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Investigation on the Effect of Neem Leaves and Garlic Bulbs Extracts on Fungi Causing Post-Harvest Spoilage of Tomatoes *in Vitro* and *in Vivo*

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Abstract: In Iraq, tomato is a highly valuable vegetable. Fungal infections result in large postharvest losses in tomato production Therefore, a studies on antifungal effect of garlic (*Allium sativum*) bulb and neem (*Azadirachta indica*) leaf extracts were determined on causative agents of post-harvest fruit rot of tomato. Three concentrations (0,100, 200mgml^{-1}) of each, in addition to 2gl^{-1} of fungicide (Carbendzim) were used. The treatments were laid out in Completely Randomized Design (CRD) with three replications. Samples of infected and non-infected tomato fruits were obtained from a local market. The infected tomatoes were directly cultured on potato dextrose agar (PDA) media for fungal growth, then incubated at room temperature with an observation for 24 hours to 7 days. Based on their morphological characteristics and an examination of their genomic DNA's internal transcribed spacer (*ITS*) sequences, funguses from tomato fruits were identified. Results revealed that when tested fungal Pathogenicity, the severity of the infection increased significantly on the fourth day, and on the fifth day the infection appeared on all tomato fruits inoculated with the fungus, as well as on the fruits of the control treatment. During in vitro and in vivo experiments, all the tested concentrations of garlic and neem significantly ($p \leq 0.05$) suppressed mycelial growth of the fungi Both garlic and neem proved effective in the control of disease severity and these natural extracts can be used as an alternative control method without posing a serious risk to human or animal health, and they provide excellent substitutes for synthetic pesticides.

Keywords: Antifungal activities; garlic, neem, Post-Harvest Spoilage, Tomatoes, *In vitro*, *In vivo*.

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Introduction

The Tomato fruits contain certain amount of nutrients such as potassium in addition to contents of acids such as ascorbic (vitamin C), citric, and malic acids as well as E vitamin (1). The tomato (*Lycopersicon esculentum Mill.*) provides consumers with vital vitamins and generates revenue for agricultural communities across the globe. The tomato plant is cultivated for its fruit, which is processed into pastes, pulps, juices, powders, and ketchup for the fresh market (2). Even if Iraq's environment and ecology are ideal for growing tomatoes, there are still a number of difficulties and crises that must be overcome, including low and unstable production, irregular supplies, post-harvest losses, rising demand, and often exorbitant prices that are out of reach for the average person. Many infections, especially those caused by fungi, can cause significant losses in the field. Chemical control of fungal diseases presents a number of challenges, including food contamination, environmental pollution,

and health risks to humans. Compared to synthetic fungicides that are hazardous to human health, natural plant protectants are widely accepted by the public (3). Therefore, as an alternative to agrochemicals, it was desirable to detect fungal pathogens, monitor fungal illnesses in tomatoes, and characterize bioactive and antifungal substances.

According to previous reports, the fact that medicinal plants contain a number of chemical compounds, they have medical importance in treating many diseases. They are used directly or indirectly (4) for bioactive compounds, including flavonoids, phenols, polyphenols, ketones, and lipophilic aromatic compounds that can quickly diffuse through the cell membrane, disturbing cellular stability (5). Pathological microorganisms, so the effect of extracts in inhibiting microorganisms varies (6). Many plants used in traditional medicine represent rich sources of natural bioactive substances with health promoting effects and no side effects (7). Natural plant products and their analogs are essential sources of agricultural biopesticides due to their antimicrobial properties (3). Overuse of fungicides has disrupted the soil's microbial balance, resulting in the proliferation of pathogens and the decline of beneficial microbes (8). Fungal rots are world-wide in occurrence and have been reported almost in all parts of the world (9). Neem leaves and garlic bulb extracts were found to have potential anti-microbial compound that inhibit the growth of pathogens isolated on tomato fruits at various concentrations (10). Few studies have evaluated natural products *in vivo* as alternative to unsafe synthetic chemical fungicides. Therefore, the present study was conducted to determine the effectiveness of neem and garlic extracts on fungi causing post-harvest spoilage *in vitro* and *in vivo*.

Materials and methods

Collection of garlic bulbs and neem leaves

Neem plants were purchased from nurseries at Babil Governorate, while garlic cloves were purchased from local markets.

Extraction of crude extracts

Weight 60 grams from each powder and placed into a separate, sterile conical flask, and methanol at a volume of 300 ml of was added, making sure the powder was fully submerged in the solvent. The flasks were then vigorously shaken, and the plants were left to stand at room temperature for two days, shaking periodically. A 500 ml conical flask was filled with a sterile funnel, and a Whitman's (No. 2) filter paper was folded and put inside the funnel. The extracts were added to the filter paper gradually and then allowed to seep into the conical flask. The filtrate was poured into a beaker and concentrated by evaporating the beaker in a water bath for 60 minutes at 50°C.

The crude extracts were concentrated and dried in an oven at 40°C for 48 hours until a powdery substance was left at the bottom of the universal bottles (11). The bottles were labeled and stored in a refrigerator at 4°C until needed.

Collection of healthy and infected tomato fruit samples

Samples of healthy and infected tomato fruit were purchased from local nearby Babylon marketplaces. The infected tomato were physically recognized and then selected at random and sealed in polythene bags, and sent to the microbiology laboratory for

isolation. The healthy fruits were ripe, firm, smooth and free of any defects.

Isolation of pathogens from infected tomato samples

Samples of infected tomato were first rinsed in running tap water and then surface sterilized for 3 minutes using 70% ethanol, after that rinsing them in three changes of sterilized distilled water. A sterilized blotting paper was used to blot the fruits dry. A sterile scalpel or scissors burned over the flame of a Bunsen burner and dipped in alcohol was used to cut 5mm x 5mm section of tissue from the tomato moving from the healthy portion to the diseased portion where fungi is likely to be more active. The tissues were dried of the juice using sterile blotting paper. The typical media used to separate the fungus from the fruits was potato dextrose agar (PDA). Before inoculating, 15 ml of previously prepared molten potato Dextrose Agar (PDA), which had been produced in accordance with the manufacturer's instructions, were added to each 9-cm Petri dish and allowed to cool. To reduce bacterial contamination, 0.16 g/L of powdered streptomycin sulphate was added. Cut portion of tomato were aseptically placed on potato dextrose agar in Petri plates and incubated for 4days at room temperature of 25C±10C. The organism was watched for 7 days until the organism became fully grown). Fungi colonies were observed on the plates following incubation (6).

Molecular detection of fungi

The fungal identity was confirmed through molecular identification by extracting fungal genomic DNA using a General DNA extraction kit (Dongsheng Biotech, Korea) per the manufacturer's instructions. The universal fungi species primers, *ITS1* (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (20). Were used. The quality and quantity of DNA were assessed using a 1% agarose gel (w/v) and a NanoDrop Spectrophotometer. Amplification was performed in 25µL reaction volume containing 12.5 Master Mix (2X), 2 µl of each primer, 3.5 Nuclease-Free water and 25-50 ng(5µl) of genomic DNA. The PCR program was as follows: initial denaturation at 94°C for 4 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 60s, extension at 72°C for 30 s, and final extension at 72°C for 7 min. 1.5% agarose gels prepared in 0.5X TBE buffer containing 0.5 µg/ml ethidium bromide were used to verify the PCR products. 5 µl of the PCR products were combined with 1 µl of 6X loading dye, loaded, and electrophoresed for approximately 90 minutes performed with 0.5X TBE buffer. The gels were visualized in a UV transilluminator and the image was taken under UV light (11).

Pathogenicity test

The pathogenicity test was performed according to Okigbo and Emoghene's (12) methods. Samples of healthy tomatoes were procured from the market and transported to the microbiology laboratory. After that, the tomatoes were cleaned under a running tap to remove any remaining dirt. For three minutes, they were surface sterilized in 1% NaoCl. They were then dried with sterile blotting paper and rinsed in three changes of sterile distilled water. To remove the discs, the tomatoes were punched using a sterile 5 mm cork borer. With a 5mm diameter flame-sterilized cork borer, cylindrical cores were removed from each fruit which were then inoculated aseptically with 5mm diameter disc from the

advancing edge of 7-day- old fungal culture. Sterile transparent adhesive tape was used to seal the inoculated tomatoes' wounds. The negative control was similarly configured, except fungal cultures were replaced with sterile PDA. As a treatment, five tomatoes were put in each sterile polythene bag, duplicated three times, and kept in the lab at room temperature (25°C). The course of the disease was monitored after two days.

Findings are documented, and a percentage is calculated Template using the technique (13). On fruit, the fungus is regarded as a disease. If fresh fungi grow out of the perforation and radiate both superiorly and radially from the first inoculated disc, the initial cut on the fruit's surface that led to its decay. Based on this, the degree of pathogenicity is noted. The fungal growth was supposed to be observed on the fruits for 7 days, but the fruits completely rotted on the fifth day. Determination of Rot Severity findings were derived using the methodology of (14). Pathogenicity and growth are categorized as follows: Low (less than 25% of the fruit's surface was covered by mold); Medium (between 25% and 50% of the fruit's surface); High (between 51 and 75%) and very high (covering 75 percent and above). The formula below is used to determine the mold severity percentage.

Mold rate = Diameter of rot covred / Diameter of fruit surface *100

Analyzing damage in tomato fruit using the separated pathogens. According to the current study, fungi were the most harmful pathogen causing fruit rot; by the 5th day of inoculation, the entire fruit crumbled.

***In vitro* antifungal assay**

For assessing the antifungal activity of neem and garlic, the extract concentrations at100 and 200 $mgml^{-1}$ for each, also fungicide cabendazim at concentration of $2gl^{-1}$ as positive control, in addition to negative control (sterile distilled water), all were added into sterilized PDA plates. The crude extracts were weighed individually to obtain 1 and 2 mg of concentrates for neem and garlic, respectively, and 2g Cabendazim, in order to provide the necessary concentrations. 3ml of each of the various extract concentrations were added to 100ml of PDA and then poured into four Petri dishes, each with four duplicates. The procedure was the same in the control experiments except that 3 ml of sterile distilled water was added to PDA instead of plant extracts. The mixture was gently swirled to obtain efficient miscibility of the agar and the extracts, the media was allowed to cool and solidify. A 5mm diameter circle that were one week old was then removed from the edges of the vigorously developing fungal colonies and moved to the middle of the medium of each Petri plate per replicate and allowed to incubate at room temperature. Measurements of the radial growth from each treatment were made every 24 hours to get data on fungal development from the second to the 7^{th} day (15). The inhibitory effect of the treatment on hyphal growth was determined for each concentration using the following formula: % inhibition = (dc- dt)/dc x 100

Where dc = the average increase in control hyphal growth.

dt = the average increase in growth in treated mycelium (16).

***In vivo* antifungal assay**

The surface of the fresh, healthy tomato fruits is sanitized with 70% ethanol and then rinsed with distilled water after being cleaned with tap water. With 6mm diameter sterile cork borer 2cm long cylindrical cores

removed from each fruit, after making holes in each fruit with a long, the fruits are allowed to dry in the open. Pathogenic fungal discs are taken out of the former farm. fungal disks measuring 6 mm. Prior to inoculation the tomato fruits is treated with 100 and 200 $mgml^{-1}$ concentrations of the plant extracts with the exception of the control which treated with ethanol or distilled water only. There is a standard fungicide used, carbendazim2 gl^{-1}. Five tomatoes are used for each treatment, with three replication of each treatment are used in a complete randomized design. The core is put back and the holes created by the drill were covered with sterile clear adhesive tape. The fruits are put in sterile polythene bags, fastened with rubber bands, and allowed at room temperature in darkness. On the second day, the diameter of the diseased tissue (rot) from each of the samples inoculated with the pathogen is measured to estimate the fruit's sensitivity to the disease's growth. Growth in radius is noted post-inoculation. For five days, weight-loss every 24 hours for tomatoes and the inhibition rate are recorded (17). The inhibitory effect of the treatment on fungi growth was determined for each concentration using the following equation(18):

Inhibition percentage % = (DC – DT / DC) x 100

Data analysis

The data collected for various parameters were subjected to ANOVA using the Statistical Analysis System-SAS program according to Completely Randomized Design-CRD with three replicates, least significant difference – LSD-Test was used to significant compare between means at $P \leq 0.05$.

Results and discussion

Identification of pathogens

Based on their morphology, the isolated pathogen microorganism from tomato fruits was determined to be fungi (Figure 1). These features were matched with standards described by Ahmad *et al.* (19). confirm the identity of the isolates as fungi. The fungal isolates were obtained from 15- day – old cultures 1 milliliter of overnight culture for pellet cells was used for DNA extraction. According to the study, tomato fruit post-harvest rot disease is associated to fungi. Tomato fruits in storage have been reported to decay due to fungal infections. This supports other studies that isolated several fungal species from tomato fruits (6, 8, 21). The identification of the isolated fungus was validated by molecular characterisation using PCR amplification and sequencing of the *ITS* region of the genome; Figure (2) shows the results of amplified products of DNA compared to a control DNA with a known number of base pairs. After amplification, 524 base pairs of DNA were present. The unique genetic marker used to identify the fungi was the *ITS* gene. The most accurate way to identify fungal isolates is to identify the *ITS* gene (15, 20, 22). It was determined by morphological examination and PCR detection that the species that was discovered was fungus. It may be necessary to isolate and identify the actual disease pathogens in order to comprehend the progression of the diseases and investigate possible remedies. They were molecularly diagnosed using the appropriate primer. After being extracted from agar plates, the eleven isolates were identified by polymerase chain reaction (PCR) with the set of primers (universal *ITS-1F/ITS-4R*). The amplified DNA

products are shown in (Figure 2). Following amplification, the concentration of DNA was 524 base pairs.Both morphological analysis and PCR detection proved that the 11 isolates were all identified as fungi.

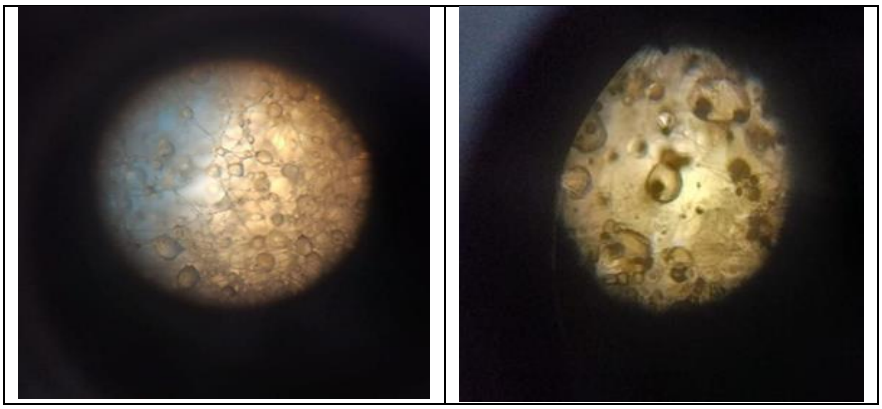

Figure (1): Morphological identification of fungi.

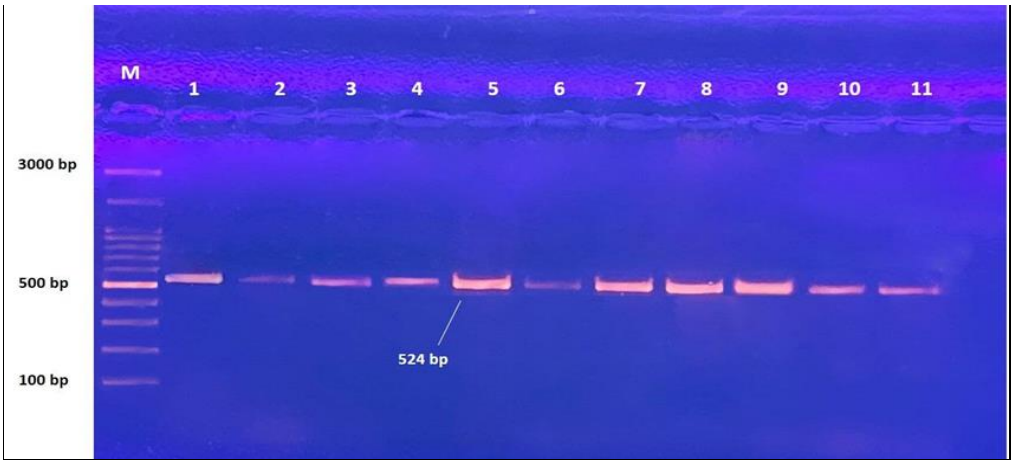

Figure (2): Agarose gel electrophoresis of PCR amplification products of fungi, *ITS* gene (524pb) run at 1.5% agarose, 75 volts for 90 min. M: 100bp ladder marker. Lane's 1-11represent *ITS* positive isolates.

Pathogenicity test

Results of pathogenicity test conducted on tomato using the inoculum collected from fungi shows that the pathogen was able to induce rot in the healthy looking tomato 3 days after inoculation (Table 1). Sunken regions were seen around the inoculation sites 72 hours after the vaccination. The sunken areas had grown into black and white lesions by the fourth day, and the infected portions showed clear evidence of mycelial structures. Healthy tomato fruits that were not inoculated with the test fungus was used as a control (inoculated with pure water) showed no symptoms of rot at the first 3 days. The Koch postulates were satisfied since the fungus could be separated again from the lesions on the contaminated tomato fruit. According to Ansari *et al.* (20), the lesion's morphology of the colony, hyphae, and conidia was the same as that of the inoculated isolates and had been previously described. As a result, the pathogen was verified and the mold's severity was calculated.

Table (1): Pathogenicity of fungal isolates on the fruits of tomato.

Fungal isolates	Days After inoculation				
	1	2	3	4	5
1	-	-	+	++++	++++
2	-	-	-	++	++++
3	-	-	+	++++	++++
4	-	-	++	++++	++++
5	-	-	+++	++	++++
6	-	-	++	++++	++++
7	-	-	++	++++	++++
8	-	-	+	+++	++++
9	-	-	+	++++	++++
10	-	-	+++	++++	++++
Control	-	-	-	+	++

+ = Low ,++ = Medium , +++ = High , ++++ =Very High

By monitoring the virulence of the fungal isolates on the fruits over the course of 5 days, the pathogen was confirmed and the severity of the mold was estimated. On the third day, an average percentage was recorded at 25–50% in isolates 1, 2, 3, 6, 8and 9, while isolates 4,5, 6 and 10 recorded 75% high compared to the control, where no appearance of mold was recorded at 3th day. The severity of the infection increased significantly on the fourth day, and on the fifth day the infection appeared on all tomato fruits inoculated with the fungus, as well as on the fruits of the control treatment, albeit with a less severe degree (Figure b3). Therefore, it was supposed to continue monitoring the severity of the infection until the seventh day, but because all the fruits were infected on the fifth day, the experiment has been terminated.

Figure (3): Severity of the fungi infection on the 5th day.

Studies on pathogenicity showed that the tested fungal isolates were pathogenic to the inoculated tomato that was the subject of this investigation. Tomatoes with wet rot symptoms were seen three days after inoculation. After five days, tomato fruits infected with all of the isolates displayed varied degrees of decaying signs (Table 1). The fruits appeared to be water soaked and had wrinkles from depression. Fruits infected with fungi exhibited lesions that were water soaked and had patches of white to pink mycelia, or they had lesions that resembled lesions (23). Fruit deterioration during storage can be attributed to microorganisms that may have entered

through surface damage from harsh handling, cracks, and inadequate road and transportation infrastructure (24). Villareal (25) asserts that a spoiled tomato fruit could have diseases that could spread and contaminate all the tomatoes in the lot.

In vitro efficacy of plant products on fungal growth

Every tested plant extracts exhibited a significant ($P \leq 0.05$) decrease in the pathogen's mycelia development compared to the control. The mycelial growth of the isolated fungi was significantly inhibited by the methanol extracts of garlic and neem, as well as by their combination. All of the extract concentrations significantly reduced the percentage of mycelial development of the fungus as compared to the control and carbendazim. However the effectiveness differed depending on the concentration employed. At all concentrations, the first three days showed the highest percentage of fungal mycelial growth suppression (Table 2). When compared to control and carbendazim, which recorded 75.33 and 46.33 mm, neem at 100 and 200 mg/ml demonstrated a substantial ($P \leq 0.05$) reduction of radial growth, recording the lowest result of 5 mm at the first 3 days. In terms of radial mycelia reduction, the results likewise showed that garlic, neem, and their combination did not vary statistically. Accordingly, the plant extracts' capacity to successfully control the infection is demonstrated by their stated ability to suppress the growth of fungus in the current investigation. As the incubation time lengthened, the extracts' effectiveness gradually declined from the fourth day until it vanished entirely on the seventh day, at which point the fungus expanded to 100% (Table 2).

Table (2): Radial growth diameter of fungi impacted by different concentrations of plant extracts and chemical fungicide after different periods *in vitro*.

	Period of incubation(day) and radial growth(mm)						
Tretment	**1**	**2**	**3**	**4**	**5**	**6**	**7**
control	20.66	24	75.33	95	100	100	100
100 N	5	5	5	6.6	32	86	100
200 N	5	5	5	10	23	66.3	100
100 G	5	7.33	26	34.66	54	100	100
200 G	5	5	16.66	20.66	34.66	81	100
100N+G	5	5	5	9.33	34.33	68	100
200N+G	5	5	5	12.33	47.66	90	100
Carbendzim	12.33	23	46.33	50.33	100	100	100
LSD value	2.04	3.75	7.62	6.21	20.01	23.91	N.S
***($p \leq 0.05$)**							

In vivo efficacy of plant products on fungal growth

The ability of plant extracts to inhibit fungus on tomato fruit in vivo was assessed by measuring the decrease in decay area, as indicated by lesion diameter, as compared to the control. A significant difference ($P \leq 0.05$) was seen between the garlic and neem treatments in comparison to the control group in the efficacy test. At all concentrations, the studied extracts significantly inhibited the fungi's in vivo mycelial growth (Table 3). The plant extracts were more effective at the beginning of the incubation period compared with the latter periods, as indicated by the mean percentage growth inhibition of two

concentrations (100 and 200 $mgml^{-1}$) of each extract on fungus during the course of the incubation period (Table 3). Regardless of the dose utilised or the duration of the incubation period, all plant extracts were able to inhibit the growth of the tested fungus for that time. Despite this, the extracts' efficacy declined as the incubation time increased. It should be observed, though, that the control treatment's fungal growth entirely encased the fruit's 55 mm diameter (Table 3). The study's findings imply that the neem and garlic crude extracts included anti-fungal substances that were able to inhibit the growth of the fungal infections under investigation.

Table (3): Radial growth diameter of fungi impacted by different concentrations of plant extracts and chemical fungicide after different periods *in vivo*.

	Period of incubation(day) and radial growth(mm)				
Tretment	**1**	**2**	**3**	**4**	**5**
control	13	45	55	55	55
100 N	0	0	8	26	26
200 N	0	0	5	10.3	12.3
100 G	0	4	5.33	12.3	12.3
200 G	0	9.33	13	16.33	19
100N+G	0	3.33	5	8.3	14.33
200N+G	0	1.66	5.33	7	11.33
Carbendzim	5.3	22	50	50	54
LSD value	1.45	6.58	3.02	3.60	2.38
***($p \leq 0.05$)**					

The current study revealed an association between fungi and tomato fruit post-harvest rot diseases. The results demonstrated that, although their effectiveness differed based on the quantities used, all crude plant extract used in the study demonstrated antifungal properties against the pathogens responsible for post-harvest loss that were investigated both *in vitro* and *in vivo*. The results showed that the neem and garlic extracts have anti-bacterial and anti-fungal properties since they were able to stop the test fungi's mycelial growth. However, the antifungal properties of the extracts varied according to the type of plant they were prepared from (26). Numerous chemical compounds that are bioactive in plants are secondary metabolites that serve as chemical messengers and offer defence against various fungal phytopathogens (27).

This study demonstrated that the extracts of neem and garlic as well as synthetic fungicide (Carbendzim), all include fungitoxic chemicals that are toxic and have the ability to control rot pathogens in vivo and suppress the growth of fungi in vitro. Plants have been shown to possess secondary chemicals that have an antimicrobial impact on a variety of fungus. Flavonoids, saponins, terpenoids, cardiac glycosides, tannins, and coumarins were detected in crude extracts of neem and garlic. The primary constituents of several anti-pathogenic substances released by plants, functioning as the primary defence mechanism, are the phenolic compounds (20). These phenolic components denaturated enzymes, which could prevent the amino acids needed for spore germination from occurring (21). In a similar line, a number of scientists have shown that

plant extracts both decreased the severity of the sickness and inhibited the growth of phytopathogenic fungi; this may be because the extracts include secondary metabolites such as phenolic, flavonoids, terpenoids, and alkaloids (28).

As the *in vitro* results, the *in vivo* results were also persuasive, which is not consistent with the results of Wens and Geuens (17) who reported in contrast to the in vitro results, which show great potential for the possibility to use the tested plant extracts as natural fungicides, the in vivo results were less persuasive. No plant extract succeeded in inhibiting the fungal growth of all fungal species under them investigation. The investigated pathogens' development was significantly impacted by garlic extracts. Although the fungus's mycelial growth was inhibited by garlic extracts, the results were not as encouraging as those of neem products. However, spores formed remained viable. But after 7 days of incubation on PDA media, the fungus showed a rise in mycelial development in the presence of both neem and garlic extracts, indicating a gradual reduction in fungitoxicity (Tables 2, 3). This shows that the extract's effects in field settings would likely wear off fairly quickly. This would suggest that the treatment would need to be applied more than once before harvest.

Conclusion

Fungi are responsible for the post-harvest rot of tomatoes, and that fungitoxic extracts from garlic and neem and synergistic effects were very effective in controlling the isolated organisms. It was found that, in comparison to their controls, the extracts' efficacy against the test pathogens differed significantly both in vitro and in vivo. These extracts can be used as an alternative control method without posing a serious risk to human or animal health, and they provide excellent substitutes for synthetic pesticides.

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