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Evaluation of some bioactive compounds of *Azadirachta indica* extracts and its application as safe fungicide against different plant pathogenic fungi

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Abstract

Extracts of leaves, seeds and twigs of *Azadirachta indica* was carried out using Soxhlet extraction technique. These extracts were subjected to percent yield, in vitro phytochemical screening and antifungal activities (minimum fungicidal concentration (MFC), minimum inhibitory concentration (MIC), zone of inhibition and spore germination assay) against plant pathogenic fungi *Cladosporium fulvum*, *Colletotrichum coccodes*, *Fusarium oxysporum* and *Rhizoctonia solani*. The Soxhlet extractive solvents were methanol, ethanol, chloroform, ethyl acetate and n–hexane. The maximum extractive yield were calculated in ethanol leaves (26±1.5%), methanol leaves (22±01%), n–hexane seeds (32±0.8%) and ethanol seeds (24±01%), while twigs calculated minimum extractive yield. The phytochemicals screening findings confirmed the occurrence of terpenoids, phenols, flavonoids, glycosides, alkaloids, tannins and saponins. The antifungal activities observed that leaves and seeds extracts of neem might cause the inhibition of mycelium and spore germination of tested fungi, though the antifungal activities rate of under study fungal strains diverse with dissimilar concentration and nature of extracts. However the entire concentrations of extract and crude extract especially the methanol and ethanol extracts of neem seeds and leaves reduce the plant pathogenic fungi growth at noteworthy rate. It was concluded from our results that neem leaves and seeds extracts were effectual as antifungal agents against the entire under study fungal strains however the most sensitive fungi were *Rhizoctonia solani* and *Fusarium oxysporum.* The findings acquired from this study revealed that chemical constituents from neem seeds and leaves may be formulated as latent agrochemical fungicides.

Keywords: Neem, phytochemicals, extraction techniques, antifungal potential, spore germination

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1. Introduction

The plant pathogenic fungi are responsible for intensive damages to plants, crops, fruits, vegetables and seeds. It is need of the day to adopt measurable methods which are environmentally safe and cheap to reduce or eliminate the plant pathogenic fungi. A chain of compounds with fungicidal properties have been originated from herbs, which are of immense significance to plants and human. Herbs are wealthy resource of biological active phytochemicals like flavonoids, alkaloids, terpenoids and tannins, which have confirmed in vitro antifungal abilities (Jeyasakthy *et al*., 2013).

The conventional application of utilizing herbs to manage the plant pathogenic fungi has grown concentration and presently the attention is on the discovery of innovative anti-fungi agents from herbs that have ecofriendly, no harm effects on human and animal health (Saha *et al*., 2005). The rigorous crop farming stresses the consumption of tough synthetic fungicides, which are hazardous for all living beings but also expensive. The explorations for non-toxic antifungal agents that do not have an environmental crash and can be concerned in sustainable agriculture are inquiry (Sheema and Durai 2015).

The *Azadirachta indica* are commonly practices in food, cosmetics, medicine, public health, agriculture and many more. Its flower, seed, bark, stem and leaves are liver tonic, expectorant, insecticidal, demulcent, refrigerant, depurative, acid, astringent, bitter properties etc. (Ijato *et al*., 2010). The current study was designed to evaluate and compare the percent extractive yield, minimum fungicidal concentration (MFC), minimum inhibitory concentration (MIC), zone of inhibition and spore germination assay of sequentially extracts of seeds, twigs and leaves of *Azadirachta indica* extracts, against plant pathogenic fungi such as *Cladosporium fulvum*, *Colletotrichum coccodes*, *Fusarium oxysporum* and *Rhizoctonia solani* and to clarify the existing bioactive compounds in the test extracts.

2. Materials and Methods

2.1 Azadirachta indica Parts Procurement

Plant materials of *Azadirachta indica* leaves, twigs and seeds were collected from Board Bazar Peshawar, Khyber Pakhtunkhwa-Pakistan. These herb materials were transport to Food Microbiology Research Section of Pakistan Council of Scientific and Industrial Research (PCSIR) Laboratories Complex Peshawar, Khyber Pakhtunkhwa-Pakistan. The leaves, seeds and twigs were carefully rinsed with H_2O and using sodium hypochlorite 1% solution for surface sterilization and then washed with H_2O. The washed processed materials were kept for drying in an Air Cabinet Dryer (England) at 40 °C and ground to form powder with the help of Standard Model No.3 Wiley Mill USA. The ground materials were packed in brown plastic bottles for further for experimental works.

2.2 Soxhlet Extraction

Thirty gram (30 g) neem leaves, seeds and stem bark powder take individually and soaked in methanol solvent (300 mL) using Soxhlet extractor apparatus (Quickfit- England). In a thimble-holder neem leaves, seeds and twigs powder materials individually kept and filled with clean methanol solvent. When the solvent arrive at the spillover point, a siphon aspirates the mixture solvent of the thimble-holder and reloads it reverses into the distillation flask, bring extracted solutes into the mass solvent. Using distillation the solute is detached from the solvent in the solvent flask. In the flask solute is left and the new solvent goes by on the neem solid bed. Approximately twelve hours is required for the complete operation extraction process. Following the extraction, it was filtered and using a Rotary Vacuum Evaporator (R-200 Buchi Rotavapor-Switzerland, B-490 Heating Bath- Buchi) to completely evaporate the solvent. The similar procedure was applied against the leftovers solvents.

2.3 Determination of Extractive % Values

The Soxhlet extractions were concentrated on a rotary evaporator (R-200 Buchi Rotavapor-Switzerland, B-490 Heating Bath-Buchi) under control pressure and temperature between 30-45°C. The semisolid extract was taken and weighed in a china dish and kept in a water bath at about 45 ºC and then dried the extract. The below formula was used to determined the yield percentage of the extract (Samuel *et al*., 2021).

Yield Percentage = Extract Weight/Plant Material Weight X 100

Qualitative assessment of phyto-compounds

The basic phytochemicals qualitative determinations were examined by the following authentic standards procedures (Khanal, 2021).

2.4 Test Fungi

The fungal pathogens, *Cladosporium fulvum*, *Colletotrichum coccodes*, *Fusarium oxysporum* and *Rhizoctonia solani* were obtained from Mycotoxin Research Section of PCSIR Laboratories Complex Peshawar, Khyber Pakhtunkhwa-Pakistan. These fungal cultures were purified on Potato Dextrose Agar (PDA) by sub-culturing technique and were preserved as slants at 10 °C in a cooled incubator (Gallenkamp-England) for experimental works.

2.5 Plant Crude Extracts Antifungal Assay

Aliquot of 100µL spore suspension ($1x10^8$cfu/mL) of each plant pathogenic fungi were in radial patterns streaked on the PDA plates. Using 4µm Millipore filter each crude extract stock solutions were filtered and sterilized. Six millimeters diameter agar wells were punched on a solidified PDA media. The neem crude extracts (500 mg/mL) of each tested extract was prepared in Dimethyl sulfoxide (DMSO) and poured in the each designated well. The negative and positive controls were used as DMSO and Mancozeb (15.6 mg/mL) respectively. The inoculated Petri plates were kept in an incubator (Memmert-Germany) at 25°C for five days. The inhibition zone radius was calculated using Vernier Caliper (Emad *et al*., 2012).

2.6 Measurements of MIC and MFC

The MIC and MFC assayed by broth macrodilution technique in 10 mL test tubes. Experiments were performed in Potato Dextrose Broth (PDB), Oxoid, Hampshire- United Kingdom to determine MIC. The plant extracts were added at concentrations of 1000, 500, 250, 125, 62.5, 31.25 and 15.625 mg/mL dissolved with 10% DMSO in the PDB. Control treatment Mancozeb (3.9, 7.8, 15.625, 31.25 mg/mL) were prepared in the same manner but without adding the tested extracts. The spore suspension (10^6

spores/mL) 100 µL in all the test tubes were cultured, mixed thoroughly and kept in an incubator (Memmert-Germany) at 25 °C for ten days. MIC was quantified as the minimum dosage of the added extracts, which was capable to restrain totally the observable expansion of the tested fungal strains. To assess MFC, 100 µL of the medium of each trial in which fungal growth was not observed was sub-growth on PDA Petri dishes and kept in an incubator (Memmert-Germany) for seven days at 25°C. The MFC was considered as the least amount has zero growth scrutinize on the plates (Loran *et al*., 2022).

2.7 Percent Spore Germination

Approximately thirty spores per microscopic field of each fungus spore suspension were prepared from 10 days old fungal stains. About 0.1mL conidia suspension of one drop was kept in a glass slid cavity holding of neem crud extracts with numerous concentration i.e. 1000, 500, 250, 125, 62.5 and 31.25 mg/mL. While the positive control 5 mg/ mL and negative control were also treated as the plant extracts. The humid chamber was prepared by keeping filter paper 2 fold in both sides of the Petri plates. These plates were kept in an incubator (Memmert-Germany) for 24 hrs at 24±2 °C for 24 hr. Spore germination percentage were calculated according to the given formula (Taskeen *et al*., 2011).

Spore Germination % = Spore germinated numbers/ Spore under Examination X 100

3. Statistical Analysis

All the experimental data were analyzed in triplicate, average, standard error of mean (SEM) and standard deviation (SD) for each analytes/tests was carry out on mean/average basis to get out the significance at $P< 0.05$.

4. Results

Herbs were synthesized miscellaneous level of therapeutic molecules, make them a wealthy resources of many kinds of products for the welfare of human beings. The selection of solvent system and plants parts for the maximum extraction yield is very important. In the current study the neem leaves, seeds and twigs were grounded and extracted in the Soxhlet apparatus with ethyl acetate, ethanol, n-hexane and methanol. Soxhlet extractive yield values (%) of *Azadirachta indica* is shown in Table 1.

Table 1. Soxhlet Extractive Yield Values (%) of *Azadirachta indica*.

Solvents	Seed	Leaves	Twigs
Methanol	09±0	22±01	08±0
n-Hexane	32±0.8	16±01	03±0.6
Ethanol	24±01	26±1.5	07±0
Ethyl acetate	18±01	18±01	04±0

Results are expressed as an average ± standard deviation (SD) with triplicate measurement (n= 3) and considered to be significantly different at $P< 0.05$.

The results show that the neem parts (30g) and solvent system (300 mL) have shown dissimilar yields. Generally, the twigs had the lowest, seeds had the moderate and leaves had the highest extract yields. Methanol and ethanol showed the best solvent for yield extraction, while n-hexane observed highest yield values for the seeds.

The phytochemicals characteristics (qualitative analysis) of the neem leaves, seeds and twigs extracts tested were summarized in Table 2.

Table 2. Phytochemicals Analysis of *Azadirachta indica* Soxhlet Extracts.

Phytochemicals	Parts	Ethyl acetate	Ethanol	n-Hexane	Methanol
Saponins	Seed	+	++	+	+++
	Leaf	+	++	++	+++
	Twig	-	+	-	+
Tannins	Seed	+	++	-	++
	Leaf	+	+++	++	+++
	Twig	-	-	-	+
Alkaloids	Seed	+	++	+	+++
	Leaf	+	+++	++	+++
	Twig	-	+	-	+
Glycosids	Seed	+	++	+	++
	Leaf	+	++	++	++
	Twig	-	-	-	+

Table 2(cont'd). Phytochemicals Analysis of *Azadirachta indica* Soxhlet Extracts.

Phytochemicals	Parts	Ethyl acetate	Ethanol	n-Hexane	Methanol
Flavonoids	Seed	+	++	+	+++
	Leaf	++	++	++	+++
	Twig	-	+	-	+
Phenols	Seed	+	+++	+	+++
	Leaf	++	+++	++	+++
	Twig	-	+	-	+
Terpenoids	Seed	+	++	++	++
	Leaf	++	++	++	++
	Twig	-	-	+	+

Explanation: High Concentration (+++); Moderate Concentration (++); Low Concentration (+); Absent (-).

The results exposed the occurrence of high concentration of bioactive compounds in the leaves, followed by seeds and twigs extracts examined. From Table 2, it could be noticed that methanol observed best solvent followed by ethanol, ethyl acetate and n-hexane in the examined parts. Table 3 is shown the antifungal activity (Zone of Inhibition) of *Azadirachta indica* crude extracts.

Table 3. Antifungal Activity (Zone of Inhibition) of *Azadirachta indica* Extract.

Solvent System	Neem Parts	Zone of Inhibition in millimeters (mm)			
		Cladosporium fulvum	***Colletotrichum coccodes***	***Fusarium oxysporum***	***Rhizoctonia solani***
Methanol	Seeds	19±1.3	21±01	19±00	20±1.5
	Leaves	21±01	22±01	24±01	23±00
	Twigs	00	11±00	13±00	09±10
n-Hexane	Seeds	20±01	18±01	20±02	19±01
	Leaves	14±1.5	12±1.5	15±00	13±00
	Twigs	00	00	00	00
Ethanol	Seeds	14±0.1	17±1.5	15±0.2	16±01
	Leaves	18±1.5	20±0.6	19±1.5	21±02
	Twigs	00	00	00	00
Ethyl acetate	Seeds	08±01	12±0.4	10±01	13±0.8
	Leaves	14±00	11±0.6	15±01	12±01
	Twigs	00	00	00	00
Positive Control (Mancozeb)		25±01	26±00	22±01	27±02
Negative Control (DMSO)		00	00	00	00

Results are expressed in an average ± Standard Deviation (SD) in triplicate (n=3) and measured considerably different level at $P< 0.05$.

The maximum zone of inhibition 24±01 mm, 23±00 mm and 22±01 mm are found against *Fusarium oxysporum*, *Rhizoctonia solani* and *Colletotrichum coccodes* respectively by methanol leaves extract. All twigs extracts have found no zone of inhibition against all the tested fungi, except the methanol extracts which have found 13±00 mm, 11±00 mm and 09±10 mm zone of inhibition against *Fusarium oxysporum*, *Colletotrichum coccodes* and *Rhizoctonia solani* respectively. Generally the methanol extracts have observed efficient extracts followed by ethanol, n-hexane and ethyl acetate. While in some cases the n-hexane seed extracts have calculated maximum zone of inhibition as compared to the extracts except methanol. Neem leaves shown maximum zone of inhibition as a whole followed by seeds and twigs. The negative control has no zone inhibition, whereas positive control observed maximum zone of inhibition as compared to all extracts. The most sensitive fungi to the extracts were *Rhizoctonia solani* and *Fusarium oxysporum*.

The antifungal activities in terms of MIC and MFC of *Azadirachta indica* extracts are shown in Figure 1. The MIC range of methanol seeds extracts are 62.5-250 mg/mL and methanol leaves extracts are 31.25 mg/mL. The n-hexane seeds extracts MIC range are 62.5-125 mg/mL and n-hexane leaves extracts are 250-500 mg/mL. The ethanol extract of seeds MIC range are 125-500 mg/mL and leaves 62.5-125 mg/mL. The ethyl acetate extracts of seed and leaves MIC range are 500-1000 mg/mL. The positive controls MIC and MFC are 7.8 and 15.6 mg/mL respectively against all tested pathogenic plant fungi. The methanol seed extracts MFC are 250 mg/mL against *Cladosporium fulvum*, *Fusarium oxysporum and Rhizoctonia solani* and 125 mg/mL is observed against *Colletotrichum coccodes.* The MFC of methanol leaves extracts are 125 mg/mL against *Cladosporium fulvum* and *Colletotrichum coccodes* while 62.5 mg/mL MFC are observed against *Fusarium oxysporum and Rhizoctonia solani.* The n-

hexane seeds and leaves extracts MFC are found in the range of 250-500 mg/mL. The ethanol seeds and leaves extracts MFC ranges are 62.5-500 mg/mL. While ethyl acetate seeds and leaves MFC is 1000 mg/mL.

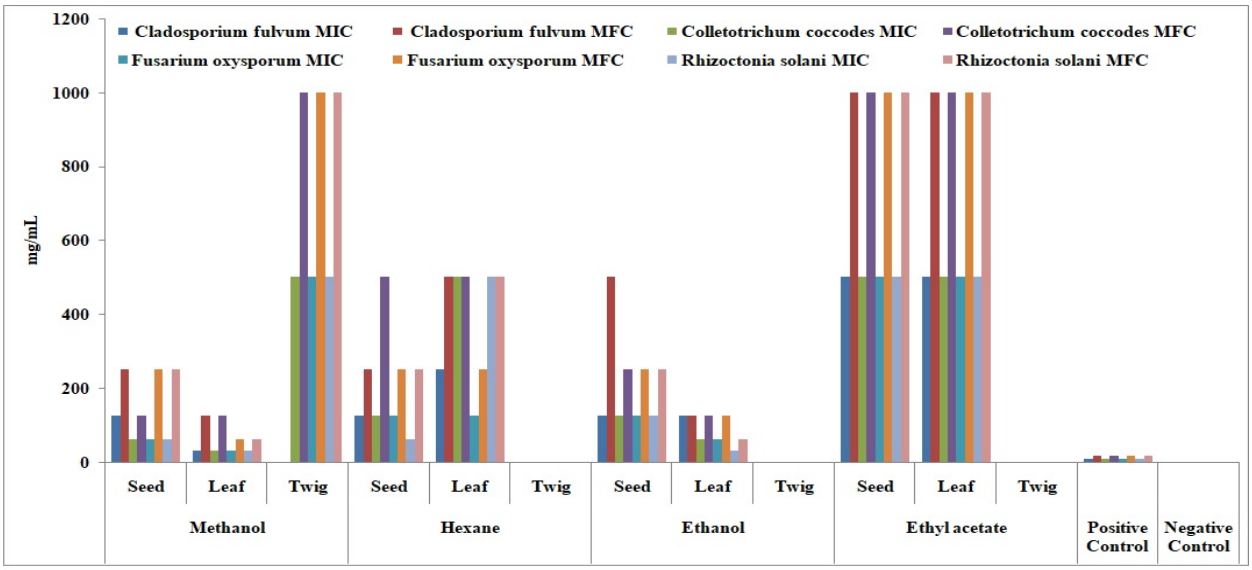

Fig. 1. Antifungal Activity (MIC and MFC) of *Azadirachta indica* Extract.

Neem crude extracts effect on spore germination: In the present work, concentrations 1000, 500, 250, 125, 62.5 and 31.25 mg/mL effects of extracts from neem leaves, seeds and twigs of methanol, ethanol, n-hexane and ethyl acetate were studied on spore germination of *Cladosporium fulvum*, *Colletotrichum coccodes*, *Fusarium oxysporum and Rhizoctonia solani* after 24 hrs treatments. The average comparison showed the germination of spores generally decreased as the extracts concentration increases and the highest inhibition usually achieved at highest concentration of extracts, but depend on the neem parts, doses and pathogenic fungi.

Percentage Conidial Germination of *Cladosporium fulvum* is shown in Table 4.

Table 4. Percentage Conidial Germination of *Cladosporium fulvum*.

Plant Parts	Extraction System	% Germination of Conidia					
		Concentration (mg/ mL)					
		31.25	62.5	125	250	500	1000
Seed	Methanol	60.0±01	40.0±0	20±0	0.0	0.0	0.0
	n-Hexane	53.3±1.5	33.3±0.5	10±0	0.0	0.0	0.0
	Ethanol	66.7±01	46.7±0.8	23.3±01	0.0	0.0	0.0
	Ethyl acetate	80.0±01	60±02	40±01	30±0	10±0	0.0
Leaves	Methanol	36.7±1.5	6.7±0.4	0.0	0.0	0.0	0.0
	n-Hexane	70.0±01	43.3±1.5	23.3±01	16.7±0	0.0	0.0
	Ethanol	46.7±01	13.3±01	0.0	0.0	0.0	0.0
	Ethyl acetate	73.3±.5	53.3±01	36.7±01	23.3±1.5	13.3±0	0.0
Twigs	Methanol	100±0	100±0	100±0	100±0	100±0	100±0
	n-Hexane	100±0	100±0	100±0	100±0	100±0	100±0
	Ethanol	100±0	100±0	100±0	100±0	100±0	100±0
	Ethyl acetate	100±0	100±0	100±0	100±0	100±0	100±0
Positive control		0.0					
Negative control		30±0					

Results are mean ± standard error of the mean (SEM). Negative control = without extract added (blank/distilled water); Positive control = Diflucan (n = 3).

The seed extracts of methanol, n-hexane and ethanol observe no percent spore germination at 250 mg/mL and ethyl acetate have no spore germination at 1000 mg/mL. The leaves extract of methanol, n-hexane, ethanol and ethyl acetate shows nil spore germination at 125, 500, 125 and 1000 mg/mL respectively.

Percentage Conidial Germination of *Colletotrichum coccodes* is shown in Table 5.

Table 5. Percentage Conidial Germination of *Colletotrichum coccodes*.

Plant Parts	Extraction System	% Germination of Conidia					
		Concentration (mg/ mL)					
		31.25	62.5	125	250	500	1000
Seed	Methanol	20±0	6.7±0	0.0	0.0	0.0	0.0
	n-Hexane	33.3±01	13.0±1.5	3.3±0	0.0	0.0	0.0
	Ethanol	40±01	26.7±01	10 ±0	0.0	0.0	0.0
	Ethyl acetate	76.7±1.5	63.3±01	50.0±02	40.0±01	16.7±01	0.0
Leaves	Methanol	23.3±01	10.0±0	0.0	0.0	0.0	0.0
	n-Hexane	30.0±01	16.7±0	10±0	0.0	0.0	0.0
	Ethanol	40.0±01	20.0±0	0.0	0.0	0.0	0.0
	Ethyl acetate	73.3±01	43.3±01	33.3±0	13.3±0	0.0	0.0
Twigs	Methanol	100±0	83.3±	60.0±	56.3±	53.3±	0.0
	n-Hexane	100±0	100±0	100±0	100±0	100±0	100±0
	Ethanol	100±0	100±0	100±0	100±0	100±0	100±0
	Ethyl acetate	100±0	100±0	100±0	100±0	100±0	100±0
Positive control		0.0					
Negative control		30±01					

Results are mean ± standard error of the mean (SEM). Negative control = without extract added (blank/distilled water); Positive control = Diflucan (n = 3).

The seeds extracts of ethanol, methanol, n-hexane, and ethyl acetate examine no germination of conidia at 250, 125, 250 and 1000 mg/mL concentration. Similar results are shown for leaves extracts except ethanol and ethyl acetate which shown no germination at 125 and 500 mg/mL concentration.

Percentage Conidial Germination of *Fusarium oxysporum* is shown in Table 6.

Table 6. Percentage Conidial Germination of *Fusarium oxysporum*.

Plant Parts	Extraction System	% Germination of Conidia					
		Concentration (mg/ mL)					
		31.25	62.5	125	250	500	1000
Seed	Methanol	20±0	10±0	0.0	0.0	0.0	0.0
	n-Hexane	43.3±0	36.7±0	0.0	0.0	0.0	0.0
	Ethanol	63.3±01	33.3±1.5	0.0	0.0	0.0	0.0
	Ethyl acetate	73.3±01	63.3±01	36.7±1.5	20±0.5	3.3±0.2	0.0
Leaves	Methanol	33.3±0.5	0.0	0.0	0.0	0.0	0.0
	n-Hexane	66.7±01	46.7±01	30±0	0.0	0.0	0.0
	Ethanol	40.0±1.5	36.7±1.5	0.0	0.0	0.0	0.0
	Ethyl acetate	66.7±01	56.7±01	33.3±01	13.3±0	0.0	0.0
Twigs	Methanol	83.3±01	60.0±01	56.3±01	36.7±1.5	10±0	0.0
	n-Hexane	100±0	100±0	100±0	100±0	100±0	100±0
	Ethanol	100±0	100±0	100±0	100±0	100±0	100±0
	Ethyl acetate	100±0	100±0	100±0	100±0	100±0	100±0
Positive control		0.0					
Negative control		30±1.5					

Results are mean ± standard error of the mean (SEM). Negative control = without extract added (blank/distilled water); Positive control = Diflucan (n = 3).

The seeds methanol, ethanol and n-hexane extracts count nil percent germination at 125 mg/mL and ethyl acetate shows less inhibit the germination and found zero percent germination at 1000 mg/mL. The leaves methanol shows excellent germination inhibition (0.0) at 62.5 mg/mL, for n-hexane, ethanol and ethyl acetate no spores are germinated at 250 mg/mL, 125 mg/mL and 500 mg/mL respectively.

Percentage Conidial Germination of *Rhizoctonia solani* is shown in Table 7.

Table 7. Percentage Conidial Germination of *Rhizoctonia solani*.

Plant Parts	Extraction System	% Germination of Conidia					
		Concentration (mg/ mL)					
		31.25	62.5	125	250	500	1000
Seed	Methanol	23.3±0.6	6.7±0	0.0	0.0	0.0	0.0
	n-Hexane	36.7±01	30.0±01	0.0	0.0	0.0	0.0
	Ethanol	56.7±01	40.0±0.8	26.7±0.4	0.0	0.0	0.0
	Ethyl acetate	70.0±02	60.0±01	50.0±01	40±0	23.3±0	0.0
Leaves	Methanol	16.7±0	0.0	0.0	0.0	0.0	0.0
	n-Hexane	40.0±0	13.3±0	16.7±0	0.0	0.0	0.0
	Ethanol	20±.8	0.0	0.0	0.0	0.0	0.0
	Ethyl acetate	66.7±01	60.0±01	46.7±0	30.0±0	13.3±0	0.0
Twigs	Methanol	76.7±02	60.0±01	53.3±1.5	23.3±0	10±0	0.0
	n-Hexane	100±0	100±0	100±0	100±0	100±0	100±0
	Ethanol	100±0	100±0	100±0	100±0	100±0	100±0
	Ethyl acetate	100±0	100±0	100±0	100±0	100±0	100±0
Positive control		0.0					
Negative control		30±2					

Results are mean ± standard error of the mean (SEM). Negative control = without extract added (blank/distilled water); Positive control = Diflucan (n = 3).

The lacking of percent germination of spores is found at 125 mg/mL for seed methanol and n-hexane, 250 mg/mL and 1000 mg/mL for ethanol and ethyl acetate respectively. While at 62.5 mg/mL leaves methanol and ethanol extracts shows absent percent germination and at 250 mg/mL and 1000 mg/mL lack of germination exhibit by n-hexane and ethyl acetate extracts respectively.

5. Discussion

The substitute management techniques for crops, plants, fruits and vegetables diseases are obligatory due to high cost of producing novel chemicals, resistance to synthetic fungicides by fungal pathogens, pollution and biohazards. Research have been carried out on the utilization of plant based products as pests management control strategy due to their broad based public acceptance, ecofriendly nature and minimal toxicity to mammals. In this connection the current study was design to assess the neem leaves, seeds and twigs Soxhlet extracts for their comparative extractive values, phytochemicals screenings, antifungal activities, MIC, MFC and spore germination assays of methanol, ethanol, n-hexane and ethyl acetate.

5.1 Extraction Yields %

The herbs raw extracts types comprise easy extraction system with production costs low and have the abilities to establish easy technology development in the agriculture industries (Maria *et al*., 2016). The mother tincture and ethanol extracts, observed maximum penetration in the culture media because of their hydrophilic nature, creating energetic chemicals molecules bio-available to fungi assayed, which characterize a significant quality to evaluate the novel molecules (Maria *et al*., 2016).

The neem leaves and seed oil yield extracts were 17.05% and 23.92% respectively (Samuel *et al*., 2021). The neem stem bark extracts yield % reported for acetone (11.7), ethanol (6.8), ethyl acetate (3.43) and methanol (9.77) reported by (Anokwuru *et al*., 2011). The seed kernels, seeds and leaves extractions % yield with n-hexane were 20.0%, 34.7%, 9.1%; methanol extractive yield were 65.7%, 52.9%, 65.2 %; chloroform % extractive yield were 10.7 %, 11.3%, 6.8 % respectively (Wafaa *et al*., 2007). A Neem leaf possesses the highest oil yield by Soxhlet technique using ethanol with the mean percentage yield of 37.73% while that of Neem bark and seed are 27.70% and 34.85% respectively (Evbuomwan *et al*., 2015). Neem bark produced maximum yield using absolute ethanol as compared to pure methanol, water methanol and ethanol (Sultana *et al*., 2009). The extractive yield of oil obtained from seeds by Soxhlet and hexane as solvent was found out to be 37% (Jessinta *et al*., 2013). Neem seed oil yield 28.4% using Soxhlet hexane extraction system (El-Mahmood *et al*., 2010). The dissimilarities in the extractive yield percentage might be due the plants drying process, fruit maturity, harvesting period, origin, geographical location, extraction methods, solvent system and mesh size (Jessinta *et al*., 2013) when compared to our findings.

5.2 Extraction Methods and Solvents system

For Polar and non-polar compounds extraction methanol is most suitable. This build the bioactive substances of non- polar and polar could be catch out conveniently (Norazlina *et al*., 2021). The neem leaves extract obtained maximum yield in Soxhlet technique using ethanol as solvent following three hours extraction time (Tesfaye and Tefera 2017). The polar solvent (ethanol) split down H_2O soluble substances (chlorophyll) and quickly blends with H_2O. The other condition like temperature and neem leaves powder mesh size during extraction should be keep in mind to achieve an improved extraction (Norazlina *et al*., 2021). Like ethanol the toxicity of ethyl acetate is less. It was reported that after extraction diverse solvents finally produced dissimilar

phytochemicals (Al-Hashemi and Hossain 2016). This is because of the polarity of the solvent has dissimilar isolation capabilities to every phytochemical. The maximum extractions of phenol compound were reported in ethyl acetate and minimum was obtained in aqueous extract (Norazlina *et al*., 2021). Moreover, for extraction hexane is applied as solvent because of its characteristics like simple recovery, more capability in selectivity to solvent, minimum latent heat of vaporization and non-polar character (Norazlina *et al*., 2021).

5.3 Phytochemical Constituents

Phytochemical components of neem leaf aqueous and methanol extract (Dash *et al*., 2017), neem leaves methanol, chloroform, water, ethyl acetate and chloroform extract (Benisheikh *et al*., 2019), methanol extract of neem leaves (Bharat *et al*., 2015), neem twigs aqueous extract (Awache *et al*., 2017), neem leaves and stem bark extracts (Effiong *et al*., 2016) were dissimilar and varied as compared to our study. These differences and deviation are because of divergence of plants parts, solvents, extraction methods, geographical location and maturity of plants parts. The detection or absence of specific category of phytochemicals occurrence in the neem might be fluctuating according to the climatic aspects and geographical position (Shuaibu *et al*., 2015; Jessinta *et al*., 2013).

5.4 Biofungicide Potential

Applications of plants for the welfare of human beings have a lot of scope; the neem potential in this regard is rising. The utilization of herbs materials for the management of plant disease is a rich field for research. Further, the production technology is easy, grounding and immersing the specific quantity in solvents, concentration and testing on vegetables, seeds and crops. Plant origin fungicides are cheap and trouble-free available in the distant rural area (Dauda *et al*., 2015). Herbs hold numerous types of bioactive compounds such as flavonoids, alkaloids, and saponins etc. generally called phytochemicals are carrying antifungal activities (Sheema and Durai 2015). These phytochemicals are the antimicrobial principles of herbs. These bioactive molecules are really the self-protective system of the herbs against diverse microbes (Shrivastava and Kshma 2014). The mode of action of the phytochemicals as antibiotic agents are; they effect and destroyed on the enzymatic processes involved in the energy synthesis (Gill and Holley 2006b), or these molecules might cause shape injury to the cell membranes resulting death of the cells (Gill and Holley 2006a). These compounds to a certain extent penetrated into the cell membrane into inner of the cell disturbing vital nucleolus functions like synthesis of nucleic acids (Cristani *et al*., 2007). The phyto-compounds act on the fungi hyphal cell wall causes failure of inflexibility and fall down of the fungal mycelium (Sharma and Tripathi, 2006).

The neem antifungal activity is accredited to dissimilar kinds of phenolics and tetra terpenoids (Khajista, 2013). Neem phytochemicals (glycosides and saponins) have antifungal activity (Bennett and Wallsgrove1994; Grayer and Harbourne, 1994). The variation in the lethal activity of the extracts might be accredited to the occurrence of the bioactive molecules that are pull out by diverse solvents, which might be affected by many factors like type of extracting solvents, method of extraction, location and age of plant (Nicolls, 1970; Qasem *et al*, 1996; Mondali *et al*., 2015).

The tannins, phenolates, flavonoids and steroids are the anti-fungal secondary metabolites found in plants extract. The development of fungal spores of *Fusarium oxysporum* could be inhibited by anti-fungal substances (Anak *et al*., 2019). Doses concentration augmentation involve amplify in the solution the bioactive compounds that play its role as antifungal on the target fungal satins. Polar extracts, like aqueous and ethanol were high energetic as compared to non–polar extracts such as chloroform (Culver *et al*., 2017). The neem leaves extracts (aqueous and ethanol) showed spore inhibition against fungal pathogens of tea (Saha *et al*., 2005). The neem extracts antifungal activities against pathogenic fungi differ with neem parts used. These make clear the disparity in toxicity to the fungal strains displayed by the fresh leaves and dry neem seed extracts (Ogechi and Marley, 2006).

A comparatively big genus of fungi is Cladosporium belong to various main ordinary outdoor and indoor molds. Numerous strains Cladosporium, together with fungi, are generally exist on dead and live plants and synthesize black or olive-green to brown colonies. This genus comprised approximately eight hundred (800) saprotrophic and pathogenic plant strains, which are frequently extremely osmotolerant and easily cultivate on general media (Rodeva *et al*., 2016). Cladosporium fulvum, among numerous strains of Cladosporium is fungal pathogen which is biotrophic in nature and produce severe diseases in various plants. In all over the world the fungus originate severe profitable losses to marketable cultivate tomato grown in greenhouses, high tunnels and open field (Rivas and Thomas, 2005). *Colletotrichum coccodes* is a fungus which causes diseases in vegetables of a family of Solanaceae. It sources fruits anthracnose of eggplant, pepper and tomato and potato black dot disease (Rodeva *et al*., 2016). It is reported as a soil-borne and seed tuber pathogen that is hard to mange (Salman *et al*., 2015).

To establish the pathogenicity, in the fungal pathogens the growth of spores are very vital as they penetrate the underlying tissue, spread and germinate. The germination and growth inhibition is very crucial to capture any disease establishment and spread. In the current study germination of conidia reduce with raise the concentration of extract and methanol and ethanol extract calculated comparatively elevated reduction ability. It was reported that hyphae were extra susceptible to herbs essential oils as compared to the spores. Spores reactive responses to the essential oil were dissimilar. Spores inhibition effectiveness was time reliant. Complete mortality of spores was observed to the formulation was time exposure extended (Zia and Cyrus, 2011).The extracts of Neem could be useful for the growth inhibition of the harmful fungus. The dissimilarities in the antifungal activities might be because of the dissimilar allocation of the bioactive substances present in the unlike extracts and parts. The current study might more show that the chemical ingredients/antifungal properties, which are moreover non-polar or polar, may be efficiently isolated by methanol and ethanol solvent.

6. Conclusion

The results revealed that methanol and ethanol leaves and seeds extracts of neem showed a significant inhibitory potential against study plant pathogenic fungi. The leaves and seeds crude extracts molecules might next be formulated into bio-fungicides which are available particularly to the small farmers. With support of policy makers and researchers the concerned stakeholders, make additional awareness regarding the need and benefits of neem based bio-fungicides and bio-pesticides. Scientists and researchers studied on similar products have responsibility to give field trials data that is reproducible and consistent. The current study provide a guideline and way forward to formulate and develop a products of bioactive nature with fungicides application, with the additional advantages of economically viable and ecofriendly.

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