TTO have focused extensively on *Melaleuca*

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alternifolia, little information is known regarding the bioactivity of oils from other tea trees, such as *Leptospermum petersonii* and *Kunzea ambigua*. Therefore, the bioactivity of oils from *L. petersonii* was investigated in the present study. Several researchers have investigated the antimicrobial activities of clove oil and TTO. The majority of studies investigating the antimicrobial activity of clove oil reported that TTO possesses antimicrobial properties against several fungal strains (Dean and Ritchie, 1987; Arora and Kaur, 1999), food-borne bacteria (Smith-Palmer *et al.*, 1998; Mytle *et al.*, 2006), and oral pathogenic bacteria (Cai and Wu, 1996). Most studies have also been focused on determining the efficacy of TTO as an antimicrobial and antifungal agent. However, little is known about the antifungal activities of either oil against dematophytes, which are known to cause superficial skin infections in humans.

The purpose of this study was to examine the antifungal activities of oils from *S. aromaticum* and *L. petersonii* toward dermatophytes, such as *Microsporum canis*, *Trichophyton mentagrophytes*, *Trichophyton rubrum*, *Microsporum gypseum*, and *Epidermophyton floccosum*. We also screened the major constituents of the oils in an effort to determine which factor was responsible for inhibiting the hyphal growth of these five dermatophytes and evaluated the changes in their morphology after treatment with essential oils.

Materials and Methods

Preparation of essential oils and test organisms

The *S. aromaticum* (clove oil) and *L. petersonii* oil (LPO) used in this experiment were purchased from Dullberg Konzentra Co. (Germany) and G. R. Davis PTY. Ltd. Co. (Australia), respectively. To examine the antifungal activity of each essential oil, five strains [*M. canis* (KCTC 6951: MC), *M. gypseum* (MG), *T. mentagrophytes* (KCTC 6077: TM), *T. rubrum* (KCCM 60443: TR), and *E. floccosum* (KCCM 11667: EF)] were prepared as test organisms. MC and TM were provided by the Center of Biological Resources at the Korea Research Institute of Bioscience and Biotechnology. TR and EF were obtained from the Korea Culture Center of Microorganisms, and MG was obtained from the Animal Hospital of Chungbuk National University. The strains were incubated on a Sabouraud Dextrose Agar (SDA; Difco, USA) plate at 28°C for 15 days.

Antifungal assay of essential oils

The antifungal activities of clove oil and LPO were assayed using agar diffusion methods. The clove oil and LPO were placed in Petri dishes (diameter: 60 mm) prepared with SDA and treated with essential oil at concentrations of 0.05, 0.1, 0.15, and 0.2 mg/ml. Three-millimeter plugs of each fungus were inoculated on the SDA plates, and the dishes were then incubated at 28°C. The antifungal index was calculated when the fungal mycelium reached the edges of the control Petri dishes (untreated). Each test was repeated three times, and the data were averaged.

Antifungal index (%) = $(1-D_s/D_c)\times100$,

where D_s is the diameter of hyphal growth on the Petri dish treated with essential oils, and D_c is the diameter of hyphal growth on the control plate (untreated).

Screening and isolation of active antifungal fractions

A TLC assay was used to identify the active antifungal fractions before fractionating the clove oil and LPO by column chromatography. A TLC assay was performed using precoated silica gel plates (silica gel 60 F_{254}: for analytical TLC aluminum sheet) (Merck, Germany). Clove oil and LPO were developed on TLC plates using the solvent system, which was prepared with hexane: acetone (4:1, v/v). The TLC plates containing clove oil were divided into three fractions (Fig. 1A): Fraction A (Rf value: 1~0.64), B (Rf value: 0.64~0.42), and C (Rf value: 0.42~0). The LPO plates were separated into six fractions (Fig. 1-B): Fraction A (Rf value: 1~0.84), B (Rf value: 0.84~0.55), C (Rf value: 0.55~0.41), D (Rf value: 0.41~0.29), E (Rf value: 0.29~0.12), and F (Rf value: 0.29~0). Spores from the five strains of fungi were individually spread on an SDA plate, and the individual TLC plates were placed on the surface of the SDA plate. The antifungal activity of the fraction was measured as the amount of hyphal growth inhibition in each of the five fungal strains.

Column chromatography was used to obtain the antifungal fractions of the essential oils, depending on the results of the TLC assay. Column chromatography was performed using silica gel 60 (70~230 mesh) (Merck Germany) as a packed resin and hexane: acetone (8:1, v/v) as a solvent system. The results obtained from TLC showed that the active antifungal fractions were successfully isolated.

Identification of active antifungal fractions by GC/MS

The essential oil fractions that showed antifungal activity were analyzed by gas chromatography/mass spectrometry (GC/MS) in order to examine their chemical structure. The GC (HP 6890) conditions for clove oil were as follows: HP-5 MS column (30 m×0.25 mm×0.25 μm); the injector and detector temperatures were maintained at 250°C and 280°C, respectively; helium was used as the carrier gas at a flow rate of 1 ml/min; the oven temperature was initially maintained at 60°C for 5 min and then raised to 280°C at a rate

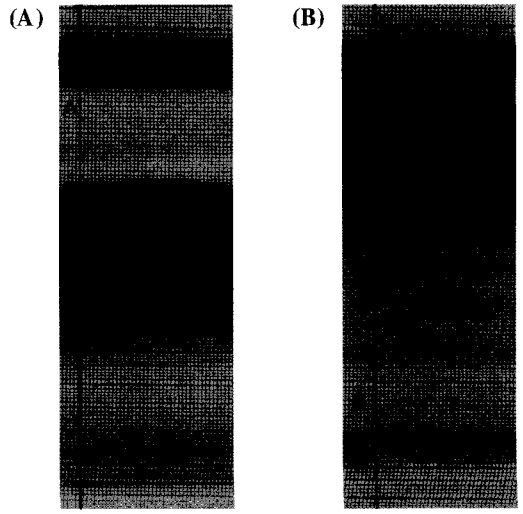

Fig. 1. TLC fractions of clove oil (A) and *L. petersonii* oil (LPO: B) used in the antifungal assay.

of 5°C/min and held for 5 min.

The conditions used for LPO were as follows: HP-5 column; the injector and detector temperatures were maintained at 240°C and 280°C, respectively; helium was used as the carrier gas at a flow rate of 10 ml/min; the initial oven temperature was 60°C for 3 min, and was then increased to 280°C at a rate of 10°C/min and maintained for 5 min.

Mass spectrometry (HP 5973) was used in EI mode: ionization voltage, 70eV; emission current, 40 μA; scan rate, 1 scan/s; scan range, 35-700 m/z; and ion source temperature, 200°C. The chemical structure of each constituent was identified by comparing the mass data for their peaks with the standard library data.

Antifungal assay of major active antifungal constituents

To investigate the antifungal activity of the major constituents of LPO, the active fraction of LPO was separated into three constituents using prep-HPLC. The prep-HPLC system (Spectra system P 2000 from Thermo Separation Products, USA) was equipped with a Waters silica gel column (19× 300 mm). The conditions used for Prep-HPLC were: solvent system, hexane: ethyl acetate (92:8, v/v); UV detector, 320 nm; and flow rate, 4 ml/min. The antifungal activities of the separated constituents against the TM, MC, and MG strains were examined using the paper disc method. For instance, spores of each strain were spread on separate SDA plates, and 8-mm paper discs treated with each constituent (2 μl) were placed on the center of the SDA plates. The plates were incubated at 28°C for 4 days until the control plate was filled with hyphae. The antifungal activities of the fractions against five strains were then measured as the amount of hyphal growth inhibition.

The antifungal activity of clove oil was compared with that of eugenol, which has been identified as a major antifungal constituent of clove oil (Sigma-Aldrich Korea, Korea). The antifungal effects of the eugenol on TM and MC were examined using the same method used for the antifungal assay of essential oil.

Transmission electron microscopy (TEM)

Hyphal specimens (1×3 mm^2, with approximately 1 mm thickness of underlying agar blocks, each) of *T. mentagrophytes* were excised from the margin of actively growing SDA culture treated with 0.11 mg/ml eugenol using a sterilized razor blade. The specimens were fixed with modified Karnovsky's fixative (Karnivsky, 1965) consisting of 2% (v/v) glutaraldehyde and 2% (v/v) paraformaldehyde in 0.05 M sodium cacodylate buffer solution (pH 7.2) at 4°C overnight. The fixed specimens were washed with the solution three times for 10 min each. The specimens were then post-fixed in the solution with 1% (w/v) osmium tetroxide at 4°C for 2 h and then washed briefly with distilled water twice each. The post-fixed specimens were *en bloc* stained with 0.5% (w/v) uranyl acetate at 4°C overnight and then dehydrated once in a graded ethanol series of 30, 50, 70, 80, and 95% and three times in 100% ethanol for 10 min each. The specimens were further treated with propylene oxide twice for 30 min each as a transitional fluid and then embedded in Spurr's resin. Ultra-thin sections (approximately 50 nm in thickness) were cut with a diamond knife using an ultra-microtome (MT-X; RMC Inc., USA). The sections were mounted on copper grids and stained with 2% uranyl acetate and Reynolds' lead citrate (Reynolds, 1963) for 7 min each. The sections were observed with a transmission electron microscope (JEM-1010; JEOL Ltd., Japan) operated at an accelerating voltage of 80 kV.

Results and Discussion

Antifungal activities of clove oil and LPO

The antifungal activities of clove oil are shown in Fig. 2. The antifungal activities of clove oil against MG, TR, and EF at concentrations of 0.05 and 0.1 mg/ml did not significantly differ from the antifungal activities of the control. The antifungal activity of the oil was approximately 14% at concentration of 0.1 mg/ml against MC. At a concentration of 0.1 mg/ml, the clove oil had an antifungal activity of 40% against TM. However, the clove oil showed better inhibitory actions against MC, TM, TR, and EF at a concentration of 0.15 mg/ml than at lower concentrations, with antifungal activities of 32%, 88%, 51% and 60%, respectively. In particular, MG showed no hyphal growth when treated with clove oil at a concentration of 0.15 mg/ml. As the concentration of clove oil increased from 0.15 mg/ml to 0.2 mg/ml, the inhibitory actions of the clove oil increased towards all strains. Interestingly, the hyphal growth of MG, TR, and TM was com-

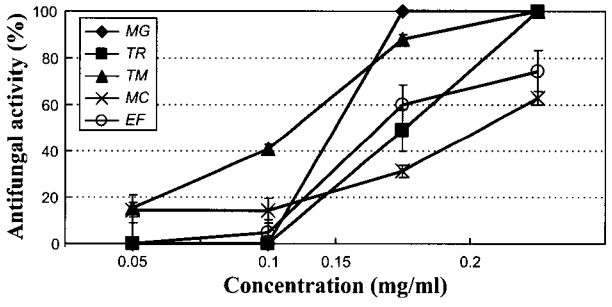

Fig. 2. Antifungal activities of clove oil against five dermatophytes: *M. gypseum* (MG), *M. canis* (MC), *T. rubrum* (TR), *T. mentagrophytes* (TM), and *E. floccosum* (EF).

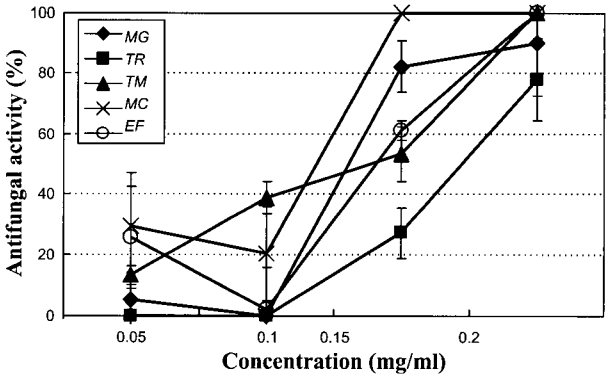

Fig. 3. Antifungal activities of *L. petersonii* oil (LPO) against *M. gypseum* (MG), *M. canis* (MC), *T. rubrum* (TR), *T. mentagrophytes* (TM), and E. *floccosum* (EF).

pletely inhibited by clove oil at a concentration of 0.2 mg/ml. Gayoso *et al.* (2005) reported that the antifungal activities of clove oil against fungus isolated from onychomycosis were 2%, indicating that the antifungal activity of clove oil is more effective against *T. rubrum* and *T. mentagrophytes* than against *Candida albicans* and *Geotrichum candidum*.

The antifungal activities of LPO varied according to the type of strain tested (Fig. 3). The hyphal growth of MG and TR was not inhibited at concentrations of 0.05 mg/ml and 0.1 mg/ml. The antifungal activities of LPO against TM and MC were 39% and 20%, respectively, at a concentration of 0.1 mg/ml. The antifungal activities of LPO increased considerably when the concentration was increased to 0.15 mg/ml. For example, MC showed no hyphal growth when treated with LPO at a concentration of 0.15 mg/ml. At the 0.2 mg/ml concentration level, however, the hyphal growth of MG, TM, and MC was not observed. MG and TR showed hyphal growth at an LPO concentration of 0.2 mg/ml, but at reduced rates of 92% and 79%, respectively. In addition, the inhibitory action of LPO was superior to that of *Melaleuca alternifolia* oil (data not shown). Griffin *et al.* (2000) indicated that the Minimum Inhibitory Concentration (MIC) of *Melaleuca alternifolia* oil against TR and TM were 1.0% and 0.3-0.4% (v/v), respectively. In comparison, the LPO tested in the present study was shown to be more effective at inhibiting the hyphal growth of TM than *Melaleuca alternifolia* oil.

Screening of active antifungal fractions

When the antifungal activities of three fractions (A, B, and C) of clove oil against TM were examined, an inhibition zone was observed in the region of Fraction B (Rf value: 0.64-0.42) (Fig. 4A). Additionally, the size of the clear zone in the plate prepared with TM was larger than that in the plate prepared with MC.

For LPO, although the size of the clear zone varied according to the type of strain tested, Fraction B (Rf value: 0.84-0.55) showed antifungal activity against all strains used in this test (Fig. 4B).

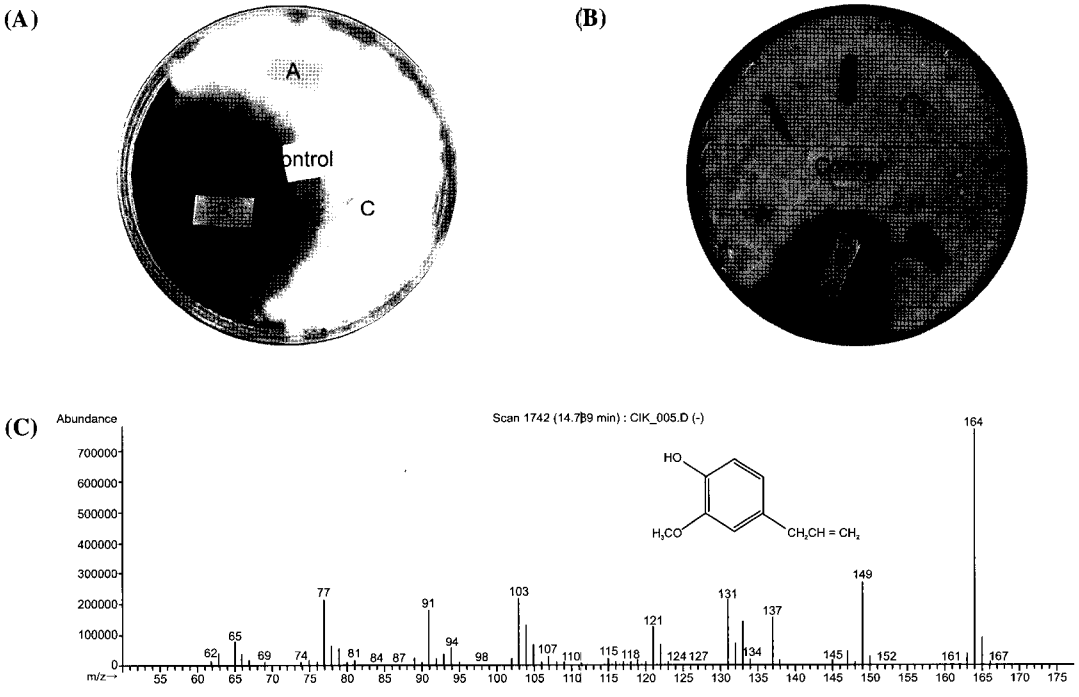

Fig. 4. Antifungal activities of TLC fractions of clove oil (A) and *L. petersonii* oil (B) toward *T. mentagrophytes* (TM) and *E. floccosum* (EF), respectively, and mass spectrum of the B fraction isolated from clove oil (C).

Table 1. Constituents of fraction B separated from *L. petersonii* oil (LPO), and the antifungal activities of each constituent against *T. mentagrophytes* (TM), *M. gypseum* (MG), and *M. canis* (MC) after incubation for 7 days

GC and GC/MS analysis of active fraction B			Antifungal activity of major constituent (clear zone: mm)		
Major constituent	Area (%)[a]	Purity (%)[b]	Against TM	Against MG	Against MC
Citronellal	21	81	10	0	0
Neral	21	90	0	0	60
Geranial	28	94	60	60	60

[a]Area percentage of each constituent in *L. petersonii* oil identified by GC
[b]Purity of each constituent isolated from fraction B by pre-HPLC

Identification of active antifungal fractions

A single major peak was observed by GC/MS analysis for the total ion chromatogram of the active antifungal fraction in clove oil. In comparison to the standard library data, the peak at m/e 164 (M) was identified as eugenol, which is a phenolic compound (Fig. 4C). Eugenol has been extensively used as a spice and fragrance due to its strong odor and as an aromatherapy oil, mouth sterilizer, and painkiller (Robenorst, 1996). Several studies have also shown that eugenol possesses antioxidant properties (Lee and Shibamoto, 2001) and can be used to alleviate fever (Feng and Lipton, 1987).

Three peaks appeared consistently on the chromatogram of the antifungal fractions in LPO, which suggests that the antifungal fraction of LPO contains three main constituents (Table 1). The three main constituents were identified as citronellal, neral, and geranial, respectively, by GC/MS analysis. The chromatogram also suggested that the contents of citronellal, neral, and geranial in LPO were 21%, 21% and 28%, respectively. In general, neral and geranial are recognized as citral due to a separation problem. Geranial and neral are used to refer to citral A and citral B, respectively (Newman, 1972). Citral exists naturally as a mixture of these isomers and accounts for 85% of lemon-grass oil. It is commonly acknowledged that citral has anti-inflammatory and anti-microbial properties (Pattnaik *et al.*, 1997; Shigeru *et al.*, 2003).

Table 2. Antifungal activities of eugenol, a major active component of clove oil, against *T. mentagrophytes* and *M. canis*.

Concentration	Antifungal activity of eugenol (%)	
	Against *T. mentagrophytes*	Against *M. canis*
0.05 mg/ml	18.32 ± 2.5	0
0.1 mg/ml	88.25 ± 1.9	7.96 ± 2.4
0.15 mg/ml	100	20.04 ± 1.2
0.2 mg/ml	100	100

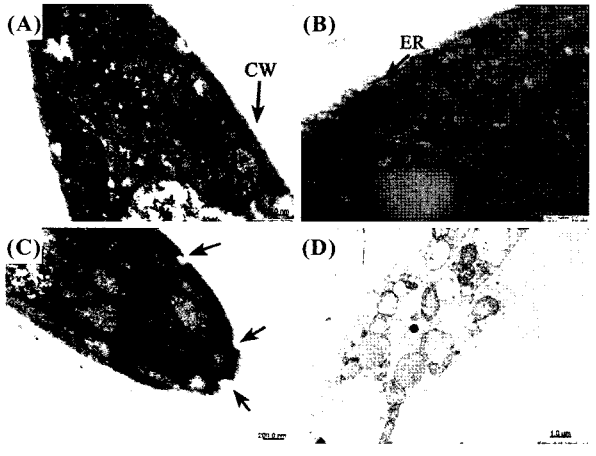

Fig. 5. Transmission electron micrographs of *T. mentagrophytes* (TM) treated with eugenol. Control (A) the expansion of endoplasmic reticulum (ER) was observed near the cell membrane (B) abnormal structure of mitochondria (wide arrows) and partial destructions of the cell membrane (arrows) (C) the complete destruction of the cell wall in certain hyphae (D). (B), (C), (D): TM exposed to eugenol. Abbreviation used throughout: CW, cell wall; M, mitochondria; ER, endoplasmic reticulum.

Antifungal assay of major active antifungal constituents

The antifungal activities of clove oil against TM and MC were compared with those of commercial eugenol using the paper disc method. As shown in Table 2, the growth of TM was inhibited when treated with eugenol at concentrations higher than 0.15 mg/ml, and the growth of MC was inhibited when treated eugenol at a concentration of 0.2 mg/ml. These antifungal activities of eugenol were relatively higher against TM and MC than that of clove oil. These results are not consistent with results presented by Rakotonirainy and Lavendrine (2005), who reported that clove oil performed better than eugenol against *Aspergillus* sp., *Cladosporum herbarum*, and *Penicillium frequentans*. The inconsistency in these results may be due to different levels of species sensitivity, as well as different strengths of the clove oil and the commercial eugenol.

Citronellal, neral, and geranial, three major constituents isolated from active fraction B of LPO, showed varying antifungal activities against TM, MC, and MG. For example, all constituents caused 100% hyphal growth inhibition of TM for 4 days of incubation. After 7 days of incubation (Table 1), the inhibition was not maintained, with the exception of geranial, suggesting that citronellal and neral have a fungistatic effect on TM rather than a fungicidal effect. The neral and geranial cultures maintained the inhibition of MC for 7 days. However, neral did not maintain the inhibition of MG for the full 7 days of incubation. Therefore, the antifungal properties of LPO against TM, MC, and MG may likely be attributed to the effects of geranial.

Morphology

In general, the mycelia of fungi have smooth cell walls with long strands of hyphae. Figure 3A shows the organelles of TM. In a hyphal specimen treated with eugenol, the expansion of its endoplasmic recticulum (ER) was observed near the cell membranes of the hyphae (Fig. 3B). The ER is the site at which xenobiotics, such as drugs or harmful pesticides, are detoxified (http://en.wikipedia.org/wiki/Endoplasmic_reticulum). In addition, the inner mitochondrial membranes were partially destroyed (Fig. 3C), with complete destruction of the cell wall observed in certain hyphae of TM (Fig. 3D). According to these results, the antifungal activity of eugenol toward TM is due to changes in fungal cell structure at the membrane level.

These results suggest that clove oil and LPO may be viable alternatives to conventional antifungal agents, with relatively minimal side effects. However, the mechanism of the antifungal activity of essential oils has yet to be fully understood, and therefore, further research is needed in order to determine these mechanisms.

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