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RESEARCH ARTICLE

Inhibitory activity of an ethanolic extract from *Azadirachta indica* (Neem) on *Fusarium* sp.

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Abstract

The intensive use of synthetic fungicides for the control of *Fusarium* has raised concerns regarding environmental impact and pathogen resistance. As a sustainable alternative, plant-derived extracts are gaining attention due to their bioactive properties. The objective of this research was to identify the mineral and phytochemical composition of an ethanolic extract from *Azadirachta indica* and to evaluate its *in vitro* fungicidal activity against *Fusarium* sp. The extract was obtained using ultrasound/microwave-assisted extraction with 70 % ethanol. Phytochemical profiling was conducted through high-performance liquid chromatography (HPLC) and Fourier-transform infrared spectroscopy (FTIR), while mineral content was assessed by X-ray fluorescence (XRF). The antifungal effect was evaluated using the poisoned medium technique, with extract concentrations of 0 (control), 1, 2, 3, 4 and 5 mL/200 mL of medium. Mycelial growth inhibition and conidia production were recorded. Data were analyzed using ANOVA (analysis of variance) and Tukey's test ($p = 0.05$). Potassium was the predominant mineral (4 %) and eight major phytochemicals were identified, including caffeic acid 4-O-glucoside, (+)-catechin, secoisolariciresinol, quercetin 3-O-xylosyl-glucuronide, quercetin 3-O-glucoside, caffeoyl glucose, *p*-coumaric acid 4-O-glucoside and *p*-coumaroyl glucose. Extract concentrations between 2 and 5 mL/200 mL reduced mycelial growth by up to 35 %, while doses of 2 and 5 mL/200 mL treatments significantly reduced conidia count to 173500 and 155500, respectively. These results suggest that *A. indica* extract exhibits fungicidal properties against *Fusarium* sp., highlighting its potential as a natural alternative to synthetic fungicides.

Keywords: FTIR; HPLC mass; inhibition; plant extracts; ultrasound/microwave

Introduction

Azadirachta indica (commonly known as neem) exhibits antimicrobial properties against a wide range of pathogens. For this reason, extracts from this plant have been utilized in various fields, including dentistry, food safety, parasitology, virology, bacteriology and mycology (1). Approximately 140 bioactive compounds have been identified from different organs of the plant (roots, stems, leaves and fruits), which exhibit antidiabetic, antioxidant, antiviral and anti-inflammatory properties, in addition to antibacterial and antifungal effects (2). Among the most notable compounds isolated from *A. indica* are azadirachtin, quercetin and limonoids such as nimbidin, nimbin and nimbinin, all of which possess documented antimicrobial activity (3). Other compounds detected in larger quantities include saponins, steroids and terpenes, while tannins and glycosides are present in moderate amounts and alkaloids, phenols, oxalic acid and flavonols are found in smaller proportions (4).

The widespread use of synthetic chemical pesticides in agriculture poses significant risks due to the presence of toxic substances that may negatively affect the environment, soil microbiota, farmers and consumers (5). As an alternative, plant extracts have been increasingly investigated for their potential to reduce dependence on synthetic pesticides. For example, that aqueous extracts of *A. indica* reduced the incidence and severity of leaf blight caused by *Stemphylium vesicarium* by 62 % and 65 %, respectively surpassing the effectiveness of certain synthetic fungicides, including organophosphates and strobilurins (6). It has been reported that *A. indica* extracts contain phytochemical compounds such as alkaloids, flavonoids and phenols (7). It was also indicated that ethanol extracts reduce mycelium growth by 88 % and decrease *Colletotrichum musae* spore production by 66 %, compared to the aqueous extract where these values were lower (8).

The application of *A. indica* extracts represents a promising alternative for managing economically important phytopathogenic fungi that affect various crops. One such pathogen is *F. oxysporum*, the causal agent of tomato wilt, a major disease of global concern (9). This fungus also infects bananas (*Musa* spp.), causing vascular wilt, root decay and bark damage and reduces yield in infected plants (10, 11). *Fusarium* spp. has also been reported to cause root and stem rot in melon plants (12). *F. solani*, *F. oxysporum* and *F. graminearum* are the species that most affect crops, in addition to producing mycotoxins (13). The objective of this study was to

identify the mineral and phytochemical composition of an ethanolic extract from *A. indica* and to evaluate its *in vitro* fungicidal activity against *Fusarium* sp.

Materials and Methods

Vegetal material

Ripe fruits of *A. indica* were collected from trees located at the School of Engineering and Sciences of the Autonomous University of Tamaulipas, Ciudad Victoria, Mexico (Fig. 1). To remove dust from the fruits, they were washed with potable water, disinfected with 3 % sodium hypochlorite for 1 min and rinsed three times with sterile water. Then they were placed in an oven for 72 hr at 50 °C. After that, the plant material was placed in a knife miller and sieved to achieve a particle size of 355 µM.

Extract obtention

The extract was obtained using simultaneously ultrasound/ microwave extraction (equipment: Nanjing ATPIO Instruments Manufacture Co., Ltd. Company, China). In this case, 83.3 g of plant material (powder) was mixed in 1 L of solvent (70 % ethanol) in an extractor reactor. For ultrasound (US) the following conditions were used: radio power 20 %, ultrasound relay on for 10 sec, ultrasound relay off for 3 sec, amplitude relay off for 25 sec and set time 20 min. While for microwave (MV): radio power 800 W, display power 0, set temperature 70 °C and set wait time 50 min. Once the extraction was complete, the plant material was separated from the extract by filtration for three times through organza cloth and filter paper (14).

Mineral content of *A. indica*

The analysis of the mineral content of *A. indica* consisted of two stages. First, the percentage of minerals was determined: magnesium (Mg), aluminum (Al), silicon (Si), phosphorus (P), sulfur (S), chlorine (Cl), potassium (K), calcium (Ca), scandium (Sc), titanium (Ti), chromium (Cr), manganese (Mn), iron (Fe), cobalt (Co), nickel (Ni), copper (Cu) and zinc (Zn) using an energy dispersive X-ray fluorescence spectrometer (Epsilon 1, Panalytical, Netherlands). For this purpose, 2 g of *A. indica* plant material was used, previously disinfected, dried in an oven for 72 hr at 50 °C and pulverized to a particle size of 355 µM. Second, the sample was placed in a crucible and placed in a furnace at 600 °C for 2 hr to obtain the ash percentage. These steps were repeated three times (15). The mineral content was obtained using the following equations (Eqn. 1 and 2):

$$\text{Mineral content} = (\text{mineral percentage} \div 100) \times \text{ash percentage} \quad \text{(Eqn. 1)}$$

$$\text{Ash percentage} = [(\text{weight of crucible with ash sample} - \text{weight of crucible without sample}) / 2\ \text{g}] \times 100 \quad \text{(Eqn. 2)}$$

Identification of phytochemical compounds

Phytochemical compounds were obtained using a Varian HPLC system, which included an autosampler (Varian ProStar 410, USA), a ternary pump (Varian ProStar 230I, USA) and a PDA detector (Varian ProStar 330, USA). A liquid chromatography ion trap mass spectrometer (Varian 500-MS IT Mass Spectrometer, USA) containing an electrospray ion source was also used. For sample preparation, 5 µL of each extract was filtered through a 0.45 µm nylon filter and placed in special HPLC system vials. Subsequently, the temperature, eluents, gradient, flow rate and ion source parameters were assigned (16). Finally, MS Workstation software (V 6.9) was used to analyze the obtained data and the samples were analyzed in a range of m/z 50-2000 in full scan mode.

Characterization by Fourier Transform Infrared Spectrometry (FTIR)

The plant material was characterized at the pilot plant of the School of Chemistry at the Autonomous University of Coahuila. A total of 2 g of plant material were analyzed using a Perkin-Elmer Nicolet Nexus 47 (USA) instrument in the range of 4000-500 cm^{-1}.

Isolation and identification of *Fusarium* sp.

To evaluate the fungicidal effect of the extract, *Fusarium* sp. was isolated from tomato leaves with characteristic symptoms of the fungus. The leaves were disinfected in 3 % sodium hypochlorite for 1 min in a laminar flow hood, rinsed three times in sterile distilled water and dried with sterile blotting paper. Leaf sections measuring 0.5 cm × 0.5 cm (healthy and diseased tissue) were then placed in a Petri dish containing potato dextrose agar (PDA) culture medium.

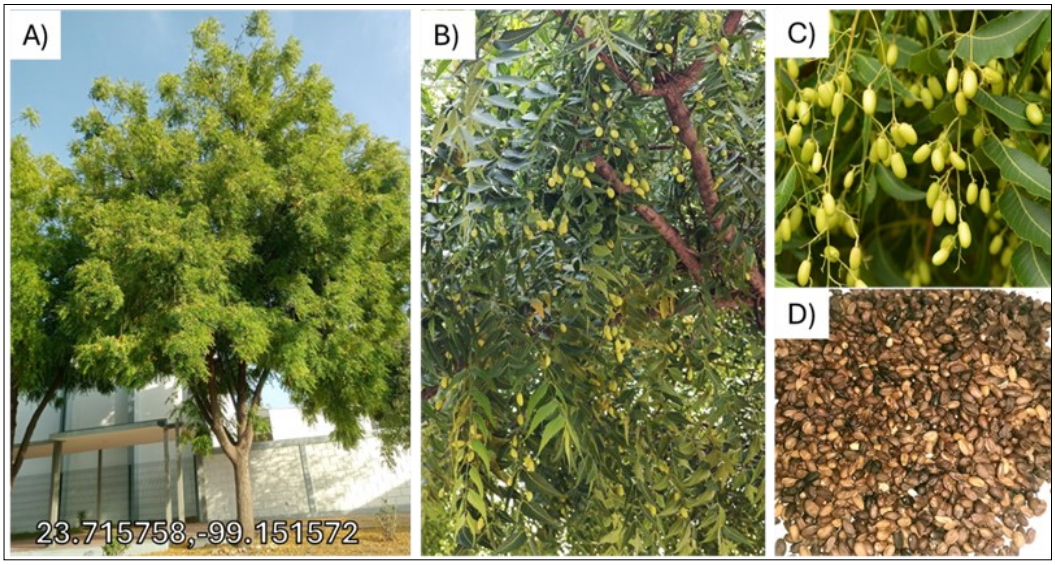

Fig. 1. *A. indica*: tree (A), branches with fruits (B), unripe fruits (C) and ripe fruits (D).

The Petri dishes were incubated at 25 ± 1 °C for three days, once mycelium growth was observed, the fungus was purified and after seven days the morphological identification was carried out at the genus level, for this purpose taxonomic keys were used, the color of the mycelium, the shape of the conidiophores, the conidia, the macro- and micro-conidia were considered (17).

Inhibition of *Fusarium* sp.

The fungicidal effect of the ethanolic extract from *A. indica* against *Fusarium* sp. was evaluated using the poisoned medium technique. First, 200 mL of PDA culture medium was prepared and mixed with the extract. Then, 20 mL of the poisoned medium was poured into 80 mm diameter Petri dishes. This procedure was repeated for each of the tested doses: 0 (control), 1, 2, 3, 4 and 5 mL of the extract. The Petri dishes were inoculated with a 5 mm diameter explant of the fungus and the 0 mL dose, i.e., a Petri dish without the extract and containing only PDA, was used as a negative control (18).

To determine inhibition, the diameter of the fungal colonies was measured every 24 hr until the control treatment, filled the Petri dish. The number of conidia was quantified after ten days. A 5 mm explant was taken at 15 mm from the center of the Petri dish and placed in microtubes with 1 mL of sterile water. The microtubes were then vortexed and the resulting solution was observed in a Neubauer chamber, where the conidia were counted. This procedure was repeated three times for each treatment. To determine the inhibition (Eqn. 3) and the number of conidia (Eqn. 4), the following equations were used (18, 19).

$$\% \text{ inhibition} = [(D1 - D2)/D1] \times 100 \qquad \text{(Eqn. 3)}$$

Where: D1 is the diameter of the untreated pathogen and D2 is the diameter of the pathogen growing in the poisoned medium with the different concentrations of the extract.

$$\text{Conidia/mL} = (\text{Number of cells} \times \text{dilution} \times 10^4) / \text{No. of mm}^2 \text{ areas counted} \qquad \text{(Eqn. 4)}$$

Statistical analysis

The experiments were carried out under a completely randomized design, with three replicates for the mineral analysis and six replicates for each dose used in the poisoned medium. A descriptive analysis of the phytochemical compounds was performed using FTIR. Analysis of variance was used for percentage inhibition and conidia counts and when needed, significant differences among treatments were found, a comparison of means was performed using Tukey's multiple range test ($p = 0.05$) using the SAS statistical package.

Results and Discussion

Mineral content of *A. indica*

A total of seven minerals were identified, of which, potassium (K) had the highest percentage (4.0 %), followed by calcium (Ca) with 1.63 %, phosphorus (P) and sulfur (S) with less than 0.5 % (Fig. 2). On the other hand, the minerals with the lowest percentages (0.03 -0.09 %) were chlorine (Cl), iron (Fe) and zinc (Zn). The minerals identified in this study are essential for plants: K, as a mineral nutrient, is involved in vital cellular processes for organisms, modulates the gene expression of virulence and resistance in microorganisms (20, 21). Among the strategies to control phytopathogenic fungi, Ca has been employed, which causes low activity of cell wall-degrading enzymes, as well as inhibition of spore germination and mycelial growth of *Botrytis cinerea* (22).

For fungal disease control, S doses have been evaluated for *F. solani* control (0, 100, 200 and 300 g/m^2), demonstrating that this mineral was effective in reducing the damage caused by the fungus to an acceptable and less expensive level compared to the fungicide Benomyl (23). Compounds containing S act by releasing sulfur dioxide (SO_2), which interferes with cellular components (24). Sodium metabisulfite inhibits cellular metabolism, energy production, protein biosynthesis and membrane synthesis (25).

Zn participates in metabolic processes and is a cofactor for various enzymes, however, at high concentrations it is toxic to living beings (26). It has been reported that Zn at concentrations between 250-500 µM favours fungal growth, as well as sporulation, but at high concentrations (1000-2000 µM), it has the opposite effect since it acts as a toxic metal (27). This effect occurs due to the slow growth of the mycelium, glucose is consumed more slowly and the mycelial growth curve reaches a stationary phase (28). In this sense, the minerals identified in the *A. indica* extract have fungicidal potential against phytopathogenic fungi.

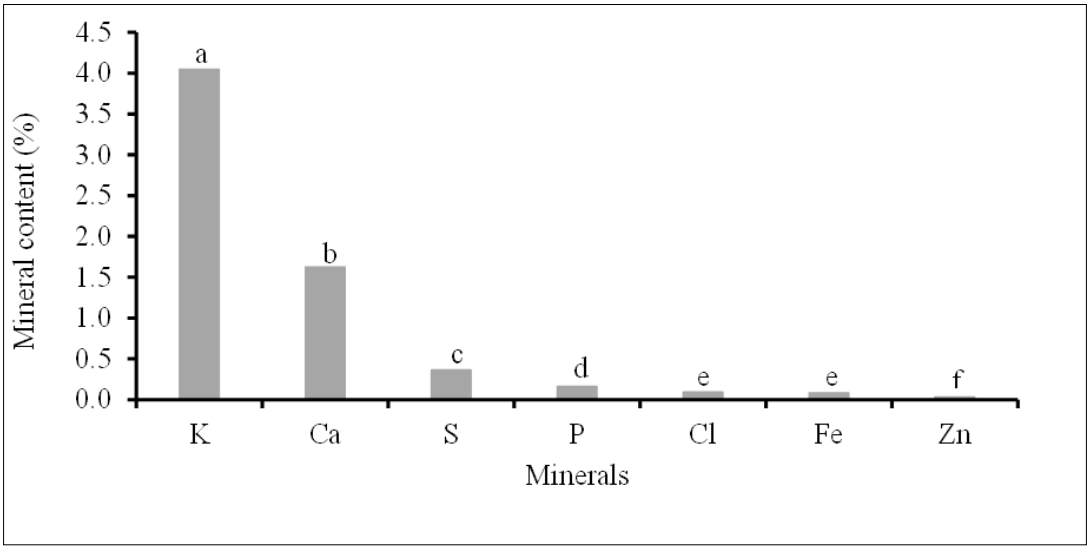

Fig. 2. Mean mineral content of *A. indica*, expressed as a percentage. Means with different letter are not statistically equal (Tukey test, $p = 0.05$). Potassium (K); calcium (Ca); sulfur (S); phosphorus (P); chlorine (Cl); iron (Fe); zinc (Zn).

Phytochemical compounds

Eight phytochemical compounds from four families were identified in the ethanolic extract from *A. indica*: hydroxycinnamic acids, lignans, flavonols and catechins (Fig. 3). Among them, hydroxycinnamic acids were found more frequently and have been identified in *A. indica* seeds shown in Table 1 (29). Likewise, the presence of flavonols such as myricetin 3-O-galactoside, quercetin and kaempferol, as well as hydroxycinnamic acids such as *p*-coumaric acid, has been observed in aqueous extracts of *A. indica* (30).

Among the compounds identified in this study, hydroxycinnamic acids have been used to inhibit the growth of *F. avenaceum* and the production of enniatins, which are a group of emerging mycotoxins produced by species of the *Fusarium* genus (31). They are present in grain tissues and contribute to the defense against diseases caused by *Fusarium*, highlighting *p*-coumaric acid, which decreased the growth of *F. graminearum* by 30 % (32). *p*-coumaric acid, has shown antifungal activity by inhibiting 64 % of the mycelial growth of *F. ananatum* at a concentration of 1000 µg g^{-1} (33).

Caffeic acid inhibited the mycelial growth of *F. graminearum* and *F. culmorum*, in addition to having a negative impact on the production of mycotoxins produced by fungi (34). This compound damages the membrane structure of fungal cells, causes thinning of the cell membrane, irregular intracellular voids and inhibits biofilm formation (35). Compounds such as *p*-coumaric and caffeic acid inhibited the growth the *F. graminearum* and *F. verticillioides* (36).

Other compounds identified in this study are lignans, of which secoisolariciresinol has shown inhibition of *F. graminearum* growth (37). Lignan-derived compounds have also shown antifungal effects, primarily against *F. graminearum*, *Pyricularia oryzae* and *Alternaria alternata* (38). Quercetin favors reduction of *Botrytis* sp., *F. oxysporum*, *Petriella setifera* and *Neosartorya fischeri* growth (39). The antifungal activity of these compounds is attributed to their ability to inhibit fungal cell wall synthesis, disrupt cell integrity, alter the cell membrane and interfere with the physiological metabolism of hyphae (40).

FTIR characterization

The FTIR spectral analysis of the *A. indica* ethanolic extract confirmed the presence of various functional groups associated with bioactive compounds of known antifungal activity (Fig. 4). The broad O-H stretching band at 3279.97 cm^{-1} suggests the presence of carboxylic acids, which are commonly reported to disrupt microbial cell walls and membranes. The C-H stretching vibrations at 2923.51 and 2853.88 cm^{-1} indicate the presence of aliphatic hydrocarbon chains, which are often components of essential oils and lipid-based bioactives.

Notably, the distinct absorption bands corresponding to C=O (1743.66 cm^{-1}) and C=N (1630.26 cm^{-1}) stretching suggest the presence of aldehydes, ketones and other carbonyl-containing compounds, many of which have been linked to antimicrobial properties through mechanisms such as protein denaturation and enzyme inhibition. The observed C-O stretches at 1152.19 and 1029.07 cm^{-1} further confirm the presence of alcohols, phenols and ethers functional groups that are commonly associated with antioxidant and antimicrobial activities.

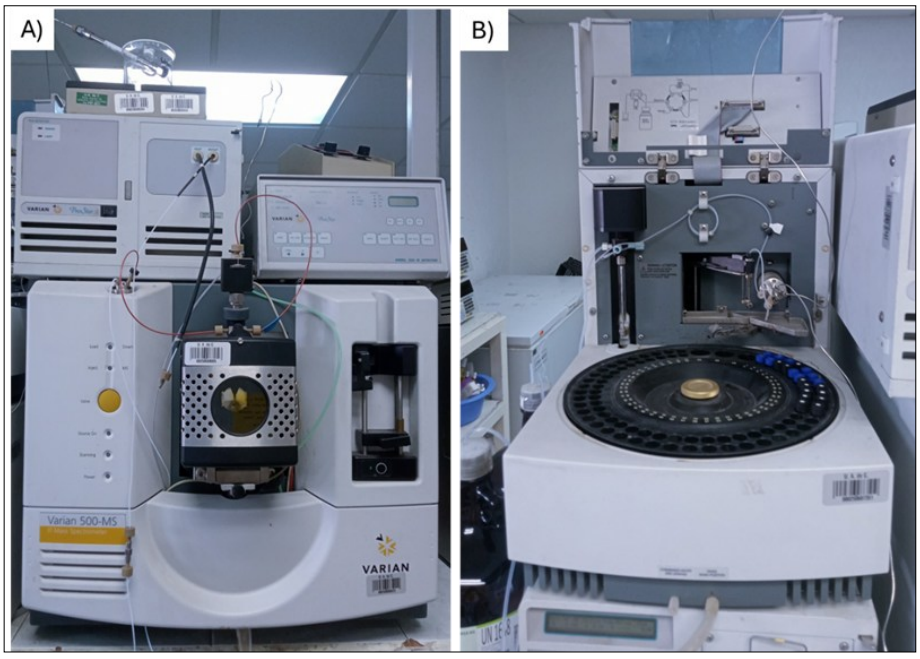

Fig. 3. Equipment for the identification of compounds in the ethanolic extract of *A. indica*: Varian 500-MS ion trap mass spectrometer (A) and Varian ProStar high performance liquid chromatography (HPLC) system (autosampler model 410) (B).

Table 1. Compounds identified in the ethanolic extract from *A. indica* by HPLC

Retention time (min)	Mass	Compound	Family
16.4	340.9	Caffeic acid 4-O-glucoside	Hydroxycinnamic acids
37.4	288.9	(+)-catechin	Catechins
43.0	364.9	Secoisolariciresinol	Lignans
50.0	608.8	Quercetin 3-O-xylosylglucuronide	Flavonols
51.4	462.9	Quercetin 3-O-glucoside	Flavonols
52.9	340.9	Caffeoyl glucose	Hydroxycinnamic acids
56.8	325.0	*p*-coumaric acid 4-O-glucoside	Hydroxycinnamic acids
58.8	325.0	*p*-coumaroyl glucose	Hydroxycinnamic acids

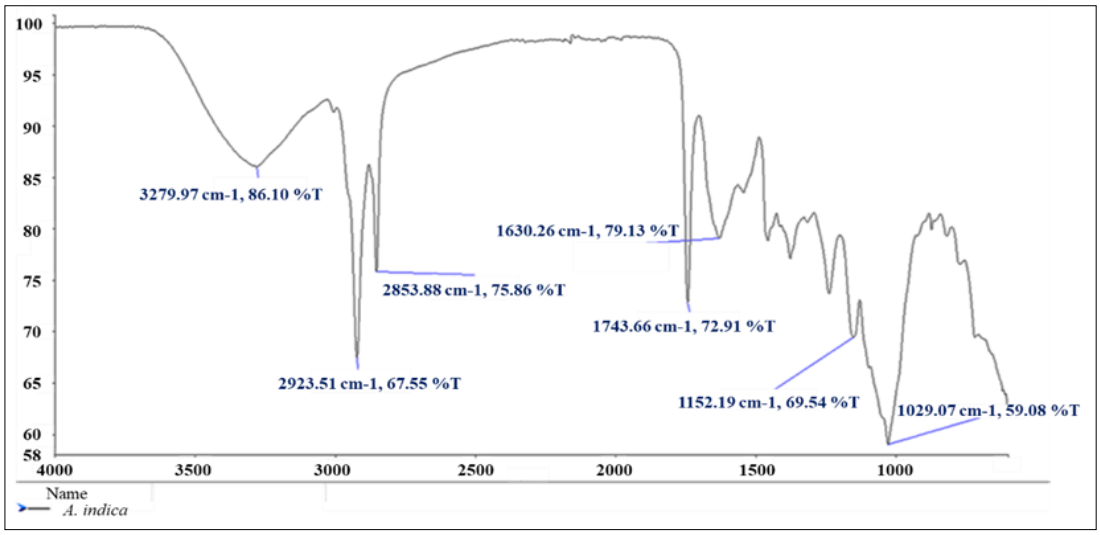

Fig. 4. FTIR of *A. indica* extract obtained using simultaneously ultrasound and microwaves.

The functional groups C-H str, C=O str and C=C str, as well as alcohols, the carboxylate ion, aliphatic and aromatic compounds, are associated with antifungal properties against phytopathogenic fungi (41). Aldehydes such as octanal (1.0 and 0.5 µL/mL), nonanal (0.5 and 2.0 µL/mL) and decanal (1 and 5 µL/mL) inhibited mycelial growth and spore germination of *Aspergillus flavus* by damaging the cell wall and membrane, as well as decreasing total lipid content and inhibiting respiratory metabolism in the fungal cells (42).

The antifungal effect of carboxylic acids, aliphatic hydrocarbons, aliphatic aldehyde groups, ketones, alcohols, phenols and ethers is related to modification of cell membranes permeability, inhibition of cell wall formation, cell division, RNA and protein synthesis, which affects growth and oxidation of lipids in the fungal cell (43). In the ethanolic extract of *A. indica* leaves, O-H and C-H stretching frequencies have been observed, as well as hydroxyl groups such as phenol, alkane, alkenes and alcohol (44).

Fusarium sp. inhibition

Table 2 shows the results obtained for the percentage of inhibition and the number of conidia of *Fusarium* sp. exposed to different doses of *A. indica* extract. Doses ranging from 2 to 5 mL/200 mL showed the greatest inhibition of mycelial growth (28 to 35 %), while the doses with the lowest number of conidia were those of 2 and 5 mL/200 mL, with 173500 and 155500, respectively. The higher fungal inhibition is due to the increase in the extract dose (45). It has been reported that the ethanolic extract of *A. indica* seeds inhibited the growth of *F. oxysporum* by presenting phytochemical compounds such as alkaloids, glycosides and flavonoids (46). In another study, 50 % inhibition of *F. oxysporum* was recorded in PDA culture medium after 120 hr (10).

Table 2. Percentage of inhibition and number of conidia of *Fusarium* sp. exposed to different doses of *A. indica* extract

Dose (mL)	Inhibition (%)	Number of conidia
0	0.0^{c}	282500^{a}
1	15.1^{b}	282000^{a}
2	27.8^{a}	173500^{b}
3	35.5^{a}	247000^{a}
4	34.7^{a}	228500^{ab}
5	34.1^{a}	155500^{b}

Means with different letter are not statistically equal (Tukey test, p = 0.05).

The extracts from *A. indica* have shown greater inhibition of *Aspergillus* spp., *Fusarium* spp. and *Penicillium* spp. in comparison to extracts from *Allium sativum*, *Zingiber officinale* and *Curcuma longa* (47). In this sense, the fungicidal potential of an extract is related to the presence of some phytochemical compounds, which are isolated using various solvents, extraction techniques, the conditions of the plant material such as age, plant organ, phenological phase, among other factors (48).

The 0 mL dose (control), radial and concentric growth was observed, with a cottony and plushy mycelium (Fig. 5A). The 1 mL dose, the mycelial growth was similar to that of the 0 mL dose (Fig. 5B). On the other hand, the 2 mL dose morphologically modified the fungal mycelium, white mycelium and a lower number of conidia were observed (Fig. 5C), compared to the other treatments, where the color of the mycelium was white the first three days, pink at five days and pink with orange at seven days, when the fungus filled the Petri dish (Fig. 5D-F). The presence of terpenoids, phenols, flavonoids, glycosides, alkaloids, tannins and saponins in *A. indica* leaf and seed extracts inhibits the germination of fungal mycelium and spores (49). Thus, the phytochemical compounds affected the growth of fungi, causing hyphae to become swollen, sunken, curled, distorted and even broken (50).

Conclusion

The ethanolic extract of *A. indica* possesses significant fungicidal activity against *Fusarium* sp., attributable to its mineral and phytochemical composition. Potassium was the predominant mineral identified and key bioactive compounds such as caffeic acid, quercetin and *p*-coumaric acid were associated with antifungal effects. *In vitro*, the surveys revealed that extract concentrations of 2 and 5 mL/200 mL effectively inhibited mycelial growth and reduced conidia production, suggesting a dose-dependent response. These findings support the potential application of *A. indica* extract as a natural alternative to synthetic fungicides for the management of *Fusarium* related diseases in crops.

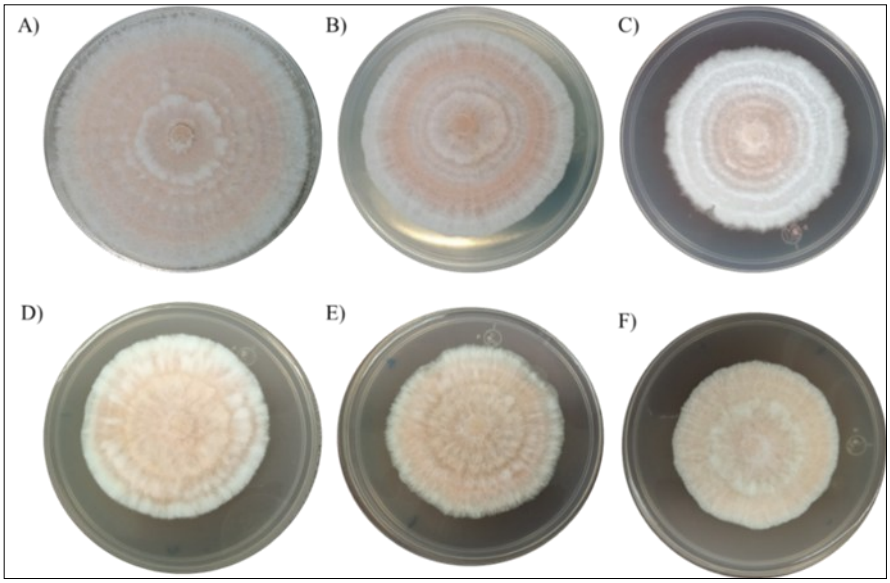

Fig. 5. Growth of *Fusarium* sp. mycelium subjected to *A. indica* extract at doses of 0 mL (A), 1 mL (B), 2 mL (C), 3 mL (D), 4 mL (E), 5 mL (F).

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Authors' contributions

AMB carried out the conceptualization, data curation, formal analysis, research, methodology and writing original draft. RRH participated in the supervision, validation, review and editing. MRR, MTJSM and LRMSC participated in the review and editing. CMLB participated in the supervision and identification of mineral content. JAAV participated in the supervision and identification of phytochemical compounds. EOH participated in the methodology, supervision, validation, review and editing. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None

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