

Potential fungicidal effect of root extracts of *Cucurbita foetidissima* (Kunth) against *Fusarium* sp.

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Abstract

The genus *Fusarium* affects a wide diversity of horticultural crops, generating significant economic losses. These fungi are capable of infecting plants during their development and even after harvest. The use of bioactive plant extracts is an environmentally friendly, non-harmful, and low-cost control strategy. Plant-based compounds have shown promise in the control of fungal diseases. *Cucurbita foetidissima* is a cucurbit with a high content of secondary metabolites present in its root with fungicidal power. The present work was conducted from 2017 to 2019 and it evaluated the antifungal effect of root extracts of *C. foetidissima* against different *Fusarium* species under *in vitro* conditions. Root ethanolic and methanolic extracts of *C. foetidissima* were obtained. The metabolites: phenols, flavonoids, terpenoids, and saponins were quantified. The antifungal capacity of the extracts on the growth of *Fusarium oxysporum*, *F. equiseti*, and *F. solani* was evaluated *in vitro*. Methanolic extracts had a higher concentration of phenols, terpenoids, and saponins compared to ethanolic extracts; the latter had a higher concentration of flavonoids. The ethanolic extract achieved a greater antifungal effect, inhibiting between 60 and 80% of the growth of the three *Fusarium* species. The root ethanolic extract of *C. foetidissima* has a moderate fungistatic capacity to inhibit the *in vitro* mycelial growth of different *Fusarium* species, suggesting the potential of its use to delay or attenuate the onset of symptoms associated with fusariosis.

Keywords:

cucurbits, fusariosis, secondary metabolites, wild squash.



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Introduction

The genus *Fusarium* includes several species of fungi that cause diseases in various horticultural crops, including the main food and cash crops, such as wheat, barley, corn, tomatoes, bananas, among others, generating significant economic losses (DGSV-CNRF, 2020). Possible symptoms of diseases caused by *Fusarium* species include root or stem rot, cankers, vascular wilt, fruit or seed rot, and leaf diseases (Ekwomadu and Mwanza, 2023).

To combat these diseases, chemical control is the most widely used practice as it is relatively inexpensive, simple to apply, and effective compared to other types of control. For years, synthetic fungicides have been used continuously and indiscriminately. However, the excessive use of these products has led to resistance in plant pathogens (Kiiker *et al.*, 2021), environmental pollution, and risks to human and animal health (Tao *et al.*, 2020).

For this reason, alternatives that are environmentally friendly and harmless to humans have been sought, such as plant compounds, which, in addition to having these characteristics, have shown potential for the control of phytopathogens. Plant extracts may contain phenols, anthocyanins, terpenoids, flavonoids, tannins, lectins, glucosinolates, saponins, among others, with biological activity (Mehmood *et al.*, 2022).

Chacón *et al.* (2021) results report that the presence of phenolic compounds, amines, flavonoids, and terpenes in the essential oil of *Piper auritum* contributed to inhibiting the growth of *F. oxysporum*. Subsequently, Isidro-Requejo *et al.* (2023) reported that tomato extract, containing phenols and flavonoids, inhibited 100% the mycelial growth of *F. oxysporum*, *F. graminearum*, and *F. verticillioides*.

Stevia rebaudiana leaf extracts, containing the austroinulin saponin, inhibited the growth of *F. oxysporum* in tomato plants (Ramírez *et al.*, 2020). In a similar study, saponins isolated from quinoa (*Chenopodium quinoa*) significantly reduced the incidence of *F. oxysporum* wilt in tomato seeds and seedlings (Zhou *et al.*, 2023).

Cucurbita foetidissima, commonly known as buffalo gourd, is a wild xerophytic vegetable from the semi-arid regions of Mexico and the southern United States. For years, it was used as food, medicine, and disinfectant (Gómez *et al.*, 2019). It is an asymptomatic host of *F. oxysporum* (Apodaca-Sánchez *et al.*, 2004), it is attributed medicinal (Mukherjee *et al.*, 2022) and antifungal properties (Mejía-Morales *et al.*, 2021).

In addition, it has several cucurbitacins and cucurbitacin glycosides in the fruit, a group of triterpene compounds that provide the bitter taste and have antimicrobial, antifungal, and cytotoxic activity (Mejía-Morales *et al.*, 2021). Rangel-Guerrero *et al.* (2018) report that extracts obtained from *C. foetidissima* fruits inhibited between 94.3 and 100% the growth of *Rhizoctonia* sp. colonies, whereas *Fusarium* sp. and *Phytophthora* sp. presented low inhibition percentages (25.7-46.7%; 25.3-42.2%, respectively). This antifungal effect may be due to the presence of tetracyclic triterpenes observed in this plant.

There are few studies on secondary metabolites present in *C. foetidissima* roots and their possible antifungal activity. Therefore, this work aimed to determine and identify the secondary metabolites present in methanolic and ethanolic extracts of *C. foetidissima* roots and their *in vitro* antifungal activity on different *Fusarium* species. The above is done in order to establish the bases that enable the development of harmless and effective natural fungicide for the control of fusariosis.

Materials and methods

Obtaining extracts from *Cucurbita foetidissima* roots

In 2017, roots of adult plants of *C. foetidissima* in the phenological stage of fruiting (summer) were randomly collected, which were from hills with slightly alkaline and saline clay-loam soils in the municipality of Ciudad Cuauhtémoc, Chihuahua, with coordinates 28° 44' 763" north latitude, 106°

82° 88' west longitude and at 2 060 masl. The samples were transported by land to the facilities of the Food and Development Research Center, AC (CIAD, for its acronym in Spanish), located in Hermosillo, Sonora, and stored in refrigeration until disinfection.

The roots were rinsed with plenty of running water to remove excess soil and then disinfected with 6% NaClO. They were cut into small pieces, dried using a forced-air convection oven (Yamato DX-600, Japan) at 60 °C for 72 h and the dry matter obtained was pulverized in an electric mill (HB 8365, China).

The dehydrated material was macerated with two different solvents (methanol and ethanol, Sigma-Aldrich, USA) at a ratio of 1:3 w/v (Chapagain *et al.*, 2007). They were then kept in continuous stirring at 120 rpm in an orbital shaker (Environ Shaker, USA) for eight days at room temperature. The extract was obtained by vacuum filtration and concentrated in a rotary evaporator (Yamato RE500, Japan) until dryness (Chapagain *et al.*, 2007). The resulting pellet was resuspended in 25 mL of methanol (MEA) or ethanol (EEA) and then stored at -80 °C in aluminum-lined tubes for further analysis. The extraction process was carried out in triplicate.

Qualitative analysis of secondary metabolites in *C. foetidissima* root extracts

In order to establish the predominant groups of secondary metabolites in the methanolic and ethanolic extracts of *C. foetidissima* roots, the presumptive tests reported by García-Granados *et al.* (2019) were applied. The FeCl₃ test was used to detect the presence of phenols (García-Granados *et al.*, 2019). Two to three drops of 12.5% FeCl₃ were added to one aliquot of each extract. The change from dark green to indigo blue indicated the presence of phenols. In flavonoids, the Shinoda test was used (García-Granados *et al.*, 2019). One milliliter of each extract was taken, 0.1 g of magnesium was added, and it was heated to 60 °C. A few drops of HCl were then added. The change in coloration to orange, red, pink, blue, or violet indicated a positive test.

Phytosterols and triterpenes were detected with the Liebermann-Burchardt test (García-Granados *et al.*, 2019). A sample of 500 mg of each extract was taken and dissolved in 10 mL of chloroform. Five milliliters were taken and 0.25 ml of acetic anhydride plus two drops of concentrated H₂SO₄ were added. The appearance of a green coloration indicated a positive test for phytosterols and a pink to purple coloration indicated a positive test for triterpenes. In addition, saponins were determined by foam testing. A 500 mg sample was taken and 10 ml of distilled water was added. It was stirred for 30 s in vortex and left to rest for 15 min. The test was positive if the foam height was 5 mm or greater.

Spectrophotometric characterization of secondary metabolites in *C. foetidissima* roots

Based on the results obtained in the previous section, the content of total phenols, flavonoids, terpenoids, and saponins was quantified. A lyophilized sample was prepared using small pieces of the cleaned and disinfected root, as described above, frozen at -80 °C for 24 h and free-dried in a lyophilizer (Yamato DC801, USA). An extract was prepared using 0.25 g of lyophilized sample, 5 ml ethanol was added, and it was cold homogenized (Ultraturax IKA, Germany) for 2 min at 3 000 rpm (Treviño *et al.*, 2012).

The supernatant was decanted and collected, 5 ml of extractor solution (ethanol or methanol) was added and the operation was repeated, and it was sonicated for 60 min at 25 °C. Subsequently, it was left to rest for 2 h, 1.5 ml was taken and it was centrifuged for 15 min at 13 000 rpm, the supernatant was separated and used in the following analyses.



Determination of the contents of total phenols, flavonoids, terpenoids, and saponins

The total phenol content was determined with Singleton *et al.* (1999) technique with slight modifications. A reaction mixture was prepared with 40 μ l of the extract, 60 μ l of ethanol, 0.4 ml of water, 5 ml of NaOH and 0.5 ml of the Folin-Ciocalteu solution (Sigma-Aldrich, USA). After 0.5 to 1 h of rest, absorbance was measured at 765 nm. It was quantified based on a calibration curve, using gallic acid (GA) as a standard. Results were reported as milligrams of gallic acid equivalents per gram of extract (meq GA g⁻¹).

Flavonoids were determined using Zhishen *et al.* (1999) technique with slight modifications. The reaction of the extracts with AlCl₃ produces a deep pink coloration that was measured at a wavelength of 415 nm. Their content was determined based on the quercetin standard. Results were expressed as milligrams of quercetin equivalent per gram of extract (meq Q g⁻¹).

Terpenoids were determined with Ghorai (2012) technique with slight modifications. An aliquot of the extract was taken and 100 μ l of concentrated H₂SO₄ was added to it, forming a red-brown complex. Absorbance was measured at a wavelength of 538 nm and quantified based on the linalool standard. The results were expressed in mg linalool g⁻¹ extract.

Total saponins were determined with Helaly *et al.* (2001) technique. Five milliliters of 72% H₂SO₄ were added to the extract and absorbance was measured at 435 nm. They were quantified according to the oleanolic acid standard. The results were reported as mg of oleanolic acid equivalents g⁻¹ extract. All the reagents and standards used in the different techniques were from the Sigma-Aldrich brand. All determinations described in this section were quantified in triplicate using a UV-Vis spectrophotometer (Hach DR 5000, Germany).

Determination of the antifungal activity of *C. foetidissima* root extracts on the *in vitro* growth of *Fusarium* sp.

Strains of *Fusarium* species

Three species of *Fusarium* (*F. oxysporum*, *F. solani*, and *F. equiseti*) previously isolated from cantaloupe melon fruits were used. The fungi were seeded in Petri dishes with potato dextrose agar (PDA, Difco, USA) for 10 days at 26 °C, to determine the characteristics of the colony: texture, color, and conidiation. Their microscopic characteristics were observed using an optical microscope (Binocular, Primo Star, Carl Zeiss). Fungi were identified to species according to the taxonomic keys reported by Leslie and Summerell (2008) and Tousson and Nelson (1976). The colonies were kept in refrigeration (4 °C).

Determination of the effect of *C. foetidissima* root extracts on the *in vitro* mycelial growth of *Fusarium* species

The ability of *C. foetidissima* extracts to inhibit the mycelial growth of *Fusarium* species was evaluated *in vitro* using the well diffusion method (Pérez-Delgado and Vallejos-Campos, 2019). The following treatments were evaluated: ethanolic extract of *C. foetidissima* root (EEA), methanolic extract of *C. foetidissima* root (MEA), control with ethanol as extract solvent (CE), and control with methanol as extraction solvent (CM). In 90 mm Petri dishes with PDA, five wells of 6 mm in diameter were drilled, located 23 mm from the center of the dish. One hundred microliters of the extract were placed in each well according to its treatment. Mycelium discs (6 mm in diameter) of each *Fusarium* species were placed in each petri dish. A negative control with 100 μ l of sterile distilled H₂O (control) was included.

Three replications were performed for each treatment. The inhibitory capacity of the extracts was determined based on the mycelial growth of the fungus, which was calculated by measuring the diameter of the mycelial growth every 24 h for 10 days with a vernier. The inhibitory capacity was expressed as a percentage of inhibition using the equation proposed by Chapagain *et al.* (2007).

$$\text{Inhibitory capacity (\%)} = \left[\frac{AC - at}{AC} \right] \times 100$$

Where: AC= average of the control area; at= individual area of the treatment. The area obtained was used to calculate the growth rate (GrR= area time, mm² day⁻¹) and growth efficiency (GrE= mm day⁻¹).

Statistical data analysis

The experiment was repeated twice and the data was analyzed using a completely randomized design. To perform the statistical analysis of the inhibition percentage, the original data was transformed by the formula $\arcsin \sqrt{\%}$. An analysis of variance (Anova) and the Tukey-Kramer test were performed to determine differences between means at a probability of 0.05%. The statistical program used was NCSS version 2017.

Results and discussion

Identification and quantification of secondary metabolites of *Cucurbita foetidissima* root extracts

Presumptive tests revealed the presence of phenols, flavonoids, terpenoids, and saponins, which have been described as antifungals in various plant tissues (Arif *et al.*, 2009). The results confirm what was reported in leaves and fruits of *C. foetidissima* by Macías *et al.* (2009), who found the presence of phenols, terpenoids, and saponins. In addition to what was obtained in leaves and fruits, they also found an abundance of these compounds in roots, along with triterpene compounds (cucurbitacins) and saponins (Mejía-Morales *et al.*, 2021).

Quantification of major secondary metabolites

Table 1 shows the concentrations of the compounds quantified in the extracts. The extractor solvent where a higher concentration of saponins was observed was methanol, which is consistent with what was reported in seed extracts of *Camellia sativa* L., where up to 50% more concentration of total phenols was obtained in methanolic extracts than in ethanolic extracts (Gupta and Kumar, 2017), whereas ethanolic extracts obtained higher concentrations only in flavonoids. Saponins were the most abundant compounds in methanolic extracts. Several authors describe the *C. foetidissima* plant as a plant that highly produces saponins and other triterpene compounds, such as cucurbitacins (Kaushik *et al.*, 2015; Mejía-Morales *et al.*, 2021).

Table 1. Secondary metabolite content in *C. foetidissima* root extracts.

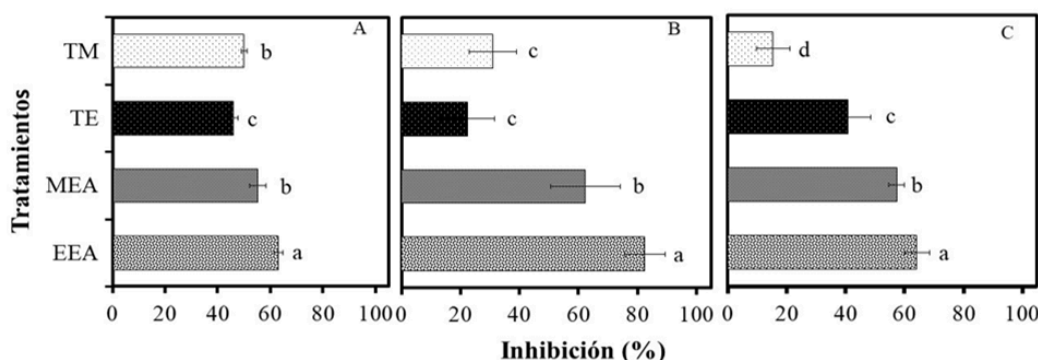
Standard used	Secondary metabolite	Drying in a forced-air oven (60 °C, 72 h)	
		Methanol (meq g ⁻¹ dw)	Ethanol (meq g ⁻¹ dw)
Gallic ac.	Phenols	92.39 ± 11.36 ^a	79.96 ± 5.02 ^a
Quercetin	Flavonoids	283.24 ± 11.79 ^b	621.62 ± 52.57 ^a
Linalool	Terpenoids	42.61 ± 3.41 ^a	38.87 ± 0.59 ^a
Oleanolic ac.	Saponins	888.2 ± 130 ^a	456.29 ± 20.33 ^b

Equal letters between rows are not significantly different (Tukey; $p(0.05, n=6)$. meq g⁻¹ dw= milligrams equivalent over grams of dry weight.

Antifungal activity of *C. foetidissima* root extracts on the *in vitro* growth of *Fusarium* sp.

The root extracts of *C. foetidissima* showed antifungal activity on the three *Fusarium* species evaluated. Figure 1 shows the effect of extracts on the inhibition of mycelial growth of *Fusarium* sp. under *in vitro* conditions. When comparing treatments using Tukey's test (0.05), it was observed that methanolic and ethanolic extracts significantly reduced ($p < 0.05$) the mycelial growth of *F. oxysporum* (Figure 1A) from the first day of evaluation.

Figure 1. Percentage of inhibition of mycelium, A) *F. oxysporum*; B) *F. equiseti* and C) *F. solani* in response to exposure to ethanolic and methanolic extracts of *C. foetidissima* roots. Bars with the same letter are not significantly different ($p \leq 0.05$).



The application of ethanolic extract (EEA) significantly reduced the growth of the fungus, achieving the maximum percentage of inhibition on day 5 with 63%, whereas the percentage of inhibition was slightly lower in methanolic extract (MEA). The EEA treatment inhibited 15% more than the control treatments (CM and CE) and 9% more compared to the methanolic extract (MEA).

Both extracts had a higher inhibitory capacity in *F. equiseti* (Figure 1B) compared to the other two *Fusarium* species. The application of the EEA inhibited mycelial growth to a greater extent, exceeding 80% inhibition, whereas the MEA extract only inhibited the growth of this fungus 60%.

The percentage of inhibition in the *F. solani* species (Figure 1C) showed a behavior similar to that in *F. oxysporum*. Again, the EEA treatment reached 65% inhibition, 10% more than the MEA extract. The inhibitory activity of *C. foetidissima* root extracts observed in the present study is higher than that reported by Rangel-Guerrero *et al.* (2018), who observed that methanolic extracts of *C. foetidissima* fruits from different locations in Mexico inhibited the growth of *Fusarium* sp., between 25.7 and 46.7% at 5 days of incubation at 27 °C.

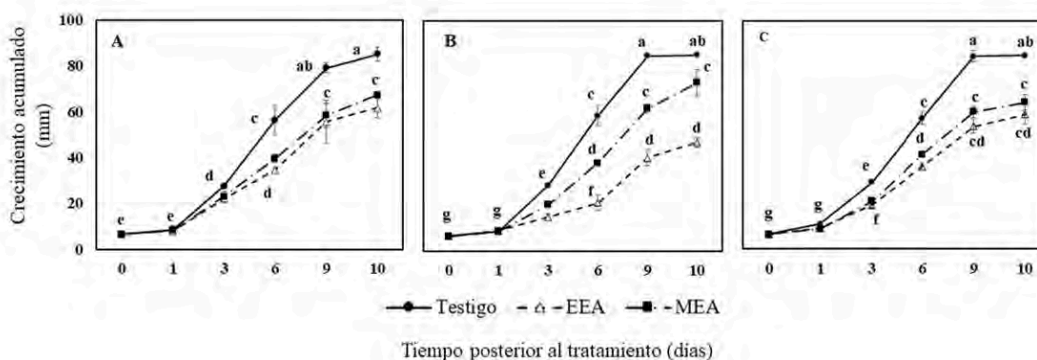
This suggests that the root of *C. foetidissima* contains secondary metabolites with greater antifungal activity than the fruit of this cucurbit. It is important to note that the ethanolic extract had a greater inhibitory capacity compared to the methanolic extract, which may be due to the high flavonoid content recorded in this study.

Findings such as Ammar *et al.* (2013) have identified flavonoids present in plants of *Tephrosia apollinea* L. as responsible for antimicrobial activity on other phytopathogenic microorganisms. Al Aboody and Mickymaray (2020) point out that flavonoids inhibit fungal growth through several mechanisms of action, causing plasma membrane disruption, induction of mitochondrial dysfunction, inhibition of cell wall formation, inhibition of cell division, and inhibition of RNA and protein synthesis.

Regarding growth kinetics, the three *Fusarium* species exposed to extracts of *C. foetidissima* showed a similar growth pattern. Figure 2 shows that both *F. oxysporum* (Figure 2A) and *F. solani* (Figure 2C) did not show significant differences in growth when exposed to both extracts, but it was significantly lower ($p \leq 0.05$) than in control.

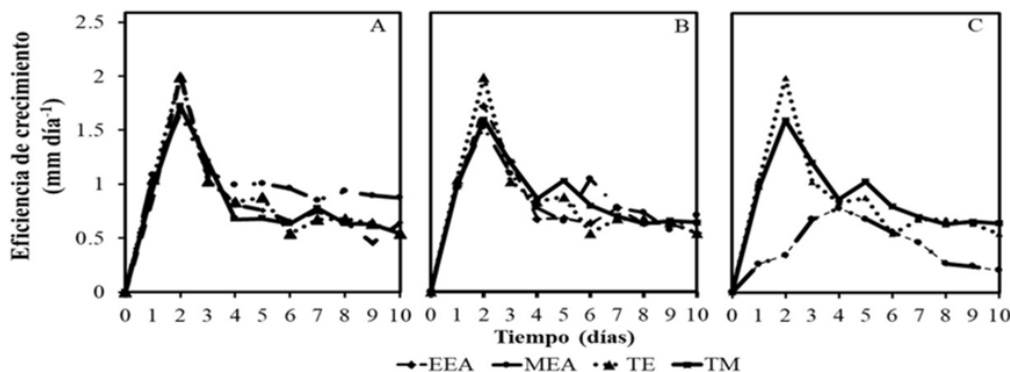
On the other hand, *F. equiseti* (Figure 2B), when exposed to the ethanolic extract, showed a lower growth compared to the other treatments and control. This suggests that *F. equiseti* is more sensitive to bioactive compounds extracted with ethanol compared to the other *Fusarium* species evaluated in the present study.

Figure 2. Growth kinetics, A) *F. oxysporum*; B) *F. equiseti* and C) *F. solani* in response to exposure to ethanolic (EEA) and methanolic (MEA) extracts of *Cucurbita foetidissima*. Means with the same letter are not significantly different (Tukey; $p(0.05, n=6)$).



On the other hand, the results obtained on the growth efficiency of *Fusarium* species in response to *C. foetidissima* extracts are presented in Figure 3. It was observed that the highest efficiency was found on the second day, with values between 1.6 and 2.09 mm day⁻¹ for *F. oxysporum* (Figure 3A) and *F. solani* (Figure 3B). From this day on, the efficiency decreased steadily until it reached the minimum value of its growth (0.21 mm day⁻¹). In the case of *F. equiseti* (Figure 3C), the growth efficiency was lower than the other *Fusarium* species.

Figure 3. Growth efficiency, A) *F. oxysporum*; B) *F. solani* and C) *F. equiseti* in response to exposure to ethanolic and methanolic extracts of *Cucurbita foetidissima*.



The behavior observed on the increase in growth efficiency to a maximum peak followed by a decrease in this could be due to the photolability of the phenolic compounds present in the extracts (Mohamed *et al.*, 2020). On the other hand, the ability of the pathogen to produce and activate detoxifying enzymes that inhibit the antifungal action of the compounds (Mahgoub *et al.*, 2023) favors their growth under adverse conditions.

Conclusions

The secondary metabolites: phenols, flavonoids, terpenoids, and saponins, were detected in ethanolic and methanolic extracts of *C. foetidissima* roots. The methanolic extract had a higher concentration of phenols, terpenoids, and saponins, whereas the ethanolic extract had a higher concentration of flavonoids. The ethanolic extract of *C. foetidissima* roots had a greater antifungal capacity to inhibit the *in vitro* mycelial growth of *F. oxysporum*, *F. equiseti* and *F. solani*, which represented a promising alternative that made it possible to delay or attenuate the onset of symptoms associated with fusariosis.

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