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Quantitative Phytochemical Analysis and Antifungal Susceptibility of *Azardirachta indica* against Some Strains of *Candida albicans*

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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 SYD and NA managed the analyses of the study. Author MG managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

This research work was focus on determining the quantitative phytochemical analysis and antifungal susceptibility of *Azardirachta indica* against some strains of *Candida albicans*. The leaves of *Azardirachta indica* was susccessively extracted using reflux method of extraction. Quantitative phytochemical screenings were done to determine the amounts of phytochemical that are present in the crude extracts. The result showed that the extract of *Azardirachta indica* (neem) possessed active principles which include: Alkaloids, saponins, flavonoids, phenols and tannin which were present in the crude extracts. The antifungal activity of the extracts was assayed against three different strains (P37005, RM1000 & SC5314) The antifungal susceptibility test of the crude extracts against the strains were determined at different concentrations of 40, 60, 80 and 100 mg/ml using the agar well diffusion method. The n- hexane and methanol crude extract showed inhibitory activity compared to that of ethyl acetate that had no inhibitory activity. The highest mean zone of inhibition (MZI) was 15.00±1.00 mm which was recorded for n-hexane leaf extract at a concentration of 100 mg/ml against strain RM1000 (isolate: S2). Although the standard antibiotics

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(Fluconazole) used in this study showed a much higher zone of inhibition than that of the n-hexane and methanol crude extract. The MIC and MFC value for n-hexane crude extracts were 12.5 mg/ml and 50 mg/ml respectively against strain P37005 (Isolate:S1)likewise, the n-hexane crude extract had a value of 6.25 mg/ml and 50 mg/ml. However, the methanol crude extract showed a value of 6.25 mg/ml and 100 mg/ml respectively against SC5314 (isolate:S3).

Keywords: Azardirachta indica; MIC; susceptibility; MFC; antifungal; crude extract; phytochemical.

1. INTRODUCTION

Azadirachta indica (Neem tree) is a tree in the mahogany family Meliaceae is evergreen tree found in most tropical countries. It is one of two species in the genus Azadirachta, native to India and Burma, growing in tropical and semi-tropical regions. It is a fast growing tree, average height 15-20 m but rarely to 35-40 m. It is evergreen but under severe drought it may shed most or nearly all of its leaves. For thousands of years the beneficial properties of neem have been recognized in the Indian tradition [1]. Each part of the neem tree has some medicinal property [2]. Almost every part of the tree has been in use since ancient times to treat a number of human ailments and also as a household pesticide [3]. The extract from bark, leaves, fruits and root have been used to control leprosy, intestinal helminthiasis and respiratory disorders in children [4]. The bark extract is also used as tonic, and useful in relieving fever, thirst, nausea, vomiting and skin diseases [5]. The immune modulatory activity of the neem bark extract has also been reported [6]. The medicinal and industrial uses of various parts of neem tree and the compounds isolated have been reviewed [7]. The bark powder contains sugar, proteins, amino acids and oil [8]. Polysaccharides such as a rabinogalactans and fucogalactoglucoarabinanes have also been isolated from neem bark [9]. Flavonoids, flavone glycosides, dihydro-chalcones, tannins and others are also important constituents of bark, leaves, fruits and flowers of neem [10]. European “Materia Medica” have acknowledged neem tree as “Panacea of all Disease”. However, in the Northern part of Nigeria, *Azardirachta indica* have been natively called Dogonyaro. In India it is famous with many other names like 'Divine Tree', "Heal All", "Nature's Drugstore" and "Village Dispensary". Traditional and Ayurvedic uses of neem include the treatment of fever, leprosy, malaria and tuberculosis. Various folk remedies use as an anthelmintic, antifeedant, antiseptic, diuretic, emmenagogue, contraceptive, febrifuge, parasiticide, pediculicide and insecticide [11]. Traditional uses of twigs for brushing teeth as effective forms of dental care. Neem oil is useful for skin care such as acne, and keeping skin elasticity. Traditionally, patients suffering from Chicken Pox sleep on the leaves in India owing to its medicinal value [3]. In Ayurvedic, Unani and folklore traditional medicine, different parts of neem were preferred in the treatment of a wide range of afflictions. Every part of the tree has been used as traditional medicine for household remedy against various human ailments.

Fig. 1. *Azardirachta indica*
Source: Field photograph

2. MATERIALS AND METHODS

2.1 Collection of Plant Materials

The Leaves of *Azardirachta indica* (neem) was collected from Maikunkele in Bosso local Government, Niger state.

2.2 Collection and Identification of Test Organisms

Three strains of *Candida albican*: SC5314, P37005 and RM1000 were collected from Federal Institute for Industrial Research Oshodi (FIIRO), Lagos, Nigeria. They were subjected to sugar fermentation test as described by Makeri et al. [11]. Germ tube test was carried out to identify and differentiate them from other species which was done according to John Thorne et al.

[12]. Their molecular characterization was also carried out to confirm the identity of the strains [13].

2.3 Identification, Authentication and Processing of the Selected Medicinal Plants

Fresh sample of the plant material (leaves) for this study were identified by the local herbal practitioners in Minna, Niger state while authentication of the plant sample was done by Mr. Lateef Akeem of the Herbarium Department of National Institute for Pharmaceutical Research and Development (NIPRD), Idu, Abuja with the following voucher numbers: (NIPRD/H/6879) and the voucher specimens were deposited in the same Herbarium Department of the Institute. The identified and authenticated medicinal plant part (leaves) were washed with distilled water to get rid of dirt. The washed leaves, were air dried under shady environment (away from sunlight). The air-dried leaves was separately grounded with the aid of a mortar and pestle and subsequently pulverized into powdered using an electric blender. The pulverized samples was further sieved with a 150µm pore size filter to obtain a fine powdered-like texture, stored in amber bottles and kept in a cool dried environment under room temperature until it was required for usage.

2.4 Extraction Procedure

The grounded plant samples (Pulverized samples) were subjected to reflux extraction according to [14] to obtain the crude extracts. The extraction was carried out beginning from non-polar solvent to polar solvent (n-hexane, ethyl acetate and methanol). 100 g of the of the plant samples was weighed and dissolve in 400 ml of the extracting solvent in a round bottom flask of 500 ml capacity. Starting with n-hexane (polarity index = 0.1p') was gradually added until a ratio of 1:4 of the pulverized samples to the extracting solvent was attain. The flask containing the mixture was then placed on the heating mantle and the opening of the flask was connected to the condenser. The power source was then switched on to supply heat and the temperature was controlled (adjusted) to 30°C. The mixture was allowed to reflux for 2hours. After refluxing, the mixture was filtered using muslin cloth and later with whatman No 1 filter paper with pore size 20 µm to obtain a clear filtrate and further concentrated to a semi solid substance with the use of a rotary evaporator at a reduced temperature of (40°C) and then dried using water bath at 60°C. The extract was then stored in an air tight amber bottle and kept in the refrigerator for further analysis. The Marc (residue) was dried at room temperature for 45 minute and was extracted with the next solvent in increasing polarity (further in succession using ethyl acetate with polarity index=4.4p'). This procedure was repeated using the last solvent and the weight of the extracts for all the solvents used were measured and recorded accordingly. Percentage yield of each of the crude extract was calculated using the formulae below:

Percentage yield (%) = Weight of extract × 100 / Weight of sample (dry plant material)

2.5 Quantitative Phytochemical Estimation of the Crude Extracts

2.5.1 Determination of flavonoid

Aluminium chloride colorimetric method was used for flavonoid determination. A 0.5 ml (1 mg/ml) of the plant crude extract was mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminium chloride, 0.1 ml of 1M sodium acetate and 2.8 ml of distilled water and kept at room temperature for 30minute. The absorbance of the reaction mixture was taken at 415nm with a double beam Shimadzu UV spectrophotometer, UV -1800. The calibration curve was prepared by using quercetin solutions at concentration of 12.5 to 100 g/ml in methanol [15].

2.5.2 Determination of total phenol

The total phenol content of the crude extract was determined according to the method described by Chang et al. [16]. A 0.5 ml (1 mg/ml) was oxidized with 2.5 ml of 10% Folin- Ciocalteau's reagent (v/v) and neutralized by 2 ml of 7.5% sodium carbonate. The reaction mixture was incubated for 40 minutes at 45°C and the absorbance was taken at 765 nm using the double beam Shimadzu UV spectrophotometer, UV- 1800. The total phenol content was subsequently calculated using Gallic acid as standard.

2.5.3 Determination of total alkaloid

A 0.5 g of the crude extract was mixed with 5 ml of 96% ethanol -20% H_2SO4 in ratio (1:1) and filter. 1ml of the filtrate was added to 5 ml of 60% H2SO4, the mixture was allowed to stand for 5 minute and 5ml of 0.5% of formaldehyde solution

was added and allowed to stand for 3hours. The absorbance was taken at a wave length of 565 nm using Shimadzu UV spectrophotometer, UV-1800. The concentration of alkaloid in the sample was calculated using the molar extinction coefficient of vincristine, ε = 15136 mol/cm [17].

2.5.4 Determination of saponin

A 0.5 g of the crude extract was mixed with 20 ml of 1MHCL and the mixture was boiled for 4 hours and allowed to cool. After cooling and filtered, 50 ml of petroleum ether was added to the filtrate, for ether layer and evaporated to dryness. 5 ml of acetone- ethanol (1:1) were added to the residue, 6 ml ferrous sulphate reagent and 2 ml of concentrated H_2SO4. The mixture was homogenized and allowed to stand for 10 minutes before the absorbance was taken at 490 nm using Shimadzu UV spectrophotometer UV-1800 [17].

2.5.5 Determination of tannin

A 0.2 g of the extract was weighed into a 50 ml beaker; 20 ml of 50% methanol was added, covered with para film and placed in a water bath at 80ºC for one hour. The mixture was shaken thoroughly and the content was transferred into a 100 ml volumetric flask. 20 ml of water, 2.5 ml of 10% Folin Denis reagent and 10 ml of 17% Na_2CO_3 was added and mixed thoroughly. The mixture was allowed to stand for 20minute. Observation for bluish green colouration was done at the end of range 12.5-100 ug/ml of tannic acid. The absorbance of tannic acid standard solution as well as sample was taken after colour development on a spectrophometer at wave length of 760nm using Shimadzu UV-spectrophometer, UV-1800 [18].

2.5.6 Phytic acid content

The phytic acid content was determined using a modified indirect colorimetric method of Emmanuel et al. [19]. The method depends on an iron phosphorous ratio of 4:6 and is based on the ability of standard ferric chloride to precipitate phytate in dilute HCL extract of the sample. 5 g of the sample was extracted with 20 ml of 3% trichloroacetic acid and filtered. 5 ml of the filtrate was used for the analysis; the phytate was precipitated as ferric phytate and converted to ferric hydroxide and soluble sodium phytate by adding 5 ml of 1 M NaOH. The precipitate was dissolved with hot 3.2M HNO_3 and the absorbance immediately at 480 nm. Preparation of standard curve for phytic acid was done as follows: Standard curve of different Fe $(NO_3)_3$ concentration was plotted against the corresponding absorbance of spectrophotometer to calculate the ferric iron concentration. The phytate phosphorous was calculated from the concentration of ferric iron assuming 4:6 Iron: phosphorous molar ratio.

2.5.7 Determination of oxalate

Oxalate was determined by permanganate titrimetric method as described by Wheeler and Ferrel [20]. Two gram (2gram) of the crude extract was suspended in 190 ml of distilled water in 250 ml volumetric Flask, 10 ml of 6M HCL was added and the suspension digested at 100°C for 1hour, cooled, then made to the mark before filtration. Duplicate portion of 125 of the filtrate were measured into beakers and four drops of methyl red indicator added. This is followed by the addition of concentrated NH_4OH solution drop wise until the test solution changes from salmon pink colour to a faint yellow colour (pH 4-4.5). Each portion is then heated to 90°C and 10 ml of 5% $CaCl_2$ solution added while being stirred constantly. After heating, it was cooled and left overnight at 5°C. The solution was then centrifuged at 2500 rpm for 5minutes, the supernatant decanted and the precipitate completely dissolved in 10 ml of 20% (v/v) H_2SO_4 solution. The total filtrate resulting from the digestion was made up to 300 ml aliquots of 125 ml of the filtrate was heated until near boiling and the titrated against 0.05M standardized $KMno_4$solution to a faint pink colour which persisted for 30seconds. The calcium oxalate content is calculated using the formula below:

$$T \times (Vme)(Df) \times 10^5 \text{ (mg/100 g)} / (ME) \times Mf$$

Where T is the titre of $KMnO_4$ (ml), Vme is the volume- mass equivalent (1 cm^3 of 0.05M $KNnO_4$ solution is equivalent to 0.00225 anhydrous oxalic acid), Df is the dilution factor V_T/A(2.5 where V_T is the total volume of titrate (300 ml) and A is the aliquot used(125 ml), ME is the molar equivalent of $KMnO_4$ in oxalate($KMnO_4$ redox reaction) and Mf is the mass of extract used.

2.6 Antifungal Activity of the Crude Extracts

2.6.1 Preparation of 0.5 McFarland standard

The population of the isolate was determined from the McFarland turbidity standard [21]. Zero point zero five milliliter (0.05 ml) of 1% $Bacl_2$ was

mixed thoroughly with 9.95 ml of 1% H_2SO_4 in a test tube. The absorbance of the mixture (white precipitate) was determined at 530 nm

2.6.2 Standardization of the test organisms

The Standardization of the test organisms was carried out as described by McFarland [22] with slight modification. The test organisms were cultured on sabouraud dextrose agar at room temperature for 24-72 hours. A loopful of the cultured organism (*Candida albicans*) was transferred into 9 ml of sterile sabouraud dextrose broth. Serial dilutions of $10^{-1} – 10^{-7}$ was made and the absorbance was determined at 530 nm using Shimadzu UV spectrophotometer. The optical density (absorbance) was compared with the optical density (absorbance) of 0.5Mcfarland standard previously obtained. The dilution corresponding to that of the 0.5 Mcfarland standard was used as the standard organism which gives a population of 1.5×10^3 cfu/ml [22].

2.6.3 Preparation of extract concentration

Two hundred milligram (200 mg), 300 mg 400 mg and 500 mg of the normal hexane, ethyl acetate and methanol extract was weighed and dissolved in 5 ml each of 10% Dimethyl sulfoxide (DMSO) to give a concentration of 40 mg/ml, 60 mg/ml, 80 mg/ml and 100 mg/ml concentrations respectively [23].

2.6.4 Determination of the antifungal activity of the crude extracts

The susceptibility test was carried out using Agar Well Diffusion Method as described by McFarland [22]. Sabouraud dextrose agar (SDA) was prepared according to the manufacturer's instruction. The prepared SDA was then inoculated with a loop full of the standardized test organism by the spread plate method using a sterile rod spreader to obtain uniform growth, wells was made using 6 mm sterile cork borer and labelled accordingly. 100 µl (0.1 ml) of the prepared crude extract of varying concentration(40, 60, 80 and 100 mg/ml) was transferred into each of the wells with a micropipette and allowed to stand for 30 minutes to 1 hour for pre- diffusion and then incubated at room temperature for 24-72 hours..100µl of 10% DMSO (free from extract) was transferred into a freshly prepared SDA (containing the test organism) to serve as negative control. Fluconazole (1 mg/ml) was used as the positive control. This was achieved by transferring 100 µl of the prepared standard antibiotics into the well and cultures were allowed to stand for 30minute after which they were incubated at room temperature for 24-72 hours. The zones of inhibition (ZI) was measured using a meter scale rule. The experiment was done in triplicates and the mean values with the corresponding standard deviation of the inhibition zone diameter (IZD was recorded. Crude extract that measured zone of inhibition ≤10 was recorded as resistant while >10 was recorded for sensitivity [24].

2.6.5 Determination of Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) crude extract

The MIC was determined using the Tube Dilution Method as described by Magaldi et al. [23]. A fourfold serial dilution of the most active crude extracts was carried to give a decrease in concentration from 100, 50, 25, 12.5, 6.25, 3.125, 1.25 and 0.78 mg/ml. The stock solution was prepared by dissolving 800 mg of the extract in 4 ml of 10% dimethylsulphoxide (200 mg/ml). Two millilitres (2 ml) of the stock concentration was transferred to a test tube labeled a containing fresh 2 ml sabouraud dextrose broth (SDB) to give a concentration of 100 mg/ml. From tube A, 2 ml will be transferred into a second tube labeled B containing 2 ml SDB to give a concentration of 50 mg/ml. This procedure continues until a concentration of 0.78 mg/ml was obtained in the last test tube labeled H. They were properly shaken to obtain a homogenous mixture and all test tubes were inoculated with 0.1 ml of the standardized test organism. Two separate test tubes containing sterile broth plus 10% DMSO and sterile broth plus test organism was prepared for negative and positive controls respectively. All test tubes were incubated at room temperature for 24 to 72 hours, after which the test tubes were compared with each control tubes. The concentration/tube without visible turbidity was taken as the MIC. The MFC was determined by subculturing from the MIC tube and other tubes that showed no turbidity onto freshly prepared SDA and incubated at room temperature for 24 to 72 hours. The concentration that showed no visible growth after incubation was taken as the MFC

2.7 Statistical Analysis

The data are presented as mean ±standard deviation; all data were analyzed by one way

Table 1. Biochemical characteristics and germtube test conducted for the fungal strains (*Candida albicans*)

				Fermentation									Assimilation									
S/N	**Isolate code**	**Gram reaction**	**Shape**	**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*

ANOVA. Differences were considered significant at $P \leq 0.05$. The analyses were carried out using Statistical Package for Social Science (SPSS) version 20.

3. RESULTS

3.1 Biochemical Characteristics and Germ Tube Test Conducted for the Fungal Strains (*Candida albicans*)

The result showed the gram reaction, biochemical and germ tube tests that was carried out to confirmed the identity of the three fungal strains as shown in Table 1. This was done in accordance with [12].

3.2 Molecular Characterization of the Fungal Strains (*Candida albicans*)

The results from the molecular analysis confirmed the identity of the strains which is represented in Table 2. The sequenced BLAST results revealed the following identities of the fungal strains (*Candida albican's*) and their accession numbers which were determined from the GENE bank through the NCBI web site [13]. Their corresponding ascension numbers were as follows: Isolate S1: P37005 (AP023893.1), Isolate S2: RM1000 (AB_017634.2) and Isolate S3: SC5314 (CP025163.1).

3.3 Percentage Yield of the Leaf Crude Extract of *Azardirachta indica*

Table 3 represents the percentage yields of the leaf crude extracts of *Azardirachta indica*. The milled plant samples were extracted with n – hexane, ethyl acetate and methanol. The leaves of *Azardirachta indica* had a percentage yield of 7.12%, 2.61% and 6.29% which were obtained in NHLE, EALE and MLE respectively. NHLE had the highest percentage yield of 7.12% and the lowest yield 2.61% was obtained in EALE.

3.4 Quantitative Phytochemical Determination of *Azardirachta indica* Leaf Crude Extracts Obtained Using Different Solvents

Table 4 shows the results obtained from the quantitative determination of n- hexane leaf extract(NHLE), ethyl acetate leaf extract(EALE) and Methanol leaf extract(MLE) of *Azardiracta indica*. The n- hexane leaf extract (NHLE) of *Azardiracta indica* had alkaloids (183.44±0.64) as the highest in amount while oxalate was present in trace amount (2.46± 0.58). Others present were flavonoid(61.66±0.58), phenols (130.81±0.58), tannins (56.66±0.58) and saponins (90.00±0.58). The ethyl acetate leaf extract (EALE) of *Azardirachta indica* had phytic acid(80.11±0.58) as the highest in amount while oxalate (2.39±0.58) was the lowest. Others were flavonoid(30.32±0.58), phenols (39.96±0.58), tannins (13.68±0.58), alkaloids (17.50±0.64) and saponins (19.11±0.58). In addition, methanol leaf extract (MLE) had alkaloids (169.80±0.64) as the highest in amount while oxalate (2.87±0.58) was still the lowest in amount. Others were flavonoid (68.61±0.58), phenols (118.51±0.58), tannins (86.80±0.58), saponins (40.20±0.58) and phytic acid (89.36± 0.58).

Table 2. Molecular characterization conducted for the fungal strain (*Candida albicans*)

Isolate code	Strains	Max score	Total score	Query cover	Expected value	Identity	Ascension number
S1	P37005	7542	5850	100%	0.0	100%	AP023893.1
S2	RM1000	700	8811	100%	0.0	100%	AB_017634.2
S3	SC5314	234	5243	100%	0.0	100%	CP025163.1

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

Plant sample	Leaf			
	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

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

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

Values are means ± standard deviation of triplicate values. Means with dissimilar letter (s) differ significantly according to the least significant different at p≤0.05

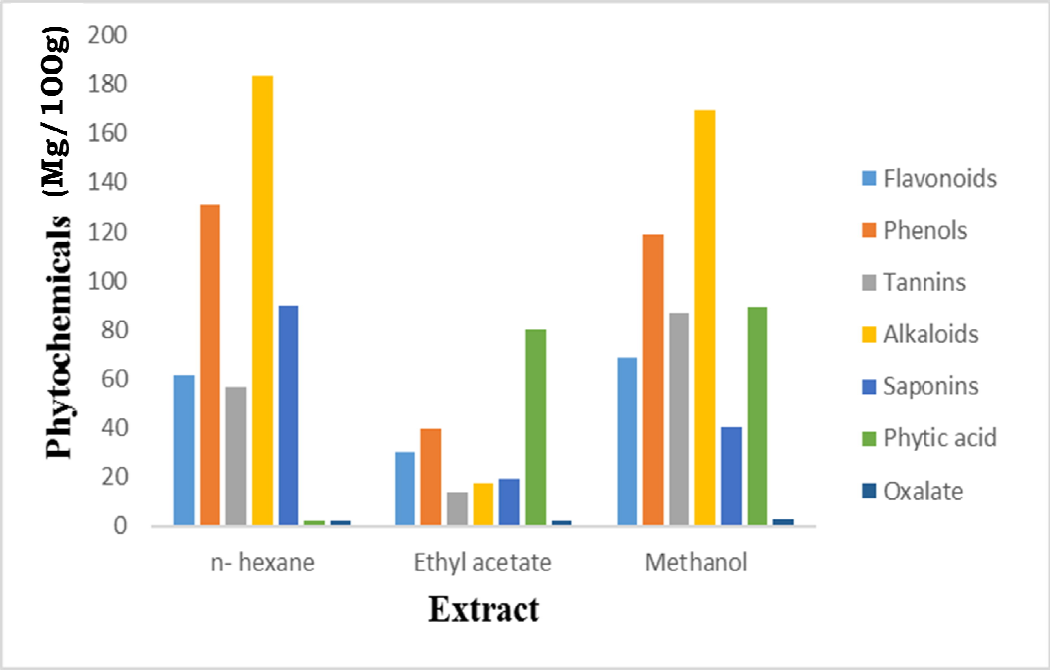

Fig. 2. Quantitative phytochemical screening of *Azardirachta indica* leaf crude extracts obtained using different solvents

Table 5. Antifungal susceptibility of the leaf crude extracts of *Azardirachta indica* 40-100 mg/ml against strain P37005 (Isolate: S1)

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

Values are means ± standard deviation of triplicate values. Means with dissimilar letter (s) in the same column differ significantly according to the least significant different at p≤0.05

3.5 Antifungal Susceptibility of the Leaf Crude Extracts of *Azardirachta indica* against Strain P37005 (Isolate: S1)

Table 5 showed the antifungal activity of the leaf of *Azardirachta indica* against P37005 (Isolate: S1). The n-hexane leaf extract (NHLE) showed no inhibitory activity at 40 and 60 mg/ml concentrations but there was inhibitory activity and the mean zone of inhibition (MZI) was 8.00±0.20 mm and 12.67±2.08 mm at 80 and 100 mg/ml respectively. Ethyl acetate leaf extract (EALE) showed no inhibitory activity at 40. 60.80 and 100 mg/ml concentration. The methanol leaf extract (MLE) also did not show inhibitory activity at 40 and 60 mg/ml but there was inhibitory activity and the mean zone of inhibition (MZI) was 6.67±1.52 mm and 8.27± 0.50 mm at 80 and 100 mg/ml respectively.

3.6 Antifungal Susceptibility of the Leaf Crude Extracts of *Azardirachta indica* against Strain RM1000 (Isolate: S2)

Table 6 showed the antifungal activity of the leaf, of *Azardirachta indica* against strain RM1000 (Isolate: S2). The n-hexane leaf extract (NHLE) showed no activity at 40 mg/ml concentrations but there was inhibitory activity and the mean zone of inhibition (MZI) was 7.00±0.20 mm,

Table 6. Antifungal susceptibility of the leaf of *Azardirachta indica* 40-100 mg/ml against strain RM1000 (Isolate: S2)

Conc. (mg/ml)	Leaf		
	n-hexane	Ethyacetate	Methanol
40	0.00±0.00[d]	0.00±0.00[b]	7.00±0.10[d]
60	0.00±0.00[d]	0.00±0.00[b]	8.00±0.10[d]
80	8.00±0.20[c]	0.00±0.00[b]	10.00±0.70[c]
100	13.00±0.70[b]	0.00±0.00[b]	15.33±1.23[b]
Fluconazole (1 mg/ml)	42.00±0.40[a]	42.00±0.40[a]	42.00±0.40[a]
DMSO(100 ul)	0.00.±0.00	0.00±0.00[b]	0.00±0.00[d]

Values are means ± standard deviation of triplicate values. Means with dissimilar letter (s) in the same column differ significantly according to the least significant different at p≤0.05

Table 7. Antifungal susceptibility of the leaf crude extract of *Azardirachta indica* 40-100 mg/ml against strain SC5314 (Isolate: S3)

Conc. (mg/ml)	Leaf		
	n-hexane	Ethyacetate	Methanol
40	0.00±0.00[e]	0.00±0.00[b]	7.00±0.20[e]
60	7.67±0.09[d]	0.00±0.00[b]	8.00±0.76[d]
80	8.00±0.76[c]	0.00±0.00[b]	10.00±0.60[c]
100	9.00±0.70[b]	0.00±0.00[b]	13.00±0.70[b]
Fluconazole (1 mg/ml)	38.00±0.60[a]	38.00±0.60[a]	38.00±0.60[a]
DMSO(100 ul)	0.00.±0.00[e]	0.00±0.00[b]	0.00±0.00[f]

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

S/No	Isolate code	Strain	Plant part	Crude extracts	MIC	MFC
1	S1	P37005	Leaf	n – hexane	12.5 mg/ml	50 mg/ml
2	S2	RM1000	Leaf	n-hexane	6.25 mg/ml	50 mg/ml
3	S3	SC5314	Leaf	Methanol	6.25 mg /ml	100 mg/ml

11.00.±1.00 mm and 15.00±1.00 mm at 60, 80 and 100 mg/ml concentrations. Ethyl acetate leaf extract (EALE) showed no inhibitory activity at 40, 60, 80 and 100 mg/ml concentration. The methanol leaf extract showed no activity at 40 and 60mg/ml concentration but there was inhibitory activity and the mean zone of inhibition (MZI) was 6.00±0.20 mm and 9.00± 0.70 mm at 80 and 100 mg/ml respectively.

3.7 Antifungal Susceptibility of the Leaf Crude Extracts of *Azardirachta indica* against Strain SC5314 (Isolate: S3)

Table 7 showed the antifungal activity of the leaf of *Azardirachta indica* against strain SC5314 (Isolate: S3). The n-hexane leaf extract (NHLE) showed no activity at 40 mg/ml concentrations but there was inhibitory activity and the mean zone of inhibition (MZI) was 7.00±0.67 mm, 8.00±0.76 mm and 9.00±0.70 mm at 60, 80 and 100 mg/ml concentrations. Ethyl acetate leaf extract (EALE) showed no inhibitory activity at 40. 60.80 and 100 mg/ml concentration. The methanol leaf extract showed no inhibitory activity at 40 and 60 mg/ml but there was inhibitory activity and the mean zone of inhibition (MZI) was 10.00±0.60 mm and 13.00± 0.70 mm at 80 and 100 mg/ml respectively.

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

The result of the Minimum inhibitory concentration (MIC) and Minimum fungicidal concentration (MFC) of the most active crude extracts is shown in Table 8. The MIC and MFC values for the most active crude extracts were 12.5 mg/ml and 50mg/ml for the n-hexane crude extract against strain P37005 (Isolate :S1), the n-hexane crude extract against RM1000 (isolate:S2) had a value of 6.25 mg/ml and 50

mg/ml. however, the methanol crude extract showed a value of 6.25 mg/ml and 100 mg/ml respectively against SC5314(isolate:S3).

4. DISCUSSION

The strains used in this study were confirmed using biochemical and molecular characterization as shown in (Tables 1-2). The leaves of *Azardirachta indica* were extracted using three different solvents namely n- hexane, ethyl acetate and methanol with different polarity index. The percentage yield of the leaf of *Azardirachta indica* is shown in (Table 3). The n-hexane leaf extract (NHLE) of *Azardirachta indica* had the highest percentage yield of 7.12%. Ethyacetate extract (EAE) was the lowest with a percentage yield of 2.16% while methanol leaf extract (MLE) had a percentage yield of 6.29%. The differences in percentage (%) might be due to the fact that each solvents used for extraction could have different volatility just like n-hexane that evaporate rapidly and as well the solubility of the different components could also affect the percentage yield of the crude extract [25]. The quantitative phytochemical analysis of *Azardirachta indica* crude extract is shown in (Table 4). Some of them present were alkaloid, phenols, flavonoid, saponin, tannin, phytic and oxalate. These phytochemicals present in *Azardirachta indica* leaf crude extract have been reported in a similar work by Oke and Aslim [26]. The quantitative phytochemical analysis indicated alkaloid (183.44± 0.64) as the highest in amount for the n-hexane crude extract while oxalate (2.46 ± 0.58) was present in trace amount. The ethyl acetate showed phytic acid(80.11±0.58) as the highest in amount while oxalate(2.39±0.58) was also the least in amount. However, the methanol crude extract also showed alkaloid (169.80±0.64) as the highest in amount while oxalate (2.87±0.58) was also the least in amount. These phytochemicals present in medicinal plants are responsible for preventing infections and also useful in treating diseases, their bio activity has shown promising pharmacological actions. The presence of these phytochemicals in good quantity is an indication of their medicinal potency. Alkaloids for example are natural product that contains heterocyclic nitrogen atoms with basic properties. Alkaloids are naturally synthesized by a large number of organisms including animals, plant, bacteria and fungi [27]. They are useful in the survival and protection of plant against microorganisms. Alkaloids have different pharmacology activities that include antihypertensive, antiarrhythmic effect (quinidine and sparein), anticancer actions and even antimalarial activity [28] other phytochemicals like flavonoids and phenols possess antioxidant potentials. Flavonoid have been shown to inhibit the initiation, promotion and progression of tumors [29]; reduction of coronary heart disease has been reported to be associated with intake of flavonoids [30]. Apart from the antioxidant properties of flavonoid, other biological functions it possesses include protection against platelet aggregation, microorganisms hepatotoxins, viruses, tumors, ulcers, free radicals, inflammation and allergies [31]. Phenol or phenolic compound are the largest category of phytochemicals and the mostly widely distributed in the plant kingdom [32]. Phenols have several properties that are beneficial to humans especially their antioxidant properties that are important in determining their role as protecting agents against free radical mediated disease processes. Various biological activities of phenol have also been reported. On the order hand the tannin contents of plant are good anti-inflammatory, antiseptic antioxidant and homoestatic pharmaceuticals. Further more Saponins consist of a group of secondary metabolite that are form a stable foam in acqueous solutions such as soap. Saponins may be describe as one of the most important part of plant defense system which have been included in a large group of protective molecules found in plants that are describe as phytoanticipins or phytoprotectants [33]. Tables 5-7 shows the antifungal activity of the leaf of *Azardirachta indica* against the three different strains (P37005, RM1000and SC5314). There was inhibitory activity for n- hexane leaf extract(NHLE) and Methanol leaf extract (MLE) for *Azardiracta indica* at different concentrations for all the strains. The highest value was 15.00±1.00mm which was recorded for n-hexane leaf extract at a concentration of 100mg/ml against strain RM1000 (Isolate:S2). The zone of inhibition (ZOI) increased as the concentrations were increased [34] reported that the activity of antimicrobial agent is concentration dependent in addition, Ewansiha et al. [24] reported that the inhibitory zones of a plant may varies with the type of solvent used for extraction. This result is in agreement with the work of Mahmoud et al. [35] and also in conformity with the work of Simhadri et al. [36] who reported the inhibitory activity of the leaf of *Azardirachta indica* against *Candida albicans*. The inhibitory activty of n-hexane and methanol crude extracts of *Azardirachta indica* might be due to the presence of higher concentration of phytochemicals

(bioactive substance) and probably the n-hexane and methanol could be good solvents that support the inhibitory activity of this test strains compare to that of the ethyl acetate crude extract. The concentration of bioactive substance are good determinant for microbial susceptibility. When the concentration of a bioactive substance is high, they might be better possibility of a higher and better zones of inhibition (ZOI). The result of the antifungal activity of the standard drug (fluconazole1 mg/ml) used in this study showed a better zone of inhibition as compared to that of the crude. Although this might be expected since this drug is in a pure and well refined form. The result of the negative control Dimethylsulphur oxide (DMSO) showed no inhibitory activity against the standardized test strains. This is suggestive that DMSO does not contain any antimicrobial agent that is capable of acting against the test strains. Table 8 shows the values of the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) that were obtained. The MIC and MFC values were different from a similar work done by Ewansiha et al. [24]. The differences might be due to geographical location of the plant, differences in laboratory procedures and reagent used [37]. Season of the plant, age of the plant and method of extraction which may affect the yield and the bioactive components of the plant [38]. The result from this study confirms the claims by local marketers and consumers of the use of the leaf either singly or in combination for the treatment of candidiasis.

5. CONCLUSION

The results of this research work reveals appreciable phytochemical properties in the leaf crude extract of *Vernonia amygdalina* which might explain it potency against the disease causing Microorganism. It is obvious from this study that the leaves of *Azardirachta indica* showed inhibitory activity against the various tested strains that they were subjected. The n-hexane and methanol crude extract were bacteriocidal in their activity against the tested strain. Although, the ethyl acetate showed no inhibitory activity. However, the positive control (fluconazole1mg/ml) used in this study had a better zone of inhibition (ZOI) compared to the crude extract obtained. The crude extracts obtained from this study should be further purified to obtain pure fractions which may enhance a better inhibitory activity and as well active components showed be characterized.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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