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Comparative bioactive compounds and fourier transform infrared spectroscopic evaluation of *Azadirachta indica* extracts and its potential as bio-fungicides against plant pathogenic fungi

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Abstract

New antifungal agrochemicals compounds discovering from natural products are a vital goal for the management of plant pathogenic fungi. As present synthetic fungicide used for the treatment of plant pathogenic fungi have negative effects on environment and human health. Therefore, it is need of the day to develop and discover new antifungal substances from natural products. The objective of the current study was to evaluate the extracts of leaves, seeds and twigs of *Azadirachta indica* using infusion, decoction and microwave extraction techniques. These extracts were subjected to phytochemical quantification, Fourier Transform Infrared (FTIR) spectroscopic analysis and antifungal assays against pathogenic fungi by well diffusion technique. The quantitative of phytochemicals screening showed that leaves had showed high concentrations of bioactive compounds, seeds showed a moderate and twigs observed the least phyto-compounds. The infusion extracts system showed high amount of active compounds, followed by microwave and decoction. Extracts of leaves and seeds showed evidence of inhibition growth of fungi. The spectra of FTIR verified the occurrence of different functional groups such as aromatic compounds, alkanes, alkyl halides, amines, carboxylic acids, phenols, alcohol and amino acids, in the neem extracts. Relevant stakeholders along with the help of policy makers and researchers should build extra understanding regarding the requirement to clinch products of organic fungicides like secure fungus management system.

Keywords: Antifungal potential, extraction techniques, functional groups, neem, phytochemicals.

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1. Introduction

The main infectious agents in plants are the pathogenic fungi, causing changes in growth phases and post-harvest stages. A broad diversity of fungi genera are attack on vegetables and fruits, which creates quality issues connected to many aspects such as limited shelf life, organoleptic characteristics, dietary values and a results a huge economic loss (Diaz *et al*., 2011). The application of plants extracts for the diseases management of crops and plants poses little environmental risk and economically viable;, the herbs are accessible to the growers in the tropics, developing countries and numerous areas of the globe that have no easy reach to the synthetics pesticides (Chukwuma *et al*., 2018). The extracts of plants derived from stem, rhizome, fruits, flowers, roots, leaves and bark consist of numerous bioactive substances that have antifungal and antimicrobial properties (Kanal, 2021). Usually, plant

pathogenic fungi are managed by man-made fungicides; though, the application of these is augmented constrained because of toxic outcomes of pesticides on environment, animals and human health. The growing demand of manufacture, rules, policies and guidelines on the application of agrochemicals and the appearance of pests' resistance to the market products, give good reason for the exploration of new bioactive substances and novel management methods (Diaz *et al*., 2011). Utilization of plant materials have a lot of scope, the *Azadirachta indica* potentials in this characteristic is budding. The plants material utilization for managing phyto diseases is a fruitful tool for study. The production technology is easy and involved grinding, extraction and testing on targeted pests and fungi. An additional importance of plant-based pesticides/fungicides is that they are substitute for synthetic fungicides/pesticides.

The neem extracts exhibits anti-hyperglycemic, antimalarial, anti-inflammatory, anticancer, antiulcer, anti-mutagenic, antioxidant, antiviral, antibacterial, antifungal, immunomodulatory immunomodulatory and pesticidal properties (Khajista, 2013). *Azadirachta indica* have bioactive compounds in nearly all parts such as branches, trunk, bark, roots, leaves and seeds and possess therapeutic properties (Kanal, 2021). Fourier Transform Infrared (FTIR) spectroscopy has contributed a significant part in the field of therapeutic herbs study. The FTIR measurements were extremely reproducible and accurate. It has prepared the application of infrared measurement virtually limitless. The Infrared absorption spectroscopy generally applied region is 4000-400 cm^{-1} because most inorganic and organic compounds absorption radiation is lies within this area. One of the main generally applied techniques to recognize functional group and identify the chemical structure of the constituents and chemical ingredients is FTIR (Lethika *et al*., 2013). FTIR measurement was applied to recognize the impact of functional groups as viable ingredients for the cure of numerous ailments (Mohan, 2001). Analysis by FTIR exposed the occurrence of numerous functional groups such as cycloalkanes groups, halogen, alkyl amines, alkanes, amides, alkynes, carboxylic acids and alcohols that might be active by yourself or in synergy, as reported by different researchers (Daoub *et al*., 2018; Diaz *et al*., 2011; Suleiman, 2017; Udo *et al*., 2017; Bashir and Javid, 2013; Javid *et al*., 2015). These functional groups reported antibacterial and antifungal potential (Basir and Javid, 2013; Javid *et al*., 2015). Although several studies have evaluated the phytochemicals, FTIR and antimicrobial activities of neem extracts against a broad range of pathogens (Ali *et al*., 2022; Amadioha, 1999; Chukwuma *et al*., 2018; Govindachari *et al*., 1998; Khanal, 2021; Khajista, 2013; Lethika *et al*., 2013; Nwali et al., 2018; Oyekanmi *et al*., 2021), but the antifungal activity against the plant pathogenic fungi and FTIR measurements together of neem extracts remains elusive. The current study was aim to find a plant product, which can be used as a substitute of commercially available chemical fungicide. The current project was design to investigate and compare the infusion, decoction and microwave extraction techniques and fungicidal potential of leaves, twigs and seeds of *Azadirachta indica* against plant pathogenic fungi, FTIR spectroscopic study and to clarify the existing bioactive substances in the extracts.

2. Materials and Methods

2.1. Azadirachta indica Parts Collection

Azadirachta indica leaves, twigs and seeds were collected from Board Bazar of Peshawar City, Khyber Pakhtunkhwa-Pakistan. These plant parts were brought to Environmental Research Section of Pakistan Council of Scientific and Industrial Research (PCSIR) Laboratories Complex Peshawar, Khyber Pakhtunkhwa-Pakistan. The collected materials were carefully washed with flowing tap H2O and 1% sodium hypochlorite solution was used for surface sterilization. Then rinsed with autoclaved H2O and kept at 40°C for dehydration in an Air Cabinet Dryer (England) and grinded to form powder with the help of Standard Model No.3 Wiley Mill USA. The powder was kept in an aluminum bags, sealed and utilized as soon as required for experimental methods.

2.2. Extraction

Infusions were carried through the addition of 50 mL of boiling distilled H2O to 05g of plant materials and the mixture enclosed for twenty minutes at room temperature. Extracts so obtained were filtered through Whatman® 40-filter paper 125 mm Ø Whatman International Ltd. Maidstone, England. The gained remains were afterward viscous on a Vacuum Rotary Evaporator (R-200 Buchi Rotavapor-Switzerland, B-490 Heating Bath- Buchi) under control temperature between 30-45°C under pressure. The high viscous extract was obtained and weighed up in a crucible and placed on a water bath with 45 ºC and subsequently the extract dried (Soberon et al., 2007). Decoctions Extraction: Decoctions were obtained through boiling Azadirachta indica leaves, twigs and seed powder (05g) with distilled H2O (50 ml) for twenty minutes and afterward kept at room temperature. Decoction extracts were gained and processed as described for infusion method (Soberon et al., 2007). Microwave Assisted Extraction (MAE): A home microwave oven (Dowlance) was utilized for extraction purposes. The operations conditions were 800 W at 2450 Hz single-phase output for the microwave oven. 05 gms of Azadirachta indica leaves, twigs and seed powder were shifted to a beaker (250 ml), then added fifty milliliters of distilled H2O. The cycle of irradiation was as follows: thirty seconds for preheated and thirty seconds for power-on. The process of extraction was afterward returning one cycle. Subsequent to the extraction process, the beaker was retained for cooling at room temperature. After that, the rest of the process was carried out as mentioned in the infusion process.

2.3. Bioactive Compounds Quantitative

Phytochemicals compounds such as alkaloids, flavonoids, saponins and terpenoids were quantified by gravimetric methods (Harborne, 1973; Kanal, 2021).

2.4. Spectroscopic Analysis by Fourier Transform Infrared (FTIR)

The FTIR Prestige -21 Shimadzu Japan was used to obtained the IR spectrum. The seeds, leaves and twigs extracts (Infusion, decoction and microwave) were examined from 200 - 4400 cm-1 and working at a 4 cm−1 resolution with ten number of scan applying IR solution software.

2.5. Antifungal Assay

2.5.1. Test Fungi

The fungal pathogens, Alternaria solani, Fusarium oxysporum, Rhizoctonia solani, Aspergillus flavus, Aspergillus parasiticus and Aspergillus niger were obtained from the Environmental Research Section of PCSIR Laboratories Complex Peshawar, Khyber Pakhtunkhwa-Pakistan. These strains were refreshes on potato dextrose agar (PDA) by slant technique of sub-culturing and placed at 10 °C in a cold incubator for the experimental work of the project.

2.5.2. Agar Well Diffusion Method

Fungal spores' suspension (0.1 mL=105 spore/mL) were swab homogeneously by means of sterile glass spreader on the PDA plate surface. Wells with a diameter of six millimeter were punched by means of sterilized cork borer. Fill the designated well with 100 µL of Azadirachta indica extracts (1000 mg/mL). The plates were kept at room temperature for seven days in an incubator (Memmert-Germany) and then around the well the zone of inhibition (ZOI) was calculated in millimeter (mm) with the help of radius scale. The Dimethyl sulfoxide (DMSO) and synthetic antifungal agent Mancozeb (40 mg/mL) were applied as negative and positive control respectively (Bashir and Javid, 2013).

2.5.3. Determination of Minimum Inhibitory Concentration (MIC)

The broth dilution technique was used to determine the MIC of the test samples. Different extracts concentration such as 1000, 500, 250, 125, 100, 50 and 10mg/mL were prepared in the Potato dextrose broth (PDB). Then added 0.1mL of the inoculums (105 spores/mL) into the extracts prepared with mentioned concentrations in the broth. Along with the tests, experimental controls were also carried out. Incubation of the test tubes was carried out for seven days at 30oC and growth turbidity observation was noted if any. The minimum dosage in the test tubes, which observed no cloud, was recorded as the MIC (Bashir and Javid, 2013).

2.5.4. Determination of the Minimum Fungicidal Concentration (MFC)

The contents of MIC were sub-cultured on to the media in the serial dilution method and incubated for seven days at 30oC. Then growth of colony was observed on the plates. The plate designates, as MFC was having the lowest tested materials concentration and with no appearance of colony (Bashir and Javid 2013).

3. Statistical Analysis

The obtained data from the tests of the neem extracts were fed into Microsoft Excel. The exported data were then entered into SPSS- version 16 to calculate statistics (descriptive) of the average and mean of standard error (SEM) diameter of inhibition zone. $p \leq 0.05$ was considered as Statistical significance.

4. Results and Discussion

4.1. Quantification of Bioactive Compounds in Azadirachta indica Parts Extracts

The results on quantitative screening of saponins, terpenoids, flavonoids and alkaloids in the neem selected parts using different solvent extraction system are shown in Table 1.

Table 1. Quantitative analysis of phytochemicals in *Azadirachta indica*

Plant Parts	Extracts	Terpenoids (%)	Saponins (%)	Flavonoids (%)	Alkaloids (%)
Twigs	Infusion	02.50±01	02.10±00	04.50±00	05.40±00
	Microwave	02.20±00	01.06±00	03.00±00	04.20±01
	Decoction	01.70±00	00.80±00	3.40±00	02.30±0.3
Seeds	Infusion	05.50±00	02.20±00	04.00±00	06.40±00
	Microwave	02.60±00	01.90±00	03.10±00	05.20±0.5
	Decoction	02.10±01	01.40±00	02.10±00	03.50±0.5
Leaves	Infusion	05.60±0.5	02.30±00	06.00±10	06.50±01
	Microwave	03.40±00	01.50±00	05.12±0.2	05.50±00
	Decoction	03.00±00	01.38±00	04.30±0.1	04.00±0.5

All values are a mean of triplicate ± SEM.

The results indicate that leaves have the maximum phytochemicals as compared to seeds and twigs extracts. Infusion was observed to be the most efficient extract system followed by microwave and decoction to extract the phytochemicals. The infusion displayed highest activity than decoction (Sandipta et al., 2021; Ali et al., 2022).

Decoction seems to affect quantity of phytochemicals and antioxidant activity that might be related to longer boiling process than infusion preparation. Due to longer boiling conditions, active constituents of the decoction might be destroyed that can affect their concurrent bioactivity (Sandipta et al., 2021). Decoction is convenient, simple and cheap in terms of equipment and reagent, when compared with other methods. Our study showed that infusion is more efficient, simple, does not use toxic and flammable solvents, and required no more heat and temperature as compared to the decoction and microwave extraction methods. The infusions were more active than decoctions; it strengthens the principle that not only the applied solvent is crucial; but also the process of extraction is also important (Ali et al., 2022). During decoction, the bioactive compounds might be damaged or partially inactivated by providing the high temperature to the plants materials. Although a maximum yield, extraction was obtained by this extraction (Soberon et al., 2007). Nine neem lamonoids were found with the capabilities to stop the growth of insects, a wide range of species found susceptible that comprises various lethal human and agriculture pests (Nwali et al., 2018). The dissimilarities and trend of phytochemicals could be because of the different geographical location of the plants, extraction methods, extraction time and solvents. The flavonoids are holding antibacterial, antiviral and antifungal activities. The principles thought to be accountable for phenol toxicity against the microorganisms comprised inhibition of enzymes through oxidized substances, probably during reaction with thiol groups or in the course of more nonspecific connections with the proteins (Hanafey and Sabry, 2013).

4.2. Fourier Transform Infrared (FTIR) Spectroscopy Analysis

The FTIR analysis was applied for the identification of functional groups of the phytochemicals present in the neem extracts based on the spectrum value in the infrared wave's area. The FTIR peaks values and functional groups were depicted in FTIR peak sketch are demonstrated in the figures 1 to 9.

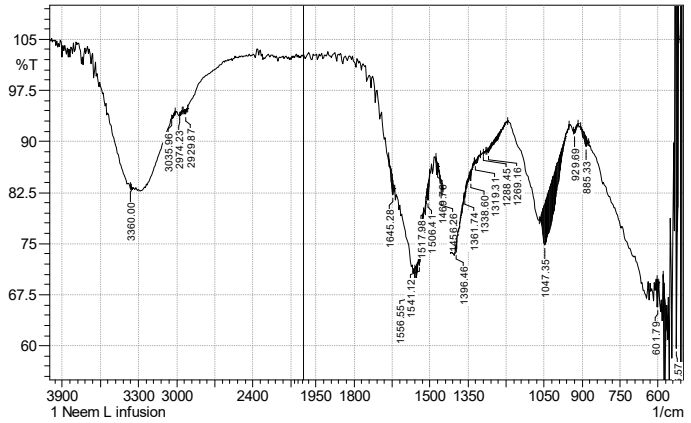

Fig. 1. FTIR Spectra of Neem Leaves Infusion Extract

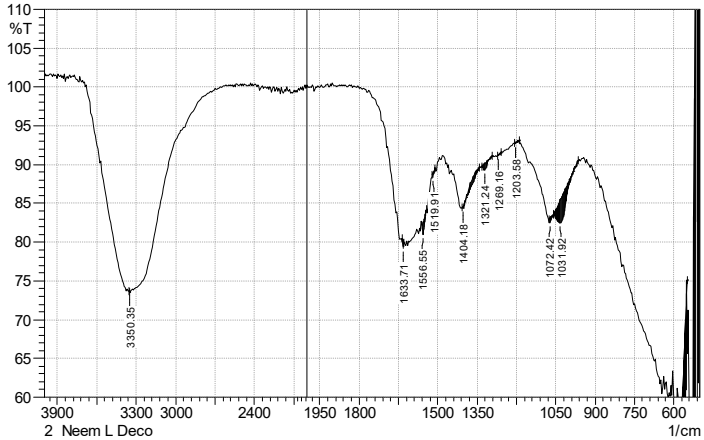

Fig. 2. FTIR Spectra of Neem Leaves Decoction Extract.

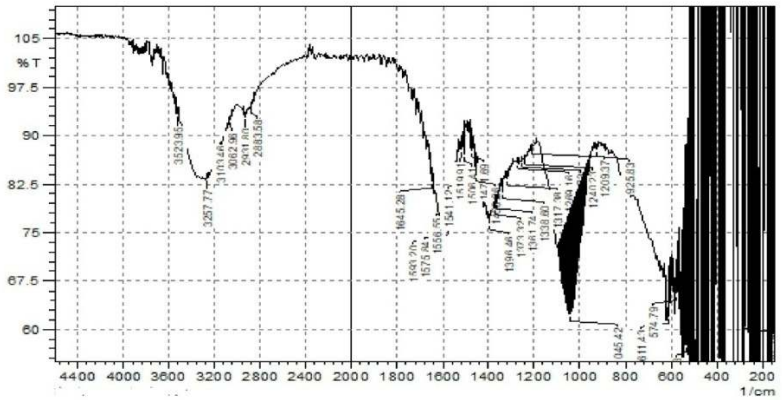

Fig. 3. FTIR Spectra of Neem Twigs Decoction Extract.

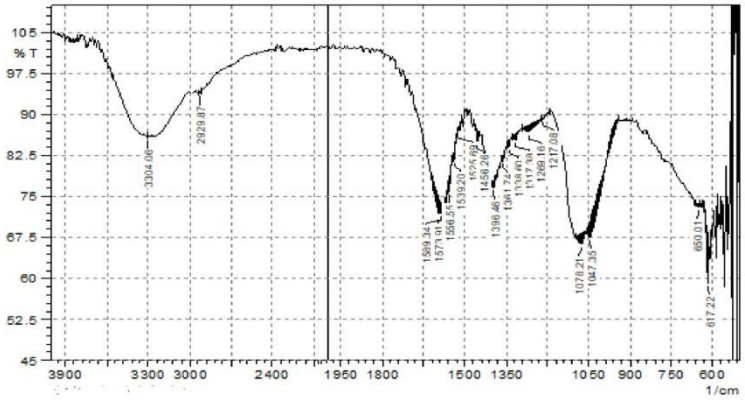

Fig. 4. Neem Twigs Infusion Extract FTIR Spectra.

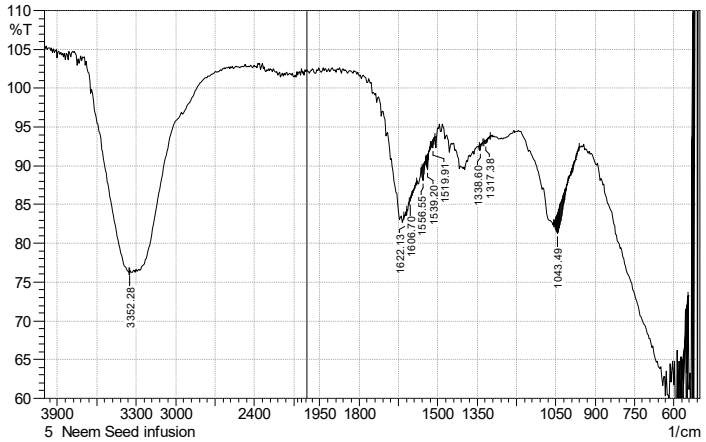

Fig. 5. FTIR Spectra of Neem Seed Infusion Extract.

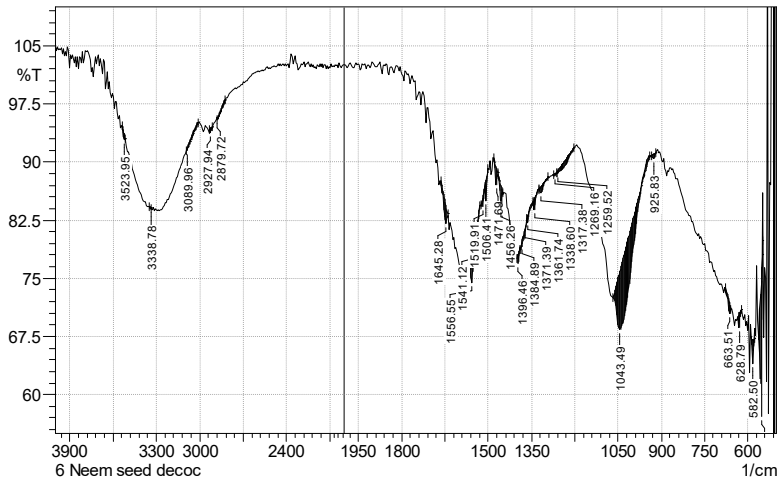

Fig. 6. Neem Seed Decoction Extract FTIR Spectra.

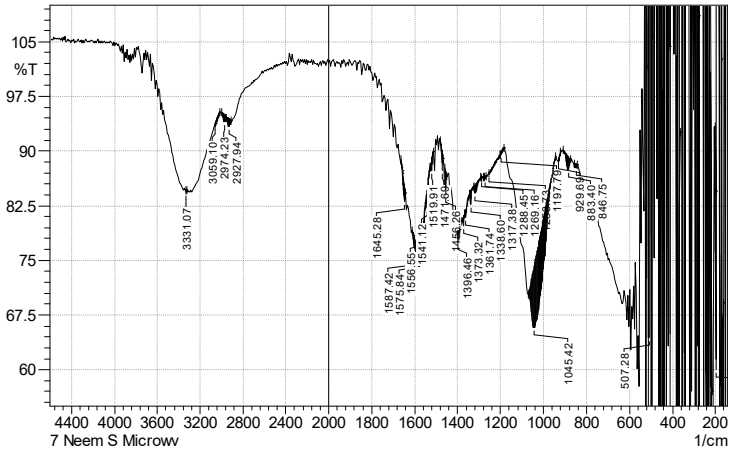

Fig. 7. Neem Seed Microwave Extract FTIR Spectra.

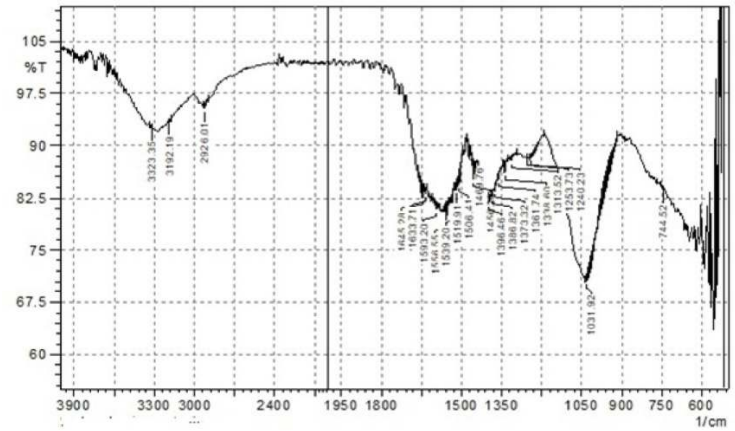

Fig. 8. Neem Twigs Microwave Extract FTIR Spectra.

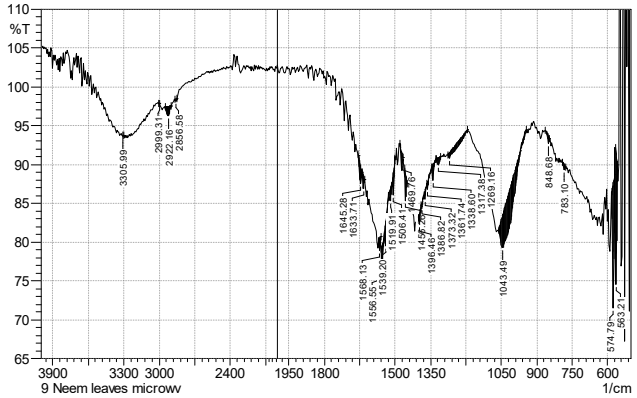

Fig. 9. Neem Leaves Microwave Extract FTIR Spectra

The FTIR spectra of neem leaves infusion extract are shown twenty one (21) peaks in figure 1. The wavelengths values are 3360.00, 3035.96, 2974.23, 2929.87, 1645.28, 1556.55, 1541.12, 1517.98, 1506.441, 1469.76, 1456.26, 1396.46, 1361.74, 1338.60, 1319.31, 1288.45, 1269.16, 1047.35, 929.69, 885.33 and 601.79 cm^{-1}. The FTIR spectra of neem leaves decoction extract (Figure 2) shows ten (10) most important peaks at the range of 3350.35, 1633.71, 1556.55, 1519.91, 1404.18, 1321.24, 1269.16, 1203.58, 1072.42 and 1031.92 cm^{-1}. Figure 3 shows the FTIR spectra of neem twig decoction extract with twenty seven (27) key peaks. These peaks ranges are 3523.95, 3257.77, 3101.46, 3062.96, 2931.80, 2883.58, 1645.28, 1593.20, 1575.84, 1556.55, 1541.12, 1519.91, 1506.41, 1471.69, 1458.26, 1396.46, 1373.32, 1361.47, 1338.60, 1317.38, 1269.16, 1240.23, 1209.37, 1045.42, 925.83, 611.43 and 574.79 cm^{-1}. Neem twigs infusion extracts FTIR bands values are shown in figure 4. Total eighteen (18) frequency bands are found in the ranges of 3304.06, 2929.87, 1589.34, 1573.91, 1556.55, 1539.20, 1525.69, 1456.26, 1396.46, 1361.74, 1338.60, 1317.38, 1269.16, 1217.08, 1078.21, 1047.35, 650.01 and 617.22 cm^{-1}. The seed infusion extract FTIR spectrum is shown in figure 5 with a nine (09) functional groups. The peaks values range are 3352.28, 1622.13, 1606.70, 1556.55, 1539.20, 1519.91, 1338.60, 1317.38 and 1043.49 cm^{-1}. The FTIR spectra of neem seed decoction extracts is shown in figure 6. The total number of functional groups are twenty five (25) and peaks ranges are 3523.95, 3338.78, 3089.96, 2927.94, 2879.72, 1645.28, 1556.55, 1541.12, 1519.91, 1506.41, 1471.69, 1456.26, 1396.46, 1384.89, 1371.39, 1361.74, 1338.60, 1317.38, 1269.16, 1259.52, 1043.49, 925.83, 663.51, 628.79 and 582.50 cm^{-1}. Neem seed microwave extract FTIR spectra is displayed in figure 7. The total number of functional groups are 26 and bands range are 3331.07, 3059.10, 2974.23, 2927.94, 1645.28, 1587.42, 1575.84, 1556.55, 1541.12, 1519.91, 1471.69, 1456.26, 1396.46, 1373.32, 1361.74, 1338.60, 1317.38, 1288.45, 1269.16, 1258.73, 1197.79, 1054.42, 929.69, 883.40, 846.75 and 507.28 cm^{-1}. Neem twigs microwave FTIR spectra are shown in Figure 8. Total numbers of functional groups present in this extract are twenty two (22). The peaks ranges are 3323.35, 31`92.19, 2926.01, 1645.28, 1633.71, 1539.20, 1593.20, 1556.55, 1519.91, 1506.41, 1469.70, 1456.0, 1396.46, 1386.82, 1373.32, 1361.74, 1338.60, 1313.52, 1253.73, 1240.23, 1031.92 and 744.52 cm^{-1}. Neem microwave extract FTIR is shown in Figure 9. The total numbers of functional groups found in the extract are 25. The wave-length ranges are 3305.99, 2999.31, 2922.16, 2856.58, 1645.28, 1633.71, 1558.13, 1556.55, 1539.20, 1519.91, 1506.41, 1469.76, 1456.20, 1396.46, 1386.82, 1373.32, 1361.74, 1338.60, 1317.38, 1269.16, 1043.49, 848.68, 783.10, 574.79 and 563.21 cm^{-1}. The spectra of FTIR verified the occurrence of amines, aromatic compounds, carboxylic acid, amino acids, alkyl halide, alkanes and phenol in the seeds, twigs and leaves of the neem.

The appearance of wide band at approximately 3300 cm^{-1} may perhaps be credited to carboxylic acid by way of stretching O-H vibration. The 1087.21 cm^{-1} wave length (Figure 4) have a C-O-C stretch (dialkyl), C-O stretch a vibration type and assigned functional group was ether and alcohol. The C-H stretching vibration modes in the hydrocarbon chains were found at 2900 cm^{-1} peak values (Oyekanmi *et al*., 2021). The peak values pointed out the occurrence of OH connecting vibrations from minor phenols, alcohol primer, alkene, C=C aromatic, carboxylic acid groups and phenolic groups that assigned (Vijayarathna and Sasidharan, 2012).Whereas, the peaks values at 1734 cm^{-1} matching to the stretching vibrations of the C=O in carboxylic acids, aldehydes and ketones (Oyekanmi *et al*., 2021). Carboxylic acid esters reported maximum antifungal activities against *Aspergillus niger*, *Candida albicans* and *Cryptococcus neoformans*. Every derivatives of carboxylic acid ester displayed maximum fungicidal activities as compared to fluconazole (Nam *et al*., 2004). The absorption bands of neem leaves at 1641 cm^{-1} was designated to the amide C=O connected to the occurrence of group of –COOH and the absorption bands at 1384 cm^{-1} were presented to the frequency stretching that comes from the C-O bonds of acetyl esters, C=O, CH_2 wagging and C-H stretching respectively (Oyekanmi *et al*., 2021). The 1600 (cm^{-1}) wave length might be ascribed to C=C stretch (alkenes) and 1400 cm^{-1} band might be recognized as alkenes with C-O-C stretch and C-H in-plane bend. It was reported that aliphatic C-C position occurred at 1249 cm^{-1} and frequency values at 1070 cm^{-1} in leaves of neem was stretching vibration C-O (Oyekanmi *et al*., 2021). The FTIR spectra verified the occurrence of halide, aliphatic amines, aromatic, carboxylic group, amides, alkynes, alkanes and alkenes. In the present study, phenolic compounds, tetra terpenoids and saponins may be the energetic phenomena accountable for the fungicidal activity (Bashir and Javid, 2013). Neem tree (Azadirachtin) extracts from the bark, leaves and seeds had been documented to possess powerful pesticide assays against *pest*, fungi and *insect*, however to ecosystem and mammals very low toxicity observed (Chukwuma *et al*., 2018). The

carboxylic acids were blame for a lot fungicidal and bactericidal activity, which are found in numerous herbs physiological molecular system (Javid *et al*., 2015).

4.3. Bio fungicide Potential of Neem

Antifungal Activity (depicted by the Zone of Inhibition) of *Azadirachta indica* is shown in Table 2.

Table 2. Antifungal Activity of *Azadirachta indica*.

Extracts System	Plant Part	Fungicide Activity	*Alternaria solani*	*Fusarium oxysporum*	*Rhizoctonia solani*	*Aspergillus flavus*	*Aspergillus parasiticus*	*Aspergillus niger*
Infusion	Leaves	IZ (mm)	17±0.5	18±01	19±1.5	23±0.6	21±0.8	20±01
	Seed	IZ (mm)	14±0	15±01	16±01	19±1.5	18±0.8	17±0.4
	Twigs	IZ (mm)	13±0.2	13±0.5	14±01	17±0.5	15±0.6	15±0.8
Decoction	Leaves	IZ (mm)	11±0.4	12±0.6	13±0.8	16±01	15±01	14±01
	Seed	IZ (mm)	08±0.2	09±0	11±0	12±0	12±0.4	10±0.5
	Twigs	IZ (mm)	--	--	--	--	--	--
Microwave	Leaves	IZ (mm)	12±0.6	13±0.5	14±0.4	17±0.3	16±0.5	15±01
	Seed	IZ (mm)	09±0	10±0	11±0.5	14±01	12±01	11±0.4
	Twigs	IZ (mm)	--	--	--	--	--	--
DMSO	-ve control	IZ (mm)	--	--	--	--	--	--
		MIC	NA	NA	NA	NA	NA	NA
		MFC	NA	NA	NA	NA	NA	NA
Mancozeb	+ve control	IZ (mm)	20±01	21±0.5	22±1.5	26±01	24±0.4	23±0.8
		MIC (mg/mL)	30	30	30	05	10	10
		MFC (mg/mL)	40	40	40	10	20	20

-- = No zone of inhibition, NA=Not applied, IZ= Inhibition zone. All values are mean of triplicates ±SEM.

Aspergillus flavus, Aspergillus parasiticus and *Aspergillus niger* were the most susceptible to the neem leaves infusion extract. Our study reported that leaves are most active against all plant pathogenic fungi, followed by seeds and twigs. On the other hand, extraction system of infusion was the most efficient, followed by microwave and decoction extract. The difference in the antifungal efficacy might be owing to uneven allocation of bioactive substances present in diverse plant extracts and parts. The neem plant extracts contain the saponins, alkaloids, terpenoids and flavonoids. The leaves of *Azadirachta indica* were found to contain steroid (0.03%), glycoside (0.27%), alkaloid (14.5%), flavonoid (2.10%) and saponin was 4.00% (Uwague, 2019). Neem leaves quantitative phytochemical (mg/ g of extract) reported by Ruth *et al*., 2021 that flavonoids were 24.45±0.02, phenolic compounds was 25.77±0.03, glycosides was 0.17±0.01, tannins was 9.15±0.02, steroids was 0.42±0.02, saponins was 0.30±0.02 and alkaloids was 5.09±0.00. The quantitative (%) phytochemicals of neem leaves, seed and stem-bark were calculated that alkaloids were (10.67±0.46, 10.73±0.29 and 10.77±0.11), flavonoids were (13.8±0.17, 13.1±0.08 and 12.8±0.15) Saponins were (2.43±0.32, 2.53±0.14 and 2.50±0.28) and Terpenoids were 13.13±0.5, 12.77±0.11 and 13.13±0.41 respectively (Khanal, 2021). The secondary metabolites ingredients of each parts of neem calculated that maximum quantities of alkaloids (%) in the roots, stem-bark and leaves were (3.79, 4.93 and 11.63), compared to flavonoids (0.92%, 2.72% and 2.19%), saponins (0.44%, 1.12% and 0.70%), tannins (0.17mg/100, 0.50mg/100 and 0.33 mg/100) and glycosides (0.19%, 0.27% and 0.23%) respectively (Nwali *et al*., 2018). Neem leaves water extracts acquired maximum amount of saponins and minimum alkaloid quantity (Biu *et al*., 2009). The antifungal and antibacterial activities of plants extracts might be because of the occurrence of secondary metabolites such as amino acids, terpenoids, tannins, flavonoids, saponins and alkaloids (Gaurav *et al*., 2019). The variation between the phytochemicals quantified in the current studies and the reported previous studies might be due to many factors such as solvent-solute ratio, temperature and period of the extraction process, extraction process solvent, ripening time of fruit, cultivars variations, geographical areas differences, soil composition where herbs grow, seasons of plant collection and growth stage of plants (Sarbaswarup *et al*., 2019; Askale *et al*., 2022).

Presently, tannins are commonly identified as significant antibiotic substances. Widespread studies have disclosed that tannins have antibiotic potential against numerous types of microorganisms such as bacteria, yeasts and fungi (Congyi *et al*., 2019). Saponins are glycosides and occurred in numerous plants, which hold surfactant characteristics. Separately from bringing indication of intoxication in elevated dosage, they possessed anti-microorganisms capabilities especially against fungus. Saponins also revealed antibiotic potential against protozoa and bacteria (Ruth *et al*., 2021). Alkaloids are organic nitrogen based compounds. These compounds are by nature alkaline and demonstrate pharmacological activities with an extraordinary array (Gaurav *et al*., 2019). The antimicrobial properties of alkaloids are reported by due to blocking enzyme activity (Askale *et al*., 2022). Phenols are a class of phytochemicals dispersed in plants that are utilize as antibiotic agents because of their capabilities to

break integrity of membrane shape in a diverted manner and stop certain enzymes of electron transport chain (Askale *et al*., 2022). Flavonoids are shape wise miscellaneous phytochemicals of plants that are recognized to stop the growth development of fungi by reducing protein synthesis, RNA synthesis, cell division, cell wall construction, efflux mediated pumping system, and inducing mitochondrial malfunction, disrupting plasma membrane (Askale *et al*., 2022). Isoprenoids or terpenoids are miscellaneous class of natural organic substances (hydrocarbon with O and hydrocarbons) with general shape units and several of the common features of fats. The general shape unit is a five (C5H5) compounds i.e. "isoprene". These substances comprise turpentine, vitamins, some growth regulators and hormones etc. Terpenoids have been recognized to be fungicide against numerous plant pathogenic fungi and saprophytic fungus (Singh *et al*., 2006).

Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of infusion extract of neem are shown in Figure 10.

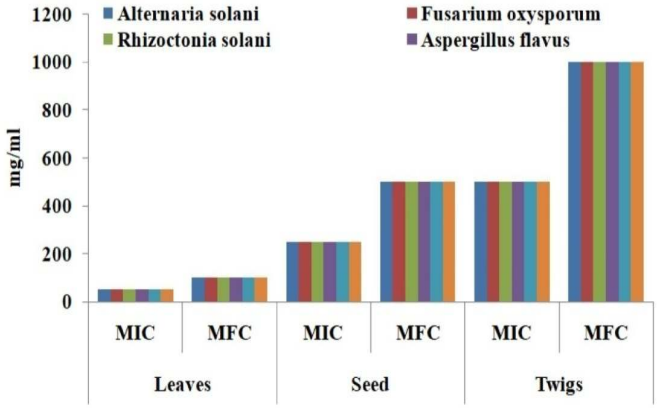

Fig. 10. Antifungal Activity of Infusion Extract of Neem.

The MIC and MFC range of infusion extract for leaf is 50-100mg/mL, for seed is 250-500 mg/mL and for twigs is 500-1000 mg/mL. Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of decoction extract of neem are revealed in Figure 11.

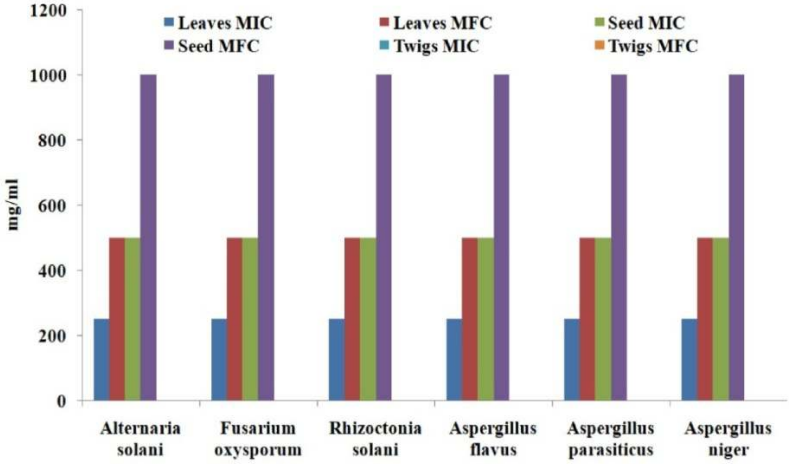

Fig. 11. Antifungal Activity of Decoction Extract of Neem.

The MIC and MFC range of neem decoction extracts for leaves is 250-500 mg/mL and seeds being 500-1000 mg/mL. The extracts of Azadirachta indica could be helpful for the inhibition growth of the plants pathogenic fungi. The Azadirachta indica microwave extract minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) are presented in Figure 12. The microwave extracts MIC and MFC ranges are 100-250 mg/mL for leaves extract and 500-1000 mg/mL for seeds. The variance in amount of responsiveness of the examined fungi could be because of the inherent acceptance of fungi and the combinations and nature of phytochemicals contents in the extracts.

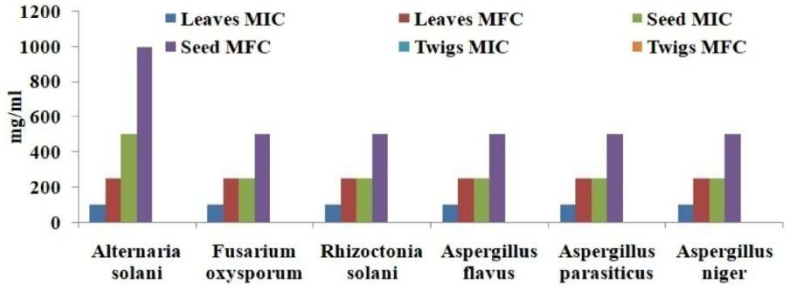

Fig. 12. Antifungal Activity of Microwave Extract of Neem.

Neem leaves extract showed high antifungal activity as compared to fruit extract against *Alternaria solani* (Khajista, 2013). The crude neem oil reported more energetic antifungal assay as compared to the isolated and individual pure compounds from crude neem oil. This displayed probably additives and synergetic effect (Govindachari *et al.,* 1998). Ethanol and aqueous leaves extracts and seed extract of neem investigated that oil extracts displayed the maximum inhibition of the fungi followed by extract of C_2H_5OH, cold H_2O and next warm H_2O (Amadioha, 1999).The greater effectiveness of C_2H_5OH as compared with H_2O extract of the neem leaf may be due to differences in constituent extraction. The dissimilarities in the activities of dissimilar extracts could be credited to the occurrence of the bioactive compounds that are achieved by diverse solvents, which might be affected by numerous factors like maturity of plants, extraction protocol and extraction solvent types (Joan, 1970; Qasem *et al*, 1996). *Azadirachta indica* related bioactive compounds such as saponins and glycosides have fungicidal potentials (Bennett and Wallsgrove, 1994; Grayer and Harbourne, 1994). The saponins antifungal abilities was recognized and observed that in healthy plants saponins quantity were suddenly increased by the fungal attack to provide resistance to pathogens (Chukwuma *et al*., 2018). Plant origin phenolic compounds possessed fungicidal potentials (Javid *et al*., 2015). Therefore, these bioactive substances might have been accountable for the fungicidal potential of the neem extracts applied in the current study. The differences in the concentration of neem extracts and Mancozeb (positive control) were that the neem extract was in raw shape, while the synthetic fungicide was in pure shaped. Consequently, extracts of plants applied in maximum dosage to act proficiently (Chiejina and Onaebi, 2016). It is important to consider the diverse genetic makeup of fungi and choice a particular plant ingredients when making a bio-fungicide. The bioactive compounds such as saponins, flavonoids, glycosides, tannins, terpenoids, phenols and alkaloids are the fungicides doctrine of plants. These are actually the self-protective system of the herbs against diverse pathogenic fungi (Chiejina and Onaebi 2016; Javid *et al*., 2015). The active ingredients occurred in the plants are affected by numerous factors, such as harvesting plant materials time, extraction methods, extracting solvents and age of plants (Chukwuma *et al*., 2018). It is not wonderful that the standard fungicide have high fungicidal activities as compared to the present study extracts. The reason behind high activity of the fungicide is preparation in sophisticated equipment and system in the factory, while the plants extracts are prepared from the raw material, which generally expose degradation and contamination (Javid *et al*., 2015). The development of such an active principle against these plant pathogenic fungi will be promising towards the quest of a natural fungicide. The current research clearly shows that the neem leaves and seeds extract can control these fungi. This study is unique and gains prominent consideration as a development of a marketable “phyto-fungicide” replacement against the synthetic fungicides.

5. Conclusion

In the present study, we use simple extraction/process of the neem parts to produce bio-fungicides. The bio-fungicides extraction process is possible and feasible for the poor and small farmers to control their crops against pathogen fungi. However, the study were limited to only three extraction system and three neem parts. There are other extraction methods. The antifungal study was limited to only mentioned the zone of inhibition, MIC and MFC, however there are many other methods also to check the antifungal potential of the neem parts. The plant-based fungicides are biodegradable in nature, but one limitation is the short shelf life. Future studies will be carry out to add the stabilizers, emulsifiers, additives and preservative to the neem seed and leaves infusion extracts to develop a long shelf life bio-fungicide products. More studies are required to investigate the bio-fungicide products by different testing system such as in-vivo, greenhouse, pot experiments and field trials on numerous crops, vegetables and fruits pathogenic fungi. Further a pilot, prototype and small industrial scale production of the neem based bio-fungicide set up will be establishes at village level for the poor farmers. Additional instigations are needed to identify the bioactive molecules producing genes through genetic engineering/breeding to improve accumulation and formation of the bioactive molecules. The augmented application of botanical fungicides to treat the pathogens of crops and fruits plants, contribute to boosting

satisfactoriness of food and agricultural goods in position markets. As a result, therefore guiding to food safety, protection of biodiversity, global business, environmental safety and organism's health.

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