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Semisynthetic eugenol derivatives as antifungal agents against dermatophytes of the genus *Trichophyton*

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Abstract

Purpose. Eugenol, the main component of clove bud essential oil (*Eugenia caryophyllus*), has been linked to antimicrobial, anti-inflammatory, insecticidal and immunomodulatory properties. The purpose of this study was to evaluate the antifungal and cytotoxic activity of eugenol, the essential oil of *Eugenia caryophyllus*, and some semisynthetic derivatives of eugenol against dermatophytes of the genus *Trichophyton*.

Methodology. We evaluated the antifungal effect of the compounds, determining the minimum inhibitory concentrations (MICs) by the microdilution method and the minimum fungicidal concentrations by cultures from the inhibitions. Additionally, the inhibition of the radial growth of the mycelium of the dermatophyte fungi was tested by poisoned substrate. Cytotoxicity was measured by the colorimetric method on Vero cells.

Results: All of the eugenol compounds tested exhibited antifungal properties, showing MICs of 62.5–500 µg ml^{-1} , determined within three dermatophyte species: *Trichophyton rubrum, Trichophyton mentagrophytes* and *Trichophyton tonsurans*. Among these derivatives, methyl isoeugenol, at concentrations of 300 and 100 µg ml^{-1}, was found to completely inhibit (100 %) radial growth of the mycelium of all three species after 20 days of treatment. Additionally, phenotypic variations related to the decrease in pigment production of *T. rubrum* were observed after treatment with O-ethyl and O-butyl isoeugenol derivatives. Meanwhile, all of the tested (iso)eugenol molecules exhibited moderate toxicity in Vero cells [50 % cytotoxic concentration (the concentration required for a 50 % reduction in cell viability; CC_{50}): 54.06–265.18 µg ml^{-1}).

Conclusion: The results suggest that the semisynthetic eugenol derivatives (SEDs) show promising antifungal activity and selectivity against dermatophyte fungi.

INTRODUCTION

Dermatophytosis, or tinea, is a superficial mycosis with tropism throughout the skin, hair and nails caused by keratinophilic fungi of the family Arthrodermataceae and classified in the genera *Trichophyton*, *Microsporum*, *Epidermophyton* and *Nannizzia* [1, 2]. These mycoses are common and frequent, and while they do not threaten a patient's life, they cause high morbidity and are a public health problem in developing countries where tropical and subtropical climates prevail and in which concern is higher due to their high propagation [3]. *Trichophyton* are the most predominant fungi in dermatophyte infections, with *Trichophyton rubrum* being the most prevalent species (69.5%), followed by *Trichophyton mentagrophytes*, *Trichophyton verrucosum* and *Trichophyton tonsurans*. Likewise, tinea corporis (70%) is considered to be the most prevalent clinical manifestation in

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Abbreviations: CC50, the concentration required for a 50 % reduction in cell viability; CC90, the concentration required for a 90 % reduction in cell viability; DMSO, Dimethyl sulfoxide; EO, essential oil; MFC, minimal fungicidal concentration; MIC, minimal inhibitory concentration; MOPS, 3-(N-morpholino)propanesulfonic acid; SED, semisynthetic eugenol derivatives.

Supplementary material is available with the online version of this article.

the infections caused by this genus [4]. Oral and topical antifungals are available for the treatment of dermatophytosis; the oral treatments (griseofulvin, itraconazole and terbinafine) are more effective, but show greater adverse side effects in the patient and require longer periods of treatment (1–6 months), depending on the anatomical part affected. On the other hand, topical antifungals (imidazoles and derivatives, allylamines, morpholines, cyclopiroxolamin, topical griseofulvin, haloprogin, tolnaftate and Whitfield ointment, among others) are mainly recommended for limited and superficial lesions, as well as for female patients during pregnancy and lactation. They are very well tolerated and are used as support for systemic treatment since their individual application does not exert the same effect as when they are used in combination [3, 4].

In these scenarios, essential oils (EOs) derived from plants and their components be used as innovative substrates for the development of new antifungals. In this context, eugenol, which is a phenylpropanoid and is the main component of essential oils such as dried clove bud essential oil (*Eugenia caryophyllus*) from clove trees *Syzygium aromaticum* (Myrtaceae), has demonstrated diverse biological properties against micro-organisms such as parasites, bacteria and fungi, as well as anti-inflammatory, antioxidant, insecticidal and immunomodulatory properties [5]. It also shows low toxicity. Additionally, there is now a special interest in obtaining derivatives of eugenol in order to enhance its effects and mechanisms of action and therefore expand its possible fields of use. The objective of this study was to evaluate the antifungal and cytotoxic activities of semisynthetic eugenol derivatives (SEDs) against dermatophyte fungi of the genus *Trichophyton* as well as epithelial cells.

METHODS

Fungal strains and mammalian cells

Trichophyton rubrum (ATCC 28188), *T. mentagrophytes* (ATCC 28185) and *T. tonsurans* (ATCC 28942) pass 3 were grown in potato dextrose broth (PDA; Oxoid Ltd, CM0139, Basingstoke, UK) at 25 °C with constant movement at 100 r.p.m. for 8 days. Subsequently, the dermatophytes were cultured for 10 days in the 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 kept in Dubelcco's Modified Eagle's Medium (DMEM; Life Technology, CA, USA) and supplemented with 5 % inactivated foetal bovine serum (iFBS; Life Technology, CA, USA) at 37 °C , 5 % CO_2 and 95 % humidity.

Chemistry

The EO was extracted from dried clove bud (*Eugenia caryophyllus*) through the microwave heating-assisted hydrodistillation (MWHD) technique.

To obtain eugenol [1], the isolated EO was dissolved in 100 ml of dichloromethane and extracted with a 5 % potassium hydroxide solution (3×50 ml). The alkaline layers were combined and extracted with dichloromethane (1×50 ml). The alkaline solution was slowly acidified to pH 1 with 5 % hydrochloric acid and extracted with dichloromethane (3×50 ml). The organic extracts were dried over sodium sulfate, and the solvent was removed by rotoevaporation. Then, a vacuum fractional distillation of crude product was carried out at 120–122 °C/5 mm Hg, providing a pure sample of (1) in 13 % (with respect to used flower buds).

The other phenols, dihydroeugenol (2), isoeugenol (3) and methyl isoeugenol (4), were acquired from Sigma-Aldrich and used as synthetic precursors for the required eugenol derivatives or model compounds for the planned bioscreening.

The reference drug, terbinafine (Novartis), was purchased in the form of a pharmaceutical preparation. Thus, it was subjected to the necessary purification processes (recrystallization, column chromatography, high-performance liquid chromatography) to ensure a purity greater than 99.0%, which allowed us to use it as a reference compound in the bioassays.

General procedure for O-alkylation (compounds 5-12)

A mixture of eugenol 1 (dihydroeugenol 2 or isoeugenol 3) (0.01 mmol) and potassium carbonate (0.08 mmol) in anhydrous acetone (25 ml) was stirred for several minutes. Subsequently, alkyl halide (ethyl bromide, butyl bromide or octyl bromide) (0.05 mmol) was added dropwise into the stirred solution at room temperature. The resulting mixture was then heated to reflux at 54–56 °C for the required time and monitored by TLC. After cooling to room temperature, the final mixture was treated with water and extracted with ethyl ether (3×15 ml). The organic layers were combined and washed with 10 % sodium hydroxide (2×15 ml), water (1×15 ml) and then finally saturated sodium chloride solution (1×15 ml). The organic extracts were dried over anhydrous sodium sulfate. The solvent was removed under reduced pressure and the residue was purified by silica gel column chromatography (CC) using mixtures of hexane and ethyl acetate. All pure products were identified by gas chromatography/mass spectrometry (GC-MS), Fourier-transform infrared (FT-IR) and nuclear magnetic resonance (NMR) experiments (See Fig. S1, available in the online version of this article).

Antifungal activity

Minimum inhibitory concentration (MIC)

Experiments were performed using the broth microdilution method, implemented according to the Clinical and Laboratory Standards Institute's (CLSI's) M27-A protocol, with the exception of the inoculum preparation [6]. Briefly, a fungal suspension was obtained from a 12–15-day colony culture in PDA medium at 30 °C with slight scraping with a 1 % Tween 80 solution in distilled water, and then finally adjusted to $0.4–5.0\times10^4$ colony-forming units (c.f.u.) ml^{-1} in RPMI-1640 culture medium (Sigma, St Louis, MO, USA) and buffered with 3-(N-morpholino)propanesulfonic acid (MOPS; Sigma-Aldrich, Darmstadt, Germany). 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^{-1}. Untreated controls were then tested. The antifungal effect was observed after 7 days of incubation at 30 °C by optical observation of turbidity. The MIC was defined as 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.

Minimum fungicidal concentration (MFC)

Subculture on Sabouraud dextrose agar medium (Oxoid Ltd, CM0041, Basingstoke, UK) was performed from the MIC and concentrations higher and less than the MIC. The dishes were incubated at 30 °C for 7 days. A control without drugs was performed. The MFC was defined as the lowest concentration of the compound at which growth was <3 c.f.u. All experiments were performed in triplicate, and the results are presented as the geometric means of the replicates.

Inhibition of mycelial radial growth

Twelve-day subcultures of each strain grown in PDA were used. The inhibition was analysed using the poisoned substrate method with some modifications [7]. Briefly, the PDA was prepared in Petri dishes and mixed with different concentrations of the compounds under study (300, 100, 64.9 and 11.1 μg ml^{-1}). Each concentration was evaluated in triplicate and a control without treatment was evaluated. Discoidal cuts with a diameter of 5 mm were made in the centre of the agar and replaced by cuts of the same diameter from the subcultures grown from 12 to 15 days. The dishes were incubated at 30 °C for 21 days and mycelial growth diameter readings were performed on day 6, 9, 13, 16 and 20. The results are expressed as the percentage of inhibition of the colony radius for each treatment with respect to the control.

Additionally, the pigmentation production was evaluated in subcultures. It was expressed according to a qualitative visual scale as similar, diminished or absent in relation to the control culture.

Cytotoxicity assay in mammalian cells

Vero cells (3×10^5 cells ml^{-1}) were placed in 96-well microplates for 24 h at 37 °C and 5 % CO_2 until the formation of the monolayer. The prepared compounds and terbinafine in concentrations of 300 to 11.1 μg ml^{-1} were then added for 72 h. Cell viability was evaluated using the MTT (3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyltetrazolium bromide; Sigma) tetrazolium salt at a concentration of 5 mg ml^{-1}. A spectrophotometric reading was performed on a microplate reader for absorbance/enzyme-linked immunosorbent assay (ELISA) (BioRad) using a wavelength of 590 nm. All assays were performed in triplicate in independent experiments.

Analysis results

The percentage of inhibition of the radial growth of the mycelium was determined by the formula: diameter of the control−diameter of the treatment/control diameter × 100. On the other hand, the 50 and 90 % cytotoxic concentrations (the concentrations required for 50 and 90 % reductions in cell viability, respectively; CC_{50} and CC_{90}) were determined by sigmoidal regression, using the statistical program XlFit5.

RESULTS

The principal components found in the 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 %.

Using dried flower buds acquired at a local market, clove EO was obtained at a yield of 15.2 % through the MWHD technique. GC-MS analysis confirmed that eugenol was a major component (71.8%). The tested compounds (5–12) (Fig. 1) were prepared easily with moderate to excellent yields (30–95 %) via alkylation reactions of eugenol(1), dihydroeugenol (2) and isoeugenol (3) with alkyl bromides (ethyl bromide, butyl bromide or octyl bromide) in the presence of Na_2CO_3 in boiling acetone (Fig. 1). All of the experimental compounds known as SEDs were purified by column chromatography and were >95 % pure according to ^{1}H and ^{13}C NMR analysis.

The *in vitro* inhibitory effect of the prepared compounds (5–12) was evaluated against *Trichophyton* ATCC species and the results are shown in Table 1. Additionally, the dried clove bud EO, eugenol (1) and commercially available isoeugenol (3) and methyl isoeugenol (O-methyl isoeugenol) (4) were also evaluated in this study, as model eugenol analogues.

Interestingly, he derivates eugenol (1), isoeugenol (3) and methyl isoeugenol (4) showed MICs of 62.5 and 500 μg ml^{-1} against three dermatophyte species studies.

These latter model compounds, isoeugenol (3) and methyl isoeugenol (4), showed the greatest inhibition of the dermatophyte *T. mentagrophytes*, with an MIC of 62.5 μg ml^{-1} and an MFC of 125 and 500 μg ml^{-1}, respectively. Likewise, clove EO, eugenol (1), O-ethyl eugenol (5) and O-butyl eugenol (7) presented an MIC of 125–250 μg ml^{-1} and an MFC of 250 to −500 μg ml^{-1}.

Increased susceptibility was observed in *T. rubrum;* seven compounds inhibited the growth of this strain with an MIC and an MFC of 250 to 1000 μg ml^{-1}. O-ethyl eugenol (5) and O-butyl isoeugenol (8), as well as isoeugenol (3), produced the greatest inhibition, with an MIC of 250 μg ml^{-1}, while eugenol (1), the main component, showed greater inhibition than the EO, with an MIC of 125 μg ml^{-1} versus one of 1000 μg ml^{-1}, respectively.

On the other hand, the SEDs (3–5) inhibited the growth of *T. tonsurans* with an MIC of 125–500 μg ml^{-1}. The EO and eugenol (1) showed similar inhibition (MIC and MFC: 250 μg ml^{-1}) in this strain. In general, the SEDs (3–5) inhibited the three *Trichophyton* species tested.

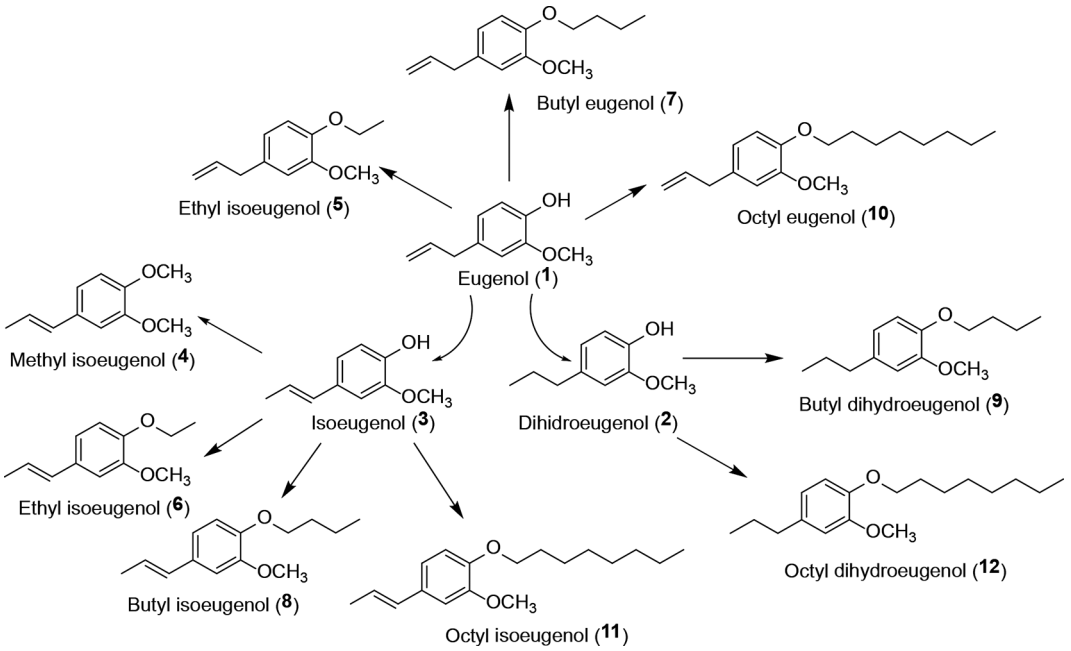

Fig. 1. Structure of the eugenols evaluated (1–12).

The results for the inhibition of the radial growth of the mycelium, expressed as percentages of inhibition, are shown in Table 2. Methyl isoeugenol (4) inhibited 100 % of the micelial growth of all of this study's strains in the tested concentrations from day 6. Similar effects were noted with eugenol (1) and its O-methyl analogues. Methyl isoeugenol (4), inhibited 100 % of the mycelial growth of *T. mentagrophytes* at concentrations of 300 and 100 µg ml^{-1}, while O-butyl eugenol (7) showed the same inhibition, but only at a concentration of 300 µg ml^{-1}. A fungistatic effect was shown by O-butyl isoeugenol (8), with it inhibiting 100 % of the mycelial growth of this dermatophyte on the sixth day, but this inhibition decreased to 30.4 % after 9 days of incubation. Similar findings were observed for *T. rubrum* when it was treated with both O-methyl (4) and O-butyl (8) isoeugenol derivatives at a concentration of 100 µg ml^{-1}. Additionally, 100 % inhibition of *T. rubrum* mycelium growth was detected after exposure to O-alkyl (iso)eugenols (4, 5 and 8) at a concentration of 300 µg ml^{-1} for 20 days.

With respect to *T. tonsurans,* O-alkyl isoeugenols (4, 6 and 8) and O-butyl dihydroeugenol (9) inhibited 100 % of mycelial growth at concentrations of 300 and 100 µg ml^{-1}, and O-butyl eugenol (7) showed the same inhibitory effect, but only at a concentration of 300 µg ml^{-1} after 20 days of treatment.

It is important to mention that SEDs did not inhibit the dermatophytes in our study at the other concentrations that were evaluated (64.9 and 11.1 µg ml^{-1}) (data not shown).

Terbinafinem, which was used as a positive control, was evaluated in concentrations of 0.3, 0.1, 0.03 and 0.01 µg ml^{-1} against *T. rubrum* and *T. mentagrophytes*. In the case of *T. tonsurans*, concentrations of 0.03, 0.01, 0.003 and 0.001 µg ml^{-1} were tested. This antifungal inhibited 100 % the hyphal growth of *T. rubrum* and *T. mentagrophytes* in the first concentrations evaluated until day 6, after which the inhibition was reduced to 70 % on day 20 of incubation. By contrast, *T. tonsurans* showed greater resistance to terbinafine, with mycelial growth only being inhibited by 36.20 % at the maximum concentration tested. A dose–response effect was observed with each of the concentrations evaluated versus the incubation time (data not shown).

Additionally, it was noted that after obtaining 100 % inhibition of hyphal growth with some of the derivatives, the fungus resumed its growth with time. Consequently, in the case of *T. mentagrophytes*, eugenol at a concentration of 64.9 µg ml^{-1} was seen to inhibit 100 % of mycelial growth until day 9. This effect decreased with incubation time and inhibition was reduced to 70 % on day 13. Likewise, derivatives 6, 7 and 9 at concentrations of 300 µg ml^{-1} were shown to inhibit 100 % of hyphal growth in *T. rubrum* until day 6. Compounds 4 and 8 inhibited 100 % of *T. rubrum* growth at a concentration of 100 µg ml^{-1} up to day 9. The same effect (100 % inhibition) was observed with compound 6 at a concentration of 100 µg ml^{-1} against *T. tonsurans* up to day 16, with the inhibition decreasing to ~80 % at day 20.

Interestingly, phenotypic changes were observed during the treatment of *T. rubrum* with O-ethyl isoeugenol (6) and its butyl analogue (8). The inhibition of the mycelial radial growth of this dermatophyte occurred in parallel with the inhibition of pigment production, which is very characteristic of *T. rubrum*; this change was observed with concentrations of 64.9 µg ml^{-1} and 100 µg ml^{-1} of the O-butyl isoeugenol (8) and O-ethyl isoeugenol (6) compounds, respectively (Fig. 2).

Table 1. Antifungal (MIC and MFC) and cytotoxic activities of SEDs, eugenol and dried clove bud oil against *T. mentagrophytes*, *T. rubrum*, *T. tonsurans* and mammalian cells

Comp.	Fungal strain						Vero cells	
	T. mentagrophytes		*T. rubrum*		*T. tonsurans*			
	MIC	MFC	MIC	MFC	MIC	MFC	CC_{50} ±SD	CC_{90} ±SD
EO	125	250	1000	1000	250	250	265.10 ±15.05	>300
1	125	500	125	125	250	250	ND	>300
3	62.5	125	250	250	125	125	105.62 ±12.75	>300
4	62.5	500	500	>1000	125	125	174.94 ±28.52	>300
5	250	500	250	250	500	500	>300	>300
6	>1000	>1000	500	>1000	>1000	>1000	>300	>300
7	250	250	500	500	>1000	>1000	>300	>300
8	>1000	>1000	250	250	>1000	>1000	>300	>300
9	>1000	>1000	>1000	>1000	>1000	>1000	>300	>300
10	>1000	>1000	>1000	>1000	>1000	>1000	76.54 ±7.86	>300
11	>1000	>1000	>1000	>1000	>1000	>1000	90.36 ±5.63	>300
12	>1000	>1000	500	1000	>1000	>1000	63.99 ±4.57	>300
TERB	0.0097	0.312	0.039	0.039	0.62	0.62	31.00 ±0.61	ND

Results are expressed as μg ml^{-1}.
CC50/CC90, cytotoxic concentration 50 and 90; Comp, compound; EO, essential oil; MFC, minimum fungicidal concentration; MIC, minimum inhibitory concentration.

For the *in vitro* cytotoxicity of the SEDs in epithelial cells (Vero), a CC_{50} of between 54.06 and >300 μg ml^{-1} was determined (Table 1). It is noteworthy that methyl isoeugenol (4) and O-ethyl eugenol (5), the most active antifungal molecules, were non-toxic compounds for these cells, with a CC_{50} of >174 μg ml^{-1} meaning that they were up to 5.6 times less toxic than the terbinafine (CC_{50}=31 μg ml^{-1}) used as a reference drug.

DISCUSSION

Eugenol is a phenolic, lipophilic, non-toxic derivative with antimicrobial, antioxidant and insecticidal properties [8–12]. It is extracted from different natural sources, such as leaves and fruits of the clove tree *Syzygium aromaticum* (Myrtaceae), the cinnamon tree *Cinnamomum verum* (formerly *C. zeylanicum* and *C. cassia J. Presl*) (Lauraceae) and species of the genus *Ocimum* (Lamiaceae), among others [7–13]. Taking into account these properties, diverse derivatives of eugenol could be interesting and important model molecules in biomedical research. Therefore, in this work, some SEDs (5–12) were prepared using eugenol (1), obtained from clove EO, as well as commercial dihydroeugenol (2) and isoeugenol (3), which were used as cheap initial materials. The preparation of the O-alkylated (iso) eugenol products (5–12) required in our study was based on the well-known Williamson reaction (O-alkylation process) of simple phenolic molecules such as (1–3) and respective alkyl (ethyl, *n*-butyl, *n*-octyl) bromides; it is noteworthy that these are all commercial, low-cost reagents. Thus, based on a common, simple and inexpensive protocol, a new series of O-alkylated (iso)eugenol molecules was generated easily at acceptable yields (30–95 %). GC-MS analysis was performed on the obtained molecules and showed high phenolic purity, while NMR experiments allowed us to confirm their structures.

Having characterized the O-alky (iso)eugenol samples, we began our biological study to evaluate their antifungal activity against fungi dermatophytes of the genus *Trichophyton* as well as their cytotoxic activity against epithelial cells (Vero).

One of the mechanisms of the pathogenicity of dermatophytes is the hyphal growth that promotes the invasion of the fungus into deep layers of the epidermis, causing the characteristic

Table 2. Inhibitory effects of SEDs and dried clove bud oil on mycelial radial growth of three *Trichophyton* strains. The results are expressed as percentages of inhibition and are the average of three independent experiments; each concentration was evaluated in triplicate

Comp.	Day	*T. mentagrophytes*		*T. rubrum*		*T. tonsurans*	
		Concentration (μg ml^{-1} ±SD)		Concentration (μg ml^{-1} ±SD)		Concentration (μg ml^{-1} ±SD)	
		300	100	300	100	300	100
EO	6	26.25±3.83	12.05±3.53	49.70±0.98	39.28±0.12	13.04±0.0	0.0
	9	12.01±2.03	8.0±0.0	35.47±0.17	33.63±0.74	0.79±0.0	0.0
	13	6.03±2.82	4.0±0.0	26.83±1.06	19.84±0.88	0.0	0.0
	16	0.0	0.0	26.47±1.94	15.08±0.71	0.0	0.0
	20	0.0	0.0	21.26±2.03	13.91±1.06	0.0	0.0
1	6	100±0.0	100±0.0	27.70±2.04	22.18±1.46	100±0.0	40.57±0.15
	9	100±0.0	100±0.0	22.03±4.01	16.81±1.15	100±0.0	18.64±2.80
	13	100±0.0	100±0.0	20.18±3.59	14.87±1.57	100±0.0	2.11±0.57
	16	100±0.0	100±0.0	17.91±1.39	11.77±2.50	100±0.0	0.0
	20	100±0.0	100±0.0	17.15±0.80	10.89±0.80	100±0.0	0.0
3	6	41.51±2.04	9.84±1.04	44.35±1.67	17.51±1.19	44.35±1.67	17.51±1.19
	9	26.16±1.09	7.40±1.84	40.86±1.82	16.17±1.39	40.86±1.81	16.17±1.39
	13	23.10±0.82	2.38±1.00	37.66±2.53	15.57±1.11	40.37±1.48	15.57±1.11
	16	18.82±3.92	1.49±0.20	36.24±1.12	14.37±1.82	37.66±2.53	14.37±1.87
	20	13.86±0.82	1.41±0.41	40.37±1.49	7.40±0.37	36.24±0.20	7.46±0.37
4	6	100±0.0	100.0±0.0	100±0.0	100±0.0	100±0.0	100±0.0
	9	100±0.0	100±0.0	100±0.0	100±0.0	100±0.0	100±0.0
	13	100±0.0	100±0.0	100±0.0	73.52±1.20	100±0.0	100±0.0
	16	100±0.0	100±0.0	100±0.0	61.42±1.40	100±0.0	100±0.0
	20	100±0.0	100±0.0	100±0.0	44.75±2.80	100±0.0	100±0.0
5	6	15.00±1.00	0.0	100±0.0	31.00±8.47	19.22±0.87	19.22±0.87
	9	10.00±0.0	0.0	100±0.0	24.90±1.58	16.61±2.02	14.84±0.21
	13	0.0	0.0	100±0.0	20.67±2.11	15.98±1.23	14.32±1.01
	16	0.0	0.0	100±0.0	18.16±1.10	12.58±1.21	11.07±1.23
	20	0.0	0.0	100±0.0	17.99±0.21	12.51±1.20	8.00±0.80
6	6	14.98±2.51	4.10±0.27	100±0.0	69.93±4.25	100±0.0	100±0.0
	9	13.43±2.82	3.09±0.85	94.80±7.35	41.08±2.48	100±0.0	100±0.0
	13	12.74±1.01	2.82±1.25	69.09±2.33	39.67±2.10	100±0.0	100±0.0
	16	6.83±0.82	2.65±1.42	45.49±2.62	29.62±1.29	100±0.0	100±0.0
	20	3.00±0.12	2.29±0.48	43.38±2.88	28.91±1.83	77.27±0.71	80.23±0.62
7	6	100±0.0	32.50±3.53	100±0.0	92.09±1.11	100±0.0	19.56±3.06
	9	100±0.0	19.00±1.41	80.40±2.77	41.10±3.25	100±0.0	8.73±0.0
	13	100±0.0	10.00±2.82	75.00±3.53	34.93±1.24	100±0.0	2.14±0.0
	16	100±0.0	5.00±1.41	49.31±3.93	28.20±2.20	100±0.0	0.0
	20	100±0.0	0.0	48.72±2.34	27.08±1.94	100±0.0	0.0

Continued

Table 2. Continued

Comp.	Day	*T. mentagrophytes*		*T. rubrum*		*T. tonsurans*	
		Concentration (μg ml^{-1} ±SD)		Concentration (μg ml^{-1} ±SD)		Concentration (μg ml^{-1} ±SD)	
		300	100	300	100	300	100
8	**6**	100±0.0	66.48±3.38	100±0.0	100±0.0	100±0.0	100±0.0
	9	30.40±0.50	19.23±1.01	100±0.0	100±0.0	100±0.0	100±0.0
	13	19.18±1.33	12.67±2.17	100±0.0	69.15±5.34	100±0.0	100±0.0
	16	12.92±0.36	3.90±0.82	100±0.0	61.03±3.82	100±0.0	100±0.0
	20	7.71±0.41	3.77±0.88	100±0.0	54.82±2.59	100±0.0	100±0.0
9	**6**	13.03±2.25	6.35±0.59	100±0.0	81.04±2.68	100±0.0	100±0.0
	9	9.98±0.50	5.28±1.50	84.20±2.23	27.70±2.20	100±0.0	100±0.0
	13	7.89±1.55	5.05±1.12	53.17±6.62	27.27±2.33	100±0.0	100±0.0
	16	5.66±1.65	4.19±1.24	43.75±4.72	27.08±1.94	100±0.0	100±0.0
	20	5.16±0.50	3.00±0.50	38.81±3.74	25.79±2.53	100±0.0	100±0.0
10	**6**	21.40±1.28	18.14±0.50	17.26±0.47	17.94±0.97	28.34±1.87	29.23±1.38
	9	19.59±1.13	16.62±0.56	20.19±2.93	15.49±1.18	27.79±1.65	27.10±1.83
	13	16.98±1.47	16.35±0.88	15.78±2.63	10.35±0.45	20.34±1.87	24.43±2.34
	16	13.34±1.15	8.00±0.82	20.52±1.86	9.47±1.31	20.16±2.00	22.10±1.83
	20	15.43±1.33	1.15±1.12	17.15±1,04	1.12±1.98	19.11±1.81	17.56±1.02
11	**6**	17.25±2.52	10.92±3.05	36.64±1.78	27.75±2.00	28.76±1.72	19.70±1.61
	9	15.08±1.60	7.19±0.31	34.50±1.69	27.04±2.28	16.90±1.36	18.82±0.31
	13	11.76±2.70	5.95±0.97	30.41±2.18	21.26±2.39	14.86±1.53	11.26±3.80
	16	9.53±1.15	3.57±0.72	30.04±1.18	20.43±1.87	13.23±1.16	10.76±2.22
	20	2.19±0.24	1.57±1.15	28.13±2.36	17.34±3.17	11.28±1.03	10.57±1.25
12	**6**	50.53±3.02	14.62±1.69	41.60±1.24	33.42±1.01	29.67±2.14	21.34±1.39
	9	27.32±1.58	12.69±0.41	35.47±5.17	30.90±2.00	27.67±2.42	17.57±3.94
	13	19.59±1.13	10.24±0.43	26.47±1.94	17.25±1.83	24.41±0.29	12.66±0.31
	16	10.05±1.90	9.65±1,87	26.83±1.06	2.07±1.50	22.25±1.87	11.80±1.20
	20	4.49±1.01	8.50±1.09	21.26±2.02	1.64±0.10	17.83±2.34	6.30±0.20

Comp, compound; EO, essential oil; SD, standard deviation.

clinical picture of these fungi (*Tineas*) [14–16]. Because of this, the inhibition of the radial growth of the mycelium is important with regard to whether the infection takes hold [16]. The semisynthetic derivatives of eugenol were shown to have some inhibitory effect on the radial growth of the mycelium of the dermatophytes tested: methyl isoeugenol (4) in particular completely inhibited the radial growth of the mycelium of *T. rubrum*, *T. tonsurans* and *T. mentagrophytes* at concentrations of 300 and 100 ug ml^{-1}. A similar effect was observed with O-butyl (8), O-ethyl (6) isoeugenols and O-butyldihydroeugenol (9) and *T. tonsurans*, with this species being more susceptible to the semisynthetic eugenol derivatives in this study, followed by *T. rubrum* and *T. mentagrophytes*, which showed high susceptibility to eugenol.

Some of the semi-synthetic compounds (4, 8, 9) that were inhibitors of hyphal growth were shown to have a fungistatic effect when tested at a concentration of 100 μg ml^{-1}. However, after 6 days of exposure to the compound, its 100 % inhibition decreased with time to <70 % inhibition, mainly in *T. rubrum*. This same effect was observed with *T. mentagrophytes* and *T. tonsurans* when using derivatives 6 and 8, respectively (Table 2). These results are important in the application of double-dose treatment during experimentation in order to

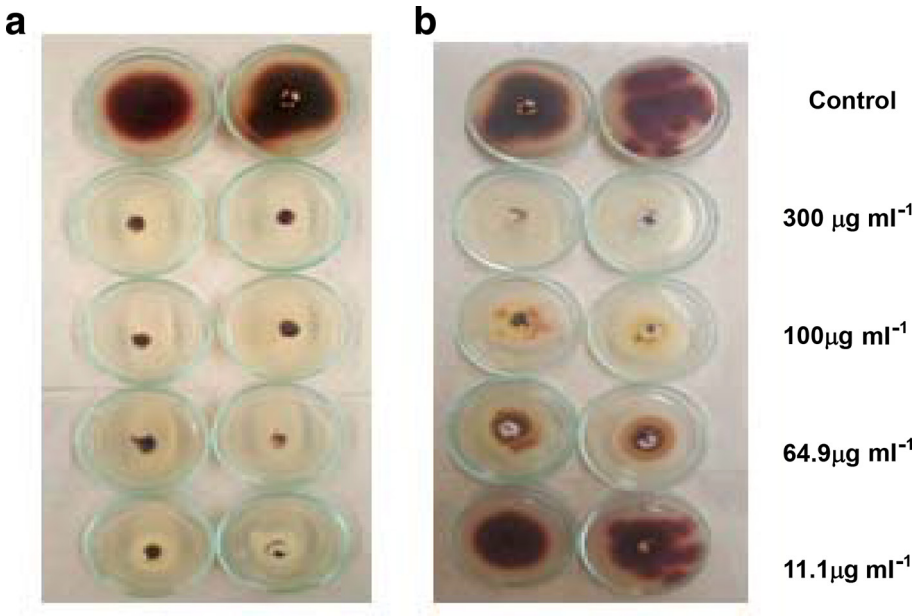

Fig. 2. Inhibition of pigment production. (a) O-butyl isoeugenol [8]. (b) O-ethyl isoeugenol [6].

obtain a constant fungicidal effect and inhibit the growth of the fungus.

In this study, terbinafine, as reference drug showed a lower MIC than the SEDs; however, it has been reported in the literature that *Trichophyton* clinical isolates are resistant to terbinafine with some frequency [17]. Further, this drug is commonly used in onychomycoses treatment, but the healing rates are low due to many factors [18]. Combining treatment with conventional drugs with natural products or alternative therapies shows potential to increase efficacy in patients with dermatophytosis, possibly related to various mechanisms of action, synergistic effects and low resistance [19]. The report on the antifungal activity of SEDs presented in this work may encourage future studies on new compounds with anti-dermatophyte activity.

Parallel to inhibition described above, a phenotypic change after treatment with O-ethyl (6) and O-butyl (8) isoeugenols was indicated by the decrease and/or absence of the pigmentation of *T. rubrum*. This is a relevant finding, because pigment, as a secondary metabolite, has been associated with preventive action against bacterial competitors present on skin and nails [20]; for instance, the literature indicates that these phenomena occur as a consequence of environmental variations and are related to genotypic modifications in functional genes [21]. This suggests that the prepared SEDs possibly interfere with biosynthetic pathways involved in the generation of pigmentation in *T. rubrum*.

The antifungal activity of eugenol against *T. rubrum* and *T. mentagrophytes* has been attributed to its lipophilicity, which facilitates its internalization through the plasmatic membrane, interfering with and causing damage to its structure [22]. Because of this, the biological activity of eugenol against *Eugenia cariophyllata* and fungi causing onychomycosis, including *T. rubrum* and *T. mentagrophytes*, has been studied extensively. This inhibition was similar to that obtained by the ketoconazole and itraconazole used as a reference [23]. Subsequently, Pinto *et al*. studied the antifungal effect of clove bud EO and eugenol on a wide variety of yeast fungi of the genus *Candida* and filamentous fungi such as dermatophytes and *Aspegillus* spp; it was shown that the EO and eugenol showed a similar antifungal effect against each of the fungal species evaluated, with an MIC of 0.12–0.64 μg ml^{-1} and an MFC of 0.32–1.25 μg ml^{-1} [24]. Similar results were reported by Rana *et al*., who demonstrated the inhibitory effect of this EO against *T. rubum* and *M. gypseum*, with MIC values of 9–12 μg ml^{-1} [25].

In contrast to the previous results, our study showed a low susceptibility of the *Trichophyton* species evaluated against eugenol and clove EO. In the case of *T. rubrum*, the essential oil did not inhibit growth, with an MIC of 1000 μg ml^{-1}. However, greater inhibition was observed with eugenol (MIC: 125 μg ml^{-1}). *T. mentagrophytes* and *T. tonsurans* presented similar inhibition after being exposed to EO and eugenol, with an MIC and an MFC of 125 and 250 μg ml^{-1}, respectively. These differences could be due to the different reference strains used in the studies, as well as the synthetic routes and commercial or natural sources used to obtain the compounds. In addition, high variability in the susceptibility results obtained for dermatophytes through methodologies such as the microdilution test (among others) has been reported. This is attributed to the various biological parameters, such as inoculum, temperature, incubation time and assessment criteria, among others, that play an important role in the standardization of

the methodologies used, even when recommended by the CLSI [26].

On the other hand, we showed a lower cytotoxic effect of the semisynthetic compounds of eugenol versus terbinafine ($P<0.05$, Table 1). This biological activity in mammalian cells has not yet been reported in the literature with our compounds. However, variability in the toxicity results for eugenol and the essential oil of *Syzygium aromaticum* using different models (genotoxicity, cytotoxicity) and cell lines [J774 macrophages, leukaemic lines, primary cultures, fibroblasts (W138, 153BR, HNDF) and endothelial cells (HMEC-1), among others], has been studied [27–29].

In conclusion, the results of this study indicate that the semisynthetic compounds of eugenol, mainly O-methyl (4), O-ethyl (6) and O-butyl (8) isoeugenol and O-butyldihydroeugenol (9), show promising antifungal activity against the radial growth of the mycelium of *T. rubrum*, *T. mentagrophytes* and *T. tonsurans*; in addition, they were not toxic to mammalian cells, showing selectivity in antimicrobial inhibition. Studies related to the evaluation of the mechanisms of action, as well as live models with active derivatives, could be taken into account for future work.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

References

1. Nenoff P, Krüger C, Schaller J, Ginter-Hanselmayer G, Schulte-Beerbühl R *et al*. Mycology - an update part 2: dermatomycoses: clinical picture and diagnostics. *J Dtsch Dermatol Ges* 2014;12:749–777.
2. de Hoog GS, Dukik K, Monod M, Packeu A, Stubbe D *et al*. Toward a novel multilocus phylogenetic taxonomy for the dermatophytes. *Mycopathologia* 2017;182:5–31.
3. Deepika TL, Kannabiran K. Review on dermatomycosis: pathogenesis and treatment. *Natural Science* 2010;2:726–731.
4. Molina de Diego A. Aspectos clínicos, diagnósticos Y terapéuticos de las dermatofitosis. *Enferm Infecc Microbiol Clin* 2011;29:33–39.
5. Marchese A, Barbieri R, Coppo E, Orhan IE, Daglia M *et al*. Antimicrobial activity of eugenol and essential oils containing eugenol: a mechanistic viewpoint. *Crit Rev Microbiol* 2017;43:668–689.
6. National Committee for Clinical Laboratory Standards. *Reference method for broth dilution antifungal susceptibility testing of yeasts*, Approved standard NCCLS document M27-A. Wayne, PA: National Committee for Clinical Laboratory Standards; 1997.
7. Leal SM, Herrera LV, Vargas LY. *In vitro* susceptibility of *Microsporum* spp. and mammalian cells to *Eugenia caryophyllus* essential oil, eugenol and semisynthetic derivatives. *Mycoses* 2018:1–10.
8. Pavela R. Acute, synergistic and antagonistic effects of some aromatic compounds on the *Spodoptera littoralis* Boisd. (Lep., Noctuidae) larvae. *Ind Crops Prod* 2014;60:247–258.
9. Chaieb K, Hajlaoui H, Zmantar T, Kahla-Nakbi AB, Rouabhia M *et al*. The chemical composition and biological activity of clove essential oil, *Eugenia caryophyllata* (*Syzigium aromaticum* L. Myrtaceae): a short review. *Phytother Res* 2007;21:501–506.
10. Gülçin İlhami, Gülçin I. Antioxidant activity of eugenol: a structure-activity relationship study. *J Med Food* 2011;14:975–985.
11. Han X, Parker TL. Anti-inflammatory activity of clove (*Eugenia caryophyllata*) essential oil in human dermal fibroblasts. *Pharm Biol* 2017;55:1619–1622.
12. Mahakittikun V, Soonthornchareonnon N, Foongladda S, Boitano JJ, Wangapai T *et al*. A preliminary study of the acaricidal activity of clove oil, *Eugenia caryophyllus*. *Asian Pac J Allergy Immunol* 2014;32:46–52.
13. Seixas FJ, da Franca Rodrigues KA, Guerra de Oliveira R *et al*. Synthesis and *in vitro* Anti *Leishmania amazonensis* biological screening of Morita-Baylis-Hillman adducts prepared from eugenol, thymol and carvacrol. *Molecules* 2016;21:1483.
14. Vermout S, Tabart J, Baldo A, Mathy A, Losson B *et al*. Pathogenesis of dermatophytosis. *Mycopathologia* 2008;166:267–275.
15. Martínez-Rossi NM, Peres NTA, Rossi A. Pathogenesis of dermatophytosis: sensing the host tissue. *Mycopathologia* 2017;182:215–227.
16. Brand A. Hyphal growth in human fungal pathogens and its role in virulence. *Int J Microbiol* 2012;166:267–275.
17. Yamada T, Maeda M, Alshahni MM, Tanaka R, Yaguchi T *et al*. Terbinafine resistance of Trichophyton clinical isolates caused by specific point mutations in the squalene epoxidase gene. *Antimicrob Agents Chemother* 2017;61:e00115–00117.
18. Christenson J, Peterson G, Naunton M, Bushell M, Kosari S *et al*. Challenges and opportunities in the management of onychomycosis. *J Fungi* 2018;4:87.
19. Gupta AK, Cernea M, Foley KA. Improving cure rates in onychomycosis. *J Cutan Med Surg* 2016;20:517–531.
20. Summerbell RC. Form and function in the evolution of dermatophytes. In: Kushwaha RKS, Guarro J (editors). *Biology of dermatophytes and other keratinolytic fungi*, Iberoamericana de Micología.
21. Guoling Y, Xiaohong Y, Jingrong L, Liji J, Lijia A. A study on stability of phenotype and genotype of *Trichophyton rubrum*. *Mycopathologia* 2006;161:205–212.
22. de Oliveira Pereira F, Mendes JM, de Oliveira Lima E. Investigation on mechanism of antifungal activity of eugenol against *Trichophyton rubrum*. *Med Mycol* 2013;51:507–513.
23. Gayoso CW, Lima EO, Oliveira VT, Pereira FO, Souza EL *et al*. Sensitivity of fungi isolated from onychomycosis to *Eugenia cariophyllata* essential oil and eugenol. *Fitoterapia* 2005;76:247–249.
24. Pinto E, Vale-Silva L, Cavaleiro C, Salgueiro L. Antifungal activity of the clove essential oil from *Syzygium aromaticum* on *Candida*, *Aspergillus* and dermatophyte species. *J Med Microbiol* 2009;58:1454–1462.
25. Rana IS, Rana AS, Rajak RC. Evaluation of antifungal activity in essential oil of the *Syzygium aromaticum* (L.) by extraction, purification and analysis of its main component eugenol. *Braz J Microbiol* 2011;42:1269–1277.
26. Fernández-Torres B, Cabañes FJ, Carrillo-Muñoz AJ, Esteban A, Inza I *et al*. Collaborative evaluation of optimal antifungal susceptibility testing conditions for dermatophytes. *J Clin Microbiol* 2002;40:3999–4003.
27. Binh LT, Beaufay C, Trong ND *et al*. *In vitro* anti-leishmanial activity of essential oils extracted from Vietnamese plants. *Molecules* 2017;22:1071.
28. Prashar A, Locke IC, Evans CS. Cytotoxicity of clove (*Syzygium aromaticum*) oil and its major components to human skin cells. *Cell Prolif* 2006;39:241–248.
29. Yoo CB, Han KT, Cho KS, Ha J, Park HJ *et al*. Eugenol isolated from the essential oil of *Eugenia caryophyllata* induces a reactive oxygen species-mediated apoptosis in HL-60 human promyelocytic leukemia cells. *Cancer Lett* 2005;225:41–52.