

Detection, quantification, and toxicity evaluation of bioactive compounds produced by *Fusarium oxysporum*

Detección, cuantificación y evaluación de la toxicidad de los compuestos bioactivos producidos por *Fusarium oxysporum*

Jaime Marcial-Quino^{1*}, Fernando Gómez-Chávez², José Alberto Mendoza-Espinoza¹ and Edgar Sierra-Palacios¹

¹ Academia de Biología, Colegio de Ciencias y Humanidades. Universidad Autónoma de la Ciudad de México, Ciudad de México 09620, Mexico.

² Laboratorio de Enfermedades Osteoarticulares e Inmunológicas, Sección de Estudios de Posgrado e Investigación, ENMH—IPN, 07320 CDMX, Mexico.

ABSTRACT

Fusarium species are common plant pathogens. In this study, a strain isolated from infected bean plants was molecularly identified as *Fusarium oxysporum* by polymerase chain reaction (PCR) amplification and sequencing of the large subunit ribosomal RNA (LSU rRNA) and triosephosphate isomerase (*tpi*) gene regions, showing 100 % identity. The fungus was cultivated in a liquid medium to produce biomass, from which intracellular metabolites were extracted using five solvents. The filtered broth was then used as an extracellular extract. Total phenol quantification indicated 5.4 mg gallic acid equivalents/g mgGAE/g in the intracellular aqueous extract and 2.8 mg GAE/g extracellularly. Similarly, flavonoid quantification revealed higher concentrations in intracellular extracts, particularly in water with 1.77 mg quercetin equivalents per gram (mg QE/g), and acetone (1.56 mg QE/g). No flavonoids were quantified in the filtered broths. Antioxidant activity was also significantly higher in aqueous intracellular extracts, with three times the activity of extracellular extracts. Furthermore, the aqueous and methanolic extracts did not show any toxic effects on *Artemia salina*. This supports their potential use for *in vitro* assays on cell lines. These findings imply that *F. oxysporum* produces antioxidants and/or metabolites that could be further investigated for application in pharmaceutical or agricultural bioproducts.

Keywords: Molecular identification; *tpi* gene; *Fusarium sp*; fungal extracts; bioactive compounds.

RESUMEN

Las especies de *Fusarium* son patógenos comunes de las plantas. En este estudio, una cepa aislada de plantas de frijol se identificó molecularmente como *Fusarium oxysporum* mediante amplificación por reacción en cadena de la polimerasa (PCR) y secuenciación de las regiones génicas de la subunidad grande del ARN ribosómico (LSU ARNr) y la triosa-fosfato isomerasa (*tim*), mostrando una identidad del 100 %. El hongo se cultivó en medio líquido para producir biomasa, y extraer metabolitos usando cinco solventes. El caldo filtrado se utilizó como extracto extracelular. La cuantificación de fenoles totales indicó 5.4 mg equivalentes de ácido gálico/g

(mg EAG/g) en extractos acuosos intracelulares y 2.8 mg EAG/g extracelularmente. Los flavonoides, registraron mayores concentraciones en extractos intracelulares, en agua 1,77 mg equivalentes de quercetina por gramo (mg EQ/g) y acetona (1,56 mg EQ/g). No se cuantificaron flavonoides en medios filtrados. La actividad antioxidante también fue significativamente mayor en los extractos intracelulares acuosos, mostrando tres veces más actividad que los extracelulares. Además, los extractos acuosos y metanólicos no mostraron efectos tóxicos sobre *Artemia salina*, lo que respalda su uso para ensayos *in vitro* en líneas celulares. Estos hallazgos implican que *F. oxysporum* produce antioxidantes y/o metabolitos que podrían investigarse más a fondo para su aplicación en bioproductos farmacéuticos o agrícolas.

Palabras clave: Identificación molecular; *tim* gene; *Fusarium sp*; extractos fúngicos; compuestos bioactivos

INTRODUCTION

Fungi play a crucial role in the environment by aiding in the decomposition of plant organic matter, which in turn helps with the recycling of nutrients (Li *et al.*, 2022). For instance, fungi known as saprophytes use various enzymatic activities to break down dead organic matter, specifically plant cell wall components like polysaccharides (chitin) and polymers such as lignin, cellulose, hemicellulose, and pectin (Kumar and Chandra, 2020; Ortega-Ávila *et al.*, 2023). In addition, fungal activity is not limited to degrading phenolic compounds present in lignin. They also have the ability to catalyze a wide range of phenolic compounds that contaminate the environment (González-González *et al.*, 2023). The function of other fungi, specifically mycorrhizal fungi present in the soil, is their symbiosis with plants. They aid in the absorption of mineral nutrients and also play a role in the carbon balance of forest ecosystems (Li *et al.*, 2022).

Within the diverse world of fungi, some species can cause damage to various plants, known as phytopathogenic fungi. Ascomycetes and basidiomycetes are among the main phytopathogens (Sharma and Marques, 2018). These microorganisms utilize different strategies to colonize plants and induce specific diseases. This effect is caused by the pre-

*Author for correspondence: Edgar Sierra-Palacios; Jaime Marcial-Quino
e-mail: edgar.sierra@uacm.edu.mx; jmarcialq@ciencias.unam.mx

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sence of compounds (enzymes, toxins, growth regulators) that are produced when phytopathogenic fungi come into contact with plants (Peng *et al.*, 2021). Therefore, these fungi represent some of the most diverse groups that cause ecological and economic problems.

About 100 species of *Fusarium* have been described (Tapia and Amaro, 2014). These fungi are cosmopolitan and are distributed in soils of tropical and subtropical areas, although they have also been found in cold climates. Out of these 100 species, more than 80 affect plants of agricultural interest. Some of the most representative species that affect plants include *Fusarium oxysporum*, *F. solani*, *F. verticillioides* and *F. moniliforme*. In addition, *F. oxysporum* and *F. graminearum* are considered to be the most pathogenic species, causing wilting and rotting in various agricultural crops (Dean *et al.*, 2012). Some examples include oats, corn, wheat, rice, barley, beans, bananas, chili, potatoes, asparagus and mango fruit trees (Amuzu *et al.*, 2024). It has been reported that toxins produced by fungi of the *Fusarium* genus are toxic to humans and animals (Tapia and Amaro, 2014). *F. oxysporum*, *F. verticillioides* and *F. solani* are pathogenic species for humans, especially immunocompromised patients, causing 70 % of infections of this type (Nucci *et al.*, 2007). These strains of *Fusarium* are identified according to certain characteristics. These fungi produce cottony mycelium which can be flat or star-shaped. When growing on a plate, they can present different colors such as purple, brown, red or colorless depending on the species (Benadof, 2010). However, these morphological characteristics alone are not enough for accurate identification and taxonomic classification. For this reason, various techniques are still being used to identify *Fusarium* species. These techniques range from their associations with host plants, other morphological characteristics like size (macro and micro) and shape of conidia, as well as absence and/or presence, including the shape of chlamydospores (Rentería-Martínez *et al.*, 2019). Biochemical tests have also been utilized, and more recently, molecular techniques have been employed. These molecular techniques are based on different markers such as the internal transcribed spacer (ITS), small subunit ribosomal RNA (SSU), and large subunit (LSU) nuclear DNA region (Hernández-Ochoa *et al.*, 2020).

Despite the various negative effects caused by fungi of the *Fusarium* genus and the importance of their identification, their relevance lies in the discovery of other properties, such as the production of secondary metabolites and their biotechnological potential (Ibrahim *et al.*, 2021). Recently, Amazu *et al.* (2024) reported that species of *Fusarium* fungi, including those from soil, phytopathogenic, plant endophytic, and marine sources, produce compounds such as terpenoids, polyketides, steroids, and phenolics. These metabolites have been found to have phytotoxic, antibacterial, antifungal, antimalarial, anti-inflammatory, and cytotoxic effects.

In this study, we were interested in evaluating the compounds produced by a phytopathogenic fungus that affects bean crops in Mexico. The strain was identified as *Fusarium*

oxysporum at the molecular level, by sequencing the large subunit of ribosomal RNA (LSU rRNA) and the triosephosphate isomerase (*tpi*) gene. It was found that in liquid culture, the fungus has the ability to produce bioactive compounds that may have biotechnological applications in the medical field. The results obtained from the strain provide valuable information that can be used to isolate specific molecules for further characterization and analysis in the future.

MATERIAL AND METHODS

Fungal strain

The fungus utilized in this study was generously provided by Dr. Marcelo Acosta Ramos from the Department of Agricultural Parasitology at the Universidad Autónoma Chapingo (UACH). The *Fusarium* sp strain was isolated from bean plants infected by this strain in crops located in the Municipio de Texcoco, Estado de México.

Culture conditions

The *Fusarium* sp strain was cultivated in Petri dishes containing Potato Dextrose Agar (PDA) medium to promote mycelial development. The mycelium grown on the plates was then used to inoculate Potato-Dextrose (PD) liquid media. The mycelium from the liquid cultures was utilized for metabolite extraction (Marcial-Quino *et al.*, 2024). The mycelium was stored at - 20 °C until needed.

DNA extraction from the fungus *Fusarium* sp

Mycelium from the fungus grown in a liquid medium (PD) was used for DNA extraction. The mycelium was washed with a 0.9 % NaCl solution, then the mycelium was transferred to a sterile mortar and ground with 2 mL of phosphate buffer (PBS). Next, 1 mL of the extract was recovered and placed in a sterile Eppendorf tube. The extraction process was then continued using the commercial AxyPrep Multisource Genomic DNA Miniprep kit, following the protocol instructions.

PCR conditions

For molecular identification of the fungus, primers targeting a fragment of the large-subunit ribosomal RNA (LSU rRNA) region were used. The primers used were LSU-F: 5'-acc-cgctgaacttaagc-3' and LSU-R: 5'-tcctgagggaaacttcg-3. The triosephosphate isomerase (*tpi*) gene was also analyzed using the primers TPI-F: 5'-atggctcgcaagttcttcgt-3 and TPI-R: 5'-ttactgcttgtagc(g/a)ttga-3' (Hernández-Ochoa *et al.*, 2020). The PCR mixture was prepared in a final volume of 25 µL consisting of: DNA (40 ng), enzyme buffer (1X), MgCl₂ (1.5 mM), dNTPs (0.2 mM), primers (each 0.2 µM) and 1.25 U of Vent polymerase enzyme (New England Biolab, USA) (Marcial-Quino *et al.*, 2016). The PCR reaction was carried out in a MaxyGene II[®] thermocycler (Axygen, USA), following the conditions previously described by Cortés-Maldonado *et al.* (2020): initial denaturation at 94 °C for 3 min, followed by 30 cycles (denaturation at 94 °C/30 seconds, annealing at 54 °C/45 seconds, polymerization at 72°C/1.5 min) and a final step at 72 °C for 5 min.

The extracted DNA and PCR fragments were run on 1.0 % (w/v) agarose gels. Genomic DNA and amplicons were stained with Gel-Red (Biotin) and documented using UVP equipment. Additionally, DNA concentration was measured with a Nanodrop (Thermo Scientific, USA). The products were then cloned into the pJET1.2/blunt vector (Thermo Scientific, USA) for sequencing (Santos *et al.*, 2017). Sequencing was carried out using the forward primer (5'-cgactcactataggga-gagcggc-3') and reverse primer (5'-aagaacatcgatttccatgg-cag-3') at the Instituto de Biología of the Universidad Nacional Autónoma de México.

Obtaining extracts from *Fusarium* sp

To obtain the fungal extract, 200 to 250 g of moist mycelium were used, obtained from a 2 L culture. The mycelium was separated from the culture broth by filtration using Whatman No. 1 filter paper. Subsequently, the mycelium was ground with 350 mL of solvent. The solvents used included acetone, ethyl acetate, methanol, hexane and distilled water. All extracts were left to macerate for 15 days at room temperature with daily manual agitation. After the maceration period, the extract was filtered twice, and the liquid fraction was then placed in a rotary evaporator (Buchi) for solvent separation. The distillation temperature varied depending on the evaporation point of each solvent used. The extracts obtained were then used for chemical evaluations (presence of anthraquinones, alkaloids, tannins, saponins and coumarins) and pharmacological evaluations (quantifying total flavonoids, total phenols, DPPH and toxicity in the *Artemia salina* model).

Qualitative detection of compounds

Anthraquinones and alkaloids were identified using thin layer chromatography (TLC) as described by Aarland *et al.* (2015). Silica gel 60F254 plates measuring 3 × 5 cm were used for TLC, with 0.5 µL of the extracts applied to the plates. The plates were developed in a solvent system of dichloromethane-methanol in a 95:5 ratio. Detection of both compounds was achieved using Dragendorff reagent. Only the plates for anthraquinones were exposed to UV light. Anthraquinones appeared as yellow or red stains, while alkaloids appeared as red-brown stains.

Saponins were detected by using 2 mL of the extract in 10 mL of distilled water. The tubes containing the extracts were heated for 30 min at 80 °C, then cooled to room temperature before being vigorously shaken. Stable foams formed over 15 to 20 min, indicating the presence of saponins (Peralta *et al.*, 2013).

To detect tannins, three different solutions were prepared, each with a final volume of 10 mL. Tube 1 contained a 1 % gelatin solution, Tube 2 contained a 1 % gelatin solution with 10 % NaCl, and Tube 3 contained only 10 % NaCl. Two mL of each fungal extract were added to their respective tubes and mixed vigorously using a vortex. The presence of tannins was indicated by the formation of a white precipitate in tubes 1 and 2 (Aarland *et al.*, 2015).

Coumarin detection was performed by dissolving 0.020 g of each extract in 10 mL of distilled water, then adding 0.1

g of NaCl. A piece of filter paper, previously moistened in a 0.5 % NaOH solution, was then placed at the mouth of the test tube. The tube was subsequently placed in a water bath until it reached boiling. After 3 min, the filter paper was removed and allowed to dry completely. The paper was then exposed to UV light; the presence of a fluorescent yellow color indicated the presence of coumarins (Peralta *et al.*, 2013).

Flavonoids quantification

The flavonoid content was assessed by quantifying quercetin as a standard, following the method outlined by Cervantes-Arista *et al.* (2020). To measure total flavonoids, varying amounts of lyophilized fungal extracts (ranging from 0.0214 to 0.121 g) were mixed with 0.4 mL of distilled water and 1.6 mL of methanol in tubes. A 0.5 mL aliquot of this mixture was then combined with 1.5 mL of methanol, 100 µL of aluminum chloride (10 % w/v), 100 µL of potassium acetate (1M), and 2,800 µL of distilled water for analysis. The tube mixtures were allowed to stand for 30 min. Absorbance was measured at 415 nm using a Jenway® spectrophotometer. The results were expressed in mg quercetin equivalents per gram (mg QE/g) of dry extract.

Total phenol quantification

Total phenols were quantified using the Folin-Ciocalteu reagent. Mushroom extracts weighing 0.0032 to 0.0101 g were mixed with 2 mL of 80 % (v/v) methanol in test tubes for one minute. Subsequently, 200 µL of the reaction mixture from each tube was combined with 1 mL of Folin-Ciocalteu reagent (diluted 1:10 in distilled water) and allowed to sit for one minute. Following this, 800 µL of 7.5 % (w/v) anhydrous carbonate were added, mixed, and left to stand at room temperature for 1 hour. The absorbance was measured at 715 nm using a Jenway® spectrophotometer. The total phenolic content was expressed as mg gallic acid equivalents per gram (mg GAE/g) of dry extract (Marcial-Quino *et al.*, 2024).

Determination of antioxidant activity using DPPH

The antioxidant capacity was determined using a colorimetric method with the reagent 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Brand-Williams *et al.*, 1995). A stock solution of DPPH (0.012 g in 50 mL of methanol) was prepared, and its absorbance was adjusted to 1.1 at 515 nm. The stock solution was diluted 1:5 for the assays.

On the other hand, lyophilized extracts (0.010 g) were diluted in 1 mL of distilled water. Fractions of 100 µL of the extracts were taken and adjusted to 1 mL with the diluted DPPH solution. The mixtures were incubated in the dark for 30 min, then measured at 515 nm. The results were reported as mg ascorbic acid equivalents per gram (mg AAE/g) of dry extract.

In vitro toxicity assessment

Artemia salina was used for the *in vitro* toxicity assessment. In a crystallizer, 185 mg of cysts were hydrated with distilled water for 1 hour with aeration. Any floating cysts were removed.

The cysts were then transferred to a decapsulating solution consisting of 1.3 mL of brine (10 g of sea salt in 500 mL of distilled water) with 0.0277 g of sodium hydroxide (NaOH) and 1.26 mL of 6 % commercial chlorine, where they were left for 7 to 10 min. Afterwards, the cysts were washed with distilled water and placed in a container with 500 mL of pH 8.0 brine (10 g of sea salt in 500 mL of distilled water) and incubated at 28 °C, with light and oxygen for 24 h (Aarland *et al.*, 2015). Three replicates were performed for each of the concentrations used. These dilutions were added to 10 mL of brine, and 10 *A. salina* nauplii were added. The samples were incubated for 24 h at room temperature. Finally, the number of dead *A. salina* nauplii was observed and quantified to determine the percentage of median lethal concentration (LD_{50}). The analyses were performed in the GraphPad PRISM program.

Sequence analysis and phylogeny

In this study, the strain was identified by analyzing the sequences of the LSU region and the *tpi* gene. The search was conducted using BLAST in GenBank (National Center for Biotechnology Information/NCBI). Multiple alignments of the LSU and *tpi* gene sequences were carried out using ClustalW from the BioEdit program (Hall *et al.*, 1999). Subsequently, MEGA X was utilized to construct the phylogenetic tree (Kumar *et al.*, 2018).

Statistical analysis

Quantitative and qualitative assays were performed in triplicate. The results were presented as mean $\pm$ standard deviation (SD). The data was analyzed using a one-way ANOVA, followed by Tukey's test. Values with $p < 0.05$ were considered significantly different. The data was analyzed and graphed using GraphPad Prism 5 software.

RESULTS

Molecular identification of *Fusarium* sp

DNA was extracted from fresh mycelium obtained from liquid cultures, resulting in approximately 22.5 $\mu\text{g mL}^{-1}$. To identify the fungus at the molecular level, nucleotide regions corresponding to LSU and *tpi* were amplified by PCR endpoint. The sequence obtained from the LSU fragment, according to BLAST analysis, showed a 97 % identity with *Fusarium oxysporum* (KF181210). However, when the nucleotide sequence of the triosephosphate isomerase (TPI) protein encoding gene was utilized as a molecular marker, the result indicated a 100 % identity with the deposited genomes of *F. oxysporum* f. sp. vasinfectum (CP130299.1), *F. oxysporum* f. sp. cubense (CP115878.1) and *F. oxysporum* f. sp. koae (CP052900.1). Moreover, an identity of 99.8 % was found with the genomes of *F. oxysporum* (CP052888.1), *F. oxysporum* Fo47 (CP052040.1) and *F. oxysporum* strain Fo5176 (CP128296.1). Even with the *tpi* gene, it was possible to generate a phylogenetic tree using the obtained sequence and sequences deposited in GenBank. This clearly showed the phylogenetic closeness of our strain to *F. oxysporum* and its separation from other *Fu-*

sarium species and other fungi (Fig. 1). Based on the sequencing results, BLAST analysis and phylogenetic relationships obtained, the strain will be referred to as *F. oxysporum* for the remainder of the study.

Qualitative chemical characterization

The biomass of *F. oxysporum* was obtained from cultures containing one liter of medium. For each liter of culture, an average of 214.16 g of wet mycelium was obtained. The biomass produced was resuspended in various in order solvents to obtain the extracts.

According to qualitative analysis, it was found that the detection of compounds changes depending on the solvent used. This is expected due to the diverse chemical properties of the solvents. The extracts obtained with solvents from biomass (intracellular fractions) showed the following data: a moderate presence of anthraquinones was recorded in ethyl acetate and acetone. However, tannin detection was moderate to high in most solvents used, including the culture broth (extracellular fraction). The lowest detection was observed in methanol. The tests indicated low detection of saponins and alkaloids, as well as no presence of coumarins (Table 1).

Quantification of total phenols

The highest concentrations of total phenols were obtained in the aqueous extract (5.4 mg GAE/g of extract), followed by the culture broth (extracellular) with a value of 2.8 mg GAE/g of extract (Fig. 2a). Both extracts were statistically different from the rest, with a p^{***} -value of 0.0001. Among the extracts obtained with the solvents, a lower amount of quantified phenols was observed, and there was a significant difference between them ($p^* = 0.0137$). The concentration of phenols obtained with solvents ranged from 1.1 to 1.8 mg GAE/g. The phenols produced by the *Fusarium oxysporum* strain are predominantly polar in nature.

Quantification of flavonoids

The highest concentrations of these compounds were found in the aqueous and acetone extracts, with values of 1.77 and 1.56 mg QE/g, respectively. The flavonoid concentrations in these extracts did not differ significantly, but they were significantly higher than those of the other extracts ($p^{***} = 0.0001$). The lowest flavonoid values were recorded in the extracts obtained with the solvents ethyl acetate, hexane, and methanol. According to statistical analysis, no significant differences were found ($p^* = 0.1240$). No flavonoids were detected in the filtered culture broth (extracellular fraction).

Antioxidant capacity

The results of this test indicated that the aqueous extract had the highest activity for DPPH, showing an antioxidant capacity value of 15.15 mg AAE/g, higher than the other extracts ($p^{***} = 0.0001$). This value was twice as high as the hexane extract (6.04 mg AAE/g) and approximately three times higher than the value obtained for the culture broth (5.6 mg AAE/g), as shown in Figure 3.

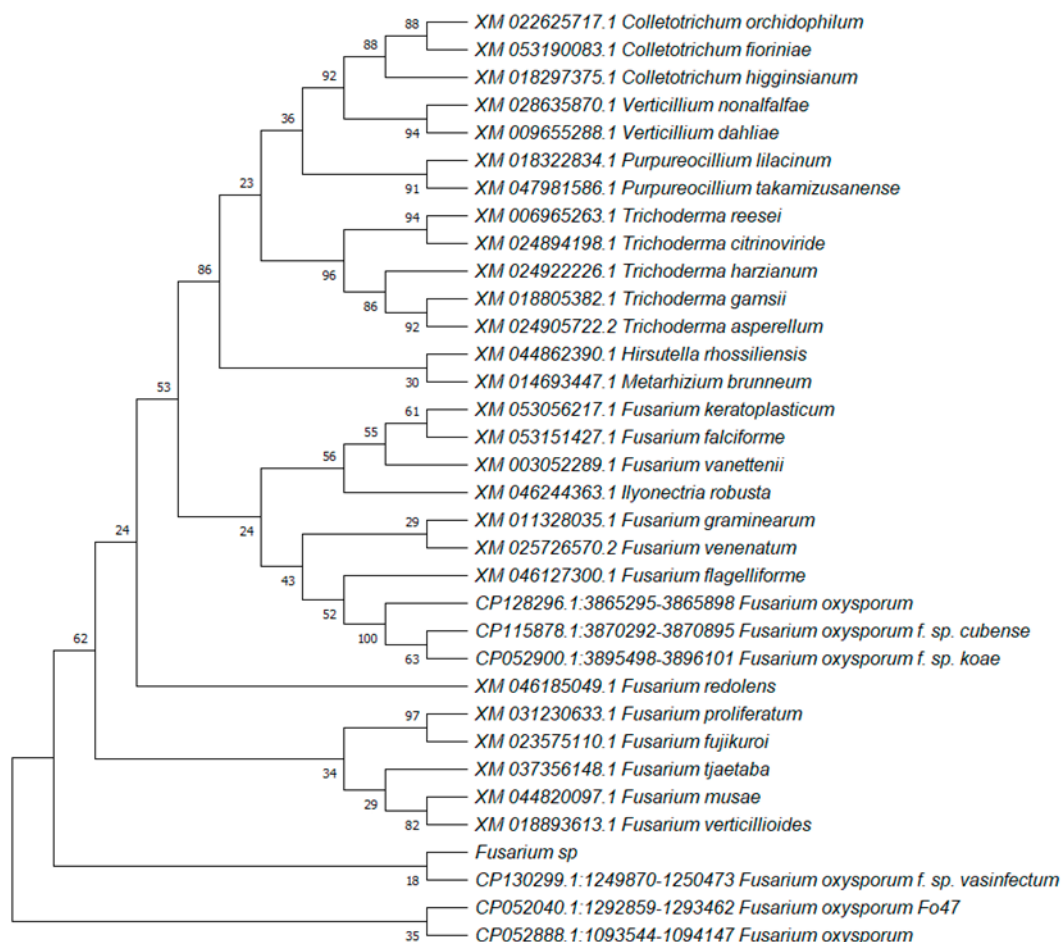


Figure 1. Phylogenetic tree inferred by Neighbor-Joining for partial *tpi* gene sequences of *Fusarium* species. The values at the nodes represent bootstrap scores based on 1,000 replicates. *Fusarium* sp refers to the strain used in this study. The evolutionary distances were obtained using the Tajima-Nei model (Tajima and Nei, 1984). The tree was constructed from 34 nucleotide sequences using MEGA X.

Figura. 1. Árbol filogenético inferido mediante el método Neighbor-Joining para secuencias parciales del *gen tim* de especies de *Fusarium*. Los valores en los nodos representan puntuaciones de bootstrap basadas en 1000 réplicas. *Fusarium* sp se refiere a la cepa utilizada en este estudio. Las distancias evolutivas se calcularon mediante el modelo Taji-ma-Nei (Tajima y Nei, 1984). El árbol se construyó a partir de 34 secuencias de nucleótidos utilizando MEGA X.

Table 1. Qualitative chemical analysis of extracts obtained from *F. oxysporum* using different solvents.

Table 1. Análisis químico cualitativo de extractos obtenidos a partir de *F. oxysporum* utilizando diferentes solventes.

Solvent	Anthraquinones	Alkaloids	Tannins	Coumarins	Saponins
Water	+	+	+++	-	++
Methanol	-	-	+	-	+
Ethyl acetate	++	+	++	-	-
Hexane	+	+	++	-	-
Acetato	++	+	++	-	-
Filtered broth	-	-	+++	-	++

Qualitative scale: Presence: low (+), moderate (++), high (+++), not detected (-)

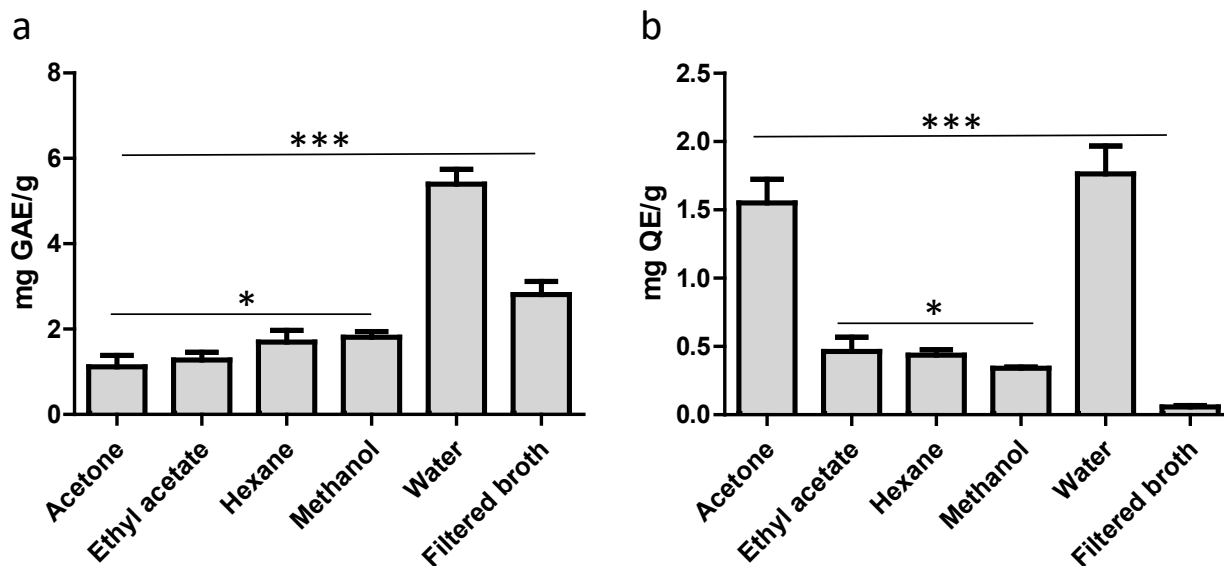


Figure 2. Measurement of compounds obtained from *F. oxysporum* biomass treated with different solvents (intracellular fractions) and the filtered culture broth (extracellular fraction). a) Concentration of total phenols and b) concentration of flavonoids. Statistical analysis was performed for all assays using ANOVA, followed by Tukey's test, with an alpha level of 0.05.

Figura 2. Medición de los compuestos obtenidos a partir biomasa de *F. oxysporum* tratada con diferentes solventes (fracciones intracelulares) y caldo filtrado (fracción extracelular). a) Concentración de fenoles totales y b) concentración de flavonoides. Se realizó un análisis estadístico para todos los ensayos mediante ANOVA, seguido de la prueba de Tukey, con alfa de 0.05.

Toxicity test on *Artemia salina*

An *in vitro* toxicity test was conducted using the *Artemia salina* model. The results showed that none of the extracts tested were lethal at concentrations up to 200 ppm. At this concentration, it was not possible to eliminate 50 % of the shrimp nauplii, as their survival rate was greater than 81 % (see Table 2). Therefore, it can be concluded that the LC_{50} is

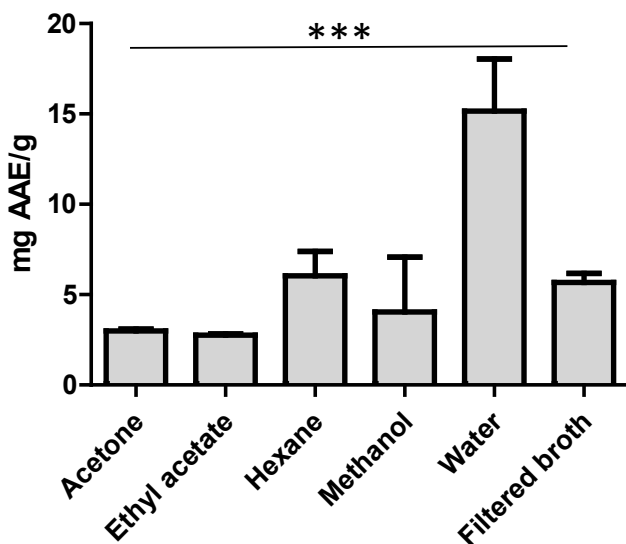


Figure 3. Quantification of antioxidant activity determined in extracts with different solvents obtained from biomass and filtered broth (extracellular). Statistical analysis was performed for all assays using ANOVA, followed by Tukey's test, ($p < 0.05$).

Figura 3. Cuantificación de la actividad antioxidante determinada en extractos con diferentes solventes obtenidos de biomasa y caldo filtrado (extracelular). Se realizó un análisis estadístico para todos los ensayos mediante ANOVA, seguido de la prueba de Tukey ($p < 0.05$).

above 200 mg/mL. These findings indicate that extracts from *F. oxysporum* are not toxic and can be safely used for testing on various cell lines.

DISCUSSION

The *Fusarium* genus is well-known for its pathogenicity on plants and is classified as having the largest number of hosts. Several of its species affect various agricultural crops, leading to significant economic losses (Sharma and Marques, 2018). Due to the diversity of species within this genus, identifying them is not easy because of their similarity to one another. Identification is typically based on microscopic and macroscopic observations, which include aspects such as the type of aerial mycelium, pigmentation, and the presence or absence of sporodochia and sclerotia, among other characteristics (Tapia and Amaro, 2014; Rentería-Martínez *et al.*, 2019). Generally, the mycelium in these species is aerial, abundant, and its color (white, cream, red, purple, etc.) depends on the species. The potato-dextrose-agar (PDA) culture medium is used as a reference for observing these characteristics (Benadof, 2010). The genus produces three types of asexual spores called macroconidia, microconidia, and chlamydospores. Another identification alternative is molecular techniques, in which specific molecular markers such as the LSU region are used. This region has been used to identify different species of fungi, including those of the *Fusarium* genus. In this work, high specificity was demonstrated when performing PCR using primers for the LSU region, resulting in the amplification of a single band of approximately 900 bp. This data was consistent with the species identification reported by Nurbaya *et al.* (2014). Although the LSU region has been

Table 2. Data obtained from the toxicity test conducted with extracts of the fungus *F. oxysporum* on *Artemia salina*.
Tabla 2. Datos obtenidos del ensayo de toxicidad realizado con extractos del hongo *F. oxysporum* sobre *Artemia salina*.

Extract	Concentration (ppm) and sobrevivence (%)					
	Control	12.5	25	50	100	200
Methanol	100 ± 0.00	100 ± 0.00	96 ± 0.57	92 ± 0.57	88 ± 0.00	88 ± 0.00
Water	100 ± 0.00	100 ± 0.00	95 ± 0.00	90 ± 0.58	86 ± 0.57	81 ± 0.00
Filtered broth	100 ± 0.00	96 ± 0.00	93 ± 0.57	86 ± 0.00	86 ± 0.00	86 ± 0.00

widely used for fungal identification, it can also present some disadvantages, such as limited resolution at the species level due to its lower nucleotide variability, which makes it less efficient for identifying closely related species (Porrás-Alfaro *et al.*, 2014).

In this study, the region corresponding to LSU did not allow differentiation between an even larger group of closely related genera. In this case, it was not sufficient to identify the *F. oxysporum* species complex. Similarly, the use of coding genes such as Translation elongation factor 1- α (*tef1*), RNA polymerase II subunit 1 and 2 (*RPB1*, *RPB2*), Calmodulin (*CaM*), among others, has been proposed for fungal identification (Raja *et al.*, 2017). Nevertheless, using the *tpi* gene sequence resulted in a 100 % similarity with sequences from *F. oxysporum* species deposited in GenBank. This data suggests that the *tpi* gene could be considered as another possible molecular marker, similar to other coding genes that have been proposed (Raja *et al.*, 2017). Furthermore, the *tpi* gene demonstrates differentiation among fungal species and *Fusarium* strains. Internal nucleotide variability between species allows for their distinction, while the conservation of flanking regions further supports this. Additionally, this gene is present in all fungal species and is readily available in databases. However, future research is needed to assess its applicability to a wider range of genera in order to validate its usefulness in the taxonomy and molecular diagnosis of plant pathogenic fungi.

On the other hand, the biological importance of this fungus lies in its pathogenicity on various plants, especially crops. As a result, most studies have focused on its pathogenic mechanisms or on finding molecules for its control (Hernández-Ochoa *et al.*, 2020). However, there are few studies related to the detection of bioactive compounds produced by *F. oxysporum*. Recently, Amuzu *et al.* (2024) reported in their review that different species of *Fusarium* produce metabolites with various biological activities including antimicrobial (on bacteria, fungi, malaria, viruses), anti-inflammatory, and cytotoxic activities.

Based on the above information, our results indicate that this strain of *F. oxysporum* has the ability to produce tannins. Besides, this strain is capable of producing phenols, although the total phenols content was lower compared to other fungi such as *Fusarium proliferatum* (21.75 mg GAE/g), *Alternaria alternata* (20.53 mg GAE/g), *Lentinula edodes* (11.7 mg GAE/g), *Hericius erinaceum* (7.8 mg GAE/g), *Agrocybe aegerita* (23.1 mg GAE/g), *Boletus edulis* (41.8 mg GAE/g) and *Boletus auranticus* (36.4 mg GAE/g) (Li *et al.*, 2015; Mujić *et al.*, 2010; Vidović *et al.*, 2010). The low amount of total phenols in *Fusa-*

rium strains was also reported by Shalapy and Kang (2022), who quantified only 2.87 ± 0.06 and 1.12 ± 0.051 $\mu\text{g}/\text{mg}$ for *F. oxysporum* and *F. solani*, respectively. *F. oxysporum* also showed presence for flavonoids. However, the data obtained for this strain were lower than those reported for some fungi such as *F. proliferatum* (8.27 mg QE/g) and *Alternaria alternata* (7.36 mg QE/g) (Li *et al.*, 2015). It is important to mention that although the concentration of phenols and flavonoids in the *F. oxysporum* strain is low compared to other fungal species, these compounds have the advantage of being extracted with water or secreted into the culture medium. This can significantly reduce the cost of obtaining these compounds. In addition, these aqueous extracts demonstrated the highest antioxidant activity compared to the data reported for these fungal strains, which had previously shown activity in ethyl acetate extracts (Putra and Karim, 2020). This confirms that obtaining the compounds will depend on each species and development condition, as well as the type of solvent used for extraction (Barros *et al.*, 2008).

Besides, it is known that in order to search for new drugs, it is necessary to evaluate the *in vitro* toxicity of molecules against organisms. *Artemia salina* has been used as one of the main models to perform lethality tests of different biological extracts for drug discovery (Banti and Hadjikakou, 2021). Recent studies have utilized the *A. salina* bioassay not only for general toxicity assessment, but also as a screening tool for compounds with cytotoxic or cellular activity. This model has enabled the detection of cytotoxic drugs with specific mechanisms of action, such as those that bind to DNA or inhibit protein synthesis, antimetotics, topoisomerase I inhibitors, and drugs that interfere with caspase signaling (Olmedo *et al.*, 2024). However, it is important to note that this model does not replace more specific assays using cell lines or higher organisms (Hendawy *et al.*, 2024). For our purposes, when testing the methanolic, aqueous and broth culture (extracellular) extracts *in vitro* on *A. salina*, these extracts did not show significant toxicity. The nauplii of the brine shrimp showed a survival rate of more than 81 % at concentrations up to 200 ppm. Based on our results, these extracts can be considered non-toxic as reported by Sosovele *et al.* (2012, 2014). It would be interesting to analyze these extracts across different cell lines to determine their cytotoxicity and potential pharmacological applications.

CONCLUSIONS

The results of this study demonstrate that analysis of the *tpi* gene allowed for precise identification of *Fusarium oxysporum*. With the phylogenetic resolution demonstrated, this

gene can be proposed as a complementary tool for taxonomic and diagnostic studies of plant pathogenic fungi.

On the other hand, this strain produces tannins, phenols and flavonoids as the main components in a liquid medium. Although the fungal biomass was treated with different solvents, the phenolic compounds and flavonoids could be efficiently extracted using only water. Notably, the aqueous extracts also showed the highest antioxidant activity in the DPPH assay compared to those obtained with other solvents. Toxicity assays using *Artemia salina* nauplii demonstrated that both extracellular and intracellular extracts were non-toxic at the tested concentrations. This finding is significant as it supports the potential for further evaluation of these compounds in cell lines to assess their therapeutic applications. Moreover, the absence of general toxicity suggests a lower risk of nonspecific cytotoxic effects in early-stage biological testing, which is advantageous in the screening of fungal metabolites with potential pharmacological activity.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest related to the content of this article.

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