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Antifungal Activities of *Vernonia amygdalina* Crude Extracts and Fractions against Strain1161, 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

This research study was focused in determining antifungal activities of the crude extracts and fractions of *Vernonia amygdalina* against strain 1161, P37005 and RM1000. *Vernonia amygdalina* leaves were extracted by the reflux extraction protocol which was done in a successive method. This study also analysed quantitatively the phytochemical that were present in the crude leaf extracts of *Vernonia amygdalina*. The study identified the presence of tannin, flavonoid, alkaloids, saponin and phenols. The three strains used in this study were tested for their susceptibility, However, activity of the crude extract was assayed at a varying concentration of 40, 60, 80 and 100 mg/ml. The methanol leaf extract (MLE) at a concentration of 100mg/ml showed the highest zones of inhibition 15.33± 1.23 mm against strain 1161(isolate S5). The result of the minimum inhibitory concentration (MIC) and minimum fungicidal concentration showed for active crude extracts were 12.5 mg/ml and 50 mg/ml presented for the n- hexane crude extract against strain 1161(IsolateS5). The value for the methanol crude extract were 12.5 mg/ml and 50 mg/ml against strain 1161 (Isolate: S5). Furthermore, the value of 12.5 mg/ml and 100mg/ml were showed for n- hexane crude extract against P37005 (isolate: B4) However, The antifungal activity of the fractions against the

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strains were determined at a concentrations of 5mg/ml and 10mg/ml. The various fractions of *Vernonia amygdalina* showed inhibitory activity against all the strains.16.00± 0.0mm was the highest value that was presented for n-hexane fraction of *Vernonia amygalina* against strain 1161 and P37005. The result of the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) for active fractions (F1) are 12.5 mg/ml and 25 mg/ml showed for n- hexane fraction of *Vernonia amygdalina* against strain P37005(Isolate:B4). The value of 12.5 mg/ml and 25 mg/ml were showed for n- hexane fraction (F4) of *Vernonia amygdalina* against strain 1161 (isolate: S5). Fraction (F4) showed 3.125 mg/ml and 6.25 mg/ml against strain P37005 (isolate: B4). The methanol fraction (F1) showed values of 3.125 mg/ml and 25 mg/ml respectively against strain RM1000(isolate B2). Fraction F6 had values of 3.125 mg/ml and 6.25 mg/ml against strain P37005 (isolate: B4). It can be deduced from this study that the n- hexane and methanol crude extracts showed a significant result than the ethyl acetate crude extracts. Furthermore, there was inhibitory activity for the n-hexane, ethyl acetate and methanol fractions against all the tested strains.

Keywords: Concentration; activity; isolate; zones fungicidal.

1. INTRODUCTION

Biological action forms the basis in herbal traditional medicine in which they exploit the natural compounds found in them that are used for treating diseases [1]. Pharmacologically, Medicinal plants are plants that have or possess substances that have the ability to treat illnesses or produce alternative drugs that can be used to alleviate symptoms of some diseases [2]. Plants are a good inexpensive source of drugs that possess therapeutic efficacy like their orthodox drugs counterpart [3]. Medicinal plant extracts and their products screening for antimicrobial and antioxidant is an indicator to show that they are primary origins of antibiotics [1]. *Vernonia amygdalina* belong to the Asteraceae family, the shrubs grow in tropical Africa to a height of 2-5m (6.6-16ft) [4]. In Nigeria, *Vernonia amygdalina* is commonly called bitter leaf in English and called ''Ewuro'' in Yoruba ''Shika'' in Hausa and in Igbo is called Olubo [5]. The leaves can be eating as vegetable and condiments, they can be soaked and washed properly to eliminate bitterness [6]. The presence of the bitter taste could be traced to the phytochemicals in them [4]. These phytochemicals are alkaloids, saponins, tannins and glycosides. Various property of *Vernonia amygdalina* have also been discovered especially its ability as antihelmitic, antimalarial, antitumourigenic, hypoglycemic and hypolipidaemic [3]. *Vernonia amygdalina* have being reported to treat stomach disorder, fever symptoms, cough, antiparasitic property and can also be used as anticancer and antibacterial. *V. amygdalina* contains active chemical structure that have pharmaceutical importance. Traditionally, it has being reported that *Vernonia amygdalina* can be used in blood clothing and can also lower blood glucose level [5].

Fig. 1. *Vernonia amygdalina*
Source: Field photograph

2. MATERIALS AND METHODS

2.1 Plant Materials

The leaves part of *Vernonia amygdalina* were taken from areas of Maikunkele in Bosso Local Government, Niger State, Nigeria.

2.2 Test Organisms

The references strains used in this study were isolated by the conventional method as described by [7] their identity were confirmed using molecular analysis [8,9]. The strains for this research study were: P37005, RM1000 & Strain 1161.

2.3 Identification, Authentication and Processing of Plant Materials

The leaves used in this research study were identified in Minna, Niger State by the local herbal practitioners. Authentication of sample

were carried out in the Herbarium Department of National Institute for Pharmaceutical Research and Development (NIPRD), Idu, Abuja by Mr. Lateef Akeem. They were assigned voucher number: (NIPRD/H/6872) and the respective voucher specimens were placed in the Herbarium Department of the institute. The medicinal plants parts(leaves) were washed carefully with distilled water to remove dirt. They were placed in a shady environment (away from sunlight). The dried leaves of the medicinal plant were ground with the aid of a mortar and pestle and afterward milled into powdered by an electric blender. The milled samples were then sieved using a filter paper to attain a refined powdered-like touch, they were kept in amber bottles and stored in a cool dried environment at room temperature before usage.

2.4 Extraction Method

The milled plant was extracted by reflux extraction method according to [10] in order to obtain crude extracts. The extraction protocol were done starting from non-polar solvent to polar solvent (n-hexane, ethyl acetate and methanol). 100 g of the milled samples were measured and dissolve in 400ml of an extracting solvent contained in a round bottom flask of 500ml capacity. The extractions begins with n-hexane (polarity index =0.1p') which was carefully added to a ratio of 1:4 of the milled samples to the extracting solvent. The round bottom flask comprising the mixture was however placed onto the heating mantle and the mouth of the flask was linked to the condenser. The source of power was then switched to supply heat and the temperature was adjusted to 30°C. The preparation was refluxed for 2hours. After complete refluxing, the preparation(mixture) was then filtered with the aid of a muslin cloth and further with a what man No 1 filter paper with pore size 20µm to attain a clear filtrate and furtherly concentrated to a semi solid substance with the aid of a rotary evaporator at a reduced temperature of (40°C) and fully dried by a water bath at a temperature of 60°C. The dried extract was kept in an air tight amber bottle and stored in the refrigerator for further procedures. The Marc (residue) were dried at room temperature for 45minute and was extracted using the next solvent in increasing polarity (further in succession using ethyl acetate with polarity index=4.4p'). The procedure continues using the last solvent and along all 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/Weight of sample (dry plant material)) ×100

2.5 Phytochemical analysis for the Crude Extracts

2.5.1 Flavonoid determination

Flavonoid determination was done by the Aluminum chloride colorimetric method. 0.5 ml (1 mg/mL) of the crude extract was added with 1.5 ml of methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1M sodium acetate and 2.8 ml of distilled water and stored at room temperature for 30 minutes. The wave length (absorbance) of the mixture was measured at 415nm with the aid of a double beam Shimadzu UV spectrophotometer, UV -1800. Whereas, the calibration curve were prepared using the quercetin solutions at concentration of 12.5 to 100 g/ml in methanol [11].

2.5.2 Total phenol determination

The total phenol content of the crude extract was estimated according to the method described by [12]. 0.5 ml (1 mg/ml) was oxidized with 2.5 ml of 10% Folin- Ciocalteau's relagent (v/v) and neutralized by 2 ml of 7.5% sodium carbonate. The reaction mixture was incubated for 40minutes at 45°C and the absorbance was taken at 765nm using the double beam Shimadzu UV spectrophotometer, UV- 1800. The total phenol content was subsequently calculated using Gallic acid as standard.

2.5.3 Total alkaloid determination

A 0.5 g of the crude extract was mixed with 5 ml of 96% ethanol -20% H_2SO_4 in ratio (1:1) and filter. 1 ml of the filtrate was added to 5ml of 60% H_2SO_4, the mixture was allowed to stand for 5 minute and 5 ml of 0.5% of formaldehyde solution was added and allowed to stand for 3hours. The absorbance was taken at a wave length of 565nm 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 [13].

2.5.4 Saponin determination

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, 6ml ferrous sulphate reagent and 2 ml of concentrated H_2SO4. The mixture was homogenized and allowed to stand for 10minutes before the absorbance was taken at 490nm using Shimadzu UV spectrophotometer UV-1800 [13].

2.5.5 Tannin determination

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 80°C for one hour. The mixture was shaken thoroughly and the content was transferred into a 100ml volumetric flask. 20ml of water, 2.5 ml of 10% Folin Denis reagent and 10ml of 17% Na_2CO_3 was added and mixed thoroughly. The mixture was allowed to stand for 20 minutes. 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 760 nm using Shimadzu UV-spectrophometer, UV-1800 [14].

2.5.6 Phytic acid content

The phytic acid content was determined using a modified indirect colorimetric method of [15]. 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. 5g 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 1M 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 Oxalate determination

Oxalate was determined by permanganate titrimetric method as described by [16]. Two gram (2 gram) of the crude extract was suspended in 190 ml of distilled water in 250ml 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 filtrates 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 5 minutes, 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 125ml 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 30 seconds. The calcium oxalate content is calculated using the formula below:

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

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 (125ml), 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 and standardization of isolates (test organisms)

The isolates population were determined by the McFarland turbidity standard [17]. The preparation proceeded by measuring Zero point zero five milliliter (0.05 ml) of 1% $Bacl_2$ which was mixed thoroughly with 9.95ml of 1% H_2SO_4 in a test tube. The absorbance of the mixture (white precipitate) was measured at 530 nm. The Standardization of the isolates were done by the method described by [18] with slight modification. The isolates (test organisms) were cultured on sabouraud dextrose agar at room temperature for 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 0.5Mcfarland standard was used as the standard organism which gives a population of 10^8 cfu/ml.

2.7 Measurement, Preparation of Crude Extracts and Fractions Concentrations

The normal hexane, ethyl acetate and methanol crude extract were weighed to 200 mg, 300 mg 400 mg and 500 mg and dissolved in 5 ml each of 10% Dimethylsulfoxoide to give a varying concentration of 40 mg/ml, 60 mg/ml 80 mg/ml and 100 mg/ml [19]. The fractions were determined by a standard chromatography method described by [20]. Concentrations of the fractions were obtained by measuring 10 mg and 20 mg of each fractions and dissolved in 2 ml of 10% DMSO to give a varying concentration of 5 mg/ml and 10 mg/ml respectively [19].

2.8 Assay of the Antifungal Activity of the Crude Extracts and Fractions

The susceptibility test was carried out using Agar Well Diffusion Method as described by [18]. The zone of inhibition (ZOI) was determined using a meter scale rule. The experiment was performed in triplicates and the mean values with their corresponding standard deviation of the inhibition zone diameter (IZD was recorded. Crude extracts and fractions that measured zones of inhibition ≤10 was recorded as resistant while >10 was recorded for sensitivity [21].

2.9 Determination of Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) of the Active Crude Extracts and Fractions

The minimum inhibitory concentration (MIC) and Minimum fungicidal concentration (MFC) was determined using the Tube Dilution Method as described by [19].

2.11 Statistical Analysis

The data are expressed as Mean ±standard deviation; all data were analyzed by one way ANOVA. Significant differences were considered at $P<0.05$. The analysis were carried out using Statistical Package for Social Science (SPSS) version 20.

3. RESULTS

3.1 Gram Reaction, Biochemical and Germ Tube Test of Fungal Strain (*Candida albicans*)

This result shows the gram reaction, biochemical and germ tube tests that was done to confirmed the identity of the three fungal strains as shown in Table 1. This was done in accordance with the method described by [22,23].

3.2 Molecular analysis Conducted for the Strains (*Candida albicans*)

The molecular analysis carried out confirmed the identity of the strains which is shown in Table 2. The sequenced BLAST results showed the various identities of the strains (*Candida albicans*) and their respective accession numbers assign to them. They were determined from the GENE bank through the NCBI web site [24]. The corresponding ascension numbers obtain are as follows: Isolate S5: Strain1161 (AFO75293.1), Isolate B4: P37005 (AP023893.1) and Isolate B2: RM1000 (AB_017634.2).

3.3 Percentage Yield of the Leaf Crude Extracts of *Vernonia amygdalina*

Table 3 showed the percentage yields of the leaf crude extracts of *Vernonia amygdalina*. The pulverized plant samples were extracted with n – hexane, ethyl acetate and methanol. The leaves of *Vernonia amygdalina* had a percentage yield of 11.42%, 0.39% and 8.28% which were obtained in NHLE, EALE and MLE respectively. NHLE had the highest percentage yield of 11.42% and the lowest yield was obtained in EALE 0.39%.

3.4 Quantitative Phytochemical Determination of *Vernonia amygdalina* Leaf Crude Extracts Obtained Using Different Solvents

Table 4 showed the results of the quantitative determination of n- hexane leaf extract(NHLE) , ethyl acetate leaf extract (EALE) and Methanol leaf extract(MLE) of *Vernonia amygdalina*. The n- hexane leaf extract (NHLE) of

Table 1. Gram reaction, biochemical and germ tube 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	S5	+	OVAL	+	+	-	-	-	-	-	-	+	+	+	-	-	-	+	+	-	+	+	*Candida albicans*
2	B4	+	OVAL	+	+	-	-	-	-	-	-	+	+	+	-	-	-	+	+	-	+	+	*Candida albicans*
3	B2	+	OVAL	+	+	-	-	-	-	-	-	-	+	+	-	-	-	+	+	-	+	+	*Candida albicans*

Table 2. Molecular analysis conducted for the strains (*Candida albicans*)

Isolated code	Strains	Max score	Total score	Query cover	Expected value	Identity	Ascension number
S5	STRAIN1161	8000	9822	100%	0.0	100%	AFO75293.1
B4	P37005	7542	5850	100%	0.0	100%	AP023893.1
B2	RM1000	700	8811	100%	0.0	100%	AB_017634.2

Table 3. Percentage yield of *Vernonia amygdalina* leaf crude extract

	Leaf			
Plant sample	**WS/DP(g)**	**NHLEg(%)**	**EALEg(%)**	**MLEg(%)**
Vernonia amygdalina	100	11.42(11.42)	0.39(0.39)	8.28(8.28)

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

Vernonia amygdalina had phytic acid (160.78±0.58) as the highest in amount while oxalate (5.65± 0.58) was the lowest. Others were flavonoid (60.88±0.58), phenols (113.46±0.58), tannin (68.32±0.58), alkaloid (132.11±0.58) and saponins (40.22±0.58). Likewise, the ethyl acetate leaf extract (EALE) of *Vernonia amygdalina* also had phytic acid (45.63±0.58) as the highest in amount while oxalate (4.55±0.58) was the lowest. Others present in amounts were flavonoid (24.96.±0.58), phenols (38.69±0.58), tannin (19.88±0.58), alkaloid (18.66.±0.58) and saponins (20.44±0.58). Methanol leaf extract had phenols (178.92±0.58) as the highest in amount while oxalate (5.30±0.58) was also present in trace amount. Others were flavonoid (79.28±0.58), tannin (98.92±0.58), alkaloid (135.65±0.58), saponins (49.16±0.58) and phytic acid (70.59±0.58).

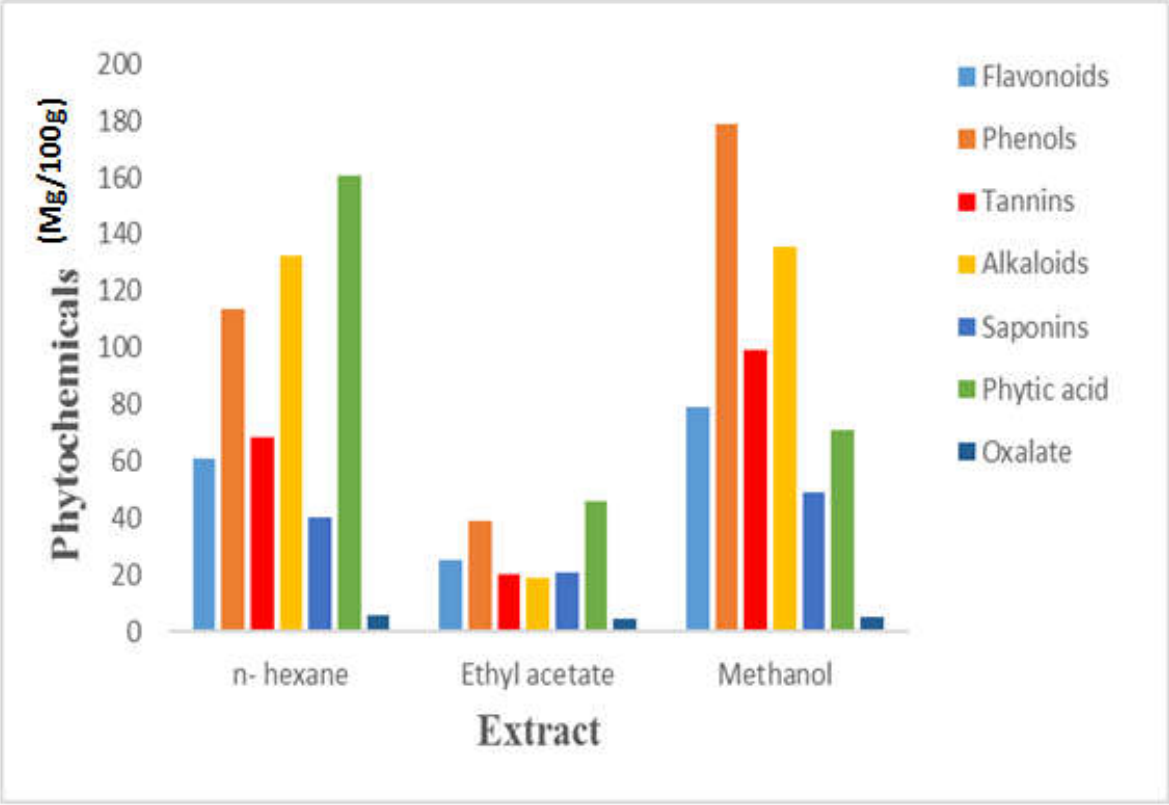

Fig. 2. Quantitative phytochemical determination of *Vernonia amygdalina* leaf crude extracts obtained using different solvents

Table 4. Quantitative phytochemical determination of *Vernonia amygdalina* leaf crude extracts obtained using different solvents

	Phytochemicals(mg/100 g)						
Extracts	**Flavonoids**	**Phenols**	**Tannins**	**Alkaloids**	**Saponins**	**Phytic acid**	**Oxalate**
n- hexane	60.88±0.58^{b}	113.46±0.58^{b}	68.32±0.58^{b}	132.11±0.58^{b}	40.22±0.58^{b}	160.78±0.58^{c}	5.65±0.58^{c}
Ethyl acetate	24.96±0.58^{a}	38.69±0.58^{a}	19.88±0.58^{a}	18.66±0.58^{a}	20.44±0.58^{a}	45.63±0.58^{a}	4.55±0.58^{a}
Methanol	79.28±0.58^{c}	178.92±0.58^{c}	98.92±0.58^{c}	135.65±0.58^{c}	49.16±0.58^{c}	70.59±0.58^{b}	5.30±0.58^{b}

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

3.5 Result of the Antifungal Activity of *Vernonia amygdalina* Leaf Crude Extracts against Strain P37005 (Isolate: B4)

The result of the antifungal activity of *Vernonia amygdalina* leaf crude extracts against strain P37005 (Isolate: B1) is shown in Table 5. The n-hexane leaf extract (NHLE) had no inhibitory activity at 40mg/ml while at 60, 80 and 100mg/ml concentrations activity was seen and their mean zone of inhibition (MZI) was 8.00±0.20mm, 9.33±0.80 and 13.00±0.70 mm respectively. Ethyl acetate leaf extracts (EALE) had no inhibitory activity for all the varying concentration used starting from 40, 60, 80 and 100 mg/ml concentrations. The methanol leaf extract (MLE) had no inhibitory activity at concentrations of 40 and 60mg/ml but at 80 and 100mg/ml concentrations activity was seen and the mean zone of inhibition (MZI) was 7.00±0.20 mm and 9.00±0.70mmrespectively.

3.6 Results of the Antifungal Activity of *Vernonia amygdalina* Leaf Crude Extracts against Strain RM1000 (Isolate: B2)

The result of the antifungal activity of *Vernonia amygdalina* leaf crude extracts against strain RM1000 (Isolate: B2) is shown in Table 6. The n-hexane leaf extract (NHLE) had no activity at 40mg/ml concentration while at 60, 80 and 100mg/ml concentrations activity was seen and the mean zone of inhibition (MZI) was 6.00±0.20 mm, 8.67±0.67 mm and 10.00±0.60 mm respectively. Ethyl acetate leaf extracts (EALE) had no inhibitory activity for all the varying concentration used at 40, 60 80 and 100 mg/ml concentrations. The methanol leaf extract (MLE) had no inhibitory activity at 40 and 60 mg/ml concentrations but at 80 and 100 mg/ml concentration inhibitory activity was seen and the mean zone of inhibition (MZI) was 6.00±0.20 mm and 8.00±0.20 mm respectively.

3.7 Result of the Antifungal Activities of *Vernonia amygdalina* Leaf Crude Extracts against Strain 1161 (Isolate: S5)

Antifungal activity of *Vernonia amygdalina* leaf crude extracts against strain 1161(Isolate: S5) is shown in Table 7.The n-hexane leaf extract (NHLE) had no inhibitory activity at a concentration of 40 mg/ml and 60 mg/ml but at 80mg/ml and 100 mg/ml concentrations there was inhibitory activity and the zones of inhibition (ZOI) recorded was 8.00±0.20 mm and 13.00±0.70mm respectively. Ethyl acetate leaf extracts (EALE) had no inhibitory activity for the different concentrations used at 40 mg/ml, 60 mg/ml 80 mg/ml and 100 mg/ml. The methanol leaf extract (MLE) had inhibitory activity at a concentrations of 40 mg/ml, 60 mg/ml, 80 mg/ml and 100 mg/ml. The inhibitory zones were 7.00±0.10 mm, 8.00±0.10 mm, 10.00±0.70mm and 15.33±1.23 mm.

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

The minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of the active crude extracts is presented in Table 8. Values for the MIC and MFC for the active crude extracts are 12.5 mg/ml and 50 mg/ml for the n-hexane crude extract against strain 1161 (isolate S5), the methanol crude extract showed a result of 12.5 mg/ml and 50 mg/ml against strain 1161 (isolate: S5) however, n- hexane crude extract had 12.5 mg/ml and 100 mg/ml respectively against strain P37005 (isolate: B4).

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

Conc. (mg/ml)	Leaf		
	n-hexane	Ethyacetate	Methanol
40	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
60	8.00±0.20^{b}	0.00±0.00^{a}	0.00±0.00^{a}
80	9.33±0.08^{b}	0.00±0.00^{a}	7.00±0.20^{b}
100	13.00±0.70^{c}	0.00±0.00^{a}	9.00±0.70^{c}
Fluconazole (1 mg/ml)	40.00±0.80^{d}	40.00±0.80^{b}	40.00±0.80^{d}
DMSO (100 ul)	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 *Vernonia amygdalina* leaf crude extracts of against strain RM1000 (Isolate: B2)

Conc. (mg/ml)	Leaf		
	n-hexane	Ethyacetate	Methanol
40	0.00±0.00[a]	0.00±0.00[a]	0.00±0.10[a]
60	6.00±0.20[b]	0.00±0.00[a]	0.00±0.10[a]
80	8.67±0.67[c]	0.00±0.00[a]	6.00±0.20[b]
100	10.00±0.60[d]	0.00±0.00[a]	8.00±0.20[c]
Fluconazole (1 mg/ml)	37.00±0.40[e]	37.00±0.40[b]	37.00±0.40[d]
DMSO(100 ul)	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 *Vernonia amygdalina* leaf crude extract (40-100mg/ml) against strain 1161(Isolate: S5)

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

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) for the active crude extracts

S/NO	Isolated code	Strain	Plant part	Crude extracts	MIC	MFC
1	S5	Stain 1161	Leaf	n – hexane	12.5 mg /ml	50 mg/ml
2	S5	Strain 1161	Leaf	Methanol	12.5 mg/ml	50 mg/ml
3	B4	P37005	Leaf	n-hexane	12.5 mg/ml	100 mg/ml

3.9 Antifungal Activity of n-hexane Fraction of *Vernonia amygdalina* against Strain 1161, P37005 and RM1000

Antifungal activity of n-hexane fraction of *Vernonia amygdalina* is presented in Table 9. The activity of fraction F1 showed zone of inhibition 11.00±0.40 mm and 15.00±0.60 mm at a concentration of 5 mg/ml and 10 mg/ml against strain P37005. Fraction F4 showed inhibitory activity 10.00±0.10 mm and 16.00±0.90 mm at the same concentration of 5 mg/ml and 10 mg/ml against strain 1161. The activity of Fraction F4 was also seen against P37005 at the same concentration which gave zones of inhibition of 12.00±30 mm and 16.00±0.80 mm respectively. Other fractions showed no inhibitory activity against the tested strains, the positive control (fluconazole 1 mg/ml) tested against the strains showed varying zones of inhibition. The negative control (DMSO) used against the tested strains showed no inhibitory activity. The activity of the fraction F1 and F4 were less compared to the activity of the positive control.

3.10 Antifungal Activity of Ethyl Acetate Fraction of *Vernonia amygdalina* against Strain 1161, P37005 and RM1000

Antifungal activity of ethyl acetate fraction of *Vernonia amygdalina* is presented in Table 10. The activity of fraction F3 showed zone of inhibition 8.00±0.10 mm and 9.00±0.10 mm at a concentration of 5 mg/ml and 10 mg/ml respectively against strain RM1000. Other fractions showed no inhibitory activity against the tested strains, the positive control (fluconazole 1 mg/ml) tested against the strains showed varying zones of inhibition. Although, the negative control (DMSO) that was tested showed no inhibitory activity.

Table 9. Antifungal activity of n-hexane fraction of *Vernonia amygdalina* against strain 1161, P37005 and RM1000

S/N	ISOLATE	STRAIN	F1		F2		F3		F4		Fluconazole (1 mg/mL)	DMSO (100 ul)
			5 mg/ml	10 mg/ml	5 mg/ml	10 mg/ml	5 mg/ml	10 mg/ml	5 mg/ml	10 mg/ml		
1	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}	10.00± 0.10^{b}	16.00± 0.90^{c}	42.00±0.40^{d}	0.00±0.00^{a}
2	B4	P37005	11.00± 0.40^{a}	15.00± 0.60^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	12.00± 0.30^{b}	16.00± 0.80^{c}	40.00±0.80^{d}	0.00±0.00^{a}
3	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}	37.00±0.40^{b}	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. Antifungal activity of ethyl acetate fraction of *Vernonia amygdalina* against strain 1161, P37005 and RM1000

Isolate	Strain	F1		F2		F3		F4		F5		F6		Fluconazole (1 mg/m)	DMSO (100 ul)
		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		
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}	42.00±0.40^{b}	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}	0.00± 0.00^{a}	0.00± 0.00^{a}	40.00±0.10^{b}	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}	8.00± 0.10^{b}	9.00± 0.10^{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}	37.00± 0.20^{c}	0.00±0.00^{a}

Table 11. Antifungal activity of methanol fraction of *Vernonia amygdalina* against strain 1161, P37005, RM1000

Isolate	Strain	F1		F2		F3		F4		F5		F6		Fluconazole (1 mg/m)	DMSO (100 ul)
		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		
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}	42.00±0.21^{b}	0.00±0.00^{a}
B4	P37005	7.00± 0.20^{b}	9.00± 0.60^{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}	8.00± 0.30^{c}	12.00± 0.70^{d}	40.00±0.90^{e}	0.00±0.00^{a}
B2	RM1000	9.00± 0.30^{b}	14.00± 0.67^{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}	0.00± 0.00^{a}	0.00± 0.00^{a}	37.00±0.80^{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 concentration (MIC) and minimum fungicidal concentration (MFC) of the most active crude extracts

S/no	Isolate code	Strain	Medicinal plant	Plant part	Fraction	MIC (mg/ml)	MFC (mg/ml)
1	B4	P37005	*Vernonia amygdalina*	Leaf	F1	12.5	25
2	S5	Strain 1161	*Vernonia amygdaina*	Leaf	F4	12.5	25
3	B4	P37005	*Vernonia amygdalina*	Leaf	F4	3.125	6.25
4	B2	RM1000	*Vernonia amygdalina*	Leaf	F1	3.125	25
5	B4	P37005	*Vernonia amygdalina*	Leaf	F6	3.125	6.25

3.11 Antifungal Activities of Methanol Fraction of *Vernonia amygdalina* against Strain 1161, P37005, RM1000

Antifungal activity of Vernonia *amygdalina* is presented in Table 10. The activity of Fraction F1 showed zone of inhibition 7.00±0.20 mm and 9.00±0.60 mm at a concentration of 5mg/ml and 10 mg/ml respectively against strain P37005. Fraction F6 also showed inhibitory activity 8.00±0.30 mm and 12.00±0.70 mm at a concentration of 5 mg/ml and 10 mg/ml respectively against strain P37005. The activity of fraction F1 was also seen against RM1000 at a concentration of 5mg/ml and 10mg/ml which gave an inhibitory zones of 9.00±0.30 mm and 14.00±0.67mm respectively. Other fraction 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 F1 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 Concentration (MIC) and Minimum Fungicidal Concentration (MFC) of the Most Active Fractions

The result of the Minimum inhibitory concentration (MIC) and Minimum fungicidal concentration (MFC) of the most active fractions is shown in Table 12. The MIC and MFC values for the most active fractions(F1) were 12.5mg/ml and 25mg/ml for the n-hexane fraction of *Vernonia amygdalina* against strain P37005 (isolate B4), the n-hexane fraction(F4) of *Vernonia amygdalina* against strain 1161(isolate S5) also had a value of 12.5mg/ml and 25mg/ml however, fraction (F4) showed value of 3.125 mg/ml and 6.25 mg/ml against strain P37005(isolate B4). The methanol fraction(F1) had an MIC and MFC values of 3.125 mg/ml and 25mg/ml respectively, against strain RM1000(isolate B2). The value of 3.125 mg/ml and 6.25 mg/ml of fraction (F6) of *Vernonia amygdalina* against strain P37005(B4) was also determined.

4. DISCUSSION

The research finding was focused in isolating, and identifying selected *Candida albicans* strains (Strains 1161, P37005, and RM1000. There susceptibility was assayed on the leave crude extracts of *Vernonia amygdalina* (Neem). The biochemical and molecular findings of the strains was in line with a similar work of Ewansiha et al. [19] as shown in (Tables 1, 2). The extraction procedure employed for this study was the reflux method of extraction which was in successive state(non- polar solvent – polar solvent).However, the solvents used were n-hexane, ethyl acetate and methanol) with varying polarity level which was used for the successive extraction of the leaves of *Vernonia amygdalina*. The percentage yield obtained from the extraction of the leave crude extract of *Vernonia amygdalina* is presented in (Table 3). The highest percentage yield was seen in the n-hexane leaf extract (NHLE) of *Vernonia amygdalina*, the least was the ethyl acetate with a percentage yield of 0.39% where as the methanol leaf extract (MLE) showed a percentage of 8.28%. The variation in percentage could be due to their differences in polarity index and as well the differences in the solvents used. Table 4 presents the quantitative phytochemical analysis of the leaf crude extract of *Vernonia amgygdalina* which was attained from the varying solvents. The quantitative phytochemical analysis was carried out to estimate the amount of bioactive substance available in them. Some of the phytochemicals presents include: Flavonoids, phenols, tannins, phytic acid and oxalate etc. This finding was similar to the study of [25] furthermore, [26] also find out the presence of alkaloids, tannin,

saponin and flavonoids. Flavonoids possess anti allergic, anti-inflammatory antiviral anti proliferative anticarcinogenic activities and antioxidants potentials and they can function in the protection of the heart against disease. The antiallergic function seen in flavonoid pose tremendous benefit since it has the abilities in the treating of miscarriages that occur with females [27]. Flavonoids possess ability to prevent platelets stickess (platelet aggregation), All types of miscarriages can be treated by flavonoids [28]. They also can thin blood which occur by their inhibition of clothing pathway. As reported by Jones et al. [29] phenolic compounds have antioxidants, antiulcer, anti–inflammatory, antispasmotic and anti depresssnt activities [30]. Tannin are used to treat nonspecific diarrhea, inflammation of mouth, throat and slightly injured skin [31]. Tannin extracts are also used against stomach and duodenal tumors and also as anti-inflammatory, antiseptic, antioxidant and homoestatic pharmaceuticals [32]. As reported by Jisika et al. [33] Alkaloids areorganic compound with various medicinal importance. Li et al. [34] reported that numerous drugs have being gotten from alkaloids because of its pharmacological importance. Although higher doses may be toxic. The oxalates and phytic acid contents of plants functions as anticancer. They play important role keeping the gut from toxins and they also function in cholesterol and blood sugar stability [35]. The antifungal activity of *Vernonia amygdalina* leaf crude extract against strainsP37005, RM1000 and strain 1161 is presented in (Tables 5-7). Inhibitory activity was seen for the n- hexane leaf extract (NHLE) and Methanol leaf extract (MLE) of *Vernonia amygalina* crude extract at varying concentrations. Methanol leaf extract (MLE) at a concentration of 100 mg/ml showed the highest value of 15.33 ±1.23 mm against strain1161 (isolate:S5). The zone of inhibition (ZI) increased as the concentrations were increased This is in line with [36] who opined that the activity of antimicrobial agent is concentration dependent Futher more, [20] reported that the inhibitory zones within a plant varied with the type of solvent used for extraction. This agrees with the similar work of [34] and also in line with the work of [37] that reported zones of inhibition of the leaf crude extract of *Vernonia amygdalina* against *Candida spp*. Values for the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) that were obtained is presented in (Table 8). The MIC and MFC values differs from the similar work done by [20]. The disparity could be due to geographical area of the plant, laboratory techniques and reagent type used [36] Season, age of plant, and method of extraction may also affect the activity and the bioactive constituents of the plant [38]. Tables 9-11 shows the antifungal activity of the fractions of n- hexane, ethyl acetate and methanol of *Vernonia amygdalina* against the three different strains(P37005, RM1000 and strain 1161) There was inhibitory activity for the n- hexane, ethyl acetate and Methanol fraction of *Vernonia amygalina* at two different concentrations(5 mg/ml and 10 mg/ml) against the tested strains but the highest value 16.00 ±0.00 mm which was record for the methanol fraction of *Vernonia amygdalina* at a concentration of 10mg/ml against strain1161 (isolate:S5) and P37005(isolate B4). Most of the inhibitory activity were seen in the n-hexane fraction of *Vernonia amygdalina* (fraction: FI and F4) which were active against P37005 and strain 1161.Methanolfraction of *Vernonia amygdalina* (F1and F6) were also active against strain RM1000 and P37005 (Table 11). The ethyl acetate fractions showed little activity which was seen in fraction F3 against RM1000 as shown in (Table 10). The fractions were less in inhibitory activity compared to the crude extracts, activity of the crude extracts showed a better inhibitory activity in terms of the zones diameter compared to the fraction. Although, the fractionation was done using column and tin layer chromatography which attain a partial purification, through this process it is likely that the fractions should be pure and refined which might improve the activity of the fractions to have a higher zone of inhibition compared to the crude extract. The higher activity of the crude extracts may be due to synergistic effect. if the fractions are further purified it may have a better activity than the crude. The activity of the fractions increased as the concentrations were increased (5 mg/ml to 10 mg/ml). This is in line with the work of [36]. The result obtained from the antifungal activity of the standard drug (fluconazole1mg/ml) used in this study showed a better zone of inhibition as than the activity of the crude extract and the fractions obtained. This may be due to the purity of the standard drugs. The negative control Dimethylsulphuroxide (DMSO) had no inhibitory activity against the strains. This implies that DMSO does not contain any antimicrobial agent.

5. CONCLUSION

This research study has proved the activity of the crude extract and fraction of *Vernonia*

amygdalina and as well the standard antibiotics against the various strains. It is cleared that the standard antibiotics (fluconazole) used for the purpose of this study showed higher zones of inhibition compared to the crude extract and the fraction. Whereas, the n-hexane leaf extracts (NHLE) and Methanol leaf extract (MLE) of *Vernonia amygdalina* showed a better activity than the activity of the fraction.

6. RECOMMENDATION

Vernonia amygdalina should be processed further by other technological means which would enhance its development for alternative drugs for curing candidiasis. The phytochemicals agents in this plant should be extracted and use as chemotherapy.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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