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Phytochemistry and antifungal activity of root and seed extracts of *Moringa oleifera*

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Abstract

The qualitative and quantitative phytochemical screening and antifungal activity of root and seed extracts of *Moringa oleifera* was investigated. Ethanolic, methanolic, hot water and cold-water extracts of *Moringa oleifera* were tested against *Candida albicans*. Results showed the presence of carbohydrate, tannins, saponins, cardiac glycosides, alkaloids and phytic acid while flavonoids, anthraquinone and steroids appeared negative. Also, quantitative estimations of 0.57% and 1.17% oxalates, 4.02% and 2.66% phytic acid, 1.92% and 3.99% alkaloid, 65.47mg/100g and 109.12mg/100g tannins for the roots and seeds respectively was recorded. Activity of the methanolic extracts was highest while the cold-water extracts had the least activity of all tested extracts. *Moringa oleifera* is a promising plant for use in antifungal infections of *Candida albicans*.

Keywords: Antifungal, *Candida albicans*, Phytochemistry, *Moringa oleifera*.

Introduction

Traditional medicine has been a reliable means of treating so many infections (Okorondu *et al*., 2015; Adeleye *et al*., 2016). Among others, *Moringa oleifera* has gained a lot of popularity in tradomedicine. It is simply one of the numerous plants with outstanding therapeutic and preventive characteristics and therefore, has been of great contribution from ancient times (Fozia *et al*., 2012; Caceres *et al*., 1991). Several reports about its antibacterial activities are available (Eilert *et al*., 1981; Nepolean *et al*., 2009; Prashith *et al*., 2010), and it has been demonstrated that extracts from the leaves, flowers had activity over different fungal species (Sahar *et al*., 2015; Chuang *et al*., 2007). Some reports have demonstrated that the extracts had no activity on *Candida albicans* but little activity on *Candida tropicalis* (Pinal *et al*., 2014; Sahar *et al*., 2015). This could be as a result of the concentrations adopted or methodology. Methods of extraction and solvent used for extraction have a major role in determining the activity of a plant material (Okorondu *et al*., 2015).

This study reports on the phytochemistry and antifungal properties of root and seed extracts of *Moringa oleifera*.

Materials and Methods

Collection of plant materials

Fresh roots and seeds of *M. oleifera* were collected from Uncle Ray Botanical Garden, Okigwe Road, Owerri Municipal Local Government Area of Imo State. This was subsequently authenticated and identified in the Department of Plant Sciences, Faculty of Science, Imo State University, Owerri, Imo State.

Preparation of Plant materials

The freshly collected roots and seeds were washed with clean tap water, air-dried under shade at ambient temperature (25^{o}C) for 3 weeks. After drying, the plant materials were pounded into powder separately using laboratory crucible and pestle and binatone blender. The powered samples were stored in nylon bags labeled adequately, and kept at room temperature (25^{o}C) until required.

Extraction of Plant materials

The active components of the plant parts were extracted with ethanol, methanol and hot and cold sterile distilled water.

a. Ethanol and methanol Extraction

Ten grams (10g) of the dried powdered sample was soaked in 100ml of ethanol, contained in 500ml flask. The flask was covered with a cotton plug and then wrapped with an aluminum foil and shaken vigorously at 3hr intervals for 24hours at room temperature (El-Mahmoud, 2009). After 24 hours, it was shaken vigorously and then filtered using Whatman's No 1 filter paper. It was then oven dried to evaporate to dryness under reduced pressure. Each extract was transferred to a glass vial and kept at 4°C until needed. The process was repeated using Methanol as solvents.

b. Hot Water Extraction

Ten grams (10g) of grinded roots and seeds of the moringa were weighed using a digital weighing balance and poured into two different 500ml conical flask. Hundred milliliters of hot water was boiled to 100^{0}C and poured into the two different 500ml Erlenmeyer flask containing the plant material. The conical flasks were stirred by vigorous shaking for 30-60 minutes on a mechanical shaker and allowed to stand for 18-24hrs. The extracts were filtered using filter paper and filtrates were oven dried at temperature of 40^{0}C in a hot air oven for 4-5hrs for water to evaporate and kept for further analysis. The process was repeated using cold water as solvent.

Qualitative and Quantitative Determination of Phytochemical Components of the Extracts of *Moringa oleifera* Roots and Seeds.

Qualitative phytochemical Analysis of the Plant material.

The qualitative methods already established to test for classes of compounds in plant extracts by Ciulei (1964) and Chitravadivu *et al*. (2009) were used. The bioactive substances that were tested included: carbohydrate, tannins, saponins, and flavonoids, cardiac glycosides, oxalate, alkaloids, anthraquinone, steroids and phytic acid (Okorondu *et al*., 2015).

a) Test for Anthraquinones

The determination of Anthraquinones was carried out by the titration method of AOAC (2010). To 1ml of solution, 5ml of 10% HCL was added and allowed to stay for 5 minutes. The solution was filtered. The filtrate was decanted into a test tube and shaken with 5ml of benzene. The upper benzene layer was pipette off and transferred into test tube containing 5ml of 10% ammonium hydroxide. Production of pink, red or violet colouration showed the presence of anthraquinones.

b) Test for Glycosides

The determination of Glycosides was carried out according to the titration method of AOAC (2010). To 1ml of the test solution, 2 drops of conc. sulphuric acid was added and placed in a water bath for about 15 minutes. KOH was added to make the solution alkaline. To this solution, a few drops of $FeCl_2$ were added. The formation of a brick red precipitate indicates the presence of glycosides.

c) Test for alkaloids

One milligram of dried extract was dissolved in 6 drops of 2% hydrochloric acid. The solution was divided into 3 aliquots; to the first portion which acted as a reference, 2 ml of distilled water was added. To the second test tube, 2 drops of Dragendorff's reagent were added. A precipitate indicated the presence of alkaloids. To the third portion, 2 drops of Mayer's reagent was added and a yellowish white precipitate indicated the presence of alkaloids (Raffauf, 1962).

d) **Test for steroids and triterpenoids**

One milligram of dried extracts was dissolved in 0.5 ml of acetic anhydride; 0.5 ml of chloroform was added. The solution was pipetted into a dry test tube and 1 ml of concentrated sulphuric acid was added at the bottom of the tube. A brown-red ring at the interface between the two liquids and a green supernatant indicated the presence of steroids and triterpenoids AOAC (2010).

e) **Test for tannins**

One milligram of plant extracts was dissolved in 1.5 ml of water; 3 drops of dilute ferric chloride were added to the extract. A blackish blue colour indicated the presence of Gallic tannins and green blackish colour indicated catechol tannins (AOAC, 2010).

f) **Test for saponins**

Three drops of dimethylsulfoxide were added to 1 mg of plant extract; 5 ml of distilled water was added and the mixture shaken. The presence of foam which persisted for more than 15 min indicated the presence of saponins. (AOAC, 2010).

g) **Test for flavones aglycones**

One milligram of dry plant extract was dissolved in 1 ml of methanol at 50°C. Metallic magnesium and 5 drops of concentrated hydrochloric acid were added. A red or orange colour indicated the presence of flavones aglycones (Shibata's reaction or Cyanidin test) (AOAC, 2010).

h) **Test for reducing sugars (Carbohydrates)**

One milligram of the extract was dissolved in 2 ml of water and 1 ml of Fehling's reagent which contained a mixture of Fehlings solution I and II was added and the mixture heated. A brick red precipitate denoted the presence of reducing sugars (AOAC, 2010).

Quantitative phytochemical screening of plant materials

a) Tannin

Tannin was determined using methods described by AOAC (1980). This method was however slightly modified. About 2g of ground plant material was defatted for 2 hours using Soxhlet extraction apparatus. The residue was dried in an oven for 12 hours at 100°C, boiled with 30ml of distilled water, diluted to 500ml in standard volumetric flask and filtered through non-absorbent cotton wool. A volume of 25ml of the infusion was measured into 2 liter porcelain dish and titrated with 0.1N potassium permanganate (0.1N potassium permanganate was standardized against 0.1N oxalic acid) until the blue solution changed to green; then few drops of 0.1N potassium permanganate were added. The difference between the two titration was multiplied by 0.006235 to obtain the amount of tannin in the sample using equation: 0.1N oxalic acid =0.0006235g tannin (constant).

b) Phytic Acid

Phytic acid (inositol hexaphosphate) content of each sample was determined by the modified method of McCance and Widdowson (1935). Ten grams aliquot of the powdered sample was extracted overnight at room temperature with vigorous shaking in an MSE orbital shaker at 200rpm for 1 hour to ensure homogeneity and then neutralized by adding about 20ml of 20% sodium hydroxide: 50m1 of 1% ferric chloride on 1N hydrochloric acid and the mixture brought just to boil. The resultant mixture was filtered through an ashless whatman No 40 filter paper. The resultant residue was dissolved in about 10ml of 20% sodium hydroxide. This procedure dissolves phytic acid while precipitating ferric hydroxide which was removed by filtration. The filtrate (containing phytic acid) was digested with a mixture of concentrated sulphuric acid and perchioric acid in the ratio 5:1 in a Kjedahl flask. The pH of the digest was adjusted to 4 and phosphate determined colorimnetricahly at 710nm.

c) Total Oxalate:

One gram of the sample was weighed into 100ml Erlenmeyer flask and 75ml 3N H_2SO4 was added and stirred intermittently with a negative stirrer for 1hr. It was then filtered using whatmann filter paper. From the filtrate, 25ml was taken and titrated while hot 80-90^0 against 0.1N $KMnO_4$ solution until a faint pink colour persisted for at least 30secs. The overall reaction is:

$$MnO_4 - + Cr_2O_4^{2-} + 8H^+ \rightarrow Mn^{2+} + 4H_2O + 2CO_2$$

$$\text{Oxalate (mg/100g)} = \frac{T \times Vme \times DF}{Me \times Ms(g)}$$

where T is the titer value of KMnO4 (ml), Vme is the volume mass equivalent. DF is the dilution factor (Vt /A) where Vt is the total volume of titrate (filtrate) and A is the aliquot used, Me is the molar equivalent of $KMnO_4$ in oxalate.

d) Alkaloid Determination

The gravimetric method of Harbone (1973) was adopted. Five grams of the sample was weighed into a 250 ml beaker and 200 ml of 10% acetic acid in ethanol was added and covered and allowed to stand for 4 h. This was filtered and the extract was concentrated on a water bath to one-quarter of the original volume. Concentrated ammonium hydroxide was added drop wise to the extract until the precipitation was complete. The whole solution was allowed to settle and the precipitated was collected and washed with dilute ammonium hydroxide and then filtered. The residue is the alkaloid, which was dried and weighed. By weight difference, the weight of alkaloid is determined and expressed as a percentage of the sample weight analyzed. Given by the formula

$$\% \text{ alkaloids} = \frac{W_2 - W_1}{W}$$

where W = weight of sample
W1 = weight of empty filter paper
W2 = weight of paper plus precipitate

Test Organism and Sources of Isolates

Candida albicans was obtained from the Department of Microbiology, Federal Medical Center, Owerri, Imo State.

Germ tube test for the identification of *C. albicans*

This was done according to methods described by Isibor *et al*. (2005). Using a sterile loop, a small portion of a pure colony of *C. albicans* was inoculation into sterile test tubes containing 0.5ml of each of the test sera. The resulting mixture was incubated aerobically at 37^{o}C for not more than 2 hrs. To 0.5ml of human serum in a separate sterile test tube was inoculated a small portion of a pure colony of yeast and incubated in a 10% CO_2 jar for not more than 2hrs. At 10minutes intervals, a drop of the yeast-serum mixture was placed on a clean microscope slide, covered with a cover slip and examined microscopically, using the x10 and x40 objective lenses. The appearance of small filaments projecting from the cell surface confirmed formation of germ tubes. The earliest time of such germ tubes production was noted for each test serum.

Standardization of Inoculum

The test organisms were sub-cultured onto a fresh plate of potato dextrose agar (PDA) and incubated aerobically at 37°C for 24hrs. After incubation, a wireloop was used to transfer a little portion to a tube containing 5ml of potato dextrose broth (PDB). The broth culture was incubated and serially diluted until it achieved a turbidity matching McFarland standard 0.5 (1.5 x 10^8CFU/ml).

Assay for antifungal activity

Agar well diffusion was used in the antimicrobial activity tests. The yeast suspensions were prepared equal to the turbulence McFarland standard and 0.1ml 0.5 (1.5 x 10^8CFU/ml) was uniformly seeded on freshly prepared surface dried Potato Dextrose Agar and spread using a sterile swabstick on the agar medium. One gram (1g) of each plant extract was reconstituted into 1ml of sterile distilled water and two-fold serial dilutions were performed to obtain dilutions of different concentrations (500mg/ml, 250mg/ml, 125mg/ml, 62.5mg/ml and 31.25mg/ml). Wells were made on the Potato Dextrose Agar using sterile cork borer of 6 mm diameter. Ranging aliquots of 0.1ml of reconstituted extracts were dropped in each labeled well ditch and then incubated at 37°C for 24hrs. Tips were discarded such that each well has the test material in serially descending concentrations. Clotrimazole (30µg/ml) and distilled water were used as positive and negative control respectively. The plates were observed for zones of inhibition and measured in millimeter with a transparent meter rule after 24hrs incubation period at 37°C.

Determination of Minimum inhibitory Concentration (MIC)

The estimation of MIC of the plant extracts were carried out using the method of Akinpelu and Kolawole (2004). Two milliliters (2ml) of nutrient broth was pipetted into several test tubes and sterilized by autoclaving at 121°C for 20 minutes at a pressure of 15psi. One gram (1g) of each extract was added into 2m1 of the nutrient broth and diluted in two-fold to obtain different concentrations. The dose levels of 500mg/ml, 250mg/ml, 125mg/ml, and 62.5mg/ml and 31.25mg/ml concentrations each of the extracts were used for MIC determination. One tenth milliliter

(0.1ml) of standardized inoculum of overnight broth culture was inoculated into the dilutions and incubated at 37°C for 24hrs. The MIC was taken as the least concentration that inhibited the growth of the test organism using spectrophotometer at wavelength (λ) =340nm to measure turbidity for yeast isolates.

Determination of Minimum Fungicidal Concentration (MFC)

The MFC of the plant extract was determined by the method of Spencer and Spencer (2004). To determine the MFC for each set of well in the MIC determination, a loopful of broth was collected from those plates, which did not show any visible sign of growth and streaked on sterile PDA. The plates were then incubated at 37°C for 24 h. After incubation the concentration at which no visible growth was seen was noted as the minimum Fungicidal concentration in mg/ml.

Statistical analysis:

The data were expressed as mean ±S.D. Biochemical parameters were analyzed statistically using one way analysis of variance (ANOVA) followed by Tukey's multiple comparison test for comparison with control group (ref). $P<0.05$ was considered as significant. The data from the MIC and MFC experiment was used to produce linear curves so as to obtain linear equation that was used to obtain the IC_{50} which is the concentration of the plant extract that could inhibit 50% of the fungal suspension (McFarland's standard 0.5).

Results and Discussion

Phytochemical analysis of the roots and seeds of *Moringa oleifera*.

Qualitative phytochemical analysis

Preliminary phytochemical analysis of the plant showed the presence of carbohydrate, tannins, saponins, cardiac glycosides, alkaloids and phytic acid while flavonoids, anthraquinone and steroids were absent.

Table 1: Preliminary phytochemical analysis of *Moringa oleifera*

Phytochemicals	Results	
	Seed	Root
Carbohydrate	+	+
Tanins	+++	++
Saponins	+	+
Flavonoids	-	-
Cardiac glycosides	+	+
Oxalate	+	+
Alkaloids	++	+
Anthraquinone	-	-
Steroids	-	-
Phytic acid	+	++

Key: + available; ++ Very available; +++ Readily available; Absent

Quantitative phytochemical analysis

The phytochemicals in the qualitative analysis with ++ were further assayed for quantitative analysis. The quantitative phytochemical analysis showed the presence of 0.57% and 1.17% oxalates, 4.02% and 2.66% phytic acid, 1.92% and 3.99% alkaloid, 65.47mg/100g and 109.12mg/100g tannins for the roots and seeds respectively (Table 2).

Table 2: Anti-nutritional factors of Moringa seeds and roots

SAMPLE	OXALATE (%)	PHYTIC ACID (%)	ALKALOID (%)	TANNIN (mg/100g)
MORINGA ROOT	0.59	4.12	1.83	68.59
	0.55	3.91	2	62.35
Mean ± SD	0.57±0.02	4.01±0.14	1.915±0.12	65.47±4.41
MORINGA SEED	1.18	2.68	4.2	112.23
	1.16	2.63	3.78	106
Mean ± SD	1.17±0.01	2.655±0.03	3.99±0.3	109.12±4.40

The phytochemical residues in plants are responsible for most of its bioactivity (Okorondu *et al*., 2015). Nweze and Nwafor (2014), had reported that aqueous extracts had higher anthraquinone (11.68 ± 0.04), alkaloid (3.07 ± 0.00), steroids (3.21 ± 0.00), terpenoids (4.84 ± 0.05), cardiac glycoside (0.36 ± 0.03), tannins (9.36 ± 0.04) and carotenoids (1.16 ± 0.05); while ethanolic extract had higher flavonoid (3.56 ± 0.03) and saponins (1.46 ± 0.03). These results also confirm that the plant contains useful phytochemicals that can be explored for phytomedicine (Okorondu *et al*., 2015). These antimicrobial phytochemicals (especially, alkaloids, tannins, anthraquinone and anthocyanin) act by binding with the cell walls and inactivate the enzymes (Tiwari *et al*., 2011). Leaves, seeds and roots of *M. oleifera* are rich in tannins. This explains why it is used in the treatment of urinary tract infection, diarrhoea, healing of wounds and dysentery (Fahey, 2005; Akaneme, 2008).

It has been reported that climatic factors and stages of maturity could cause variations in distribution of these phytochemicals in leaves of *M. oleifera* (Bamishaiye *et al*., 2011) as well as the choice of solvent as different solvents have different extraction capabilities and spectrum of solubility for phytoconstituents (Handa *et al*., 2008). Therefore, the choice of the solvent for extraction, the time of harvest of plant material and climatic factors need to be explored so as to confirm the best period of plant harvest for phytomedicine.

Antifungal property of ethanolic root and seed extracts of *Moringa oleifera*.

Figure 1 shows the antifungal activities of root and seed extracts of *Moringa oleifera*. Best activity was recorded in the methanolic extracts while the least activity was recorded in the cold-water extracts. The activities of the extracts increased with increase in concentration. The results also depicted that the root extracts had better activities on *Candida albicans*. Compared to the control(Nystatin), the extracts had lower activities. Opposed to results from this work, reports from Pinal*et al*. (2014) and Sahar *et al*. (2015) reported no activity against *Candida albicans*.

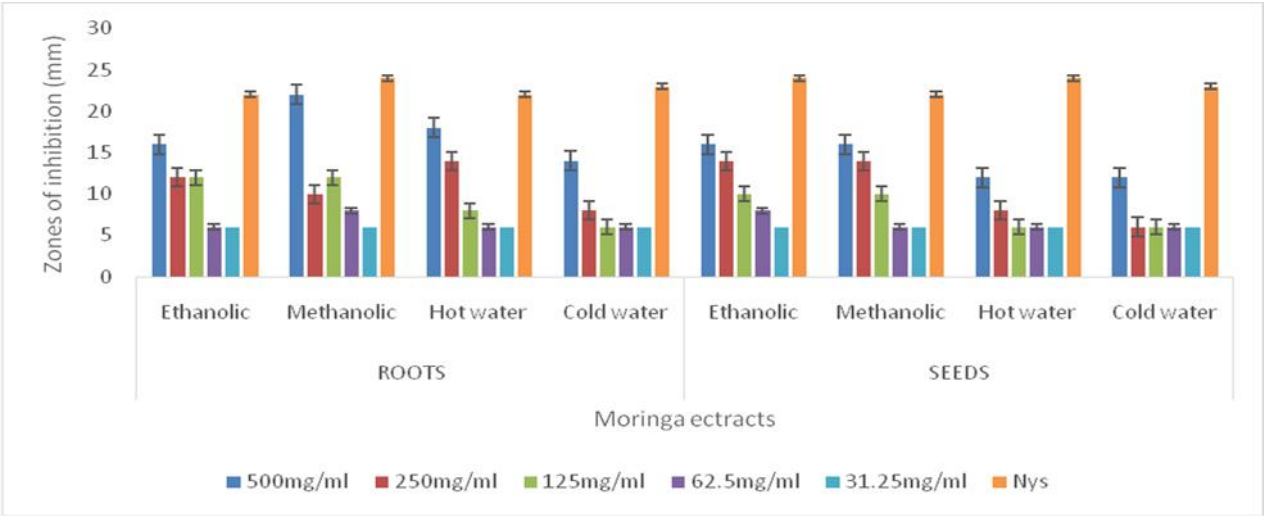

Figure 1: Antifungal activities of Root and seed extracts of *Moringa oleifera*.

Results from the MFC and IC_{50} also demonstrated that the Methanolic and ethanolic roots and seed extracts had MFC values of 125 mg/ml and 250 mg/ml respectively (Figure 2). They were however distinguished in their IC_{50} values of 200.46 mg/ml and 320.26 mg/ml for root extracts and 390.21 mg/ml and 442.77 mg/ml for seed extracts. Lower values were recorded in hot water and cold-water extracts, however, they had higher MFC values.

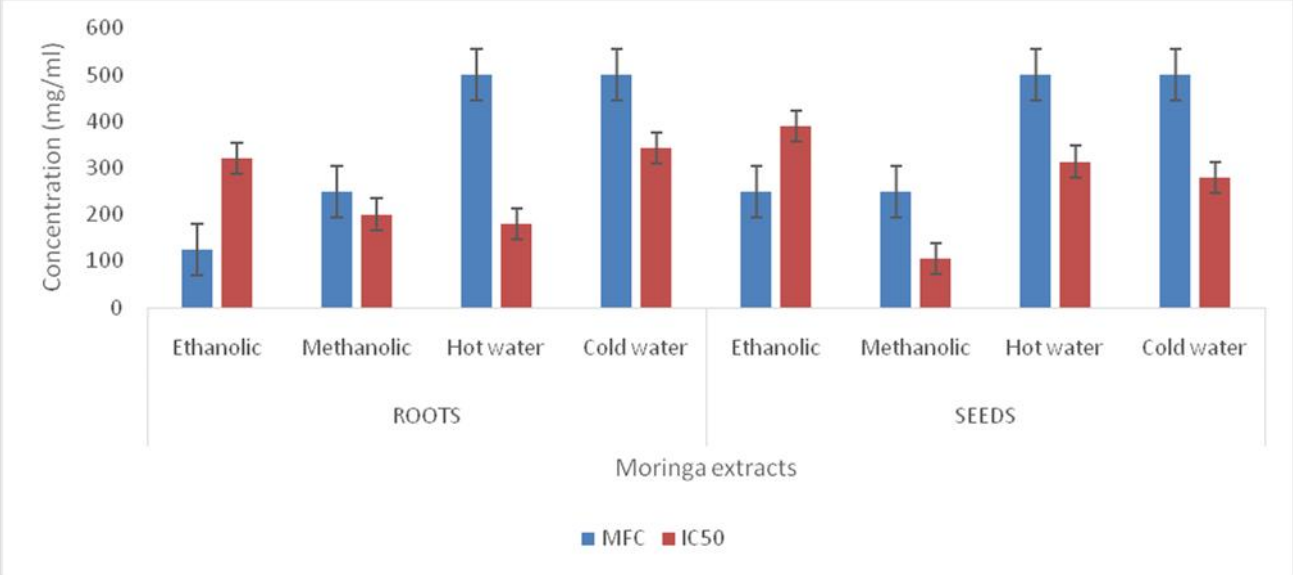

Figure 2: MIC and IC_{50} values of Root and seed extracts of *Moringa oleifera*.

Conclusion

The activity of the plant materials tested had varied activities which were dependent on the extract used. Although methanolic extracts had the best activity, proper care should be taken in its use due to methanol toxicity. More so, the plant material is a good source of antimicrobial variables and should be encouraged. More work has to be done to evaluate the mechanism or resistance as reported by previous work as well as reasons for susceptibility reported in this work.

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