Quantitative phytochemical analysis and antifungal susceptibility of *Vernonia amygdalina* against some strains of *Candida albicans*

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ABSTRACT

This study was aimed at determining the quantitative phytochemical analysis and antifungal susceptibility of *Vernonia amygdalina* against some strains of *Candida albicans*. Reflux method of extraction was used for the successive extraction of the leaves of *Vernonia amygdalina*. Quantitative phytochemical screenings were done to determine the amounts of phytochemicals that are present in the crude extracts, the study revealed that phytochemicals which include flavonoids, tannins, alkaloids saponins and phenol were present in the crude extracts. Three different strains (P37005, RM1000 and SC5314) were subjected for antifungal susceptibility test, the antifungal susceptibility test of the crude extracts against the strains were determined at different concentrations of 40,60, 80 and 100mg/ml using agar well diffusion method. The highest zone of inhibition (ZOI) was 21.00 ±0.30mm which was recorded for methanol leaf extract (MLE) at a concentration of 100mg/ml against SC5314 (isolate:B3). The MIC and MFC values for the most active crude extracts were 12.5mg/ml and 100mg/ml for the n-hexane crude extract against strain P37005 (isolate B1), the value of 12.5mg/ml and 100mg/ml was also revealed for the n-hexane crude extract against SC5314 (isolateB3) however, the methanol crude extract showed a value of 12.5mg/ml and 50mg/ml respectively against SC5314 (isolate :B3). The results from this study suggest that n-hexane and methanol crude extract have a better antifungal activity than the ethylacetate crude extract. This study also validate the claim of the local herbal practitioners of the use of the leaves of *Vernonia amygdalina* in curing candidiasis.

Key words: Phytochemical, *Vernonia amygdalina,* Antifungal, Susceptibility, Strains, Crude extract.

INTRODUCTION

Vernonia amygdaline is a member of the Asteraceae family is a small shrub that grows in tropical Africa having a height of 2–5 m (6.6–16.4 ft). The leaves are elliptical and up to 20 cm (7.9 in) long. Its bark is rough [1] *V. amygdalina* is commonly called bitter leaf in English. "Oriwo'' in Edo "ewuro'' in Yoruba, "Shikaa'' in Hausa and olubu in Igbo[2] . The leaves are consumed as vegetable and condiments, after macerating and washing thoroughly to remove the bitterness. *Vernonia amygdalina* have been reported to have Antihelmitic antimalarial, antitumourigenic hypoglycemic and hypolipidaemic properties [3]. Athough, Medicinal plants are known to contain substances which could be used for treating purposes or used to produce drugs, many of such plants known to be used primitively to alleviate symptoms of illness have been screened to have medicinal importance, some of which include : *Vernonia amygdalina* (Bitter leaf), *Allium sativum* (Garlic), and *Zingiber officinale* (Ginger) These plants have been reportedly used in the treatment of ailment such as stomach disorder, fever, symptoms and cough traditionally[4] . *Vernonia amygdalina* is a valuable medicinal plant that is widespread in West Africa. It is known as bitter leaf due to its characteristics bitter taste and flavor, and can be used as an active anticancer, antibacterial, antifungal and anti-parasitic agent .This plant contains complex active components that are useful pharmacologically. In ethno medicine, the roots and the leaves are used to treat fever, Hiccup, Kidney problems, and stomach discomfort. Many West African countries such as Cameroon, Ghana and Nigeria use the stem and root as chewing sticks [5].It is also documented that *Vernonia amygdalina* has been used traditionally in blood clothing and has elicited a substantial reduction in the level of glucose in the body[5]

Fig1:*Vernonia amygdalina* Source: field photograph

MATERIALS AND METHODS

Plant materials

The leaves of *Vernonia amygdalina* (bitter leaf) was collected from Maikunkele in Bosso local Government, Niger state

Collection and identification of test organisms

Three strains of *Candida albican*: SC5314, P37005 and RM1000 were collected from Federal Istitute for Industrial Research Oshodi (FIIRO), Lagos, Nigeria. They were subjected to sugar fermentation test as described by [6].Germ tube test was carried out to identify and differentiate them from other species which was done according to[7] . Their molecular characterization was also carried out to confirm the identity of the strains [8] .

Identification, Authentication and processing of plant materials

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/6872) and the voucher specimens were deposited in the same Herbarium Department of the institute. The identified and authenticated medicinal plants parts 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 of the medicinal plant 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 is required for usage.

Extraction Procedure

The grounded plant samples (Pulverized samples) were subjected to reflux extraction according to [9] in order to obtain the crude extracts. The extraction was carried out beginning from non-polar solvent to polar solvent (n-hexane, ethyl acetate and methanol). 100g of the plant samples was weighed and dissolve in 400ml of the extracting solvent in a round bottom flask of 500ml 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^{o}C. The mixture was allowed to reflux for 2hours. After refluxing, the mixture was filtered using muslin cloth and later with what man No 1 filter paper with pore size 20µm 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^{o}C) and then dried using water bath at 60^{o}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 45minute 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:

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

Determination of Flavonoid

Aluminium chloride colorimetric method was used for flavonoid determination. A 0.5ml (1mg/mL) of the plant crude extract was mixed with 1.5ml of methanol, 0.1ml of 10% aluminium chloride, 0.1ml of 1M sodium acetate and 2.8ml 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 100g/ml in methanol[10] .

Determination of total phenol

The total phenol content of the crude extract was determined according to the method described by [11]. A 0.5 ml (1mg/ml) was oxidized with 2.5ml of 10% Folin- Ciocalteau's reagent (v/v) and neutralized by 2ml of 7.5% sodium carbonate. The reaction mixture was incubated for 40minutes at 45^0C 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.

Determination of total alkaloid

A 0.5g of the crude extract was mixed with 5ml of 96% ethanol -20% H_2SO4 in ratio (1:1) and filter. 1ml of the filtrate was added to 5ml of 60% H2SO4, the mixture was allowed to stand for 5minute 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 565nm using Shimadzu UV spectrophotometer, UV- 1800. The concentration of alkaloid in the sample was calculated using the molar extinction coefficient of vincristine, ε=15136mol/cm[12].

Determination of Saponin

A 0.5g of the crude extract was mixed with 20ml of 1MHCL and the mixture was boiled for 4 hours and allowed to cool. After cooling and filtered, 50ml of petroleum ether was added to the filtrate, for ether layer and evaporated to dryness. 5ml of acetone- ethanol (1:1) were added to the residue, 6ml ferrous sulphate reagent and 2ml 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[12].

Determination of Tannin

A 0.2g of the extract was weighed into a 50ml beaker; 20ml of 50% methanol was added, covered with para film and placed in a water bath at 80^{o}C for one hour. The mixture was shaken thoroughly and the content was transferred into a 100ml volumetric flask. 20ml of water, 2.5ml of 10% Folin Denis reagent and 10ml of 17% Na_2 CO_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 12.5-100ug/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 [13]

Phytic acid content

The phytic acid content was determined using a modified indirect colorimetric method of [14]. 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 20ml of 3% trichloroacetic acid and filtered. 5ml 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 5ml of 1M NaOH. The precipitate was dissolved with hot 3.2M HNO_3 and the absorbance was taken immediately at 480nm. 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.

Determination of Oxalate

Oxalate was determined by permanganate titrimetric method as described by [15]. Two gram (2gram) of the crude extract was suspended in 190ml of distilled water in 250ml volumetric Flask, 10ml of 6M HCL was added and the suspension digested at 100^{0}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^{0}C and 10ml of 5% $CaCl_2$ solution added while being stirred constantly. After heating, it was cooled and left overnight at 5^{o}C. The solution was then centrifuged at 2500rpm for 5minutes, the supernatant decanted and the precipitate completely dissolved in 10ml of 20% (v/v) H_2SO_4 solution. The total filtrate resulting from the digestion was made up to 300ml 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 30seconds. The calcium oxalate content is calculated using the formula below:

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

Where T is the titre of $KMnO_4$ (ml), Vme is the volume- mass equivalent (1cm^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 (300ml) 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.

Antifungal activity of the crude extracts

Preparation of 0.5Mcfarland Standard

The populations of the clinical isolate were determined from the McFarland turbidity standard [16]. Zero point zero five milliliter (0.05 ml) of 1% $Bacl_2$ was mixed thoroughly with 9.95ml of 1% H_2SO_4 in a test tube. The absorbance of the mixture (white precipitate) was determined at 530 nm

Standardization of the Test Organisms

The Standardization of the test organisms was carried out as described by [17] with slight modification. The test organism was 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 0.5Mcfarland standard was used as the standard organism which gives a population of 1.5×10^3 cfu/ml .

Preparation of Extract Concentration

Two hundred milligram (200mg), 300mg 400mg and 500mg 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 40mg/ml, 60mg/ml,80mg/ml and 100mg/ml concentrations respectively[18] .

Determination of the antifungal activity of the crude extracts

The susceptibility test was carried out using Agar Well Diffusion Method as described by [17]. 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 (0.1ml) of the prepared crude extract of varying concentration (40, 60, 80 and 100mg/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 while Fluconazole (1mg/ml) was used as the positive control. This was achieved by transferring 100µl 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 24-72hours. The zone of inhibition (ZOI) 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[19].

Determination of Minimum inhibitory concentration (MIC) and Minimum fungicidal concentration (MFC) of the most active crude extract.

The MIC was determined using the Tube Dilution Method as described by [18]. A four fold serial dilution of the most active plant extract was carried to give a decrease in concentration from 100, 50, 25, 12.5, 6.25, 3.125, 1.25 and 0.78mg/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 0.78mg/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 0.1ml 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.

Statistical Analysis

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

RESULTS

Biochemical characteristics and germtube test of fungal strain(*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 [7].

Table1: 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	B1	+	OVAL	+	+	-	-	-	-	-	-	+	+	+	-	-	-	+	+	-	+	+	*Candida albican*
2	B2	+	OVAL	+	+	-	-	-	-	-	-	+	+	+	-	-	-	+	+	-	+	+	*Candida albican*
3	B3	+	OVAL	+	+	-	-	-	-	-	-	-	+	+	-	-	-	+	+	-	+	+	*Candida albican*

Molecular characterization conducted for 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 strain (*Candida albicans*) and their accession numbers which were determined from the GENE bank through the NCBI web site [8].Their corresponding ascension numbers were as follows: Isolate B1: P37005 (AP023893.1), Isolate B2: RM1000 (AB_017634.2) and Isolate B3: SC5314(CP025163.1).

Table 2: Molecular characterization conducted for the fungal strains (*Candida albicans*)

Isolated code	Strains	Max score	Total score	Query cover	Expected value	Identity	Ascension number
B1	**P37005**	**7542**	**5850**	**100%**	**0.0**	**100%**	**APO23893.1**
B2	**RM1000**	**700**	**8811**	**100%**	**0.0**	**100%**	**AB_017634.2**
B3	**SC5314**	**234**	**5243**	**100%**	**0.0**	**100%**	**CP025163.1**

Percentage yield of the leaf crude extracts of *Vernonia amygdalina*

Table 3 represents the percentage yields of the leaf crude extracts of *Vernonia amygdalina* .The milled 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%.

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

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

Table 4 showed the results obtained from 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 *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).

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

	Phytochemicals(mg/100g)						
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^{a}	5.65±0.58^{a}
Ethyl acetate	24.96±0.58^{c}	38.69±0.58^{c}	19.88±0.58^{c}	18.66±0.58^{c}	20.44±0.58^{c}	45.63±0.58^{c}	4.55±0.58^{a}
Methanol	79.28±0.58^{a}	178.92±0.58^{a}	98.92±0.58^{a}	135.65±0.58^{a}	49.16±0.58^{a}	70.59±0.58^{b}	5.30±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 $p \leq 0.05$

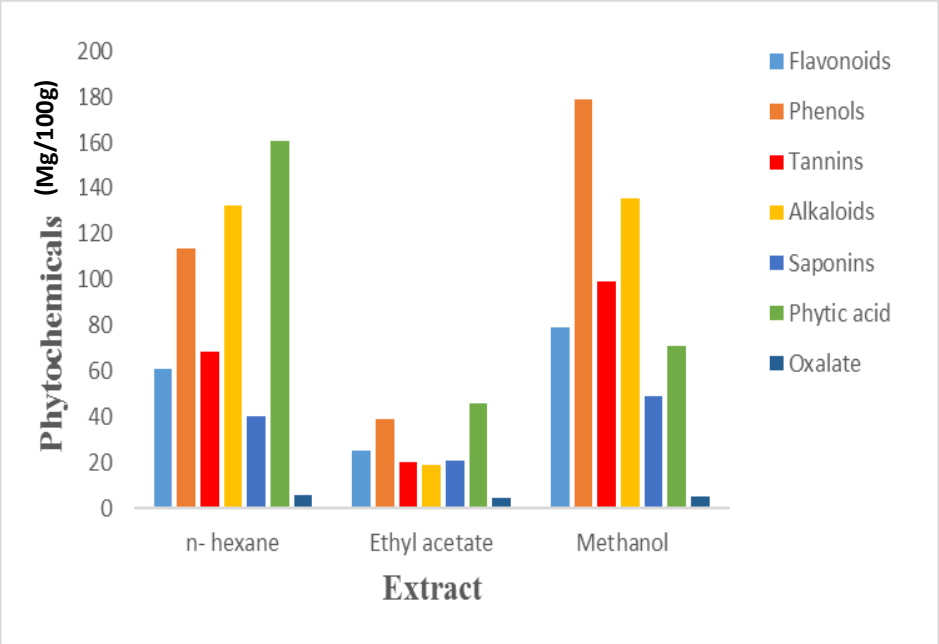

Fig 2: Quantitative phytochemical determination of *Vernonia amygdalina* leaf crude extracts obtained using different solvents.

Antifungal susceptibility of the leaf, crude extracts of *Vernonia amygdalina* against strain P37005 (Isolate: B1)

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

Table 5: Antifungal susceptibility of the leaf of *Vernonia amygdalina* 40-100mg/ml against strain P37005(Isolate: B1)

	Leaf		
Conc. (mg/ml)	n-hexane	Ethyacetate	Methanol
40	0.00±0.00^{d}	0.00±0.00^{b}	0.00±0.00^{d}
60	8.00±0.00^{c}	0.00±0.00^{b}	0.00±0.00^{d}
80	9.00±0.20^{c}	0.00±0.00^{b}	7.00±0.20^{c}
100	13.00±0.70^{b}	0.00±0.00^{b}	9.00±0.70^{b}
Fluconazole (1mg/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) differ significantly according to the least significant different at $p \leq 0.05$

Antifungal susceptibility of the leaf crude extracts of *Vernonia amygdalina* against strain RM1000 (Isolate: B2)

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

Table 6: Antifungal susceptibility of the leaf crude extract of *Vernonia amygdalina* 40-100mg/ml against strain RM1000 (Isolate: B2)

	Leaf		
Conc. (mg/ml)	n-hexane	Ethyacetate	Methanol
40	0.00±0.00^{e}	0.00±0.00^{b}	0.00±0.10^{d}
60	6.00±0.20^{d}	0.00±0.00^{b}	0.00±0.10^{d}
80	8.67±0.67^{c}	0.00±0.00^{b}	6.00±0.20^{c}
100	10.00±0.60^{b}	0.00±0.00^{b}	8.00±0.20^{b}
Fluconazole (1mg/ml)	37.00±0.40^{a}	37.00±0.40^{a}	37.00±0.40^{a}
DMSO(100ul)	0.00.±0.00^{e}	0.00±0.00^{b}	0.00±0.00^{d}

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

Antifungal susceptibility of the leaf crude extracts of *Vernonia amygdalina* against strain SC5314 (Isolate: B3)

Antifungal susceptibility of the leaf crude extracts of *Vernonia amygdalina* against strain SC5314(Isolate: B3) is presented in Table 7. The n-hexane leaf extract (NHLE) showed no activity at 40 mg/ml concentration but at 60, 80 and 100mg/ml concentration there was inhibitory activity and the mean zone of inhibition (MZI) was 8.00±0.76mm, 12.67±0.76mm and 14.00±0.60mm respectively. Ethyl acetate leaf extracts (EALE) showed no inhibitory activity at varying concentration used at 40, 60 80 and 100 mg/ml. The methanol leaf extract (MLE) had no inhibitory activity at 40 and 60mg/ml concentration but at 80 and 100mg/ml concentrations there was inhibitory activity and the mean zone of inhibition (MZI) was 16.00±0.70 mm and 21.00±0.30mm,

Table 7: Antifungal susceptibility of the leaf of *Vernonia amygdalina* 40-100mg/ml against strain SC5314 (Isolate: B3)

	Leaf		
Conc. (mg/ml)	n-hexane	Ethyacetate	Methanol
40	0.00±0.00^{e}	0.00±0.00^{b}	0.00±0.00^{d}
60	8.00±0.76^{d}	0.00±0.00^{b}	0.00±0.00^{d}
80	12..67±0.76^{c}	0.00±0.00^{b}	16.00±0.70^{c}
100	14.00±0.60^{b}	0.00±0.00^{b}	21.00±0.30^{b}
Fluconazole (1mg/ml)	38.00±0.60^{a}	38.00±0.60^{a}	38.00±0.60^{a}
DMSO(100ul)	0.00±0.00^{e}	0.00±0.00^{b}	0.00±0.00^{d}

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

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 12.5mg/ml and 100mg/ml for the n-hexane crude extract against strain P37005(isolate B1), the value of 12.5mg/ml and 100mg/ml was also revealed for the n-hexane crude extract against SC5314 (isolateB3) however, the methanol crude extract showed a value of 12.5mg/ml and 50mg/ml respectively against SC5314 (isolate :B3).

Table 8: Minimum inhibitory concentration (MIC) and Minimum fungicidal concentration (MFC) for the most active crude extract

S/N0	Isolated code	Strain	Plant part	Crude extracts	MIC	MFC
1	B1	P37005	Leaf	n – hexane	12.5mg /ml	100mg/ml
2	B3	SC5314	Leaf	n – hexane	12.5mg/ml	100mg/ml
3	B3	SC5314	Leaf	Methanol	12.5mg/ml	50mg/ml

DISCUSSION

The result of the biochemical, germ tube test and molecular characterization confirmed the three isolates collected. The use of biochemical and molecular characterization in identifying isolates is in agreement with a similar work of [18] as shown in Table 1-2. Three different solvents namely (n- hexane, ethyl acetate and methanol) with different polarity index were used for the extraction of the leaves of *Vernonia amygdalina***.** The percentage yield of *Vernonia amygdalina* is shown in Table 3. The n-hexane leaf extract (NHLE) of *Vernonia amygdalina* had the highest percentage yield of 11.42(%), Ethyacetate extract (EAE) was the lowest with a percentage yield of 0.39(%) while methanol leaf extract (MLE) had a percentage yield of 8.28% . The differences in percentage (%) might be as a result of the different solvents used, solubility of the different components in them and as well the availability of the different extractable components in them.[18,20]. Table 4 shows the quantitative phytochemical analysis of *Vernonia amygdalina* leaf crude extract obtained from different solvents. The quantitative phytochemical was done to determine the amount of constituents that are present in each of them. Several of them which include flavonoid, phenols, tannins and alkaloids was determined. A study conducted by [12]

on *Vernonia amygdalina* (bitter leave) revealed that the plant contain flavonoids, saponins, alkaloids, tannins and anthraquinones. Similarly,[21] also detected the presence of alkaloids, tannins, saponins and Flavonoids. Flavonoids generally are also reported to be present in glycosylated forms in plants and the sugar moiety has been found to be an important factor in determining their bioactivtity. Flavonoids have antioxidants potentials hence could offer protection against heart disease and cancer probably by enhancing the body defense against pathology induced free –radicals [22]. As reported by [23] phenolic compounds are some of the most widespread molecules among plant secondary metabolites which are known to act as natural antioxidants, antiulcer, anti–inflammatory, antispasmotic and antidepresssnt activities[24].The tannins containing plant extracts can be used against stomach and duodenal tumors and as anti-inflammatory, antiseptic, antioxidant and homeostatic pharmaceuticals [25]. Furthermore it have been reported by[26] that alkaloids potentials which tend to be organic and natural ingredients that have nitrogen and are also physiological active together with sedative and analgesic roles. Oxalates and phytic acid are said to be antinutrients but they have proven potentials, especially phytic acid which was found to be anticancer against bone, prostate, ovarian, breast, liver, cholesterol, leukemia, sarcoma and skin cancers. They are also important in protection of the gut from toxins and may have a positive impact on cholesterol and blood sugar [27] .Table 5-7 shows the antifungal activity of the leaf of *Vernonia amygdalina* against the three different strains (P37005, RM1000and SC5314).There was inhibitory activity for n-hexane leaf extract (NHLE) and Methanol leaf extract (MLE) of *Vernonia amygalina* at different concentration of the crude extracts for all the strains but the highest value 21.00 ±0.30mm was recorded for methanol leaf extract (MLE) at a concentration of 100mg/ml against SC5314 (isolate:B3). The zone of inhibition (ZI) increased as the concentrations were increased. Accordng to Prescott *et al.* [28] reported that the activity of antimicrobial agent is concentration dependent in addition, Edeoga *et al.*[19] reported that the inhibitory zones varies with the type of solvent used for extraction. This result is in agreement with the work of [29]. and also in conformity with the work of [30] who reported inhibitory activity of the leaf of *Vernonia amygdalina* against *Candida albicans*. The activity of n-hexane and methanol crude extracts of *Vernonia amygdalina* could be due to the presences of higher concentrations of bioactive substances that were found in both of them. The concentration of the bioactive substance eg saponin, alkaloids might have been in sufficient concentration for both the n-hexane and methanol crude extract compared to that of the ethyl acetate. Concentration of bioactive substance is one of the factors that affect microbial susceptibility, the higher the concentration the higher the activity of the chemical substances [31] These bioactive substances are mostly the antimicrobial agents that carry out inhibitory activity for both bacterial and fungal. There importance in medicinal plant extracts cannot be overemphasized. The result of the antifungal activity of the standard drug (fluconazole1mg/ml) used in this study showed a better zone of inhibition as compared to the crude. However, the negative control dimethylsulphur oxide (DMSO) showed no inhibitory activity against the standardized test strains. This suggests that DMSO does not contain any antimicrobial agent. 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[19] . The differences might be due to geographical location of the plant, differences in laboratory procedures and reagent used [32]. The season of the plant, age of the plant and method of

extraction may also affect the yield and the bioactive components of the plant [33]. 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

.Conclusion

The leaves of *Vernonia amygdalina* used in this study have shown promising pharmacological prospect. The crude extracts of n-hexane and methanol had inhibitory activity against the tested strains of *Candida albicans* (P37005, RM1000 and SC5314). However, the results from this study also indicates that the leaves of *Vernonia amygdalina* might have contained sufficient amount of bioactive substance that have antifungal potentials against the tested strains. The extracts from the leaves of *Vernonia amygdalina* should be further subjected to invivo trials inorder to evaluate their toxicity profile on organ system, which would ascertain their safety to humans. Active components should be characterized as well as elucidation of structure of the components should be done.

Conflict of interest

There was no conflict of interest among the authors.

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