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Antifungal Activities of *Azardirachta indica* Crude Extracts and Fractions against Strain 1161, P37005 and RM1000

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Authors' contributions

This work was carried out in collaboration among all authors. Author ADMO designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors MG and NA managed the analyses of the study. Author SYD managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Azardirachta indica (neem) has served as an alternative medicine in the treatment of some common illnesses that have being associated to man. *Azardirachta indica* have proven effectively against certain fungi species that can infect human beings and cause disease. This study was aimed at determining the antifungal activities of *Azardirachta indica* crude extracts and fractions against certain *Candida albicans* strains: P37005, RM1000 and Strain1161. The reflux method was used for successive extraction of *Azardirachta indica* leaves which was done by three solvents namely: n-hexane, ethyl acetate and methanol. The leaves of *Azardirachta indica* was assayed for its phytochemicals components which was determined quantitatively. However, the various strains were subjected to the crude extracts of *Azardirachta indica* at a varying concentration of 40, 60, 80 and 100 mg/ml. The activity of n- hexane and methanol crude extracts had higher zones of inhibition than that of ethyl acetate crude extracts. The n- hexane crude extract showed a minimum inhibitory

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concentrations (MIC) and minimum fungicidal concentration (MFC) value of 12.5 mg/ml and 50 mg/ml respectively against the tested strain of P37005(Isolate B4). The value of 6.25 mg/ml and 50 mg/ml were also observed for the n- hexane crude extract against strain RM 1000(Isolate B2). Whereas, the methanol crude extract presented a value of 6.25 mg/ml and 100mg/ml respectively against strain RM1000(Isolate B2). More so, the antifungal activities of the fractions were determined at a concentration of 5mg/ml and 10 mg/ml which showed inhibition for all the strains. The n-hexane fraction F3 of *Azardirachta indica* had an MIC and MFC value of 12.5 mg/ml and 25 mg/ml against P37005(IsolateB4). The value for the methanol fractions(F6) of *Azardirachta indica* against strain 1161(Isolate S5) was 3.125 mg/ml and 6.25 mg/ml. It is obvious from this study that the antifungal activities of the crudes and fractions used were less in activity compared to the standard antibiotics(fluconazole).

Keywords: Azardirachta indica; fractions; bioactive; inhibition strains.

1. INTRODUCTION

Medicinal plants are plants that have bioactive components in them and are capable of therapeutic abilities especially in the treatment of common illnesses. *Azardirachta indica* is a plant that belongs to the Maliaceae family [1]. The Leaves are greenish and they are found more in a tropical environment. They grow faster and have a minimum height of 15-20mm [1]. The plant possesses various parts that include the leaves, fruit, bark, stem and root. *Azardirachta indica* (neem leaves) have been used locally to treat respiratory disorders, malaria and intestinal helminthosis. However, the traditional and Ayurvedic uses of *Azardirachta indica* involve the treatment of fever, leprosy and tuberculosis [2] Other remedies of neem include, antifeedant, antiseptic, diuretic, anthelmintic emmenagogue, pediculicide, parasiticide and insecticide. Its leaves have also been used in the treatment of skin infection especially treat acne, chicken pox etc [3]. They have also been used for the treatment of a large range of affliction and focus have being on every part of the tree because of its importance [3] more so, the leaves of neem plant can be used in inflammation of gums, gingivitis, boils etc. Other important product of neem plant like the oil can be used for female contraceptive and as well for treatment of vaginal infection. The oil can also serve for the eradication of mosquitoes. Neem oil are also useful in keeping skin elasticity. The tree have being used as a medicine for house hold against different human diseases [4]. In Nigeria, most especially the northern part of the country, *Azardirachta indica* are found most often and they are called with the native name Dogonyaro [5]. In India, it has been described as divine tree, nature drug store and as well village dispensary [6]. The Ayurvedic and traditional and uses of neem plant have proven effectively for the treatment of fever, leprosy, malaria and tuberculosis [6]. The twigs can also be used for brushing and also for dentalcare [6]. The plant has also been useful in treating people with chicken pox by which they sleep on it [4]. *Candida albicans* is a dimorphic fungus that grows both as yeast and filamentous cells and one of the few species of *Candida* that cause the infection candidiasis in humans [7]. *Candida albicans* have emerged as important causes of morbidity and mortality in immunocompromised patients (e.g., AIDS, cancer, chemotherapy, organ or bone marrow transplantation). *Candida albicans* represents the most common fungal pathogens that affects human and produce a wide spectrum of disease ranging from superficial mucocutaneous to invasive illness such as hepatosplenic candidiasis [7]. However, some of the drugs of choice that have being used for treatment have shown toxicity to the body and as well there is tendency for future resistance by this organism. More so, the economic predicament of most developing countries like Nigeria necessitate the search for new herbal drugs which can serve as an alternative source that are cheap, less toxic antimycotic that have structures and mode of action that are distinct from those of the current drugs in use.

1.1 Research Objectives

1. Isolate and identify *Candida albicans* from clinical samples
2. Screen quantitatively for phytochemical components of the selected crude plant extracts`
3. Determine antifungal activities of the crude extracts and fractions
4. Determine the minimum inhibitory/minimum fungicidal concentrations of the active crude extracts and fractions

Fig. 1. *Azardirachta Indica* Source: field photograph

2. MATERIALS AND METHODS

2.1 Collection of Plant Samples

The *Azardirachta indica*(neem) leaves used in this study were obtained from Maikunkele at Bosso Local Government, Niger state, Nigeria.

2.2 Test Organisms

2.2.1 Isolation of *Candida albicans*

2.2.1.1 Culturing on sabouraud dextrose broth (SDB)

High vaginal Swab (HVS) and urine samples were cultured each onto sabouraud dextrose broth supplemented with chloramphenicol to suppress the growth of bacteria and increase selectivity. The cultures were then incubated at room temperature for 72 hours [8].

2.2.1.2 Culturing on Sabouraud dextrose agar (SDA)

Candida isolates from sabouraud dextrose broth were furtherly sub cultured onto SDA containing chloramphenicol at room temperature for 72hours. Mixed culture were then subcultured onto freshly prepared SDA to have a pure culture [8].

2.3 Identification

2.3.1 Macroscopic examination

Cultural identification of the pure culture plates was based on colony colour and consistency of their colonies. This was then compared with known taxa of Candida species [9].

2.3.2 Microscopic examination

Microscopic examination was carried out to determine the morphology of the isolates.

2.3.2.1 Gram staining procedure

A sterile wire loop was used to transfer a small portion of the isolate onto a clean grease free slide containing a loopful of normal saline and a thin smear was made by emulsification. The smear was allowed to air dry and then heat fixed by passing the slide through a bursen flame two – three times. The smear was then flooded with crystal violet dye and allowed to stand for one minute. The slide was washed briefly with water. Grams iodine was added and allowed to stand for one minute. The slide was also washed with water and decolorized using 95% alcohol until no further colour came off. The alcohol was rinsed off immediately. The slide was finally stained using Safranin for two minutes before it was washed off with water and allowed to air dry. After it was airdried, oil immersion was added and then viewed under the objective lens (×100) of the compound microscope. When viewed the cells appeared purple/blue which was indicative of gram positive organisms and their morphology revealed were oval in shape [10].

2.4 Biochemical Test

The suspected yeast culture were subjected to sugar fermentation test (glucose, fructose, D-mannitol, Mannose, sucrose, galactose, maltose,

arabinose, lactose and sorbitol). Ten gram (10 gram) of each sugar was weighed together with 15 gram of peptone water, 0.4 gram of phenol red and dissolved into 1000 ml of distilled water. 10 ml of the solution was dispensed into tubes containing inverted vials known as Durham tubes. The test tubes were autoclaved at 121°C for 15 minute. After cooling the tubes were inoculated with the test organism (yeast isolate). A control test was also prepared (uninoculated) both the inoculated and uninoculated tubes were incubated at 28±2°C for 72 hours. The result was observed and recorded as positive or negative. Positive result indicated fermentation shown by colour change (acid production) and (gas production) which was determined by its accumulation in the Durham tubes while negative result showed the absence of acid and gas production [11]

2.5 Germ Tube Test

Germ tube test was carried out to differentiate the suspected yeast cells (*Candida albicans*) from other yeast cells. A Small inoculum of suspected yeast cultures was inoculated into 500 ul (0.5 ml) of human serum in a small test tube and incubated at 37°C for 3 hours. After incubation, a Pasteur pipette was used to transfer a drop of the serum yeast culture to a glass slide, overlaid with a cover slip and examined microscopically for the presence or absence of germ tubes [10].

2.6 Molecular Identification of Test Organisms

The molecular confirmation of the test organisms (*Candida albicans*) was carried out according to the method stated in Promega Technical Manual #TM050 (www.promegacorperation.com) [12]

2.7 Identification of Plant Materials (Leaves)

This research study focused on the leaves of *Azardirachta indica* plant. The leaves were thoroughly identified by the local herbal practitioners (kasuwan gwari) In Minna, Niger State Nigeria.

2.8 Authentication and Processing of Plant Material

The authentication of the leaves of *Azardirachta indica* was carried out by Mr. Lateef Akeem in the Herbarium Department of National institute for pharmaceutical research and Development (NIPRD), Idu Abuja with the following voucher numbers (NIPRD/H/6879). The identified and authenticated plant materials were rinsed with distilled water in order to remove dirt. After washing they were dried at room temperature away from sunlight for a period of 21days. The leaves were ground to particles with a mortar and pestle and further reduced by an electric blender. The ground samples were sieved with filter paper in order to derive fine powdered particle. The fine particles were kept at amber bottles under room temperature for further processes.

2.9 Extraction Protocol

Three different solvents namely: n-hexane, ethyl acetate and methanol were used for the extraction of the pulverized samples of *Azardirachta indica* plant using the reflux extraction protocol. Thou a successive method which employs its extraction from a non-polar solvent to a polar solvent. In this method, 100gram of the ground particles were measured in a weighing balance and further mixed into a 400ml of an extracting organic solvent inside a round bottom flask of 500 ml. The n- hexane solvent which began the extraction first was added in a ratio of 1:4 of the ground samples. The round bottom flask was placed onto the heating mantle while the position of the flask that was opened was connected to a condenser. The source of power was on to supply the required electricity while the temperature was regulated to 30°C. This process was refluxed for 2 hours, the refluxed mixture was filtered and concentrated to a semi solid substance with the aid of a rotary evaporator at 40°C and further dried with water bath at a temperature of 60°C. The concentrate was kept in an amber bottle and finally placed in the refrigerator for further procedure. The residue was stored at room temperature for 45minute. The ethyl acetate which serves as the second solvent was used in succession, the method continues until the last solvent was used after the entire extraction process was over the weight of the crude extract was measured and recorded [13]. The percentage yield of the crude extract was calculated using the formula below.

$$\text{Percentage yield (\%)} = \frac{\text{Weight of extract} \times 100}{\text{Weight of sample (dry plant material)}}$$

2.10 Quantitative Phytochemical Methods Used for the Determination of the Extracts

2.10.1 Flavonoid

Aluminium chloride colorimetric procedure was employed for flavonoid estimation which was described by Chang et al. [14].

2.10.2 Total phenol

The total phenol determination of the crude extract was carried out as described by Singleton et al. [15]

2.10.3 Total alkaloid

Total alkaloid determination was carried out by the method described by Oloyed [16].

2.10.4 Determination of Saponin

Saponin determination was carried out by the method described by Oloyed [16].

2.10.5 Determination of Tannin

Determination of tannin was done by the method described by Emmanuel et al. [17].

2.10.6 Phytic acid content

The phytic acid content was determined using a modified indirect colorimetric method of Wheeler and Ferrel [18].

2.10.7 Determination of Oxalate

Oxalate was determined by permanganate titrimetric method as described by Oke [19].

2.11 Determination of the Antifungal Activity of the Crude Extracts

2.11.1 0.5 McFarland Standard and standardization of isolates

The isolates population were determined by the McFarland turbidity standard [20]. The standard was prepared by mixing zero point zero five milliliter (0.05 ml) of 1% $Bacl_2$ together with 9.95 ml of 1% H_2SO_4 in a clean test tube. Absorbance of the mixture (white precipitate) was measured at 530 nm by the aid of Shidmazu UV spectrophotometer UV – 1800. The Standardization of the isolates were carried out by the method described by Magaldi et al. [21] with slight modification. The isolates were cultured onto a freshly prepared sabouraud dextrose agar at room temperature for 72 hours. A loopful of the isolates (*Candida albicans*) were transferred into 9 ml of sterile sabouraud dextrose broth. A Serial dilutions of 10^{-1} – 10^{-7} was prepared and their absorbance was measured at 530 nm with the aid of Shimadzu UV spectrophotometer. After this process were carried out, the absorbance obtained were compared with the 0.5Mcfarland standard obtained previously. Dilution that were corresponding to the 0.5Mcfarland optical density (absorbance) was used as the standardized organism.

2.12 Extracts and Fractions Concentration

The extracts of n- hexane, ethyl acetate and methanol were measured to achieve 200mg, 300 mg, 400 mg and 500 mg each, they were dissolved in 5 ml each of 10% Dimethyl sulfoxide to obtain a concentration of 40 mg/ml, 60 mg/ml, 80 mg/ml and 100 mg/ml respectively [22]. The fractions were obtained using standard chromatography methods as described by Fair and Kosmos [23]. The concentration of the fractions was determined by measuring 10 mg and 20 mg of each fraction obtained and dissolved in 2 ml of 10% DMSO to give a concentration of 5 mg/ml and 10 mg/ml [22].

2.13 *In vitro* Determination of the Antifungal Activity of the Crude Extracts

The antifungal activity of the crude extracts was performed by the Agar Well Diffusion method as described by Magaldi et al. [21]. The media used for this process were prepared according to the manufacturer's instruction. The freshly prepared sabouraoud dextrose agar (SDA) was inoculated-by a Loopful of the standardized test organism using the spread plate method. A sterile rod spreader was used to obtain a uniform growth, wells was made using 6 mm sterile cock borer and labelled properly. 100 ul(0.1 ml) of the crude extracts of different concentrations of 40, 60, 80, & 100 mg/ml was placed into each of the separate wells using a micropipette. They were left for 30 minutes to 1hour for pre-diffusion before incubation at room temperature for 72 hours. Img/ml of fluconazole (standard antibiotics) served as the positive control which was done by placing 100 ul(0.1 ml) of the standard antibiotics into the well and the culture were left for 30 minutes, the negative control was carried out by measuring 100 ul of DMSO free

from extract which was placed into SDA that contain the standardized organism. The positive and negative control plates were incubated at room temperature for 72hours. Their diameters were measured with the aid of a meter scale rule. All the experiment was performed in triplicates and their mean values together with the corresponding standard deviation were recorded. The zones of inhibition with value reading of ≤10 was recorded as resistant while value reading of >10 was recorded as sensitive [24].

2.14 *In vitro* Determination of the Antifungal Activity of the Fractions

The antifungal activity of the fractions was performed using the Agar Well Diffusion method as described by Magaldi et al. [21].

2.15 *In vitro* Determination of the Minimum Inhibitory Concentration and Minimum Fungicidal Concentration of the Crude Extracts and the Fractions

The determination of the minimum inhibitory concentration and minimum fungicidal concentrations of the crude extracts and fractions were done by the Tube Dilution Method described by Ewansiha et al. [22]

2.16 Statistical Analysis

The statistical analysis was carried out using SPSS VERSION 20. The data were expressed as mean ±standard deviation, they were analyzed using the one-way ANOVA method. Differences in values were expressed as significant at $P<0.05$.

3. RESULTS

3.1 Gram Staining, Biochemical Behaviour and Germ Tube Results of the Fungal Strains (*Candida albicans*)

The result showed the gram reaction, biochemical and germ tube tests that were carried out to confirmed the identity of the three fungal strains as shown in Table 1. This was done in accordance with the method described by Cheesbrough [10,11].

3.2 Molecular Characteristic of the Strains (*Candida albicans*)

All the strains used for this study were confirmed using molecular method which is shown in (Table 2) After the molecular analysis, they were sequenced BLAST to know their various identities and ascension number from the gene bank via NCBI website [25]. The ascension number of the strains are: Isolate S5: Strain1161 (AFO75293.1), Isolate B4: P37005 (AP023893.1) and Isolate B2: RM1000 (AB_017634.2)

3.3 Percentage Yield of *Azardirachta indica* Leaf Crude Extract

The percentage yield of *Azardirachta indica* leaf crude extract is shown in Table 3. *Azardirachta indica* leaves had a percentage yield of 7.12%, 2.61% & 6.29% from the extracts of NHLE, EALE and MLE respectively. The NHLE showed the highest percentage of 7.12% and the lowest was 2.61% from EALE.

3.4 Quantitative Phytochemical Analysis of the Leaf Crude Extracts of *Azardirachta indica*

Table 4 shows the quantitative analysis of n-hexane, ethyl acetate and methanol leaf crude extract of *Azardirachta indica* . The crude extract of n- hexane (NHLE) of *Azardirachta indica* had alkaloid (183.44± 0.64) as the highest value, the least was oxalate with a value of 2.46±0.58). Other phytochemical present were flavonoid (61.66±0.58), phenols (130.81±0.58), tannins (56.66±0.58), saponins (90.00±0.58) and phytic acid (2.14±0.58).

3.5 Antifungal Activity of *Azardirachta indica* Leaf Crude Extracts (40-100 mg/ml) against Strain P37005 (Isolate: B4)

Table 5 represent the antifungal activity of *Azardirachta indica* leaf crude extract against P37005(Isolate B4). The extract of n-hexane leaf (NHLE) had no inhibitory activity at a vary concentrations of 40 mg/ml and 60 mg/ml. At 80 mg/ml and 100 mg/ml there was inhibitory activity with a mean zone of inhibition of 8.00±0.20 mm and 12.67±2.08 mm respectively. There was no inhibitory activity for the ethyl acetate leaf extract for all the varying concentrations used (40 mg/ml, 60 mg/ml, 80 mg/ml, and 100 mg/ml) for the methanol leaf extract but at concentrations of 80 mg/ml and 100 mg/ml there was inhibition and the mean zone of inhibition was 6.67± 1.52mm and 8.27± 0.50 mm) respectively.

Table 1. Gram reaction, biochemical behaviour and germ tube test conducted for the fungal strains (*Candida albicans*)

S/N	Isolate code	Gram reaction	Shape	Fermentation									Assimilation									
				Glucose	Fructose	Sorbitol	D-mannito	Lactose	Sucrose	Mannose	Arabinose	Galactose	Glucose	Fructose	Sorbitol	D-mannito	Lactose	Sucrose	Mannose	Arabinose	Galactose	Germtubetest	Inference
1	S1	+	Oval	+	+	-	-	-	-	-	-	+	+	+	-	-	-	+	+	-	+	+	*Candida albican*
2	S2	+	Oval	+	+	-	-	-	-	-	-	+	+	+	-	-	-	+	+	-	+	+	*Candida albican*
3	S3	+	Oval	+	+	-	-	-	-	-	-	-	+	+	-	-	-	+	+	-	+	+	*Candida albican*

Table 2. Molecular Characteristics of the Strains (*Candida albicans*)

Isolate code	Strains	Max score	Total score	Query cover	Expected value	Identity	Ascension number
B4	P37005	7542	5850	100%	0.0	100%	AP023893.1
B2	RM1000	700	8811	100%	0.0	100%	AB_017634.2
S5	Strain 1161	8000	9822	100%	0.0	100%	AF075293.1

Table 3. Percentage yield of *Azardirachta indica* (neem) leaf

	Leaf			
Plant sample	**WS/DP(g)**	**NHLEg(%)**	**EALEg(%)**	**MLEg(%)**
Azardirachta Indica	100	7.12(7.12)	2.61(2.61)	6.29(6.29)

Key: WS/DP: Weight of sample/ weight of dried powered material, NHLE: n-hexane leaf extract, EALE: Ethylacetate leaf extract, MLE: Methanol leaf extract

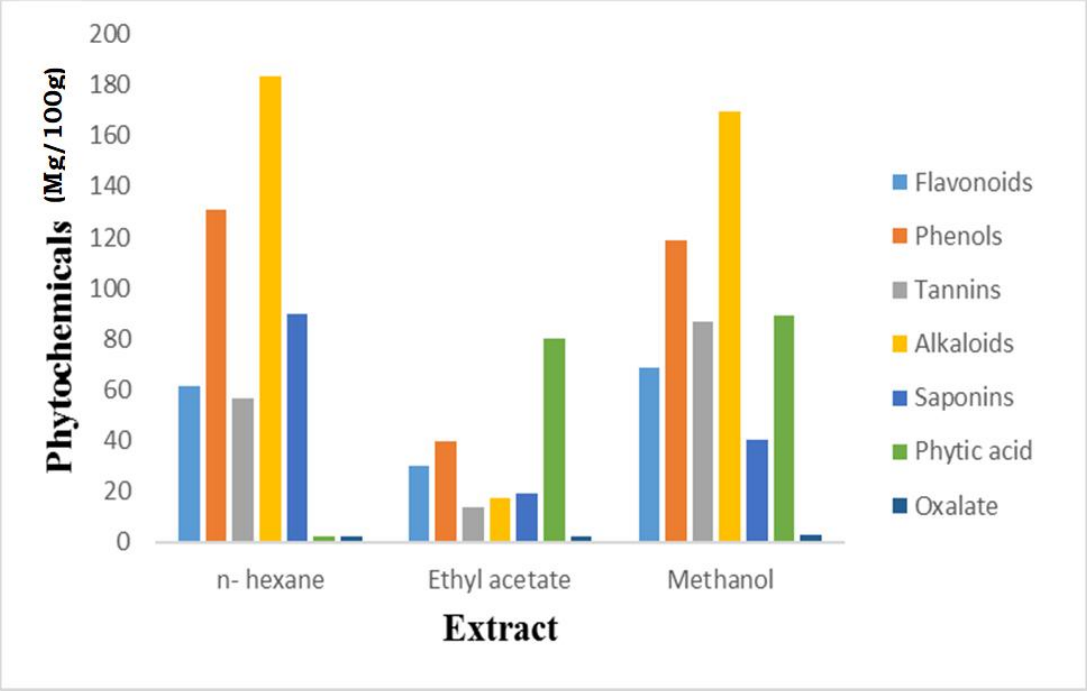

Fig. 2. Quantitative phytochemical analysis of the leaf of *Azardirachta Indica* (Neem)

3.6 Antifungal Activity of *Azardirachta indica* Leaf Crude Extracts (40-100 mg/ml) against Strain RM1000 (Isolate: B2)

Table 6 represent the antifungal activity of *Azardirachta indica* leaf extract against strain RM1000(Isolate B2). The extract of n- hexane leaf had no inhibitory activity at a concentration of 40 mg/ml but at 60 mg/ml, 80 mg/ml and 100 mg/ml concentrations there was inhibitory activity and their zone of inhibition were 7.00±0.20 mm, 11.00±1.00 and 16.00±1.00 mm respectively. There was no activity for the ethyl acetate leaf extract at all the varying concentrations used. At 40 mg/ml and 60mg/ml concentrations, there was no activity for the methanol leaf extract while there was inhibitory activity at 80 mg/ml and 100mg/ml concentrations. There means zone of inhibition was 6.00± 0.20 mm and 11.00±0.70 mm respectively.

3.7 Antifungal Activity of *Azardirachta indica* Leaf Crude Extract (40- 100 mg/ml) against Strain 1161 (Isolate: S5)

Table 7 represent the antifungal activity of *Azardirachta indica* crude extract against strain 1161 isolate: S5). The extracts of n-hexane leaf (NHLE) showed no inhibitory activity at 40 mg/ml concentration but at 60 mg/ml, 80 mg/ml and 100 mg/ml concentrations. There was inhibitory activity and there mean zone of inhibition were 7.00±0.02 mm, 9.00±0.70 mm and 10.00±2.08 mm respectively. There was no inhibitory activity for the methanol leaf extract at 40 mg/ml and 60mg/ml but at 80 mg/ml and 100 mg/ml there was activity and the mean zone of inhibition was 6.00±0.20 mm and 9.00±0.70 mm respectively.

3.8 Results of the Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) of the Active Crude Extracts

The values of the MIC and MFC of the active crude extracts is presented in Table 8. The n-hexane crude extract had values of 12.5 mg/ml and 50 mg/ml respectively for their MIC and MFC against strainP37005(Isolate: B4). However, Values of 6.25 mg/ml and 50 mg/ml was also observed for the n-hexane crude extract against strain RM1000(isolate B2). Where as the methanol crude extract presented a value of 6.25 mg/ml and 100 mg/ml respectively against strain RM1000 (isolate B2)

Table 4. Quantitative phytochemical screening of *Azardirachta indica* leaf crude extracts obtained using different solvents

	Phytochemicals (mg/100g)						
Extracts	**Flavonoids**	**Phenols**	**Tannins**	**Alkaloids**	**Saponins**	**Phytic acid**	**Oxalate**
n- hexane	61.66±0.58^{b}	130.81±0.58^{b}	56.66±0.58^{b}	183.44±0.64^{c}	90.00±0.58^{c}	2.14±0.58^{a}	2.46±0.58^{b}
Ethylacetate	30.32±0.58^{a}	39.96 ±0.58^{a}	13.68±0.58^{a}	17.50±0.64^{a}	19.11±0.58^{a}	80.11±0.58^{b}	2.39±0.58^{a}
Methanol	68.61±0.58^{c}	118.51±0.58^{c}	86.80±0.58^{c}	169.80±0.64^{b}	40.20±0.58^{b}	89.36±0.58^{c}	2.87±0.58^{c}

Results are expressed as mean ± standard deviation for triplicate determination. Values with the same superscript letter on the same column do not differ significantly at p≤0.05

Table 5. Antifungal activity of *Azardirachta* indica leaf crude extract (40-100mg/ml) against strain P37005 (Isolate: B4)

	Leaf		
Conc. (mg/ml)	**n-hexane**	**Ethyl acetate**	**Methanol**
40	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.10^{a}
60	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.10^{a}
80	8.00±0.20^{b}	0.00±0.00^{a}	6.67±1.52^{b}
100	12.67±2.08^{c}	0.00±0.00^{a}	8.27±0.50^{c}
Fluconazole (1mg/ml)	40.00±0.80^{d}	40.00±0.80^{b}	40.00±0.80^{d}
DMSO(100ul)	0.00.±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}

Results are expressed as mean ± standard deviation for triplicate determination. Values with the same superscript letter on the same column do not differ significantly at p≤0.05

Table 6. Antifungal activity of *Azardirachta indica* leaf crude extract (40-100mg/ml) against strain RM1000 (Isolate: B2)

	Leaf		
Conc. (mg/ml)	**n-hexane**	**Ethyacetate**	**Methanol**
40	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.10^{a}
60	7.00±0.20^{b}	0.00±0.00^{a}	0.00±0.10^{a}
80	11.00±1.00^{c}	0.00±0.00^{a}	6.00±0.20^{b}
100	16.00±1.00^{d}	0.00±0.00^{a}	11.00±0.70^{c}
Fluconazole (1mg/ml)	37.00±0.40^{e}	37.00±0.40^{b}	37.00±0.40^{d}
DMSO(100ul)	0.00.±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}

Results are expressed as mean ± standard deviation for triplicate determination. Values with the same superscript letter on the same column do not differ significantly at p≤0.05

Table 7. Antifungal activity of *Azardirachta indica* leaf crude extract (40-100mg/ml) against strain1161 (Isolate: S5)

	Leaf		
Conc. (mg/ml)	**n-hexane**	**Ethylacetate**	**Methanol**
40	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]
60	7.00±0.02[b]	0.00±0.00[a]	0.00±0.00[a]
80	9.00±0.70[c]	0.00±0.00[a]	6.00±0.20[b]
100	10.00±2.08[d]	0.00±0.00[a]	9.00±0.70[c]
Fluconazole (1mg/ml)	42.00±0.40[e]	42.00±0.40[b]	42.00±0.40[d]
DMSO(100ul)	0.00.±0.00[a]	0.00±0.00[a]	0.00±0.00[a]

Results are expressed as mean ± standard deviation for triplicate determination. Values with the same superscript letter on the same column do not differ significantly at p≤0.05

Table 8. Minimum inhibitory concentration (MIC) and Minimum fungicidal concentration (MFC) of the active crude extracts

S/N0	Isolate code	Strain	Plant part	Crude extracts	MIC	MFC
1	B4	P37005	Leaf	n – hexane	12.5 mg/ml	50 mg/ml
2	B2	RM1000	Leaf	n-hexane	6.25 mg/ml	50 mg/ml
3	B2	RM1000	Leaf	Methanol	6.25 mg /ml	100 mg/ml

3.9 Results of the Activity of n- hexane Fractions of *Azardirachta indica* Leaf against Strain 1161, P37005, RM1000

Antifungal activity of *Azardirachta indica* is presented in Table 9. The activity of fraction F3 showed zone of inhibition 9.00±0.40 mm and 11.00±0.78 mm at concentrations of 5 mg/ml and 10 mg/ml respectively against strain P37005, fraction F3 also showed inhibitory activity at the same concentrations against strain RM1000, the zones of inhibition were 6.00±0.20 mm and 7.00±0.20 mm respectively. Fraction F7 showed inhibitory activity 6.00±0.2 mm and 9.00±0.40 mm at concentrations of 5 mg/ml and 10 mg/ml respectively against strain 1161, there was also activity 7.00±0.10 mm and 8.00±0.10 mm against strain P37005 at the concentrations of 5 mg/ml and 10 mg/ml respectively. The activity of fraction F7 also showed inhibitory activity 7.00±.0.30 mm and 9.50±0.50 mm against strain RM1000 at concentrations of 5 mg/ml and 10 mg/ml. Other fractions showed no inhibitory activity against the tested strains although, the positive control (fluconazole 1 mg/ml) tested against the strains showed a varying zone of inhibition. The activity of the fraction F3 and F7 were less compared to the activity of the positive control. The negative control (DMSO) used against the tested strains showed no inhibitory activity.

3.10 Results of the Activity of Ethyl Acetate Fractions of *Azardirachta indica* Leaf against Strain 1161, P37005, RM1000

Antifungal activity of *Azardirachta indica* is presented in Table 10. The activity of fraction F1 showed zone of inhibition 8.00±0.30 mm and 9.00±0.30 mm at a concentrations of 5 mg/ml and 10 mg/ml respectively against strain 1161, fraction F6 showed inhibitory activity at the same concentrations against strain P37005, the zones of inhibition were 7.00±0.20 mm and 10.00± 0.60 mm respectively. Fraction F7 showed inhibitory activity 6.00±0.20 mm and 9.00±0.56 mm at concentrations of 5mg/ml and 10mg/ml respectively against strain 1161, there was also activity 6.00±0.40 mm and 8.00±0.50 mm against strain RM1000 at the concentrations of 5 mg/ml and 10 mg/ml respectively. The activity of fraction F7 also showed inhibitory activity 7.00±0.10 mm and 9.00±0.30 mm against strain RM1000 at concentrations of 5 mg/ml and 10mg/ml. Other fractions showed no inhibitory activity against the tested strains although, the positive control (fluconazole 1 mg/ml) tested against the strains showed a varying zones of inhibition. The activity of fraction F1, F6 and F7 were less compared to the activity of the positive control. The negative control (DMSO) used against the tested strains showed no inhibitory activity.

Table 9. Results of the activity of n- hexane fraction of *Azardirachta indica* leaf against strain 1161, P37005 and RMI000

		F1		F2		F3		F4		F5		F6		F7			
Isolate	**Strain**	**5 mg/ml**	**10 mg/ml**	**5 mg/ml**	**10 mg/ml**	**5 mg/ml**	**10 mg/ml**	**5 mg/ml**	**10 mg/ml**	**5 mg/ml**	**10 mg/ml**	**5 mg/ml**	**10 mg/ml**	**5 mg/ml**	**10 mg/ml**	**Fluconazole (1 mg/m)**	**DMSO (100ul)**
S5	strain1161	0.00± 0.00^{a}	0.00 ± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	6.00± 0.2^{b}	9.00± 0.40^{c}	42.00±0.3^{d}	0.00±0.00^{a}
B4	P37005	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	9.00± 0.40^{b}	11.00± 0.78^{c}	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	7.00± 0.10^{d}	8.00± 0.10^{d}	40.00±0.60^{e}	0.00±0.00^{a}
B2	RM1000	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	6.00± 0.20^{b}	7.00± 0.20^{b}	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	7.00± 0.30^{c}	9.00± 0 .50^{d}	37.00±4.00^{e}	0.00±0.00^{a}

Results are expressed as mean ± standard deviation for triplicate determination. Values with the same superscript letter on the same row do not differ significantly at p≤0.05

Table 10. Results of the activity of ethyl acetate fractions of *Azardirachta indica* leaf against strain 1161, P37005and RM1000

		F1		F2		F3		F4		F5		F6		F7			
Isolate	**Strain**	**5 mg/ml**	**10 mg/ml**	**5 mg/ml**	**10 mg/ml**	**5 mg/ml**	**10 mg/ml**	**5 mg/ml**	**10 mg/ml**	**5 mg/ml**	**10 mg/ml**	**5 mg/ml**	**10 mg/ml**	**5 mg/ml**	**10 mg/ml**	**Fluconazole (1mg/m)**	**DMSO (100ul)**
S5	Strain1161	8.00± 0.30^{b}	9.00± 0.30^{b}	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	6.00± 0.20^{c}	9.00± 0.56^{d}	42.00±0.40^{e}	0.00±0.00^{a}
B4	P37005	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	7.00± 0.20^{b}	10.00± 0.60^{c}	0.00± 0.00^{a}	0.00± 0.00^{a}	40.00±0.80^{d}	0.00±0.00^{a}
B2	RM1000	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	6.00± 0.40^{b}	8.00± 0.50^{c}	7.00± 0.10^{d}	9.00± 0.30^{e}	37.00±0.40^{f}	0.00±0.00^{a}

Results are expressed as mean ± standard deviation for triplicate determination. Values with the same superscript letter on the same row do not differ significantly at p≤0.05

Table 11. Antifungal activity of methanol fraction of *Azardirachta indica* leaf against strain 1161, P37005 and RM1000

		F1		F2		F3		F4		F5		F6		F7			
Isolate	**Strain**	**5 mg/ml**	**10 mg/ml**	**5 mg/ml**	**10 mg/ml**	**5 mg/ml**	**10 mg/ml**	**5 mg/ml**	**10 mg/ml**	**5 mg/ml**	**10 mg/ml**	**5 mg/ml**	**10 mg/ml**	**5 mg/ml**	**10 mg/ml**	**Fluconazole (1 mg/m)**	**DMSO (100 ul)**
S5	Strain116	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	8.00± 0.40^{b}	12.00± 0.60^{c}	0.00± 0.00^{a}	0.00± 0.00^{a}	42.00±0.40^{d}	0.00±0.00^{a}
B4	P37005	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	9.00± 0.30^{b}	11.00± 0.60^{c}	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	40.00±0.42^{d}	0.00±0.00^{a}
B2	RM1000	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	6.00± 0.50^{c}	7.00± 0.80^{b}	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	37.00±0.40^{d}	0.00±0.00^{a}

Results are expressed as mean ± standard deviation for triplicate determination. Values with the same superscript letter on the same row do not differ significantly at p≤0.05

Table 12. Minimum Inhibitory and Minimum Fungicidal Concentrations of active fractions (mg/ml)

S/NO	Isolate	Strains	Medicinal plant	Plant part	Fractions	MIC (mg/ml)	MFC (mg/ml
1	B4	P37005	*Azardirachta indica*	Leaf	F3	12.5	25
2	S5	Strain1161	*Azardirachta indica*	Leaf	F6	3.125	6.25

3.11 Antifungal Activity of Methanol Fraction of *Azardirachta indica* Leaf against Strain 1161, P37005, RM1000

Antifungal activity of methanol fraction of *Azardirachta indica* is presented in Table 11. The activity of Fraction F3 showed zones of inhibition 9.00±0.30 mm and 11.00±0.60 mm at concentrations of 5 mg/ml and 10 mg/ml against strain P37005, fraction F3 also showed inhibitory activity 6.00±0.50 mm and 7.00±0.80 mm against strain RM1000. Fraction F6 showed inhibitory activity 8.00±0.40 mm and 12.00±0.60 mm at the same concentrations of 5 mg/ml and 10mg/ml against strain 1161. Other fractions showed no inhibitory activity against the tested strains, although, the positive control (fluconazole 1 mg/ml) used against the tested strain showed a varying zone of inhibition. The activity of fraction F3 and F6 were less compared to the activity of the positive control. The negative control (DMSO) used against the tested strains showed no inhibitory activity.

3.12 Minimum Inhibitory Concentrations (MIC) and Minimum Fungicidal Concentrations (MFC) of Active Fractions

The values of the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of the active fractions is shown in Table 12. The MIC and MFC values for the n-hexane fraction F3 of *Azardirachta indica* were 12.5 mg/ml and 25 mg/ml against strain P37005 (isolate B4). The methanol fraction(F6) of *Azardirachta indica* against strain 1161(isolate S5) showed a value of 3.125 mg/ml and 6.25 mg/ml for their respective MIC and MFC values.

4. DISCUSSION

This research study was aimed at determining the antifungal activities of *Azardirachta indica* crude extracts and fractions against strain P37005, RM1000 and Strain 1161. The various strains were confirmed by biochemical and molecular characterization as presented in (Table 1-2). The extraction of the leaves of *Azardirachta indica* was carried out using three different solvents namely: n- hexane, ethyl acetate and methanol with various polarity index. The various percentage yield of the crude extracts of *Azardirachta indica* is presented in (Table 3). The highest percentage yield was observed in n- hexane leaf extract (NHLE) while ethyl acetate was the least with a percentage occurrence of 2.61%. The methanol leaf extract (MLE) showed a percentage yield of 6.29%. The variation could be as a result of the solubility of the different components in them [22]. However, the quantitative phytochemical analysis of the various crude extracts is presented in (Table 4). The quantitative phytochemical analysis was carried out to quantify the bioactive substances present which would be an indicator to measure the level of bioactivity. This phytochemicals present in the leaf extract of *Azardirachta indica* were principally the source of its bioactivity. Alkaloids, saponin and tannin are good groups that are essentials in bioactivity. The results from the phytochemical analysis showed the presences of Phenol, Saponin, alkaloids, tannin, phytic acid and oxalate. The presence of these phytochemicals is similar to the work that was carried out by Mahmoud et al. [26] Alkaloid was the highest with a value of 183.44±0.64. In the n-hexane crude extract, oxalate was the least with a value of 2.46± 0.58 thus in trace amount. Methanol crude extract also had alkaloid (169.80± 0.64) as the highest in value while oxalate (2.87± 0.58) was the least in amount. Phytic acid (80.11±0.58) was the highest value of ethyl acetate and oxalate (2.87.58) was the least in amount. The presence of this bioactive substance indicate their medicinal value and their capability to prevent some infections that could affect man. These bioactive substances can also be useful in the treatment of diseases. Some of the phytochemicals like alkaloids which are natural products that are produced by different kinds of organisms like plant, animal, fungi and bacteria etc. Alkaloids have antihypertensive, antarrhythmic, anticancer and antimalarial properties, they also function against heart disease and cancer by enhancing the body defense against pathology induced free radicals generation. Phenols possess certain properties that are useful to man. These properties include antiulcer, anti-inflammatory, antioxidant, cytotoxic properties, antiplasmodic etc [26] However, the tanning bioactive substance also serves as antiseptic, anti-inflammatory, antioxidant and haemotic pharmaceuticals. The saponin content possess secondary metabolite that can serve as a good form in water solution such as soap. They also serve as phytoprotectants [27,28]. The antifungal activity of *Azardirachta indica* leaf crude extracts against the three different strains used in this study is presented in (Table 5-7). The n- hexane and methanol leaf extract of *Azardirachta indica* showed inhibitory activity at different concentrations against the strains used.

The value of 16.00±1.00 mm was the highest value recorded for n- hexane leaf extract at a concentration of 100 mg/ml against strain RM1000 (isolate: B2) which might suggest that there are more or sufficient bioactive components in the n- hexane leaf extracts (NHLE). Although there zone of inhibition increased as the concentration were increased, the activity were concentration dependent. Furthermore, Prescott et al. [29] reported the inhibitory activity of an antimicrobial agent to be concentration dependent. Edeoga et al. [24] stated that the inhibitory activity of a plant differs with the kind of extracting solvents used. This finding is in agreement with the work of Mahmud et al. [26] Simhadri et al. [30] The n-hexane and methanol crude extracts showed a better inhibitory activity against the various strains than the ethyl acetate extract, this may be as a result of sufficient amount of bioactive substance that were present in them than that of the bioactive substance in ethyl acetate. It could also be that the both (n- hexane and methanol) are solvents that support good extraction of the active principles of *Azardirachta Indica* plant. It is obvious from this study that the higher the amount of bioactive substance the higher the inhibitory activity. The values for the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) are presented in (Table 8). The result obtained from the MIC and MFC differs from a similar work done by [30] this could be due to the disparity in laboratory procedure and reagent used during experiment, geographical area of plant and as well the kind of extraction procedure [31]. However, the activity of n-hexane, ethyl acetate and methanol fractions of *Azardirachta indica* is presented in (Table 9-11). All the fractions (n- hexane, ethyl acetate and methanol) showed inhibitory activity at a varying concentration of (5 mg/ml and 10 mg/ml) against the tested strains. The activity of the fractions were also increased as the concentrations were increased. The methanol fraction showed the highest zone of inhibition with a value of 12.00±0.00 mm at a concentration of 10mg/ml against strain 1161. Most of the inhibitory activity were seen in the n- hexane fraction of *Azardirachta indica* (fraction F3 and F4) which were active against all the strains. Methanol fraction of *Azardirachta indica* (F3 and F6) were also active against all the strains (Table 11). The ethyl acetate fractions showed little activity which was seen in fraction (F1, F6 and F7) against the various strains (Table 10). The fractions were less in inhibitory activity than that of the crude extracts, activity of the crude extracts showed a better inhibitory activity in terms of the zones diameter compared to the fractions. Although, after fractionation using column and tin layer chromatography thou a partial purification, it is expected that the fractions should be pure and refined which should enhance the activity of the fractions to have a better or higher zone of inhibition compared to the crude extract. The activity of the crude extracts could be due to synergistic effect [32], perhaps if the fractions are further purified it may have a better activity than the crude extracts.

5. CONCLUSION

This research study has shown noticeable phytochemical properties of *Azardirachta indica* leaf crude extracts. The presence of this bioactive substance could be a major reason for its antifungal activity. From this study, it is clear that the leaves of *Azardirachta indica* crude extract and fractions inhibited the growth of the different strains that were subjected for trials. The activity of the n-hexane and methanol crude extracts showed better zones of inhibition against the different strains. The phytochemical presents could be a determinant of their bioactivity. However, alkaloids had a content of 132.11 mg/100 g which was seen as the highest value followed by phytic acid which had 160.78 mg/100 g. Oxalate was seen as the least with an amount of 5.65 mg/100 g in n-hexane crude extract. The ethyl acetate presented phytic acid 45.63 mg/100 g followed by phenol which had 38.69 mg/100 g while oxalate was seen as the least with amount of 4.55 mg/100 g. The methanol had phenol 178.92 mg/100 g as the highest followed by the tannin content which was 98.92 mg/100 g and oxalate was the least with 5.30 mg/100 g. The Minimum inhibitory concentration (MIC) of the extracts and fractions showed a varying concentrations against the tested strains. Although, which was low at that concentration to inhibit the growth of the tested strains. It can be deduced from this study that the lower the MIC values the better the activity of the plant. However, the ethyl acetate crude extract showed no inhibitory activity. Although, the fraction of n- hexane, ethyl acetate and methanol had activity against all the listed strains. Activity of the positive control (fluconazole1 mg/ml) showed zones of inhibition that were higher than that of the crude extracts and fractions.

6. RECOMMENDATION

The fractions obtained from this study should be further purified to have a higher inhibitory activity further more active principles obtained should be characterized.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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