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Scientific Article

In vitro effect of polar extracts from *Annona glabra* seeds on *Fusarium solani*

Avaliação da atividade fungicida dos extratos polares de *Pond apple* para o controle de *Fusarium*

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ABSTRACT - *Fusarium* species are the cause of diseases in various crops throughout the world and cause significant economic losses. Chemical fungicides are the main strategy for control, but their intensive and long-term use generates resistance, toxic residues, and environmental contamination. The biological control of bioactive substances has wide applications. In this study, the fungicidal activity of the extract obtained from *Annona glabra* L. seeds was evaluated as a control alternative for *Fusarium solani*. In addition, chemical characterization was carried out to identify the groups of metabolites present in the extract. The fungicidal activity against *F. solani* was evaluated using the microdilution method, and the minimum inhibitory concentration (MIC) was established, as well as the mean inhibitory concentration (IC_{50}). The IC_{50} of the extract was 972.24 mg L^{-1}, and the MIC was 12.02 mg L^{-1}. Using chemical characterization by ultrahigh-efficiency liquid chromatography coupled with mass spectrometry, the presence of THF acetogeniones, isoquinolinic alkaloids, and kaurene-type diterpenes, which were the main compounds responsible for the activity evaluated, was confirmed. The results obtained provide evidence that *A. glabra* seed extracts can be a potential source of metabolites with fungicidal activity.

Keywords: Annonaceae. Acetogenins. Alkaloids. Biological control. Terpenoids.

RESUMO – O gênero *Fusarium* é causador de doenças em diversas culturas agrícolas em todo o mundo com perdas econômicas significativas. Os fungicidas químicos são a principal estratégia de controle, mas o seu uso intensivo e prolongado gera resistência, resíduos tóxicos e contaminação ambiental. O controle biológico a partir de substâncias bioativas tem ampla aplicação. Neste estudo, a atividade fungicida do extrato obtido de *Annona glabra* L. como alternativa ao controle de *Fusarium solani*. Além disso, foi realizada uma caracterização química com o intuito de identificar os grupos de metabólitos presentes no extrato. A atividade fungicida contra *F. solani* foi avaliada pelo método de microdiluição e foi estabelecida a concentração inibitória mínima (CIM) e a concentração inibitória média (IC_{50}). Os resultados da atividade fungicida mostraram que o IC_{50} do extrato foi de 972.24 mg L^{-1} e o MIC foi de 12.02 mg L^{-1}. Através da caracterização química por cromatografia líquida de ultra-alta eficiência acoplada à espectrometria de massas (UHPLC-MS/MS), foi confirmada a presença de acetogênios THF, alcaloides isoquinolínicos e diterpenos do tipo caureno, que são os principais responsáveis pela atividade avaliada. Os resultados obtidos fornecem evidências de que os extratos de sementes de *A. glabra* podem ser uma fonte potencial de metabólitos com atividade fungicida contra *F. solani*.

Palavras-chave: Annonaceae. Acetogeninas. Alcaloides. Controle biológico. Terpenoides.

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INTRODUCTION

Fusarium is a genus of filamentous ascomycete fungi that are distributed in the soil as saprophytes and associated with plants as parasites. Worldwide, these microorganisms have negative impacts on crops and mainly affect agricultural and ornamental plants (XIA et al., 2020). Diseases caused by these microorganisms include wilt, blight, rot, and cankers in different parts of the plant, such as stems, roots, and fruit (GONZÁLEZ-RUÍZ et al. 2021). It also produces a wide variety of toxins that can contaminate agricultural products, making them unsuitable for food or feed (MA et al., 2013).

The wide distribution of the genus *Fusarium* has been attributed to the ability of these fungi to grow on different substrates and to their efficient mechanisms for spore dispersal (OKUNGBOWA; SHITTU, 2012). This genus contains more than 20 identified species (LI et al., 2022), and the most common species include *F. solani*, *F. oxysporum*, *F. equisetti*, and *F. chlamydosporum*.

Fusarium solani is a filamentous fungus found on the roots, stems, fruit, seeds, and soil of susceptible plants, and it can cause root or stem rot in crops, forests, and vegetables. It is a specific pathogen that affects several plants of agricultural importance, such as peas, cucurbits, sweet potatoes, papayas, cucumbers, and tomatoes. However, it has been associated with infections in humans and other animals, especially with invasive mycoses, causing systemic infections with a high mortality rate (LI et al., 2022).

The control of species of the genus *Fusarium* is generally carried out by chemical methods, such as fumigation of the soil with 1,3-dichloropropene,

chloropicrin, and benomyl (NEL et al. 2007). This generates negative impacts on the ecosystem and the development of resistant strains (FU et al., 2017). Therefore, the use of bioactive substances has been evaluated as a strategy for their control and management (LI et al., 2022).

In this context, some properties and compounds of different plants have been studied, such as those of the Annonaceae family, which are characterized as containing metabolites, such as acetogenins, phenolic compounds, alkaloids, and terpenes (LEITE et al., 2020), with possible antimicrobial, antiprotozoal, insecticidal, and larvicidal activities (LEITE et al., 2020). Therefore, the objective of this study was to evaluate the fungicidal activity of the polar extracts of *Annona glabra* (common name pond apple) seeds against *F. solani* and their corresponding chemical characterization since studies have demonstrated their antimicrobial power (KHALAF et al., 2023).

MATERIALS AND METHODS

Plant material

Annona glabra seeds supplied by the company WANACOL SAS from the Samaria farm located in the north of the valley were used with georeferencing (4° 41 '11.77 N; 76° 2' 42.05 W). The fruit was transported to the laboratory of the Biotechnology and Natural Products Research Group (GBPN) of the Universidad Tecnológica de Pereira. The taxonomic identification of the plant was carried out by the Herbarium Universidad del Quindío (HUQ).

Obtaining the extract

The seeds were washed with TEGO 51 soap, oven dried, processed in an electric knife mill, and stored at −4°C. The ground seeds were subjected to a passive maceration process for one week with regular stirring at room temperature. Ethanol was used as the solvent in a sample:solvent ratio of 1:4. The extract was subsequently rotated under reduced pressure and stored at −4°C for further analysis (GIRALDO-RIVERA; GUERRERO-ÁLVAREZ, 2018).

Evaluation of fungicidal activity

Preparation of the inoculum: The inoculum was prepared using *F. solani* (ATTC 11712) incubated on Sabouraud agar at 29°C for 7 days with a 12 h photoperiod. The inoculum was standardized in a concentration range of 1×10^6 to 5×10^6 CFU mL^{-1} (ROBLES-YERENA et al., 2016), as determined by turbimetry in DensiCHEK TM Plus.

Determination of fungicidal activity: Fungicidal activity was determined following the methodology described by the Clinical and Laboratory Standards Institute (CLSI, 2008) for filamentous fungi. A stock solution of 2000 mg L^{-1} of the *A. glabra* L. seed extract was prepared using a solution of dimethyl sulfoxide (DMSO) and distilled water in a 1:10 ratio as the solvent. Two hundred microliters of the stock solution were deposited in 96-well sterile plates, and successive dilutions were prepared with Sabouraud liquid medium until the working concentration range (60–1000 mg L^{-1}) was obtained.

Once the plates were prepared with the extract, each well was inoculated with 10 µL of the spore suspension. The volume of the mixture was 210 µL (10 µL inoculum, 100 µL extract, and 100 µL culture medium). The plates were maintained at 26°C for 48 h in the dark. The commercial antifungal ketoconazole was used as a positive control at a concentration of 1000 mg L^{-1}. A mixture of water and DMSO in a 1:10 ratio was used as a solvent control. Sabouraud broth without extract or antifungal compounds was used as a negative control, and a sterility control (without inoculation) was used.

Each bioassay was performed in triplicate in a completely randomized design. The results were determined by the spectrophotometric method from the optical density (OD) at 450 nm for each of the wells using the UV–Vis spectrophotometer for Multiskan GO microplate with a cuvette 100–240 V (Thermo Fisher Scientific Corporation). The effect of the extracts was established as the percent inhibition of the growth of the fungus based on optical density. The minimum inhibitory concentration (MIC) and mean inhibitory concentration (IC_{50}) were determined; additionally, analysis of variance (ANOVA) was performed to establish differences between the evaluated concentrations.

The percentage of inhibition of the growth of the fungus was determined based on the optical density. For this, the optical density value of the growth control well was taken as 100% growth, and the value of the solvent blank was subtracted from the extracts. The MIC and IC_{50} were determined using the microdilution method. MIC was defined as the lowest concentration inhibiting visible fungal growth after incubation, and IC_{50} was determined by probit regression analysis and defined as the concentration at which the extract was capable of inhibiting 50% of the growth of the fungus.

Chemical characterization

Analysis by ultrahigh resolution liquid chromatography coupled to UHPLC–MS/MS mass spectrometry: For the analysis, an UltiMate 3000 UHPLC system (ThermoFisher, San José, CA, USA) was used, coupled to a UHR-QqTOF Impact II mass spectrometer (BRUKER, Billerica, MA, USA) with an electrospray ionization source in positive ion mode (ESI^+). A Hydro-RP 100° column (2 × 100 mm × 2.5 µm) was used. Acetonitrile (CH_3CN):water (H_2O) (both solvents acidified with 0.1% formic acid) was used as the mobile phase, using the following gradient: 75% CH_3CN for 10 minutes; subsequently, an increase was made linear until reaching 95% acetonitrile in minute 12 and was kept in this composition for 4 min, for a total running time of 16 minutes. The flow rate was 400 µL min^{-1}, and the injection volume was 10 µL. The samples were dissolved in acetonitrile grade HPLC–MS with 0.1% formic acid and prepared at a concentration of 500 mg L^{-1}.

The TOF data were collected at an accessible mass/load (m/z) that ranged from 50 to 1200. The source parameters were established as follows: gas temperature set at 200°C, drying gas at 8 L min^{-1}, nebulizer pressure at 2 bar, capillary voltage at 4500 V, and fragmenting voltage at

2000 V. It was calibrated with a sodium formate solution infused after the column using reference masses between m/z 90.9766 and 1518.7125 to obtain high precision. Each ion chromatogram was obtained using a theoretical m/z with a symmetric window width of ± 5 ppm.

A search was conducted on the main compounds reported to have fungicidal activity (D'ELIA OLIVEIRA et al., 2022). The precursor ions were searched according to the m/z ratio of the analytes of interest, the fragmentation patterns of the most abundant ions were analyzed, and they were compared with the theoretical structures according to the Human Metabolome Database (HMDB).

Chemical identification of the extract by UHPLC–MS/MS was performed by selection of chromatographic peaks, with subsequent determination of the formula according to the exact mass and isotope pattern (MS1) and comparison of the spectrum database of composite fragments (MS2) (METLINE, GNPS, MassBank).

RESULTS AND DISCUSSION

Fungicidal activity against *Fusarium solani*

The *A. glabra* seed extract exhibited activity against *F. solani* using the microdilution method in a concentration range of 60–1000 mg L^{-1} (Figure 1). Based on these results, the IC_{50} was determined. The extract reached the highest fungicidal activity at a concentration of 1000 mg L^{-1}, with 50.58% inhibition.

There was a linear relationship between the concentration and the percentage of inhibition, and the higher the concentration, the higher the percentage of inhibition, as shown in Figure 1.

Through probit regression analysis, the IC_{50} was determined, finding a value of 972.24 mg L^{-1}. Through the ANOVA with a significance of 5%, it is evident that there were significant differences in the evaluated concentrations of the seed extract.

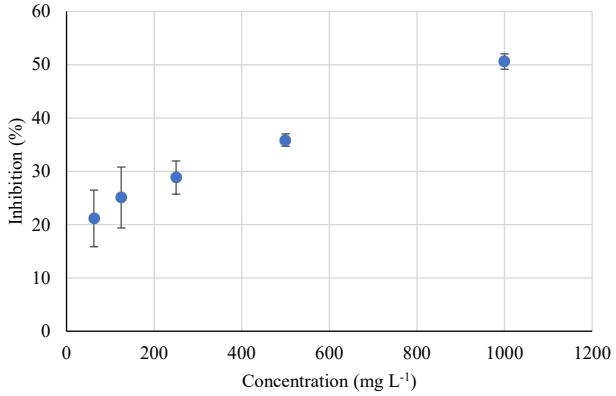

Figure 1. Inhibitory activity of Annona glabra seed extract against Fusarium solani.

Figure 2 presents the results for the determination of MIC in a concentration range of 0.9–60 mg L^{-1}. An MIC of 12.02 mg L^{-1} was obtained. According to da Silva Andrade et al. (2018), the antimicrobial efficacy of the extract could be classified as good because it presented an MIC < 100 µg mL^{-1}.

As shown in this study, the species *A. glabra* showed antifungal activity against *F. solani*. In the same way, various studies on the same plant species have reported inhibitory effects on the mycelial growth of *Fusarium oxysporum* (NGUYEN; NGUYEN; LAM, 2017) and inhibition of spore germination of *Fusarium lateritium* (PADMAJA et al., 1995).

However, for the control of *F. solani*, different extracts from species of the Annonaceae family have been evaluated, among which *Annona muricata* (CASTILLO-REYES et al., 2022), *Annona squamosa* (KALIDINDI et al.,2015), and *Annona cherimola* (OCHOA-FUENTES et al., 2012), stand out.

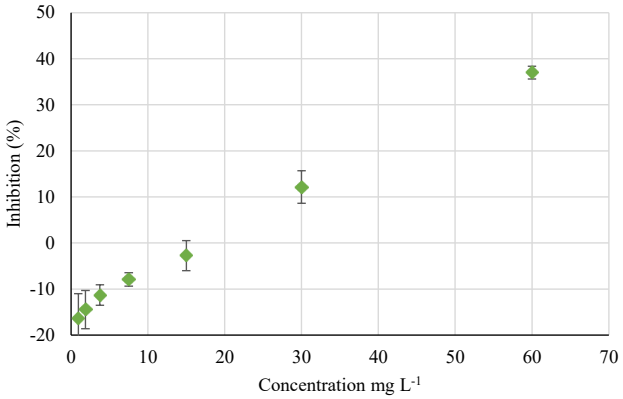

Figure 2. Analysis of the inhibitory activity for the determination of the minimum inhibitory concentration (MIC).

Chemical characterization

The results obtained from the chemical characterization by UHPLC–MS/MS the *A. glabra* seed extract are shown in Table 1. By means of chemical analysis, 3 acetogenins, 3 isoquinolinic alkaloids, and 2 terpenoids were identified.

Based on the results obtained in this study (Table 1), it was not possible to precisely define the compounds as acetogenins. However, a tentative identification of the structures was proposed based on the crude extract of *A. glabra* seeds and results reported by other authors for the same species.

Glabrazin A and glabrazin B are bis-THF acetogenins reported by Liu et al. (1998a). However, this same chemical formula may correspond to Compound 27-hydroxybulatacin, which has been reported by Liu, Pilarinou, and Mclaughlin (1999) for this same species.

Liu et al. (1998b) identified glacin A and glacin B, which are mono-THF acetogenins in *A. glabra,* which may correspond to the chemical formula defined in Table 1.

Table 1. Tentative identification of compounds in Annona glabra seed extract by HPLC–MS/MS.

Compound type	Retention time (Rt)	[MH]	Calculated mass	Theoretical mass	Fragments	Formula	Tentative Identification
Acetogenins	18–18.7	639.483	638.4752	638.4752	(621.4723) (603.4689)	$C_{37}H_{66}O_8$	27-hydroxybullation glabration-A glabration-B
	20.2–21	595.4567	594.4488	594.4496	(577.4429) (559.4275) (541.4248)	$C_{35}H_{62}O_7$	6-OH-desacetyl-uvaricin
	22.3	597.4722	596.4652	596.4643	(561.4465) (543.4409) (525.4299)	$C_{35}H_{64}0_7$	glacin-A glacin-B
Isoquinolin Alkaloids	8.3–8.6	268.133	267.1252	267.1259	(251.1064) (219.0799) (189.0699) (165.0714)	$C_{17}H_{17}NO_2$	Asimilobin
	10.1–10.4	284.1282	283.1204	283.1208	(147.0440) (121.0670) (91.0538)	$C_{17}H_{17}NO_3$	Anaxagorein
	10.4–10.6	314.1385	313.1306	313.1314	(177.0550) (121.0646) (91.0521)	$C_{18}H_{19}NO_4$	Muricinin
Terpenoids	11–11.1	363.2526	362.2447	362.2457	(287.2379) (193.1205) (93.0695)	$C_{22}H_{34}O_4$	Anoglabasin B
	17.7–17.8	407.2426	407.2347	406.2355	(301.2156) 287.2034) (241.1950)	$C_{23}H_{34}O_5$	Anoglabasin C

Although the Formula $C_{35}H_{62}O_7$ corresponds to different acetogenins reported in different Annonaceae species, it has been identified as 6-OH-desacetyl-uvaricin in *A. glabra* by Liu, Pilarinou and Mc Laughlin (2000) and corresponds to an adjacent bis-THF acetogenin.

The difficulty in proposing a single name for the mentioned compounds is due to the differences that exist between the substituents along the chain, as well as the spatial differences and configurations of the THF rings that these types of compounds present (MÉNDEZ-CHÁVEZ et al., 2022).

Acetogenins can cause microbial inhibition by adhesion to the cell surface or diffusion into fungal cells (LÓPEZ-ROMERO et al., 2022). Antifungal substances in an extract can inactivate the function of the genetic material by interfering with the formation of nucleic acids (DNA and RNA) (LÓPEZ-ROMERO et al., 2022). Higher antifungal activity could be attributed to bis-type acetogenins tetrahydrofuran having two tetrahydrofuran (THF) rings, which could be because the function of the THF ring is to anchor the glycerol region contained in the liposomal membrane (MÉNDEZ-CHÁVEZ et al., 2022).

However, chemical studies of the Annonaceae family have shown that alkaloids and acetogenins are the main chemical constituents detected in this family (LÚCIO et al., 2015).

The tentative identification of the alkaloids in *A. glabra* for compounds such as asymolobin has been reported by Lee et al. (2015). Anaxagorein has been identified in species such as *Anaxagorea dolichocarpa* (HOCZUEMILLER; RASAMIZAFYS; MORETTI, 1981) and *Cananga odorata* (HSIEH, CHANG; WU, 1999). Muricinin has been identified in *Annona muricata* (MANSKE, 1954). All of these species belong to the Annonaceae family. Regarding the mode of action of isoquinolinic alkaloids against different fungi, the quaternary nitrogen atom, alkyl substituent, and methylenedioxy are key factors for their activity (QING et al., 2017).

Finally, the presence of two kaurene-type diterpenes was identified, which correspond to anoglabasin B (D'ELIA OLIVEIRA et al., 2022) and anoglabasin C (CHEN et al., 2000) and have been previously reported in seeds of *A. glabra*.

According to previous studies, diterpenes can be directly or indirectly involved in blocking Ca^{2+} channels to exert their fungicidal activity (VILLA-RUANO; LOZOYA-GLORIA; PACHECO-HERNÁNDEZ, 2016).

CONCLUSION

The seed extract of *A. glabra* showed activity against *F. solani* with an IC_{50} of 972.24 mg L^{-1}. In addition, the MIC was in the range of 10–20 mg L^{-1}.

The seed extract of *A. glabra* can be an important source of natural compounds with antifungal potential against *F. solani*. This activity is possibly attributed to the presence of compounds such as THF acetogeniones, isoquinolinic alkaloids, and kaurene-type diterpenes.

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