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In Vitro Antifungal Potential of *Barkleyanthus salicifolius* and *Punica granatum* Extracts Against Crop-Associated Pathogens

Martha Salinas-Sandoval [1,2], Gildardo Rivera [2], Luis Fernando Ceja-Torres [1], Martha-Isabel González-Domínguez [3], Alma D. Paz-González [2], Janneth López-Mercado [3] and Dioselina Álvarez-Bernal [1,*]

[1] Instituto Politécnico Nacional, Centro Interdisciplinario de Investigación para el Desarrollo Integral Regional, Unidad Michoacán, Justo Sierra 28, Jiquilpan 59510, Michoacan, Mexico; msalinass2300@alumno.ipn.mx (M.S.-S.); lfceja@ipn.mx (L.F.C.-T.)

[2] Pharmaceutical Biotechnology Laboratory, Genomic Biotechnology Center, Instituto Politécnico Nacional, Boulevard del maestro S/N esq, Elías Piña, Reynosa 88710, Tamaulipas, Mexico; giriveras@ipn.mx (G.R.); apazg@ipn.mx (A.D.P.-G.)

[3] Ingeniería en Nanotecnología, Universidad de la Ciénega del Estado de Michoacán de Ocampo, Avenida Universidad #3000, Colonia Lomas de la Universidad, Sahuayo 59103, Michoacan, Mexico; migonzalez@ucemich.edu.mx (M.-I.G.-D.); jannlopezm@gmail.com (J.L.-M.)

* Correspondence: dalvarezb@ipn.mx

Abstract

The potential of methanolic extracts from jara (*Barkleyanthus salicifolius*) and pomegranate carpel membranes (*Punica granatum*) as biological alternatives for the control of phytopathogenic fungi was evaluated against pathogens associated with commercially important crops in the Ciénega de Chapala region. Extracts were assessed in vitro against *Botrytis cinerea* and *Rhizoctonia solani* (strawberry), *Curvularia* sp., *Pestalotiopsis* sp., and *Fusarium oxysporum* (blackberry), *Pythium* sp. and *Fusarium* sp. (tomato), and *Sclerotium rolfsii* (onion). Antifungal bioassays demonstrated that the *B. salicifolius* extract inhibited the mycelial growth of *R. solani*, whereas the pomegranate extract inhibited seven of the eight species tested, with the exception of *S. rolfsii*. Phytochemical screening revealed the presence of alkaloids, flavones, flavonols, chalcones, and quinones in pomegranate, and flavones, flavonols, alkaloids, and sterols in jara. Additionally, phytol and caryophyllene were identified in the latter via GC–MS.

Keywords: antifungal activity; biological control; plant extracts

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1. Introduction

For decades, crop disease management has relied on eradication or prevention strategies based on synthetic chemical compounds with fungicidal activity [1]. However, the indiscriminate use of these inputs has led to significant consequences for agricultural sustainability, including increased production costs, complexities in risk management, the presence of pesticide residues in agricultural products, the deterioration and alteration of ecosystems, the development of fungicides-resistant strains, and potential impacts on human health. This situation has driven the search for safer and more environmentally compatible alternatives for phytosanitary control [2,3].

Among phytopathogenic microorganisms, fungi represent one of the most diverse and agronomically relevant groups. More than 8000 fungal species have been reported to cause damage, often spreading to multiple hosts [4]. Berry, tomato, and onion crops in

Mexico, particularly in Michoacán, are exposed to a wide variety of plant-pathogenic fungi, including species of *Fusarium*, *Rhizoctonia*, *Phytophthora*, *Pythium*, *Colletotrichum*, *Botrytis*, *Alternaria*, and *Stemphylium* [5–7]. The etiological complexity of diseases such as strawberry blight, which is associated with pathogen complexes, complicates their management and increases their impact on agricultural production, with these genera having the greatest economic impact and prevalence in fruits and vegetables [8]. Consequently, fungal diseases can significantly limit agricultural production globally, with particularly severe impacts in developing countries, where production systems tend to be more vulnerable and have less access to integrated management technologies [9,10].

In this context, plant extracts have gained interest as an alternative for controlling phytopathogenic fungi due to their chemical diversity and biological potential. These extracts constitute a rich source of metabolites with antifungal activity, such as essential oils, terpenoids, glucosinolates, flavonoids, and phenols, among others [11]. In addition to their direct action on the pathogen, their chemical complexity may contribute to reducing the likelihood of resistance development, unlike what has been observed with single-target synthetic molecules [12,13]. Therefore, recent research has focused on utilizing plant extracts and developing plant secondary metabolites as an alternative for producing biomolecules with fungicidal properties and potential application in integrated disease management programs [14].

Secondary metabolites have been associated with a wide range of biological activities, including antibacterial, antioxidant, and anticancer properties, which has encouraged the exploration of natural compounds with specific functions. Experimental evidence supports their usefulness in controlling fungal pathogens, for instance, extracts such as that of *Jatropha curcas* inhibit the mycelial development of *Colletotrichum musae*, the causal agent of anthracnose in banana crops; likewise, their effectiveness has been reported for the control of *Sclerotium* spp., related to *Azolla* disease [15]. Similarly, essential oils, methanolic extracts, and other fractions obtained from *Metasequoia glyptostroboides* show high potential against *Fusarium oxysporum*, *Fusarium solani*, and other fungi of phytopathological importance. Additionally, antifungal activity has been documented in species belonging to various botanical families, including *Umbelliferae*, *Cucurbitaceae*, *Celastraceae*, and *Poaceae*, reinforcing the taxonomic breadth of plants with biofungicidal potential [14,16–18].

Various plant species have attracted interest as potential sources of antifungal compounds for the management of phytopathogenic fungi of agricultural importance. Among them, *Barkleyanthus salicifolius* has shown promising results in vitro assays, particularly against *Fusarium oxysporum* and *Colletotrichum gloeosporioides*. The observed activity has been associated primarily with aqueous and ethanolic extracts obtained from different plant organs, with ethanolic extracts generally exhibiting higher levels of phenolic compounds and flavonoids, as well as a greater inhibitory effect on fungal growth. Although the available evidence for this species is still limited and focuses mainly on preliminary studies, the results suggest that *B. salicifolius* constitutes a plant source with potential for the development of naturally derived antifungal alternatives [19].

In addition, *Punica granatum*, particularly through its peel extracts, has been shown to have antifungal activity against various plant pathogens, including *Botrytis cinerea*, *Aspergillus flavus*, *Fusarium proliferatum*, *Fusarium oxysporum*, as well as species of *Colletotrichum*, *Penicillium*, *Rhizoctonia*, and *Monilinia* [20,21]. Its biological effect has been linked to the presence of bioactive phenolic compounds, such as punicalagins, ellagic acid, and tannins, which have demonstrated the ability to inhibit germination, mycelial growth, and, in some cases, mycotoxin production [22]. Taken together, these findings support the interest in *P. granatum* as a plant-based alternative with potential application in sustainable control strategies for diseases caused by phytopathogenic fungi.

Based on the available evidence regarding the biological activity of plant extracts and their secondary metabolites, the objective of this study is to evaluate the in vitro antifungal activity of extracts obtained from plant species with potential phytosanitary applications, with a view to developing more sustainable alternatives for crop disease management. In this context, *Barkleyanthus salicifolius* was selected due to its wide distribution and abundance in the study area, where it is commonly considered a ruderal species or weed, making it an accessible and underutilized plant resource. Likewise, *Punica granatum*, particularly its fruit, was included due to its regional relevance as a raw material in the preparation of "pomegranate punch," a traditional beverage associated with local festivities, which highlights its availability and sociocultural value.

2. Materials and Methods

2.1. Places Where Plant Material Was Collected to Obtain Extracts

Samples of rockrose (*B. salicifolius*) plants and pomegranate (*Punica grantum*) fruits were collected during July–August (summer of 2024) at random in the municipality of Venustiano Carranza, Michoacán (20°06′33″ North latitude and 102°38′56.69″ West longitude, at an altitude of 1533 m above sea level in Jiquilpan, Michoacán, México (19°59′28″ north latitude and 102°43′5″ west longitude, at an altitude of 1560 m above sea level; municipalities that are part of the Ciénega de Chapala in Michoacán. Strawberries with gray rot disease were collected in the municipalities of Zamora, Mich., as well as plants with symptoms of "dry rot" in Los Reyes, Mich. blackberry plants with obvious signs of disease, necrosis, and dieback were obtained, and in Cojumatlán, Mich., wilted tomato plants and onion bulbs showing signs of "white rot" disease were collected.

2.2. Isolation, Purification and Identification of Phytopathogenic Fungi

Diseased plant material, including roots, branches, bulbs, and fruits, was washed with water and cut into small pieces (1–2 cm). These pieces were disinfected with 3% sodium hypochlorite for two minutes and then rinsed three times with sterile distilled water. After removing excess moisture, the pieces were inoculated onto Petri dishes containing potato dextrose agar (PDA) and incubated at 27 °C (Felisa SI-1, Zapopan, Mexico). The colonies obtained after 5–10 days were transferred to water agar (WA) to promote the development of isolated hyphae. Four days later, pure cultures were obtained using the hyphal tip technique and transferred back to PDA. Morphological identification was performed both microscopically and macroscopically based on the growth characteristics of each strain, using analytical keys [23–25].

Pathogenicity was confirmed by fulfilling Koch's postulates, whereby fungi isolated from diseased tissue and purified in culture were inoculated into healthy hosts and subsequently re-inoculated from tissues that developed symptoms similar to those originally observed. This was carried out on previously disinfected strawberry fruits and onion bulbs, placing a 5 mm diameter PDA disc containing the corresponding fungus on the tissue. In the case of strawberry and tomato seedlings, incisions were made at the base of the stem, where a fungal disc was applied. In blackberry plants, incisions were made in the upper section of the stems; the PDA discs were placed and secured with Parafilm® to ensure contact. All tests were performed in triplicate, using a sterile PDA disc without fungus as a negative control [26].

2.3. Preparation of Methanol Extracts

Extracts were obtained from dried rockrose leaves (*B. salicifolius*) and from the pomegranate (*Punica granatum*) carpels and pericarp. For each sample, 100 g of plant material were weighed and macerated in 300 mL of 70% methanol. Following the method

described by Cheong et al. [27], the mixtures were shaken for 15 min and allowed to stand for 48 h at 25 °C in the dark. The resulting solutions were individually filtered through Whatman® No. 4 paper. To reduce the volume, the supernatants were concentrated using a vacuum rotary evaporator (Labconco, 2078A, KC USA) at 45 °C and 150 rpm until a concentrated liquid was obtained without reaching complete dryness.

2.4. Inhibitory Activity of Methanolic Extracts, Using the Disc Assay Technique

A suspension of conidia and mycelium was made in 10 mL of sterile distilled water from each of the fungal colonies. Next, 5 µL of each suspension was transferred to Petri dishes containing PDA culture medium using the spread plaque technique. Once the agar had solidified, five sterile discs were placed inside each Petri dish to add three concentrations of each extract (50 mg/mL, 100 mg/mL, and 200 mg/mL), a positive control (thiabendazole, 1 mg/mL)—determined based on the average recommended dose for the application of the commercial product, used to eradicate these plant pathogens in crops—and a negative control (methanol, 100 µL), which was performed in triplicate. The plates were then incubated at 25 °C, and the inhibition zones were measured between 5 and 10 days later [28–30].

2.5. Inhibitory Activity of Methanolic Extracts of Pomegranate (Punica granatum) and Rockrose (Barkleyanthus salicifolius) Using the Microdilution Method

Microdilution tests were performed according to the NCCLS (National Committee for Clinical Laboratory Standards) M38-A protocol for filamentous fungi, with modications to the dilution range [31]. Although this protocol is standardized for pure compounds using low concentrations, higher concentrations were required here due to the complex mixture of compounds present in natural extracts. Each fungus was cultured in potato dextrose broth (PDB) as a negative control, and calculations were performed to inoculate each well of a 96-well microplate with 1×10^6 CFU. The fungicide thiabendazole (1 mg/mL) served as the positive control for each phytopathogenic isolate. Five extract concentrations (12.5 µL, 25 µL, 50 µL, 100 µL, and 200 µL) were tested to evaluate growth inhibition. The plates were incubated for 48 h, and absorbance readings were taken at 595 nm using a spectrophotometer iMark™ #168-1130XTU (Bio-Rad Laboratories, Inc., Hercules, CA, USA.) at 0, 24, and 48 h [32–34].

2.6. Qualitative Tests for Alkaloids and Sterols

To detect alkaloids, the extract was acidified with HCl (5:5, *v*/*v*), followed by the addition of a few drops of Wagner's reagent. A positive result was indicated by the formation of brown precipitates. A second test was performed using Dragendorff's reagent, where the formation of red, orange, or brown precipitates, persisting for 24 h, confirmed the presence of alkaloids. Sterols were determined by adding Liebermann–Burchard reagent (1 mL of acetic anhydride, 1 mL of chloroform, and one drop of sulfuric acid) to the extract. The test was considered positive if a coloration ranging from green to red and yellow was observed after 15 min [35,36].

2.7. Qualitative Tests for Flavones, Flavonols, Chalcones, and Quinones

Salkowski's reagent was employed for these analyses. Briefly, 1 mg of extract was dissolved in 1 mL of chloroform, followed by the addition of 1 mL of sulfuric acid. The presence of flavones and flavonols was indicated by a yellow coloration, while chalcones were identified by a red-blue tint, and quinones by a red-purple coloration [35,36].

2.8. GC-MS Analysis of the Methanol Extract (Barkleyanthus salicifolius)

The *Barkleyanthus salicifolius* extract was analyzed using an Agilent gas chromatograph (HP6890, DE, USA) equipped with a mass selective detector (HP5973), following established methodologies for bioactive compound analysis [37,38]. Helium was used as the carrier gas at a flow rate of 1 mL/min. Samples were introduced via split injection (50:1 ratio) at 250 °C onto a non-polar HP-5MS capillary column (25 m × 0.25 mm × 0.25 µm). The oven temperature program was set as follows: an initial temperature of 50 °C, increased at a rate of 5 °C/min to 280 °C (held for 1 min), followed by a second ramp of 25 °C/min up to a final temperature of 300 °C (held for 3 min). The mass spectrometer was operated in SCAN mode at 70 eV, with an interface temperature of 250 °C and a mass range of 50–500 m/z. Compound identification and purity were confirmed using the NIST02 (National Institute of Standards and Technology v2) database. Only peaks with a purity index of 1 and a spectral match confidence between 80% and 100% were accepted.

2.9. Statistical Analysis

A one-way analysis of variance (one-way ANOVA, $p \leq 0.05$, SAS v9.0 and OriginPro v8) will be performed on the results obtained, using the diameter of the inhibition zones as the response variable, in order to identify statistically significant differences in activity against plant pathogenic fungi among the evaluated treatments, doses, and the positive control. The aim is to simultaneously compare both extracts and independent doses, determining whether the variations observed in the inhibition zones were due to the effect of the treatments and not to random variability.

3. Results

3.1. Fungal Isolation and Identification

Eight fungal genera were isolated from diseased plant material at the study site and identified based on their microscopic and macroscopic morphological characteristics (Table 1). From strawberry fruits exhibiting gray mold, *Botrytis cinerea* was isolated, and from necrotic roots of plants showing "dry rot," *Rhizoctonia solani* was identified. From necrotic roots and dieback in blackberry, *Curvularia* sp., *Pestalotiopsis* sp., and *Fusarium oxysporum* were isolated. From necrotic roots of tomato plants with wilt symptoms, *Pythium* sp. and *Fusarium* sp. were isolated. Finally, *Sclerotium rolfsii* was isolated from onion bulbs affected by southern blight. Koch's postulates were fulfilled for all isolates, confirming their pathogenicity and establishing a causal relationship with the diseases observed in the crops of interest [39,40].

Table 1. Morphological characteristics of each identified species.

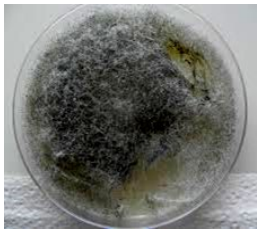
Gender	Characteristics of the Colony	Microscopic Characteristics	Isolation
Botrytis cinérea	Produces large amounts of gray or dark gray mycelium.	Long, branched conidiophores, whose rounded apical cells produce clusters of ovoid, unicellular, colorless or gray conidia. The conidiophores and conidia clusters resemble a bunch of grapes. The fungus readily releases its conidia when the weather is humid, and they are then spread by the wind. The fungus often produces irregular, flat, hard, black sclerotia. Some species sometimes produce a perfect phase of Sclerotinia, in which ascospores are formed on an apothecium [23,24].	

Table 1. *Cont.*

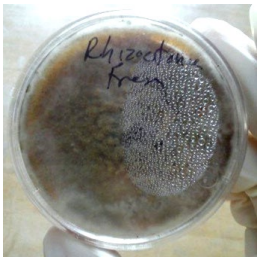
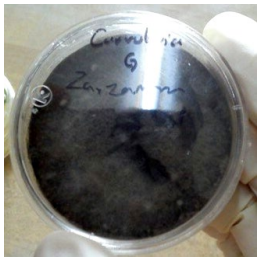
Gender	Characteristics of the Colony	Microscopic Characteristics	Isolation
Rhizoctoniasolani	It is characterized by dark brown pigments in the hyphae.	Branching near the central septum in the young vegetative hypha. Constriction of the hypha and formation of septa a short distance from the point where the hyphal branches originate, distal septum. Multinucleate cells in the young vegetative hypha [23,24].	
Phytium sp.	White, cottony-looking colonies.	It is characterized by the fact that in the sexual structures, the antheridium surrounds the oogonium (paragynion). Reproductive structures and oospore formation occasionally develop depending on the medium in which the strain is cultivated; immature sporangia can be observed [23–25].	
Fusarium sp. *Fusarium oxysporum*	Colonies grow moderately to profusely, displaying various colors of white, pale pink, red, orange, purple, light blue, olive green, or brown, especially on the underside of the colony, except for dark brown or black. The mycelium is sparse or dense, either cottony, felt-like, or with a central zone of funicles, but in some cases it is slimy. There are orange-colored fusaria. The pigments that diffuse in the agar tend to vary in color or tone with the pH. Some species have concentric zones of different macroscopic morphology due to the light-dark sequence.	The spores are dispersed in the aerial mycelium or in sporodochia or slimy masses (pionotes). The macroconidia are curved, pluriseptate, with a more or less pointed apical cell and, in many species, with a foot-shaped basal cell. Microconidia are commonly unicellular, ellipsoidal, fusiform, clavate, pyriform, or subglobose, similar in width to macroconidia, with a rounded or truncated base, usually forming mucous heads, but in some species in basipetal chains. The conidiophores of the aerial mycelium in some cases consist only of a conidiogenous cell, in others they are branched, sometimes in whorls [41–43].	
Curvularia sp.	Cottony colonies, white to gray in color, turning olive green, brown to black as they mature. Dark brown to black reverse side.	Septate dematiaceous hyphae. Conidiophores straight, brown, multicellular, simple or branched, bent at the tips where the conidia originate (porogenous conidiogenous cell), with sympodial proliferative growth. Conidia 8.14 × 21–35 µm, dematiaceous, multicellular, ellipsoidal, curved in the central cell, with 3 to 4 transverse septa [41–43].	

Table 1. *Cont.*

Gender	Characteristics of the Colony	Microscopic Characteristics	Isolation
Pestaloptiosis sp.	The colony is white and cottony, with diffuse aerial mycelium, advancing irregularly toward the edge and denser in the older parts of the colony. Colonies usually show daytime growth of mycelial zones and acervuli in formation, and the acervulus develops from a small yellowish clump of hyphae and gives rise to protruding masses of spores ranging from green to black. On the reverse side, the colony shows slight pigmentation with concentric grayish rings.	The reproductive bodies vary depending on the species and can be acervular or pycnidial, with most measuring 75–35 µm in diameter, like small black horn-shaped tabs (cirri) that can reach lengths of 2 mm [41–43].	
S. rolfsii	Feathery white colonies	The fungus produces abundant rounded sclerotia, ranging in color from tan to reddish-brown or dark brown, similar in size to mustard seeds. The sclerotia are attached to the mycelium on the surface of the plant [41–43].	

3.2. Identification of Secondary Metabolites

Qualitative tests revealed the presence of six types of metabolites groups in the methanolic extracts of pomegranate (*Punica granatum*): alkaloids, flavones, flavonols, chalcones, and quinones. Collectively, these results confirm that the extract primarily composed of phenolic and flavonoid compounds-groups frequently associated with biological activity in plant matrices. In the methanolic extract of rockrose (*Barkleyanthus salicifolius*), qualitative tests revealed the presence of four classes of metabolites: sterols, flavones, flavonols, and, predominantly, alkaloids (Table 2). Consistent with these findings, phytochemical studies of *B. salicifolius* have identified phenolic compounds and flavonoids as the primary bioactive metabolites, notably, ferulic acid and naringenin in ethanolic root extracts [19].

Table 2. Secondary metabolites in methanolic extracts of pomegranate (*Punica granatum*) and rockrose (*B. salicifolius*).

Extract	Alkaloids	Sterols	Flavones and Flavonols	Chalcones	Quinones
Pomegranate	+	−	+	+	+
Rockrose	+++	+	+	−	−

Reference: (+) presence of compounds, (+++) strong positive reaction (−) absence of compounds.

GC-MS analysis of the methanolic extract of rockrose (*B. salicifolius*), identified compounds with a high probability of correspondence (80–100). Two of these were particularly relevant due to their documented antifungal properties: phytol, which accounted for 24.8% of the extract, and the terpenoid caryophyllene at 5.6% (Figure 1).

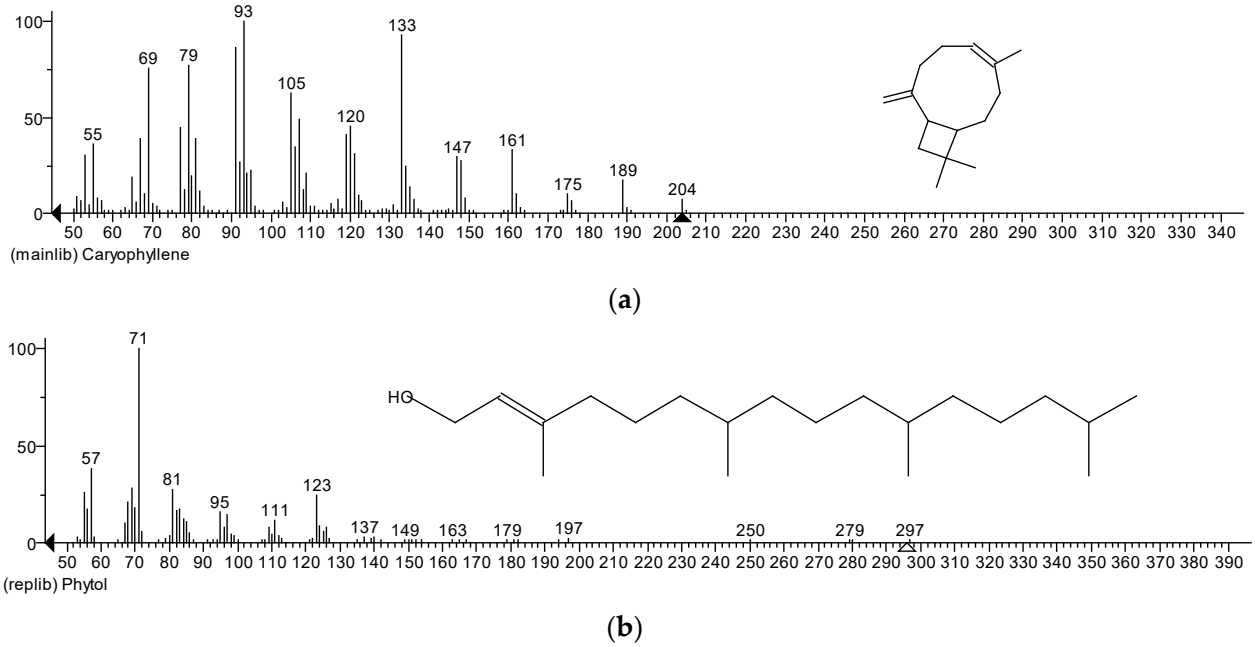


Figure 1. CG-MS spectra of the structure of phytol (**a**) and caryophyllene (**b**).

3.3. Antifungal Activity

The methanolic extract of pomegranate (*Punica granatum*) inhibited seven of the isolated fungi: *B. cinerea* (Figure 2a), *R. solani* (Figure 2b), *Curvularia* sp. (Figure 2c), *Pestalotiopsis* sp. (Figure 2d), *Fusarium oxysporum* (isolated from blackberry) (Figure 2e), *Pythium* sp. (Figure 2f), and two strains of *Fusarium oxysporum* (isolated from blackberry and tomato, respectively) (Figure 2h). However, it showed no activity against *S. rolfsii* (Figure 2). It should be noted that the fungicide thiabendazole also failed to inhibit *S. rolfsii* (Figure 2g).

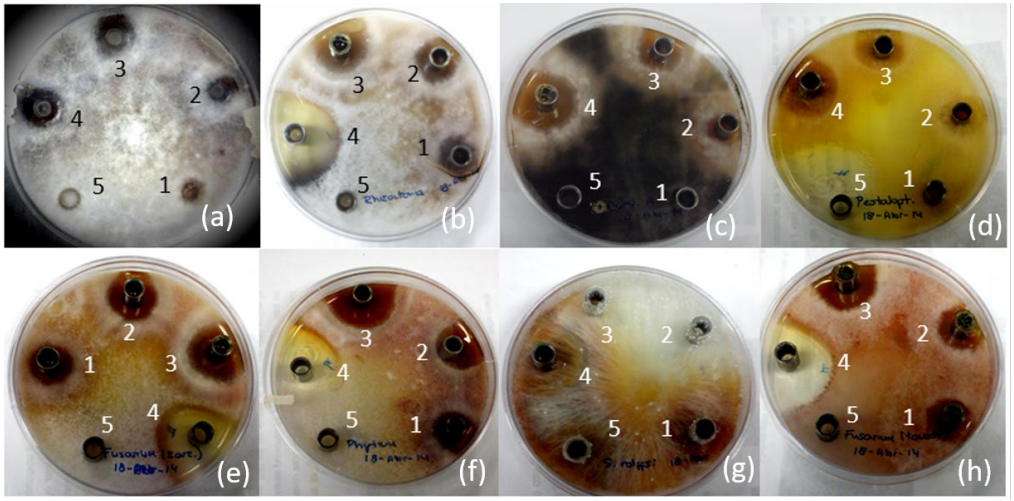


Figure 2. In vitro inhibition tests of pomegranate extract on isolated fungi. (**a**) *Botrytis cinerea*, (**b**) *Rhizoctonia solani*, (**c**) *Curvularia* sp., (**d**) *Pestalotiopsis* sp., (**e**) *Fusarium oxysporum* (blackberry), (**f**) *Pythium* sp., (**g**) *Sclerotium rolfsii*, and (**h**) *Fusarium* sp. (tomato); 1 = 50 µL, 2 = 100 µL, 3 = 200 µL of methanolic pomegranate extract, 4 = Thiabendazole, and 5 = Methanol.

Regarding the methanolic extract of rockrose (*B. salicifolius*), it only inhibited the growth of *Rhizoctonia solani*; however, this effect was not persistent up to the 96 h mark. Fungal colony diameters were measured after 5–10 days of incubation. Antifungal activity was expressed as the inhibition of mycelial growth, determined by measuring the diameter of the inhibition zone (mm).

The extract of *Punica granatum*, showed a higher level of activity compared to the positive control (tiabendazole) against *Curvularia* sp. and *Pestaloptiosis* sp., and a similar level of significance ($p \leq 0.05$) against *Phytium* sp., indicating greater activity than the positive control used to treat these types of conditions. In the case of *B. salicifolius*, no high level of significance was observed compared to the positive control (thiabendazole), but similar activity was observed against *Rhizoctonia solani* (Table 3).

Using the microdilution method, the inhibitory activity of pomegranate (*Punica granatum*) extract against *Curvularia* sp. was evident (Figure 3). The absorbance of the *P. granatum* extract against *Fusarium oxysporum* (Figure 3a). varied depending on the concentration and the time point of evaluation. At all three time points analyzed, the negative control exhibited the highest values, with a marked increase at 24 and 48 h, indicating greater fungal growth in the absence of treatment. In contrast, the positive control showed considerably lower values, indicating an inhibitory effect on fungal growth. Among the concentrations evaluated, the response varied over time; at 24 and 48 h, the 12.5 µL dose tended to record the lowest absorbances, while the 25 µL dose showed higher values compared to the other doses. Grouping letters indicated significant differences among various treatments, confirming that both concentration and time significantly influenced the observed response. The absorbance of *Curvularia* sp. treated with *P. granatum* extract (Figure 3b), was influenced by treatment concentration and evaluation time. In the initial measurement at 0 h, absorbance values were low across all treatments, although the 100 µL dose exhibited the highest value among the concentrations evaluated. At 24 h, the positive control recorded the highest absorbance, followed by the negative control, while among the extract doses, 100 µL stood out with the highest value and 12.5 µL with one of the lowest. At 48 h, the negative control showed the highest absorbance, followed by the positive control; among the concentrations, the 100 µL dose maintained the highest response, while 200 µL and 50 µL had the lowest values. Significant differences ($p \leq 0.05$) were observed between treatments at the various evaluation time points, suggesting a differential effect of the concentrations on fungal growth.

The inhibitory activity of rockrose extract (*B. salicifolius*) was observed exclusively against *Rhizoctonia solani*. This activity was influenced by the treatment concentration and the time point of evaluation. At all three time points analyzed, the negative control consistently exhibited the highest mean values. At the initial measurement (0 h), absorbance values were low in most treatments, although the 25 µL treatment showed the highest response among the doses. At 24 h, the 100 µL concentration recorded the highest absorbance among the extract treatments, while 200 and 50 µL showed the lowest values. At 48 h, the 50 µL dose exhibited the highest absorbance among the evaluated concentrations, followed by 100 µL, while 200 µL maintained the lowest response. Showing statistical differences ($p \leq 0.05$) between treatments, demonstrating a differential effect of concentration and time on fungal growth (Figure 4).

Table 3. Inhibition zones (cm) of the methanolic extract of pomegranate (*Punica granatum*) and rockrose (*B. salicifolius*) on the isolated fungi.

Inhibition of Mycelial Growth "In Vitro" Activity of Methanolic Extract (cm)								
	Punica granatum						*Barkleyantus salicifolius*	
Treatments	*Botrytis cinerea*	*Rhizoctonia solani*	*Curvularia* sp.	*Pestalotiopsis* sp.	*Fusarium* sp.	*Pythium* sp.	*Fusarium oxysporum*	*Rhizoctonia solani*
(50 mg/mL)	1.03 ± 0.057^{D}	1.35 ± 0.050^{C}	1 ± 0.095^{C}	1.16 ± 0.152^{C}	1.25 ± 0.040^{C}	1.47 ± 0.040^{C}	1.06 ± 0.115^{C}	0.00
(100 mg/mL)	1.64 ± 0.121^{C}	1.58 ± 0.102^{C}	1.62 ± 0.064^{B}	1.74 ± 0.052^{B}	1.96 ± 0.057^{B}	1.96 ± 0.057^{B}	1.56 ± 0.057^{B}	1.06 ± 0.115^{C}
(200 mg/mL)	2.46 ± 0.152^{B}	2.28 ± 0.080^{B}	2.28 ± 0.080^{A}	2.25 ± 0.050^{A}	2.06 ± 0.115^{B}	2.6 ± 0.264^{A}	1.68 ± 0.028^{B}	1.83 ± 0.288^{B}
(C +) *	3.06 ± 0.115^{A}	3.35 ± 0.160^{A}	0.00^{D}	1.83 ± 0.288^{B}	2.83 ± 0.288^{A}	2.8 ± 0.288^{A}	2.76 ± 0.251^{A}	3.45 ± 0.104^{A}
(C −) **	0.00^{E***}	0.00^{D}	0.00^{D}	0.00^{D}	0.00^{D}	0.00^{D}	0.00^{D}	0.00^{D}

* Positive control corresponds to thiabendazole (1 mg/mL). ** Negative control corresponds to methanol (100 µL). *** Figures with the same letter in each column are statistically equal (DMS $p \leq 0.05$). Each value corresponds to the mean of three replicates ± standard deviation.

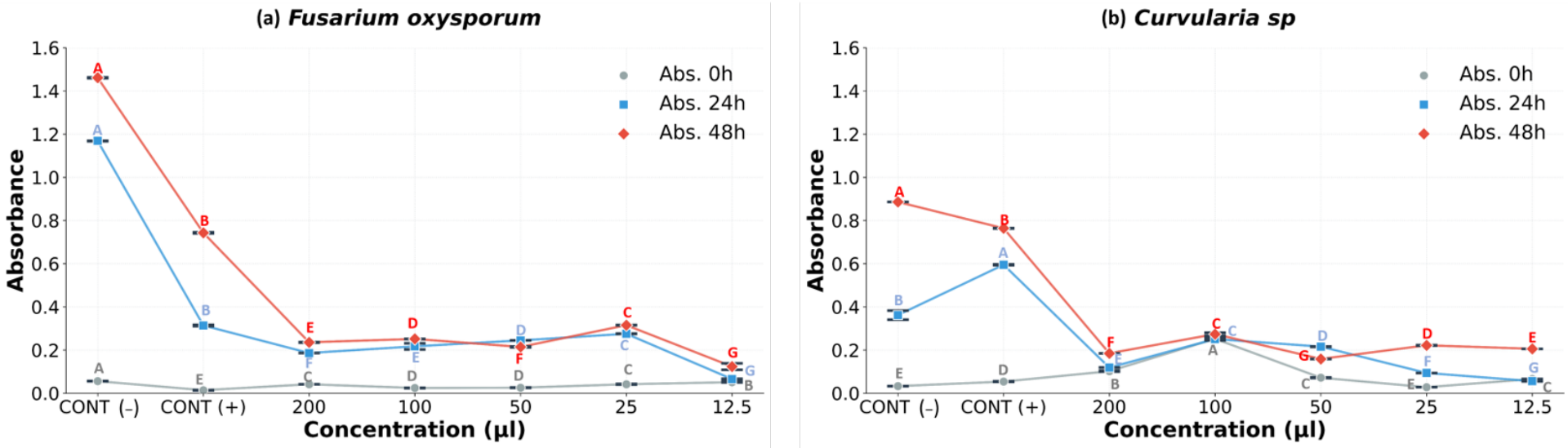


Figure 3. Effect of pomegranate extract (*Punica granatum*) concentration on *Fusarium oxysporum* (**a**) and *Curvularia* sp. (**b**) inoculum.

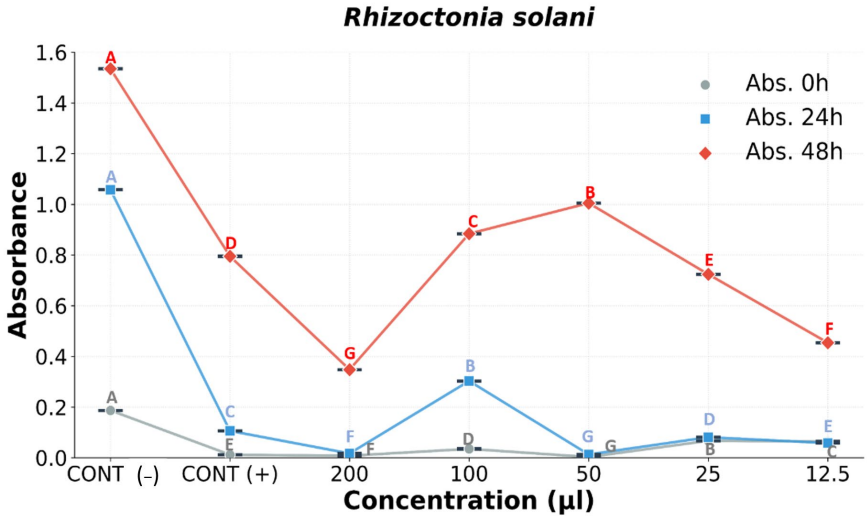


Figure 4. Effect of rockrose extract *(B. salicifolius*) concentration on *Rhizoctonia solani* inoculum.

4. Discussion

Through the isolation and identification of plant-pathogenic fungi in affected fruit and vegetable crops, we have found that many of these diseases are caused by multiple pathogens rather than by individual species. Strawberry blight, for example, is caused by a group of causal agents that includes *Fusarium oxysporum*, *Rhizoctonia fragariae*, *Pythium aphanidermatum*, and *Phytophthora* sp. This etiological complexity has important implications for management, as control strategies must be effective against multiple pathogens with different biological and epidemiological characteristics, which requires accurate diagnoses to implement appropriate management strategies [44].

Phytochemical tests support us as a first experimental filter to identify the presence of classes of non-volatile metabolites in plant extracts and prioritize fractions for isolation and subsequent characterization, facilitating a correlation between chemical composition and biological activity that could be exerted; this includes flavonoids (quercetin and hesperetin) and phenolic acids such as gallic acid [45], which are detected in *Punica granatum* extract, supporting the phytochemical content. These phenolic compounds, including ellagitannins (punicalagin and punicalin), ellagic acid, and other polyphenols, have shown significant antifungal activity against commercially important phytopathogens through multiple mechanisms. In particular, it has been proposed that punicalagin directly inhibits topoisomerases I and II, interfering with essential processes of fungal DNA replication and segregation [46]. Additionally, molecular dynamics analysis suggests that these compounds may interact with ergosterol in the fungal cell membrane, causing disturbances in membrane integrity similar to those observed with conventional antifungal agents [47]. The efficacy of pomegranate peel extracts has been documented in vitro and in vivo studies against agriculturally relevant pathogens, where structural alterations in the fungal cell wall, increased production of reactive oxygen species (ROS), and activation of host defense pathways were observed [48–50]. These findings position pomegranate secondary metabolites as a promising alternative for the development of biofungicides with multiple and specific mechanisms of action at the molecular level, providing an important contribution to the conditions associated with the diseased material collected in this study.

In accordance with this phytochemical study, it has been reported that *B. salicifolius* contains phenolic compounds and flavonoids as its main bioactive metabolites, notably ferulic acid and naringenin in ethanolic root extracts, which have been positively correlated with antioxidant activity. Likewise, the presence of pyrrolizidine alkaloids and phenolic compounds (including phenolic acids and flavonoids) has been reported in this botanical

genus, with evidence of in vitro antifungal activity against phytopathogenic fungi such as *Colletotrichum gloeosporioides* [19]. At the molecular level, it has been proposed that the phenolic compounds of *B. salicifolius* may interact with fungal membrane proteins, while some flavonoids inhibit cytochrome P450-dependent steroid hydroxylases and oxidases, altering key pathways for sterol biosynthesis. However, specific evidence on the mechanisms of action of *B. salicifolius* and its direct effects on phytopathogenic fungi is still limited [51].

The phytol found in the extract of *B. salicifolius* through GC-MS analysis is an acyclic diterpene alcohol and a component of chlorophyll and various essential oils, with the same aliphatic structure that could facilitate interaction with biological membranes [52], which would increase and demonstrate its potential as an alternative bioactive compound. Therefore, its use as an additive and disinfectant has been evaluated [53]. Although current references on the specific effects of phytol on fungal membranes are scarce, existing evidence comes mainly from studies on mixtures or extracts. Such is the case with studies on *Ballota hisurta* oil, where Z-phytol was shown to inhibit the growth and sporulation of *Botrytis cinerea* and *Penicillium expansum*. Furthermore, its potential binding to 14α-sterol demethylase suggests an impact on sterol synthesis, which would theoretically compromise the integrity of the fungal cell membrane [54].

Among the various applications of caryophyllene, also found in *B. salicifoluis*, it is widely used as a preservative in food, medicine, and cosmetics. Its antifungal efficacy has been demonstrated in vitro against dermatophytes, showing activity comparable to that of ciclopirox olamine and sulconazole [55]. In addition, active derivatives have been evaluated against *Fusarium proliferatum*, and β-caryophyllene has been shown to exert direct molecular mechanisms on histone regulation and gene expression, which in turn affects spore germination and ribosomal biosynthesis. Although there is currently no evidence of a direct effect on the cell wall [56], as noted above, these compounds represent a promising biological alternative for the conditions associated with the diseased material collected in this study.

Based on the results obtained from the evaluation of inhibitory activity using the plate pour plate method, it was observed that the activity of *P. granatum* was very similar to that of the antifungal agent thiabendazole (positive control) and that this extract also exhibited activity against *Curvularia* sp., whereas the antifungal agent showed no activity against this species. In the case of the rockrose extract, activity was observed only against the genus *Rhizoctonia* sp. at concentrations of 100 µL and 200 µL, with the inhibition zone at the 200 µL concentration being similar to that of the positive control (thiabendazole), corresponding to moderate antifungal activity. Inhibitory zones of 0.3–1.7 cm were considered to indicate moderate antifungal activity (concentrations of 50 µL and 100 µL), while inhibitory zones of 2.0–3.5 cm were considered to indicate significant antifungal activity (concentrations of 200 µL), which were observed in the *P. granatum* extract [57].

The microdilution method, using the M38 A protocol, provides reproducible reference conditions for determining the in vitro susceptibility of filamentous fungi, helping to detect resistance and guide possible treatments that have a direct effect on the fungi studied. This allows us to demonstrate that the activity of *Punica granatum* has a greater effect on *Fusarium* sp. and *Curvularia* sp.

The response observed in *Fusarium oxysporum* was influenced by both the concentration tested and the exposure time, suggesting a significant interaction between these two factors on fungal growth. The fact that the negative control consistently exhibited the highest absorbance values at all three measurement time points confirms that, in the absence of an inhibitory agent, the pathogen maintained sustained and progressive growth. In contrast, the positive control showed a significant decrease in absorbance compared to the negative control, supporting the efficacy of the reference compound in limiting fungal

growth. However, a strictly linear concentration-dependent response was not observed among the evaluated extract doses, as some intermediate or low doses exhibited more favorable behavior than higher concentrations. In particular, the 12.5 µL dose tended to record the lowest absorbance values, while the 25 µL dose exhibited relatively high absorbance at later time points, which could indicate that the extract's antifungal effect does not depend solely on the amount applied, but also on factors such as the availability, stability, diffusion, or interaction of the active compounds in the medium. Furthermore, statistical analysis of the differences between treatments confirms that the observed variations were not random but rather the result of the differential effect of each concentration over time. Taken together, these findings suggest that the extract has an effect on the development of *F. oxysporum*, although its effect varies depending on the dose and the evaluation period, whereas in *Curvularia* sp., it was observed that the results were influenced by both the treatment concentration and the exposure time. In general, the highest absorbance values were observed in the control treatments, particularly at 24 and 48 h, suggesting greater fungal development under conditions where the inhibitory effect was absent or less evident. This behavior confirms that pathogen growth varied over time and that the system's response was sensitive to the conditions of each treatment. Among the concentrations evaluated, a strictly linear dose-dependent relationship was not observed, as some intermediate doses, such as 100 µL, exhibited relatively high absorbance values, while 200 and 50 µL tended to show lower responses at different evaluation times. This suggests that the antifungal activity of the treatment does not depend exclusively on the amount applied, but also on the interaction of the active compounds with the medium and the exposure time. Taken together, these results demonstrate a differential effect of the treatment on the growth of *Curvularia* sp.

While for *B. salicifilus*, its main activity is manifested on the inoculum of *Rhizoctonia solani*, was influenced by both the treatment concentration and the exposure time. The negative control consistently exhibited the highest absorbance values at all three evaluation time points, confirming sustained fungal growth in the absence of an inhibitory agent. In contrast, the extract treatments showed a variable reduction in absorbance, suggesting a differential effect on the development of the phytopathogen.

No linear dose-dependent response was observed among the concentrations evaluated, as higher concentrations did not always produce the lowest absorbance. In fact, at 24 h, the 100 µL dose showed a relatively high response, while at 48 h, the 50 µL concentration exhibited one of the highest values among the extract treatments. On the other hand, the 200 µL dose tended to record the lowest absorbances, particularly at the 24 and 48 h time points, suggesting a greater inhibitory effect under the test conditions. This behavior indicates that the antifungal activity of the treatment may depend not only on the applied concentration but also on the interaction of the active compounds with the medium and the exposure time. Overall, these findings indicate that the treatment evaluated inhibits the growth of *R. solani*, although its efficacy varies depending on the dose and the time of assessment.

5. Conclusions

Our study confirms that methanolic extracts from pomegranate (*P. granatum*) and jara (*B. salicifolius*) possess significant antifungal properties. Pomegranate extract showed broad-spectrum inhibition against a diverse range of pathogens isolated from strawberry, blackberry, and tomato crops; this extract reveals the ability to modify the phytopathogen's response, although that response did not follow a linear dose-dependent pattern, suggesting that the extract's biological activity may depend not only on the amount applied, but also on the nature, stability, and availability of its active metabolites in the test system.

B. salicifilus extract specifically targeted *Rhizoctonia solani*, where the treatment evaluated had a differential effect on the growth of *Rhizoctonia solani*, which was determined by the concentration applied and the duration of exposure.

These biological responses indicate that the secondary metabolites within these plants effectively suppress mycelial growth, offering a promising, eco-friendly alternative to traditional synthetic fungicides. As global agriculture seeks to mitigate the challenges of pesticide resistance and chemical toxicity, these extracts emerge as strong candidates for the development of biopesticides. Subsequent studies are necessary to isolate the responsible bioactive compounds, optimize application concentrations, and validate these findings in field trials.

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