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Original Article

Antifungal activity and phytochemical screening of inflorescences, leaves, stems, and roots extracts of *Verbesina sphaerocephala* (Asteraceae) against two phytopathogenic fungi

Atividade antifúngica e triagem fitoquímica de extratos de inflorescências, folhas, caules e raízes de *Verbesina sphaerocephala* (Asteraceae) contra dois fungos fitopatogênicos

K. Y. Rodríguez-Valdovinos[a], R. Salgado-Garciglia[a], J. R. Medina-Medrano[b], P. D. Loeza-Lara[b], J. E. López-Meza[c], R. E. N. del Río-Torres[a], A. Saavedra-Molina[a] and A. Hernández-García[a]*

[a]Universidad Michoacana de San Nicolás de Hidalgo, Instituto de Investigaciones Químico Biológicas, Morelia, Michoacán, México
[b]Universidad de La Ciénega del Estado de Michoacán de Ocampo, Licenciatura en Genómica Alimentaria, Sahuayo, Michoacán, México
[c]Universidad Michoacana de San Nicolás de Hidalgo, Centro Multidisciplinario de Estudios en Biotecnología, Morelia, Michoacán, México

Abstract

Verbesina sphaerocephala A. Gray (Asteraceae) is an endemic plant species with wide distribution in Mexico. This plant is used in traditional medicine and mainly has antioxidant and antifungal activity, due to its phenolic composition. The present study aimed to analyze the antifungal activity and perform a phytochemical screening of aqueous, methanol, ethyl acetate, and chloroform extracts of the inflorescences, leaves, stems, and roots of *V. sphaerocephala*. Antifungal activity was evaluated using the well diffusion method against two phytopathogenic fungi: *Botrytis cinerea* and *Fusarium oxysporum*. Phytochemical screening was performed for phenolic compounds, flavonoids, coumarins, quinones, saponins, and terpenes. Aqueous stem extract exhibited the highest antifungal activity, resulting in a 100% reduction in the mycelial growth of the two fungi at 10 mg/mL. This study revealed that aqueous stem extract suppressed the growth of *B. cinerea* at a concentration of 1 mg/mL and that this extract contains a high content (++) of phenolics, coumarins and quinones, with and flavonoids and terpenes slightly present (+). Our study showed that the aqueous stem extract of *V. sphaerocephala* contains phytochemical compounds with fungicidal properties, mainly against *B. cinerea*.

Keywords: aqueous stem extract, *Botrytis cinerea*, *Fusarium oxysporum*, polyphenols, terpenes.

Resumo

Verbesina sphaerocephala A. Gray (Asteraceae) é uma espécie de planta endêmica com ampla distribuição no México. Essa planta é utilizada na medicina tradicional e possui principalmente atividade antioxidante e antifúngica, devido à sua composição fenólica. O presente estudo teve como objetivo analisar a atividade antifúngica e realizar a triagem fitoquímica de extratos aquosos, metanólicos, de acetato de etila e clorofórmicos de inflorescências, folhas, caules e raízes de *V. sphaerocephala*. A atividade antifúngica foi avaliada pelo método de difusão em poço contra dois fungos fitopatogênicos: *Botrytis cinerea* e *Fusarium oxysporum*. A triagem fitoquímica foi realizada para compostos fenólicos, flavonoides, cumarinas, quinonas, saponinas e terpenos. O extrato aquoso de caule exibiu a maior atividade antifúngica, resultando em 100% de redução do crescimento micelial dos dois fungos a 10 mg/mL. Este estudo revelou que o extrato aquoso de caule suprimiu o crescimento de *B. cinerea* na concentração de 1 mg/mL e que esse extrato contém alto teor (++) de fenólicos, cumarinas e quinonas, com flavonoides e terpenos levemente presentes (+). Nosso estudo mostrou que o extrato aquoso de caule de *V. sphaerocephala* contém compostos fitoquímicos com propriedades fungicidas, principalmente contra *B. cinerea*.

Palavras-chave: extrato aquoso de caule, *Botrytis cinerea*, *Fusarium oxysporum*, polifenóis, terpenos.

1. Introduction

Species of the genus *Verbesina* belonging to the Asteraceae family, such as *V. macrophylla*, *V. lanata*, *V. encelioides* and *V. persicifolia*, have antifungal activities (Divya-Ramakrishnan et al., 2017; Ramseyer et al., 2017; de Veras et al., 2021). *Verbesina* represents the second largest genus of the Asteraceae family in Mexico, with 164 native and 138 endemic species (Villaseñor, 2004), several of which are used in traditional Mexican medicine because of their antioxidant, antifungal, antibacterial, and antitumor properties (Jain et al., 2008; Sindhu et al., 2011).

*e-mail: alejandra.hernandez@umich.mx
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Verbesina sphaerocephala A. Gray is a common plant in Michoacán, Mexico, and is abundant mainly on dirt roads or undisturbed areas. Various communities in these regions use it as a medicinal plant. *V. sphaerocephala* is also used as a melliferous plant in honey production (Villaseñor and Ortiz, 2012). Although there have been few phytochemical and biological activity studies on this species, it has recently been reported that this plant contains sesquiterpenes (e.g., cadinene) and flavonoids, among other phenolic compounds (e.g., rutin) that are related to antifungal activity (Arciniegas et al., 2020; Rodríguez-Valdovinos et al., 2021). Rodríguez-Valdovinos et al. (2021) demonstrated the antibacterial activity of methanolic extracts against *Staphylococcus aureus* and *Escherichia coli*. However, their antifungal properties and the identification of their main terpene and phenolic compounds are unknown.

Terpenes and phenolic compounds are among the most abundantly produced by plants, and due to their broad biological activities, such as antifungal properties, they are considered metabolites with a high potential to inhibit the growth of phytopathogenic fungi that cause various diseases, such as grey mould and root or stem rot caused by *Botrytis cinerea* and *Fusarium oxysporum* in berries. Owing to these characteristics, studying plants that produce these types of metabolites is important for searching alternatives for new antifungals to reduce the use of chemically synthesized fungicides (Mahlo et al., 2010).

Among polyphenols, phenolic acids and flavonoids exhibit antifungal activities; some can inhibit the germination of fungal spores, whereas others cause cell deformation and lysis (Pusztahelyi et al., 2015). Flavonoids (e.g., quercetin) and phenolic acids (e.g., ferulic acid) have shown antifungal activity against phytopathogens, including *Alternaria alternata*, *Rhizoctonia solani*, *F. oxysporum*, and *B. cinerea* (Wianowska et al., 2016). Similarly, various terpene compounds, such as monoterpenes, diterpenes, triterpenes, and sesquiterpenes, exhibit antifungal activity. These metabolites are major components of essential oils from various plants, such as those of the Asteraceae family, containing both terpenes and phenolics compounds that are responsible for antifungal activity (Gavrilović et al., 2016).

This study aimed to evaluate the antifungal activity of extracts of different polarities from inflorescences, leaves, stems, and roots of *V. sphaerocephala* against the phytopathogenic fungi *B. cinerea* and *F. oxysporum*, and determine the presence of polyphenols and terpenes in the extract with the highest antifungal activity.

2. Materials and Methods

2.1. Plant material

The complete plants specimens of *V. sphaerocephala* were collected in Pajacuarán, Michoacán, México (20° 05' 54.8'' N Latitude and 102° 33' 19.2'' W Longitude) on October 2020. The plant samples were authenticated and deposited in the Biology Faculty Herbarium (EBUM) of Michoacan University of San Nicolás of Hidalgo (UMSNH) (Accession: 31383). All plant parts (inflorescences, leaves, stems, and roots) were separated and dried at room temperature. Subsequently, the samples were crushed and stored separately in the dark for extract preparation.

2.2. Fungal strains

Botrytis cinerea and *Fusarium oxysporum* belong to the fungal collection from the Plant Biotechnology Laboratory of the Chemical Biological Research Institute (IIQB-UMSNH), and were isolated from diseased blackberries, fruits and roots, respectively (Huerta-Olalde et al., 2022). They previously have been molecularly characterized with the confirmation of genus and species, *B. cinerea* (GenBank access code PQ636509) (Loeza et al., 2024a), and *F. oxysporum* (GenBank access code PQ641222) (Loeza et al., 2024b). Each fungi strain was subculture and maintained on Potato Dextrose Agar (PDA) medium and incubated at 25 °C to obtain inoculum for testing.

2.3. Chemicals and reagents

Methanol and ethyl acetate, ammonium hydroxide and sulfuric acid were analytical grade of Meyer® (Mexico City, Mexico). Potato Dextrose Agar was of Bioxon® (Mexico City, Mexico). Benomyl 50% WP® (Taicang Pesticide Factory, China). Acetic anhydride, hydrochloric acid, and chloroform were from JT Baker® (Mexico City, Mexico). Mercuric chloride was obtained from Fermont® (Mexico City, Mexico). Potassium iodide from Monterrey Chemical Products® (Monterrey, Mexico). Lead acetate form Sigma Aldrich® (Germany). Sodium hydroxide from Jalmeck® (Mexico City, Mexico). The standards gallic acid, naringenin, stigmasterol, linalool, and methoxycoumarin were obtained from Sigma Aldrich® (Germany); digitonin, from Fluka® (Switzerland), and coenzyme Q10, from Qunol® (USA).

2.4. Preparation of plant extract

All extracts were prepared by cold maceration (4 °C). The dry-milled materials (100 g dry weight each) were extracted with 500 mL of each solvent (water at 90 °C, methanol, ethyl acetate, and chloroform) and maintained for 5 days with constant stirring (150 rpm) in the dark (Abah and Egwari, 2011). They were then filtered at room temperature using a funnel with Whatman paper No 1 (Whatman Ltd., England), and the solvents containing the extracted materials, concentrated until dryness in a rotary evaporator Buchi® under reduced pressure and 50 °C. The total solid test was used to determine the extract yield, which was expressed as milligrams of dry solids concentrated at 100 mg/mL. Finally, the total extract was stored in an amber flask at 4 °C until further use.

2.5. Antifungal activity assay

Antifungal activity was determined using the well diffusion method (Magaldi et al., 2004), using Petri dishes with PDA medium, in the center of the plate a 5 mm hole was made with the upper end of a sterile Pasteur pipette. Each well was filled with either 100 µL of the extracts (10 mg/mL); Benomyl 50® (10 mg/mL), used as a positive control; each solvent (water, methanol, ethyl acetate and chloroform) used as a negative control, allowing its volatilization to stand on the bench for 30 min. Subsequently, 5 mm discs of mycelia from each fungus were inserted into the wells. All the plates were incubated for 7 days, at 25 °C both for *B. cinerea* and *F. oxysporum*.

The zone of inhibition was measured using a set of Vernier calipers and recorded in millimeters. The assay was repeated twice for confirmation. The percentage inhibition of mycelial growth was calculated by the following Formula 1:

$$Inhibition(\%) = \left(\frac{R_1 - R_2}{R_1}\right) x\ 100 \quad (1)$$

whereas, R1: the average diameter (mm) of mycelial growth in control plate. R2: the average diameter (mm) of mycelial growth in treatment plate.

Three concentrations (1, 5, and 10 mg/mL) of the extract with the highest antifungal activity were assayed using the well-diffusion method, following the methodology described above.

2.6. Phytochemical screening

After the bioassay tests, the extract with the highest antifungal activity was selected for the qualitative determination of polyphenols (phenolics, flavonoids, coumarins, and quinones), saponins, and terpenes using established procedures (Ortega-Medrano et al., 2023) with modifications. For these tests, extracts were prepared at a concentration of 5 mg/mL. The criterion reported in the work of García-Granados et al. (2019) was used to evaluate the qualitative presence of the various compounds in the extracts of *V. sphaerocephala*: (+++) indicates abundant presence, (++) indicates notable presence, (+) indicates minimal presence and (-) indicates absence.

2.6.1. Tests for phenolics

Lead acetate test: 500 µL of 10% lead acetate solution was added into 500 µL extract in a glass test tube. The presence of phenolics was indicated by a white precipitate. Gallic acid was used as the standard.

2.6.2. Test for flavonoids

Ammonium hydroxide test: 400 µL of 5% ammonium hydroxide was added into 400 µL extract. The presence of flavonoids was indicated by yellow coloration using naringenin as the standard.

2.6.3. Test for coumarins

Sodium hydroxide test: 400 µL of 10% sodium hydroxide was added to 400 µL extract. The presence of flavonoids was indicated by yellow coloration, using methoxycoumarin as the standard.

2.6.4. Test for quinones

Sulfuric acid test: 400 µL of sulfuric acid was added into 400 µL extract. The presence of quinones was indicated by a soft red coloration using ubiquinone as the standard.

2.6.5. Test for saponins

Froth formation test: A total of 500 µL of plant extract was thoroughly mixed with 1.5 mL distilled water in a test tube, for a 5 min. The presence of saponins was confirmed by the formation of a stable and persistent froth.

2.6.6. Test for terpenes

Salkowski test: A total of 500 µL of plant extract was mixed with chloroform (1.5 mL) in a test tube, and 500 µL of sulfuric acid was added. The formation of the red phase denotes the presence of terpenes, using linalool as the standard.

2.7. Statistical analysis

Results were reported as mean ± standard deviation of three independent replicates. An analysis of variance (ANOVA) was used to assess statistical significance. Differences between values with a $P < 0.05$ were considered statistically significant. Tukey's test was performed for the comparison of means for the corresponding results. These analyses were performed with the SPSS software version 29.0 (IBM, Armonk, NY, USA).

3. Results

3.1. Yields of the plant extracts

The yields of crude extracts from 100 g of dried plant material extracted from *V. sphaerocephala* are shown in Table 1. The results showed that the chloroformic inflorescence and aqueous stem extracts produced the highest amount of crude extract, with 5.54 g/100 g dry weight (dw) and 5.49 g/100 g dw, respectively. The lowest yields were obtained from the root and ethyl acetate extracts.

3.2. Antifungal activity of the plant extracts

All extracts used in this study exhibited varying degrees of antifungal activity against the two fungi (Table 1). However, the aqueous extract of *V. sphaerocephala* stems was the most effective among the extracts tested. In fact, this extract resulted in a 100% inhibition percentage against *B. cinerea* and *F. oxysporum* assayed compared to the positive control, Benomyl 50 (10 mg/mL). The other extracts did not have the ability to inhibit fungal growth above 33% and root extracts had the lowest antifungal activity (Table 1). The effects of the extracts were compared with those of the respective solvents (water, methanol, ethyl acetate, or chloroform) as negative controls, which did not show any antifungal activity. To determine the lower inhibitory concentrations of the aqueous stem extract of *V. sphaerocephala* against the two fungi, seven days after culture, the effects of three concentrations (1, 5, and 10 mg/mL) were observed, using water as a negative control and Benomyl 50 as a positive control (10 mg/mL) (Table 2).

The mycelial growth of the two fungi was 100% inhibited at concentrations of 5 and 10 mg/mL; however, the concentration of 1 mg/mL was effective in a 100% only against *B. cinerea* and very low against *F. oxysporum*. The treatment with water did not show any inhibition, and with Benomyl 50, 100% inhibition was obtained in the two fungi (Table 2). These results demonstrate that the aqueous stem extract of *V. sphaerocephala* contains compounds with high antifungal activity against *B. cinerea* at the lowest concentration tested (1 mg/mL).

3.3. Phytochemical screening

The present study revealed that aqueous stem extract form *V. sphaerocephala* shown the presence of phytochemical constituents, such as phenolics, flavonoids, coumarins, quinones, and terpenes. In this extract, the phenolic, coumarin, and quinone contents were higher than those of the flavonoids and terpenes, and saponins were not detected (Table 3).

4. Discussion

Various factors have been reported to affect the extract yield, particularly plant materials, extraction time, temperature, and solvent polarity (Zhang et al., 2018; Truong et al., 2019). The extraction yields obtained from *V. sphaerocephala* in this study were significantly influenced by solvent polarity and the specific plant part used.

Table 1. Extract yield and antifungal activity of *Verbesina sphaerocephala* crude extracts against *Botrytis cinerea* and *Fusarium oxysporum* using well diffusion method.

Crude extract [10 mg/mL]	Extract yield (g)	Inhibition (%)	
		B. cinerea	***F. oxysporum***
Aqueous			
Inflorescences	2	1.11 ± 1.92^{c}	25.56 ± 1.92^{b}
Leaves	1.39	0 ± 0^{d}	5.56 ± 1.92^{g}
Stems	5.49	100 ± 0^{a}	100 ± 0^{a}
Roots	3.64	0 ± 0^{d}	1.11 ± 1.92^{h}
Methanolic			
Inflorescences	3.59	0 ± 0^{d}	28.89 ± 3.85^{b}
Leaves	3.26	0 ± 0^{d}	30 ± 3.33^{b}
Stems	1.52	32.22 ± 1.92^{b}	11.11 ± 1.92^{e}
Roots	0.74	0 ± 0^{d}	12.22 ± 1.92^{e}
Ethyl acetate			
Inflorescences	3.81	0 ± 0^{d}	20 ± 3.33^{c}
Leaves	2.17	4.94 ± 1.92^{c}	31.11 ± 1.92^{b}
Stems	0.71	0 ± 0^{d}	7.78 ± 1.92^{f}
Roots	0.41	0 ± 0^{d}	0 ± 0^{i}
Chloroformic			
Inflorescences	5.54	1.11 ± 1.92^{c}	7.78 ± 1.92^{f}
Leaves	4.21	0 ± 0^{d}	20 ± 3.33^{c}
Stems	1.03	3.33 ± 3.33^{c}	17.78 ± 1.92^{d}
Roots	0.43	2.22 ± 1.92^{c}	2.22 ± 1.92^{h}
Benomyl 50		100 ± 0^{a}	100 ± 0^{a}

Mean ± SD in a column superscripted with different lowercase letters are significantly different (P<0.05).

Table 2. Inhibition of mycelium growth of *B. cinerea* and *F. oxysporum* with different concentrations of the aqueous stem extract from *V. sphaerocephala* (7 days of culture).

Treatment (mg/mL)		Inhibition (%)	
		B. cinerea	***F. oxysporum***
Water		0 ± 0^{c}	0 ± 0^{c}
Stem aqueous extract	**1**	100 ± 0^{a}	10.42 ± 1.82^{b}
	5	100 ± 0^{a}	100 ± 0^{a}
	10	100 ± 0^{a}	100 ± 0^{a}
Benomyl 50	**10**	100 ± 0^{a}	100 ± 0^{a}

Mean ± SD in a column superscripted with different lowercase letters are significantly different (P<0.05).

Table 3. Phytochemical screening of aqueous extract from *Verbesina sphaerocephala* stems.

Chemical group	Test	Response
Phenolic	Lead acetate	++
Flavonoid	Ammonium hydroxide	+
Coumarin	Sodium hydroxide	++
Quinone	Sulfuric acid	++
Saponin	Froth	-
Terpene	Salkowski	+

Note: - Absence, + slightly present, and ++ present.

The high yields observed with both aqueous (polar) and chloroform (non-polar) solvents suggest a diverse profile of secondary metabolites within the species, ranging from hydrophilic to lipophilic compounds. While polar solvents typically yield higher amounts of biomass in many medicinal plants (Nawaz et al., 2020), our findings regarding chloroform yields align with observations in other Asteraceae species, such as those in the *Achillea* genus (Altemimi et al., 2017, Kaczorová et al., 2021). However, from a sustainable and green chemistry perspective, the high yield and efficiency of the aqueous stem extract of *V. sphaerocephala* is particularly promising. The use of water as a solvent offers clear advantages in terms of low toxicity, cost-effectiveness, and safety for potential applications in the food and pharmaceutical industries (Choi and Verpoorte, 2019).

A few studies have confirmed the antifungal activity of *Verbesina* species against phytopathogenic fungi. An ethyl acetate extract from the inflorescences of *V. lanata* showed significant inhibitory activity *in vitro* against grapevine downy mildew (*Plasmopara viticola*) (Ramseyer et al., 2017) and an acetone bark extract of *V. turbacensis* inhibited the opportunistic fungal pathogen *Cryptococcus neoformans* (Powers et al., 2022). One of the most studied species is *V. encelioides* that presents antifungal activity, and stem and leaf powders in silver nanoparticles have shown activity against *Aspergillus niger* and *A. flavus* (Kushwaha and Malik, 2013), hydroalcoholic extracts of the aerial branches, roots, and flowers were effective against *Aspergillus fumigatus* (Albalawi et al., 2015), and a protein extract showed biological activity against *A. flavus* and *F. oxysporum* (Divya-Ramakrishnan et al., 2017). The antifungal effect of the aqueous extract of *V. sphaerocephala* stems is consistent with that reported for the polar extracts of *V. encelioides* (Albalawi et al., 2015).

Aqueous extracts of different plants have been shown to inhibit phytopathogen fungi (Sayago et al., 2012; Ngegba et al., 2018), noting that antifungal activity is concentration dependent. Alotibi et al. (2020) studied the antifungal activity of the aqueous extracts of *Rumex vesicarius* stems, observing that at a concentration of 50 mg/mL, obtained about 50% inhibition of *Exserohilum rostratum* and *F. oxysporum*; the aqueous extract of *Moringa oleifera* leaves was effective against *A. niger*, *A. flavus*, *A. fumigatus* and *Rhizopus oryzae* at values ranged from 31.25 to 62.5 mg/mL (Oniha et al., 2021); aqueous extracts from *Arum cyreniacum* tubers, leaves and flowers shown a low inhibition against *F. oxysporum* with only 5.8, 10.5 and 47% of inhibition with 200 mg/mL, respectively (Abdulrraziq et al., 2021); whereas against *F. solani*, the aqueous extract from *Annona squamosa* leaves exhibited intermediate effect with percentage inhibition of 33.33% at 1 mg/mL and 64.58% at 2 mg/mL (Kalidindi et al., 2015).

The aqueous extract of stems of *V. sphaerocephala* showed an antifungal activity of 100% on the two fungi assayed at concentration of 5 mg/mL. *B. cinerea* was the pathogenic strain with greater susceptibility to mycelial growth inhibition at 1 mg/mL, an inhibitory concentration considered optimal because the antifungal effect of this extract was 10 times more active than that of the commercial fungicide Benomyl 50 (10 mg/mL), which was used as the positive control. This suggests that the stems of this species possess highly potent or synergistic antifungal constituents that are efficiently captured through aqueous extraction.

The phytochemical screening revealed a rich presence of phenolics, coumarins, quinones, and terpenes in the *V. sphaerocephala* aqueous stem extract. The antifungal success of this extract is likely not the result of a single compound but rather a synergistic effect between these metabolites. These groups of compounds can be attributed to the antifungal activity of the two fungi under study, mainly against *B. cinerea,* as they can act alone or in synergy, as demonstrated in other studies (Pusztahelyi et al., 2015; Salhi et al., 2017; Al Aboody and Mickymaray, 2020). Mechanistically, the presence of polyphenols and coumarins may cause irreversible damage to the fungal cell membrane and induce oxidative stress via ROS accumulation (He et al., 2021). Simultaneously, quinones and terpenes may disrupt mitochondrial function and enzymatic activity, such as ATPases, leading to programmed cell death (Guerra et al., 2015; Zaker, 2016; Kuete, 2017; Shang et al., 2019; He et al., 2021). In particular, the hydroalcoholic extracts of *Tagetes patula* and *Juniperus communis* have antifungal activity because of their polyphenol and terpene (Politi et al., 2016; Fierascu et al., 2018).

In order to investigate the antifungal compounds of the aqueous stem extract of *V. sphaerocephala*, in our working group was carried out Ultra-High Performance Liquid Chromatography-Tandem Mass Spectrometry analysis (Rodríguez-Valdovinos et al., 2024), this study revealed a diverse array of bioactive metabolites, that include phenolic acids (such as chlorogenic acid derivatives), glycosylated flavonoids (quercetin and kaempferol derivatives), and specific sesquiterpene lactones typical of the *Verbesina* genus.

The high concentration of these bioactive groups explains why the aqueous extract was more effective than the more specialized organic extracts. These results position *V. sphaerocephala* as a strong candidate for developing bio-fungicides. Future research, including the Gas Chromatography-Mass Spectrometry (GC-MS) analysis currently planned by our group, will be crucial to pinpoint the specific volatile compounds contributing to this high inhibitory capacity.

5. Conclusions

The present study demonstrates that *V. sphaerocephala* is a promising source of natural antifungal agents, with the aqueous stem extract standing out for its exceptional biological activity. This extract achieved total mycelial inhibition (100%) against *B. cinerea* and *F. oxysporum* at low concentrations, notably outperforming the commercial fungicide Benomyl 50. The high yield obtained through aqueous extraction, combined with its non-toxic and cost-effective nature, highlights the potential of this species for sustainable agricultural applications.

The potent antifungal effect is attributed to the presence of key phytochemical groups—primarily phenolics, coumarins, and quinones—which likely act through synergistic mechanisms to disrupt fungal integrity. These findings validate the importance of exploring *V. sphaerocephala* as a viable, eco-friendly alternative to synthetic pesticides in the management of phytopathogenic fungi. Future characterization of its volatile compounds will further elucidate the specific molecules responsible for this high bioactivity.

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Data Availability Statement

The full set of data supporting the results of this study was published in the article itself.

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