

INVITRO STUDY OF ANTIBACTERIAL POTENTIAL OF *Moringa oleifera* ETHANOLIC LEAF EXTRACT ON BACTERIA ISOLATED FROM *Clarias gariepinus*

ABSTRACT

The aim of this study was to determine the in vitro antibacterial potential of ethanolic leaf extract of *Moringa oleifera* on bacteria organisms isolated from at 25mg/ml, 50mg/ml, 100mg/ml and 200mg/ml concentration. The antibacterial potential of ethanolic leaf extracts of *Moringa oleifera* were determined against bacteria isolated from the body of *Clarias gariepinus*. The leaf of *Moringa oleifera* was extracted with 99.9% ethanol and distilled deionized water and reconstituted at 25mg/ml, 50mg/ml, 100mg/ml and 200mg/ml concentrations. The phytochemical analysis carried out on the leaf extract of *Moringa oleifera* reveals the presence of alkaloids, tannins, flavonoids, saponins, oxalates and phytate. The micro-organisms isolated include; *Escherichia coli*, *Staphylococcus aureus* and *Salmonella spp*. Antibacterial testing was by agar well diffusion method. The highest zone of inhibition $2.14 \pm 2.10\text{mm}$ achieved with ethanolic extract of *Moringa oleifera* at concentration of 200mg/ml against *Staphylococcus aureus* while the lowest zone of inhibition was $1.66 \pm 1.52\text{mm}$ at 50mg/ml concentration. The highest and lowest zone of inhibition 1.68 ± 1.70 and $1.58 \pm 1.51\text{mm}$ at concentrations of 200mg/ml and 50mg/ml was achieved with ethanolic leaf extract of *Moringa oleifera* against *Escherichia coli*, the highest and lowest zone of inhibition was 0.98 ± 1.36 and $0.72 \pm 1.02\text{mm}$ at concentrations of 200mg/ml and 100mg/ml respectively. There was no susceptibility to the organisms at concentration 25mg/ml and 50 mg/ml for *Salmonella spp* with *Moringa oleifera* leaf extract. The most susceptible organism was *Staphylococcus aureus* and the least susceptible was *Salmonella spp*. The presence of the phytochemicals in *Moringa oleifera* supports the use of this leaf extract as an antibacterial agent at higher concentration which might justify its ethno-medicinal use.

1. introduction

Moringa oleifera is a fast growing, drought-resistant tree of the kingdom Plantae (Vascular plant), Division Magnoliophyta (Flowering plants), Family Moringaceae Martionov (Horse-radish tree family), Genus *Moringa* Adans and Specie *Moringa oleifera* Lam.

Common names: Ben oil tree of benzo live tree, *Moringa* drumstick tree (from the long, slender, triangular seed pods), Horse-radish tree (from the taste of the root, which resembles horse-radish). In local Nigerian languages, *Moringa* is known as 'Barambo' in Hausa, 'Odudu Oyibo' in Igbo and 'Ewele' in Yoruba. It is widely cultivated for its young seed pods and leaves used as vegetables and for traditional herbal medicine. *Moringa* is economically important in the production of several commodities, such as oils, foods, condiments and medicine (1).

Moringa oleifera have been used in the treatment of hard water, and proved that hardness removal efficiency of *M. oleifera* increases with increasing dosage. *Moringa* seed powder is a natural alternative to imported alum (2)

Bacteria disease and its effect on all species of fish have become a concern in fish industries and its effect on the consumers. Fish living in natural environments are known to harbor pathogenic Enterobacteriaceae (3). Water of poor physico-chemical quality has adverse effects on fish and fish consumers thereby resulting in serious economic and human loss (4), although there are vaccines that have been developed and marketed but these vaccines cannot be used as a universal control measure in aquaculture.

During the last decades, antibiotics were not only used as a traditional strategy for fish disease management but also for the improvement of growth and efficiency of feed conversion (5). On the other hand, antibiotics inhibit or kill beneficial microbiota in the gastrointestinal ecosystem but it also makes antibiotic residue accumulated in fish products to be harmful on human consumption (6). Bacteria can enter the fish body through the gills or skin or it can stay on the surface of the body (7), Animal scientist and Veterinarians are now turning attention towards alternative sources of natural ingredients like plants or herbs to replace antibiotics (8).

Plant oil from seeds and leaves such as *Moringa oleifera* are in high demand for their medicinal value. Apart from the medicinal uses *M. oleifera* was reported to be a good source of vitamin and amino acids (9). Its extract was reported to have antibacterial properties and a conclusion was

made to investigate it as a Phyto-therapeutic agent to combat infectious agents (10). The views by Cowan (11, 12) that medicines produced from raw plants are safe is in agreement with this study. The growing interest in plant's usage for medicinal purposes due to the presence of several antibacterial compounds presence in them, the safety of plant extracts and the economically friendly cost necessitates this study.

2.0 MATERIALS AND METHODS

2.1 Collection and Identification of Plant Material

The fresh leaves of *Moringa oleifera* used in this study was collected from in and around Umudike and its environment on 16th June 2023.

2.2 Processing of The Plant Material

The leaves of *M. oleifera* were thoroughly cleaned and dried at room temperature for three weeks at the Veterinary Biochemistry Laboratory bench in the College of Veterinary Medicine, MOUAU. The dried plant material was then pulverized to fine powder using an electric blender and stored in a sterile container until the time of extraction and analysis.

2.3 Preparation of The Plant Sample

The leaves were carefully separated from the stalk, air dried for 3weeks. The dried leaves were grounded with an electric blender to a fine powder form for extraction and was stored in an airtight container.

2.4 Extraction Procedure

Two solvents; ethanol and distilled water were used for the extraction of the grounded plant leaves. These solvents were chosen to yield the most active extracts (13) with documented advantageous properties (14, 15).

The ethanoic extraction was done using Soxhlet extractor and reflux method in the report of (16). Using electronic weighing balance, 200.54g of the stored dried powder of *M. oleifera* were collected into the Soxhlet apparatus with 250ml of ethanol. External heat source was attached to the extractor with temperatures between 30⁰C to 60⁰C. The extraction ran for some days with anti-pumping silicone beads added each day before the apparatus was turned on. The extracts were collected into a clean beaker and filtered with a Whatman No.1 filter. The ethanol solvent was then evaporated in *vacuum* and later in a water bath at 4⁰C until completely dry crude extract was obtained. The whole extract was refrigerated at 4⁰C until further use. The store extract was reconstituted using distilled water to obtain extracts of several concentrations 25, 50, 100, and 200 mg/ml.

2.5 The Qualitative Phytochemical Screening of leaf extract of *M. oleifera*

Qualitative phytochemical analysis of aqueous and ethanolic extracts of *M. oleifera* leaf was carried out as per method (17).

2.5.1 Detection of Flavonoids

To 2.0mL of aqueous and ethanolic extracts, few drops of sodium hydroxide solution were added. Formation of intense yellow color, which became colorless in addition to dilute HCL indicated the presence of flavonoids.

2.5.2 Detection of Tannins

To 2.0mL of aqueous and ethanolic extracts, 3drops of 1% ferric chloride was added. Appearance of blue-green color indicated the presence of tannins.

2.5.3 Detection of Phytate

The method reported (18) was adapted for phytate quantification. Powdered samples (4g) were soaked in 100 cm³ of 2% HCl (w/v) and allowed to stand for over 3 hours before filtration. From the filtrate, 25 cm³ was taken and placed in a conical flask, 5 cm³ of 0.3% NH₄SCN (aq) and 53.5 cm³ of distilled water were mixed together and titrated against standard FeCl₃ (aq) having 0.00195g Fe/cm³ and observed the formation of brown-yellow color which may persist for 5 minutes. Blank was treated in a similar manner.

2.5.4 Detection of Saponins

Two mL of aqueous and ethanolic extracts were diluted with 10mL of distilled water and mixed for 15min. formation of layers of foam which remain for 10min indicated the presence of saponins.

2.5.5 Detection for Alkaloids

To 2.0ml of aqueous and ethanolic extracts, 2ml of picric acid (Hager's reagent) was added. Formation of orange or yellow color precipitate indicated the presence of alkaloids.

2.5.6 Detection for Oxalate

Detection of oxalate according to (19) proposed a method for determination of oxalate. The leaves sample should be (1g) and put inside a 100cm³ flask. 75cm³ of 3MnH₂SO₄ was measured, placed into the same conical flask and stirred for about 1 hour. The filtration of the solution was carried out using a Whatman No 1 filter paper. 25cm³ of the filtrate was measured

and titrated over 0.05N potassium permanganate (KMnO₄) solution till the appearance of pale-pink color. 1ml of 0.05m KMnO₄ was used to calculate the oxalate content.

2.6 Quantitative determination of Phytochemical Constituents of leaf extract of *M. oleifera*.

2.6.1 Flavonoids

Flavonoids in plant extracts were determined by aluminum chloride colorimetric method (20). About 0.25 ml of extract (10mg/ml) was mixed with 0.75 ml of ethanol. 0.005 ml of 10% aluminum chloride, 0.02 ml of 1M potassium acetate and 1.4 ml of distilled water. The reaction mixture was incubated at 37⁰C for 30 minutes. The absorbance of the mixture was measured at 415 nm using UV- VIS spectrophotometer.

2.6.2 Tannins

Tannin content was estimated using the Person method, about 1.0 g of the sample was dispersed on 10 ml distilled water and agitated. This was left to stand for 30 minutes at room temperature and shaken every 5 minutes. After 30 minutes, it was centrifuged and the extract was obtained. About 2.5 ml of the supernatant extract was dispensed into a separate 50 ml volumetric flask. Similarly, 2.5 ml of standard tannic acid was dispensed into a separate 50 ml flask. The absorbance was measured at 250 nm.

2.6.3 Saponin

Saponin content was estimated as per the method of (21). About 2 g of dried plant sample was dissolved in 50 ml of petroleum ether. The suspension was heated over a hot water bath at

55°C for 1 hour with continuous stirring. The mixture was filtered and the residue was re-extracted in 50 ml of methanol. The combined filtrates were reduced to 10 ml by placing a water bath at about 90°C. The concentrate was transferred into a 250 ml separating funnel and acetone was added slowly and shaken well. The aqueous layer was recovered and the purification process was repeated. The remaining solution which contains saponin was heated in a water bath and the sample was dried in the oven and weighed. The saponin content was calculated in percentage.

$$\% \text{ saponin} = (\text{weight of saponin}) / (\text{weight of sample}) * 100$$

2.6.4 Alkaloids

The quantity of alkaloids was estimated as per the method of (22). About 1 g of powdered sample was mixed with 40 ml of 10% acetic acid and allowed to stand for 4 hours. It was filtered and concentrated on a water bath to one fourth of its original volume then concentrated ammonium hydroxide was added drop by drop to the extracts until the precipitation was completed. The whole solution was allowed to settle and the precipitate was collected and washed with dilute ammonium hydroxide and then filtered. The residue is the alkaloid, which was dried and weighed. The percentage of alkaloid is expressed mathematically as;

$$\% \text{ Alkaloid} = (\text{weight of alkaloid}) / (\text{weight of sample}) * 100$$

2.6.5 Phytate

The method reported by (17)) was adapted for phytate quantification. Powdered samples (4g) were soaked in 100 cm³ of 2% HCl (w/v) and allowed to stand for over 3 hours before filtration. From the filtrate, 25 cm³ was taken and placed in a conical flask, 5 cm³ of 0.3% NH₄SCN (aq) and 53.5 cm³ of distilled water were mixed together and titrated against standard FeCl₃ (aq) having 0.00195g Fe/cm³ and observed the formation of brown-yellow color which may persist for 5 minutes. Blank was treated in a similar manner.

2.7 Preparation of Crude Plant Extract

0.5 g of extract was weighed out using an electronic weighing balance and then dissolved in 20 ml, 10 ml, 5 ml and 2.5 ml of distilled water to obtain 25, 50, 100, and 200 mg/ml concentrations.

2.8 Media Preparation

All media used were prepared according to the manufacturer's directives.

2.8.1 Nutrient Agar (Fluka)

It was prepared according to (23), by suspending 28 grams of the medium in one liter of distilled water and sterilized by autoclaving at 121⁰C for 15 mins and checked for sterility at 37⁰C for 24 hours. The nutrient agar was used for plating out the organisms as well as storing them in slants and also was used for sensitivity tests.

2.8.2 Mac-Conkey's Agar

It was prepared as described by (23), Mac-Conkey agar is a differential medium used to distinguish lactose-fermenting from non-lactose fermenting bacteria. 51.53grams of Mac-Conkey agar was suspended in the demineralized water. The powder dissolved completely by heating in a water bath sterilized by autoclaving for 15 minutes at 121⁰C. It was then poured into petri-dishes, about 16-18 ml in each and allowed to solidify at room temperature. The shelf life is about one month. It is stored at 2-8⁰C.

2.8.3 Blood Agar

It was prepared according to (23), Blood agar is an enriched medium. It can also be made selective by adding some antibiotics like Kanamycin. When blood agar is heated, the red blood cells are lysed and the media becomes brown, called Chocolate agar. Dissolve 40.0g in 1 liter of purified water, heat in boiling water and agitate frequently until completely dissolved. Autoclave for 15minutes at 121⁰C, it was then poured into petri-dishes, about 16-18 ml in each and allowed to solidify at room temperature. The shelf life is about one month. It was stored at 2-8⁰C.

2.8.4 Eosin Methylene Blue Agar (EMB)

It was prepared according to (23), to suspend 36 grams of EMB agar in 1000 ml of distilled water. Heat to dissolve the medium completely. Dispense and sterilize by autoclaving at 15 lbs, pressure (121⁰C) for 15 minutes. Avoid overheating. Cool to 50⁰C and shake the medium in order to oxidize the methylene blue (i.e to restore its blue color) and to suspend the flocculent precipitate and store the dehydrating medium at 24⁰C and the prepared medium at 2-8⁰C.

2.8.5 Salmonella Shigella Agar (Ss-Agar)

It was prepared according to (23), suspending 63 grams in 1 liter of distilled water. Bring to boiling with frequent agitation and allow to simmer gently to dissolve the agar. Do not autoclave. Cool to about 50⁰C, mix and pour into petri-dishes and store at 8⁰C.

2.8.6 Gram- Staining Technique

The technique was introduced by (24). Make a smear and fixed, by passing the slide rapidly over a flame. Cover the slide with crystal violet and allow it to act for 30 seconds. Pour off and wash freshly with iodine solution. Cover with fresh iodine solution and allow it to act for 30 seconds. Pour off the iodine solution and wash freely with acetone iodine. Cover with acetone iodine and allow it to act for about 30 seconds (until the stain stops carrying out). Wash thoroughly with water, counterstain with safranin for 30 seconds and wash with water, blot and dry. See under the microscope with an oil immersion lens.

2.8.7 Catalase Test

Test was carried out as described by (25). Pour 2-3 drops of fresh hydrogen peroxide solution on a glass slide. Using a sterile wooden or glass rod or wire loop, remove a good piece of growth of the test organism and immerse it in 3% hydrogen peroxide solution. Look for immediate

bubbling, which indicates the production of oxygen. Active bubbling produced is a positive result.

2.8.8 Oxidase Test

The test was performed as described (26), by the indirect paper strip procedure in which a few drops of the reagent were added to a filter paper strip. A loop full of suspected colonies was smeared into the reagent's zones of the filter paper. Bacterial colonies having cytochrome oxidase activity developed a deep purple color at the site of inoculation within 10 seconds. Precautionary measures were taken so as not to use stainless steel inoculating loops or wires so as to avoid surface oxidation products formed by metals when flamed for sterilization which gives a false positive reaction.

2.9 Isolation of Sample Organism

Samples were taken from the body of fishes using sterile swab sticks and inoculated into nutrient agar plates, incubated for 48 hours at 37⁰C. The samples were later subcultured into different medias like eosin methylene blue, salmonella shigella agar, blood agar and Mac-Conkey agar and incubated at 37⁰C for 48 hours. The presence of green metallic sheen was confirmatory of *Escherichia coli*, the presence of incomplete zones of hemolysis confirms *Staphylococcus aureus* on blood agar, discrete raised colonies with black centers in SSA is suggestive of *Salmonella spp.*

2.9.1 Preparation of Test Organisms and Test for Potency of Bacteria Pathogen

Microorganisms were obtained from the prepared and stored stock cultures of test organisms in the Department of Veterinary Microbiology, MOUAU. The organisms are as follows; *Salmonella spp.* *Staphylococcus aureus* and *Escherichia coli*

Bacteria organisms were sub-cultured in nutrient agar. Overnight cultures were prepared by inoculating approximately 2 ml nutrient broth with colonies of the appropriate organism taken

from the agar slant. Broth was incubated overnight at 37⁰C, bacteria inoculum was prepared by diluting overnight cultures in saline to approximately 10 raise to power 8 CFU/ml for each of the organisms.

2.9.2 Determination of Antimicrobial Activity of the Extract

The antimicrobial screening of the aqueous and ethanolic extract was carried out using the agar well diffusion method as described by (27). Nutrient agar was poured into sterile petri-dishes and allowed to solidify. About 1.0 ml of the test culture was dropped on the appropriate solidified agar and spread over the surface of the medium using sterile swab stick. Wells of approximately 6 mm in diameter were made in the agar medium using sterile cork borer. Each well was filled with 0.2 ml of the appropriate concentration of extract. The dishes were allowed to stand for 40 minutes at room temperature to allow proper diffusion of the extract to occur. Control experiments were set up with 0.2 ml of 99% ethanol and 0.2 ml distilled water in separate wells. The plates were incubated for 24 hours. All tests were performed in 5 replicate and antimicrobial activity was expressed as the mean diameter of the clear zone (mm) produced by the plant extract. Zones of clearance around each well means inhibition and the diameter of each zone are measured in millimeters (mm).



Figure 1: Ethanolic extract of *M. oleifera* at concentration 25, 50, 100 and 200 mg/ml against *E. coli*

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Figure 2: Ethanolic extract of *M. oleifera* at concentration 25, 50, 100, and 200



Figure 3: Ethanolic extract of *M. oleifera* at concentration 25, 50, 100 and 200 mg/ml

RESULTS

3.0 Yield from Aqueous and Ethanolic Extractions

The leaves were extracted with 99.9% ethanol and distilled water. The highest yield was achieved with ethanol *M. oleifera* 6.63%, while the highest yield achieved with distilled deionized water was 6.56%.

3.1 Table 1: The qualitative analysis of *Moringa oleifera*

Sample	Alkaloid	Saponins	Tannins	Flavonoids	Oxalate	Phylate
<i>Moringa oleifera</i>	+	+	+	++	+	+

Key: ++ = highly present, + = present

Moringa oleifera used in this research contained Alkaloid, saponins, tannins, flavonoids, oxalates and phytate in different proportions in ethanolic extraction with higher levels of Flavonoids followed by tannins.

3.2 Table 2: The aqueous quantitative analysis of alkaloid, saponins, tannins, oxalate, flavonoids and phytate in *Moringa oleifera*.

Sample	Alkaloid	Saponins	Tannins	Flavonoids	Oxalate	Phylate
<i>Moringa oleifera</i>	0.59 ± 0.62	1.68 ± 1.72	3.51 ± 3.66	6.60 ± 6.63	0.35 ± 0.28	2.69 ± 1.25

Measurement are in mg/100g

3.3 Table 3: The ethanolic quantitative analysis of alkaloids, saponins, tannins, flavonoids, oxalates and phytate in *Moringa oleifera*.

Sample	Alkaloid	Saponins	Tannins	Flavonoids	Oxalate	Phylate
<i>Moringa oleifera</i>	0.66 ± 0.45	1.70 ± 0.20	3.58 ± 0.75	6.59 ± 0.35	0.37 ± 0.20	2.71 ± 0.28

Measurement are in mg/100g

3.4 Table 4: Anti-microbial activity of ethanolic leaf extract of *Moringa oleifera* with zones of inhibition measured in (mm) at different concentrations. Zone of inhibition(mm)

Test Organisms	Ethanol	25 mg/ ml	50 mg/ml	100 mg/ml	200 mg/ml
<i>Staphylococcus aureus</i>	0	0	1.66 ± 1.52	1.66 ± 1.54	2.14 ± 2.10
<i>Escherichia coli</i>	0	0	1.58 ± 1.51	1.60 ± 1.47	1.68 ± 1.70
<i>Salmonella spp</i>	0	0	0	0.72 ± 1.02	0.98 ± 1.36

The inhibition diameter of the aqueous leaf extract of *Moringa oleifera* against *Staphylococcus aureus* ranges from 1.66 ± 1.52 to 2.14 ± 2.10, *Escherichia coli* ranges from 1.58 ± 1.51 to 1.68 ± 1.70, and *Salmonella spp* ranges from 0.72 ± 1.02 to 0.98 ± 1.36.

4.0 DISCUSSION

The susceptibility of the test microorganisms is related to the inhibition zone size in millimeters via agar well diffusion assay. The phytochemical screening conducted on the leaf extract of *Moringa oleifera* revealed the presence of phytochemical constituents such as alkaloids, saponins, tannins, flavonoids, oxalates and phytate which have been shown to possess some pharmacological activities. According to Duke (28), Evans (29), Lawal *et al* (30), Wasaguet *al* (31), Ibrahim *et al* (32) as well as Magaji and Yaro (33), phytochemical components are responsible for both pharmacological and toxic activities in plants. The ethanolic quantitative analysis composition of the phytochemical in *Moringa oleifera* revealed flavonoids 6.59 ± 0.35 as highest phytochemical followed by tannins 3.58 ± 0.75 , phytate 2.71 ± 0.20 , saponins 1.70 ± 0.20 , alkaloid 0.66 ± 0.45 and the lowest oxalates 0.37 ± 0.20 . Several functions and roles are attributed to flavonoids in humans and animals; this includes protection and fight against inflammatory disorders, allergies, diarrhea, microbes' invasion, platelet aggregation, ulcers, hepatotoxins, viruses, and tumors Kumar *et al.*, (34). Flavonoids were able to achieved the aforementioned properties because of their antipyretic (fever-reducing), antioxidant, analgesic (pain-relieving), and spasmolytic (spasm-inhibiting) activities Kokoska *et al.*, (35) also known to possess some pharmacological activities. Saponins are used medically for the treatment of increased blood cholesterol and are beneficial to patients with arteriosclerosis and hypertension and in the control of post-menopausal syndrome Akpuaka, (36).

The ethanolic extract of *Moringa oleifera* gave the yield of 0.00 at the concentration of 25 mg/ml. At concentrations 50 mg/ml, 100 mg/ml and 200 mg/ml there were little zones of inhibition (reactions, as shown in the pictures later) against *Escherichia coli*, same reaction (zone of inhibition) was also achieved against *Staphylococcus aureus*. Little reaction or zone of inhibition was observed in *Salmonella spp* at the four different concentrations. Ethanol has been shown to be a stronger extractant than water Brain and Turner (37), Akinyemi (38). The presence of ethanol in addition to achieving better extraction may also enhance the efficacy of the active principle or ingredients. This may justify the traditional use of alcohol in extracting the leaf component of the medicinal plants from application against pathogens Panditt and Langfield (39). Earlier, Eloff in 1998 reported that due to the inability of water to extract nonpolar compounds, the water preparation is usually not suitable for antibacterial discovery. Another

reason for organic extract to be more active than water extract is due to the better solubility of the active components in organic solvents which is in agreement with the work done by Doughariet *al* (40). The presence of phytochemicals supports the use of this plant as antibacterial agent and therefore can be used as antibacterial agent in higher concentrations; this is in agreement with the work done by Gupta et al (41).

Salmonella spp was found to show no inhibitory activity and this disagrees with the study of Siddhuraju and Becker, (42) that indicated significant inhibitory effects of *Salmonella spp* on the plants extract. *Salmonella spp* was the least test organism with zone of inhibition while *Staphylococcus aureus* had the highest zone of inhibition in this study which is not up to the standard inhibition diameter. The statistical analysis was insignificant since none of the three organisms used in this study exhibited a clear zone of inhibition by the extract at different concentrations.

5.1 CONCLUSION

From the study, it has been revealed that *Moringa oleifera* leaf extracts may possess antibacterial properties when used at a higher concentration. The extracts showed little antibacterial effect on pathogens that affect *Clarias gariepinus*.

RECOMMENDATION

Further work should be carried out and there should be an increase the concentration of the leaf extract in order to verify the ethnomedicinal claim.

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