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Chorisia speciosa Extract Induces Systemic Resistance against Tomato Root Rot Disease Caused by *Rhizoctonia solani*

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Abstract: Chemical pesticides and fungicides are used extensively, negatively affecting people's health and the environment. Reducing synthetic pesticides and increasing the efficiency of sustainable food production using plant extracts as natural chemicals is a win–win. Here, we first describe and evaluate an ethanolic extract of *Chorisia speciosa* for its protective and curative activities against *Rhizoctonia solani* in greenhouse-grown tomato plants. The results showed that the mycelial growth of *R. solani* was completely suppressed in vitro by *C. speciosa* extract (10 µg/mL). Twenty days after fungal inoculation, the results demonstrated that using *C. speciosa* extract (10 µg/mL) in vivo significantly improved shoot and root growth parameters in protective and curative treatments. Further, the protective and curative treatments decreased the disease index by 26.67% and 53.33%, respectively. *C. speciosa*-treated tomato plants showed significantly increased antioxidant enzyme production (PPO, CAT, and SOD) and up-regulated *PR-1*, *PR-2*, *PR-3*, *PAL*, and *CHS* expression levels compared to untreated plants. According to HPLC examination, the most prevalent phenolic acids or flavonoid components quantities (µg/mL) noticed in *C. speciosa* extract were 7-OH-flavone (10.36), kaempferol (9.23), *p*-coumaric acid (8.65), ferulic acid (8.14), caffeic acid (7.59), gallic acid (6.33), and iso-ferulic (5.71). Our findings are the first to demonstrate that a *C. speciosa* extract can assist plants in combating fungal infestation. Therefore, the data imply that *C. speciosa* extract, as a natural and renewable product, could be adopted as a long-term approach for regulating plant fungus.

Keywords: tomato; *Chorisia speciosa*; *Rhizoctonia solani*; HPLC; gene expression; defense-related enzymes



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1. Introduction

Tomato (*Lycopersicon esculentum* L.) is one of the world's most widely cultivated fruits and vegetables. It is a member of the Solanaceae family and includes the beneficial amino acids tryptophan and tomatin (a glycoalkaloid major component). Tomatoes are the world's second-largest processed vegetable and the second-largest growing vegetable. FAOSTAT (2017) estimates global tomato production at 178.24 million US tons in 2017, with a value of USD 59 billion. Crop production and environmental stability are threatened by harmful pathogens [1]. *Rhizoctonia*, *Verticillium*, and *Fusarium* are soil-borne pathogens that cause different plant diseases [2]. *Rhizoctonia solani* is the causal agent of plant damping-off and root rot diseases, which have various symptoms that lead to tomato seedling death [3].

Finding new ways to stop plant diseases is difficult because physiological pathogen races have become more resistant to fungicides [4].

As a result, natural substances may be able to combat a wide variety of plant diseases without creating harmful environmental effects. Many plant extracts' antimicrobial and antifungal activities have gained considerable popularity and scientific interest [5]. Plant chemicals, such as natural antifungal chemical compounds, are considered safer and more acceptable than synthetic counterparts [6]. Many plants' antimycotic properties and extracts have been investigated [7]. *Chorisia speciosa* A. St. Hil (Syn. *Ceiba speciosa*) is a member of the family Bombacaceae, which contains about 28 genera and about 200 species [8]. These plants traditionally cure many health disorders, e.g., headaches and diabetes, and pathogens, such as *Bacillus cereus*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Psuedomonas aeruginosa* [9,10]. A few *Chorisia* species were subjected to phytochemical analyses that yielded a number of flavonoids, triterpenes, and carbohydrates [11]. Anti-inflammatory, hepatoprotective, cytotoxic, antioxidant, hypoglycemic, and antibacterial actions with high safety margins have been observed in various *Chorisia* species, according to biological studies [12–15].

Because of their safety, environmental friendliness, and public acceptance, plant extracts have been studied for plant diseases control [16,17]. The current study aimed to assess the growth antifungal activity of *C. speciosa* ethanol extract against *R. solani* and its protective and curative properties in vivo as this has not previously been completed. Screening and introducing potential plant extracts with promising antimicrobial properties for agricultural applications is critical. In addition, the levels of expression of defense-related genes, such as *PR-1*, *PR-2*, *PR-3*, *PAL*, and *CHS*, as well as tomato growth parameters, were measured to see how the extract affected them. In addition, the main phytochemical components of *C. speciosa* ethanolic extract were identified using HPLC analysis.

2. Materials and Methods

2.1. Isolation and Characterization of Fungal Isolate

Tomato-root-rot-disease-like signs were collected from an Egyptian field in El-Gharbia governorate. The symptomatic plant parts were cut and washed with tap water to remove the dust, kept in 2% sodium hypochlorite for 2 min, then rinsed in sterile water 2–3 times before drying in laminar flow. PDA medium was utilized in the isolation and cultivation processes. The hyphal tip technique was used to purify the fungus. The fungus was identified based on morphological and microscopic properties, according to Alexopoulos et al. [18]. Furthermore, the internal transcribed spacer (ITS) region was amplified by ITS1 and ITS4 primers to identify the fungal isolate at the molecular level (Table 1). Heflish et al. [19] previously described the PCR settings reaction as containing 0.4 µL of each primer, 12.5 µL of 2x *Taq* PCR Ready Mix, and 1 µL of DNA, and the molecular grade H₂O was added to obtain a final volume of 25 µL. The ITS region was amplified using a TECHNE Prime PCR with the following settings: 94 °C for 5 min, then 40 cycles of 94 °C, 55 °C, and 72 °C for 40 s each, and a final elongation at 72 °C for 7 min. The PCR reaction was separated on an agarose gel, refined, and sequenced. The retrieved nucleotide sequences were aligned and compared to those of other related species using the MEGA X program and the Genbank database. The nucleotide sequence of the fungus was then deposited in the NCBI database entry portal to obtain an accession number.

2.2. Preparation of *C. speciosa* Extract

Chorisia speciosa plant leaves were collected from the Faculty of Agriculture, Saba Basha, Alexandria, Egypt and dried at room temperature (25 °C) for two weeks before being crushed to a fine powder with a grinding mill (Moulinex AR1044, Paris, France). 100 g of the powder was immersed in 200 mL of methanol for six days. The methanol mixture was put through a filter, and the extract that came out was then placed in a rotary vacuum evaporator to concentrate it [17].

Table 1. Oligonucleotide primers used in this study.

Gene	Abbreviation	Nucleotide Sequences
Internal Transcribed Spacer	ITS	ITS1-TCCGTAGGTGAACCTGCGG ITS4-TCCTCCGCTTATTGATATGC
Pathogenesis-related protein-1	PR-1	For-GTTCCTCCTTGCCACCTTC Rev-TATGCACCCCCAGCATAGTT
Endoglucanase	PR-2	For-TATAGCCGTTGGAACGAAG Rev-CAACTTGCCATCACATTCTG
Chitinase	PR-3	For-ATGGAGCATTGTGCCCTAAC Rev-TCCTACCAACATCACCACCA
Phenylalanine ammonia-lyase	PAL	For-GTTATGCTCTTAGAACGTCGCCC Rev-CCGTGTAATGCCTTGTTCCTTGA
Chalcone Synthase	CHS	For-CACCGTGGAGGAGTATCGTAAGGC Rev-TGATCAACACAGTTGGAAGGCG
β -actin	β -actin	For-TGGCATACAAAGACAGGACAGCCT Rev-ACTCAATCCCAAGGCCAACAGAGA

2.3. The Antifungal Activity of *C. speciosa* Extract against Root Rot Fungus In Vitro

The *C. speciosa* extract was investigated for its effectiveness against the root rot pathogen using the food poisoning technique [20]. Different amounts of *C. speciosa* extract (1, 2, 4, 8, and 10 $\mu\text{g/mL}$) were mixed with PDA dishes and compared to fungicide (Rizolex 2 $\mu\text{g/mL}$) and the negative control (PDA-free medium from additives). Fungal round discs were cut from the edges of one-week cultures, seeded in the center of the treated pouring PDA Petri plates, and cultured for a week at room temperature. All the treatments are triplicated. The effectiveness of *C. speciosa* extract on fungal linear growth was determined as a percentage of growth inhibition (%) = $[(Tr - Ts) / Tr] \times 100$, where *Tr* is the fungal growth length control and *Ts* is the length of fungal hyphae growth in the treatment [21].

2.4. Greenhouse Experimental Design and Growth Parameters Assessment

A greenhouse experiment was conducted under controlled conditions ($27 \pm 3^\circ\text{C}$, 80% relative humidity, 14 h of light, and 10 h of darkness) to investigate the efficacy of *C. speciosa* extract in controlling *R. solani* and boosting tomato development. Seedlings of the 023 variety of tomato, aged 20 days, were transplanted in sterile soil in each of the 20-cm pots. *C. speciosa* extract (10 $\mu\text{g/mL}$) was sprayed on the leaves of the transplanted plants a week after they were established. An *R. solani* inoculum was created by inoculating 250 g of pre-autoclaved, watered barley grains in a bag with a 0.5-cm-diameter plug of the fungus and incubating for 7 days at room temperature. The grain inoculum was then air-dried, crushed into a powder, and distributed as 10 g/kg of soil in pots, where it was to be applied to the plant root zone [22]. There were five independent sets of treatments. Treatments I and II involved spraying the extract on plants two days after (curative) or before (protective) inoculation with *R. solani*; treatment III involved only inoculating plants with *R. solani*, and treatment IV involved treating plants with *C. speciosa* extract. The plants were inoculated with potato dextrose broth in the control treatment. Each treatment has nine pots; each pot has three plants. After one month of planting, the plants were removed to evaluate root rot severity on a scale from 0 to 5 [23]. The data were expressed as a percentage of the disease index.

$$(DI)\% = \left[\frac{\text{Sum of scale number of infected plants}}{\text{maximum scale rate number} \times \text{total plants number}} \right] \times 100$$

The effects of the *C. speciosa* extract on the height (cm), root length (cm), fresh and dry weight (g), and root fresh and dry weight (g) of the uprooted plants were also evaluated (g). Leaves were also obtained from each treatment to analyze the expression of defensive

scheme genes and the total phenolic compounds. Twenty days after being inoculated with *R. solani*, plant leaves were picked.

2.5. Total Phenolic Content (TPC) in Tomato Plants

TPC levels in tomato extracts were evaluated using the Folin–Ciocalteu (FoC) procedure. 0.1 mL μ L of 1 mg/mL tomato extract was added to 0.75 mL of Folin–Ciocalteu (aqueous diluted ten times). After five minutes at room temperature, 0.75 mL of CaCO_3 was added, and the combination was carefully mixed. After 90 min, the mixture was measured at 725 nm with 6305 UV/Visible Spectrophotometer (Cole-Parmer Instrument Company, Vernon Hills, IL, USA). Gallic acid concentrations between 0.01 and 0.05 mg/mL were used to produce a calibration graph. TPC levels were calculated using the method provided by Velioglu et al. [24] to measure gallic acid content (μ g GAE/100 g extract).

2.6. Oxidative Stress Markers

2.6.1. Malondialdehyde Assay (MDA)

Thiobarbituric acid (TBA) was used to evaluate the malondialdehyde level (MDA) in all treatments. Plant leaves were pulverized in 0.1% trichloroacetic acid (TCA) and spun down at $12,000 \times g$ for 30 min. Afterward, one milliliter of the obtained liquid was combined with a solution comprising TCA (40%):TBA (10%) in a water bath (95 °C, 30 min). The combination was quickly placed on ice to cool. The sample's wavelength absorbance (WA) was measured at 600 nm, and the MDA concentration was represented as μ M/g F.Wt. [25].

2.6.2. Hydrogen Peroxide Assay (H_2O_2)

Velikova et al. [26] determined the H_2O_2 concentration of tomato leaves. Little pieces of leaves were homogenized with 0.1 TCA (%) and spun with a centrifuge. The supernatant, KH_2PO_4 (10 mM, pH 7), and 1 M KI were combined in equal parts. The WA was calculated at 390 nm. The H_2O_2 amount is expressed as μ M/g F.Wt. [26].

2.7. Antioxidant Enzymes

For all enzyme activity experiments, leaves were mashed in potassium phosphate buffer pH 7 and spun down with a centrifuge at $10,000 \times g$ for 10 min, with the supernatant used for further analysis. All enzymes are reported as μ M/g F. Wt.

2.7.1. Polyphenol Oxidase (PPO)

Polyphenol oxidase (PPO) activity was determined using the Cho and Ahn [16] technique. 500 μ L of enzyme extract was treated at 25 °C for 10 min with 1 mL of Tris-HCl and quinone (50 mM, pH 6). At 420 nm, all measurements were taken three times [27].

2.7.2. Catalase (CAT) Activity

CAT activity was quantified using the Cakmak and Marschner technique with slight adjustments by measuring the rate of disappearance of H_2O_2 [28]. 50 μ L enzyme extract was treated with 1 mL of a K-phosphate buffer (25 mM, pH 7) and H_2O_2 combination (10 mM as final concentration). CAT activity was detected at 240 nm.

2.7.3. Superoxide Dismutase (SOD) Activity

The efficacy of SOD to prevent the photochemical reduction of nitro blue tetrazolium (NBT) was tested using a slightly modified Beauchamp and Fridovich method. KH_2PO_4 (50 mM, pH 7.8), EDTA (0.1 mM), L-methionine (10 mM), NBT (75 mM), and riboflavin (20 mM) were combined in a tube with 100 μ L of enzyme extract. Before being placed in the dark, the tubes were kept at 25 °C for 15 min under two 15-watt fluorescent lamps. The WA at 560 nm was then measured [29].

2.8. Real-Time Quantitative Reverse Transcription PCR Analysis (RT-qPCR) of Defense-Related Genes

Further, 0.1 g of plant leaves was taken at 20 dpi and subjected to a manual whole RNA extraction using a customized guanidium isothiocyanate (GITC) technique [30]. The quality and quantity of the extracted RNA were evaluated using the 6305 UV/Visible Spectrophotometer (Cole-Parmer Instrument Company, Vernon Hills, IL, USA). By utilizing a reverse transcriptase (RT) enzyme, the cDNA from the extracted RNA was synthesized [31]; the conventional reverse transcriptase PCR was carried out in a Bio-Rad cycler (Bio-Rad Laboratories Inc. Hercules, CA, USA) at 45 °C for 1 h and then inactivated at 85 °C for 3 min. The template cDNA was reserved in a −40 refrigerator until used in quantitative real-time PCR.

Using quantitative polymerase chain reaction (qPCR), the relative transcription of three genes is involved in tomato disease (*PR-1*, *PR-2*, and *PR-3*) and two phenolic genes (*PAL* and *CHS*). The housekeeping β -*actin* gene was used to normalize the data obtained. Sequences of the oligonucleotides employed as primers in this study are presented in Table 1. Experiments were conducted three times for each biological sample. Q-PCR was performed using a Bio-Rad real-time cycler (Bio-Rad laboratories Inc., Hercules, CA, USA) and TB Green Advantage qPCR Premix (Takara Bio USA Inc., San Jose, CA, USA) [32]. The PCR reaction mixture was performed using 10 μ L of TB green Advantage qPCR Premix, 0.4 μ L of each for/rev primer, 2 μ L of template cDNA, and 7.2 μ L of dH₂O for a total of 20 μ L. The real-time PCR conditions were as follows: the initial denaturation step at 95 °C for 10 s, denaturation at 95 °C for 3 s, and 40 cycles of annealing/extension at 60 °C for 20 s. The relative transcription levels of all genes were measured according to the $2^{-\Delta\Delta CT}$ method [33].

2.9. Antioxidant Activity of *C. speciosa* Extract

The Shimada et al. [34] technique was applied to assess scavenging free radical efficiency. In 100 mL of methanol, 3.94 mg of DPPH (1,1-diphenyl-2-picrylhydrazyl) was diluted to 0.1 mL. 2 mL of diluted DPPH was combined with 6 mL of each concentration sample (1000, 500, 250, 125, and 62.5 μ g/mL). The mix was vigorously stirred and held at 25 °C for 30 min. Each combination's absorbance value (V_a) was measured at 517 nm. The DPPH inhibition was computed as $D\% = [(D_i - D_s) / D_i] \times 100$, where D_s is the sample V_a and D_i is the control V_a reaction (which contains all reagents except the sample). The antioxidant activity was measured according to the 50% DPPH (IC₅₀) scavenging calibration curve compared to butylated hydroxytoluene (BHT).

2.10. HPLC Analysis of Polyphenolic Components for the Obtained Plant Extract

Using an HPLC-Agilent 1260 with an Infinity II analytical quaternary pump (Santa Clara, CA, USA) and an Eclipse Plus C18 column with a particle size of 3.5 μ m, polyphenolic components of the *C. speciosa* plant extract were identified [35]. The following standards were bought from Merck KGaA in Darmstadt, Germany: catechin, pyrogallol, ellagic acid, kaempferol, ferulic acid, iso-ferulic acid, gallic acid, rutin, caffeic acid, naringenin, *p*-coumaric acid, hesperidin, 7-OH flavone, apigenin, and myricetin. The 14 common phenolic and flavonoid components were the basis for the analysis.

2.11. Statistical Analysis

The data were processed with the Costat program, and the means were compared with post hoc Tukey's honest differences (HSD) at $p \leq 0.05$. The standard deviation (SD) was displayed at the top of the column bar. Up-regulated genes indicated that the relative transcription values are more than 1, whereas down-regulated gene levels indicated less than 1.

3. Results and Discussion

3.1. *Rhizoctonia Solani* Fungus Identification

The morphological examination of the isolated root rot fungus was congruent with *Rhizoctonia* genus features because it is an anamorphic hyphal septate pathogen that does not form asexual spores [19]. An amplified fragment of the ITS region was carried out and sequenced to verify the morphological identity of the *Rhizoctonia* isolate. The sequence of the amplified amplicon was retrieved and blasted in the NCBI-GeneBank database, where it was recognized as *Rhizoctonia solani* and accessioned with the number MW664426 and provided strain identification RS35. The matching of the obtained sequence of Rs35 with the other relative NCBI sequences revealed the genomic similarity closest to 100% with *R. solani* (MH172550 and MT408040) and an isolate of *Rhizoctonia* sp. (MK084681) Figure 1.

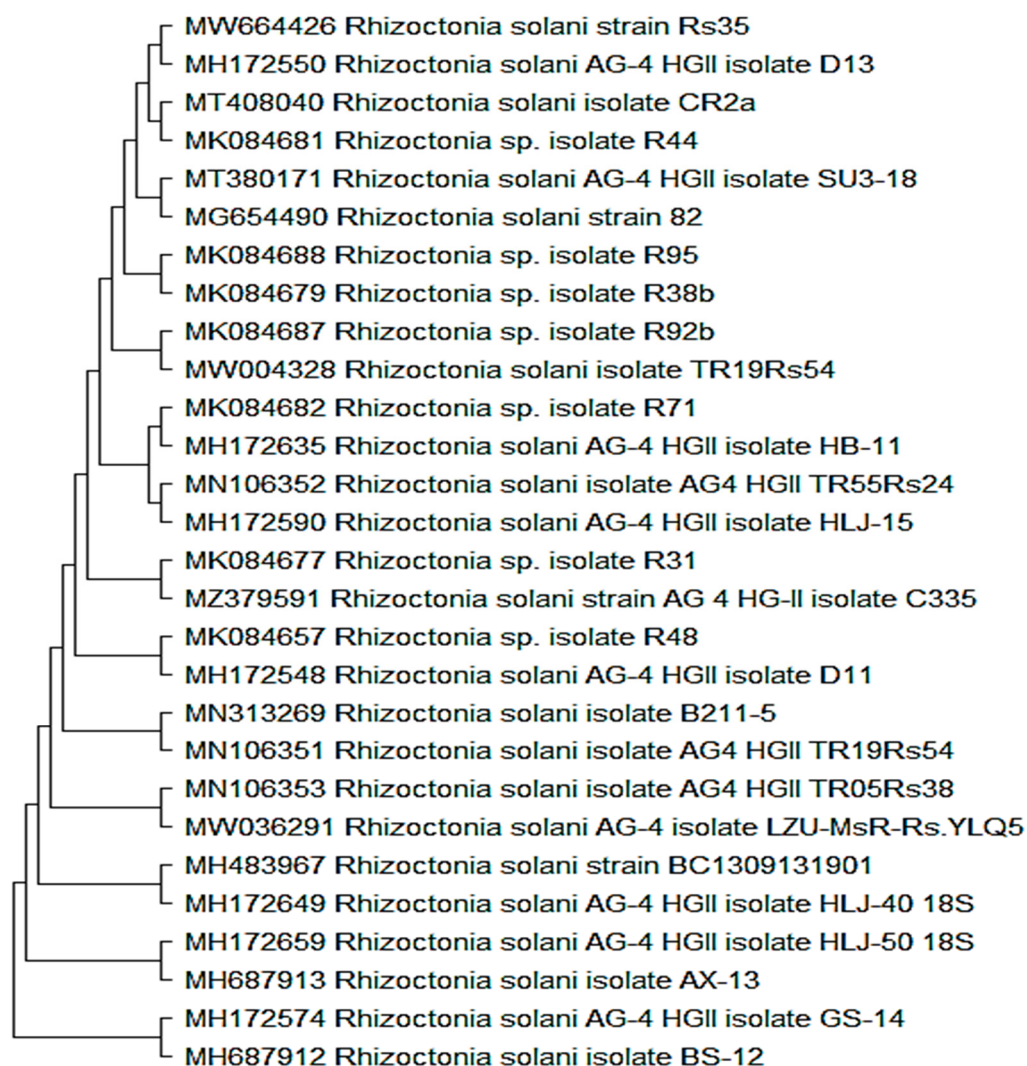


Figure 1. The evolutionary dendrogram displays the similarity of *Rhizoctonia solani* Rs35 (MW664426) to other *R. solani* nucleotide ITS sequences.

3.2. *C. speciosa* Extract inhibitory Effect In Vitro

Table 2 and Figure 2 show the growth inhibition of *R. solani* regarding the evaluated *Chorisia speciosa* extract. The fungal growth was reduced as the concentration of *C. speciosa* extract was increased from 1 to 10 µg/mL. The results showed that, at 10 µg/mL, *C. speciosa* extract inhibited *R. solani* radial growth completely. Simultaneously, there was no effect on *R. solani* hyphal development at 1 µg/mL when compared to the Rizolex fungicide (2 µg/mL). As reported before, different methods, including chemical fungi-

cides and biological control agents, work well to minimize crop loss resulting from fungal infections [36,37].

Table 2. In vitro growth inhibition (%) of *R. solani* in response to *Chorisia speciosa* extract. The different letters mean the data values were significantly different at $p \leq 0.05$.

Treatment ($\mu\text{g/mL}$)	Growth Inhibition %
Negative control	00.00 ± 0.00 e
1	00.00 ± 0.00 e
2	40.37 ± 0.29 d
4	89.77 ± 0.12 c
8	92.43 ± 0.15 b
10	100.00 ± 0.00 a
Fungicide (Rizolex, $2 \mu\text{g/mL}$)	100.00 ± 0.00 a

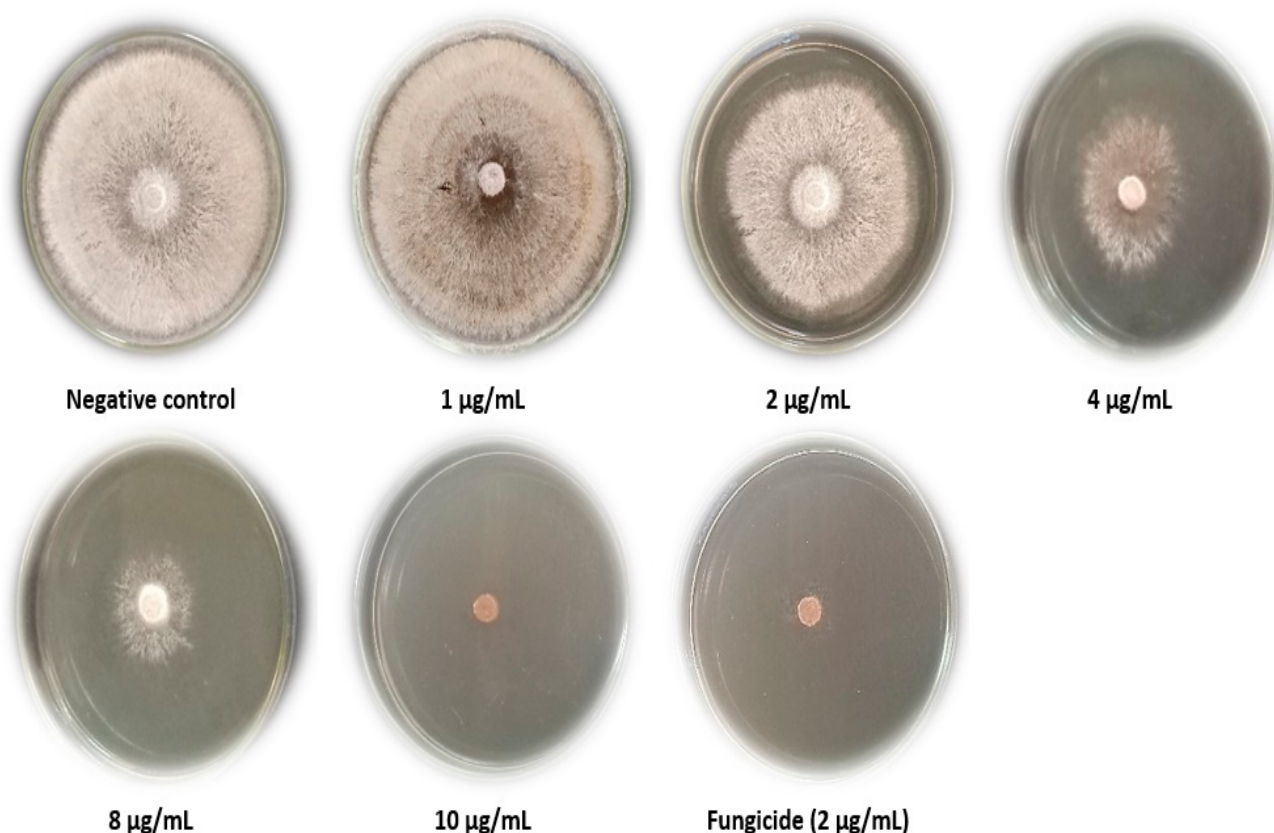


Figure 2. Antifungal activity of different concentrations of *Chorisia speciosa* extract against *Rhizoctonia solani* fungus compared with negative control and fungicide (Rizolex).

In our previous study, the growth of *Fusarium culmorum*, *Rhizoctonia solani*, and *Botrytis cinerea* was suppressed by 38.5%, 64.4%, and 100%, respectively, when using the ethanol extract of *Coccoloba uvifera* at $30 \mu\text{g/mL}$ [38]. *R. solani* and *F. culmorum* growth were highly inhibited at a $30 \mu\text{g/mL}$ concentration of an n-hexane *Eucalyptus camaldulensis* extract [39]. *Acacia saligna* aqueous extract also stifled *F. culmorum* and *R. solani* fungal development [40]. It was found that the oil extracted from *Chorisia speciosa* leaves had varying degrees of inhibition against bacteria, including *S. aureus*, which showed the most sensitivity (25 mm), *Escherichia coli* showing intermediate activity (15 mm), and *S. typhi* showing no sensitivity at all at the concentration of 3.64 mg [41]. The ether and ethyl acetate fractions of the 70% ethyl alcohol extract of *Chorisia insignis* leaves had strong antimicrobial properties against *Bacillus subtilis* and *B. cereus* [42].

3.3. Disease Index of *R. solani* Pathogen in Response to *C. speciosa* Extract

Under greenhouse conditions, the extract of *C. speciosa* was evaluated on *R. solani* to determine whether it promoted or suppressed the development of the fungal pathogen. Root discoloration browning symptoms and their severity (measured on a scale from 0 to 5) were used to calculate the disease index (DI%) for each treatment. Compared to the control treatment, *C. speciosa* extract significantly decreased the DI %. The DI % was reduced by 26.67% in the protective and 53.33% in the curative treatments. DI% was 93.33% in the Rhizoctonia treatment, and no disease symptoms were observed in the control and plant extract treatments (0%). In our investigation, *C. speciosa* extract resulted in a statistically significant decrease in disease symptoms. Similarly, Al-Askar and Rashad [43] found that clove extract reduced the occurrence of Pea root-rot infection in a greenhouse trial. Both clove extract at 4% concentration and the fungicide significantly reduced disease occurrence and increased the percentage of surviving plants (from 40% to 48%). Numerous research studies have compared the antimicrobial properties of organic and water extracts and realized that the alcoholic is better than the water extracts [44–48].

3.4. In Vivo Effect of *C. speciosa* Extract on Tomato Plants

Tomato plant growth was significantly increased ($p \leq 0.05$) in a pot trial when *Chorisia speciosa* extract was used in either protective or curative treatments (Figure 3). In addition, the treatment with *C. speciosa* extracts considerably affected plant length. The *C. speciosa* extract treatment resulted in a height of 38.66 cm, preceded by the protective treatment with 38.51 cm. Root length raised with the *C. speciosa* extract treatment, but not much more so than in the protective or curative treatments, and quite different from the control. Treatment with *C. speciosa* resulted in greater fresh shoot weight (15.88 g) and fresh root weight (8.14 g) than other treatments. Dry shoot weights were also measured and found to show no statistically significant differences between treatments and the control. The *C. speciosa* extract treatment affected the root dry weight compared to the control (Figure 3). Different studies found that using *Moringa oleifera* leaves extract improved tomato and bean growth and yield [49,50], as well as growth characteristics and photosynthesis in *Eruca vesicaria* [51]. The development and biomass of maize roots were boosted by blueberry, red grape, and hawthorn leaf extracts [52]. Many researchers have demonstrated that bioactive compounds, such as terpenoids, flavonoids, alkaloids, and polyphenols, are functional as promoters or antagonists of plant development [53–55].

3.5. Oxidative Stress Markers Assay

Rhizoctonia solani fungi increased the quick buildup of H_2O_2 in infected plants by 1.6 times at 20 dpi (fungi, $9.1 \mu\text{M/g f. wt.}$) compared to the non-inoculated control (control, $5.6 \mu\text{M/g f. wt.}$). In general, the H_2O_2 content in all treatments was significantly higher than in the control group, except for the plant extract treatment in which the level was expressed down ($4.2 \mu\text{M/g f. wt.}$). The H_2O_2 levels in protective and curative treatments (Figure 4A) were non-significantly increased by 1.19–1.29 times compared to the control (6.7 and $7.2 \mu\text{M/g f. wt.}$, respectively). Compared to uninoculated controls ($128 \mu\text{M/g f. wt.}$), the fungal treatment had 1.84 times higher MDA levels (Figure 4B). On the other hand, MDA buildup was shown to be minimally raised in protective and curative treatments (139 and 141, respectively). Lipid peroxidation levels in the protective, curative, and control treatments did not significantly change with the plant extract treatment. According to the data found in our study, the fungal infection was responsible for a substantial rise in the amount of lipid peroxidation that MDA measured. Similar findings were reported previously in different plant fungal infections: Refs. [56,57] reported similar findings in bananas infected with *Fusarium oxysporum*. Mondal et al. [58] reported that cellular oxidation was prevented in infected tomato leaves due to the enhanced biosynthesis of peroxidases, which regulate the highest MDA and H_2O_2 levels. Enhanced levels of MDA are indicative of severe oxidative stress in plants. When pathogens attack plants, MDA levels may be a superb indicator of membrane damage [59]. In the context of plant–

pathogen interactions, H_2O_2 has several functions. It is a systemic acquired resistance (SAR) signal molecule that controls the defense machinery system, inhibits the transmission of pathogens, and lignifies cell walls [60–62].

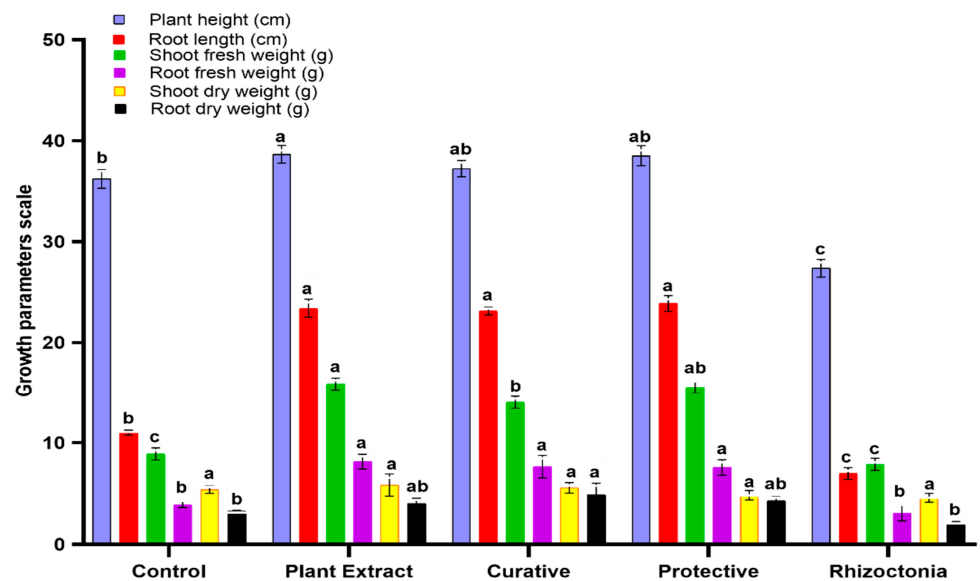


Figure 3. Effect of treatment with *Chorisia speciosa* extract on growth parameters of tomato plants. The different letters on top of the columns mean the data values were significantly different at $p \leq 0.05$.

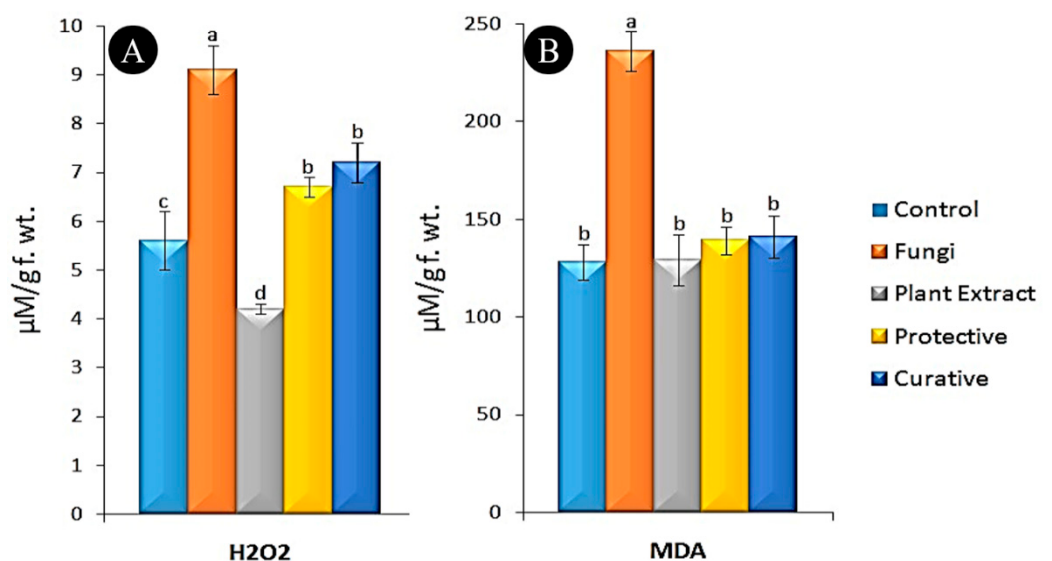


Figure 4. H_2O_2 activities (A) and MDA content (B) are expressed in different treatments as $\mu\text{M/g}$ f.wt. The different letters on top of the columns mean the data values were significantly different at $p \leq 0.05$.

3.6. Defense-Related Genes' Relative Expression Levels

The relative expression levels of four defense-related genes (*PR-1*, *PR-2*, *PR-3*, *PAL*, and *CHS*) were studied at 20 days post-inoculation. The qPCR results confirmed *C. speciosa*'s antifungal efficacy by showing an impressive upregulation of defense genes within the treated plants (Figure 5). It has been known for over a couple of decades that *PR-1* is an important regulator of SAR and may act as a signal for early responses of plant defense [63]. SA induction in response to pathogenic organisms is related to the buildup and activation of *PR-1*, an SA biomarker signal gene [32,64]. All treatments, including

extract, showed up-regulation of *PR-1* compared to controls, while non-treated plants showed down-regulation. The extract treatment alone was observed to have the best average transcript levels (2.98-fold), followed by the protective (2.15-fold) and curative treatments (1.66-fold). Elevated *PR* transcription promotes resistance to microbial infections, which agrees with previous reports [65,66]. Therefore, it is feasible that *C. speciosa* can alter the plant's responses, boost resistance, and stop *R. solani* from silencing defensive genes. Inhibition of fungal cell proliferation is caused by sterol-binding *PR-1* proteins [67], while *PR-2*-1,3-glucanases and *PR-3*-chitinases cause lysis of the fungus wall. In all *C. speciosa* extract treatments, *PR-2* upregulation rose (Figure 5). Relative expression level reports showed that curative treatment was 2.94-fold higher than the control, with protective treatment reporting a 2.75-fold increase. A 1.27-fold decrease in regulatory activity was observed between the control plants and those exposed to the fungus (Figure 5). Multiple studies have shown that SAR inducers, particularly SA, activate the *PR-2* protein family, which then participates in various physiological plant defense activities [68]. Plants given an extract of *C. speciosa* may have shown an increase in *PR-2* activity due to the presence of fungal metabolites [69]. The current study suggests that microbe elicitors increase *PR-2* mRNA in plants [70]. Enhanced *PR-2* activity in the plant cell has been demonstrated to improve the number of oligosaccharides generated, stimulating defense mechanisms.

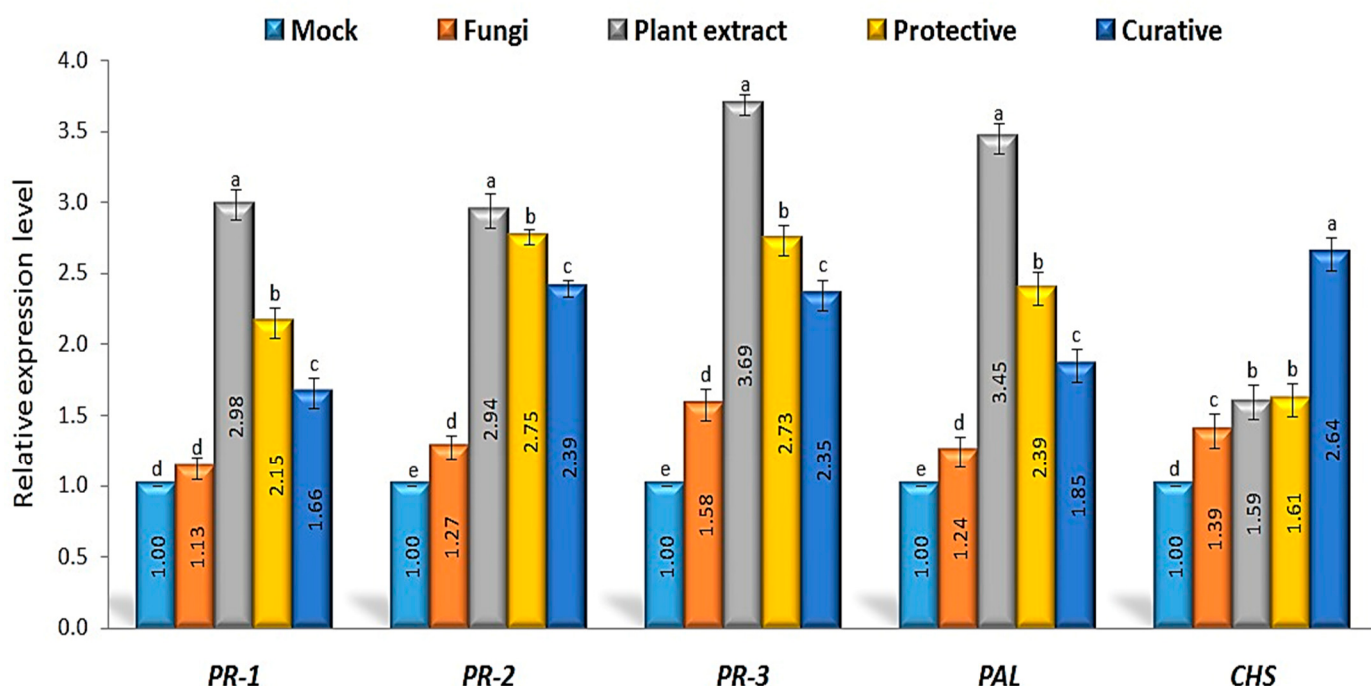


Figure 5. Relative expression levels of five genes: *PR-1*, *PR-2*, *PR-3*, *PAL*, and *CHS* at 20 dpi. The different letters on top of the columns mean the data values were significantly different in each gene at $p \leq 0.05$.

When comparing treated and control plants (mock plants), *PR-3* relative expression levels were significantly higher in all cases (Figure 5). Treatment with plant extract alone resulted in the greatest transcriptional level (3.69-fold), followed by protective treatment (2.73-fold), curative treatment (2.35-fold), and mock plants (1.58-fold). Chitinase, an enzyme that catalyzes the breakdown of chitin and protects plants from fungal attacks, is encoded by the *PR-3* gene [17]. In this study, the *PR-3* gene was induced in tomato plants in response to fungal infection, *C. speciosa*, and curative and protective applications. The results demonstrate that *PR-3* is a gene that works to improve plant tolerance to fungal invasions. When *C. speciosa* is applied, numerous defense genes are activated in plant leaves, including *PR-3*, which results in greater resistance to pathogens [19]. All treatments

increased *CHS* gene transcription levels when comparing treated and mock plants. *CHS* converts *p*-coumaroyl CoA to naringenin chalcones [71,72] and is thus a necessary first step in the plant flavonoid biosynthetic pathway. The curative application showed the highest transcript levels (2.64-fold) compared to the other treatments (protective, plant extract, and mock plants; Figure 5). After treating with *C. speciosa* extract, curative, and protective treatments [17,73], *CHS* was found to be most activated, which is needed for flavonoid formation. Extensive flavonoids with broad antifungal effects against a variety of plant diseases have been observed to be produced by *CHS* over-transcription in prior studies [74,75]. For this reason, using *C. speciosa* on tomato plants as a protective or curative application may lead to greater production of flavonoid molecules. Thus, we hypothesize that the *C. speciosa* extract includes elicitor compounds that can trigger SAR and enhance plant resistance to fungal infection. In many cases of host–pathogen interactions, the primed state is characterized by the subsequent activation of defense-related *PR* genes upon pathogen attack [76,77]. This is also the case in inducible systemic resistance (ISR) against hemitrophic and necrotrophic diseases, as shown by the priming of defense-related genes by *Harpophora oryzae* in the rice–*Magnaporthe oryzae* interaction [65] and by *B. subtilis*-induced related proteins in tomato confronted by *Pectobacterium carotovorum* [66]. However, *C. speciosa* can potentially be employed as a biocontrol agent to combat *R. solani* infections. However, further studies, such as the fractionated compounds from the extract, are required for field applications in the future.

3.7. TPC Accumulation

The accumulation of polyphenolic compounds in plants treated with plant extract is associated with a robust biochemical defense against pathogenic diseases. About 6.3 times more phenolics were present in the protective treatment than in the control, whereas *C. speciosa* extract and *R. solani* treatments produced 3.9 and 4.0 times the phenolic content, respectively (Figure 6). Several investigations [78–80] found that infections with fungi or bacteria significantly increased total phenolic content. In addition, the curative treatment had the highest phenolic content, nearly 2.8-fold higher than control plants, despite being lower than all treatments except the control (0.066 g GAE/100 g). Therefore, the phenolic compound chemicals that build in plants treated with plant extracts may act as proton donors, protecting root cells from oxidative damage caused by microbial infection [81]. Tomato plants would be protected against plant diseases if their TPC increased as a plant response to the administration of plant extract in protective treatment only. According to Mikulic-Petkovsek et al. [82], these TPC variations result from biosynthesis phenylpropanoid pathway regulation.

3.8. Antioxidant Enzymes Activity

The current study found that inoculating tomatoes with *R. solani* significantly increased the levels of three antioxidant enzymes (PPO, CAT, and SOD). Antioxidant enzyme PPO activity was 1.25-fold higher in the fungal treatment (0.15 $\mu\text{M/g f.wt.}$) compared to the control (0.12 $\mu\text{M/g f.wt.}$), but this difference was not statistically significant. The enzyme value was raised 2.16 times over the control after treatment with the plant extract. Enzyme levels in preventive and curative treatments (0.18 and 0.22 $\mu\text{M/g f.wt.}$, respectively) differed significantly from the control (Figure 7). PPO's consumption of reactive oxygen species (ROS) as a substrate has also been linked to the production of lignin within the cell wall, which acts as a protective border against the spread of plant diseases [83]. Figure 7 shows that accumulated CAT was 0.63 M/g f.wt. greater after treatment with plant extract compared to the control treatment. The accumulation rates for the preventative and curative treatments were drastically different. However, when comparing the fungal treatment (0.45 $\mu\text{M/g f.wt.}$) to the control treatment (0.38 $\mu\text{M/g f.wt.}$), there was a statistically significant increase in CAT activity levels. It has been established that CAT plays a crucial role in shielding plant cells from the damage generated by reactive oxygen species (ROS) under plant stress [84]. SOD activity did not increase substantially in any of the treatments'

leaf tissues except for the plant extract. In tomato plants, the extract alone increased enzyme activity by 1.29-fold compared to the control (0.66 $\mu\text{M/g f.wt}$). The SOD enzyme has been shown to prevent pathogen penetration by fortifying cell walls [85], a role that has been previously reported. Our findings indicate that pre- or post-fungal inoculation treatment with *C. speciosa* extract significantly alters tomato plants' PPO, CAT, and SOD activities. Based on their findings, Sobhy et al. [56] conclude that the extract has a high phenolic content and a potent ability to quench radicals. *C. speciosa* extract has a wide variety of phenolic and flavonoid components with antioxidant properties, which may account for the lack of antioxidant enzyme activity observed in the treatments. In contrast, increasing CAT levels can reduce H_2O_2 -induced oxidative stress and mimic H_2O_2 signaling in disease progression [86]. Therefore, foliar application of *C. speciosa* extract before fungal inoculation may be an option for reducing the negative consequences of fungal infections. Our results suggest that *C. speciosa* extract enhances defense and detoxification systems, leading to more rapid and effective responses to fungal inoculation. Moreover, flavonoids, phenolics, PPO, CAT, and SOD activities may play an important role in tomato survival when exposed to fungal stress. By improving growth parameters, boosting antioxidant status, and modulating gene expression in fungal-stressed plants, foliar application of *C. speciosa* extract before *R. solani* inoculation greatly protected tomato plants from fungal infection problems. Thus, this research may have inspired the development of a new extract–host–pathogen system, albeit the underlying mechanism of how this would work is now unknown. More research is needed in the future to understand the changes to the proteome in extract-primed tomato.

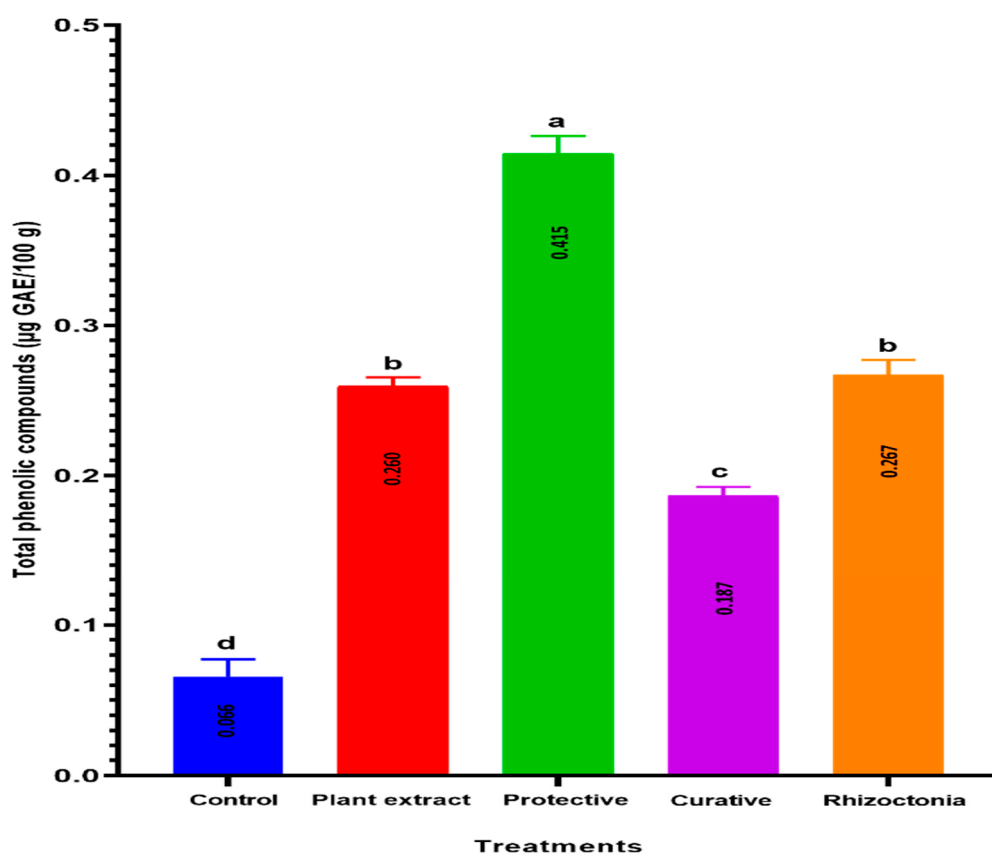


Figure 6. Total phenolic compounds of all treatments used in this study. The different letters on top of the columns mean the data values were significantly different at $p \leq 0.05$.

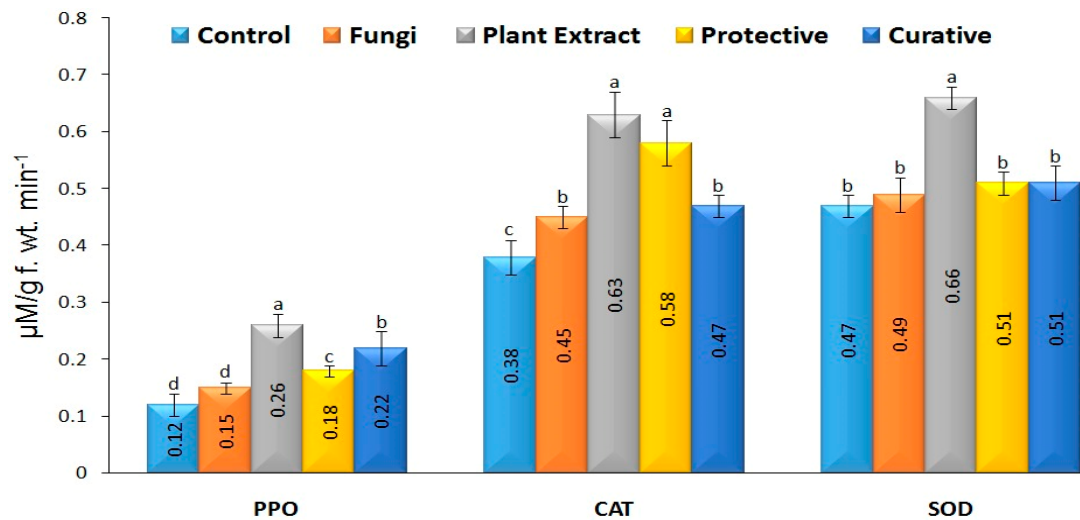


Figure 7. PPO, CAT, and SOD enzyme activities under the studied different treatments. The post hoc test analysis of each antioxidant enzyme was conducted separately. The different letters on top of the columns of each antioxidant enzyme mean the data values were significantly different at $p \leq 0.05$.

3.9. Characterization of *C. speciosa* Extract

3.9.1. Antioxidant Activity

The ability of *C. speciosa* extract to scavenge free radicals was measured using the DPPH (1,1-diphenyl-2-picrylhydrazyl) technique. As shown in Figure 8, the extract's antioxidant activity value (IC_{50}) was 93.46 $\mu\text{g/mL}$ compared with butylated hydroxytoluene (BHT, 5.04 $\mu\text{g/mL}$). Our findings demonstrated that *Chorisia* species possessed antioxidant properties consistent with previous research. Few studies showed high antioxidant activity for different fractions of *Chorisia speciosa* leaves and stem ethanol extract [87]. In addition, Refaat et al. [14] showed that different plant parts' ethyl acetate, aqueous, and chloroform fractions of both *C. speciosa* and *C. chodatii* plants exhibited the most extraordinary free radical scavenging properties and the highest polyphenol contents.

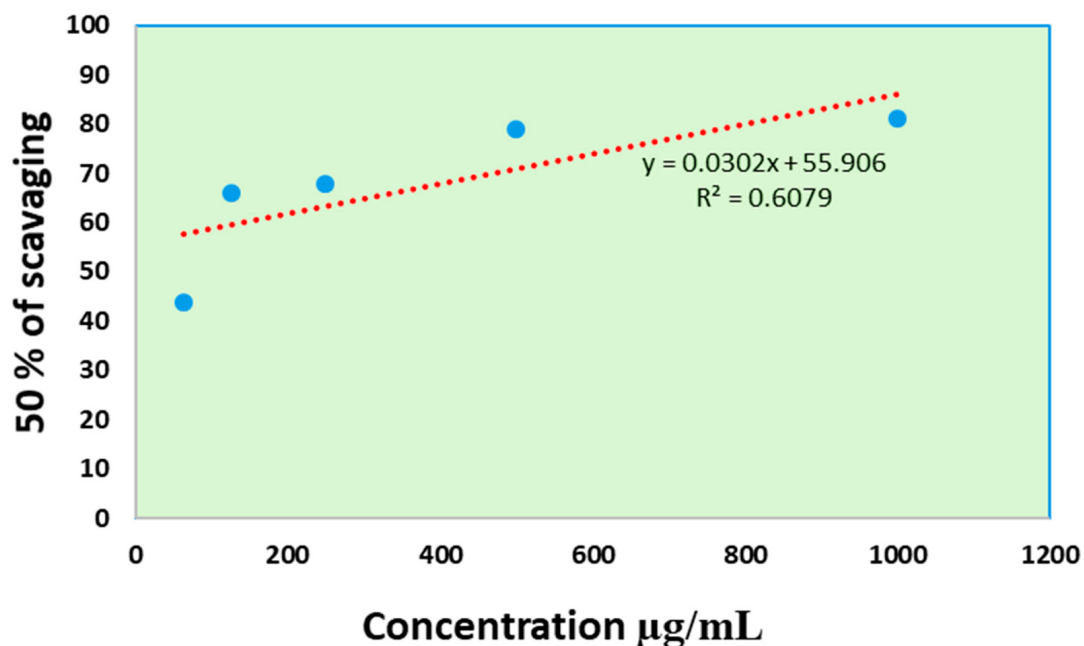


Figure 8. The scavenging percentage of 50% inhibition of DPPH (IC_{50}) of the *Chorisia speciosa* extract.

3.9.2. HPLC of *C. speciosa* Extract

HPLC was used to analyze the *C. speciosa* extract and revealed a list of chemical compounds in the graphs shown in Figure 9 and Table 3. The main compounds were determined as $\mu\text{g/mL}$: 7-OH-flavone (10.36), kaempferol (9.23), *p*-coumaric acid (8.65), ferulic acid (8.14), caffeic acid (7.59), gallic acid (6.33), iso-ferulic (5.71), apigenin (5.47), catechin (3.51) and naringenin (3.14), pyrogallol, ellagic acid, hesperidin, and myricetin. Many plants used for medical purposes for many years contain flavonoids and polyphenols. Ferulic acid, quercetin, ellagic acid, chlorogenic acid, catechins, gallic acid, caffeic acid, and myricetin are all forms of polyphenols that have antibacterial, antiviral, and antioxidant properties [88,89]. Our data suggest that polyphenolic molecules serve critical functions in SAR as elicitor compounds. The antimicrobial activity of *C. insignis* ethyl acetate extract may be due to flavonoids and kaempferol derivatives, as previously reported by El Sawi et al. [90]. Previous studies have identified various phenolic compounds in medicinal plants, many of which contribute to the plants' antioxidant and antibacterial activities [91,92].

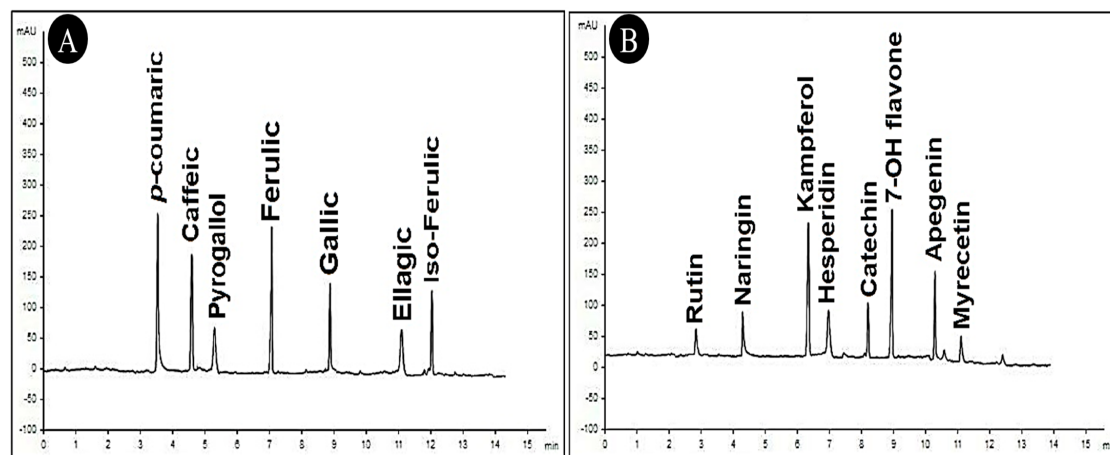


Figure 9. HPLC chromatograms of the phenolic (A) and flavonoid (B) chromatograms identified in *Chorisia speciosa* extract.

Table 3. Polyphenolic compounds identified in *Chorisia speciosa* extract.

Compounds	Retention Time (min.)	Amount ($\mu\text{g/mL}$)
<i>p</i> -Coumaric acid	3.5	8.65
Caffeic acid	4.8	7.59
Pyrogallol	5.2	1.22
Ferulic acid	7.0	8.14
Gallic acid	9.0	6.33
Ellagic acid	11.0	1.18
Iso-Ferulic	12.0	5.71
Rutin	3.0	0.88
Naringenin	4.1	3.14
Kaempferol	6.1	9.23
Hesperidin	7.0	2.89
Catechin	8.1	3.51
7-OH flavone	9.0	10.36
Apigenin	10.1	5.47
Myricetin	11.0	0.79

4. Conclusions

Applying 10 µg/mL of a *C. speciosa* extract to the leaves of tomato plants increased their growth, induced systemic resistance, and reduced the incidence of root rot disease caused by the *R. solani* RS 35 pathogen. Our results showed that *C. speciosa*-treated plants had a lower disease index than control plants, and the expression levels of *PR-3*, *PAL*, and *CHS* genes had increased significantly at 20 dpi. The HPLC examination of the *C. speciosa* extract showed that the main compounds were 7-OH-flavone, kaempferol, *p*-coumaric acid, ferulic acid, caffeic acid, gallic acid, iso-ferulic, apigenin, catechin, naringenin, pyrogallol, ellagic acid, hesperidin, and myricetin. Our findings indicate that curative treatment is the most effective way of minimizing root rot. Consequently, the evaluated extract could be a suitable source for the production of antifungal agents to combat plant diseases. However, further studies are needed for open field applications.

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