




Article

Efficacy of *Azadirachta indica* and *Punica granatum* Extracts in the Control of *Cuminum cyminum* Wilt Disease Caused by *Fusarium oxysporum* f. sp. *cumini*

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Abstract: *Punica granatum* and *Azadirachta indica* are plants rich in phytochemicals, which directly contribute to antioxidant activity. The aim of this study was to test *A. indica* and *P. granatum* against *Fusarium oxysporum* f. sp. *cumini* (Foc), the causal pathogen of Fusarium wilt in cumin plants, in vivo and in vitro. After screening different concentrations of both plants, three concentrations (250, 500, and 1000 $\mu\text{g}\cdot\text{mL}^{-1}$) of *P. granatum* and *A. indica* were selected to study their effectiveness against Fusarium wilt in cumin plants. The in vitro study showed that both extracts have the ability to reduce mycelium growth of the pathogen with different degrees of efficacy, but less than the positive control. Under greenhouse conditions, all treatments of cumin plants significantly reduced Fusarium wilt compared to the infected control. The most effective concentration for *P. granatum* was 1000 $\mu\text{g}\cdot\text{mL}^{-1}$. The use of both extracts significantly increased the fresh and dry weight of cumin plants (g plant^{-1}) compared to infected plants. Total phenols and flavonoids increased in inoculated cumin plants after treatment with both extracts. The results revealed that both extracts are rich in phytochemicals and possess potent in vitro antioxidant activity. Both are rich in carbohydrates, saponins, amino acids, proteins, alkaloids, and terpenoids. In conclusion, the application of methanolic extracts of *P. granatum* and *A. indica* can provide an alternative to chemical fungicides to mitigate the Fusarium wilt of cumin and, therefore, future studies should focus on the study of both extracts on different pathogens, as well their ability to reduce disease under field conditions.

Keywords: antifungal activity; cumin wilt; plant extract; phenol compounds; peroxidase



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1. Introduction

Many vegetables, field crops, flowers, and plantation crops suffer severe losses due to Fusarium wilt [1]. According to previous studies, losses from cumin wilt have been estimated to range from 5 to 60% [1]. Warm winter climates are known to favor Fusarium wilt, especially when conditions are dry. Fusarium wilt disease is one of the most important cumin diseases [2]. The pathogen is seed- and soil-borne, and it is widely prevalent in almost all cumin-growing countries [1]. Chemical fungicides, Vitavax (carboxin)-thiuram or Vitavax-captan, are methods that provide a major deterrence to Fusarium wilt [3], but they are expensive and are known to be hazardous to human health and the environment [4]. Therefore, there is a pressing urgency for less expensive, more environmentally friendly methods of controlling Fusarium wilt.

Certain higher plant products, most notably, phytochemicals, can be a source of antimicrobial resistance, and have attracted attention due to their biodegradability and safety for human health, among other attractive properties [5]. According to Sharma and Bohra [6], *Boerhavia diffusa* leaf extract effectively inhibited the growth of *Fusarium*

oxysporum f. sp. *cumini* (Foc) and decreased the severity of cumin wilt. Similar to *Datura stramonium*, fresh leaf extracts of *Datura procera* and *Calotropis procera* demonstrated the highest antifungal efficacy against Foc [7].

Punica granatum is a plant that is rich in phytochemicals, which directly contribute to antioxidant activity. The color of *P. granatum* is due to the presence of a biochemical pigment called anthocyanin. Anthocyanins are a group of compounds that are rich in ascorbic acid and phenolic acids, and phytochemicals such as gallic acid, flavonoids, amino acids, and saponins. Accumulation of anthocyanins in the vacuole gives *P. granatum* its red color [8].

The peel fruit of *P. granatum* is rich in tannins, flavonoids, and phenolic acids, and has various biological functions, including activity against pathogenic microorganisms [9]. Several studies have evidenced the antimicrobial activity of the pomegranate extract against phytopathogenic fungi and bacteria, suggesting a high potential source of natural antifungal and antibacterial agents [10–12]. Dahham et al. [13] tested pomegranate extract against certain phytopathogenic fungi, *Aspergillus niger*, *Rhizopus oryzae* and *Penicillium citrinum*, and found that the peel extract can reduce the mycelia growth of all pathogens tested. Additionally, Mangang and Chhetry [14] found that the extract of pomegranate reduced linear growth of *Sclerotium rolfsii*, *Phoma destructiva*, *Alternaria alternata*, *R. solani*, and *F. oxysporum*, with different degrees of activity. The metabolites of *P. granatum* include various types of glucose, fatty acids, vitamins, and polyphenols, and *P. granatum* also has the ability to prevent diseases such as lung cancer, anemia, myocardial infarction, infertility, and atherosclerosis [15].

Azadirachta indica is a member of the mahogany family, Meliaceae. Recently, several biological activities have been proved for various parts of neem trees, including antibacterial, antifungal, antimalarial, and antioxidant activities [16]. Several researchers have used it as method of control for many plant diseases, including bacterial wilt of tomatoes [16] and soil-borne pathogens such as *Fusarium oxysporum*, sp. *ciceri*, *Rhizoctonia solani*, and *Sclerotium rolfsii* [17]. There are thousands of different chemical components in neem; however, Azadirachtin is the most well-known. It belongs to a class of compounds called terpenoids that are also found in other parts of the neem tree [18].

To the best of our knowledge, there have been no studies on the effectiveness of *A. indica* and *P. granatum* on Fusarium wilt in cumin; therefore, the present work aimed to study the effectiveness of different concentrations of methanol leaf extracts of *P. granatum* and *A. indica* against the causal pathogen of cumin wilt, *F. oxysporum*, under in vitro conditions, and to study their effect on the control of cumin wilt in a greenhouse experiment.

2. Materials and Methods

2.1. Cumin Seeds and Growth of Plants

The cumin seeds (*Cuminum cyminum* L. cultivar Balady) used in this study were obtained from the Department of Ornamental Plants, Faculty of Agriculture, Egypt. Ten seeds were sown in each plastic pot filled with a pasteurized mixture of soil and sand (4:1 w/w), measuring 30 cm in diameter (2.4 kg). The seedlings were kept in the greenhouse at 50–70% relative humidity (RH) and temperatures between 25 and 30 °C, and they received about 12 h of natural light and 12 h of darkness daily. Each pot of plants was given roughly 50 mL of NPK (12:4:6) fertilizer suspension (1 g L⁻¹) each week, in addition to the necessary amounts of water.

2.2. Source of Pathogen

A highly virulent strain was identified as *Fusarium oxysporum* f. sp. *cumini* (FO7) and deposited in the GenBank sequence database under accession number OM918708.1. This isolate was isolated previously by the authors. The isolate was grown on potato dextrose agar (PDA) obtained from HiMedia company, India, on plates (15 mL/plate) incubated at 27 °C for 7 days.

2.3. Preparation of Plant Extracts

Two plant species, *Azadirachta indica* and *Punica granatum*, were collected from different places from the Assiut Governorate, Egypt. The *Azadirachta indica* leaves and the fruit peel of *Punica granatum* were used. Plant materials were dried at room temperature for 15 days, then ground into fine powders in a grinder and used for the preparation of the methanolic extracts by maceration in 80:20 methanol:water at a 1:10 *w/v* ratio of sample to solvent, and kept under continuous shaking for three days at room temperature. Whatman No. 1 filter paper was used to filter the macerated materials after they had been filtered through two layers of cheesecloth. Using a rotary evaporator (Heidolph VV2000), the filtrates were mixed, concentrated, and then freeze-dried with a Telstar-LyoQuest plus-55 lyophilizer. The extract's yield was calculated and kept in opaque glass tubes at $-20\text{ }^{\circ}\text{C}$ [19,20].

2.4. Evaluation of Plant Extracts

2.4.1. In Vitro Antifungal Activity

Different volumes of crude methanol extracts were added to the PDA media before being poured into Petri plates in order to achieve extract concentrations of 250, 500, and $1000\text{ }\mu\text{g}\cdot\text{mL}^{-1}$. The plates were then inoculated with 2 mm fungal plugs in the center and cultivated for 10 days at $28\text{ }^{\circ}\text{C}$ [21]. Calculations were used to determine the pathogen's percentage of inhibited radial growth. Five plates per replication were used to replicate each treatment three times. The commercial fungicide hymexazol [3-hydroxy-5-methylisoxazole 5-methylisoxazol-3(2H)-one hymexazole] was used as the positive control ($1000\text{ }\mu\text{g}\cdot\text{mL}^{-1}$). Methanol at 8% was used as the negative control. The percentage of reduction (Mr) of the colony diameter by each extract was computed following the technique used by Nduagu et al. [22]:

$$\text{Growth inhibition (Mr)} = \left(\frac{M1 - M2}{M1} \right) \times 100 \quad (1)$$

where Mr = % reduction in colony diameter, M1 = colony diameter in the untreated medium (negative control), and M2 = colony diameter in the treated medium.

2.4.2. Greenhouse Conditions

The fungal inoculum of *F. oxysporum* f. sp. *cumini* (Foc) was prepared as follows: The Foc isolate was grown on a sterilized grain sorghum medium (200 mL + 150 g + 4 g + 4 g + 50 g of water, grain sorghum, sucrose, and clean sand, respectively) and incubated for two weeks at $27\text{ }^{\circ}\text{C}$ [23]. The inoculum was mixed with the soil at 3% (*w/w*), and then irrigated three times over one week before transplanting to ensure an even distribution and growth of the pathogen isolate. Seven days before planting, sterilized sandy clay soil that had been inoculated with 3% (*w/w*) fungal inocula was placed in plastic pots (30 cm in diameter) [16]. Disinfected cumin seeds were soaked in the solutions of each peel methanol extract of *P. granatum* (Pme) and leaf methanol extract of *A. indica* (Lme) for 12 h before being seeded at a rate of 10 seeds per infested pot. Cumin seeds were also soaked in 8% methanol for the same amount of time and sown at the same rate in pathogen-infested soil as part of the control treatment. Each treatment consisted of five pots used as replicates. After 50 days from planting, the severity of the wilt was noted according to the method described by Ghoneem et al. [24]. Wilt severity was recorded 40 days after sowing. The disease index (%) = $\{[\sum (\text{number of plants for each disease severity} \times \text{disease rank})] / (\text{total number of plants} \times \text{the highest level})\} \times 100$. The severity of Fusarium wilt in cumin was classified into five grades (0–5) according to the criteria of disease grades [24].

2.5. Phytochemical Screening Test

This was determined according to Reddy et al. [25].

2.5.1. Test for Phlobatannins

A total of 1 mL of 1% HCl was added to 1 mL of the extract before it was heated for 10 min. Phlobatannins were present when a precipitate of a red color formed.

2.5.2. Test for Carbohydrates

A total of 1 mL of the extract was mixed with three to five drops of Molisch reagent, and then 1 mL of concentrated sulfuric acid was carefully introduced via the test tube. After standing for two minutes, the liquid was diluted with 5 mL of pure water. Carbohydrates were detected by the formation of a red or dim violet ring at the liquid–liquid interface.

2.5.3. Test for Flavonoids

A total of 1 mL of the extract was added to a test tube, along with a few drops of 1% liquid ammonia, which caused the extract to become yellow, showing the presence of flavonoids.

2.5.4. Test for Alkaloids

A total of 2 mL of the sample and 2 mL of HCl were mixed. Following the addition of six drops of HCN and an additional two drops of picric acid, a pale-yellow cream color suggested the presence of alkaloids.

2.5.5. Test for Terpenoids

A total of 3 mL of conc. H_2SO_4 and 2 mL of the sample were added, along with 2 mL of chloroform. The resulting red color showed the presence of terpenoids.

2.5.6. Test for Proteins

A small quantity of extract was added after 1 mL of ninhydrin had been dissolved in 1 mL of acetone. Protein was detected by the development of a purple color.

2.5.7. Detection of Saponins

Test for foam: a portion of the extract was rapidly shaken with water and any persisting foam was noted.

2.5.8. Test for Steroids

A total of 1 mL of concentrated sulfuric acid and 1 mL of chloroform were combined with 1 mL of extract. Ten drops of acetic anhydride were also added. The development of dark-red or dark-pink pigments was a sign that steroids were present.

2.6. Non-Enzymatic Assays

For the sample preparation, 1 gram of the root sample of each treatment was collected 35 days after the sowing date and washed with tap water extracted in 10 mL methanol:water (8:2 *v/v*). The suspension was shaken at 120 rpm for 2 h at 30 °C, then centrifuged at 1013 g for 5 min. The supernatant was collected and used for the determination of the total contents of phenols and flavonoids.

2.6.1. Total Phenol Content (TPC)

The level of TPC was determined in the prepared methanolic extract in accordance with the method reported by Malik and Singh [26]. All chemicals and reagents used in this experiment were purchased from Sigma-Aldrich (Waltham, MA, USA). The reaction mixture contained 50 μ L of the sample methanolic extract mixed with distilled H_2O (850 μ L) and Folin–Ciocalteu reagent (100 μ L). An aliquot of 500 μ L of sodium carbonate (Na_2CO_3 , 20%) was added to the mixture and left to react for 30 min before measuring the absorbance at 750 nm. A calibration curve of gallic acid was prepared using different concentrations, following the same procedure as for the sample analysis. TPC was expressed as mg gallic acid equivalent/g fresh weight of cumin samples. All treatments were repeated three times.

2.6.2. Total Flavonoid Content (TFC)

TFC was evaluated using a modified method reported by Zhishen et al. [27]. An aliquot of 250 μL of the methanol extract was combined with 75 μL of 5% sodium nitrate (NaNO_2) solution and 1.25 μL of sterile distilled water in a 75 μL flask. After 6 min, 150 μL of 10% aluminum chloride (AlCl_3), 0.5 μL of 1 M sodium hydroxide (NaOH), and 275 μL of sterile distilled water were added to the solution. A calibration curve was created using known catechin concentrations analyzed the same way as the samples. The absorbance was recorded at 510 nm for the samples and the standard catechin, and the TFC was calculated and displayed as mg catechin equivalent/g fresh weight of orange samples.

2.7. Statistical Analysis

All tests were set up in a completely randomized design, and each experiment consisted of three replicates repeated twice. Using the statistical analysis method, data were subjected to an ANOVA [28]. The LSD test was used to compare the means at $p < 0.05$ levels.

3. Results and Discussion

Currently, the main method for disease control is through the use of fungicides [29], but chemical control is harmful to the environment and human health, and the control effects decrease gradually due to drug resistance in the pathogens. Therefore, it is necessary to search for natural and safer alternatives to chemicals. Many studies have recommended plant extracts to control fungal, bacterial, and helminthic diseases [16,30].

3.1. In Vitro Antifungal Activity

In this study, the antifungal activity of methanol extracts of two plant extracts, *Azadirachta indica* and *Punica granatum*, was tested. All concentrations of both plant extracts were effective in reducing the mycelial growth of *Foc* (Table 1). In general, *P. granatum* (Pme) exhibited the highest reduction in mycelium growth of the pathogen at 1000 $\mu\text{g}\cdot\text{mL}^{-1}$, followed by 1000 for *A. indica* (Lme). The lowest reduction was achieved by *A. indica* at 250 $\mu\text{g}\cdot\text{mL}^{-1}$.

Table 1. Antifungal activities of methanol extract of leaf extract *Azadirachta indica* and peel extract of *Punica granatum* against *Fusarium oxysporum* f. sp. *cumini*.

Treatments	Dilutions ($\mu\text{g}\cdot\text{mL}^{-1}$)	Mycelial Growth (cm)	Mycelial Reduction %
<i>Azadirachta indica</i> (Lme)	250	5.2 b	42.2
	500	4.8 c	46.7
	1000	4.1 d	54.4
<i>Punica granatum</i> (Pme)	250	4.2 d	53.3
	500	4.0 d	55.6
	1000	3.5 e	61.1
Negative control	Methanol at 8%	9.0 a	-
Hymexazole	1000 $\mu\text{g}\cdot\text{mL}^{-1}$	1.8 f	80.0

The same letters within each treatment represent values that were not significantly different at the $p < 0.05$ level of confidence according to a least significant difference (LSD) test.

Tehraniifar et al. [31] reported that the phenolic content of pomegranate peel extract was 2.8-fold higher compared to the leaf extract, and the high concentrations probably explained the antifungal activity of the extract. They found a number of biologically active compounds, such as flavonoids (quercetin, rutin, luteolin, pelargonidin, prodelfphinidin, apigenin, etc.) and tannins (gallic acid, ellagic acid, punicalagin, and ellagitannin) [32]. Along the same lines, various studies have reported that plant extracts affected the growth of phytopathogenic fungi, as well as reduced disease severity, which may have been due to the secondary metabolites (phenolics, flavonoids, terpenoids, and alkaloids) [33–35].

Previous studies [36–38] mentioned that peel extracts of *P. granatum* were effective against many bacteria streptococci strains. Antibacterial activity may be due to the presence of the hydrolysable tannins and polyphenolics in the pomegranate extract, specifically, punicalagin and gallic acid [39,40]. This phenolic compound also has directly antifungal activity against *Phytophthora* spp. in vitro [41].

Azadirachtin is the main constituent of neem-based products which helps in the protection of crop plants from several pests and diseases [42]. Neem extracts are used to kill soil-borne pathogens, as well as seed-borne pathogens when used for seed treatment [42]. Plant extracts contain a mixture of many components, and their antifungal activity is probably not attributable to a single mechanism.

Values in the same column followed by different letters indicate significant differences among treatments according to the LSD test ($p = 0.05$).

3.2. Effect of Methanol Extract of *Azadirachta indica* and Peel Extract of *Punica granatum* on Disease Reduction under Greenhouse Conditions

The results, presented in Figure 1, show that the tested methanol extract of both plants at different concentrations significantly reduced the disease severity of Fusarium wilt disease in cumin plants under greenhouse conditions. The highest reduction was achieved by *P. granatum* at a concentration of $1000 \mu\text{g}\cdot\text{mL}^{-1}$, which showed nearly the same reduction in disease of 75% as hymexazole and *A. indica* at $1000 \mu\text{g}\cdot\text{mL}^{-1}$. The other concentrations of both extracts also could reduce the disease severity, but with the highest concentration ($1000 \mu\text{g}\cdot\text{mL}^{-1}$). Pme and Lme reduced the disease severity of Fusarium wilt of cumin (Figure 1). Our findings were in general agreement with those of Hassenein et al. [43], who found that neem leaf extract sprays on tomato plants reduced the severity of Fusarium wilt. In the present study, both extracts reduced the Fusarium wilt of cumin when compared with the untreated control, and the same effect as fungicides was observed in the reduction of disease severity.

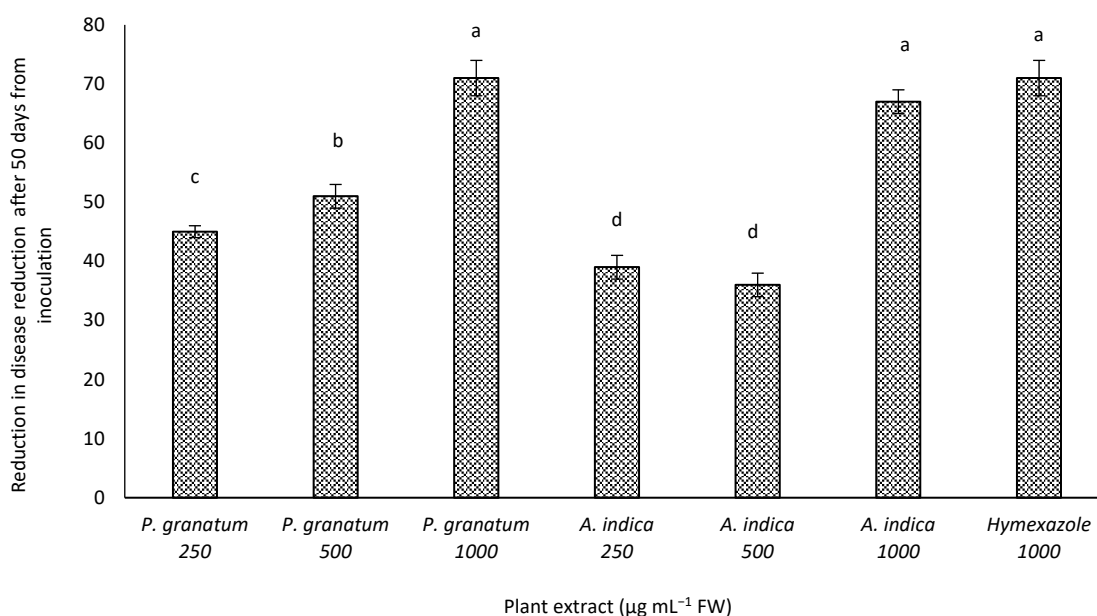


Figure 1. Effect of different concentrations of methanol extracts of *Azadirachta indica* and *Punica granatum* (250 , 500 , and $1000 \mu\text{g}\cdot\text{mL}^{-1}$) and fungicide (hymexazole, $1000 \mu\text{g}\cdot\text{mL}^{-1}$) on Fusarium wilt under greenhouse conditions. Values in columns followed by different letters indicate significant differences among treatments according to a least significant difference test ($p = 0.05$). Bars indicate the standard error.

3.3. Vegetative Growth

The fresh weight (FW) and dry weight (DW) of the cumin plants increased significantly with both extracts at different concentrations compared to the control group (Figure 2). The height of the cumin plants increased significantly in all treatment groups compared to the control group (Figure 2). The highest increase was found with *P. granatum* and *A. indica* treatments at 1000 $\mu\text{g}\cdot\text{mL}^{-1}$, followed by 500 and 250 $\mu\text{g}\cdot\text{mL}^{-1}$ of *P. granatum* (98.8 and 96.7%, respectively). The same trend was observed for fresh and dry weight, which increased by 88 and 85.6%; this maybe correlated with the reduction in the disease severity. Many studies have noted that plant extracts increase the seed germination, as well as the vigor index, of ginger, garlic, and neem [44–46]. Additionally, Abo-Elyousr et al. [4], in their work on *Calotropis procera*, reported that tomato plants treated with an aqueous extract of *C. procera* showed increased fresh and dry weights of the tomato seedlings.

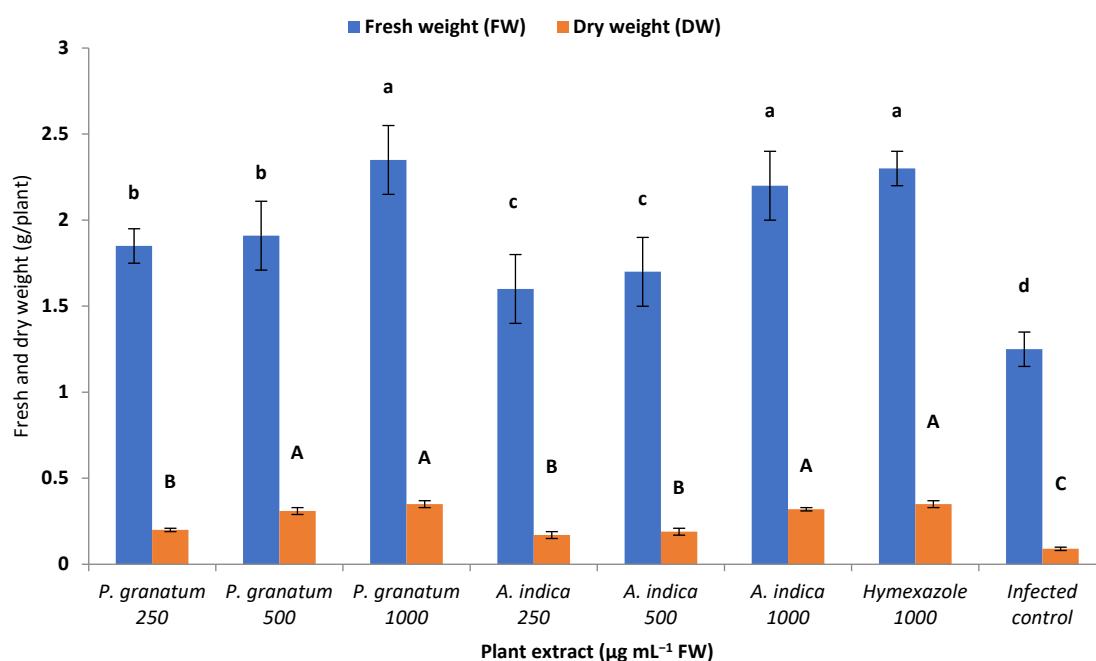


Figure 2. Effect of different concentrations of methanol extracts of *Azadirachta indica* and *Punica granatum* (250, 500, and 1000 $\mu\text{g}\cdot\text{mL}^{-1}$) and fungicide (hymexazole, 1000 $\mu\text{g}\cdot\text{mL}^{-1}$) on fresh and dry weight of whole plants (g plant^{-1}) under greenhouse conditions. Values in columns followed by different letters indicate significant differences among treatments according to a least significant difference test ($p = 0.05$). Bars indicate the standard error.

3.4. Effect of Different Concentrations of *Azadirachta indica* and *Punica granatum* on Phenol and Flavonoid Contents

From the data illustrated in Figure 3, the treatment of cumin plants with Pme and Lme at 1000 $\mu\text{g}\cdot\text{mL}^{-1}$ gave the highest flavonoid and phenol contents in inoculated or uninoculated plants, followed by 500 $\mu\text{g}\cdot\text{mL}^{-1}$ and the lowest concentration of 250 $\mu\text{g}\cdot\text{mL}^{-1}$, relative to the infected control plants.

Obtained results revealed that *A. indica* and *P. granatum* (250, 500, and 1000 $\mu\text{g}\cdot\text{mL}^{-1}$) increased the phenol content. In agreement with this, similar results have been reported in the literature. The authors of [4] found that the application of a plant extract (*Calotropis procera*) to tomato plants significantly reduced wilt severity compared to untreated controls. In this context, phytosterols, a group of steroidal alcohols, and phytochemicals naturally present in plants, have been found to display antifungal activity against *Aspergillus*, *Penicillium*, and *Fusarium* [47]. Phenolic compounds are toxic to phytopathogenic fungi, and the accumulation of these bioactive ingredients at infection sites has been associated with pathogen development restriction [48].

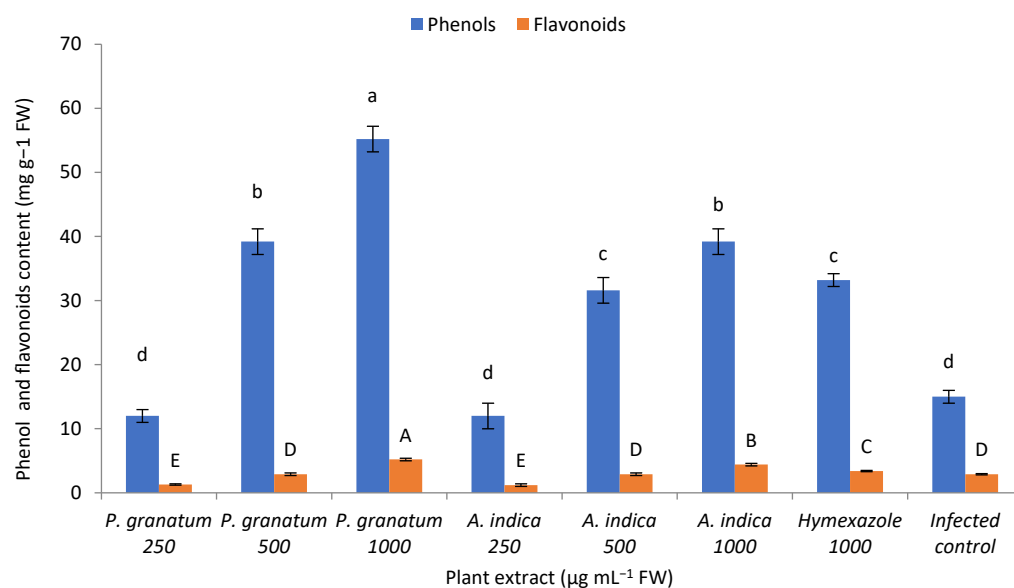


Figure 3. Effect of different concentrations of methanol extracts of *Azadirachta indica* and *Punica granatum* (250, 500, and 1000 $\mu\text{g}\cdot\text{mL}^{-1}$) and fungicide (hymexazole, 1000 $\mu\text{g}\cdot\text{mL}^{-1}$) on phenol content (phenols; mgg^{-1} FW) and flavonoids (mg g^{-1} FW) in root extracts of cumin plants after 35 days of treatment with both extracts and inoculation with *F. oxysporum*. Values in columns followed by different letters indicate significant differences among treatments according to a least significant difference test ($p = 0.05$).

3.5. Phytochemical Screening Test

The results of the qualitative phytochemical analysis (Table 2) of *P. granatum* peel and *Azadirachta indica* extracts showed that Pme and Lme are rich in carbohydrates, saponins, amino acids, proteins, alkaloids, and terpenoids. *P. granatum* peel extract is rich in carbohydrates, amino acids, proteins, alkaloids, and terpenoids. Phytochemical screening results showed that the methanol peel and leaf extract of both plants are rich in amino acids, flavonoids, alkaloids, and terpenoids, and fairly rich in saponins and flavonoids. Peel extract is rich in carbohydrates and saponins, and fairly rich in flavonoids. The secondary metabolites contribute significantly towards the biological activities, such as antioxidant and antidiabetic activities. Phytochemicals are mostly rich in green leaves, where they prevent free-radical formation. Hence, the presence of phytochemicals in the extracts might contribute to their beneficial activities [49].

Table 2. Qualitative phytochemical analysis of *Azadirachta indica* and *Punica granatum* extracts.

Phytochemical	<i>Azadirachta indica</i> Leaf Extract	<i>Punica granatum</i> fruit Peel Extract
Carbohydrates	++	++
Saponins	++	+++
Steroids	++	+++
Amino acids	+++	+++
Proteins	+++	++
Flavonoids	+	+++
Alkaloids	+++	++
Terpenoids	+++	++

(+++)= strong color format, (++) = moderate color format, (+) = weak color format.

Methanol extracts of *P. granatum* exhibited in vitro antioxidant activity in a concentration-dependent manner. Ascorbic acid was used as the standard chemical for comparing the antioxidant activity. Dietary antioxidants such as ascorbate, carotenoids, and tocopherol from natural compounds could reduce oxidative stress. Medicinal plants are a natural

source for antimicrobial substances, which have fewer side effects and an ability to scavenge free radicals.

4. Conclusions

The seeds of cumin plants treated with the methanol extract of *Azadirachta indica* and *Punica granatum* at different concentrations reduced *F. oxysporum* f. sp. *cumin* radial growth in vitro; thus, the cumin seed pretreatment reduces the disease severity and increases the vegetative growth of cumin seedlings under greenhouse conditions. Seeds treated with both extracts had increased flavonoid and phenol contents in cumin roots at 35 days after the sowing date. Our results show that *Azadirachta indica* and *Punica granatum* at different concentrations are promising ecofriendly substances for use against *F. oxysporum* under greenhouse conditions.

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