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Sunflower hulls as a source of phenolic-rich extracts with antifungal activity against phytopathogenic fungi

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Abstract

Plant-derived compounds represent a promising source of antifungal agents against phytopathogenic fungi. Sunflower hulls, an underutilized agro-industrial by-product, are a potential source of bioactive molecules with applications in crop protection. This study aimed to characterize a phenolic-rich extract from sunflower hulls (EPC), evaluate its antifungal activity against phytopathogenic fungi, and explore its mode of action by analyzing the cellular responses associated with its fungicidal effects. LC-MS/MS analysis identified chlorogenic acid as the major phenolic compound, together with smaller amounts of caffeic and coumaric acids, as well as other constituents. EPC also exhibited concentration-dependent antioxidant activity. At 0.4 mg/mL, the extract showed fungicidal activity against the tested fungi. Further analyses revealed increased plasma membrane permeabilization and hydrogen peroxide accumulation in fungal cells exposed to EPC, linking its antifungal activity to membrane damage and alterations in cellular oxidative status. These findings provide insight into the antifungal effects of a chemically characterized, multicomponent plant extract and highlight sunflower hulls as a promising source of bioactive compounds for further investigation in crop protection.

Keywords

Agro-industrial by-product, Antifungal activity, Phenolic compounds, Phytopathogenic fungi, Sunflower hulls

Introduction

The environmental contamination and soil degradation associated with the intensive application of synthetic pesticides and inorganic fertilizers have increased the need to develop safer and more sustainable alternatives for agricultural production. In this context, growing attention has been directed toward the identification of natural protectants and biostimulants capable of enhancing crop growth, productivity, and quality while minimizing environmental impact (Guo and Lin 2021). Sustainable agricultural practices increasingly rely on bio-based products that combine efficacy with low toxicity and environmental compatibility.

Plants synthesize a broad diversity of secondary metabolites, including phenolic compounds, which play a central role in plant defense against microbial pathogens. Beyond their defensive function, these metabolites participate in numerous biological processes such as signaling, structural support, and adaptation to both biotic stresses, including pathogen infection and nutrient imbalance, and abiotic stresses such as ultraviolet radiation, visible light exposure, and extreme temperatures (Khan et al. 2025; Valenciene et al. 2020). Due to their antimicrobial properties, several naturally occurring phenolic compounds have been proposed as promising alternatives to synthetic fungicides for disease management in agriculture (de Oliveira et al. 2025; Dembińska et al. 2025). In addition, a variety of plant-derived products, including extracts, essential oils, and resins, have demonstrated biological activity both *in vitro* and *in vivo* and are currently being explored as biofungicides with reduced adverse effects on humans, animals, and ecosystems (Rivas et al. 2025).

Seed hulls from numerous plant species are recognized as valuable sources of phenolic compounds involved in natural protective mechanisms (Shahidi et al. 2019). Among these, sunflower seeds represent a particularly rich source of phenolics, containing approximately 1–4% phenolic compounds by weight. Chlorogenic acid (CGA) has been identified as the predominant component, accounting for nearly 80% of the total phenolic fraction (Denić et al. 2026). Other phenolic acids reported in sunflower seeds include caffeic, cinnamic, coumaric, ferulic, sinapic, and hydroxycinnamic acids, as well as lower concentrations of vanillic, syringic, and hydroxybenzoic acids (De Nobili et al. 2021; Da Silva et al. 2025; Sripad et al. 1982; Li et al. 2025). Notably, between 0.7% and 5.4% of these phenolic compounds are concentrated in sunflower hulls, a major by-product generated by the oil-processing industry

(Tsvetanova et al. 2025; Nemli et al. 2025). Although commonly treated as industrial waste, sunflower hulls constitute a potentially valuable and underutilized source of bioactive molecules.

Despite previous evidence of chlorogenic acid activity against several phytopathogenic fungi (Martínez et al. 2017), less is understood about the antifungal properties of phenolic-rich extracts obtained directly from sunflower hulls and the cellular responses associated with their activity. A better understanding of the antifungal potential of these extracts and their effects on fungal cells could help establish their value as sources of bioactive compounds for crop protection. Therefore, this study aimed to chemically characterize a phenolic-rich extract obtained from sunflower hulls, assess its antifungal activity against phytopathogenic fungi, and investigate the cellular responses associated with its fungicidal effect. By integrating chemical characterization with antifungal assays and cellular analyses, this work contributes to the evaluation of sunflower hulls as a source of bioactive extracts with potential agricultural applications.

Materials and Methods

Sunflower hulls extract enriched in phenolic compounds

Sunflower seed hulls were supplied by Oleaginosa Moreno Hnos. (Quequén, Argentina). To obtain the polyphenolic extract, the hulls were mechanically ground into fine particles and subjected to extraction with 80% ethanol at room temperature under continuous agitation for 1 h, using a ratio of 10 mL of solvent per gram of plant material (Ordóñez et al. 2003). The resulting extract was sequentially filtered through cheesecloth and Whatman No. 12 filter paper to remove solid residues. Subsequently, the filtrate was concentrated tenfold using a speed-vac system (Automatic Environmental SpeedVac System AES1010 SAVANT Thermo Fisher Scientific) and centrifuged at 4°C to clarify the extract and eliminate lipid material. The resulting extract enriched in phenolic compounds (EPC) was stored at 4°C and protected from light until further use. Total phenolic content was quantified spectrophotometrically at 725 nm using the modified Folin–Ciocalteu method (Singleton et al. 1999).

Identification of the EPC components

To analyze the phenolic compound composition of the EPC, a Prominence Shimadzu HPLC system equipped with a diode-array detector (DAD; SPD-M20A) was employed. The extract was fractionated using DAD-HPLC on a C18 Phenomenex column (250 × 4.6 mm i.d.; 5 µm particle size) equilibrated in 0.1% trifluoroacetic acid (TFA). The elution was performed at a flow rate of 1.5 mL/min, employing a linear gradient from 5 to 80% acetonitrile 0.1% TFA in 12 min. Solutions of chlorogenic acid (CGA), caffeic acid, and coumaric acid (5 mg/mL in 80% ethanol) were used as standards. Detection of phenolic compounds was performed at 330 nm.

To determine the molecular identity of the compounds present in the EPC, the extract was analyzed by liquid chromatography–tandem mass spectrometry (LC-MS/MS). Chromatographic separation was performed using a C18 Luna Phenomenex column (100 × 2 mm i.d., 3 µm particle size) at a flow rate of 0.3 mL/min, employing a linear gradient of 5–50% acetonitrile over 40 min. Mass spectrometric analysis was carried out using a micrOTOF-Q II mass spectrometer equipped with an orthogonal Z-spray electrospray ionization (ESI) interface operating in negative ionization mode (UBA-UMYMFOR Service). Argon (Ar) was used as collision gas at a pressure of 30 psi in the collision cell. The capillary voltage was set at 4.5 kV, and the source temperature was maintained at 200°C. Data acquisition and processing were performed using Hystar PP software version 3.2.44.0 (Bruker Daltonik GmbH). The obtained mass spectra were compared with entries available in the MassBank database (https://massbank.eu/MassBank) for compound identification.

Antioxidant Capacity Assay

The antioxidant capacity of the EPC was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay as described by Chan et al. (2014). Briefly, different dilutions of EPC, chlorogenic acid (CGA), and caffeic acid prepared in 96% (v/v) ethanol were dispensed into 96-well flat-bottom microplates (50 µL per well), followed by the addition of 150 µL of freshly prepared 0.25 mM DPPH solution in 100% methanol. The reaction mixtures were incubated for 30 min at room temperature in the dark, after which absorbance was measured at 595 nm using a microplate spectrophotometer (ELx800, BioTek). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; Sigma) was used as a positive control. Antioxidant activity was determined based on the decrease in absorbance resulting from DPPH reduction

and expressed as the percentage of radical-scavenging activity. All measurements were performed in triplicate in three independent experiments.

Fungi

Fungal strains from the collection of Instituto de Investigaciones Biológicas (UNMdP, Mar del Plata, Argentina), including *Fusarium solani eumartii, Fusarium oxysporum, Botrytis cinerea, Verticillium dahliae,* and *Sclerotinia sclerotiorum,* were used in this study. Fungi were cultured on potato dextrose agar (PDA) plates supplemented with 100 µg/mL ampicillin and 100 µg/mL chloramphenicol and incubated at 25°C for 15 days. To obtain spores from *Fusarium, B. cinerea, and V. dahliae,* plates were washed with 1 mL of sterile distilled water. The resulting suspensions were filtered through cheesecloth to remove residual mycelial fragments. Ascospores of *S. sclerotiorum* were provided by Advanta Semillas S.A.I.C. (Balcarce, Argentina) and collected from Petri dishes containing apothecial imprints. Spore suspensions were quantified using a Neubauer chamber under light microscopy. Freshly collected spores were used immediately or stored at 4°C for up to 5 days before use.

Antifungal activity assays

To evaluate the antifungal activity of EPC, fungal spore germination inhibition assays were performed on microscope slides as previously described by Regente and de la Canal (2000). The incubation mixture consisted of $1x10^4$ spores, 2% sucrose, and EPC at final concentrations equivalent to 0.4 or 0.7 mg/mL of total phenols, in a final volume of 20 µL. In control treatments, EPC was replaced with an equal volume of sterile water. Spore germination and hyphal growth were assessed after 16 h of incubation at 25°C and 100% relative humidity using a light microscope (Eclipse Microscope E200, Nikon).

Quantitative fungal growth assays were performed in flat-bottom 96-well microplates containing EPC at increasing concentrations, 1×10^4 spores, and Sabouraud broth in a final volume of 100 µL, as previously described by Del Rio et al. (2018). Fungal growth was monitored by measuring optical density at 595 nm during 3 days of incubation at 25°C. Growth inhibition percentage in the presence of EPC was calculated relative to the control treatment, which was defined as 100% growth. All assays were conducted in triplicate and independently

repeated at least twice. The IC_{50} value was defined as the EPC concentration required to inhibit 50% of fungal growth relative to the control.

To evaluate the effect of EPC on the integrity of the cell wall and plasma membrane of *Fusarium solani*, spore germination inhibition assays were performed on microscope slides using agents known to disrupt these structures (Del Rio et al. 2019). The incubation mixture contained 1×10^4 cells/mL in Sabouraud broth, EPC (0.05 mg/mL), and either 20 µg/mL Calcofluor White (CFW) or 20 µg/mL Congo Red (CR), both used as cell wall–perturbing agents (Ansari et al., 2016); or 0.02% sodium dodecyl sulfate (SDS) as a plasma membrane–perturbing agent (Heerklotz, 2008). Spore germination and hyphal growth were evaluated after 16 h of incubation at 25°C and 100% relative humidity using light microscopy. All assays were performed in triplicate, with two independent biological replicates.

Viability assays

To evaluate the fungicidal activity of EPC, radial fungal growth assays were performed on PDA plates. Briefly, 1×10^4 spores of *F. solani* or *S. sclerotiorum* were incubated in 2% sucrose for 24 h at 25°C in the presence of EPC at final concentrations of 0.4 or 0.7 mg/mL, or sterile water as a negative control. Subsequently, 20 µL of each treatment were inoculated onto the center of PDA plates and incubated at 25°C. Radial fungal growth was recorded at 24, 48, and 72 h post-inoculation.

Cell viability was further assessed using the Evans blue uptake assay, based on the selective penetration of the dye into non-viable cells. Following antifungal activity assays on microscope slides, Evans blue was added to a final concentration of 0.05% and incubated for 10 min at room temperature. Positively stained fungal cells were visualized by light microscopy. In addition, the cytotoxic activity of the extracts was determined by the MTT assay (Berridge and Tan, 1993). Following quantitative fungal growth assays, MTTreagent (Sigma-Aldrich) was added at a concentration of 1 mg/mL and incubated at 37°C for 4 h. Formazan crystals were dissolved in 100% DMSO and measured at 570 nm in a microplate spectrophotometer (ELx800, BioTek). The % of cellular viability was obtained normalizing to the values in the absence of treatments.

Detection of hydrogen peroxide

Hydrogen peroxide production in phytopathogenic fungal spores was detected by staining with 3,3′-diaminobenzidine (DAB), as previously described by Giudici et al. (2004). Briefly, 1×10^4 fungal spores were incubated on microscope slides with either sterile water (negative control) or EPC (0.4 mg/mL) in the presence of 0.5 mg/mL DAB. Hydrogen peroxide production was visualized by light microscopy as the formation of a brown precipitate. Quantification was performed by determining the percentage of DAB-positive cells relative to the total number of cells. Cell counts were obtained from at least 10 microscopic fields for each treatment across three independent experimental replicates.

Results

Characterization of EPC

To obtain an extract enriched in phenolic compounds (EPC), 80% ethanol was selected as the extraction solvent, as previously reported by Oluwaseun et al. (2021). EPC was obtained by hydroalcoholic maceration of sunflower hulls, yielding a total phenolic content of 2.48 ± 0.21 mg/mL. The composition of EPC was analyzed by reverse-phase DAD-HPLC using an increasing acetonitrile gradient (Fig. 1). Phenolic compounds were tentatively identified by comparing their retention times (RT) with those of commercial standards, including chlorogenic acid (CGA), caffeic acid and coumaric acid. Three major peaks were detected with RT values corresponding to CGA (7.1 min), caffeic acid (7.8 min), and coumaric acid (8.8 min). Quantitative analysis of the DAD-HPLC chromatogram revealed concentrations of 1.075 mg/mL for CGA, 0.03 mg/mL for caffeic acid, and 0.11 mg/mL for coumaric acid. Among these compounds, CGA was the predominant phenolic constituent, representing approximately 88% of the total phenolic content of the extract.

To confirm the molecular identity of the compounds detected by reverse-phase DAD-HPLC and to identify additional constituents present in the extract, EPC was further analyzed by LC-MS/MS (UBA-UMYMFOR Service). The main compounds detected are summarized in Table 1 and Fig. S1. Among them, chlorogenic acid (RT 9.7 min) and caffeic acid (RT 12.3 min) were successfully identified, thereby confirming the results obtained by DAD-HPLC analysis. In contrast, coumaric acid could not be conclusively identified by LC-MS/MS.

Notably, MS/MS analysis of the peak eluting at RT 18.8 min led to the identification of azelaic acid. Since azelaic acid is not a phenolic compound, it was not detected in the DAD-HPLC analysis performed at 330 nm. In addition, another major non-phenolic compound eluting at RT 29.5 min was identified as oleic acid, one of the predominant fatty acids present in sunflower seeds.

Table 1. LC-MS^2 characteristics of compounds detected in EPC.

Id. Compound	RT	Molecular ion	Ion	Intensity
Chlorogenic Acid	9.7 min	353.09116	191.05413	100
Caffeic Acid	12.3 min	179.03556	179.03498	100
Azelaic Acid	18.8 min	187.09795	97.06848	26
			123.07401	24
			125.09275	81
			126.09647	17
Oleic Acid	29.5 min	329.40155	127.10981	10
			139.09241	17
			171.09571	82
			211.12392	52
			212.12881	10
			229.14093	22
			329.23002	20

Several bioactive properties of polyphenols are closely associated with their strong free radical scavenging capacity. Therefore, the total antioxidant activity of EPC and selected reference phenolic compounds was evaluated using the DPPH assay (Fig. 2). As expected, EPC as well as the commercial phenolic standards, chlorogenic acid (CGA) and caffeic acid, exhibited dose-dependent antioxidant activity. Notably, EPC displayed an antioxidant capacity comparable to that of pure CGA, consistent with the high abundance of this compound in the extract.

Antifungal activity of EPC on phytopathogenic fungi

In this study, we evaluated the antifungal activity of EPC against phytopathogenic fungi of major relevance to horticultural crops, characterized by their broad host range and their capacity to cause severe agricultural losses. As an initial approach, spores of *Sclerotinia sclerotiorum, Fusarium solani, Fusarium oxysporum, Verticillium dahliae,* and *Botrytis cinerea* were incubated for 16 h with different concentrations of EPC and subsequently analyzed by light microscopy. As shown in Fig. 3, spores incubated with water (negative control) germinated normally and developed hyphae. In contrast, complete inhibition of spore germination was observed in *S. sclerotiorum, F. solani, V. dahliae,* and *F. oxysporum* when treated with EPC at both tested concentrations (0.4 and 0.7 mg/mL). In the case of *B. cinerea*, fungal growth comparable to the negative control was detected even at 0.7 mg/mL EPC, indicating lower sensitivity to the extract. Nevertheless, EPC-treated *B. cinerea* hyphae displayed marked morphological alterations, including an increased degree of branching compared with the control treatment.

To further characterize the antifungal activity of EPC, *Fusarium solani* was selected as a model organism to quantitatively assess fungal growth inhibition by determining the IC_{50}, defined as the EPC concentration required to inhibit 50% of fungal growth. For this purpose, *F. solani* spores were cultured in the presence of increasing concentrations of EPC or water as a control. The resulting dose–response analysis yielded an IC_{50} value of 0.05 mg/mL (Fig. 4).

Mechanism of action of EPC

To gain further insight into the antifungal mechanism of EPC, we investigated whether the extract affects the viability of phytopathogenic fungi. For this purpose, Evans blue staining was performed, taking advantage of the ability of the dye to penetrate non-viable cells with compromised membrane integrity. After 24 h of incubation with EPC, *F. solani* conidia failed to germinate and exhibited intense blue staining, indicating loss of plasma membrane integrity and fungal cell death (Fig. 5A). To validate and quantitatively assess this effect, *F. solani* cell viability was evaluated using the mitochondrial dehydrogenase-dependent MTT assay, which selectively measures metabolically active cells. EPC treatment induced a marked and dose-dependent reduction in fungal viability, with the magnitude of the effect directly correlating with the polyphenol concentration of the extract (Fig. 5B). These findings highlight the strong

cytotoxic activity of EPC against fungal cells and reinforce the relevance of its polyphenolic components in mediating this antifungal effect. To confirm whether EPC exerts fungicidal activity, plate viability assays were performed. Spores of *F. solani* and *S. sclerotiorum* were incubated with EPC for 24 h at 25°C and subsequently inoculated onto the center of PDA plates to assess spore viability and their capacity to develop radial growth. As shown in Fig. 6, EPC completely inhibited fungal growth in both *F. solani* and *S. sclerotiorum*, even at the lowest concentration tested, as no mycelial development was observed after 72 h of incubation. If the spores had remained viable, recovery of fungal growth would have been expected following transfer to fresh PDA medium. Therefore, these results confirm that EPC acts as a fungicidal agent rather than merely inhibiting fungal growth transiently.

To further investigate the mechanism of action of EPC and its effects on the integrity of the cell wall and plasma membrane of *F. solani*, spore germination inhibition assays were conducted in the presence of agents known to disrupt these cellular structures. *F. solani* spores were incubated with a subinhibitory dose of EPC that does not lead to inhibition of spore germination, together with either Calcofluor White (CFW) or Congo Red (CR), both recognized as cell wall–perturbing agents, or sodium dodecyl sulfate (SDS), a plasma membrane–perturbing agent. This approach is based on sensitizing a potential cellular target—in this case, the cell wall or plasma membrane—to identify the site of action of the compound under study. If the targeted structure is affected by the treatment, the presence of a sensitizing agent is expected to enhance cellular destabilization, leading to increased inhibition of microbial growth. As shown in Fig. 7, fungal growth in the presence of EPC at 0.05 mg/mL combined with the cell wall–perturbing agents CFW or CR was comparable to that observed in the control treatments, suggesting that EPC does not significantly affect cell wall integrity. In contrast, the combination of EPC with SDS resulted in a marked inhibition of spore germination and fungal growth, indicating that the plasma membrane is likely the primary target of EPC. This finding is consistent with the membrane permeabilization observed using Evans blue staining.

Although the biological activities of polyphenols are commonly associated with their antioxidant properties, under certain environmental conditions or at high concentrations they may also exert pro-oxidant effects by promoting the generation of reactive oxygen species (ROS) (Miranda et al. 2021). To gain deeper insight into the mechanism of action of EPC, we evaluated the oxidative status of fungal cells exposed to the extract. To determine whether the

cytotoxic activity of EPC involves oxidative stress, hydrogen peroxide production in *F. solani* spores was assessed using diaminobenzidine (DAB), which forms a dark brown precipitate upon oxidation. Microscopic analysis revealed intense brown staining in spores treated with EPC for 4 h (Fig. 8A). Quantification of DAB-positive cells demonstrated significantly higher peroxide levels in EPC-treated samples compared with the negative controls (Fig. 8B). These results indicate that EPC disrupts the oxidative balance of fungal cells by increasing peroxide production, which may contribute to its cytotoxic and antifungal effects.

Discussion

A major limitation of synthetic pesticides is their progressive loss of efficacy due to the emergence of resistant pathogen populations. In this context, the exploitation of agro-industrial residues as sources of bioactive compounds represents a sustainable and strategically relevant alternative. Among these, sunflower hulls, an abundant by-product of the edible oil industry, constitute a valuable yet underutilized source of phenolic compounds with potential agrobiotechnological applications.

Polyphenols comprise a structurally diverse class of plant secondary metabolites, including flavonoids and phenolic acids, which are involved in multiple physiological functions and have been widely associated with antimicrobial activity (Mandal and Domb, 2024; Zagoskina et al., 2023). In the present study, we demonstrate that an extract enriched in phenolic compounds (EPC) obtained through a simple hydroalcoholic maceration of sunflower hulls exhibits potent antifungal activity. Chemical characterization by DAD-RP-HPLC and LC-MS/MS identified chlorogenic acid (CGA) as the major component, together with minor phenolics such as caffeic and coumaric acids, as well as non-phenolic compounds including oleic and azelaic acids.

The antifungal activity of chlorogenic acid (CGA) was previously demonstrated in our laboratory, where CGA exhibited broad-spectrum activity against several phytopathogenic fungi, including *Sclerotinia sclerotiorum, Fusarium solani, Verticillium dahliae, Botrytis cinerea, and Cercospora sojina* (Martínez et al., 2017). However, the concentrations of CGA required to achieve antifungal effects (5–15 mg/mL) were substantially higher than the concentration of total phenolics in EPC required to produce comparable effects in the present study (0.4–0.7 mg/mL), even against the same fungal species. This difference suggests that the

antifungal activity of EPC cannot be explained solely by its CGA content and may instead reflect the combined effects of several compounds, including additive or synergistic interactions among its constituents. Similar enhancement effects have been described for other natural mixtures, where the combined activity exceeds that of individual constituents (Pintos et al., 2018; Rajčević et al., 2022). Moreover, the presence of multiple bioactive compounds targeting different cellular structures may reduce the probability of resistance development, an advantage over single-compound fungicides (Yiu et al., 2024). Beyond the potential benefits associated with its multicomponent nature, EPC also exhibited considerable antifungal potency against *Fusarium solani*, as reflected by its low IC_{50} value. The IC_{50} value of 0.05 mg/mL was approximately one order of magnitude lower than the concentration required to achieve complete inhibition of spore germination (0.4 mg/mL). This difference is consistent with previous reports for other antifungal agents, in which partial growth inhibition occurs at substantially lower concentrations than those needed to completely suppress spore germination (Del Rio et al., 2018).

Mechanistically, our results indicate that EPC exerts a fungicidal effect primarily through disruption of plasma membrane integrity. The increased uptake of Evans blue observed in treated spores supports membrane permeabilization as a key event in EPC-induced cytotoxicity. This finding is further reinforced by the enhanced antifungal effect observed in the presence of sodium dodecyl sulfate (SDS), a known membrane-perturbing agent, whereas no evident effect was detected with cell wall–perturbing agents such as Calcofluor White or Congo Red. Together, these observations indicate that the plasma membrane, rather than the cell wall, is the primary target of EPC.

In addition to membrane disruption, our data suggest that oxidative stress contributes to EPC-mediated fungal cell damage. The induction of hydrogen peroxide production in treated cells indicates an imbalance in redox homeostasis. While polyphenols are widely recognized for their antioxidant capacity, including radical scavenging activity as confirmed here by the DPPH assay, they can also exhibit pro-oxidant behavior depending on environmental conditions and concentration (Liang and Kitts, 2015; Miranda et al., 2021). For instance, CGA has been shown to generate reactive oxygen species (ROS) under specific conditions, contributing to cytotoxicity (Hou et al., 2017). In line with these observations, our results support a dual

mechanism in which EPC modulates ROS levels, leading to oxidative damage that, in combination with membrane permeabilization, leads to fungal cell death.

From an applied perspective, these findings highlight the potential of EPC as a source of bioactive compounds for further investigation in crop protection. Beyond the valorization of an agro-industrial by-product, this study contributes to the understanding of the antifungal effects of a chemically characterized, multicomponent plant extract against several phytopathogenic fungi. The association between EPC-induced fungal damage, plasma membrane permeabilization, and increased hydrogen peroxide accumulation provides insight into the cellular responses underlying its fungicidal activity. These findings extend the characterization of sunflower hulls beyond their established value as a source of phenolic compounds and antioxidant activity, supporting further investigation of their antifungal properties and mechanisms of action. More broadly, plant-derived by-products such as citrus peels, grape skins, and apple pomace are increasingly recognized as sources of bioactive compounds (Palos-Hernández et al., 2022; Panzella et al., 2020; Singh et al., 2020), although their application in crop protection remains underexplored compared to their use in human health. The present study therefore links agro-industrial by-product valorization with the investigation of antifungal activity and associated cellular responses, contributing to the search for plant-derived alternatives for more sustainable agricultural systems.

Conclusion

This study demonstrates that sunflower hulls are a readily accessible source of phenolic-rich extracts with antifungal activity against several phytopathogenic fungi. The EPC exhibited antifungal activity at concentrations of 0.4 mg/mL, with its fungicidal effects associated with plasma membrane permeabilization and alterations in cellular oxidative status. These findings provide insight into the cellular responses associated with the antifungal activity of a chemically characterized, multicomponent plant extract. The antifungal activity of EPC may reflect the combined contribution of its major phenolic compounds, particularly chlorogenic acid, caffeic acid, and coumaric acid, together with other constituents identified, such as azelaic and oleic acids, within the complex chemical composition of the natural extract. Overall, this work advances the characterization of the antifungal properties of sunflower hull extracts and

supports further investigation of their potential as plant-derived agents for crop protection, while promoting the valorization of an agro-industrial by-product.

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Conflict of interest

The authors declare that they have no conflict of interest.

Credit authorship contribution statement

Guadalupe Martínez: Conceptualization, Investigation, Methodology, Validation, Visualization, Writing - Review and Editing

Marianela Del Rio: Conceptualization, Investigation, Methodology, Validation, Visualization, Writing - Review and Editing

Melisa Radicioni: DPPH analysis, Methodology, Visualization, Writing - Review and Editing

Guadalupe Rodriguez: Antifungal mechanism analysis, Methodology

Ximena Silveyra: DPPH analysis, Writing - Review and Editing

Marcela Pinedo: LC- MS/MS analysis

Mariana Feldman: Writing - Review and Editing

Candela Lobato: Writing - Review and Editing

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Debora Soto: Methodology

Laura de la Canal: Conceptualization, Investigation, Funding acquisition, Project administration, Supervision

Mariana Regente: Conceptualization, Investigation, Funding acquisition, Project administration, Supervision, Writing – original draft

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Figure captions

Fig. 1. EPC analysis by reverse phase DAD-HPLC. Fractionation of phenolic compounds from sunflower hull extract. The sample was subjected to a gradient of 5-80% acetonitrile in 0.1% TFA. The intensity at 330 nm was determined. The table indicates the retention times of the EPC peaks and standard phenolic compounds, and the content of each peak in the extract. These results are representative of 5 biological replicates.

Fig. 2. Antioxidant activity of EPC. DPPH radical reduction produced by different concentrations of phenolic compounds (CGA, caffeic acid) and EPC. The reaction mixture contained DPPH (0.25 mM) alone or in the presence of increasing phenolic compounds or EPC concentrations. Activities were determined in triplicate for 3 biological replicates.

Fig. 3. Antifungal effect of EPC. Spores of different phytopathogenic fungi were incubated in the presence of water (negative control) or EPC (0.4 mg/mL and 0.7 mg/mL) for 16 h. Images were obtained with a 40x magnification. They are representative of 3 biological replicates.

Fig. 4. Quantification of the antifungal activity of EPC: determination of the IC_{50}. Increasing concentrations of EPC were evaluated, and fungal growth was determined by optical density readings at 595 nm after 3 days of incubation. The percentage of growth inhibition in the presence of EPC was calculated relative to the control, which was considered as 100% growth.

Fig. 5. Cytotoxic effect of EPC. A. Effect of EPC on the permeability of the F. solani plasma membrane. Fungal spores were incubated in the presence of water (negative control) or EPC (0.4 mg/mL) for 24 h and were subjected to direct observation or previously stained with 0.05% Evans blue. 40x magnification. The images are representative of 3 biological replicates. B. Effect of EPC on fungal cell metabolic activity. F. solani spores were incubated for 24 h with increasing concentrations of EPC, expressed as polyphenol content, and cell viability was evaluated by measuring mitochondrial dehydrogenase activity using the MTT assay. Cellular viability (%) was calculated relative to the untreated control, which was considered 100% viability.

Fig. 6. Effect of EPC on the viability of phytopathogenic fungi. Fungal survival of F. solani and S. sclerotiorum after incubation in the presence of water (negative control), or EPC (0.4 mg/mL 0.7 mg/mL) for 24 h. The images are representative of 3 biological replicates.

Fig. 7. Effect of EPC on the integrity of the cell wall and plasma membrane of F. solani. Susceptibility assay of cellular structures in the presence of cell wall–perturbing agents (CFW and CR) and the plasma membrane–perturbing agent (SDS). The images are representative of two independent biological replicates.

Fig. 8. Effect of EPC on the F. solani oxidative balance. A. Microscopic images of spores incubated in the presence of water (control) or EPC (0.4 mg/mL) for 4 h and detection of hydrogen peroxide with DAB. B. Quantification of brown-stained spores by the presence of peroxides. The images are representative of 3 biological replicates.

Graphical Abstract

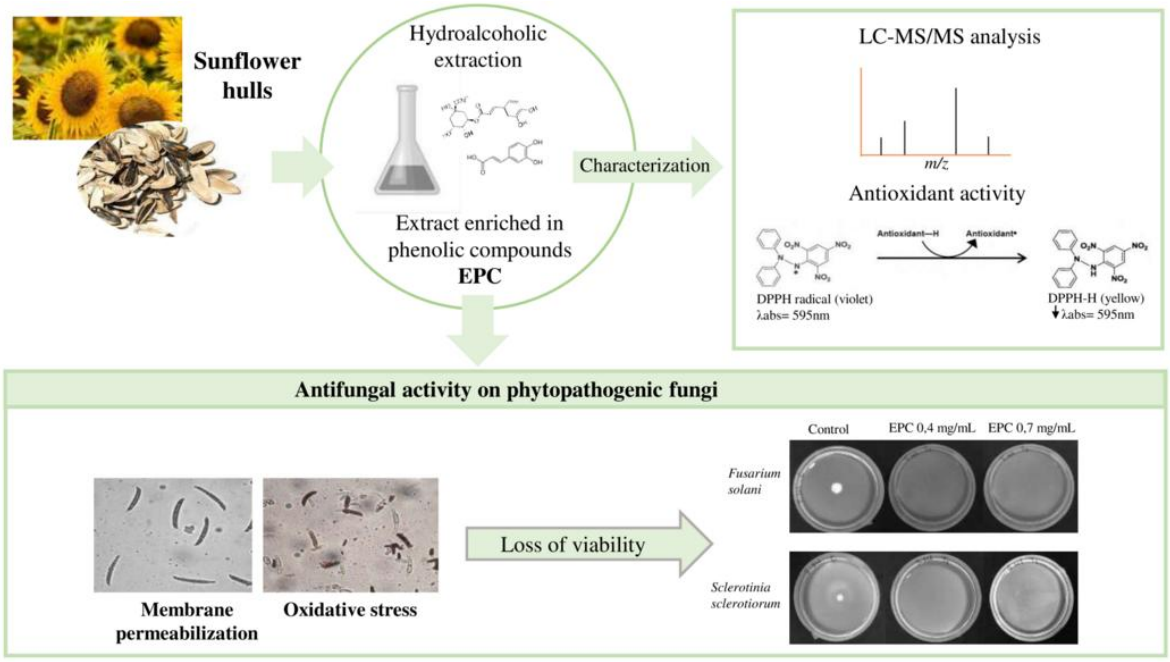
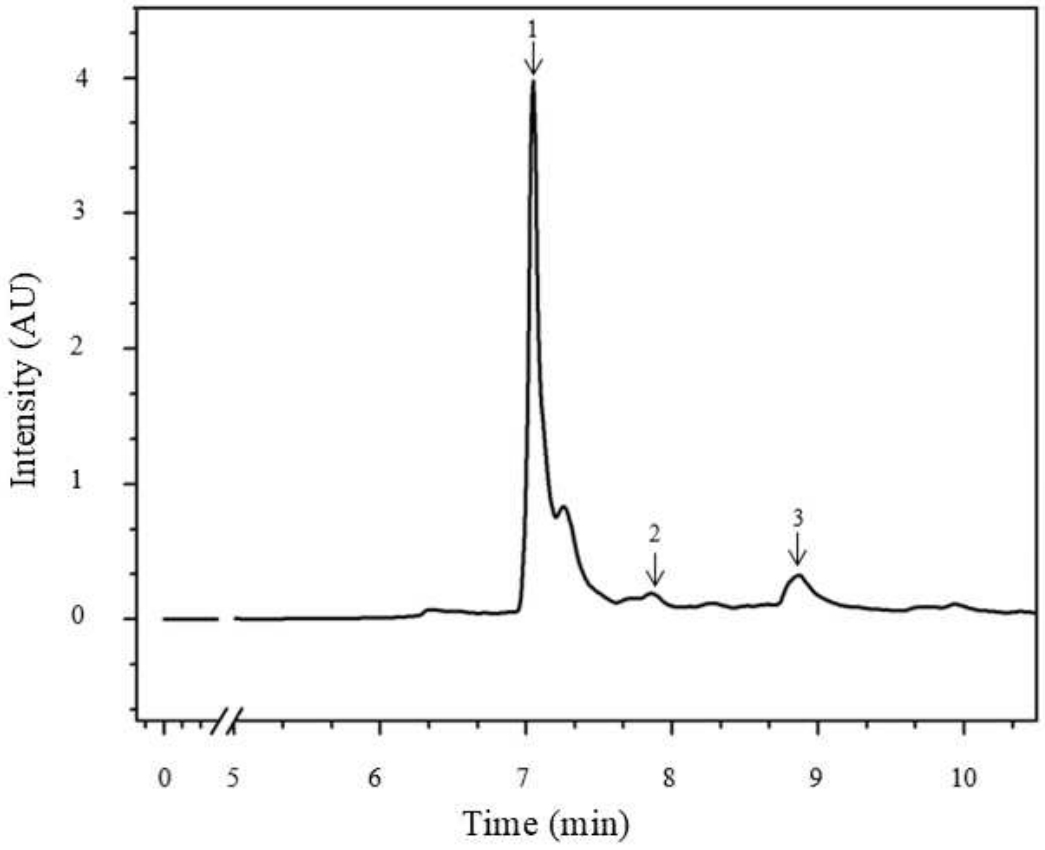


Figure 1

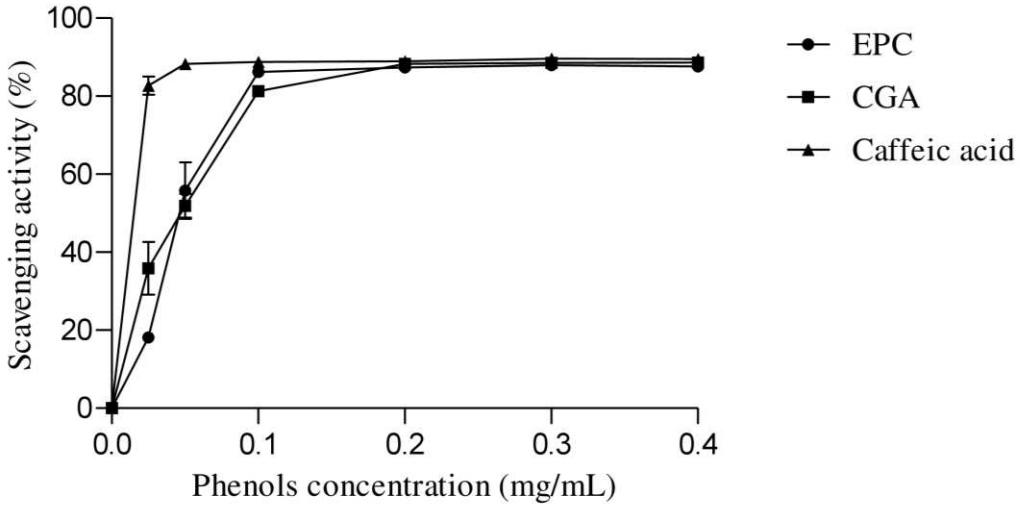
Fig. 1. EPC analysis by reverse phase DAD-HPLC. Fractionation of phenolic compounds from sunflower hull extract. The sample was subjected to a gradient of 5-80% acetonitrile in 0.1% TFA. The intensity at 330 nm was determined. The table indicates the retention times of the EPC peaks and standard phenolic compounds, and the content of each peak in the extract. These results are representative of 5 biological replicates.



Standard Name	Retention Time (min)	Peak Name	Retention Time (min)
Chlorogenic acid	7.169	1	7.105
Caffeic acid	7.894	2	7.845
Coumaric acid	8.814	3	8.888

Figure 2

Fig. 2. Antioxidant activity of EPC. DPPH radical reduction produced by different concentrations of phenolic compounds (CGA, caffeic acid) and EPC. The reaction mixture contained DPPH (0.25 mM) alone or in the presence of increasing phenolic compounds or EPC concentrations. Activities were determined in triplicate for 3 biological replicates.



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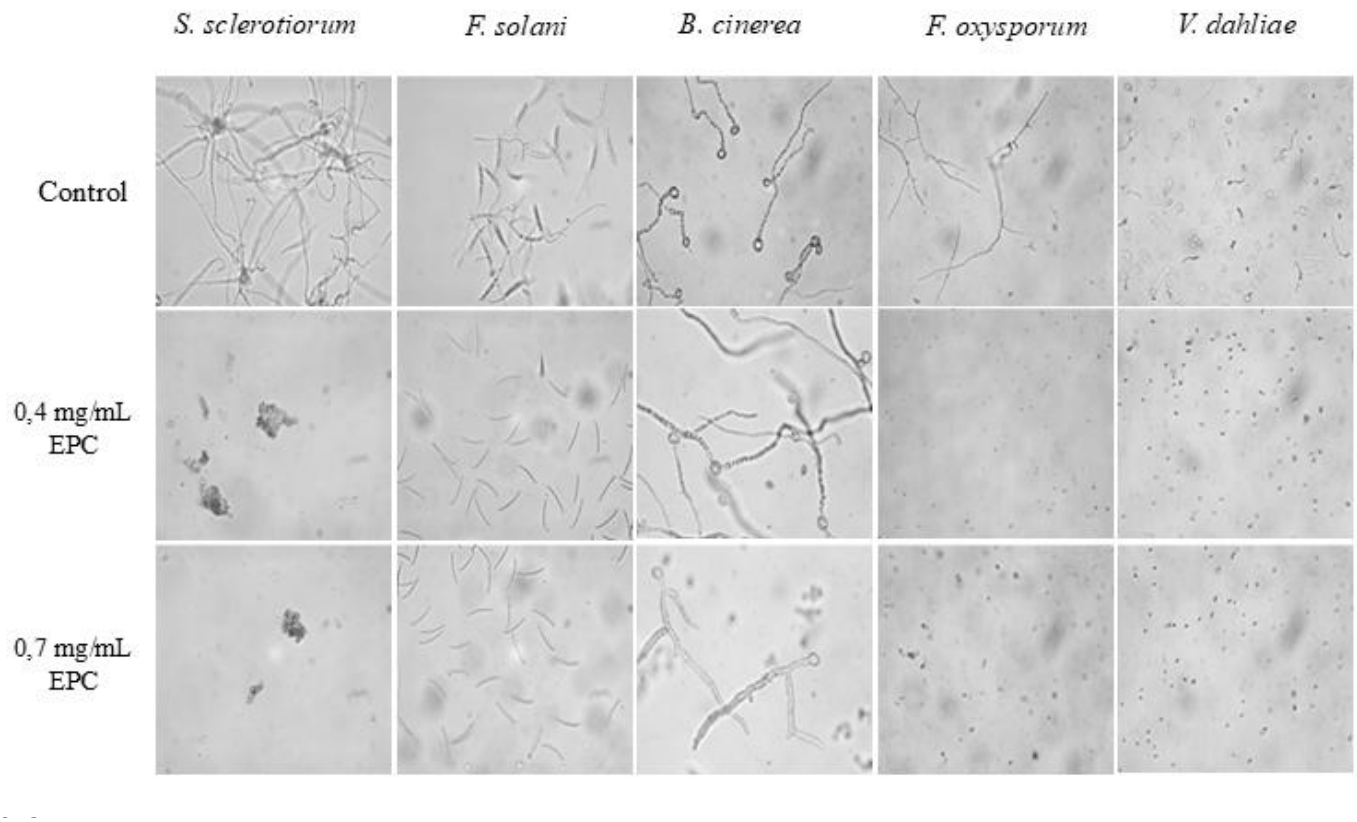

Figure 4

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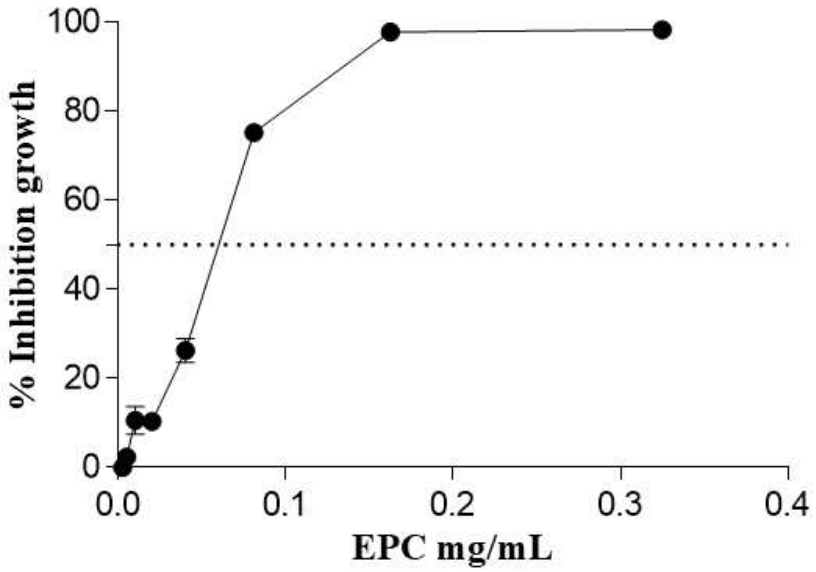

Figure 5 A

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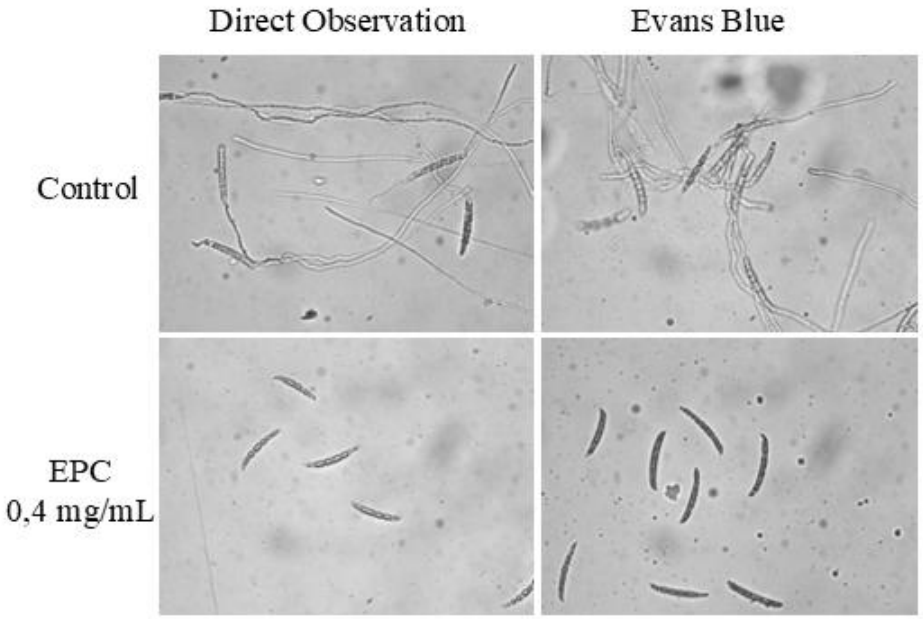

Figure 5 B

Fig. 5B. Cytotoxic effect of EPC. Effect of EPC on fungal cell metabolic activity. F. solani spores were incubated for 24 h with increasing concentrations of EPC, expressed as polyphenol content, and cell viability was evaluated by measuring mitochondrial dehydrogenase activity using the MTT assay. Cellular viability (%) was calculated relative to the untreated control, which was considered 100% viability.

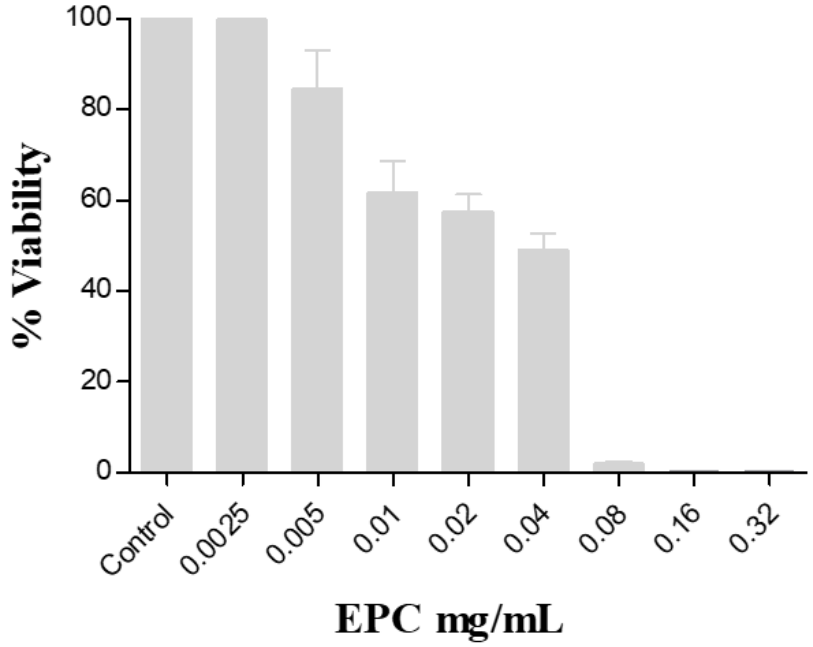

Figure 6

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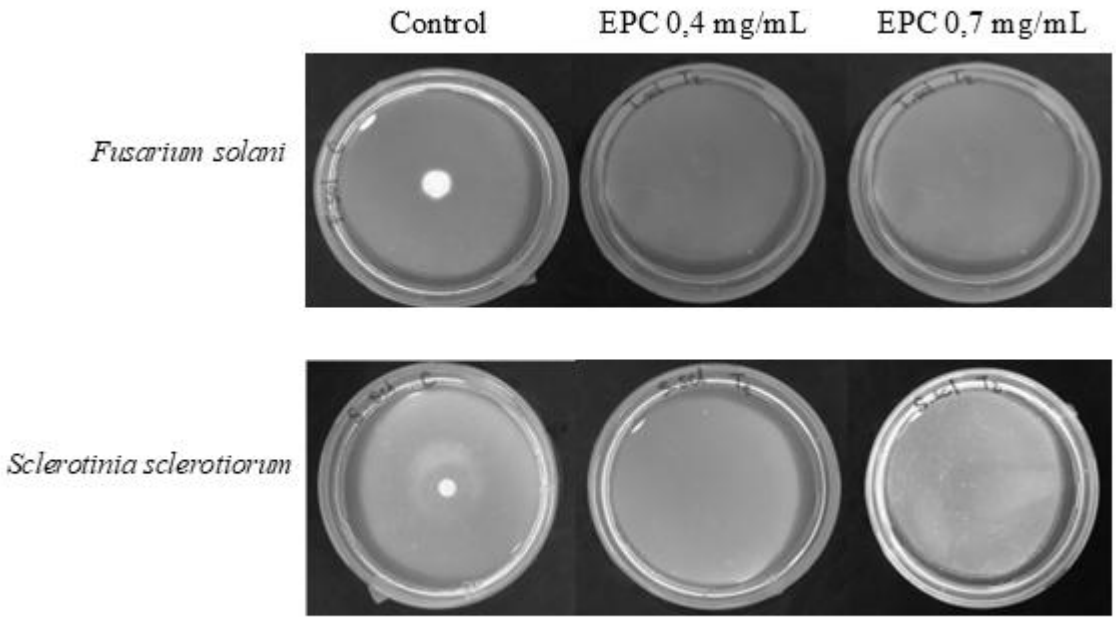

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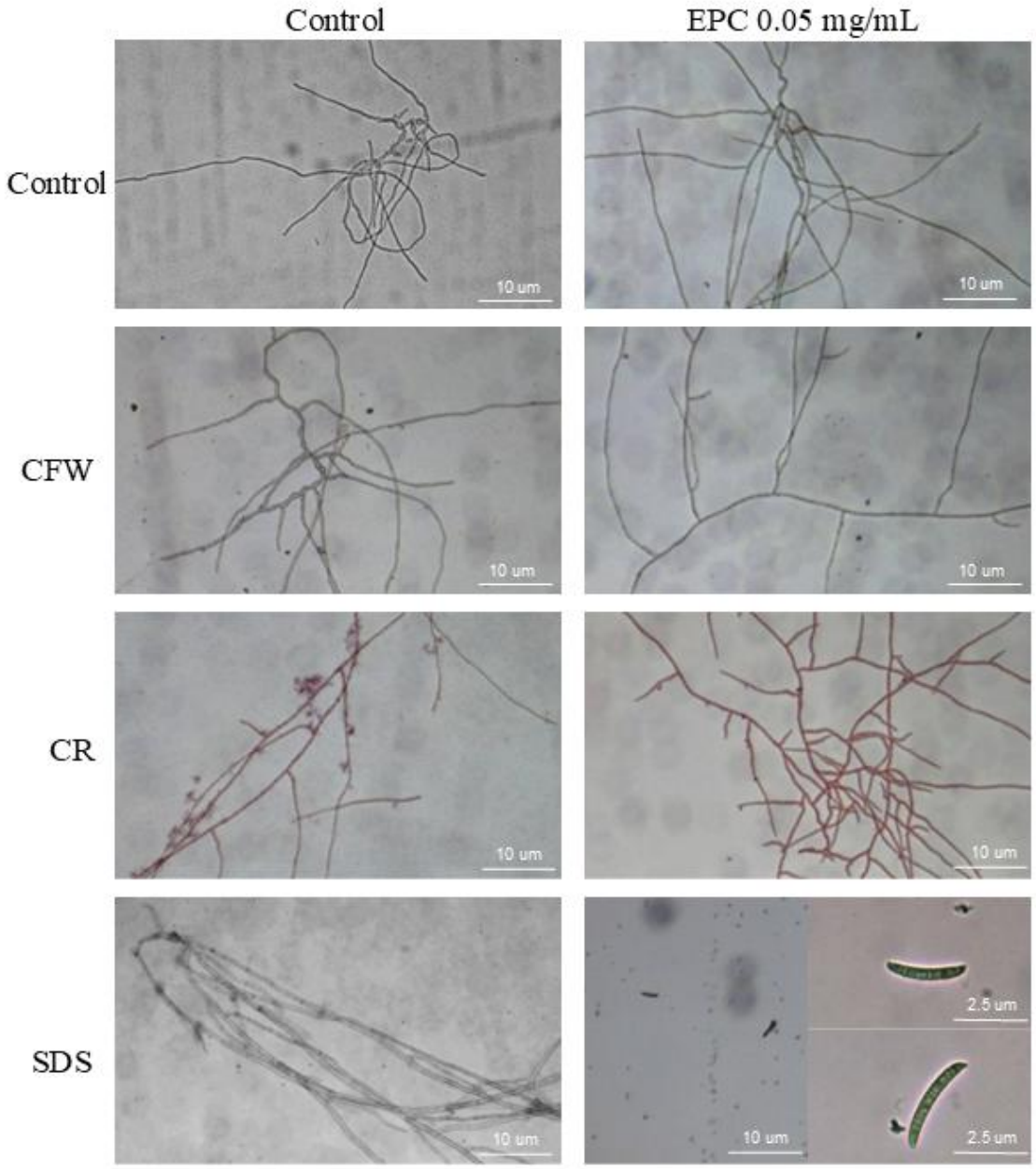

Figure 8

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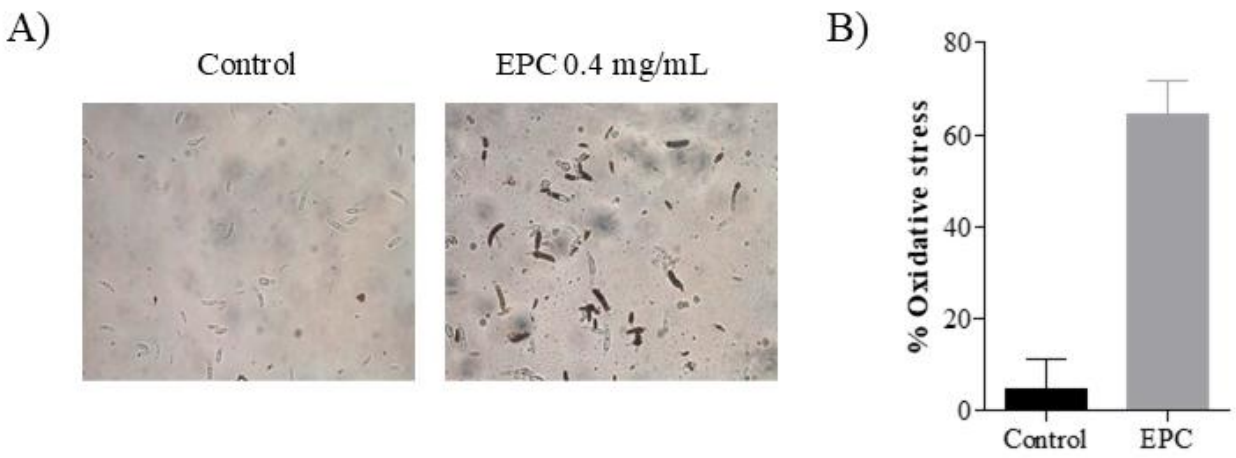
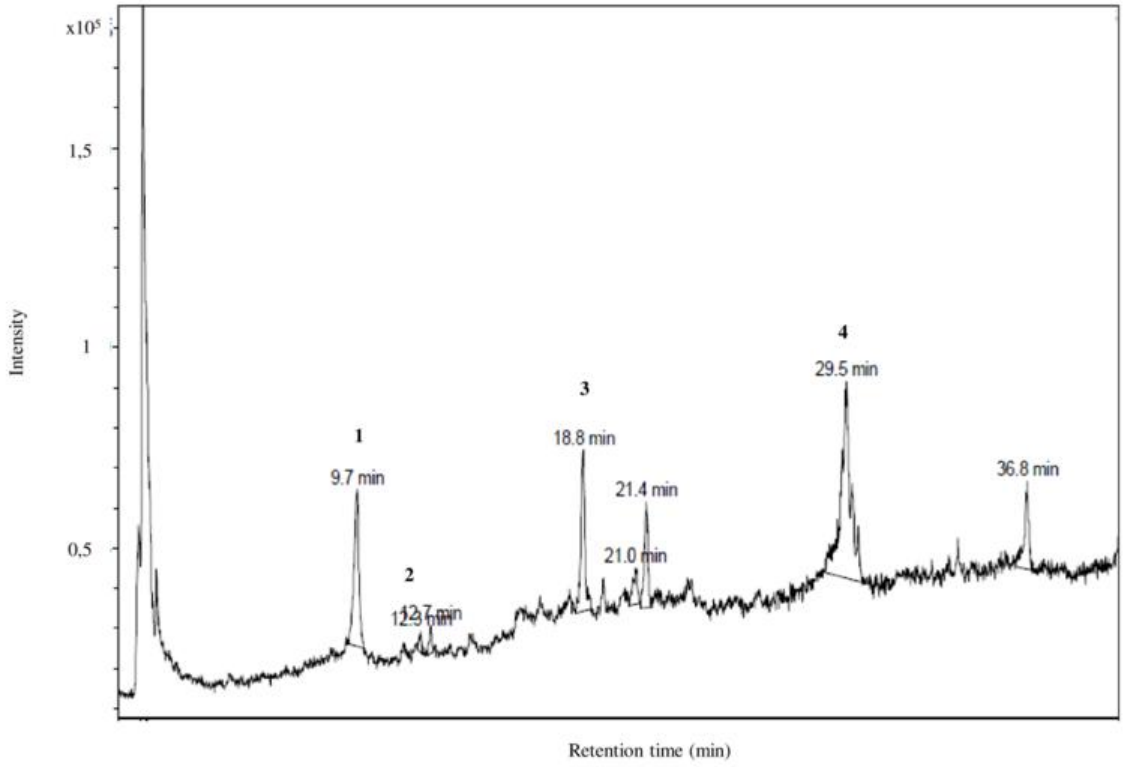
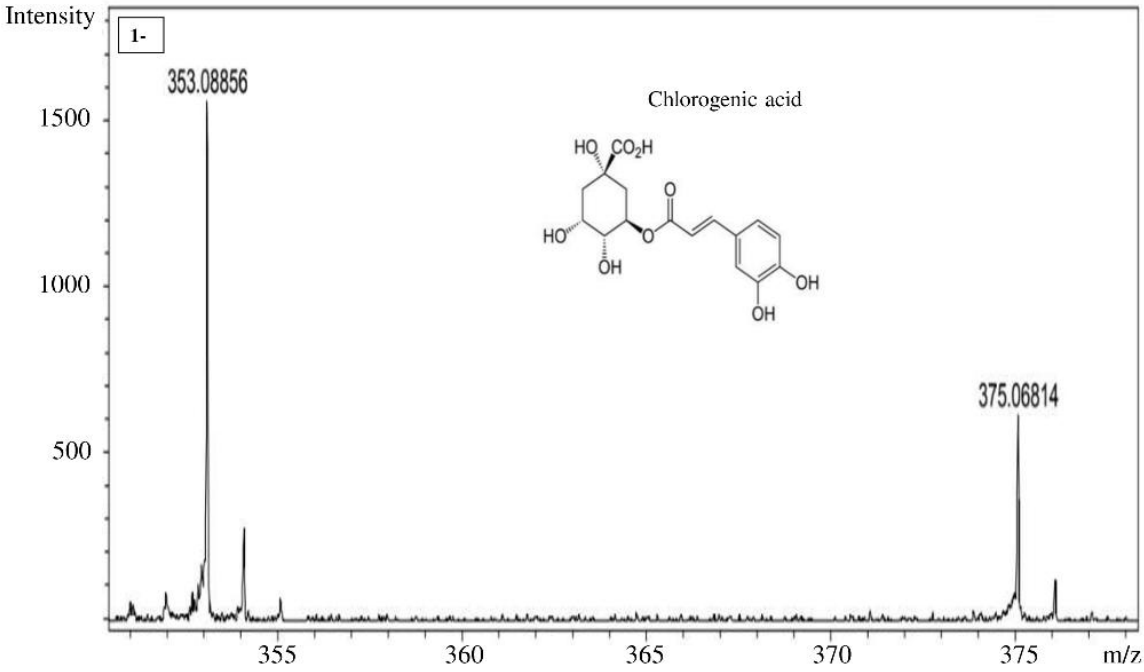


Fig. S1. EPC analysis by LC-MS/MS. A: Total chromatogram. B: Mass spectrum of the compounds identified: 1) chlorogenic acid, 2) caffeic acid, 3) azelaic acid; the inset corresponds to the MS2 of m/z 187.09816 peak, 4) oleic acid; the inset corresponds to the MS2 of m/z 329.22908 peak.

A)



B)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CO2H
HO
OH
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OH
OH
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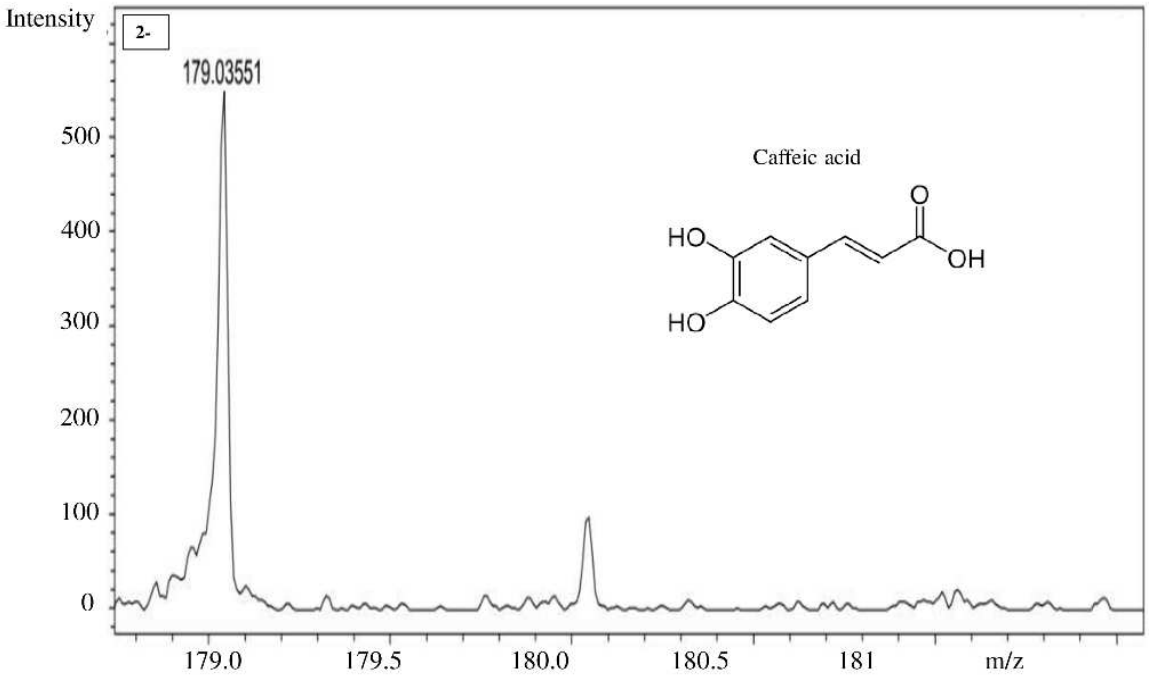
500
355
360
365
370
375
m/z

686

687

688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300
200
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179.0
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180.0
180.5
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m/z
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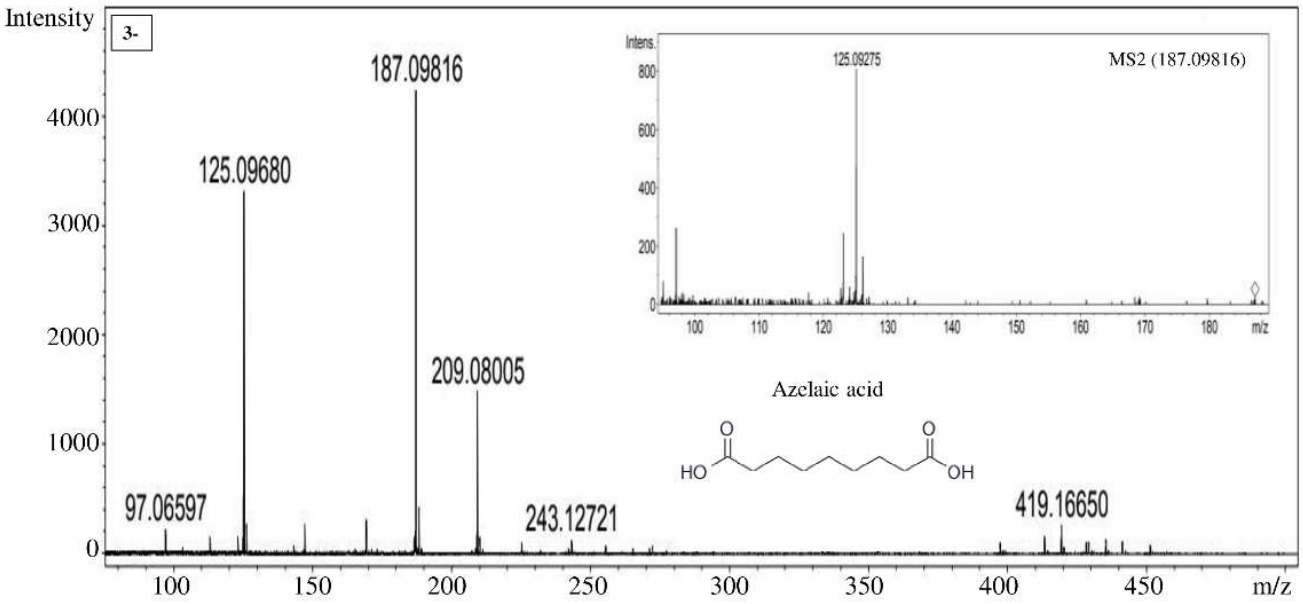
HO
O
OH

690

691

692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0
100
150
200
250
300
350
400
450
m/z
Intens.
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MS2 (187.09816)
800
600
400
200
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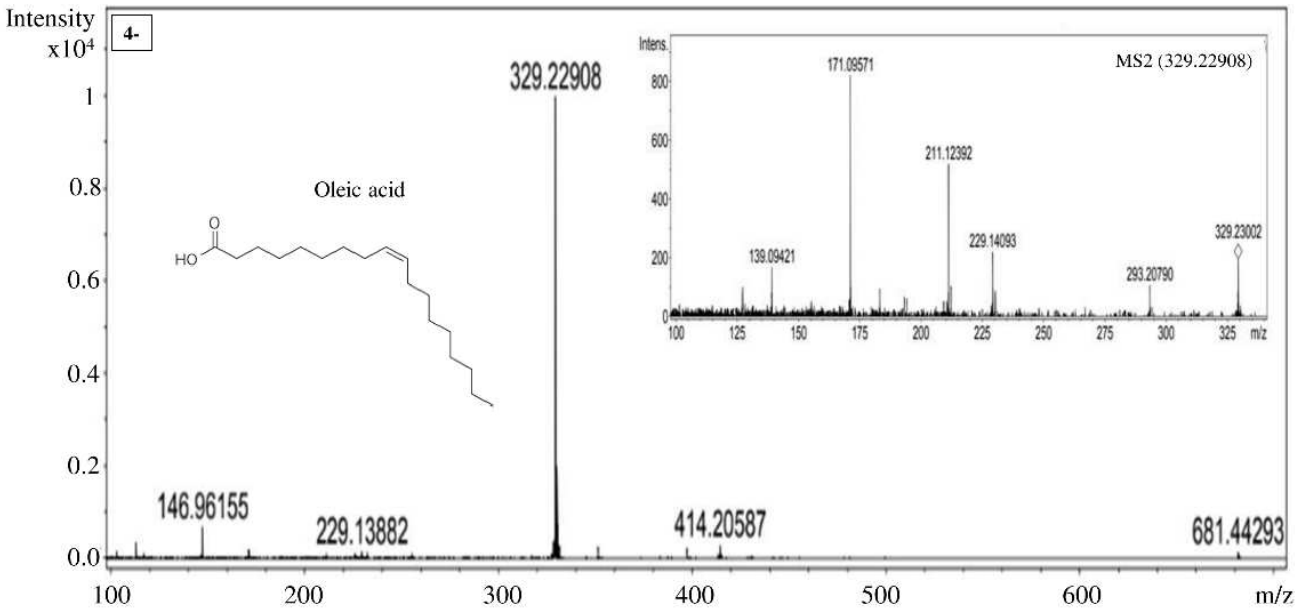

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