

Chemical compounds of *Tagetes lucida* essential oil and effects against *Botrytis cinerea*

Miguel Ángel Ruíz-González¹

Roney Solano-Vidal¹

Ernestina Valadez-Moctezuma¹

Miguel Ángel Serrato-Cruz^{1,§}

1 Universidad Autónoma Chapingo. Carretera México-Texcoco km 38.5, Chapingo, Estado de México, México. CP. 56230. (miguelruiz93@live.com.mx; roneysv@hotmail.com; nestty56@yahoo.com.mx).

Autor para correspondencia: serratocruz@gmail.com.

Abstract

The environment influences the chemical composition of plant essential oil and its biological effect. The purpose of the study was to describe the chemical profile of *Tagetes lucida* essential oil and to evaluate the *in vitro* biological effect against *B. cinerea*. The study was conducted in Texcoco, Mexico in October 2021. Essential oil was obtained from flowering plants by hydrodistillation; the identification of chemical compounds was carried out using the GC-MS technique. The *in vitro* bioassay employed the method of poisoned agar and mycelium of *B. cinerea* of three days of growth. Twelve treatments were evaluated: essential oil 0.1, 0.5, 1, and 2%, Tween 20 at 0.1, 0.3, 0.5, 0.8, 1 and 2%, commercial fungicide, and absolute control (only with sterile double-distilled water). Every 24 h, radial growth of the fungus was measured with a digital vernier and growth rate and inhibition of mycelial growth were estimated. Thirty-one chemical compounds were identified, (1S)-(-)- β -Pinene (36.4%), 1, 3, 5, 7-Cyclooctatetraene (12.7%), eucalyptol (10.6%), and o-Cymene (6.1%). The concentrations of 0.1, 0.5, 1 and 2% inhibited the mycelial growth and sporulation of *B. cinerea*. The commercial fungicide and the 2% concentration totally inhibited the growth of the fungus. Tween 20 also inhibited mycelial growth. The LC₅₀ was 0.06% and the LC₉₅ was 1.69%. The abundance of terpenes in the essential oil of *T. lucida* showed a fungicidal effect against *B. cinerea*. The surfactant had minor effects.

Keywords:

Tagetes lucida, *B. cinerea*, essential oil.



Introduction

The Yiahtli, pericón, or Santa Maria herb (*Tagetes lucida* Cav.) is a plant distributed in temperate and transition zones, at altitudes of 1 400 to 2 800 m (Serrato, 2014), grows in pine, oak, pine-oak, and broadleaf forest vegetation (Turner, 1996). Its height is from 40 cm to 1.8 m and its flowering begins in mid-August until October (Serrato, 2014).

Current traditional knowledge about *T. lucida* reveals various uses: as a relaxant in cancer situations, cleaning of C-section wounds, remedy against scorpion and snake bites, to reduce rheumatic pain, control ticks in birds, soften the effect of hangovers, scent washed clothes, eliminate lice and flea eggs, make cane alcohol with anise flavor, and flavoring of various foods (Serrato, 2014). It has a biocidal effect against pathogens that affect humans (Céspedes *et al.*, 2006; Torres-Martínez *et al.*, 2022) and other biological effects against nematodes (Omer *et al.*, 2015) and some phytopathogenic fungi (López *et al.*, 2018).

All these effects are attributed to the chemical compounds contained in *T. lucida*, such as terpenoids, coumarins and flavonoids (Gutiérrez *et al.*, 2018). Around 40 to 44 secondary metabolites of the class of monoterpenes, sesquiterpenes, and phenylpropanoids have been detected in the essential oil (Regalado *et al.*, 2011; Zárate-Escobedo *et al.*, 2018), which means a wide variability in types and quantity of molecules. In *T. lucida* essential oil, the abundance of monoterpenes and phenylpropanoids is related to climate and soil variability (Zárate-Escobedo *et al.*, 2018).

Studies conducted with *T. lucida* essential oil against fungi used plant populations from cold temperate and semi-cold climates (Céspedes *et al.*, 2006; Barajas *et al.*, 2011; López *et al.*, 2018), in which phenylpropanoids (anethole and estragole) are the main components and are responsible for biological effects against fungi.

T. lucida oil that has this origin inhibits mycelial growth, sporulation, and sclerotia formation in *Sclerotium rolfsii* and *Monilinia fructicola* (Barajas *et al.*, 2011). It also inhibits the mycelial growth of *Aspergillus niger*, *Fusarium oxysporum*, *Penicillium janthinellum*, and *Rhizoctonia solani* (López *et al.*, 2018). In hot weather, the secondary metabolites in the essential oil correspond mostly to terpenes (Zárate-Escobedo *et al.*, 2018). Therefore, the evaluation of the biological effect of the essential oil of this species grown in warm temperate climates is important.

The variation in the chemical composition of essential oils depends on various factors, including genetic and environmental factors (Acero-Godoy *et al.*, 2019). Therefore, toxicological studies with *T. lucida* oil should consider the characteristics of the habitat in which populations of this species develop naturally.

The evaluation of *T. lucida* terpenes in fungal growth will complement the available information on the fungal effects of phenylpropanoids contained in the same species grown in temperate environments of cold subtypes. The distribution of natural populations of *T. lucida* in altitudinal levels (Serrato, 2014), associated with contrasting temperatures, represents a rich environmental diversity for *T. lucida* and also of biomolecules.

Although several essential oils have been evaluated against *B. cinerea* (Tan#inová *et al.*, 2022), *T. lucida* essential oil has not yet been evaluated against this fungus. This work aimed to describe the chemical profile of *T. lucida* essential oil and to evaluate the *in vitro* biological effect against *B. cinerea*. It is expected that the composition of the essential oil is specific and that this oil has a biological effect against the aforementioned phytopathogenic fungus.

Materials and methods

Biological material

In October 2021, samples of *Tagetes lucida* Cav. in flowering stage (stems, flowers, and leaves) were collected in the municipality of Pilcaya, Cacahuamilpa, Guerrero (18° 40' 42.6" north latitude,

99° 32' 31.3" west longitude) at 1 479 m altitude and in a temperate climate with rainfall in summer (Cw₁) (Köppen, 1948). The flowering stems were cut 10 cm above the ground to avoid the loss of plants in their habitat since this is perennial and sprouts again. Specimens of these samples were deposited in the Jorge Espinosa Salas JES Herbarium-Hortorio of the Department of Agricultural High School of the Chapingo Autonomous University, State of Mexico (record 35872).

Essential oil extraction

Two liters of drinking water were added to an Italian-type glass distiller, with a capacity of 6 kg; then, 2 kg of fresh plants previously crushed with pruning shears was placed in the balloon flask; essential oil was extracted by hydrodistillation for 45 min (Rodríguez *et al.*, 2012). The essential oil was preserved in amber glass bottles in refrigeration until use; 1.6 ml of essential oil per kilogram of dry weight of plant tissue was obtained.

Identification of chemical compounds

The essential oil samples were analyzed with the solid-phase microextraction technique (Zhao *et al.*, 2022); to do this, 5 µl of essential oil was added to an amber bottle and a solid-phase microextraction fiber (PALsystem Ingenious Simple Handling) was placed for 1 min; after that, the sample was injected into a gas chromatograph coupled to a mass spectrometer (GC-MS Agilent Technologies 7890 A GC System); two replications were performed and a VF-5ms GC column 30 × 0.25 (0.25) was used.

The temperature of the column oven started at 60 °C, then at 120 °C for 15 min, and a final temperature of 230 °C. The temperature increase was 15 °C min⁻¹ and helium were used as a carrier gas at a constant flow of 0.5 ml min⁻¹. The compounds were identified by comparing the retention time and the mass spectrum with the NIST spectral library and corroborated with Kovats and R match indices greater than 800. N-alkanes were used as references in the calculation of the Kovats indices.

In vitro bioassay

The evaluation was made in potato dextrose agar culture medium (PDA 39 g L⁻¹) with the poisoned agar method (Dikshit and Husain, 1984); an emulsion was prepared with 100 ml of double-distilled water, 100 µl of Tween 20, and essential oil of *T. lucida* to obtain four concentrations (0.1, 0.5, 1, and 2% v/v). Separately, six concentrations were prepared with Tween 20 (0.1, 0.3, 0.5, 0.8, 1, and 2% v/v), and a commercial fungicide was used: (1 g L⁻¹): Cabrio C (Boscalid 25.2% + Pyraclostrobin 12.8%).

Sterilization was carried out in an autoclave at 120 °C for 20 minutes. The PDA medium was then poured into sterile 90 mm glass Petri dishes. After 24 h, *B. cinerea*, obtained from the Genetic Resistance Laboratory of the Universidad Autónoma Chapingo (UACH), for its acronym in Spanish, was inoculated. The strain of *B. cinerea* used has molecular identification with NL4 and ITS5HP indicators (Toju *et al.*, 2012) and GenBank registration PP401673.1.

Experimental design and data analysis

The experiment was established with a completely randomized design (CRD) with 12 treatments: four concentrations of essential oil, six concentrations of Tween 20, 1 g L⁻¹ of Cabrio C fungicide (Boscalid 25.2% + Pyraclostrobin 12.8%) and one control, each treatment with five replications. A digital vernier was used to measure the radial growth of the fungus every 24 h for five days. Growth rate (GR) (Sinclair and Cantero, 1989) and mycelial growth inhibition (%I) (Kagezi *et al.*, 2015) were evaluated.

A stereo microscope was utilized to observe if there was sporulation from the fifth to the eighth day after the fungus was seeded. A nominal scale was employed. Data were analyzed with Tukey mean tests ($p \leq 0.05$) through the SAS academic software (SAS Institute Inc, 2023). The results of the essential oil concentrations evaluated in vitro were used to determine the Probit concentrations (LC50 and LC95) in the SAS academic software (Finney, 1971; Castillo, 2007; SAS Institute Inc, 2023).

Results and discussion

Chemical compounds

Thirty-one compounds were identified in the essential oil, with four of them being the major ones: (1S)-(-)- β -pinene (36.4%), 1, 3, 5, 7-cyclooctatetraene (12.7%), eucalyptol (10.6%), and o-cymene (6.1%) (Table 1), of all the compounds, sesquiterpenes represented 40%, monoterpenes 30%, other terpenes 3%, and the rest corresponded to less abundant alcohols. Compounds such as estragole, anethole, methyl eugenol (Bicchi *et al.*, 1997), β -ocimene, β -cubebene, β -myrcene, germacrene, caryophyllene, nerolidol (Regalado *et al.*, 2011) and geranyl acetate (Zárate-Escobedo *et al.*, 2018) have been reported as major compounds in *T. lucida*, which means that the secondary metabolites that were the majority in the Pilcaya population (Table 1), other than those reported previously in other studies (Table 2), expand the knowledge of the phytochemistry of this species.

Table 1. Relative abundance (%) of chemical compounds in essential oil of *Tagetes lucida*, using gas chromatography/mass spectrometry (GC/MS) and the Kovats index (KI).

Compound	(%)	KI	Compound	(%)	KI
(1S)-(-)- β -Pinene	36.4	961	2-Furanmethanol, 5-ethenyltetrahydro- $\alpha,\alpha,5$ -trimethyl-, cis-	0.4	1070
1,3,5,7-cyclooctatetraene	12.7	880	5-Hepten-2-one, 6-methyl-	0.4	958
Eucalyptol	10.6	1032	Acetic acid, hexyl ester	0.4	1010
o-Cymene	6.1	1024	δ -Elemene	0.4	1361
(-)- β -Bourbonene	2.4	1390	Cyclosativene	0.4	1379
Cubenol	2.1	1651	Cyclotrisiloxane, hexamethyl-	0.4	825
Cyclohexene, 1-methyl-4-(1-methylethenyl)-(S)-	1.7	1029	2-furancarboxaldehyde, 5-methyl-	0.2	913
4-Hexen-1-ol, (4E)-, acetate	1.6	1003	α -Amorphene	0.1	1453
trans- β -Ocimene	1.5	1044	α -Murolene	0.1	1504
β -acorenol	1.3	1598	1,3,5-cycloheptatriene	0.1	771
(1S)-2,6,6-Trimethylbicyclo[3.1.1]hept-2-ene	1.2	931	3-Penten-2-one, 4-methyl-	0.1	778
Dihydroedulan II (cis)	1.1	1193	Trans-calamenene	0.1	1537
N-amyl isovalerate	0.8	1103	Gleenol	0.1	1630
Caryophyllene oxide	0.7	1598	γ -Murolene	0.09	1522
Cyclotetrasiloxane, octamethyl-	0.7	1004	Aromandendrene	0.05	1439
2-Butenal, 3-methyl-	0.5	748			

Table 2. Major chemical compounds found in the essential oil of *T. lucida* evaluated by Zárate-Escobedo *et al.*(2018).

Compound	Relative abundance (%)
β -Ocimene	24
Geranyl acetate	19.2
Nerolidol	12.5
β -Cubebene	7.3
Caryophyllene	6.7
1,3,6,10-Dodecatetraene,3,7,11-trimethyl-, (E,E)-	4.3
Verbenone, (L)-	3.5
Caryophyllene oxide	2.6
β -pinene-(1S)-(-)	1.1

When comparing the compounds found in the study population (Table 1), called Pilcaya (1 479 masl), with those found by Zárate-Escobedo *et al.* (2018) in the locality called El Mogote-Pilcaya (1 493 masl), both points nearby, the main compounds in the essential oil of both populations do not coincide (Table 2). The compound (-)- β -pinene, which had 36.4% abundance in Pilcaya (Table 1), had an abundance of 1.1% in El Mogote-Pilcaya (Table 2) (Zárate-Escobedo *et al.*, 2018).

The Pilcaya site is warmer, possibly by 2 °C, and at a lower altitude (1 479 m) compared to the El Mogote-Pilcaya site. In other species, temperature, water regime and other factors have been recorded as influencing the synthesis of secondary metabolites (Karalija *et al.*, 2022; Valkovszki *et al.*, 2023). The result is valuable for selecting areas for collection and possible cultivation due to the importance that changes in temperature and altitude can have on the type and quantity of chemical compounds.

***In vitro* bioassay**

Regarding the biocidal effect of the essential oil, it was found that, with 1 and 2%, the mycelial growth of *B. cinerea* was inhibited, with statistical differences compared to the concentration of 0.1% (Table 3), and there was no sporulation of the fungus. Therefore, the concentration of 1% is adequate to verify whether the effect observed at the *in vitro* level could also be reproduced at the *in vivo* level with due care not to entail phytotoxicity effects.

Table 3. Mean values of the variables of percentage of inhibition and growth rate.

Treatments	Inhibition (%)	Growth rate (mm)
Absolute control	0 e	10.54 a
CF	100 a	0 e
TL2	100 a	0 e
TL1	94 ab	2.83 d
TL0.5	72.46 bc	4.9 cd
TL0.1	66.15 c	6.33 bc
Tween 20 (2%)	24.02 d	6.51 bc
Tween 20 (1%)	21.65 de	6.58 bc
Tween 20 (0.8%)	15.14 de	6.76 bc
Tween 20 (0.5%)	14.74 de	6.86 bc
Tween 20 (0.3%)	12.68 de	7.2 bc
Tween 20 (0.1%)	5.15 de	8.03 ab
CV	18.28	20.4
LSD	23.72	2.69

Means with equal letters are not statistically different (Tukey, 0.05), CV= coefficient of variation; LSD= least significant difference; *= significance level ($p \leq 0.05$); TL2= essential oil 2%; TL1= essential oil 1%; TL0.5= essential oil 0.5%; TL0.1= essential oil 0.1%; CF= commercial fungicide Cabrio C.

T. lucida essential oil also inhibits *Penicillium notatum*, *Fusarium moniliforme*, *Fusarium sporotrichum*, *Trichophyton mentagrophytes* (Céspedes *et al.*, 2006), *Sclerotium rolfsii* and *Monilinia fructicola* (Barajas *et al.*, 2011), *Aspergillus niger*, *Fusarium oxysporum*, *Penicillium janthinellum* and *Rhizoctonia solani* (López *et al.*, 2018). This work presents results against *B. cinerea* for the first time (Table 3). The biological effect of 1% *T. lucida* oil is highlighted since, statistically, it equals the commercial fungicide and this opens up technical-economic possibilities to analyze a possible use of the aforementioned natural resource.

The inhibitory effect caused by the essential oil against fungi is because they cause damage to the cytoplasmic membrane and interrupt several layers of polysaccharides, fatty acids, and phospholipids (Helal *et al.*, 2006; Rammanee and Hongpattarakere, 2011). They also damage hyphae, vacuolation, protoplast leakage or mitochondrial destruction (Rasooli *et al.*, 2006). Soylu *et al.* (2010) reveal morphological alterations in the hyphae of *B. cinerea* caused by the application of essential oils of *Origanum syriacum*, *Lavandula stoechas*, and *Rosmarinus officinalis*.

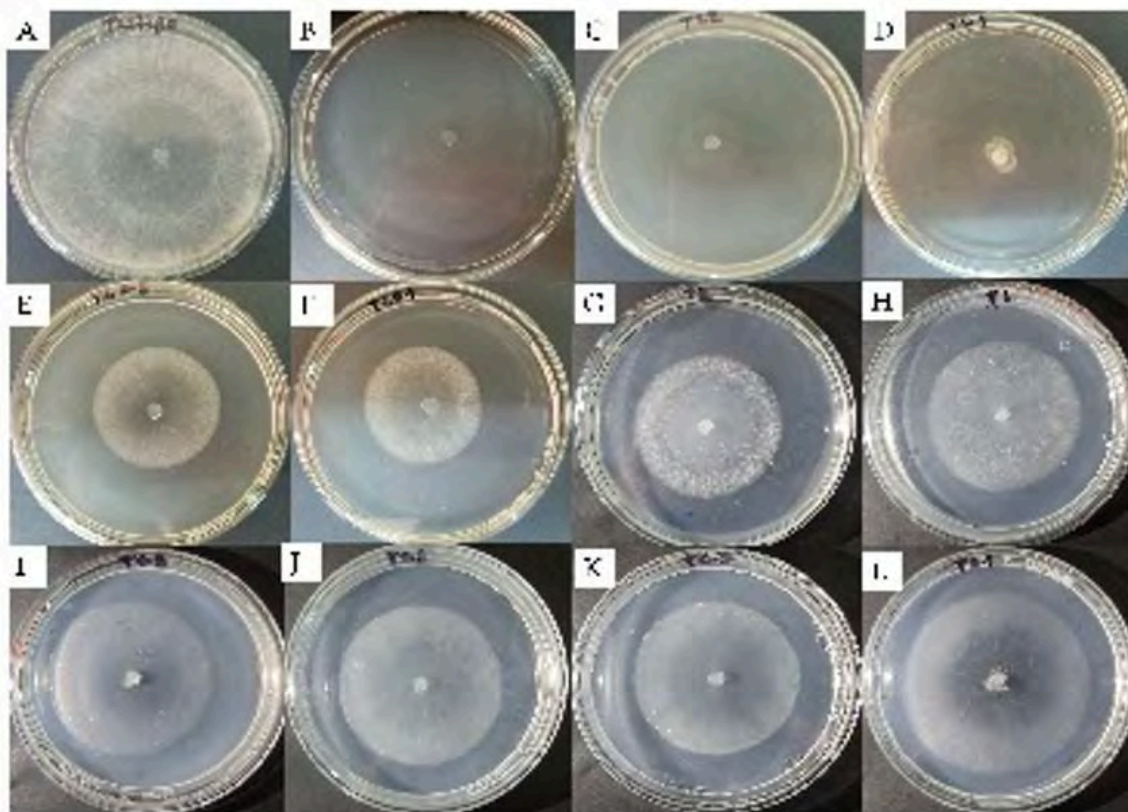
Treatment with Tween 20 (2%) showed inhibition of 24.02% (Figure 1) with a mycelium growth rate of 6.51 mm d⁻¹, whereas with the lowest concentration (0.1%), the inhibition was 5.15% and the growth rate increased to 8.03 mm d⁻¹ (Table 3); the concentrations of 0.1, 0.5, 0.8, and 1% of the surfactant that were tested were not statistically different from the control.

Apparently, the effect of Tween is antifungal-dependent and could be related to the solubility of the antifungal in the medium used; therefore, it is important to standardize the concentration of surfactant for use in the preparation of the inoculum or when making the emulsion in order to ensure reliable results (Gómez-López *et al.*, 2005), since it has been observed that Tween 20 (0.1%) inhibits the germination of *Beauveria bassiana* conidia by 20% (Mwamburi *et al.*, 2015). In the present experiment, the use of Tween 20 at 0.1% had virtually no inhibitory effect. However, higher concentrations (>0.1%) did inhibit *B. cinerea*.

In treatments with essential oil and commercial fungicide, there was no sporulation of *B. cinerea* (Figure 1), whereas in the control and treatment with Tween 20, there was sporulation until the eighth day, which reinforces the biological activity of the *T. lucida* oil that was evaluated.



Figure 1. Mycelial growth of *B. cinerea* after five days in Petri dishes with the treatments. A= control; B= commercial fungicide; C= essential oil 2%; D= essential oil 1%; E= essential oil 0.5%; F= essential oil 0.1%; G= Tween 20 2%; H= Tween 20 1%; I= Tween 20 0.8%; J= Tween 20 0.5%; K= Tween 20 0.3%; L= Tween 20 0.1%.



The *in vitro* application of essential oils of *Thymus vulgaris*, *Origanum vulgare*, *Origanum dictamnus*, and *Origanum majorana* also reduced the production and germination of conidia in *Penicillium digitatum* (Daferera *et al.*, 2000). The antisporulating effect in *Aspergillus fumigatus* was related to respiration-inhibiting activity due to the effect of essential oils (Inouye *et al.*, 1998).

The Probit concentrations of the essential oil of *T. lucida* from Pilcaya were 0.06% (v/v) for LC₅₀ and 1.69% (v/v) for LC₉₅ (Table 4), references that allow us to suggest that *T. lucida* oil is a candidate plant substance for the biocontrol of *B. cinerea*. In perspective, it is contemplated to evaluate *in vivo* the best treatment considering the reference of the Probit analysis and to verify the biological effect of each major compound.

Table 4. Lethal concentrations (LC) and confidence limits obtained with the essential oil of *T. lucida* evaluated in *B. cinerea*.

Essential oil (v/v)	Lethal concentrations	Confidence limits (95%)
<i>T. lucida</i>	LC ₅₀ 0.06	0.02704-0.09868
	LC ₉₅ 1.69	1.07514-3.5546

The natural populations of *T. lucida* in Pilcaya, Cacahuamilpa, Guerrero are abundant and constitute a promising natural resource for their sustainable use at the level of the communities that own these lands, especially if the exploration of biological effects is extended to insect pests, various phytopathogenic fungi, mites, bacteria, and other organisms of importance in human and veterinary medicine, it will yield favorable results.

Of course, it is advisable to assess the financial viability of the oil distillation process in order to have a real picture of the potential for exploiting the plant genetic resource in question. It must be considered that the yield of 1.6 ml is obtained from 1 kg of dry tissue, so to obtain 1 L of oil, 625 kg of dry tissue would be used, which would be approximately equivalent to a volume of 5 t of fresh plant, a scenario that leads us to reflect on possible agroecological management of native populations or take them to conventional cultivation.

Conclusions

T. lucida essential oil contains thirty-one chemical compounds. The major compounds are terpenes and their abundance is associated with the warm temperate climate from which the plant material comes. The essential oil evaluated has an *in vitro* fungicidal effect against *B. cinerea* and was validated with the Probit evaluation. Tween 20 partially inhibits growth, so the amount of surfactant to dissolve the oil must be adjusted.

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