Antifungal potential and chemical composition of *Tagetes lunulata* Ort. essential oil for the control of *Trichophyton rubrum* Malmsten

Rodríguez-Juárez, Mitzy I.[1]; Arjona-Suárez, Enrique[2†]; Cadena-Íñiguez, Jorge[3]; Guevara-Olivar, Brenda Karina[4]; Ruiz-Posadas, Lucero del Mar[1*]

[1] Colegio de Postgraduados Postgrado en Botánica, Campus Montecillo.
[2] Colegio de Postgraduados, Campus Montecillo Postgrado en Estadística, km 36.5 carretera México-Texcoco, Montecillo, Texcoco, Estado de México, C.P. 56264.
[3] Colegio de Postgraduados, Campus San Luis, Iturbide No. 73, Salinas de Hidalgo, San Luis Potosí, C.P. 78600.
[4] Facultad de Estudios Superiores Aragón, Área de Ecología, Avenida Universidad Nacional s/n, Bosques de Aragón, Nezahualcóyotl, México, C.P. 57171
* Correspondence: lucpo@colpos.mx

ABSTRACT
The essential oils of aromatic and medicinal plants are an important resource used to control several health conditions; however, information about their composition and antimicrobial activity is scarce. This study used a gas chromatography-mass spectrometry (GC-MS) to analyze the composition of the essential oil (EO) of *Tagetes lunulata* Ort., a Mexican endemic plant, known as wild *cempaxúchitl*. The major components of the EO include: verbenone (47.17%), α-pinene (10.93%), 1,1,1-Trifluoro-2-hexanone (9.63%), β-caryophyllene (6.10%), germacrene-D (4.99%), L-verbenone (4.89%), and E-tagetone (4.44%). The disk agar diffusion method was used to evaluate the antimicrobial activity of *T. lunulata* against *Trichophyton rubrum* (athlete's foot). A significant antimicrobial activity was observed with a ≥60% EO concentration. The dilution method was used to determine the minimum inhibitory concentration (MIC): 200 μg ml^{-1}. The *T. lunulata* EO recorded a strong antimicrobial activity against *T. rubrum*; therefore, it is a natural alternative for the control of natural antifungals.

Keywords: Essential oil, *Tagetes*, antimicrobial activity, athlete's foot.

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INTRODUCTION

The use of traditional medicinal and aromatic plants (MAPs) and the isolation of their phytochemical components have allowed the discovery of drugs aimed to control several diseases (Ojah, 2020). Consequently, they are an excellent option to treat some infectious diseases and to control resistant strains (Chouhan *et al.*, 2017). Several studies have proven the antimicrobial efficiency of essential oils (EO) extracted from MAPs against fungi, bacteria, and viruses (Swamy *et al.*, 2016).

Mexico has 23,314 native plant species, 49.8% of which are endemic plants. The Asteraceae family has the highest species diversity (Villaseñor, 2016). Mexico is the center of diversity of the *Tagetes* species, which has shown a biological activity against several

organisms (Barajas-Pérez *et al.*, 2011). However, its chemical composition and its use as a potential antifungal are not fully understood. In addition, information about its effects against dermatophytes that impact human health is scarce. The *Tagetes lunulata* Ort. species belongs to the Asteraceae family and it is endemic to Mexico. It is an annual, wild, and aromatic plant, with bright yellow or orange flowers and a red marking in its base. *Tagetes lunulata* Ort. is commonly known as wild *cempaxúchitl*, *clamol*, *flor de muerto*, or *cinco llagas* and it is found from northern Mexico to Central America, particularly in the central-south region and some states of northern Mexico (Serrato-Cruz, 2009). It is associated with shrubs, pastures, or *Quercus-Juniperus* woodlands (Rzedowski and Rzedowski, 2005), mainly in ruderal vegetation or disturbed fields located at 2,250 and 3,000 m.a.s.l. Serrato-Cruz (2004) has proved its antimicrobial activity against phytopathogen fungi and bacteria, applying aqueous extracts.

The *Trichophyton rubrum* fungus is the most common dermatophyte and it is the causative agent of tinea corporis, tinea pedis (athlete's foot), and onychomycosis. It causes 60% of superficial infections (Graser *et al.*, 2000; Wang *et al.*, 2006). During the last few years, an increase in global infections has been recorded (Arenas, 2002; Hernández-Salazar *et al.*, 2007), as a result of fungi resistance to antifungal medication; in addition, there has been a relapse in the number of cases (Méndez-Tovar *et al.*, 2007). Consequently, seeking new control alternatives to guarantee the elimination of the pathogenic agent and a decrease of the side effects (such as hepatoxicity caused by several drugs) is fundamental. One of these alternative treatments is the use of photodynamic therapy: a combination of photosensitizing agent, an appropriate light wavelength, and molecular oxygen. Although this treatment has been successfully used against several pathogens (Smijs and Pavel, 2011), it is only applied in specialized centers and is therefore unavailable for the general population. Consequently, affordable control alternatives without side effects are required.

Therefore, the objective of this research was to determine the chemical characterization of the essential oil extracted from the flowers of wild *cempaxúchitl* (*Tagetes lunulata*) and to evaluate its antimicrobial activity against *Trichophyton rubrum*.

MATERIALS AND METHODS

EO extraction

The collection of *Tagetes lunulata* was carried out in October, during its flowering stage. The plants were found in agricultural areas, located within the Teuhitli volcano (19° 14' 03.1" N and 99° 01' 02.41" W, at 2,500 m.a.s.l.), in Milpa Alta, Mexico City. The plant material was place in cotton fabric bags and transported to the biological assays with medicinal plants lab of the Colegio de Postgraduados, where they were put on newspaper sheets, in order to divide the inflorescence from the stems and leaves. The botanical identification was carried out at the herbarium-hortorium of the Postgrado en Botánica of the Colegio de Postgraduados. Steam hydro-distillation was used to extract the essential oil from the fresh flowers. A semi-industrial stainless-steel distiller, with a 5 kg capacity, was used to process the plant material for 3 h at 80 °C. The output of the essential oil was determined following the method proposed by Quert *et al.* (2001).

Chemical composition of the cempaxúchitl EO

The chemical composition of the *cempaxúchitl* EO was analyzed through a gas chromatography-mass spectrometry (GC-MS), using a LECO Pegasus® BT 4D (St. Joseph, MI, USA), with a time-of-flight mass spectrometer coupled to an Angilet 6890N network gas chromatograph (Shanghai, China). A 10 m×0.18 mm×0.18 μm HP-5ms (DB5) capillary GC column (phase) (Shanghai, China) was used. Helium was the carrier gas; it had a flux speed of 1 ml min^{-1}. The sample was diluted in methylene chloride. An Agilent 7683B automatic liquid sampler (Wilmington, DE, USA) was used to inject 1 μl of the sample. The mass analyzer was the time-of-flight. Perfluorotributylamine (PFTBA) was used as calibration standard. The C_8, C_9, C_{10}, C_{12}, C_{14}, C_{16}, C_{18}, C_{20}, C_{22}, and C_{24} lineal saturated hydrocarbons were used as standards of Kovats retention indexes.

Inoculum preparation

The Mycology Laboratory of the Facultad de Medicina of the Universidad Nacional Autónoma de México (UNAM) provided the *Trichophytum rubrum* dermatophyte. Subsequently, it was cultured in a Sabouraud dextrose agar growing medium and distributed applying the striated technique with an inoculation loop, at 32 °C for 15 d until sporulation. Afterwards, 1 ml of distilled and sterile water was poured into the Petri dish containing the fungus. An inoculation loop was used to scrap the sample. The suspension was then collected using a micropipette and was adjusted with a spectrophotometer, at 0.5 in the McFarland scale, in a saline solution (1×10^6 UFC ml^{-1}).

Antimicrobial activity evaluation

The completely randomized design was made up of 10 treatments (10-100% EO dilutions) and two control treatments (1% terbinafine and distilled water). Each treatment had six repetitions and each repetition was a Petri dish. Dimethyl sulfoxide (DMSO, Sigma Aldrich) was used to dilute the EO. The statistical analysis consisted of an analysis of variance ($\alpha=0.05$ significance level); the SAS statistical package (SAS®, 2013) was used for this purpose. The comparison of means was determined with a Tukey's test.

The antimicrobial activity was evaluated using the disk agar diffusion method, according to modifications made to the method proposed by Khadka (2017) for filamentous fungi. A 6 mm wide filter paper disk was saturated with 10 μL of each treatment. It was then placed in the center of a Petri dish with a Sabouraud medium, which had been previously inoculated with 100 μL of the fungi suspension, adjusted to 0.5 in the McFarland scale, at 32 °C for 15 d. The diameter of the inhibition halo was measured using the ImageJ2 analysis software (Rueden *et al.*, 2017), based on scanning images of the Petri dishes that were calibrated with a scale graduated in millimeters.

Minimum inhibitory concentration (MIC)

MIC was determined using the dilution method, based on the CLSI standard for filamentous fungi (Cantón-Lacasa *et al.*, 2007). Sterile test tubes (11×70 mm) with screw caps and 1 ml of culture medium were used. Different concentrations of EO diluted with DMSO (Sigma Aldrich) were added to the test tubes. Control treatments consisted

of the inoculating medium, terbinafine, and 1% of DMSO. Each test tube contained 9 ml of growing medium, inoculated with 5×10^3 UFC mL^{-1}. One-hundred μL of the EO concentration solutions under evaluation (1600-3.12 μg mL^{-1}) were poured into the said test tubes. Afterwards, the test tubes were incubated at 37 °C for 48 h. Subsequently, a 100 μL aliquot from each tube was taken and read with a spectrophotometer at 530 nm. The MIC was the lowest EO concentration that inhibited fungal growth.

RESULTS AND DISCUSSION

The output of *Tagetes lunulata* EO was 0.11% higher than fresh weight. This result is 10 times higher than the findings of Zarate-Escobedo *et al.* (2018), who reported 0.008-0.01% fresh weight for the *T. lucida* populations. Consequently, this research obtained a good output, considering that it involved a wild species, to which it would provide an added value.

The GC-MS analysis identified 15 chemical components (Table 1), mainly: verbenone (47.17%), α-pinene (10.93%), 1,1,1-Trifluoro-2-hexanone (9.63%), β-caryophyllene (6.10%), germacrene-D (4.99%), L-verbenone (4.89%), and E-tagetone (4.44%). The main chemical component of the *T. lunulata* EO is monoterpene verbenone, which is also the main chemical component (22% concentration) of *T. lacera* (Díaz-Cedillo *et al.*, 2012). Several studies about this terpene recorded antimicrobial activity against gram-positive and gram-negative bacteria, as well as fungi and yeasts (Santoyo *et al.*, 2005; Scollard *et al.*, 2016; Petrovic *et al.*, 2022). Consequently, the recorded verbenone concentration would seem to be the chemical component with the biological properties needed for antifungal activities.

Table 1. Chemical components of the essential oil of the flowers of *Tagetes lunulata* Ort. identified by the GC-MS.

Chemical compound	Retention time (s)	Kovats Retention Index	Relative Peak Area (%)
Verbenone	489.7	1239.4	47.17
α-Pinene	385.9	1035.2	10.93
1,1,1-Trifluoro-2-hexanone	394.4	1051.3	9.63
β-Caryophyllene	568.3	1425	6.10
Germacrene D	591.7	1487.2	4.99
L-Verbenone	484.5	1227.2	4.89
E-Tagetone	447.3	1151.7	4.44
Binapacryl	818.1	2199.9	1.82
allo-Ocimene	434.8	1127.9	1.56
Isopiperitonone	503.4	1271.1	1.54
α-Phellandrene	365.6	997.48	1.54
Phytol	709.2	1826.9	1.52
Cyclobutane, 1,2-bis(1-methylethenyl)-, trans-	380.6	1025	1.47
3,3-Dimethylacryloyl chloride	427	1113.2	1.25
6-Methyl-6-hepten-4-yn-3-ol	442.4	1142.4	1.15

Additionally, the α-pinene monoterpene has shown a strong antimicrobial action against fungi (Rivas da Silva *et al.* 2012). Verbenone is generated by an auto-oxidation process of α-pinene (Lajunen and Koskinen, 1994), which could explain the high concentration of both components in the *T. lunulata* EO. The main components found in *T. lucida* were sesquiterpene germacrene D and β-caryophyllene (Zárate-Escobedo *et al.* 2018), while E-tagetone and allo-ocimene were found in other *Tagetes* species (Muthee *et al.*, 2016; Álvarez *et al.*, 2016; Lizárraga *et al.*, 2017).

Regarding antimicrobial activity, high concentrations (90% and 100%) of the *T. lunulata* EO recorded a total growth inhibition of *T. rubrum*. The 1% terbinafine antifungal control has a similar effect; terbinafine is the conventional drug used to control *T. rubrum*. However, an important antifungal activity was detected with a ≥60% concentration, when >4 cm *in vitro* inhibition diameters were recorded (Figure 1). According to the Duraffourd *et al.* (1986) scale, this result falls within the very sensitive category regarding the antifungal agent. Based on the analysis of variance and the Tukey's mean comparison test (α=0.05), significant differences were recorded between the treatments 60, 70, 80, 90, 100, and control (1% terbinafine). Some of the treatments recorded a zero-standard error, given the total inhibition caused by the antifungal agent. Meanwhile, a total growth within the Petri dish was recorded in the distilled water treatment.

The MIC concentration of *T. lunulata* EO was 200 μg mL^{-1}, the lowest concentration at which the *T. rubrum* dermatophyte did not record any growth. This concentration was lower than the one reported by Lima *et al.* (2009), who recorded 500 μg ml^{-1} for the *T. mendocina* EO used against *T. rubrum*. This result could also be consequence of a high verbenone concentration in the EO. Several studies have proven that the antifungal action mode of essential oils is a result of their capacity to penetrate and break cell walls and cytoplasmatic membranes, which leads to the disintegration of the mitochondrial membranes (Swamy *et al.*, 2016). Consequently, the *Tagetes lunulata* EO has antifungal activity because it breaks the three-layered cell wall of *T. rubrum*, which is made up of

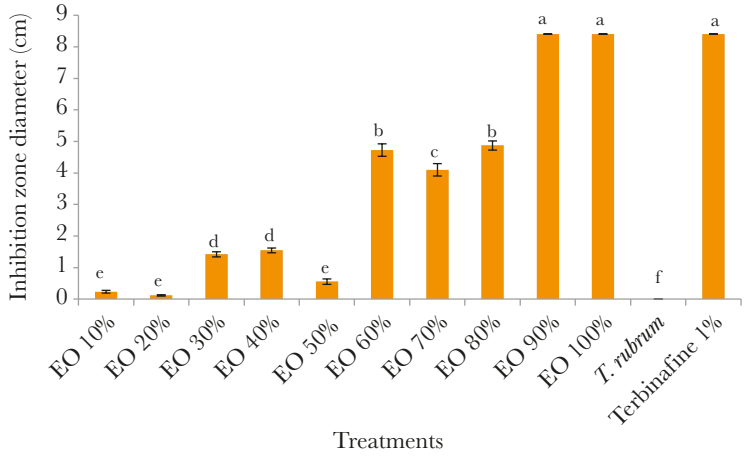

Figure 1. Average diameter of the inhibition halo of *T. rubrum*, recorded at 15 d of exposure to the *T. lunulata* essential oil. Different letters are statistically different (p>0.0001, α=0.05, Tukey). The vertical bars show the ± ES (SE).

β-glucan, galactomannan, and chitin. Additionally, its cell membrane contains ergosterol. New antifungal control alternatives should be focused on the destruction of growing cells and the conidia, which are responsible for the spreading of fungi. Consequently, the antifungal treatment will be shorter and more successful, while the relapse of the infection will be null (Smijs and Pavel, 2011).

CONCLUSIONS

The results suggest that the *T. lunulata* EO is an efficient natural antifungal against *T. rubrum*, proving that it can be used as an alternative to conventional antifungals. Verbenone —the main phytochemical component that provides the EO with its antifungal properties— is the main component of the essential oil extracted from the flowers of *T. lunulata*; therefore, further studies about its capacity to control other type of microorganisms, such as viruses and bacteria, should be carried out. The combination of the main components of *T. lunulata* (verbenone, α-pinene, 1,1,1-Trifluoro-2-hexanone, β-caryophyllene, germacrene-D, L-verbenone, and E-tagetone, which have proven to have antifungal activity) provide the resulting EO with outstanding antimicrobial properties against *T. rubrum*. Further clinical evaluations should be carried out to determine its behavior and its application in the human health sector. In addition, organs of the plant should be studied to determine the chemical composition of their EO. Furthermore, the constitution of the EO extracted from plants from other areas must be established, particularly to determine verbenone concentration, which plays a key role in their antifungal activity.

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