DR SANDRA MILENA LEAL (Orcid ID : 0000-0002-0120-8060)

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In vitro susceptibility of Microsporum spp and mammalian cells to *Eugenia caryophyllus* essential oil, eugenol and semisynthetic derivatives

Sandra Milena Leal Pinto[1], Laura Viviana Herrera Sandoval[2], Leonor Yamile Vargas[3]

[1]Facultad de Ciencias de la Salud, Universidad de Santander, Bucaramanga, Colombia

[2]Departamento de Ciencias Básicas, Grupo de investigación Sistema Estomatognático y morfofisiología, Universidad Santo Tomás, Bucaramanga, Colombia

[3]Facultad de Química Ambiental, Grupo de Investigaciones Ambientales para el Desarrollo Sostenible, Universidad Santo Tomás, Bucaramanga, Colombia

Correspondence: Sandra Milena Leal Pinto, Universidad de Santander, Bucaramanga, Colombia, sandramilena20@gmail.com, phone +57 3167946429

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ABSTRACT

Background: Microsporum spp are keratinophilic dermatophytes that mainly invade the stratum corneum of the skin and hair causing clinical symptoms associated with *tinea*. Its treatment has several limitations and the search for new active molecules is necessary. Objetive: To evaluate the antifungal and cytotoxic potential of *Eugenia caryophyllus* essential oil (EO), eugenol, isoeugenol and methylisoeugenol against *Microsporum canis*, *M. gypseum* and Vero cells. Methods: The EO was extracted by conventional heating assisted hydrodistillation, the eugenol obtained commercially and the derivatives through Williamson synthesis. Minimal Inhibitory Concentration (MICs), minimum fungicidal concentration, inhibition of radial mycelial growth and germination inhibition were used to evaluate the antifungal activity. In addition, a colorimetric test was conducted to evaluate cytotoxic activity. Results: MIC and MFC values for all compounds were 62.5 to 500 µg/ml for both of the species of *Microsporum* evaluated. Also, concentrations of 300 µg/ml of the compounds inhibited 100% of *M. canis* mycelium. The inhibition of germination was observed after 6 hours of treatment (11.86 ±3.46-85.31 ±0 %). No cytotoxicity was observed in Vero cells (CC_{50} > 105 µg/ml), whereas terbinafine showed CC_{50} 31.00±0.61 µg/ml. Conclusions: Our study indicates an interesting bioactivity of isoeugenol and methylisoeugenol against *M. canis, M. gypseum* and mammalian cells.

KEY WORDS: citotoxicity, eugenol, isoeugenol, methylisoeugenol*, Microsporum canis*, *Microsporum gypseum*

1. INTRODUCTION

Dermatophytoses are cutaneous mycoses caused by keratinophilic fungi that have the ability to invade the stratum corneum of the skin, hair and nails. They produce differing clinical manifestations that may vary according to the immunological state of the host; they can be mild or cause suppurative and inflammatory lesions, known as *Tinea*[1].

Three genera of dermatophytes have been reported to cause this mycosis: *Trichophyton spp, Microsporum spp* and *Epidermophyton spp*, in order of prevalence. *M. canis and M. gypseum* are the zoophilic and geophilic species, respectively, which are most frequently isolated in Latin America as the cause of *tinea capitis*, *tinea faciei* and *tinea corporis* in humans; mainly children, adolescents, pregnant mothers, animal caregivers and veterinarians[2]. *M. canis* particularly is an important public health concern because it more frequently generates zoonotic infections[3]. In Colombia, dermatophytosis is very common and it is estimated that *M. canis* is associated with 62.5% of *tinea capitis*[4].

Treatment of dermatophytosis caused by the genus *Microsporum* depends on the age and clinical condition of the patient. Generally, topical and systemic medications are used in treatment or combination therapies. Griseofulvin is indicated for cases of *tinea capitis* caused by *M. canis* while terbinafine is a second option for oral administration that is contraindicated in children under 3 years and has low effectiveness against *M. canis* and *M. gypseum*. Azoles such as ketoconazole, fluconazole, and itraconazole are also used as elective therapies, but like terbinafine, the latter two do not have pediatric formulations. Although there are various medications for these cutaneous mycoses, a number of factors may limit the ability to achieve resolution of the infection including long periods of treatment (> 30 days),

hepatotoxicity and other adverse effects (headache, gastric disorders, dizziness). Based off of this information, the search for new active molecules is a priority.

The use of plants and their products as substrates of molecules with biological activity is a leading strategy in research due to its lower toxicity and greater bioavailability[6]. Eugenol is a phenolic monoterpene with anesthetic, bactericidal, fungicidal and antioxidant properties, among others[7,8,9,10] ; It is extracted as the main component of clove essential oil (*Syzygium aromaticum*), honey, bay leaves and cinnamon[6,11]. Its antifungal activity against dermatophyte fungi was reported by Pinto *et al.* demonstrating an inhibitory effect on the growth of *E. floccosum, T. rubrum, T. mentagrophytes* and *M. gypseum*[12]*,* Additionally, Escobar *et al.* propose three possible mechanisms of action exerted by eugenol in the cells: 1) imbalance in ionic homeostasis , 2) alterations in the cell membrane and 3) generation of oxidative stress[13]. These mechanisms have also been reported in fungi exposed to eugenol: de Oliveira 2013 reported the mechanism of action of eugenol on *T. rubrum*, a species of highly prevalent dermatophyte related to chronic cases and resistant to local therapies. They showed that eugenol acts on the wall and cell membrane of this dermatophyte by inhibiting ergosterol synthesis[11]. In this sense, eugenol becomes a platform for the study of semisynthetic derivatives that can exert a greater fungicidal effect with less toxicity. Our objective was to evaluate the antifungal and cytotoxic effect of clove essential oil, pure eugenol and two semisynthetic derivatives of eugenol against *Microsporum canis, Microsporum gypseum* and mammalian cells.

2. MATERIALS AND METHODS

2.2 Fungal strains and mammalian cells

Microsporum gypseum (ATCC, 7911), *Microsporum canis* (ATCC, 8137) pass 3, were grown in potato dextrose broth (PDA, OXOID LTD, CM0139, Basingstoke, Hampshire, England), at 25°C with constant movement at 100 rpm for 8 days. Subsequently, the dermatophytes were cultured for 10 days on PDA agar at 30°C.

African green monkey kidney epithelial cells "*Cercopithecus actiops*" (Vero, CCL-81) donated by Dr. José Arteaga of the Universidad Industrial de Santander, were maintained in Dubelcco's Modified Eagle Medium (DMEM, Life Technology, CA, USA) and supplemented with 5% inactivated fetal bovine serum (FBSi, Life Technology, CA, USA) at 37°C , 5% CO2 and 95% humidity.

2.3 Compounds

The compounds were prepared by green chemistry processes, from renewable, biodegradable, economical and commercially available raw material. To summarize; the essential oil (EO) was extracted from clove bud dried floral buttons of *Eugenia caryophyllus* though the hydrodistillation technique (HD) and assisted by microwave heating. It was characterized qualitatively and quantitatively using gas chromatography coupled to mass spectrometry, and employing the ChemStation data system G17001DA and its database as characterizing criteria (NIST 2002, NBS 75K and WILEY 138K). The extraction yield was 15.2 %. The principal components finding of EO and their relative amounts were: eugenol 71.8%, eugenyl acetate 18.4%, trans-β-caryophyllene 7.5%, α-humulene 1.1%, caryophyllene oxide 0.5%,

methyl salicylate 0.2%, chavicol 0.2%, α-copaene 0.1%, benzyl acetate 0.1%, 2-heptanone <0.1%, 2-nonanone <0.1%, and ethyl benzoate <0.1%.

To isolate the eugenol, the essential oil was dissolved in 100 mL of dichloromethane and extracted with a 5% potassium hydroxide solution (3 x 50 mL). The alkaline layers were combined and re-extracted with dichloromethane (1 x 50 mL). The alkaline solution was acidified slowly to pH 1 with 5% hydrochloric acid, and extracted with dichloromethane (3 x 50 mL). The organic extracts were dried over sodium sulfate, and the solvent was removed by rotoevaporation.

To purify the crude eugenol, a fractional distillation was carried out under reduced pressure, eugenol distilled at 120-122 °C/5 mmHg (with a yield of 13% with respect to the flower buds used). IR (thin film): 3512 (ν_{OH}), 1510 ($\nu_{C=C}$), 1260 (ν_{OCH3}) cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$), δ (ppm): 3.32 (2H, d, *J* = 6.8 Hz, 1´-CH_2), 3.83 (3H, s, -OCH_3), 5.06 (1H, dd, *J* = 4.2, 0.4 Hz; 3´=CH_{cis}), 5.08 (1H, dq, *J* = 12.0, 4.2 Hz; 3´=CH_{trans}), 5.55 (1H, s, OH), 5.95-6.01 (1H, m, 2´-CH), 6.69-6.71 (2H, m, 5,6-H_{Ar}), 6.85 (1H, dd, *J* = 8.0, 0.77 Hz, 3-H_{Ar}). CG: t_R 34.82 min. MS (EI) *m/z* (relative intensity): 164 ($M^{+\bullet}$, 62), 149 (100), 131 (36), 77 (10).

Other natural phenols in essential oils, like isoeugenol and methyl isoeugenol, were obtained from Sigma-Aldrich. The reference medicine, terbinafine (Novartis), was purchased in the form of pharmaceutical preparations; it was subjected to necessary purification processes (recrystallization, column chromatography, high performance liquid chromatography) to ensure a purity greater than 99%, which permitted its use as a reference compound in the bioassays. The composition and structure of each was corroborated with spectrochemical methods: nuclear magnetic

resonance of ^{1}H, ^{13}C and 2D, gas chromatography coupled to mass spectrometry and infrared, and with physical methods of analysis: melting point, boiling, density and refractive index.

2.4 Antifungic activity

2.4.1 Minimum inhibitory concentration (MIC) assay

Experiments were performed using the broth microdilution method according to CLSI M38-A protocols for filamentous fungi. Briefly, a fungal suspension was obtained from a colony of 10 day-culture in PDA medium at 30°C. Then, 3mL of a 1% solution Tween 80 in distilled water was added to the colony, a slight scraping was then performed to obtain the fungal structures and adjusted per McFarland standard 5. The suspension was diluted 1:50 (v/v) in RPMI-1640 culture medium (Sigma, St. Louis, MO, USA) and buffered with 3 (N-morpholino) propanesulfonic acid (MOPS; Sigma-aldrich, Darmstadt, Germany) resulting in a suspension containing 0.4-5.0×10^{4} CFU/mL. The compounds were prepared in RPMI medium supplemented with MOPS. Serial dilutions 1: 2 were performed on the microplates and evaluated at concentrations of 1000-0.97 µg/mL. Untreated controls were then tested and 100 µL of the initially prepared inoculum was added to the microplates. The antifungal effect was observed after 7 days of incubation at 30ºC by optical observation of turbidity. MIC was the lowest concentration of compound capable of inhibiting observed fungal growth in the wells by 100%. Terbinafine was used as a reference drug. All experiments were performed in triplicate, and the results are presented as the geometric means of the replicates.

2.4.2 Minimum fungicidal concentration (MFC) analysis

After reading the corresponding MIC values, 10 µL samples from clear wells, the last tube showing growth was subcultured on Sabouraud dextrose agar medium (OXOID LTD, CM0041, Basingstoke, Hampshire, England). The dishes were incubated at 30°C for 7 days. A control without drugs was performed. MFC was defined as the lowest concentration of the compound at which growth was <3 CFU. All experiments were performed in triplicate, and the results are presented as the geometric means of the replicates.

2.4.3 Inhibition of mycelial radial growth

The effect of the compounds on the radial growth of mycelia of *M. canis* and *M. gypseum* was evaluated by the impregnated substrate method.[14] To summarize, the culture media (PDA) was mixed with different concentrations of essential oil, the compounds of interest (300, 100, 64.9, 11.1 µg/mL) and terbinafine (0.3, 0.1, 0.03, 0.01 µg/mL) in triplicate. Afterwards, 5 mm diameter cuts were made in the middle of the agars where the dermatophytes (same diameter) were extracted from 10-day PDA cultures of the reproductive zone. Agars were incubated at 30°C for 21 days by performing mycelial growth diameter readings on days 6, 9, 13, 16 and 20. Controls without treatment were evaluated. The results are expressed as percent inhibition of the colony radius for each treatment with respect to the control.

2.4.4 Inhibition of conidia germination

The inoculum was prepared from cultures with 7-15 days of growth in PDA medium, the colonies were immersed in 0.1% distilled water with Tween 80 and scraped with the Driblaski handle, the suspension was centrifuged at 2500rpm for 15

minutes and the pellet resuspended in potato broth. The final concentration was $2x10^5$ conidia/mL. The compounds were prepared in a culture medium in concentrations of 200, 100, 69.4, and 50 µg/mL and 1 mL aliquots of each concentration were mixed with 1 mL of the inoculum and incubated in constant agitation at 28°C for 84 hours. During this period, the amount of germinated conidia (generation of a germ tube) were measured within a total of 100 conidia using a haemocytometer. Controls without treatment were also evaluated.

2.5 Cytotoxicity test on mammalian cells

Vero cells ($3x10^5$ cells/mL) were added in 96-well microplates for 24 hours at 37°C and 5% CO_2 until the formation of the monolayer. Compounds and terbinafine in concentrations of 100 µg / mL to 3,7 µg / mL were then treated with serial dilutions 1:3 for 72 hours. Cell viability was evaluated using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma) and tetrazolium salt at a concentration of 5mg/mL. A spectrophotometric reading was performed on a microplate reader for absorbance/ELISA (BIO-RAD) using a wavelength of 580 nm. All assays were performed in triplicate in independent experiments.

2.6 Result analysis

The inhibition percentage of the radial growth of the mycelium was determined by the formula: diameter of the control - diameter of the treatment / control diameter x 100. On the other hand, the cytotoxic concentrations 50 and 90 (CC_{50} and CC_{90}) were determined by sigmoidal regression, using the statistical program XlFit5.

For the statistical analysis of the data, a Shapiro-Wilk test ($p < 0.05$) was used to determine the nature of the data taking into account the above results, analysis of

variance (ANOVA) or its equivalent nonparametric test using the statistical software SPSS 15.0 for Windows. For the analysis, we worked with a confidence percentage of 95%.

3. RESULTS

Table 1 summarizes the MIC and MFC values of *M. canis* and *M. gypseum* against test compounds. MIC and MFC values for all compounds were in the range of 62.5 to 500 µg/ml for both of the species of *Microsporum* evaluated. In general, eugenol and the EO of *Eugenia caryophyllus* showed lower MIC and FMC values (62.5 µg/ml) in *M. canis*. However its semisynthetic derivatives showed fungiestatic effects at higher concentrations (>125 µg/ml). To exert the fungicidal effect on *M. canis* and *M. gypseum*, 125 and 250 µg/ml concentrations of the semisynthetic derivatives of, respectively, were necessary. Terbinafine used as a reference drug showed an MIC and MFC of between 0.312 to 0.625 µg/ml and 0625 µg/ml for *M. canis* and *M. gypseum*, respectively.

Table 1. Antifungic (MIC and MFC) and cytotoxic activity of EO of *Eugenia caryophyllus*, eugenol and semisynthetic derivatives of eugenol against *M. canis, M. gypseum* and mammalian cells

Table 2 shows the percent inhibition of radial mycelial growth. The inhibition of radial mycelium growth of *M. canis* was 100%, during the 21 days of incubation with concentrations of 300 µg/mL of EO, eugenol and semisynthetic derivatives. Contrary to the above, inhibitions of less than 55% were observed in the other concentrations

evaluated (100, 64.9 and 11.1 µg/mL). Figure 1 shows the inhibition of radial mycelial growth of the four compounds under study when testing 100 µg/mL of each until the ninth day of incubation. The essential oil extracted from *Eugenia caryophyllus* showed the greatest inhibitory effect on *M. canis* at 100 µg/mL compared to the other compounds ($p < 0.05$). After 9 days of incubation, no significant changes in radial mycelium inhibition of *M. canis* were observed compared to the untreated control. On the other hand, terbinafine, used as a reference drug, showed 100% inhibition at concentrations of 0.3 and 0.1 µg/mL and 59.27 ± 3.31 and 27.19 ± 2.42% at concentrations of 0.03 and 0.01 µg/mL respectively after nine days (9) of incubation.

The radial growth of mycelium of *M. gypseum* showed a 100% inhibition until day nine (9) with 300 µg/mL of the EO, eugenol and methylisoeugenol. This inhibition was maintained until the end of the experiment with eugenol (21 days). However, when concentrations of 100 µg/mL EO and eugenol were tested, 51.22 ±6.34% and 53.48 ±10.28% of inhibition were observed at day nine of incubation. Concentrations <100µg/mL were found to be insufficient to inhibit the fungus. In addition, isoeugenol showed low inhibition of *M. gypseum* in less than 60% at the maximum concentration evaluated. Similarly, terbinafine at day 9 was shown to inhibit 100% of the radial mycelium growth of *M. gypseum* at concentrations of 1 and 0.3 µg/mL, 52.56 ±1.11% and 8.97 ±1.11% at 0.1 and 0.03 µg/mL, respectively.

Figure 1. Percent inhibition of radial mycelium growth of *Microsporum spp* after 9 days of treatment with 100 µg/ml of the compounds under study. (a): *M. canis*; (b): *M. gypseum*

Table 2. Percentages of radial mycelium growth inhibition of *M. canis* and *M. gypseum*. Results are expressed as percent ± standard deviation and are shown as the average of three independent experiments

We also evaluated the germination inhibition of the two species of *Microsporum* treated with the compounds (Table 3). Germination percentages of <30% were observed in negative controls (*M. canis* 26.50 ± 5.19 and *M. gypseum* 17.0 ± 4.04). The maximum germination was obtained after 84 hours of incubation with methylisoeugenol compared to the control in both of the species of dermaphytes studied. (53.0±1.15 %). The EO, eugenol and isoeugenol showed percentages of conidia germination inhibition of *M. canis* at 6h between 33.30 - 82.35% in all the evaluated concentrations. The inhibition decreased depending on the time and dose of the compound, and an inhibition percentage of <5.2% was observed at 84h after treatment. Methylisoeugenol did not inhibit the conidia germination of *M. canis*.

In the case of *M. gypseum,* the germination inhibition was observed after 37 hours of incubation with the EO (8.82-32-35%). Eugenol, isoeugenol and methylisoeugenol inhibited germination from 6 hours post treatment (11.76- 8.71%). Figure 2 shows percentages of germination inhibition at 24 hours of incubation.

Figure 2. Effect of the essential oil of *Eugenia caryophyllus*, eugenol and its derivatives on the germination inhibition of *Microsporum spp* at 24 hours post treatment. (a): *M. canis*; (b): *M. gypseum*

In addition, the in vitro cytotoxicity of compounds on Vero cell was evaluated. The results are shown in Table 1. The EO, eugenol and semisynthetic derivatives, exhibited CC_{50} between 105.62 ±12.75 to > 300 µg/mL, showed a low toxicity on these mammalian cells. The EO and eugenol was shown to be less toxic with CC_{50}> 265 µg/ml ($p > 0.05$). However, Terbinafine was shown to be toxic to this non-tumor cell line with CC_{50} of 31.00 ± 0.61 µg/mL.

Table 3. Percent inhibition of germination of *Microsporum spp* after treatment with EO of *Eugenia caryophyllus*, eugenol and its derivatives

4. DISCUSSION

M. canis and *M. gypseum* are considered zoophilic and geophilic pathogens that cause *tinea* in animals (dogs and cats) and humans (*tinea* capitis, mainly children, 97%). *M. canis* is the dermatophyte species most frequently isolated from patients with this clinical manifestation. On the contrary, *M. gypseum* occurs less frequently[15,16]. This data is supported by a retrospective study conducted in the laboratory of the Medical and Experimental Mycology Unit of the Biological Research Corporation, in Medellin, Colombia. It was reported that during 19 years, from 1994-2013, the two main species of dermatophytes causing suspected cases of *tinea capitis* were *M. canis* (86.44%, 102/118 cases) and *M. gypseum* (4.23%, 5/118 cases). A case of co-infection by these two species was also reported[16].

In general, the treatment for dermatophytosis usually consists of topical and/or oral therapy, which mainly depends on the location of the lesion in the body, severity of the infection, fungal species causing the pathology, and age of the patient. Fluconazole, itraconazole, terbinafine and griseofulvin are the available oral

antifungals; However, the first two have shown interaction with other drugs and their use during pregnancy is prohibited in any systemic therapy[17]. On the other hand, topical formulations such as creams, gels, solutions, shampoo, ointments or myconazole, clotrimazole, ciclopirox and amorolfine sprays, among others, are commonly used for these pathologies; however, the low penetration in the skin is associated with ineffectiveness and causes difficulties. In addition, the inadequate use and free sale (non-prescription) of antifungals, have led to variability in the effectiveness of therapies and often is associated with the generation of resistance[15,17]. In the case of infections caused by the genus Microsporum, griseofulvin is the drug of choice; however, some studies report the effective use of itraconazole and topical terbinafine (shampoo) for *tinea* capitis cases in humans and domestic animals[18,19]. Griseofulvin is considered more effective but with greater adverse effects compared to itraconazole and terbinafine[19]. Because of this, it is considered important to study new therapies and compounds with antifungal activity that promote a better treatment panorama for these infections.

EO of *Eugenia caryophyllus* and eugenol, have been reported for their biological activity[20-24]. Also, eugenol has been isolated from different natural sources such as pepper, bay leaves and cinnamon and is a major phenolic constituent of clove[20,25].

In this study we demonstrated the antifungal and cytotoxic activity of EO of *Eugenia caryophyllus*, its main component (Eugenol, 4-allyl-2-metoxy-phenol) and two semi-synthetic derivatives of eugenol (Isoeugenol and Methylisoeugenol). A fungicidal effect on the mycelial growth of dermatophytes (day 21) was observed when concentrations of 300 µg/ml were tested, however, when the concentration decreased to 100 µg/ml, this effect was observed only after day 6 of treatment, after

which the behavior of the fungus's growth was similar to the control, possibly suggesting a fungistatic effect of the compounds under these conditions.

Previous studies have demonstrated the activity of EO rich in eugenol and pure eugenol on some dermatophyte fungi, Lee *et al.* reported the antifungal activity of eugenol extracted from Japanese cypress oil against *M. gypseum* in an in vivo model, suggesting eugenol as a supplement for antifungal therapy in lesions caused by this dermatophyte[26]. Pinto E *et al*. evaluated the effect of the essential oil of *Syzygium aromaticum* (L.) and eugenol on different species of dermatophytes (*Trichophyton rubrum, T. mentagrophytes, M. canis, M. gypseum* and *Epidermophyton floccosum*), determining values of MIC between 0.08 -0.16 µl/mL (v/v) for these fungi, when treated with both essential oil and pure eugenol; *M. canis*, unlike the other dermatophytes, presented a MIC of 0.08 µl/mL (v/v) against eugenol[12]. In contrast to these results, this study showed a lower susceptibility of the two species of *Microsporum spp* evaluated against eugenol and EO of *Syzygium aromaticum* (L.) with MIC of 62.5 and 0.16 µg/ml respectively. These differences could be caused by the different strains used in the studies, synthetic routes and the commercial and/or natural sources from which the compounds were acquired. Similarly, Rana *et al.* reported the inhibitory effect of the essential oil of *Syzygium aromaticum* (L.) on *T. rubum* and *M. gypseum* with MIC between 9-12 µL/mL[27]. A short study by Gayoso *et al.* also demonstrated the inhibitory effect of *E. cariophyllata* essential oil (MIC 2%) and eugenol (MIC 4%) on dermatophytes and yeasts causing onychomycosis (*Candida albicans, Candida tropicalis, Candida krusei, T. rubrum, T. mentagrophytes* and *Geotrichum candidum*)[28]. Other reports corroborate this eugenol antifungal effect against fungi such as *Candida spp, Aspergillus spp* and *Fusarium spp*[12,27,29].

We evaluated the antifungal properties of isoeugenol and methyisoeugenol; two semisynthetic derivatives that demonstrated similar antimicrobial effects with MICs between 250-500 µg/mL. Methylisoeugenol (300µg/mL) showed to have a fungiesthetic effect on *M. gypseum* with a 100% inhibition of the radial mycelial growth observed until the ninth day of incubation. This inhibition decreased to 67.33% at day 12. The results allow us to suggest that reinforcement with new doses of methylisoeugenol would be necessary at day 9 to maintain the fungicidal effect on this dermatophyte. On the other hand, 300µg/mL concentrations of the two eugenol derivatives inhibited 100% of the mycelial growth of *M. canis*. However, concentrations of ≤100µg/mL did not significantly inhibit the growth of this dermatophyte and showed inhibition rates of <20%. There are still no reports in the literature about the antifungal activity of these semisynthetic derivatives of eugenol. However, the insecticidal properties of isoeugenol (LD_{50} 44.0µg/L) have been demonstrated for the control of *Spodoptera littoralis*; an insect considered to be a pest for tobacco crops, corn, cotton and some vegetables[30]. Additionally, the antibacterial effect of methylisoeugenol, isoeugenol and eugenol against *Campylobacter jejuni*, the main causative agent of gastroenteritis in humans, was reported with MIC values of 250, 125 and 250 µg/mL, respectively[31].

Conidia play an important role in the adhesion processes during the infection of dermatophytes; they make the first contact with the stratum corneum of the host. This union, mediated by adhesins of the fungus and host receptors, establishes the infection through germination and subsequent invasion of hyphae on keratinized tissues[32]. In this study, we observed <25% of germinated conidia in our untreated controls, contrary to the results obtained by Silva *et al.* on *M. canis* and *M. gypseum* which showed > 80% germination of these fungi. However, it is important to highlight

that the strains evaluated by them came from clinical isolates and ours were reference strains which would support the variability. The germination of the macroconidia of both species of *Microsporum* was observed from 6 hours of incubation in our assays with maximum germination at 84 hours. Models ex vivo in reconstructed interfollicular feline epidermis demonstrated the adhesion of conidia of *M. canis* dependent on time between 2-6 hours postinoculation[33]. Despite the low germination, the compounds under study generated inhibition after 24 hours post treatment (see Table 3).

We used terbinafine as a reference drug; a broad spectrum antimycotic derived from allylamine. This drug is usually selected as the second option for the treatment of cutaneous mycosis in cases of therapeutic failure with azoles. However, it comes with different adverse effects such as: pain muscular, rash, headache, nausea, diarrhea and abdominal distention, among others[5]. Concentrations of <0.3 µg/mL were evaluated, showing a considerable antifungal effect; however, their toxicity was demonstrated in our *in vitro* cellular model (see Table 1). The compounds under study did not show to be more active than terbinafine, but we presented results of *in vitro* cytotoxicity in a non-tumor cell line widely used as an in vitro model for screening studies of compounds with potential antimicrobial activity. Toxicity results in these mammalian cells demonstrated a low in vitro cytotoxic effect of EO, eugenol, isoeugenol and methylisoeugenol with CC_{50}> 105.62 µg/mL (See Table 1). The eugenol demonstrated lower toxicity compared to semisynthetic derivatives ($p < 0.05$). It was not possible to determine the CC_{50} at the highest evaluated concentration (300µg/mL) of the EO, demonstrating minimal toxicity for these cells. Thus, concentrations higher than 300µg/mL should be tested to achieve a cytotoxic EO effect. The in vitro toxicity of eugenol extracted as a major component

of EO of *Ocimum gratissimum* was evaluated by Binh Le T *et al.* on the human non-cancer fibroblast cell line WI38 and macrophage-like murine cell line J774, demonstrating 80% cell viability when they were exposed for 72 hours with 50 nL/mL[34]. Contrary to these results, a high toxicity and genotoxicity of eugenol was reported on human dental pulp fibroblasts of primary teeth at concentrations of 0.06 µM[13]. Similarly, Prashar A *et al.* reported high cytotoxicity the EO of *Syzygium aromaticum* and eugenol on human fibroblasts (153BR), human normal dermal fibroblasts (HNDF) and a primary culture obtained from biopsy and HMEC-1 cells; a transformed human dermal microvascular endothelial cell line. The viability of the three cell lines tested decreased by 60-90% when the oil concentration increased from 0.01% to 0.03%. Also, by increasing the eugenol concentration from 0.03% to 0.06%, cell viability decreased from 60% to <20%[35]. However, clove oil, isoeugenol and eugenol continue to be recognized as 'safe' by the Food Drug Administration (FDA), and may be used in dental cement or food additives[36].

In conclusion, we demonstrated the antifungal activity of two semisynthetic derivatives of eugenol: isoeugenol and methylisoeugenol, which were shown to inhibit *M. gypseum* and *M. canis*; dermatophyte fungi of clinical interest that cause cutaneous mycoses. Additionally, we demonstrated its low in vitro cytotoxic effect on a non-tumoral lineage (Vero cells). Other toxicity studies and formulation enhancement should be encouraged. Consequently, the findings of the present study indicate that these compounds have interesting potential as a therapeutic option against this type of fungi.

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6. CONFLICT OF INTEREST

The authors declare no conflicts of interest

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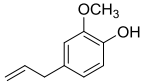
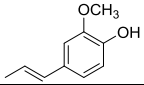
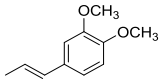
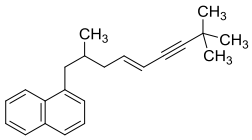
Table 1. Antifungic (MIC and MFC) and cytotoxic activity of EO of *Eugenia caryophyllus*, eugenol and semisynthetic derivatives of eugenol against *M. canis, M. gypseum* and mammalian cells

COMPOUNDS	*M. canis*		*M. gypseum*		Vero cells	
	MIC	MFC	MIC	MFC	CC_{50}	CC_{90}
EO	62.5 – 125	125	125	500	265.10 ±15.05	ND
Eugenol 	62.5	62.5	125-250	125	ND	ND
Isoeugenol 	500	500	250-500	250	105.62 ±12.75	ND
Methylisoeugenol 	125 – 250	250	250-500	500	174.94 ±28.52	ND
Terbinafine 	0.312 - 0.625	0.625	0.625	0.625	31.00 ±0.61	ND

MIC: Minimum inhibitory concentration; MFC: Minimum fungicidal concentration; CC_{50} /CC_{90}: Citotoxic concentration 50 and 90. ND: not determined (CC_{50}/$_{90}$ >300 μg/mL), Results are expressed as μg/mL

Table 2. Percentages of radial mycelium growth inhibition of *M. canis* and *M. gypseum*. The results are expressed as percent ± standard deviation and are the average of three independent experiments.

M. canis																
Compound	**OE**				**Eugenol**				**Isoeugenol**				**Methylisoeugenol**			
Day	**300**	**100**	**64,9**	**11,1**	**300**	**100**	**64,9**	**11,1**	**300**	**100**	**64,9**	**11,1**	**300**	**100**	**64,9**	**11,1**
6	100 ±0.0	100 ±0.0	16.47 ±2.68	NI	100 ±0.0	10.62 ±4.7	NI	NI	100 ±0.0	21.54 ±7.13	16.47 ±2.48	NI	100 ±0.0	29.69 ±8.68	18.25 ±3.87	10.10 ±2.38
9	100 ±0.0	61.43 ±2.13	12.40 ±2.78	NI	100 ±0.0	6.75 ±3.5	NI	NI	100 ±0.0	16.91 ±2.67	7.33 ±2.4	NI	100 ±0.0	17.94 ±3.23	10.0 ±3.51	4.43 ±1.26
13	100 ±0.0	52.22 ±3.12	9.13 ±2.30	NI	100 ±0.0	NI	NI	NI	100 ±0.0	7.18 ±0.98	NI	NI	100 ±0.0	9.49 ±2.70	4.24 ±1.63	3.38 ±2.02
16	100 ±0.0	28.23 ±5.75	3.49 ±1.37	NI	100 ±0.0	NI	NI	NI	100 ±0.0	6.66 ±2.82	NI	NI	100 ±0.0	7.81 ±1.37	4.53 ±2.24	3.37 ±1.81
20	100 ±0.0	22.71 ±2.88	1.48 ±1.0	NI	100 ±0.0	NI	NI	NI	100 ±0.0	NI	NI	NI	100 ±0.0	6.97 ±3.8	0.47 ±0.0	NI

M. gypseum																
Compound	**OE**				**Eugenol**				**Isoeugenol**				**Methylisoeugenol**			
Day	**300**	**100**	**64,9**	**11,1**	**300**	**100**	**64,9**	**11,1**	**300**	**100**	**64,9**	**11,1**	**300**	**100**	**64,9**	**11,1**
6	100 ±0.0	70.0 ±2.50	60.0 ±4.33	5.83 ±1.15	100 ±0.0	73.33 ±2.88	42.50 ±6.61	NI	63.55 ±1.29	26.66 ±5.67	6.66 ±2.88	1.66 ±0.10	100 ±0.0	23.33 ±2.88	13.33 ±2.88	4.16 ±0.60
9	100 ±0.0	53,48 ±10.28	45,96 ±3.79	NI	100 ±0.0	51,22 ±6.34	11,57 ±3.79	NI	57,32 ±6.38	12,57 ±6.34	8,77 ±0.5	NI	100 ±0.0	16,08 ±0.5	11,57 ±2.78	4,91 ±0.10
13	65.33 ±9.00	33.33 ±5.77	1.33 ±1.11	NI	100 ±0.0	8.00 ±3.46	Ni	NI	55.00 ±5.0	11.38 ±4.09	NI	NI	67.33 ±1.15	7.38 ±3.07	2.00 ±1.37	NI
16	50.66 ±8.26	11.05 ±3.53	NI	NI	100 ±0.0	10.00 ±0.0	NI	NI	19.33 ±9.01	NI	NI	NI	47.33 ±3.05	7.33 ±3.05	NI	NI
20	21.43 ±2.34	NI	NI	NI	100 ±0.0	4.44 ±0.21	NI	NI	2.33 ±1.20	NI	NI	NI	25.64 ±2.40	NI	NI	NI

OE: essential oil; NI: no inhibition

Table 3. Percent inhibition of germination of *Microsporum spp* luego el tratamiento con EO of *Eugenia caryophyllus*, eugenol and its derivatives

Concentration (µg/mL)	*M. canis*					*M. gypseum*				
	Percent inhibition of germination ± SD									
	0 h	**6 h**	**24 h**	**37 h**	**84 h**	**0 h**	**6 h**	**24 h**	**37 h**	**84 h**
200	NI	81.57 ±0.57	52.83 ±0.57	21.42 ±1.73	11.76 ±9.81	NI	NI	NI	32.35 ±4.04	42.85 ±1.15
100	NI	76.31 ±1.73	43.39 ±6.92	1.96 ±2.30	NI	NI	NI	NI	14.7 ±5.19	32.14 ±0.57
64.9	NI	76.31 ±0.57	30.18 ±6.35	NI	NI	NI	NI	NI	14.7 ±4.04	NI
50	NI	71.05 ±0.57	15.09 ±4.04	NI	NI	NI	NI	NI	8.82 ±2.88	NI
200	NI	78.43 ±1.73	73.68 ±0.57	73.58 ±1.15	21.42 ±1.73	NI	64.28 ±0.0	61.76 ±0.57	50.0 ±1.73	32.14 ±0.57

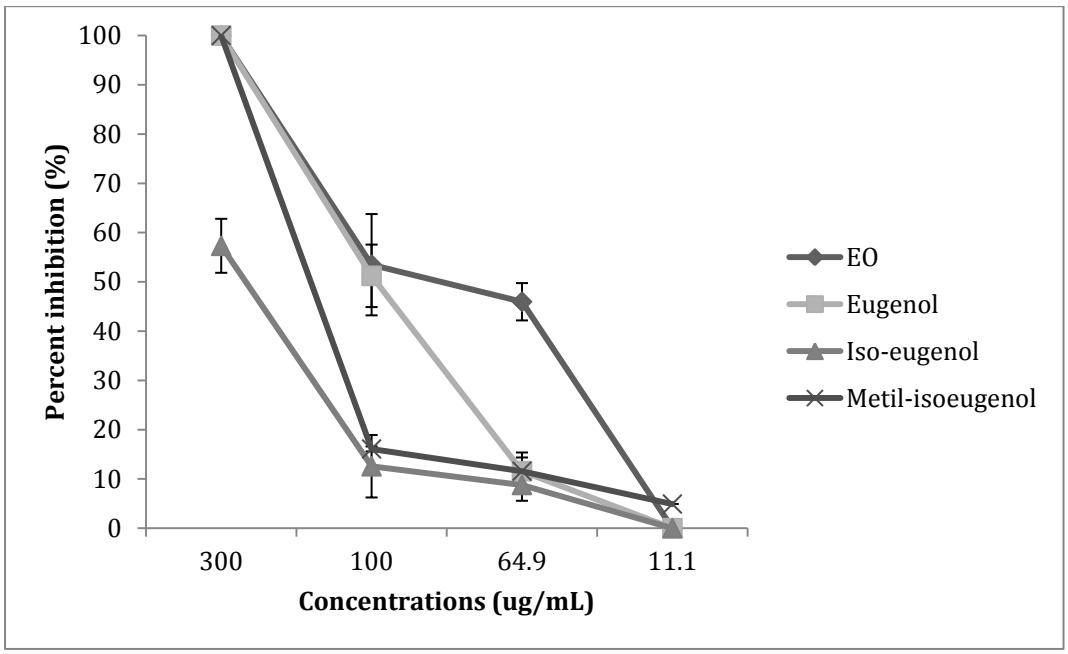
100	NI	72.54 ±2.30	73.68 ±0.0	60.37 ±1.15	21.42 ±0.57	NI	61.76 ±0.57	60.71 ±0.57	35.29 ±2.30	27.77 ±0.57
64.9	NI	50.98 ±1.73	45.28 ±0.57	28.3 ±2.30	NI	NI	50.0 ±0.0	42.85 ±0.0	32.35 ±0.57	NI
50	NI	37.25 ±4.61	31.57 ±2.30	21.05 ±0.0	NI	NI	35.71 ±0.57	28.57 ±0.0	22.22 ±1.15	NI
200	NI	82.35 ±0.57	73.68 ±1.54	73.58 ±1.15	28.57 ±1.15	NI	85.71 ±0.0	71.42 ±0.0	64.7 ±0.0	61.11 ±0.57
100	NI	64.7 ±3.46	64.15 ±0.57	50.0 ±0.57	14.28 ±1.57	NI	71.42 ±0.0	64.7 ±2.30	64.28 ±0.57	38.88 ±0.57
64.9	NI	41.17 ±3.46	30.18 ±5.19	14.28 ±1.15	10.52 ±2.30	NI	57.14 ±1.15	46.42 ±1.73	50.0 ±2.88	33.33 ±0.0
50	NI	33.33 ±4.61	28.3 ±8.08	7.14 ±0.67	5.26 ±1.15	NI	50.0 ±0.0	46.42 ±0.57	47.05 ±1.15	5.55 ±0.57
200	NI	NI	NI	NI	28.3 ±1.15	NI	28.57 ±1.15	23.52 ±3.46	NI	NI

100	NI	NI	NI	NI	9.43 ±8.08	NI	28.57 ±1.15	11.76 ±4.16	NI	NI
64.9	NI	NI	NI	NI	NI	NI	11.76 ±3.46	NI	NI	NI
50	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI

NI: no inhibition; SD: Stándar deviation; h: hours; EO: Essential oil

Figure 1. Percent inhibition of radial mycelium growth of *Microsporum spp* after 9 days of treatment with 100 µg/ml of the compounds under study. (a): *M. canis*; (b): *M. gypseum*

(a)



(b)

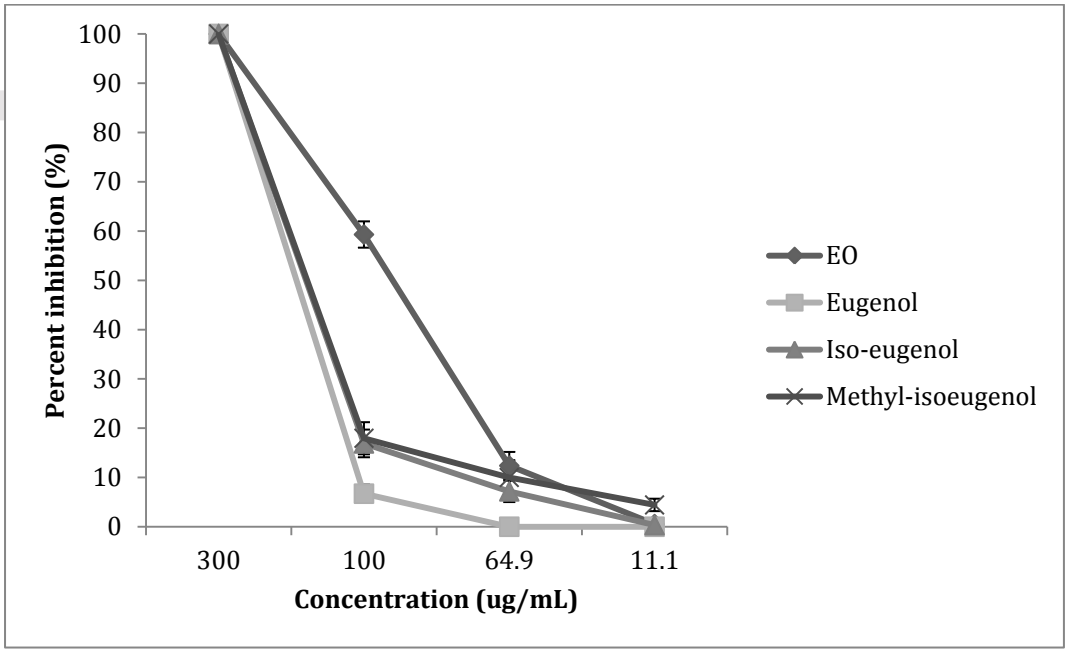
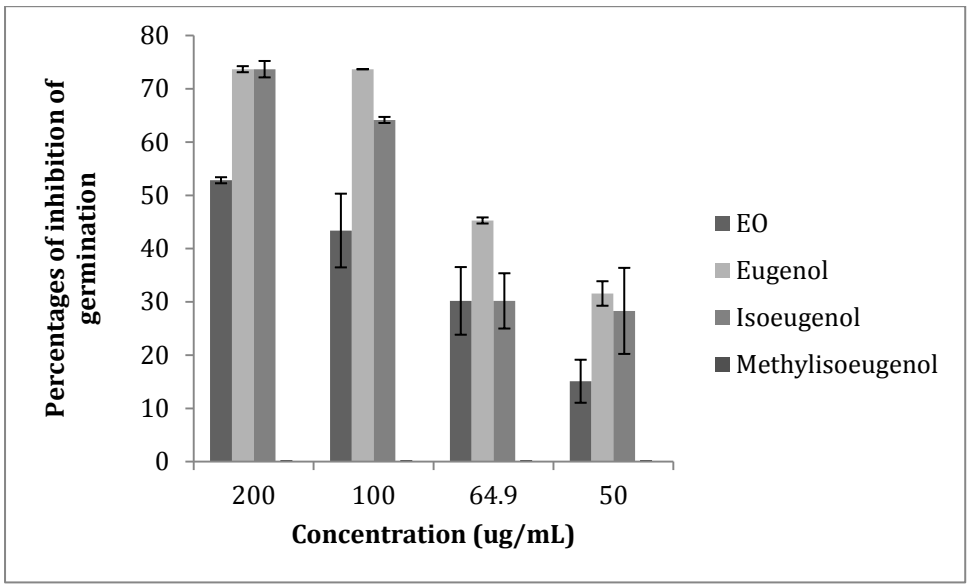


Figure 2. Effect of the essential oil of *Eugenia caryophyllus*, eugenol and its derivatives on the inhibition of the germination of *Microsporum spp* at 24 hours post treatment. (a): *M. canis*; (b): *M. gypseum*

(a)



(b)