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Comparative Phytochemical screening and invitro Antimicrobial Activity of Aqueous, Ethanolic, and Ethyl Acetate Extracts of Stem Bark and Leaves of Horse Radish (*Moringa oleifera*) Plant

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Abstract: This study compared the phytochemical composition of *Moringa oleifera* leaf and stem bark in vitro antimicrobial potential of its aqueous, ethanolic, and ethyl acetate extracts tested on selected pathogens; E. coli, Staph. aureus, P. aeruginosa, A. niger, A. fumigatus and C. albicans. Plant materials were sampled and prepared as described by Gwana, *et al.* [1]. Phytochemicals were screened by using the methods as described by Harbone [11], Sofowora [4], Tease and Evans [12]. Antimicrobial activity of the extracts of the leaves and stem bark were assayed, using the agar - disc diffusion method as described by Joanne, *et al.* [13] and Kirby - Bauer classification method. The study revealed the presence of alkaloids, flavonoids, glycosides, saponins, steroids and tannins. The stem bark extracts has been shown to contain more phytochemicals, which reflects in their ability to show more antimicrobial activity. The aqueous leaf and stem bark extract inhibited all the fungi isolates. Minimum inhibition concentration (MIC) and minimum bactericidal concentration were determined (MBC). The ethanol extracts of the leaf and stem bark at a MIC at l00 mg / ml exhibited antimicrobial activities against all the microorganisms. Ethyl acetate stem bark showed the highest zone of inhibition (16 mm) against E. coli, this is owing to the fact that more secondary metabolites are released. Aqueous leaf and stem extracts gave the highest MBC at 25 mg / ml and 50 mg / ml for A. niger. The variations in the presence of the phytochemicals may be due to the choice of the solvent used in the extraction. The necessity to search for plant - based antimicrobials is increasing due to high cost, reduced efficacy and increased resistance to conventional medicines and there is need to proffers solutions these problems matter.

Keywords: Antimicrobial; Extracts; Inhibition; In Vitro; *Moringa oleifera*; Phytochemical.

1. Introduction

The natural products have been interesting and important sources of biologically active (antimicrobial) substances and the major sources of which are still left undiscovered [1-3]. The existence of plants with therapeutic uses (medicinal plants) could be traced to as far back as the time of creation [4-7]. The plant constituents which function to cure diseases are termed secondary metabolites [2, 3, 8]. Plants have been major source of medicine and

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presence of plant secondary metabolites (Phytochemicals) has been implicated for most plants therapeutic activities [6, 9, 10].

There are more than 35,000 plant species with various phytochemicals in them being used in various human cultures around the world for medicinal purpose [3, 5, 8]. Phytochemicals are biologically active compounds found in plants such as vegetables and grains in small amounts, these compounds are not established nutrients, but significantly protect the development of lots of degenerative diseases [3, 12, 14]. However, the presences of the phytochemical constituents such as alkaloids, flavonoids, tannin, and phenolic, etc, compounds have been reported to be important compounds in many other medicinal plants [1, 2, 9].

Drumstick or horseradish tree (*Moringa oleifera*) is the most widely cultivated species of the genus *Moringa*, which is the only genus in the family Moringaceae. This rapidly growing, drought - resistant tree is native to the southern foothills of the Limalayas in northwestern India and widely cultivated in tropical (including Nigeria) and subtropical areas where its young seed pods and leaves are used as vegetables. It is a perennial softwood tree with timber of low quality but which for centuries has been advocated for traditional medical and industrial uses.

Moringa is derived from the Tamil word, Murungai [15, 16]. It is a fast - growing, deciduous tree and can reach a height of 10 to 12 m (32 - 40 Ft.) with a trunk diameter of 45cm (1.5 ft). The bark has a whitish - grey colour and is surrounded by thick cork. Young shoots have purplish or greenish-white, hairy bark. The tree has an open crown of drooping, fragile branches and the leaves build up feathery foliage of tripinnate leaves. The flowers are fragrant and bisexual, surrounded by five unequal, thinly veined, yellowish-white petals and grow on slender airy stalks in Spreading or drooping later flower clusters which have length of 10 - 25cm [5, 17, 18].

Moringa oleifera tree is one such plant which is reported to possess several medicinal properties [8]. The different parts of this plant viz. leaves, stem bark, root bark, flowers, fruits and seeds are used in the indigenous systems of medicine for the treatment of variety of human ailments and some parts are also eaten as vegetable [15, 19]. Due to advancements in technology and improved scientific separation techniques, many more phytochemicals have been discovered to have therapeutic values. As the global scenario is now changing towards the use of non-toxic and eco-friendly products, development of modern drugs from traditional medicinal plants should be emphasized for the control of various human and animal diseases [3, 4, 20].

Other chief medicinal properties of the plant include antispasmodic, diuretic, antimicrobial activities, etc. *M. oleifera* parts are being employed for the treatment of different ailments in the indigenous system of medicine, particularly in South Asia [19, 21]. The use of plant extracts, and pure compounds isolated from natural sources has always provided a foundation for modern pharmaceutical compounds [11]. In recent years considerable work has been done to investigate the pharmacological actions of the leaves, stem barks and other parts *of Moringa oleifera* on scientific lines [16].

The aims of this research study are to screen and compare the activities of the stem bark and leaf of *Moringa oleifera* plant extracts using different solvents with emphasis on their antimicrobial effects on *Escherichia coli, Staphylococcus aureus, pseudomonas aeruginosa, Aspergillus niger, Aspergillus flavus* and *Candida albicans.* The necessity to search for plant - based antimicrobials is increasing due to high cost, reduced efficacy and increased resistance to conventional medicines and the harvested leaves and stem bark from *Moringa oleifera* trees (known in Hausa language as *Zogale*; in Kanuri language as *Allam*; in Fulfulde language as *Kubibu*) served a wide variety of purposes are important agents of healing and nourishment to both human and animals.

2. Materials and Methods

The research was conducted between April and December, 2015, at Biotechnology Research Centre of Nnamdi Azikiwe University, Awka, Nigeria. All materials and reagents used were standard of high grade, and some of the materials and reagents (Biotec, H & B Warners, Merck and Pfizer Product) for this study were of analytical grade and were commercially obtained. Standard Operation Procedures are absolutely being observed.

2.1. Samples Collection

The plant stem bark and leaves were collected from *Moringa oleifera* plantation aseptically, at number 10, Ubaka lane, by works road, Awka, Anambra State of Nigeria.

2.1.1. Plant Sample Identification

The plant was identified as *Moringa oleifera* by Okigbo, R.N. of Botany department, Nnamdi Azikiwe University, Awka, Anambra State, Nigeria. The age of the plant was estimated at about two years old and authenticated with reference to the herbarium sheets (Voucher number 024), kept at the Herbarium of the Botany Department of the same University.

2.1.2. Preparation of Sample Plant Materials

The plant parts materials obtained were prepared, standard operation procures (SOP) are absolutely being observed as described by Gwana, *et al.* [1].

The leaves were destalked and the stem bark was cut into pieces, washed with running tap water for several minutes, deionised water for few minutes and air - dried each separately for three weeks at an average room temperature of 30 – 34^{o}C. Continuous turning of the plants materials were done to avert fungal growth for eighteen

days. The leaves and the stems samples were kept away from high temperatures and direct sunlight to avoid destroying active compounds.

The reduced chopped leaves and stems bark materials were pulverized reduced to fine powder using an electronic blender. This was done in order to increase the surface area so that the solvents could act on the bonds in the plants thus releasing the photo - constituents. The fine powdered plant material was then each separately transferred in to sterilized brown plastic bottles, labeled, screwed - capped and stored in a dry – cool, and away from direct sunlight (this is to avoid the denaturalizing the prepared plant material sample and its components) until when ready for extractions.

2.2. Photochemical Screening

The qualitative screening was carried out on the both the stem bark extracts and leaves of the three (3) solvents (water, ethanol and ethyl acetate) to test for the present of alkaloids, terpenoid, cardiac glycoside, polyphenol, reducing sugar, tannin, hydroxyl methyl anthraquinones, steroid, flavonoid and saponin in the leaves and stem bark of *Moringa oleifera* plant as follows. Phytochemical Screening was carried out using established methods of Harbone [11] and Sofowora [4] and Tease and Evans [12].

2.2.1. Test for Alkaloids

Procedure: 2mls of the extract was added into a test tube and the mixture was heated for 20 minutes using water bath. The heated mixture was filtered and 1ml of the filtrate was measured into a test tube; 0.5ml of Wagner's reagent was added to it. A reddish brown colouration was observed which is indicative of alkaloid [11].

2.2.2. Test for Saponin

Procedures: This is divided into two; frothing and emulsion tests.

2.2.2.1. Frothing Test

Procedure: 3mls of the extract was pipette into a test tube, 2mls of distilled water was added to it, and then it was shook vigorously. A persistent frothing movement at least for 15 minutes was observed.

2.2.2.2. Emulsion Test

Procedure: 3mls of the extract was pipette out into a test tube and 5 drops of olive oil was also added and it was shook vigorously. Emulsification was observed [11].

2.2.3. Test for Steroid

Procedure: 1ml of the extract was treated with 0.5ml of acetic acid, 0.5ml of chloroform and 1ml of concentrated hydrogen tetraoxosulphate. A reddish brown ring was formed at the separating level of the two liquids indicating the presence of steroids [11].

2.2.4. Test for Flavonoid

Procedure: 3ml of the extract was pipette out and 10ml of distilled water was added to it and it was shaken and 1ml of 10% Sodium hydroxide was also added to the mixture. A yellow colouration was observed showing the presence of flavonoid [11].

2.2.5 Test for Tannin

Procedure: 1ml of extract was measured into a test tube and it was heated. A drop of 10 % ferric chloride was added to it. The mixture showed a green coloration.

2.2.6 Test for Anthraquinones

Procedure: 2mls of extract was shaken with 5ml of 10% ammonia solution. The presence of a pink red to violet colour in the ammoniacal (lower) phase indicated the presence of anthraquinones.

2.2.7. Test for Hydroxyl Methyl Anthraquinones

Procedure: 2mls of extract was shaken with 5ml of 10% ammonia solution. The formation of a red colouration or precipitate indicates its presence [12].

2.2.8 Test for Reducing Sugar

Procedure: 2mls of the extract in a test tube was added to 5ml of Fehling solutions and heated hi a water bath at 80°C for 10 minutes. The presence of brick red precipitate indicates the presence of reducing sugar.

2.2.9. Test for Polyphenol

Procedure: 5mls of distilled water was added to 2mls of the extract and heated in a water bath for 10 minutes. 1ml of ferric chloride was added to the mixture followed by 1ml of 1% potassium ferro cyanide. The formation of a green-blue precipitate indicated the presence of polyphenol [12].

2.2.10. Test for Terpenoid

Procedure: 5mls of extract was mixed with 2mls of chloroform in a test tube. 3mls of concentrated hydrogen tetraoxosulphate was carefully added to the mixture which then forms an interface of reddish brown colouration. This is indicative of the presence of terpenoid [4].

2.2.11. Test for Cardiac Glycoside

Procedure: 0.5 grams of the sample was dissolved in 2mls of chloroform. 2m!s of concentrated sulphuric acid was carefully added to form a reddish brown colour at the interface which is indicative of the presence of steroidal ring (a glycone portion of the cardiac glycoside) [4].

2.3. Preparations of Culture Media

The experiments were carried out under sterile condition and hygienic environment. The media used were Mac Conkey agar (Biotech product), Centrimide agar (Biotech), Sabouraud Dextrose Agar (Merck product) Nutrient Broth (Biotech) Sabouraud Dextrose Broth (Merck product) and Muller Hilton Agar (Biotec product). The standard antimicrobial drugs used were Fluconazole (Pfizer product) for fungi and Tetracycline (Pfizer product) for bacterial isolates. The media were prepared according to the manufacturer's instructions. The Sabouraud Dextrose Broth and Sabouraud Dextrose Agar were used for the fungi, while the Nutrient Broth, Mac Conkey agar and Centrimide agar were used for the bacteria. After the preparation of the media, were allowed to cool to 45^{o}C before were dispensed aseptically in to the plastic Petri dishes each and were allowed to solidify before inversion respectively.

2.3.1. Source, Collection and Confirmation of Isolates

The pure culture of microorganisms used for the evaluation of the antimicrobial potential of the leaves and stem bark extracts of *Moringa oleifera* were *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Aspergillus fumigatus, Aspergillus niger* and *Candida albicans.* The isolates were all locally isolated pure cultures obtained from the Glanson Diagnostic Laboratory, Awka, Anambra State of Nigeria. The isolates were identified using various standards of biochemical tests. All bacterial isolates were maintained on nutrient agar slants and fungal isolates on Sabouraud dextrose agar at a temperature of 4°C. The isolates were confirmed using morphological and biochemical examinations. The morphological examinations include culture of microorganism and Gram staining tests. Biochemical Tests, these include Coagulase test, Catalase test and Indole test.

2.3.2. Maintenance of Microorganisms (Bacteria and Fungi)

i. Bacterial Isolates

All bacterial isolates were maintained on nutrient agar slants wrapped with aluminium foil and kept in refrigerator at a temperature of 4^{o}C respectively.

ii. Fungal Isolates

All fungal isolates were maintained on Sabouraud dextrose agar slants, they were carefully wrapped with aluminium foil, also kept in the refrigerator at a temperature of 4°C.

2.3.3. Isolates Culture

The isolates were cultured on their respective selective media. Mac Conkey agar, Centrimide agar, Sabouraud Dextrose Agar and confirmed with the Gram Staining reaction, Indole, Catalase, Coagulase, Motility tests, while slide culture test, Germ tube test were used for the confirmation of fungi isolates.

2.3.4. Inoculums Preparation

The bacterial and the fungal inoculums were prepared by inoculating a loopful of test organisms in 10 ml of nutrient broth in to three separate Bijou bottles for the bacterial isolates and another 10 ml of the Sabouraud dextrose broth in to three separate Bijou bottles for the fungal isolates. They were incubated at 37^{o}C and 25^{o}C for 4 to 6 hours for bacteria and fungi till a moderate turbidity were developed respectively.

2.3.5. Standard Antibiotic Drug Used

The standard drugs of choice of quality were; Tetracycline and Fluconazole (Pfizer product) were obtained commercially as a standard for the working concentration and antimicrobial activity test.

2.4. Preparation of Extract for Antimicrobial Assay

Procedure: 1600 mg of the crude ethanolic extract was dissolved with 16 ml of 50 % V/V Dimethyl sulphoxide (DMSO) giving a concentration of 100 mg / ml, was further reduced from 100 mg / ml, 25 mg / ml and 12.5 mg / ml using a two – fold dilution with DMSO as solvent. This procedure was carried out same (with both solvents) for ethyl acetate extract and aqueous extract using DMSO and water as solvent respectively.

2.4.1. Antimicrobial Bioassays

The antibacterial activity of the aqueous, ethanolic and ethyl acetate extracts of the leaves and stem bark were assayed, using the agar - disc diffusion method as described by Joanne, *et al.* [13] and Kirby Bauer classification method.

The plates were seeded with the test organisms of *Pseudomonas aeruginosa*, *Escherichia coli* and *Staphylococcus aureus*. The concentrations of the leaves and stem plant extracts used were; 150 mg / ml, 125 mg / ml, 100 mg / ml, and 50 mg / ml respectively. Sterile filter paper discs of 6 mm in diameter were impregnated with various concentrations of the extract and were placed on the seeded plates. The inoculated plates were allowed to stand for 10 minutes for diffusion to occur. The plates were incubated for 24 hours at 37^0C. The relative susceptibility of the microorganisms in the various extracts was indicated by clear zones of growth inhibition around the well and measures in millimeter. The standard drug test was also treated as the same as the extracts method was carried out in triplicates and the results were taken and recorded.

2.4.2. Determination of Minimum Inhibitory Concentration (MIC)

The MIC was determined by the broth macro dilution method; 1ml of the 100 mg / ml of the extract was added to 1ml of broth for bacteria, thus reducing the concentration to 50 mg / ml, using a two-fold dilution series. The same procedure was done with Sabouraud dextrose broth. The bacteria and fungi were inoculated into the mixtures of NB and SDB respectively. Positive controls were prepared using 100 mg / ml of tetracycline for bacteria and 100 mg / ml of Fluconazole for fungi in place of the extracts while water was used as the negative control for the aqueous, ethanolic and ethyl acetate extracts respectively. Culture medium without sample, but with the organism, the different extracts and the culture medium with the standard drugs and also culture medium alone were also prepared and incubated and observed after 24 hrs for turbidity.

2.3.5. Minimum Bacterial Concentration (MBC) and Minimum Fungicidal Concentration (MFC)

The tubes that were not turbid were further streaked on a solid medium to determine the lethal concentration, while the clear tubes of the bacterial mixtures were streak on MHA; Fungal Mixtures were streaked on SDA; with positive and negative controls were carried out. The inoculated plates were incubated at 37^oC for 24 hrs to observe the MBC and 37^oC for 3 days (72 hours) to observe the MFC.

2.4. Data Analysis

Data obtained from this research study were subjected to statistical tools of analysis using percentage, mean for the measurement of central tendency, and standard deviations for measurement of dispersion and or discrepancy within the variables being obtained and its' significance, as described by Stroud and Booth [22]

3. Results

The analysis on some plants that produces a very range of bioactive molecules which are rich in phytonutrients and with different types of medicines and food products becomes necessary; produces such products, such as antimicrobials activities should be tested and examined against microbes to confirm these activities associated and to ascertain the parameters associated with it. This leads to the results obtained from this research study work on screen for phytochemicals and compare the activities of the stem bark and leaf of *Moringa oleifera* plant extracts using different solvents (aqueous, ethanolic and ethyl acetate) with emphasis on their antimicrobial effects and were done on six organisms; three bacterial and three fungal isolates, (*Escherichia coli, Staphylococcus aureus, pseudomonas aeruginosa, Aspergillus niger, Aspergillus flavus* and *Candida albicans*). The results obtained are presented in tables below:

Table 1 showed the biochemical identification and the motility test of the bacterial isolates involved that; *E. coli* was Catalase, Indole, motility positive and Coagulase negative. *P. aeruginosa* was Catalase, motility positive and Coagulase, Indole negative, while *S. aureus* was Catalase, Coagulase positive and Indole, motility negative.

Table 2 showed the mean percentage yield and the appearance of the crude extracts of *M. oleifera* that; the leave aqueous crude extract appeared light green solid with 11 %, ethanolic crude extract appeared dark green semi – solid with 16 % and the ethyl acetate crude extract appeared dark green solid and had a mean percentage of 14. While the stem bark aqueous crude extract was light brown solid in colour appearance and had 12 %, ethanolic crude extract of the stem bark appeared dark brown solid with mean percentage of 10 and the ethyl acetate stem bark crude extract appeared lemon green solid and had the mean percentage of 12 respectively.

Table 3 showed the qualitative screened of the phytochemicals of the different leave extracts of *M. oleifera* involved in the analysis that; Anthraquinones and reducing sugar were not detected. Alkaloid was present in both

ethanolic and ethyl acetate but not detected in aqueous extract. Cardiac glycoside was present in ethanolic extract only. Cyanogenic glycoside was present in aqueous leave extract only. Flavonoid was detected in aqueous and ethanolic extracts of the *M. oleifera* leave, also same with Phenol and terpenoid. Saponin (Emulsion technique) was detected in ethyl acetate extract and Saponin (Frothing technique) was detected in the ethanolic extract of the leave, also same with steroid and tannin respectively.

Table 4 showed the qualitative screened of phytochemicals of the different stem bark extracts of *M. oleifera* that were carried out that; Anthraquinones, Polyphenol, Reducing sugar and Tannin were not detected in the both extracts. Alkaloid, Cardiac glycoside and Saponin (Emulsion technique) were present in ethyl acetate extract of the stem bark only. Cyanogenic glycoside was detected in aqueous and ethyl acetate extracts in abundant. Flavonoid was present in ethanolic extract only. Saponin (Frothing technique) was detected in both the aqueous and ethanolic extracts of the plant stem bark. Steroid and Terpenoid were detected in ethanolic and ethyl acetate extracts of the stem bark of *M. oleifera*.

Table 5 showed the antimicrobial activity of the leaves and stem bark ethyl acetate extracts of *M. oleifera* on both the bacterial and fungal microbes that were determined. Ethyl acetate stem bark extract at concentration of 100 mg / ml had the mean zone of inhibition for bacteria; the highest was 15 mm for *E. coli*, 11.5 mm for *P. aeruginosa* and the lowest was 11 mm for *S. aureus*. While fungi; was 10 mm for *A. fumigatus*, *A. niger* and *C. albicans* each. At 50 mg / ml for bacteria, the mean zone of inhibition ranges from 9 mm to 13 mm and for fungi, the mean zone of inhibition ranges from 6 mm to 8 mm. At 25 mg / ml for bacteria was 6 mm to 10.5 mm and for fungi was 0 mm (no inhibition). At a concentration of 12.5 mg / ml for bacteria was 6 mm to 8 mm and no inhibition (0 mm) of the any fungi involved. Ethyl acetate stem bark extract at concentration of 100 mg / ml had the mean zone of inhibition for bacteria range from 13 mm to 16 mm and for fungi was 10 mm to 15 mm. At a 50 mg / ml for bacteria was ranged from 11.5 mm to 13.5 mm and that of fungi was 9.5 mm to 13 mm. At concentration of 25 mg / ml the bacteria mean zone of inhibition ranged from 9 mm to 10 mm, while for fungi was 8 mm to 1o mm. finally at a concentration of 12.5 mg / ml, for bacteria was 6 mm to 8 mm and for fungi was 0 mm to 6 mm (where 0 mm no inhibition) respectively.

Table 6 showed the antimicrobial activity assay of the ethanolic stem bark and leaves extracts of *M. oleifera* on both the bacterial and fungal microbes. At concentration of 100 mg / ml of the ethanolic leaf tract, bacteria mean zone of inhibitions were 13 mm, 10.5 mm and 11 mm for *E. coli*, *P. aeruginosa* and *S. aureus* (range between 10.5 mm to 13 mm), that fungi were 15.5 mm, 14.5 mm and 11 mm for *A. fumigatus*, *A. niger* and *C. albicans* (11 mm to 15.5 mm). At 50 mg /ml, bacteria were ranged from 11 mm to 14.5 mm and fungi were from 0 mm to 13 mm (where 0 mm showed no inhibition). The activity of the assay at the concentration 25 mg / ml, bacteria were ranged from 6 mm to 9 mm, while fungal were 0 mm to 10 mm. At the concentration of 12.5 mg / ml, bacteria were ranged from 0 mm to 7 mm, while the fungi were at the ranged from 0 mm to 8 mm. For the ethanolic stem bark extract concentration at 100 mg / ml, bacterial mean zone of inhibitions were ranged from 13.5 mm to 15 mm, while that of fungi were 0 mm to 15 mm. At the concentration of 50 mg / ml of the extract, bacteria were ranged from 11 mm to 14.5 mm, while the fungi were ranged from 0 mm to 13 mm. At 25 mg / ml of concentration, the mean inhibitions of bacteria were from 9.5 mm to 12 mm, and the fungi were ranged from 0 mm to 10 mm, and lastly at the concentration of 12.5 mg / ml of the stem bark extract, bacteria were ranged from 9 mm to 10 mm, while the fungi were from 0 mm to 8 mm respectively.

Table 7 showed the antimicrobial activity assay of the aqueous stem bark and leaves extract of *Moringa oleifera* on both the bacterial and fungal microbes. At concentration of 100 mg / ml, all the bacteria mean zone of inhibitions were 0 mm, (no inhibition at all), fungi were ranged from 7 mm to 15 mm. At 50 mg /ml, bacteria were 0 mm and fungi were from 0 mm to 11 mm (where 0 mm showed no inhibition). The activity of the assay at the concentration 25 mg / ml, bacteria were 0 mm (no inhibition), while fungal were ranged from 0 mm to 10 mm. At a concentration of 12.5 mg / ml, bacteria were found no inhibition (0 mm), while the fungi were at the ranged from 0 mm to 8 mm. For the aqueous stem bark extract concentration at 100 mg / ml, bacterial mean zone of inhibitions were no inhibition (0 mm), while that of fungi were 7 mm to 15.5 mm. At the concentration of 50 mg / ml of the extract, bacteria were 0 mm (no inhibition), while the fungi were ranged from 0 mm to 14 mm. At 25 mg / ml of concentration, the mean inhibitions of bacteria were 0 mm (no inhibition), and the fungi were ranged from 0 mm to 10 mm, and lastly at the concentration of 12.5 mg / ml of the stem bark extract, both bacteria and fungi had no shown any inhibition.

Table 8 showed the antimicrobial activity of the standard drugs (Tetracycline and Fluconazole) used. Tetracycline on both the bacterial and fungal microbes at a concentration of 150 mg / ml, bacterial inhibitions were 23 mm, 27.5 mm and 21.5 mm for *E. coli*, *P. aeruginosa* and *S. aureus*, and that of fungi tested were no inhibition at all. While the Fluconazole at a concentration of 150 mg / ml, bacteria had no inhibition at all, and the fungi had 17.5 mm, 21 mm and 24 mm for *A. fumigatus*, *A. niger* and *C. albicans* respectively.

Table 9 showed the minimum inhibition concentration for ethyl acetate stem bark and leaves extract of *Moringa oleifera* on both the bacterial and fungal microbes. The concentration of turbidity (mg / ml) for the leave and stem bark at 100 mg / ml and 50 mg / ml, both bacteria and fungi were negative (no inhibition). At a concentration of turbidity of 25 mg / ml of the leave extract, bacteria had no inhibition, while the fungi were all positive. At 12.5 mg / ml of concentration of turbidity of the leaves extract, only *E. coli* was positive and in fungi all were positive. At a turbidity concentration of 6.25 mg / ml, both bacteria and fungi involved in this experiment were positive. At turbidity concentration of 25 mg / ml, the stem bark extract, only *E. coli* and *P. aeruginosa*, *A. niger* and *C. albicans*

were positive, except *S. aureus* and *A. fumigatus* that were negative. At the turbidity concentrations of 12.5 mg / ml and 6.25 mg / ml of the stem bark extract, both bacteria and fungi involved in this experiment were positive respectively.

Table 10 showed the minimum inhibitory concentration for ethanolic stem bark and leaves extract of *Moringa oleifera* on both the bacterial and fungal microbes. At turbidity concentrations of 100 mg / ml and 50 mg / ml of the extract of leaves and stem bark, both bacteria and fungi were negative. At turbidity concentration of 25 mg / ml of the leaves extracts, among the bacteria only *P. aeruginosa* was positive, while all the fungi were positive. Only *E. coli* was negative, but all the microbes involved were positive at a 12.5 mg / ml turbidity concentration of the leaves extract, and at a concentration of 6.25 of the both leaves and stem bark extracts, all the microbes involved were positive. At a 25 mg / ml of turbidity concentration of stem bark extract, among the microbes involved in this study, only *P. aeruginosa* and *C. albicans* were positive and all rest were negative. At a 12.5 mg / ml, the turbidity concentration of the stem bark extract showed only E. coli was negative, but the rest of microbes involved were positive.

Table 11 showed the minimum inhibitory concentration for aqueous stem bark and leaves extract of *Moringa oleifera* on the fungal microbes. At 100 mg / ml and 50 mg / ml of concentration of the leaf extract, all the fungi involved were negative. At the turbidity concentration of 25 mg / ml of the leaf extract, only *C. albicans* was positive and at a concentration of 12.5 mg / ml, only *A. niger* was negative, but all the fungi involved in this experiment were positive at a concentration of 6.25 mg / ml of the leaf extract. At 100 mg / ml of the stem bark extract, all the fungi involved were negative, 50 mg / ml only *A. fumigatus* was negative, at 25 mg / ml of the extract only A. niger was negative. Both at 12.5 mg / ml and 6.25 mg / ml of turbidity concentration of the stem bark extract, the pathogenic fungal microbes involved in this study were positive respectively.

Figure I showed graphical representation of antimicrobial activity of ethyl acetate extract of *Moringa oleifera* stem bark.

Figure II showed graphical representation of antimicrobial activity of ethanol extract of *Moringa oleifera* stem bark.

Figure III showed graphical representation of antifungal activity of aqueous extract of *Moringa oleifera* stem bark.

Figure IV graphical representation of antimicrobial activity of ethyl acetate extract of *Moringa oleifera* leaf.

Figure V graphical representation of antimicrobial activity of ethanolic extract of *Moringa oleifera* leaf.

Figure VI graphical representation of antifungal activity of aqueous extract of *Moringa oleifera* leaf.

Table-1. Biochemical Identification and Motility Test of Bacterial Isolates

Type of Bacteria	Type of Test			
	Catalase	Coagulase	Indole	Motility
E. coli	+	-	+	+
P. aeruginosa	+	-	-	+
S. aureus	+	+	-	-

KEYS: + = positive, - = negative

Table-2. Mean Percentage Yield and Appearance of the Crude Extracts *of Moringa oleifera*

Type Plant Part	Type of Extract	Crude Extract Appearance	Mean Percentage Yield (%)
Leaf	aqueous	light green solid	11 ± 0.29
	ethanolic	dark green semi-solid	16 ± 0.29
	ethyl acetate	dark green solid	14 ± 0.58
Stem bark	aqueous	light brown solid	12 ± 0
	ethanolic	dark brown solid	10 ± 0
	ethyl acetate	lemon green solid	12 ± 0.58

Table-3. Qualitative Screening of Phytochemicals of the Different Extracts *of Moringa oleifera* Leaf

Type of Phytochemical	Method of Test	Type of Extract		
		Aqueous	ethanolic	Ethyl acetate
Alkaloid	Harbone (1998)	-	++	+
Anthraquinones	Trease and Evans (1989)	-	-	-
Cardiac Glycoside	Sofowara (1993)	-	+	-
Cyanogenic Glycoside	Sofowara (1993)	++	-	-
Flavonoid	Harbone (1998)	+	+	-
Phenol	Trease and Evans (1989)	+	++	-
Reducing sugar	Harbone (1998)	-	-	-
Saponin (Emulsion)	Harbone (1998)	-	-	+
Saponin (Frothing)	Harbone (1998)	-	+	-
Steroid	Harbone (1998)	-	+	-
Tannin	Trease and Evans (1989)	-	+	-
Terpenoid	Sofowara (1993)	+	+	-

KEYS: - = absent, + - slightly present, ++ = moderately present

Table-4. Qualitative Screening of Phytochemical of the Different Extracts *of Moringa oleifera* Stem Bark

Type of Phytochemical	Method of Test	Type of Extract		
		Aqueous	ethanolic	Ethyl acetate
Alkaloid	Harbone (1998)	-	-	++
Anthraquinones	Trease and Evans (1989)	-	-	-
Cardiac Glycoside	Sofowara (1993)	-	-	+
Cyanogenic Glycoside	Sofowara (1993)	+++	-	++
Flavonoid	Harbone (1998)	-	+	-
Polyphenol	Trease and Evans (1989)	-	-	-
Reducing sugar	Harbone (1998)	-	-	-
Saponin (Emulsion)	Harbone (1998)	-	-	++
Saponin (Frothing)	Harbone (1998)	+	++	-
Steroid	Harbone (1998)	-	+ +	+
Tannin	Trease and Evans (1989)	-	-	-
Terpenoid	Sofowara (1993)	-	++	+

KEYS: - = absent, + - slightly present, ++ = moderately present, +++ = abundantly present.

Table-5. Antimicrobial Activity Assay of Ethyl Acetate Stem Bark and Leaves Extracts of *Moringa oleifera*

Extract	Concentration (mg / ml)	Mean Zone of Inhibition of each Microbe (mm)					
		E. coli	*P. aeruginosa*	*S. aureus*	*A. fumigatus*	*A. niger*	*C. albicans*
Leaf	100	15	11	11.5	10	10	10
	50	13	9	10	7	6	8
	25	10.5	6	8	0	0	0
	12.5	8	0	6	0	0	0
Stem Bark	100	16	15	13	15	10	12
	50	13.5	12	11.5	13	11	9.5
	25	10	9.5	9	10	9	8
	12.5	8	8	6	0	6	6

KEYS: 0 = No inhibition, 0.5 -10 = moderately sensitive, 10 - 20 = very Sensitive, 20 and above = highly sensitive
The negative control – ethyl acetate solvent, gave no zone of inhibition for all the isolates.

Table-6. Antimicrobial Activity Assay of Ethanolic Stem Bark and Leaves Extracts of *Moringa oleifera*

Extract	Concentration (mg / ml)	Mean Zone of Inhibition of each Microbe (mm)					
		E. coli	*P. aeruginosa*	*S. aureus*	*A. fumigatus*	*A. niger*	*C. albicans*
Leaf	100	13	10.5	11	15.5	14.5	11
	50	11.5	10	8	13.5	11.5	0
	25	9	8.5	6	10	10	0
	12.5	7	7	0	0	8	0
Stem Bark	100	15	13.5	13.5	15	0	9
	50	14.5	11	11	13	0	0
	25	12	9.5	10.5	10	0	0
	12.5	10	9	9	8	0	0

KEYS: 0 = No inhibition, 0.5 -10 = moderately sensitive, 10 - 20 = very Sensitive, 20 and above = highly sensitive
The negative control - ethanol solvent, gave no zone of inhibition for all the isolates.

Table-7. Antimicrobial Activity Assay of Aqueous Stem Bark and Leaves Extracts of *Moringa oleifera*

Extract	Concentration (mg / ml)	Mean Zone of Inhibition of each Microbe (mm)					
		E. coli	*P. aeruginosa*	*S. aureus*	*A. fumigatus*	*A. niger*	*C. albicans*
Leaf	100	0	0	0	15	13.5	7
	50	0	0	0	11	11	0
	25	0	0	0	10	9	0
	12.5	0	0	0	0	8	0
Stem Bark	100	0	0	0	15	15.5	7
	50	0	0	0	12	14	0
	25	0	0	0	10	10	0
	12.5	0	0	0	0	0	0

KEYS: 0 = No inhibition, 0.5 -10 = moderately sensitive, 10 - 20 = very Sensitive, 20 and above = highly sensitive

Table-8. Antimicrobial Activity Assay of Standard Drugs (Tetracycline and Fluconazole) Used

Extract	Concentration (mg / ml)	Mean Zone of Inhibition of each Microbe (mm)					
		E. coli	*P. aeruginosa*	*S. aureus*	*A. fumigatus*	*A. niger*	*C. albicans*
Tetracycline	150	23	27.5	21.5	-	-	-
Fluconazole	150	-	-	-	17.5	21	24

KEYS: 0 = No inhibition, 0.5 -10 = moderately sensitive, 10 - 20 = very Sensitive, 20 and above = highly sensitive

Table-9. Minimum Inhibitory Concentration for Ethyl Acetate Stem Bark and Leaves Extract of *Moringa oleifera*

Extract	Concentration Turbidity mg/ml	Inhibition of each Microbe (mm)					
		E. coli	*P. aeruginosa*	*S. aureus*	*A. fumigatus*	*A. niger*	*C. albicans*
Leaf	100	-	-	-	-	-	-
	50	-	-	-	-	-	-
	25	-	-	-	+	+	+
	12.5	+	-	-	+	+	+
	6.25	+	+	+	+	+	+
Stem Bark	100	-	-	-	-	-	-
	50	-	-	-	-	-	-
	25	+	+	-	-	+	+
	12.5	+	+	+	+	+	+
	6.25	+	+	+	+	+	+

KEYS: - = No growth; + = turbidity

Table-10. Minimum Inhibitory Concentration for Ethanolic Stem bark and Leaves Extract of *Moringa oleifera*

Extract	Concentration Turbidity mg/ml	Inhibition of each Microbe (mm)					
		E. coli	*P. aeruginosa*	*S. aureus*	*A. fumigatus*	*A. niger*	*C. albicans*
Leaf	100	-	-	-	-	-	-
	50	-	-	-	-	-	-
	25	-	+	-	+	+	+
	12.5	-	+	+	+	+	+
	6.25	+	+	+	+	+	+
Stem Bark	100	-	-	-	-	-	-
	50	-	-	-	-	-	-
	25	-	+	-	-	-	+
	12.5	-	+	+	+	+	+
	6.25	+	+	+	+	+	+

KEYS: - = No growth; + = turbidity

Table-11. Minimum Inhibitory Concentration for Aqueous Stem bark and Leaves Extract of *Moringa oleifera*

Extract Type	Concentration of Turbidity (mg/ml)	Inhibition of each Microbe		
		A. fumigatus	*A. niger*	*C. albicans*
Leaf	100	-	-	-
	50	-	-	-
	25	-	-	+
	12.5	+	-	+
	6.25	+	+	+
Stem Bark	100	-	-	-
	50	-	+	+
	25	+	-	+
	12.5	+	+	+
	6.25	+	+	+

KEYS: - = No growth; + = turbidity

Figure-1. Graphical Representation of Antimicrobial Activity of Ethyl acetate Extract of *Moringa oleifera* Stem bark..

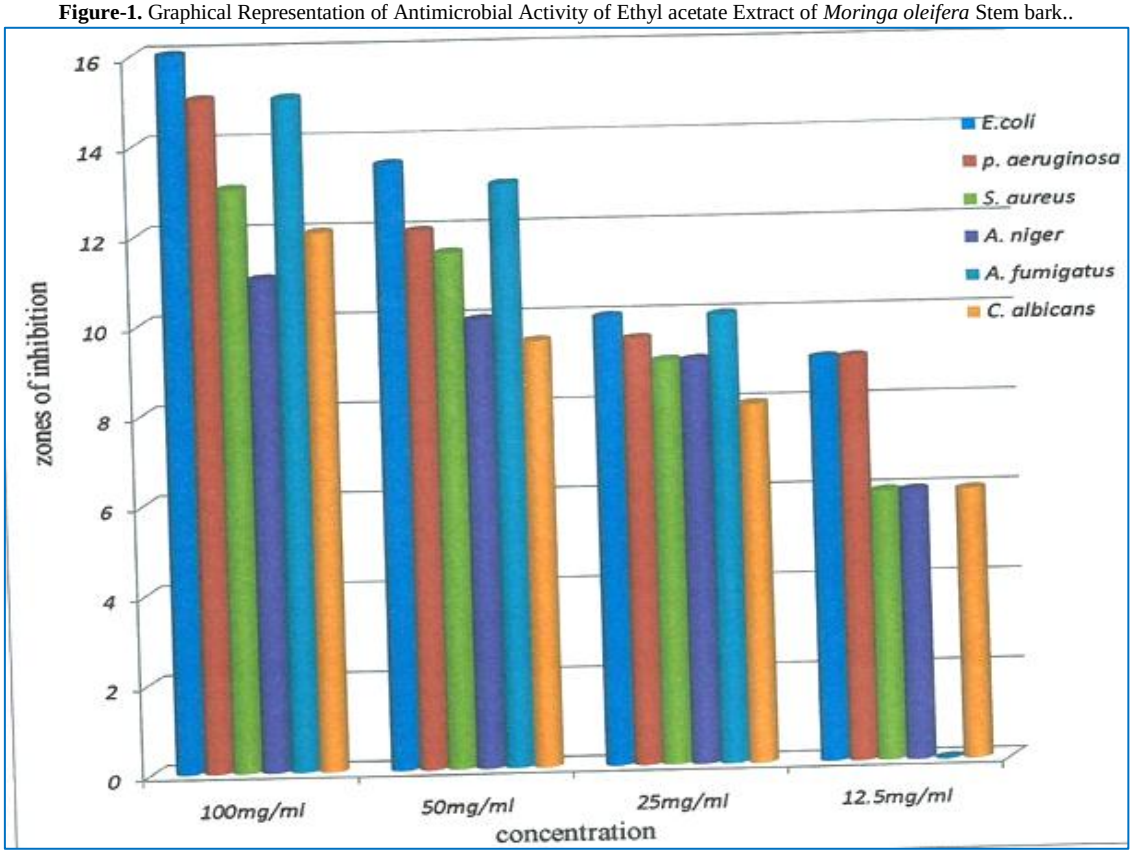


Figure-II. Graphical Representation of Antimicrobial Activity of Ethanol Extract of *Moringa oleifera* Stem bark.

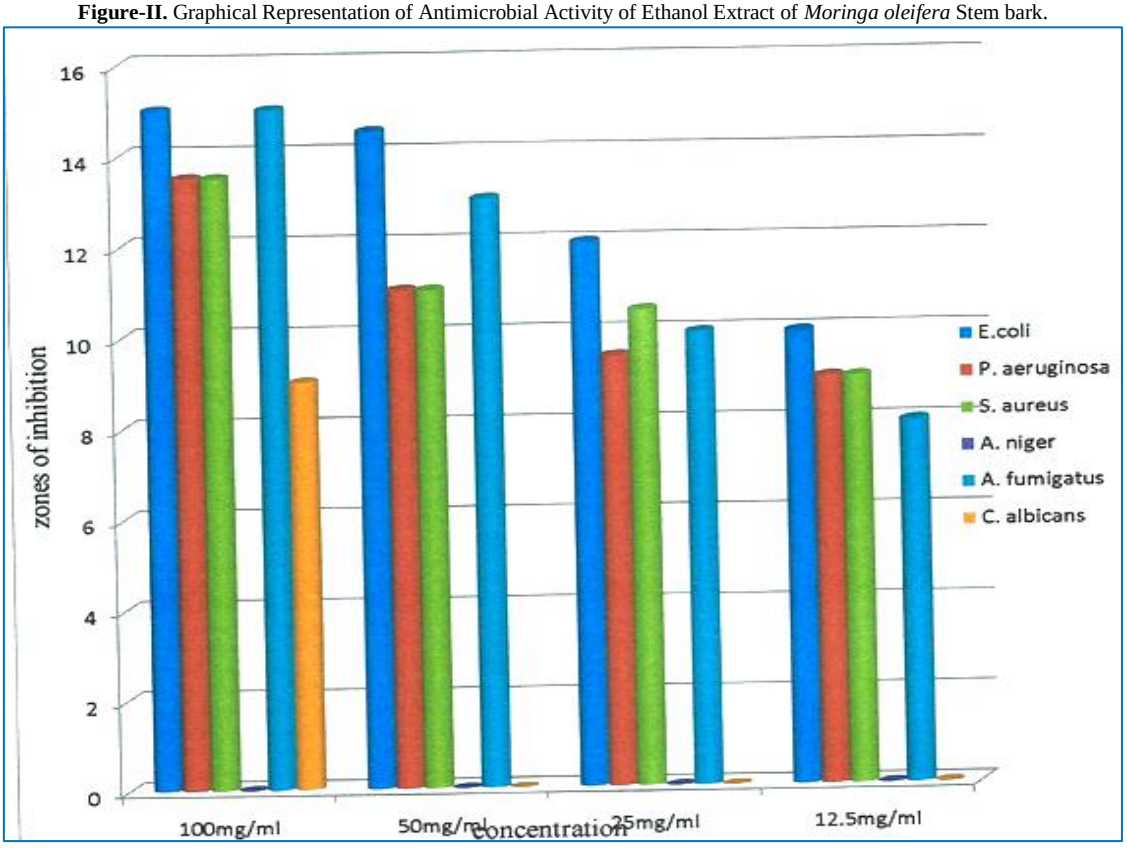


Figure-III. Graphical Representation of Antifungal Activity of Aqueous Extract of *Moringa oleifera* Stem bark.

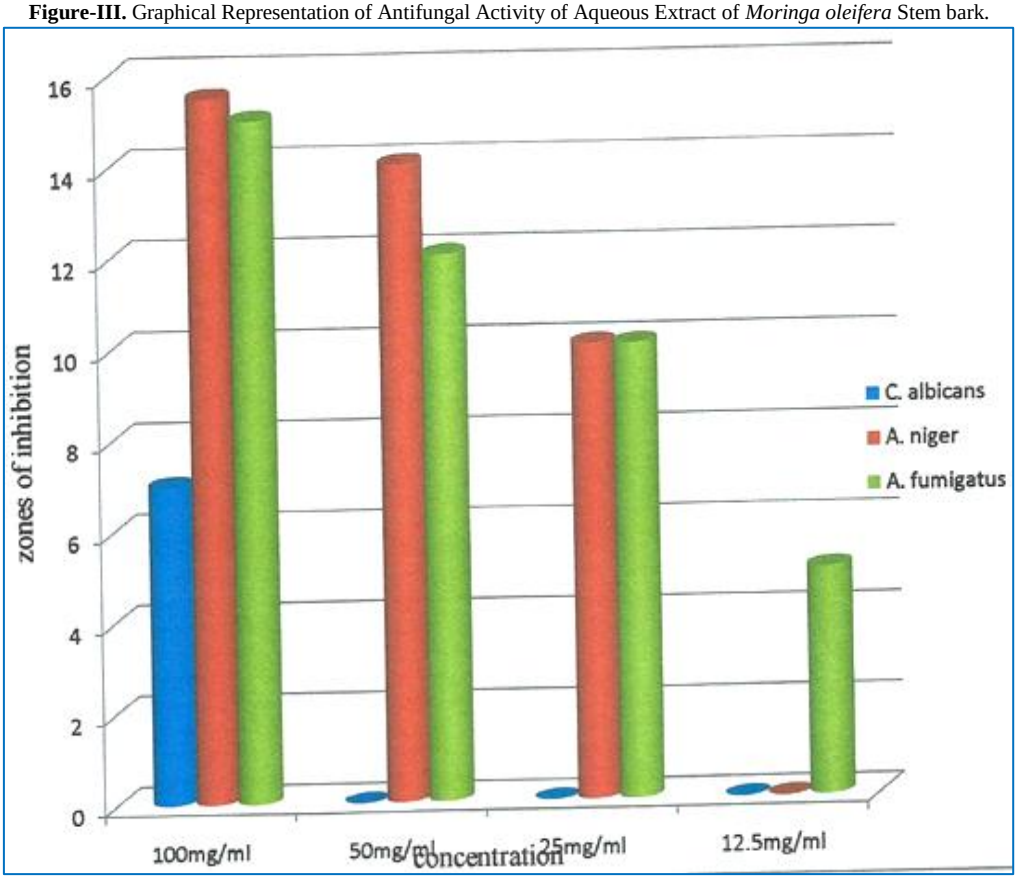


Figure-IV. Graphical Representation of Antimicrobial Activity of Ethyl acetate Extract of *Moringa oleifera* Leaf.

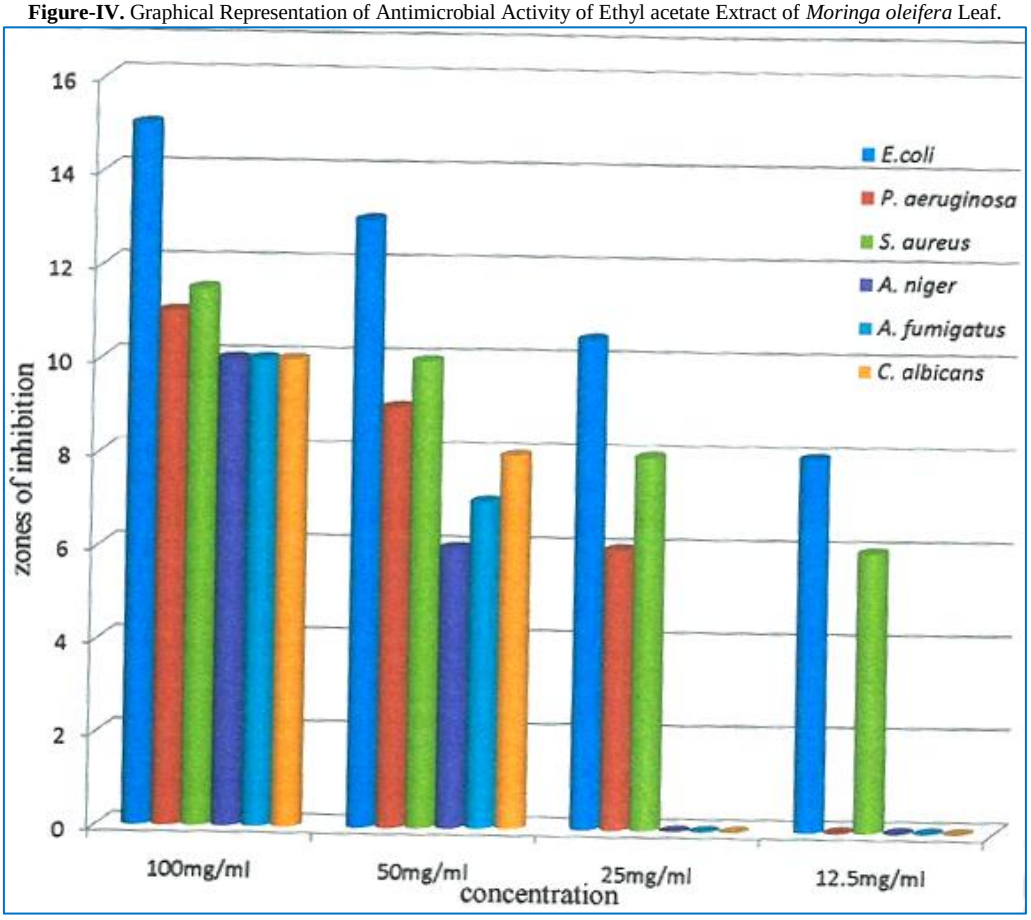


Figure-V. Graphical Representation of Antimicrobial Activity of Ethanolic Extract of *Moringa oleifera* Leaf.

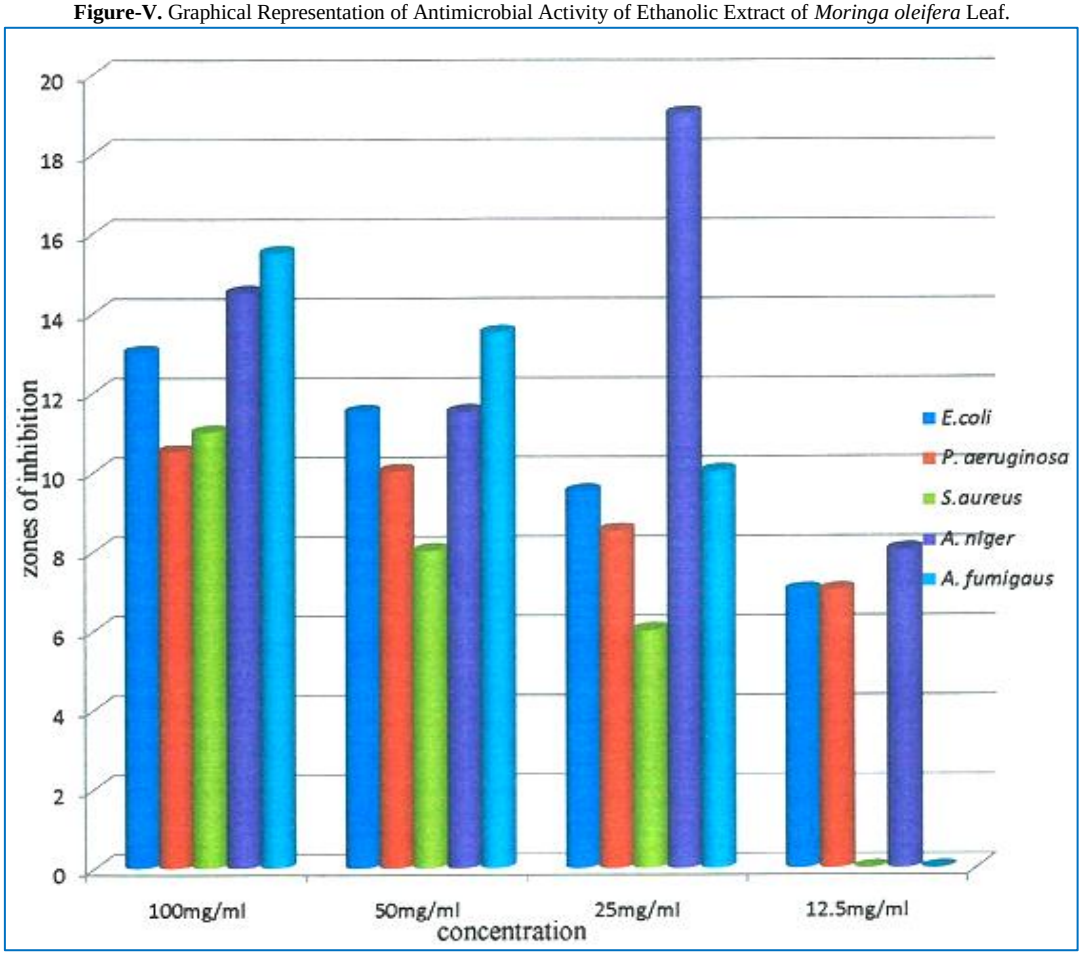
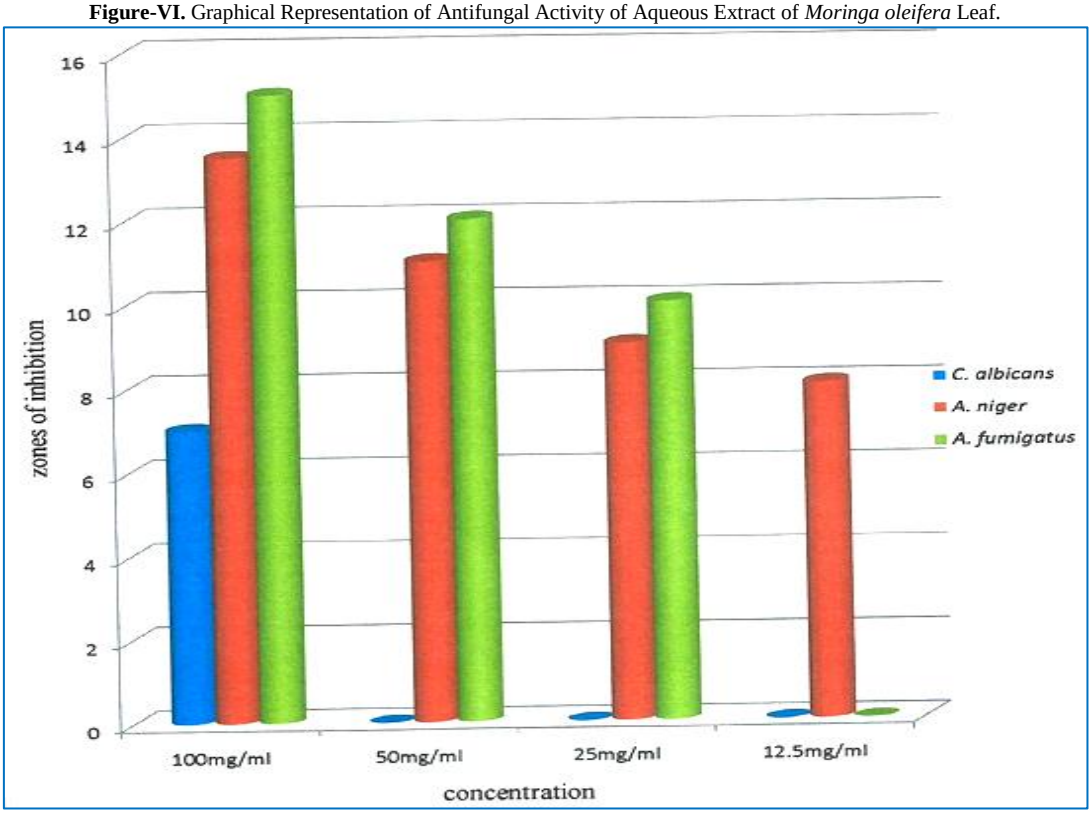


Figure-VI. Graphical Representation of Antifungal Activity of Aqueous Extract of *Moringa oleifera* Leaf.



4. Discussion

Natural products from plants are known to control some infectious diseases. The finding of the active compound may be interesting in the search for new efficacious and safe antimicrobial agent against a variety of pathogenic

bacteria and fungi. It is necessary to investigate those plants scientifically, which have been used in traditional medicine to improve the quality of healthcare. According to some scientists and researchers, such as [1, 3, 23, 24] who stated that plant extracts are potential sources of novel antimicrobial compounds especially against bacterial pathogens.

The present investigation confirms that there are antibacterial and antifungal properties in the crude and diluted extracts of M. *oleifera* stem bark. Due to several intricacies of modern or the orthodox antibiotics, there has been a significant shift towards alternative therapy and herbal remedies. Antibiotic screening on natural products obtained from *M. oleifera* used in the Complementary and Alternative Medicine (CAM) is a major thrust of research and development. Therefore, in a bid to discover new antimicrobials that would be effective against multi-drug resistant microbial strains, phytochemical screening and investigations into the antimicrobial profile of the leaves and stem bark extracts of *M. oleifera* were carried out.

During the course of this research, investigations have afforded many phytochemicals with promising antibacterial properties. It is suggested that this plant drug would have enormous health benefits with little untoward effects that is common with the synthetic drugs. The variations in the presence of the phytochemicals may be due to the choice of solvent used in extraction. During extraction, solvents may have diffused into the plant material and solubilised compounds with similar polarity. The age of the plant has been reported not to affect the phytochemicals present in the plant, thus, at every stage of growth, the phytochemicals in the plant remains the same, this is in line with [25]. Percentage yield of extracts were calculated dividing the weight of extract obtained by the weight of powdered sample and multiply one hundred divide by one. Mathematically expression;

$$\% \text{ Yield} = \frac{\text{Weight of extract obtained}}{\text{Weight of powdered sample}} \times \frac{100}{1}$$

It indicates that ethanol gives the maximum yield for the leaves (16%) and ethyl acetate gives the maximum yield for stem bark at 12%.

The Phytochemical screening of the leaf and stem bark *of Moringa oleifera* revealed the presence of the different phytochemical components. The leaf and stem bark contain a number of phytochemicals such as alkaloids, saponins, tannins, terpenoid, glycosides and steroids. Saponin was largely present in both the leaf and stem bark ethanolic extract. Methyl acetate extract showed that terpenoid is largely present in stem bark and also the presence of flavonoids, steroids and phenol in the leaf. The Cyanogenic glycoside was shown to be largely present in aqueous extract.

The antibacterial activity of the extracts were investigated using agar well diffusion method, against the selected human pathogens such as *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Aspergillus niger, Aspergillus fumigatus* and *Candida albicans.* All the examined extracts (ethanolic, ethyl acetate and aqueous) showed varying degrees of antibacterial and antifungal activities against the tested organisms.

Minimum Inhibitory Concentration; this refers to the lowest concentration that can inhibit growth of an organism. The MICs *of E. coli, P. aeruginosa, A. niger, C. albicans* is at 50 mg / ml, *A. fumigatus and S. aureus* have their MICs at 25 mg / ml for extract of ethyl acetate stem bark. Ethanolic stem bark extract was able to inhibit growth of *E. coli, P. aeruginosa, A. fumigatus, C. albicans* at 50 mg / ml and *A. niger, S. aureus* at 25 mg / ml. The aqueous extract showed no zone of inhibition for bacteria isolates thus, MIC was not carried out for bacteria isolates. Meanwhile the aqueous stem bark extract gave positive MIC result for *A. niger* and *C. albicans* at 50 mg / ml while *A. fumigatus* was 25 mg / ml. S. aureus and A. fumigatus have MICs at 25 mg / ml for the ethyl acetate leaf extract and *S. aureus* and *C. albicans* have their MICs at 25 mg / ml for the ethanolic leaf extract *E. coli* has its MIC at 12.5 mg / ml at both ethanolic extracts of leaf and stem bark.

Minimum Lethal Concentration; this includes both the minimum bactericidal concentration and minimum fungicidal concentration. It refers to the least concentration that can kill 99.9% of the organism. The stem bark and leaf ethyl acetate gave no MBC for *E. coli* but showed a cidal effect at l00 mg / ml for both ethanolic extracts of leaf and stem bark. *P. aeruginosa* was killed totally at l00 mg / ml of both ethyl acetate and ethanolic extracts of leaf but no killing activity was observed by both ethyl acetate and ethanolic extracts of stem bark. Ethanolic leaf and stem bark extract of gave the highest MBC of 50 mg / ml, 100 mg / ml, for ethyl acetate stem bark extract and no effect in the ethyl acetate leaf extract.

C. albicans was not killed by both aqueous leaf and stem bark extracts, and all the extracts of stem origin but had MBCs for ethyl acetate and ethanolic leaf extracts at l00 mg / ml. *A. fumigatus* had MBCs for ethanolic leaf and stem bark extracts, and ethyl acetate stem bark extract at 50 mg / ml; l00 mg / ml for ethyl acetate leaf extract and no MBC at both aqueous leaf and stem bark extracts. Aqueous leaf and stem extracts gave the highest MBC at 25 mg / ml and 50 mg / ml respectively for *A. niger,* ethanolic and ethyl acetate leaf extract gave MBC at l00 mg / ml while the ethanolic stem bark extract gave no MBC.

These findings provide an interesting view to traditional method (decoction or boiling of the plant or plant parts) of treating bacterial infection. The leaf and stem bark contain a number of phytochemicals such as alkaloid, saponin, tannin, terpenoid, glycoside and steroid. The presence of these phytochemicals is responsible for antimicrobial activities of the plant. Tannins are polyphenol with pronounced ability to suppress bacterial cell proliferation by blocking essential enzymes of microbial metabolism such as the proteolytic macerating enzymes. Saponins might act by altering the permeability of cell walls and hence exert toxicity on all organized tissues. They exert some antibacterial activity by combining with cell membranes to elicit changes in cell morphology leading to cell lyses

(Cytolysis). The extract of both leaf and stem bark showed the highest levels of phytochemicals present in the plants. The high extraction of phytochemical in ethanolic extract is suspected to be due to the high polarity of the solvents. The mechanism of action by which the phytochemical constituents of *M. oleifera* exert their antibacterial activity might be attributable to bacterial enzyme inhibition such as the sortase inhibitory effect, DNA replication, bacterial toxin action and causing the cytolysis of bacterial cells. These phytochemicals released are secondary metabolites of the plants, thus, they act as inhibitory substances to organisms.

However, the results of the susceptibility assay shows that the antimicrobial activity is limited since all the extracts are very active only at high concentrations such as l00 mg / ml, their activities reduces drastically as the concentration reduces. Aqueous extract has no antibacterial activity; this is due to the fact that water being a universal solvent does not extract alkaloid which is the main antimicrobial agent. Alkaloids are basically Nitrogen containing naturally occurring compounds commonly found to have antimicrobial properties due to their ability to intercalate with the DNA of microorganisms. Aqueous significantly extracted Cyanogenic glycosides from both the leaf and stem bark, this compound to a large extent contributes to the inhibition of molds.

Ethyl acetate stem bark showed the highest zone of inhibition (16 mm), this is owing to the fact that more secondary metabolites are released such as the Cyanogenic glycosides, steroid, terpenoid, alkaloids and saponin. These released phytoconstituents are thought to be responsible for the antimicrobial activities. The orthodox medicine (Tetracycline and Fluconazole) were found to be more active than the plant extracts, hence purification of the phytoconstituents of the plants will enhance its antimicrobial activities.

Generally, Gram negative bacteria are known to be resistant to the action of most antibacterial agents including plant based extracts and these have been reported by many researchers. Gram negative bacteria have an outer phospholipids membrane with the structural lipopolysaccharide components, which make their cell wall impermeable to anti-microbial agents. *E. coli* was inhibited at low concentrations of ethyl acetate stem bark extracts while showing the highest zone of inhibition, this can be connected to the presence of saponin which causes injury to cells of the organism thus prevent formation of the peptidoglycan of Gram negative which further cause death and damage to the cell. *S. aureus* being a Gram positive have a thick outer peptidoglycan layer, and has been shown to have the least zone of inhibitions in all extracts compared to *E. coli* and *P. aeruginosa.* Ethyl acetate stem bark extract displayed notable antibacterial activity against *Pseudomonas aeruginosa.* This is of great important because the infections caused by this bacterium are known to be difficult to control. It is an opportunistic organism which has been reported many researchers such as Sankar [19] and Cowan [20], to readily receive resistance carrying plasmid from other bacteria species.

The ethanolic leaf extract have significant inhibitory zones on *A. niger* while the ethanolic stem bark extract showed no inhibitory potential, this could be attributed to the presence of alkaloid, phenol and the glycosides in the leaf extract as opposed to that present in the ethanolic stem extract. Comparing the phytochemicals of the stem bark and leaf extracts in general, the stem bark extracts has been shown to contain more phytochemicals thus these phytochemicals reflects in their ability to show more antimicrobial activity unlike the leaf extracts and is in conformity with work of Bassey, *et al.* [2] who reported similar reports of their findings.

Escherichia coli is the pathogenic organism responsible for the intestinal disorder, gastroenteritis, meningitis, while *Aspergillus* species are opportunistic fungi which cause aspergilloma and *Candida albicans* causes candidiasis, vaginitis and thrush. This proves that *Moringa oleifera* leaves could be used in the treatment of infection caused by such pathogens as *Staphylococcus aureus, E. coli, Candida albicans* and *Aspergillus species.*

The MIC obtained shows that different concentrations were effective against some of the test organisms. The most susceptible organisms to the antibacterial activity was *E. coli* at 12.5 mg / ml of the ethanolic leaf and stem bark extract while the least susceptible was *P. aeruginosa* which was not inhibited by any of the extract at concentration of more than 50 mg / ml. The MBC determined revealed that at a concentration of 50 – 100 mg / ml microbicidal effect was observed for most of the extracts. C. *albicans* was not killed by both aqueous leaf and stem bark extracts, and all the extracts of stem origin but had MBCs for ethyl acetate and ethanolic leaf extracts at l00 mg / ml. This also reflects in the zones of inhibition the organism gave, it gave the lowest zones as a fungi compared to other fungi which in it case inhibition zones are usually seen at l00 mg / ml while the other lower concentrations have no inhibitory zones or are less sensitive. Aqueous leaf and stem extracts gave the highest MBC at 25 mg / ml and 50 mg / ml respectively for A. *niger*, the fungus is very sensitive to the aqueous extract of *M. oleifera* plant. The reason not yet verified.

5. Conclusion

Moringa oleifera is indeed a very useful breakthrough in the demand of alternative natural medicine for the treatment of various disease activities by pathogenic organisms. This is proved by the good antimicrobial activity and the presence of secondary metabolites showed by the stem bark and leaf extract.

The phytochemical screening and investigation into the antimicrobial potential of the different leaves and stem bark extract of *M. oleifera* plant showed or highlighted the antibiotic spectra of the plant extract under assay, suggesting a promising lead as an alternative antibiotic and it yielded scientific support to their use in traditional ayurvedic medicine.

The generated information of this study will provide data which is helpful in the correct identification and authentication of this medicinal plant. The leaf of *Moringa oleifera* has been known to be used in the treatment of dental caries, common cold, Fever, Diarrhea, flatulence and Edema. There is an increasing awareness that many

components of traditional medicine are beneficial while others are harmful, hence World Health Organization encourages and supports countries to identify and provide safe and effective remedies for use in the public and private health services. This study demonstrated the antimicrobial activity of leaves and stem bark of *Moringa oleifera* plant which could potentially serve to treat those infections that otherwise has become highly resistant to most of the conventional antibiotics used for its treatment. Moreover, that the plant is available, cheaper and affordable makes it an alternative for conventional antibiotics provided lexicological investigations is further carried out. Its limitation is that it is required in high concentrations depending on the microbial load.

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