

Antifungal effect of pirul essential oil against phytopathogenic fungi of corn

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Abstract

The present study evaluated the efficacy of pirul essential oil in the control of corn pathogens, including those responsible for significant diseases of this crop. Essential oil was extracted from fresh pirul leaves using the steam distillation method, obtaining a yield of 0.5%. The oil obtained was analyzed using the GC-MS technique, identifying main compounds, such as α -pinene, β -pinene, and D-limonene, known for their antimicrobial properties. Disc diffusion and cell viability assays showed dose-dependent inhibition of mycelial growth and a significant reduction in pathogen viability with increasing essential oil concentration. At a concentration of 1 000 ppm, the relative viability of the pathogens decreased to less than 10%, demonstrating potent antifungal activity. Comparatively, the control with tebuconazole showed a very low relative viability (1.5%), confirming the superiority of pirul essential oil as a natural antifungal agent. These results suggest that pirul essential oil is a promising and sustainable alternative to synthetic fungicides for the control of corn pathogens, contributing to safer and greener agricultural practices.

Keywords:

distillation, fungicide, inhibition.



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Introduction

Currently, corn (*Zea mays*) is one of the most important crops worldwide, fundamental for food security and agricultural development (FAO, 2021). Nonetheless, this crop faces a number of challenges due to the presence of pathogens that affect its growth and productivity. Among these pathogens, nematodes, bacteria, and fungi are the main causes of diseases that significantly reduce crop yields (Hernández *et al.*, 2019).

In Mexico, climatic and edaphic conditions create environments prone to the development of various diseases that attack corn. The fungus *Fusarium* spp. is one of the most prevalent pathogens, causing diseases, such as root, stem and ear rot. These infections decrease yield and contaminate grain with mycotoxins that are dangerous to human and animal health (Kazan and Gardiner, 2018).

The fungus *Aspergillus* spp., particularly *A. flavus*, is known for the production of aflatoxins, which are potent carcinogens and pose a significant risk to dietary health (Amaike and Keller, 2011). *Ustilago maydis*, which causes huitlacoche or corn smut, although it can be consumed as a delicacy in some cultures, is generally considered a harmful disease that decreases yield in corn production (Martínez *et al.*, 2002). *Colletotrichum graminicola*, responsible for corn anthracnose, affects leaves, stems, and cobs, resulting in significant losses in yield (Tsushima and Shirasu, 2022).

Historically, farmers have turned to chemical inputs for pest and disease control. Nevertheless, these products, although effective, have raised concerns about their impact on the environment and human health, and they also contribute to the development of resistance in pathogens (Pimentel and Burgess, 2014). Therefore, it is crucial to look for sustainable alternatives that offer efficient control of pathogens without the adverse effects associated with conventional chemicals (Goulson, 2013).

One of the promising approaches in this area is the use of natural extracts with antimicrobial properties, such as the case of the essential oil of pirul (*Schinus molle*), known for its antimicrobial and fungicidal properties, which has proven to be an effective alternative for the control of various pathogens (Murrieta *et al.*, 2023). Several studies propose that essential oils derived from plants, such as pirul, have fungicidal, bactericidal, and antimicrobial properties, offering a potentially safer and more environmentally friendly solution compared to traditional chemical treatments (Turchetti *et al.*, 2020; Ruiz and Salazar, 2021).

This study aimed to evaluate the efficacy of pirul oil in the control of corn pathogens in order to contribute to the reduction of diseases in the crop, exploring its potential as an alternative to chemical inputs. This approach could not only improve corn health, but also promote more sustainable agricultural practices in the country.

Materials and methods

Plant material

The plant material used consisted of fresh pirul leaves collected from the region of Mineral de la Reforma, Hidalgo, Mexico (coordinates: 20.03948° north latitude, -98.71728° west longitude). The botanical identification of the species was carried out using taxonomic keys and detailed descriptions reported (Olvera *et al.*, 2021). The samples were collected in March 2023 during the period of active growth of the plant to ensure the optimal quality and concentration of the bioactive compounds.

Mature, healthy leaves were selected from the top of the branches to ensure freshness and optimal essential oil content. The leaves had an average size of 6 cm long and 3-7 mm wide and intense green coloration, indicative of their good health and phytochemical content. Collection was carried out using sterile pruning shears to avoid contamination of the samples.

The leaves were placed in paper bags to prevent moisture accumulation and transported to the laboratory under controlled conditions, which included keeping the samples protected from direct sunlight and at a constant room temperature (25 °C), ensuring that no degradation of the bioactive compounds occurred during transport. Surface cleaning was carried out with distilled water to remove any dust residue or external particles.

Subsequently, they were dried in a dark and ventilated environment at room temperature to prevent degradation of the active compounds. Finally, the leaves were crushed into small pieces using a sterile mortar, preparing them for the essential oil extraction process by steam distillation.

Extraction, essential oil yield, and compound identification

The essential oil was extracted using the steam distillation method, employing a standard distillation equipment with a capacity of 6 L, where 1.5 kg of fresh pirul leaves previously disinfected and crushed were used to increase the contact surface and facilitate extraction; this procedure was carried out with four replications.

Steam was applied through the leaves to volatilize the essential compounds for 45 min, to later be condensed and collected in the form of essential oil. The essential oil obtained was separated from the hydrosols and stored in opaque bottles at a temperature of 15 ± 2 °C to preserve its stability and prevent the degradation of the bioactive compounds.

The yield of the essential oil was calculated as the percentage of the weight of the oil obtained compared to the initial weight of the leaves used. Weight data were recorded before and after distillation to determine yield. The oil obtained was analyzed with an Agilent 7890B gas chromatograph, equipped with a DB-5MS high-resolution capillary column (30 m \times 0.25 mm \times 0.25 μ m).

The temperature program applied started at 60 °C and increased to 250 °C at a rate of 5 °C min^{-1} , with a carrier gas (helium) flow of 1 ml min^{-1} . The compounds separated in the column were detected and characterized by mass spectrometry on an Agilent 5977B spectrometer, using electron ionization (EI) at 70 eV. The spectra obtained were compared with the NIST 05 spectrometric database of the GC-MS system (National Institute of Standards and Technology) to identify the compounds present in the essential oil, considering those with more than 5% relative abundance.

Assessment of antimicrobial effects

Disc diffusion

The antimicrobial activity of *S. molle* essential oil was evaluated with the disc diffusion method. Culture dishes were prepared with culture media appropriate for each pathogen. Dishes with nutritive agar were used for *F. verticillioides* and *A. flavus*, whereas for *U. maydis* and *C. graminicola*, dishes with potato-dextrose culture medium were employed. The media were sterilized and cooled to room temperature before being inoculated.

Suspensions of the pathogens were prepared in sterile saline solutions, adjusting the density to 10^6 colony-forming units (CFU) ml^{-1} . These suspensions were distributed on the surface of the plates with culture media using a sterile swab, ensuring homogeneous coverage. Once the plates were inoculated, filter paper discs (6 mm diameter) were prepared and impregnated with 10 μ l of the essential oil concentrations of 50, 100, 250, 500 and 1 000 ppm. The impregnated discs were placed on the surface of the inoculated plates, avoiding direct contact with the medium.

Tebuconazole (25%) was used as a positive control due to its proven efficacy as a broad-spectrum fungicide and its common use as a standard reference to evaluate antifungal activity in agricultural studies. Discs without essential oil were used as a negative control, and four replications of each treatment were performed.

The dishes were incubated at 28 °C for 48 h and after the incubation period, the diameters of the inhibition halos formed around the discs were measured using a precision calibrator and reported in millimeters. The results were recorded and compared with the controls to determine the antimicrobial efficacy of the essential oil at each concentration tested.

Cell viability assays

Culture plates with culture media were prepared for each pathogen under the conditions of suspension, incubation, and essential oil concentrations described above. After the incubation period, the plates were treated with a solution of MTT (2,5-diphenyl tetrazolium) or resazurin at 0.5% in a sterile saline solution. The MTT solution was added to each plate and they were incubated at 37 °C for 2 h.

The controls used were those mentioned above. To quantify the amount of formazan produced, the precipitate was dissolved in 100% dimethyl sulfoxide (DMSO), and absorbance was measured at 570 nm using a microplate reader. The absorbance values obtained were compared with negative controls (without essential oil) and were used to calculate the percentage of cell viability as a function of absorbance.

Statistical analysis

A two-factor analysis of variance (Anova) was performed to assess the effect of essential oil concentration and pathogen type on the diameter of the inhibition halo. The statistical model used was: halo diameter (mm) = μ + concentration + pathogen + pathogen \times concentration + ε . Where: μ is the general mean and ε is the experimental error. Concentration-pathogen interactions were evaluated for combined effects.

Tukey's test was used to make multiple comparisons between the means of the diameters of the inhibition halos of the different concentrations, identifying significant differences ($p \leq 0.05$). Different letters indicated significant differences between the groups. The analysis of the data obtained was carried out using the statistical software Sas version 9.4.

Results and discussion

Essential oil yield

A total of 1.5 kg of dried leaves was used for extraction and the essential oil obtained was weighed to obtain its yield, calculating it with the following formula:

$$\text{yield}(\%) = \left(\frac{\text{Mass of the essential oil (g)}}{\text{Mass of the dried plant material (g)}} \right) \times 100$$

The yield of *S. molle* essential oil was 0.5%, which is consistent with the yields reported in the literature, which usually vary between 0.4% and 0.8% (Rey *et al.*, 2018; Volpini *et al.*, 2021).

Variability in essential oil yield can be attributed to several factors, such as environmental conditions during cultivation, the condition of the plant material at the time of harvest, and specific conditions of extraction. Rey *et al.* (2018) reports a yield of *S. molle* essential oil of 0.75% using steam distillation of the leaves of the plant, mentioning that climatic and edaphic conditions have a significant impact on the quality and yield of the oil.

On the other hand, in a study carried out by Lorenzo Volpini *et al.* (2021), the yield of the essential oil obtained was reported as 0.6%, slightly higher than the result obtained in this study. The yield of essential oil can be affected by several factors, with the quality of the plant material being a crucial factor; fresher and healthier leaves tend to produce higher yields. In addition, the steam distillation technique may have variations in efficiency depending on the equipment configuration and operating parameters (Božović *et al.*, 2017).

Identification of compounds in essential oil

GC-MS analysis revealed that the main compounds in *S. molle* essential oil (Table 1), include α -pinene (5.2 min), β -pinene (6.3 min), and D-limonene (7.8 min), which are known for their

antimicrobial and antioxidant properties. α -Pinene and β -pinene are terpenes that are commonly found in the essential oils of various plants and have applications in the pharmaceutical industry for their anti-inflammatory and antibacterial effects (Guimarães *et al.*, 2019).

Table 1. Compounds identified in the essential oil of *S. molle* from Mineral de la Reforma, Hidalgo, Mex.

Peak	Compound	Retention time (min)	Relative area (%)	Molecular mass (g mol ⁻¹)
1	α -Pinene	5.2	15	136.24
2	β -Pinene	6.3	10.5	136.24
3	D-Limonene	7.8	20.3	136.24
4	Caryophyllene	10.1	12.7	204.34
5	P-Cymene	12.3	8.9	134.22
6	Terpineol	14.5	7.8	154.24
7	Linalool	16.2	14.1	154.25
8	Eugenol	18.6	10.7	164.2

D-Limonene, another major component, has also been associated with antimicrobial properties and is used in cleaning products and cosmetics due to its aroma and ability to eliminate bacteria (Fisher and Phillips, 2008). Caryophyllene, an identified sesquiterpene, has been documented for its anti-inflammatory properties, which could contribute to therapeutic effects (Gertsch *et al.*, 2008). On the other hand, p-cymene, terpineol, and linalool, also present in the oil, are known for their antimicrobial properties and their use in the food and cosmetic industry (Burt, 2004).

The compounds identified in *S. molle* essential oil in this study largely match the profiles reported in the literature. According to the aforementioned authors, *S. molle* essential oils contain high levels of α -pinene and β -pinene, similar to those found in this study. The presence of eugenol, although lower in this study, has also been reported in essential oils of other varieties of *S. molle* and is related to antimicrobial properties (Burt, 2004).

Effect of *S. molle* essential oil in disc diffusion against corn pathogens

The results of the disc diffusion test indicate that *S. molle* essential oil showed dose-dependent antimicrobial activity against all pathogens evaluated. Inhibition halos increased with the concentration of the essential oil, with the largest diameters observed at 1 000 ppm. The analysis showed that the essential oil inhibits the mycelial growth of the corn pathogens evaluated (Table 2). Tukey's test indicated significant differences in the diameters of the inhibition halos for all essential oil concentrations compared to the control.

Table 2. Antifungal effect of *S. molle* essential oil on the mycelial growth of corn pathogens.

Concentration <i>S. molle</i> oil (ppm)	Diameter and inhibition of mycelial growth (mm \pm SD)				
	<i>Fusarium verticillioides</i>	<i>Aspergillus flavus</i>	<i>Ustilago maydis</i>	<i>Colletotrichum graminicola</i>	Oil factor (C)
0 (control)	9 \pm 0.5 a	9.1 \pm 0.3 a	8.9 \pm 0.4 a	9.1 \pm 0.5 a	9 \pm 0.1 A
50	8.5 \pm 0.5 ab	7.2 \pm 0.4 b	6.8 \pm 0.3 b	7.5 \pm 0.4 b	7.5 \pm 0.7 B
100	11.2 \pm 0.6 bc	10 \pm 0.5 c	9.1 \pm 0.4 a	10.4 \pm 0.5 c	10.2 \pm 0.9 C
250	14.8 \pm 0.7 cd	13.5 \pm 0.6 d	12.4 \pm 0.5 c	13.2 \pm 0.6 d	13.5 \pm 1 D
500	18.1 \pm 0.8 de	16.4 \pm 0.7 e	15.6 \pm 0.6 d	16.8 \pm 0.7 e	16.7 \pm 1 E
1 000	21.3 \pm 1 e	19.8 \pm 0.9 f	18.3 \pm 0.7 e	20.1 \pm 0.8 f	19.9 \pm 1.2 F
Control (tebuconazole)	1.5 \pm 0.5 f	1.4 \pm 0.6 f	1.6 \pm 0.3 f	1.5 \pm 0.1 f	1.5 \pm 0.1 G

Different letters in the column of each pathogen indicate significant differences according to Tukey's test ($p \leq 0.05$). The values in parentheses represent the percentage of mycelial growth inhibition compared to the control. The oil factor (C) shows the average diameter of the inhibition halo for each concentration, considering all pathogens.

The diameter of the inhibition halo for *F. verticillioides* increased with the concentration of the essential oil, from 8.5 mm at 50 ppm to 21.3 mm at 1 000 ppm, suggesting a dose-dependent inhibition of the mycelial growth of this pathogen, as confirmed by Achar and Sreenivasa (2021), who, with essential oils of oregano, thyme, and eucalyptus, have demonstrated high efficacy in the inhibition of *F. verticillioides*, with reductions in mycelial growth of up to 86% at similar concentrations.

For *A. flavus*, there was an increase in the diameter of the inhibition halo from 7.2 mm at 50 ppm to 19.8 mm at 1 000 ppm, so the effectiveness of *S. molle* essential oil against this pathogen highlights its potential in preventing aflatoxin contamination in corn crops, as suggested by Kohiyama *et al.* (2015) for the case of thyme oil in the inhibition of *A. flavus* significantly.

As for *U. maydis*, the inhibition halo increased from 6.8 mm (50 ppm) to 18.3 mm (1 000 ppm), being slightly lower compared to other pathogens, proving to be effective in inhibiting the growth of this causative agent of corn head smut, as reported by Falcão *et al.* (2010) with mint and citronella oils to significantly reduce the growth of *U. maydis*.

In the case of *C. graminicola*, the diameter of the inhibition halo increased from 7.5 mm at 50 ppm to 20.1 mm at 1 000 ppm, demonstrating a high effectiveness of the essential oil against this pathogen, which suggests its potential use in the management of corn anthracnose, as reported by Perczak *et al.* (2019) by using basil and clove oils to inhibit the growth of *Colletotrichum* spp. effectively.

In the control with tebuconazole, there were smaller inhibition halos, with approximately 1.5 mm for all pathogens, confirming the superiority of the essential oil as a natural antifungal agent. The results obtained suggest that *S. molle* essential oil may be a promising alternative to synthetic fungicides for pathogen control in corn crops, contributing to agricultural practices that are more sustainable and less dependent on chemicals.

Cell viability assays

The results of the assays show a reduction in the cell viability of the pathogens evaluated with the increase in the concentration of *S. molle* essential oil, as shown in Table 3. At concentrations of 1 000 ppm, the relative viability of all pathogens decreased to less than 10%, highlighting an antifungal activity of the essential oil compared to the positive control of tebuconazole.

Table 3. Cell viability results of corn pathogens in the presence of *S. molle* essential oil by MTT assay.

Pathogen	Concentration (ppm)	Absorbance (570 nm)	Relative viability (%)
<i>Fusarium verticillioides</i>	0 (control)	1.2 ±0.05	100 ±0 a
	50	1.02 ±0.04	85 ±3 b
	100	0.84 ±0.03	70 ±4 c
	250	0.66 ± .05	55 ±5 d
	500	0.48 ±0.03	40 ±3 e
	1 000	0.06 ±0.02	5 ±2 f
	Control (tebuconazole)	0.018 ±0.01	1.5 ±0.5 g
<i>Aspergillus flavus</i>	0 (control)	1.25 ±0.06	100 ±0 a
	50	1 ±0.05	80 ±4 b
	100	0.81 ±0.04	65 ±3 c
	250	0.625 ±0.05	50 ±4 d
	500	0.438 ±0.03	35 ±3 e

Pathogen	Concentration (ppm)	Absorbance (570 nm)	Relative viability (%)
<i>Ustilago maydis</i>	1 000	0.063 ±0.02	5 ±2 f
	Control (tebuconazole)	0.019 ±0.01	1.5 ±0.6 g
	0 (Control)	1.18 ±0.05	100 ±0 a
	50	1.04 ±0.04	88 ±2 b
	100	0.885 ±0.03	75 ±3 c
	250	0.71 ±0.05	60 ±5 d
	500	0.53 ±0.03	45 ±3 e
	1 000	0.12 ±0.02	10 ±2 f
<i>Colletotrichum graminicola</i>	Control (tebuconazole)	0.018 ±0.01	1.5 ±0.3 g
	0 (Control)	1.22 ±0.05	100 ±0 a
	50	1.02 ±0.04	84 ±3 b
	100	0.83 ±0.04	68 ±4 c
	250	0.635 ±0.05	52 ±4 d
	500	0.465 ±0.03	38 ±3 e
	1 000	0.098 ±0.02	8 ±3 f
	Control (tebuconazole)	0.018 ±0.01	1.5 ±0.1 g

Different letters in the column of each pathogen indicate significant differences according to Tukey's test ($p \leq 0.05$).

S. molle essential oil reduced the viability in *F. verticillioides* from 85% at 50 ppm to 5% at 1 000 ppm, in *A. flavus* from 80% at 50 ppm to 5% at 1 000 ppm, in *U. maydis* from 88% at 50 ppm to 10% at 1 000 ppm, and in *C. graminicola* from 84% at 50 ppm to 8% at 1 000 ppm, indicating a dose-dependent antifungal activity, which can be attributed to compounds such as α -pinene and limonene, known to be antimicrobial (Prado *et al.*, 2019; Tian *et al.*, 2022).

Positive control with tebuconazole showed a low relative viability for all pathogens tested, with values around 1.5%, indicating that it is effective as an antimicrobial agent; however, its use in agricultural applications is limited due to negative effects, such as the accumulation of residues in the soil, which can reduce microbial biodiversity, and the development of resistance in the treated pathogens, which compromises its long-term effectiveness (Han *et al.*, 2021).

Conclusions

The study of the antifungal effect of the essential oil of *S. molle* (pirul) demonstrated antimicrobial activity against *Fusarium verticillioides*, *Aspergillus flavus*, *Ustilago maydis*, and *Colletotrichum graminicola*. The results showed a dose-dependent inhibition of mycelial growth and a significant reduction in the cell viability of pathogens, highlighting the potential of the essential oil as an effective and sustainable alternative to synthetic fungicides.

The chemical composition of the oil, rich in bioactive compounds, such as α -pinene, β -pinene, and D-limonene, supports its potential use in safe and ecological agricultural practices to contribute to the cultivation of corn and the reduction of the use of conventional chemicals. This approach not only improves crop health but also promotes agricultural sustainability in regions affected by these pathogens.

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