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IN VITRO CONTROL OF POST-HARVEST FRUITS ROT PATHOGENIC FUNGI USING *AZADIRACHTA INDICA* (NEEM) SEEDS AND LEAVES EXTRACTS

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Abstract *The present study was carried out to assess the neem seeds and leaves ethanol and aqueous extracts against post-harvest fruits rot pathogenic fungi i.e. Alternarta citrt, Dothiorella dominicana, Lasiodiplodia theobromae, Colletotrichum loeosporioides, Phomopsis caricae-papayae and Colletotrichum musae. The mycelial growth inhibition assay, spore germination, and spore inhibition germination assay in groove slides were determined. The results showed that the neem seed ethanol extracts showed maximum mycelium growth reduction of 88.16% against Colletotrichum musae, while the minimum mycelium growth inhibition was 51.32% against Lasiodiplodia theobromae. The neem leaf extracts maximum and minimum spore inhibition was calculated 66% (ethanol) and 40% (aqueous) against Colletotrichum musae and Lasiodiplodia theobromae respectively. The neem leaf ethanol extracts showed maximum (70%) spores' inhibition germination against Colletotrichum musae and minimum (38%) spores' inhibition germination was observed against Lasiodiplodia theobromae. It was noted that neem seeds and ethanol extracts were the most effective antifungal parts and solvents as compared with leaves and aqueous extract. The suggested that the neem extract many be used for the control of pathogenic fungi species.*

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Keywords: *Plant pathogenic fungi; post-harvest fungal diseases; spore inhibition; colony diameter; mycelial growth inhibition*

Introduction

The most significant plant pathogens are viruses, phytoplasmas, bacteria, and fungi, which cause huge economic losses to plants, crops, and their products. Amongst them, fungi comprise the highly abundant class of plant pathogens and could frequently be sources of harsh diseases in fruits, crops, vegetables, and plants (Change et al., 2008). For more than decades, numerous efforts have been carried out to control, eradicate, or prevent plant diseases and the production of synthetic pesticides was especially in manufacturing (Lee et al., 2009). These insecticides, fungicides, and pesticides are recognized to be extremely efficient in the prevention of different postharvest diseases of plants, fruits, and vegetables. While effectual, their repeated or continued utilization may imbalance the equilibrium of the environment, causing remarkable disease outbreaks, extensive pathogens resistance growth to one or more pesticides, poisonous to non-specific pests, and ecosystem issues (Lee et al., 2009). Occasionally, they mount up as a residue above safe standards in the food chain (Lee et al., 2008). In addition, the residue of pesticides in food poses a higher carcinogenic danger than herbicides and insecticides (Lee et al., 2009). An observed pesticide inefficiency and environmental pollution concern of the present synthetic fungicides as well as a spectacular decline of the saleable ones because of the current European laws and regulations, have pointed out a call to formulate substitute prevention ways or inventive crop defense and postharvest techniques of fruits decay management with decrease application of harmful synthetic fungicides or with no conventional agrochemicals at all (Kim et al., 2003).

Plant origin fungicide research is now being exaggerated, as it has grown to be proved that these materials have huge efficacy to enhance the upcoming agrochemicals technology. In reality, there are good causes to presume that secondary herbal metabolism has naturally been produced to energetically safeguard fruits and vegetable species from the attack of microbial pathogens (Kim et al., 2003). Because secondary botanical metabolites are usually energetic against a small number of particular target pest species and are bi-degradable to harmless products, they are latently beneficiary in integrated pest control systems: Furthermore, they might permitted to prepare a novel group of probably secure pest control compounds. Consequently, research has been spotlighted on plant-based compounds for their efficacy utilization as marketable fungicides or as guide substances (Lee et al., 2001).

The *Azadirachta indica* (neem) have been exercised for centuries in numerous facets for man usefulness,

like wood, soil amendment, heating source, food, an agrochemical pesticide, and important curative means for allegedly over a hundred ailments (Dauda et al., 2015). The extracts of neem possess immunomodulatory, antifungal, antibacterial, antivirus, antiradical, anti-mutagenic, anti-ulcer, anticancer, anti-inflammatory, anti-malaria, and anti-hyperglycaemic and as well as pesticides for farming apply (Khajista 2013). A massive number of bioactive substances are occurred in neem such as aliphatic, tannins, coumarins, dihydrochalcone, glycosides, flavonoids, polyphenols, sulfur, polysaccharides, amino acids, non-isoprenoids, salanin, nimbin, azodirachtin, C-secomeliacins, vilasinin, gedunin, limonoids, promeliacin and azadirone compounds and may more (Biswas et al., 2002). The present study is carried out to investigate the neem seed and leaves aqueous and ethanol extracts against post-harvest plant pathogenic fungi using mycelial growth inhibition assay, spore germination, and spore inhibition germination assay.

Materials and Methods

Collection of Neem Parts and Its Extraction

The leaves and seeds of *Azadirachta indica* (Neem), were obtained within the premises of the Pakistan Council of Scientific and Industrial Research (PCSIR) Laboratories Complex Peshawar-Pakistan. They were dipped in clean drinking H2O to remove dust and any foreign and unwanted particles. The water was decanted and rinsed with clean water and again dipped for five minutes dipping in one percent NaOCl for sterilization. After sterilization the materials were washed five times with running clean and sterile water, decanted the water, spread on a clean stainless steel tray, and kept in a dehydrator (Air Cabinet Dryer –UK) for 24 @45° to obtain complete dry materials. The dry materials were ground individually to obtain powder using Laboratory Blender (Waring® Commercial)- USA. To obtain the extracts we used aqueous and ethanol as solutions, taking hundred grams (100g) of individual materials and adding 300mL of each solvent in a 01L conical flask. The mixtures were energetically vibrated and kept undisturbed for twenty-four hours. Then the mixture was filtered using Whatman® Filter Paper-UK. These practices were repeated three times with fresh solvents to obtain the maximum extractive compounds. Finally, the filtrate was concentrated using a Vacuum Rotary Evaporator (B-490 Heating Bath- Buchi, R-200 Buchi Rotavapor-Switzerland).

Antifungal Assay

Collection and Maintenance of Fungal Stains

The plant pathogenic fungi such as *Alternarta citrt* (*A. citrt*), *Dothiorella dominicana* (D. *dominicana*), *Lasiodiplodia theobromae* (L. *theobromae*), *Colletotrichum loeosporioides* (C. *loeosporioides*), *Phomopsis caricae-papayae* (P. *caricae-papayae*) and *Colletotrichum musae* (C. *musae*) were obtained from Mycotoxin Research Section of PCSIR Peshawar-Pakistan. These fungi were grown and re-activated on Potato Dextrose Agra (PDA) slants and Petri dishes @28°C for seven days in an incubator (Memmert-Germany). After every week the strains were re-strike and cultured following the same procedures. The PDA slants were kept in a cooled incubator (Gallenkamp- England) for storage, and long-term usage in the experimental work of the present study.

Mycelial Inhibition Activities

Growth development inhibition activities were carried out by applying the Food Poison Technique. The pre-sterilized media was kept in the Water bath (Colra-Germany) at 45°C. The neem extract was mixed with liquefied media to gain a 250mg/mL final concentration for each sample. The standardized and uniform blends were dispensed in sterile Petri dishes. The mycelium agar discs of six millimeters from 07 days old fungal culture of tested fungi were kept in each Petri plate middle point. The Petri plates without neem extracts having mycelium discs were considered as negative control. The inoculated and control Petri Plates were kept in an Incubator (Memmert-Germany) for four days at 28 ± 2 °C. The growth of fungi was calculated as the diameter of the fungi colony and the antifungal activities of neem extracts against experimented fungi were considered as mycelium inhibition percent assay using the following Equation (Zohra and Fawzia 2013):

Inhibition of Growth (%) = $[(D_c - D_t)/D_c] \times 100$

Wherever, D_c: Control Colony Diameter (mm), D_t: Treatment Colony Diameter (mm)

Properties of Neem Extracts on Germination and Inhibition of Spore Assay

Spores germination and spore germination inhibition capability neem extracts were carried out using spores suspension 10µL of concentration 10^5 spores/mL of the experimented fungi were blended with neem extracts (10µL=250mg/mL) in a cavity of sterilized eight-cavity slides. Spores in slides remained humid by keeping them on L-natured glass rods on wet paper towels using Petri dishes closed up through Parafilm. The spores' germination was calculated after incubation for eight hours at 24°C and a hundred spores were calculated in every experimental trial. The spores' germination observation was scored whilst the germ tube enlarged to at smallest amount twice the size of the spores itself or a spore is assumed germinated but it observed observable development or extension from the body of the spore such as in fungi a hyphal tip. To produce a negative control in this experiment, distilled water was used in the treatment. The spores' germination percentage was measured using the below formula (Jalal, 2019):

PS (%) = S/B×100

Where, PS (%) = Spore germination (%), S= No. of germinated spores, and B= No. of Observed spores.

Spore germination inhibition (%) was measured by applying the bellow equation (Jalal, 2019):

Inhibition of spores Germination (%) = (C–T)/C×100

Wherever, T= Spores germination in the treatment, C= Spores germination in negative control.

Statistical analysis

The experimental parameters were run in triplicate (n=3), determining mean and standard deviation (SD) using Statistical Package for the Social Sciences (SPSS) software version 21.0 (NY-USA).

Results

The main post-harvest illnesses caused by fungi in literature and previous studies are shown in Table 1. These diseases are very serious and cause a huge loss to the farmers, dealers, and the country. The mode of action of botanicals and bioactive compounds, substances, and molecules reported in the literature are shown in Table 2. Generally, these bioactive substances are present in the study pants parts. Antifungal activities of neem leaf extracts (250mg/mL) against post-harvest fungi are shown in Table 3. The neem leaves aqueous extracts at 250mg/mL concentration shows the mycelium growth inhibition maximum rate of 84.21% was observed against *Colletotrichum musae*, followed by 72.00%, 71.23%, 67.53%, 60.00%, and 51.32% against *Colletotrichum loeosporioides*, *Phomopsis caricae-papayae*, *Alternarta citrt*, *Dothiorella dominicana* and *Lasiodiplodia theobromae* respectively. While the neem leaves ethanol extracts show the mycelium growth inhibition is 86.84% against *Colletotrichum musae*, 78.08% against *Colletotrichum loeosporioides*, 77.00% against *Phomopsis caricae-papayae*, 74.03% against *Alternarta citrt*, 62.00% against *Dothiorella dominicana* and 55.26% against *Lasiodiplodia theobromae.*

Antifungal activities of neem seed Extracts (250mg/mL) against post-harvest fungi are shown in Table 4. The neem seeds' aqueous extract shows significant mycelium growth reduction against the tested post-harvest plants' pathogenic fungi. The results display that a maximum growth reduction is 86.84% against *Colletotrichum musae* and the minimum is 53.95% against *Lasiodiplodia theobromae*. The other values are 74.36%, 73.97%, 70.13 and 64.00% against *Colletotrichum loeosporioides*, *Phomopsis caricae-papayae*, *Alternarta citrt*, and *Dothiorella dominicana* respectively. While the neem seed ethanol extracts show a maximum 88.16% mycelium growth inhibition *Colletotrichum musae* followed by 83.56%, 79.49%, 76.62%, and 66.70% against *Phomopsis caricae-papayae*, *Colletotrichum loeosporioides*, *Alternarta citrt*, and *Dothiorella dominicana* respectively.

The effect of neem leaf extracts (250mg/mL) on spore germination and inhibition is shown in Figure 1. The spore germination inhibition results of neem leaf aqueous extracts showed that the maximum inhibition of 63% was observed against *Colletotrichum musae* and minimum inhibition was 40% recorded against *Lasiodiplodia theobromae.* The moderate results were shown by the rest of the tested post-harvest pathogenic fungi. The neem leaves ethanol extracts have maximum spore germination inhibition at 66% followed by 64% (*Colletotrichum loeosporioides*), 63% (*Phomopsis caricae-papayae*), 58% (*Alternarta citrt*), 56% (*Dothiorella dominicana*) and 45% (*Lasiodiplodia theobromae*).

Effect of neem seeds extracts ((250mg/mL) on spore germination and inhibition are shown in Fig.2. The neem seed aqueous extract showed minimum spore inhibition germination against *Lasiodiplodia theobromae* (38%), followed by *Dothiorella dominicana* (55%), 62% (*Phomopsis caricae-papayae*), 58% (*Alternarta citrt*), 64% (*Colletotrichum loeosporioides*) and 65% (*Colletotrichum musae*). The seeds ethanol extracts followed the same trend but with a high inhibition rate. The results showed that neem seeds are more effective against the tested fungi as compared with leaf extracts. The ethanol solvent proved itself better extraction solvents as compared with aqueous extracts. The ethanol extracts have found more bioactive compounds which showed antifungal activities in contrast with water extracts which have extracted less bioactive molecules which are fungicides in nature.

Table 1. Main post-harvest illnesses caused through fungi (Mohammad & Narendra 2014)

Fungal pathogen	Host fruit	Disease
Alternarta citrt	Citrus	Black centre rot
Dothiorella dominicana	Mango	Stem
Lasiodiplodia theobromae	Mango	Stem
Colletotrichum loeosporioides	Mango	Anthracnose
Alternaria citri	Lemon	Leaf spot of
Alternaria alternata	Apple	Core rot
Monilinia fructicola	Peach	Brown rot
Phomopsis caricae-papayae	Papaya	Phomopsis rot
Penicillium expansum	Peach	Blue mould
Botrytis cinerea	Apple	Gray mold
Colletotrichum musae	Banana	Crown rot
Rhizopus stolonifer	Peach plums	Rhizopus rot
Penicillium spp.,	Grape	Gray mold

Botrytis cinerea	Grape	Gray mold

Table 2. Mode of Action of Botanicals (Parthasarathy *et al*., 2016)

Class	Sub-class	Mechanism
Phenolic acids	Phenolic acids	Inactivate enzymes, bind to adhesins, complex with cell wall
Alkaloids	Alkaloids	Intercalate into cell wall
Coumarins	Coumarins	Interaction with eucaryotic DNA
Phenolics	Simple phenols	Disruption of membrane, substrate deprivation
Lectins and polypeptides	Lectins and polypeptides	Form disulfide bridges
Terpenoids, essential oils	Terpenoids, essential oils	Membrane disruption
Tannins	Tannins	enzyme hang-up, bind to proteins, substrate deprivation

Table 3. Antifungal Activities of Neem Leaves Extracts against Post Harvest Fungi

Fungal pathogens	Aqueous Extracts		Ethanol Extracts	
	Diameter of Colony (mm)	Inhibition (%)	Diameter of Colony (mm)	Inhibition (%)
Alternarta citrt	25±01	67.53	20±00	74.03
Dothiorella dominicana	30±0.1	60.00	28±0.5	62.00
Lasiodiplodia theobromae	37±0.5	51.32	34±0.1	55.26
Colletotrichum loeosporioides	22±0.4	72.00	16±0.2	78.08
Phomopsis caricae-papayae	21±0.5	71.23	18±0.4	77.00
Colletotrichum musae	12±00	84.21	10±00	86.84

Results values are average of three replications (n=3); P<0.01.

Table 4. Antifungal Activities of Neem Seed Extracts (250mg/mL) against Post Harvest Fungi

Fungal pathogens	Aqueous Extracts		Ethanol Extracts	
	Diameter of Colony (mm)	Inhibition (%)	Diameter of Colony (mm)	Inhibition (%)
A. citrt	23±01	70.13	18±0.5	76.62
D. dominicana	27±1.5	64.00	25±0.6	66.70
L. theobromae	35±0.5	53.95	33±01	57.90
C. loeosporioides	20±0.4	74.36	16±0.4	79.49
P. caricae-papayae	19±0.5	73.97	12±0.1	83.56
C. musae	10±0.2	86.84	09±00	88.16
Control	100	00	100	00

Results values are average of three replications (n=3); P<0.01.

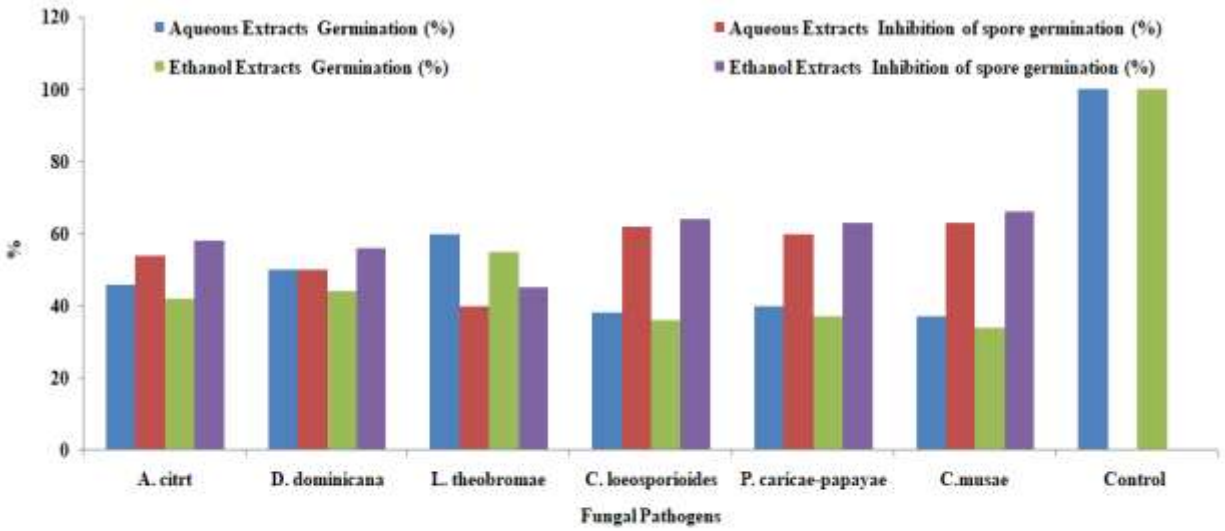

Figure 1. Effect of Neem Leaves Extracts on Spore Germination and Inhibition

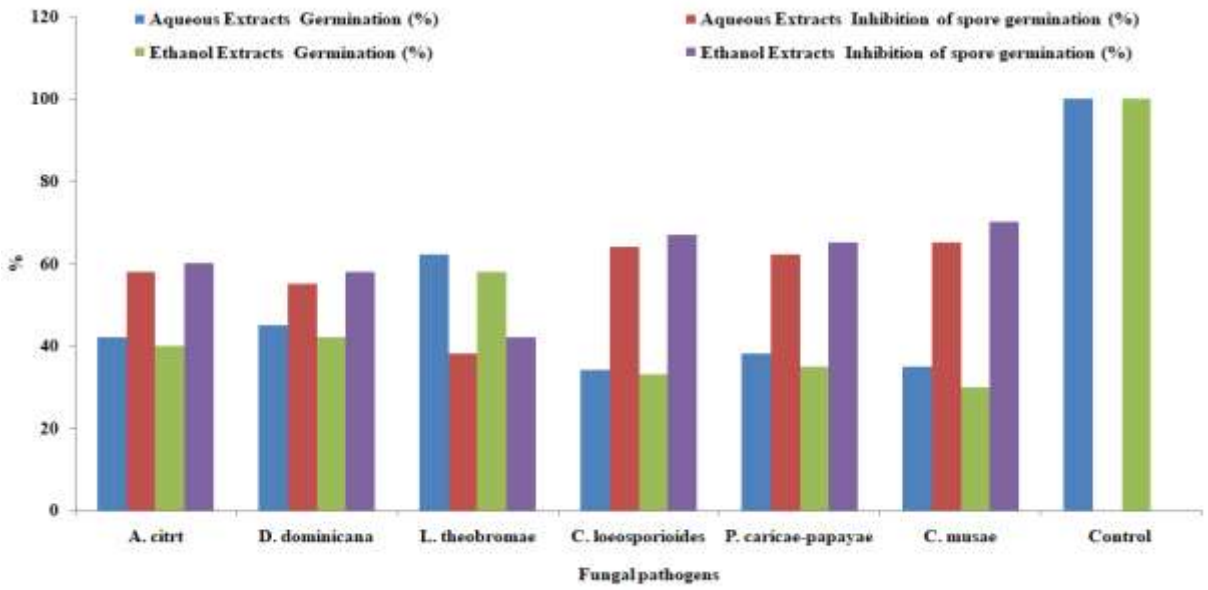

Figure 2. Effect of Neem Seeds Extracts on Spore Germination and Inhibition

Discussion

The current *In-Vitro* study showed that neem seed and leaf extract demonstrated reliable antifungal activities against all the trial fungi. The outcome observed that ethanol and aqueous extract types differ in their efficiency in controlling fungal growth. The ethanol extracts displayed greater absorbance in the in-culture media due to their hydrophilic nature, delivering bioactive chemical agents bioavailable to the tested pathogenic fungi, which symbolized significant properties in the assessment of novel drugs, agrochemicals, and other compounds.

Phytochemicals are plant chemicals with non-nutritive that have defensive or aliments prevention capabilities (Jagessar et al., 2015). There has been a current revival of attention in the application of plant-origin products in agriculture pest management due to the synthetic agrochemicals issues such as environmental pollution, and human and animal health effects (Isman 2008). Botanicals are known to possess antimicrobial activities. Herbs are rich in broad types of phytochemicals that have been involved in their *In-Vitro* antimicrobial activities (Cowan 1999). The phenolic substances have been categorized into three main classes such as tannins, flavonoids, and phenolic acids (Chung et al., 1998). It was reported that various plant species can be utilized as botanicals in the promising natural food production sector in advanced industrial countries and more notably in source-lacking developing countries where customary plants utilized are extensive but deficient scientific underlying principles (Isman 2006). Plants are a wealthy origin of therapeutic substances like flavonoids, alkaloids, saponins, terpenoids, tannins, and other substances observed to possess In-vitro antifungal activities (Arif et al., 2009). It was reported that various types of saponins exhibited antifungal activities (Shimoyamada et al., 1990). Saponins come out to perform by unsettling the fungal cell's membrane integrity (Arif et al., 2009). The fungicidal characteristics of plants could be attributed to the presence of various phenolic and flavonoid compounds (Zidon et al., 2005), which can crack the cytoplasmic covering of the fungi cells and injure the intracellular ingredients (Chen et al., 2003) or they might act together with lipid bilayers or stop the nucleic acid and protein synthesis of the fungi cells (Adetumbe et al., 1986). Fungicidal characteristics are most likely accredited to the occurrence of phytochemicals present in the plants' aqueous, ethanol, and hexane extracts or antifungal natural products (Jagessar et al., 2015). The majority of findings showed that the antifungal activities amplify as the plant extract's polarity rises i.e. alcohol extracts are more potent as compared with hexane extract (Jagessar et al., 2015). Conversely, this is not for all time because various molecules could be more potent in the hexane extract than they found in the extracts of ethanol (Jagessar et al., 2015).

The antifungal activities have been accredited to the occurrence of phenolic molecules in the plant extracts that could function to denature spore germination-involved enzymes or block the amino acids connected to the process of germination (Nychas, 1995). Further study showed that the phenolic compounds production augmentation in plant extracts could be connected with the stimulation of antagonism in treated herbs against plant pathogenic fungi (Hussin et al., 2009). It was described that various classes of flavonoids exhibit antibacterial, antiviral, and antifungal properties (Cushnie and Lamd 2005). The phenomenon considered to be accountable for phenolic compound's poisonous properties towards microbes comprised inhibition of enzymes through oxidized molecules; might be in the course of reaction with a group of sulfhydryl or utilizing more

nonspecific contacts with proteins (Arif et al., 2009). The total phenolic content (g GAE/100gdw) of neem stem bark was calculated in methanol (3.77), ethanol (7.47), ethyl acetate (13.5), and acetone (15.10); while flavonoid contents (gQE/100G) in methanol (5.14), acetone (5.15), ethyl acetate (5.23) and ethanol (8.70) (Anokwuru et al., 2011).

The herbal extracts derived from ethanol, its fractions, essential oils, and resins have been observed to exhibit antifungal activities and displayed capabilities to manage the plant pathogenic fungi (Maria et al., 2016). These extraction methods comprise easy and simple extraction techniques with little manufacturing (Maria et al., 2016), and have the ability for scientific and technical development effortlessly put into practice in agrochemicals industries. Of the neem leaves aqueous extracts 80% showed more mycelial growth inhibition % in contrast to 60% aqueous extracts (Ijato et al., 2010). While the ethanol extract of 30% showed greater mycelia growth inhibition as weighed against to aqueous extract of neem leaves (Ijato et al., 2010). In the current study, we noted that the neem ethanol extracts were more antifungal as compared with aqueous extracts and seed showed a high inhibitory effect as compared with leaves. The neem leaves aqueous extracts (80%) exhibited mycelia growth inhibition against tomato rot fungi such as *Aspergillus niger*, *Fusarium oxysporum*, *Rhizopus stolonifer*, and *Geotrichum candidum* were 60.0%, 62.50%, 65.20% and 59.20% respectively (Ijato et al., 2010).

The neem leaves ethanol extracts of 30% were found to have more inhibitory effect against Aspergillus *niger*, *Fusarium oxysporum*, *Rhizopus stolonifer*, and *Geotrichum candidum* were 83.30%, 79.80%, 83.60%, and 80.20% respectively (Ijato et al., 2010). Seed oil extract of neem, aqueous, and ethanol extracts of neem considerably decreased the *Pyricularia oryzae radial growth In-vitro and the growth and expansion of rice blast disease in the greenhouse.* The best control of pathogens and subsequent disease was exhibited by oil extracts, followed by ethanol extracts, cooled aqueous, and after that hot H_2O extracts (Amadioha, 1999). In the current study, we also found that the seed extracts are more effective against the tested fungi as compared to the leaf extracts. The neem oil at 10% concentration showed 100% growth control against *Macrophomina phaseolina, Drechslera rostrata, Aspergillus niger*, and *Fusarium moniliforme* (Vir and Sharma 1985). Similarly, neem oil (2-10%) completely holds up the expansion of *Alternaria alternata, Aspergillus niger*, and *Fusarium oxysporum* (Locke, 1995). The neem oil displayed remarkable antifungal activities against Aspergillus species (Niaz and Kazim 2005). The pesticidal properties own the plant commonly possess the secondary metabolite classless as revealed in the *Azadirachta indica* aqueous extracts (Kosma et al., 2011). The neem stem bark extracts at 15%, 105 and 5% concentrations on Aspergillus flavus a causative agent of decay in fruits of tomato observed mycelia % reduction were 34.77, 29.22, and 16.88 respectively (Chigoziri et al., 2017). The neem extracts showed antifungal activities against *Fusarium* spp. and *Curvularia lunata* on wheat seeds (Khan and Kumar 1992). The neem extracts accredited the mycelia growth inhibition of Aspergillus flavus to the occurrence of Azadirachtin in the neem extract (Okwute, 1992; SaiRam et al., 2000). Neem leaf extracts displayed the inhibition effect on seed-borne fungal strains Rhizopus and Aspergillus (Mondall et al., 2009). The neem leaves and seeds extracts had 100% inhibitory effects, while the neem leaves aqueous extracts showed 87.60% inhibition effects against *Fusarium oxysporum*. The seeds and leaves ethanol extracts showed the highest antifungal activities (*Fusarium oxysporum* and Pythium spp), while the leaves aqueous extracts exhibited the minimum inhibition against Fusarium oxysporum (Ezeonu et al., 2019).

Water extracts of numerous neem plant parts illustrated reduction effects against fungi spore germination (Farag et al., 2011). Neem aqueous leaf extract revealed noteworthy control of the causative agent (*Fusarium oxysporum*) of banana development disease (Singh et al., 1993). Moreover, various scientists reported that the utilization of some herbal extracts could stimulate systemic resistance in various plants in the course of a buildup of pathogenesis like proteins-PR proteins (Sateesh et al., 2004). Some plant treatments with aqueous extracts of neem granted control of many fungi diseases in the course of metabolic alteration in plants comprising stimulation of enzymes of phenol biosynthesis, phenol accumulation, and antioxidant defensive enzymes (Farag et al., 2011). The variation in the bio-ingredients of the chemical compositions of herbs extracts such as plant's secondary metabolites, yet those collected from similar species, might outcome in variant responses, particularly with a look upon the potential for microbe's embarrassment. Other liked features are the type of microorganisms under experiments, diffusion properties in the growth medium, volatility, pH, and solubility (Maria et al., 2016). Certain herbs phytochemicals and extracts perform in various means on numerous kinds of aliments composite and might be utilized in agriculture and food industries in the way as other similar synthetic pesticides. The preparation of natural fungal pesticides and antimicrobials could assist in reducing the harmful impact of artificial compounds, like environmental pollution, resistance, and residue issues. In this scenario, organic, plant-origin, and bio-based fungicides may be effectual, less toxic to food, agriculture industries, and the environment, biodegradable, and selective.

Conclusion

The current research study is one of the leading antifungal activities of neem extracts against tested plant pathogenic fungi. These findings are distinctive

and achieve important reflection as to innovate and formulate a marketable "phyto-fungicide" reserve against highly toxic (environment and human) and banned synthetic fungicides. Further studies are suggested to scale up the extraction process more easily, optimize the extraction process, describe the controlling, molecular mechanisms, and isolate the controlling components in neem and filed study.

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Authors' Contributions

JA designed the study and collected the literature, AH performed the statistical analysis, wrote the protocol, A.S wrote the first draft of the manuscript, JA, and JA has done the analyses of the study and proofread the manuscript draft. All authors read and approved the final manuscript.

Conflict of Interest

The authors have declared no conflict of interest.

Declaration of Interest Statement

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work.

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