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Antimicrobial Activity of Moringa Oleifera Seed On Beta-Lactam Resistant Bacteria

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ABSTRACTS

The antimicrobial activity and cytotoxicity of aqueous methanolic extracts of *Moringaoleifera* seeds and leaves was examined against some beta lactam resistant bacteria (*E. coli*, *Shigella* and *Staphylococcus aureus*). The antimicrobial activity of the seed extracts was evaluated by the zone of inhibitions. Bioassay was also used to evaluate the cytotoxicity of the leaf and seed extracts. Seed extracts at 1000µg/ml has the highest activity against *Salmonella spp*(20mm), *Shigellaspp*(16mm), *E.coli*(24mm) and *Staphylococcus aureus*(19mm).The LD_{50} of both the seed extracts at $P \leq 0.05$. Toxicity increases as the concentration increases. The high antimicrobial activity of the extracts and its inherent low cytotoxic effect makes *Moringaoleifera* a potential substitute to combat the Beta lactam resistance menace.

KEYWORD:*Moringaoleifera*, Beta lactam resistance, Cytotoxicity, Antibiotics, Antimicrobials.

I. INTRODUCTION

Natural products, such as plants extract, either as pure compounds or as standardized extracts, provide unlimited opportunities for new drug discoveries because of the unmatched availability of chemical diversity (Cos *et al.*, 2006). According to the World Health Organization (WHO), more than 80% of the world's population relies on traditional medicine for their primary healthcare needs. The use of herbal medicines in Asia represents a long history of human interactions with the environment. Plants used for traditional medicine contains a wide range of substances that can be used to treat chronic as well as infectious diseases (Duraipandiyan*et al.*, 2006).

Due to the development of adverse effects and microbial resistance to the chemically synthesized drugs, men turned to ethnopharmacognosy. They found literally thousands of phytochemicals from plants as safe and broadly effective alternatives with less adverse effect. Many beneficial biological activity such as anticancer, antimicrobial, antioxidant, antidiarrheal, analgesic and wound healing activity were reported. In many cases, people claim the good benefit of certain natural or herbal products. However, clinical trials are necessary to demonstrate the effectiveness of a bioactive compound to verify this traditional claim. Clinical trials directed towards understanding the pharmacokinetics, bioavailability, efficacy, safety and drug interactions of newly developed bioactive compounds and their formulations (extracts) require a careful evaluation. The premier steps to utilize the biologically active compound from plant resources are extraction, pharmacological screeni g, isolation and characterization of bioactive compound, toxicological evaluation and clinical evaluation.

*Moringaoleifera*widely used in African communities belongs to the family *Moringaceae*, widely cultivated in the tropical areas of Africa (Fahey *et al.*, 2005). It has been shown to be an excellent nutrient source and also illustrated many medicinal properties (Nikon *et al.*, 2003; Fahey *et al.*, 2005; Chuang *et al.*, 2007). The medicinal, nutritional and therapeutic potential of the plant has become part of the belief and culture of the native people (Fahey *et al.*, 2005). Various parts of this plant such as the roots, leaves, fruit, bark, immature pods and flowers are used for health and dietary purposes (Fahey *et al.*, 2005). It is rich in calcium, potassium, iron, Vitamin C and A and its high protein content rivals with that of eggs and milk (Fahey *et al.*, 2005).

II. MATERIALS AND METHOD

Source of *Moringaoleifera* seeds

Moringaoleifera seeds used in this study were collected into clean polythene bags at the Kogi State University Agricultural farm, Anyigba.

Preparation of Crude Methanol Extract

The Moringa seeds were carefully crushed using a pestle and mortar to de-hull the seeds. Thereafter, the seeds were manually cleaned by separation from the hulls. The seeds was ground to make fine powder and 50.0g was weighed for extraction using the Soxhlet apparatus. 50g of the powdered seed sample was put into a porous thimble and placed in a soxhlet extractor, using 300ml of methanol as extracting solvent for six (6) hours repeatedly until the required quantity was obtained. The oil was obtained after evaporation using a hot plate at 70^oC to remove the excess solvent from the extracted oil. The oil was kept in the refrigerator without further treatment until needed for further analysis.

Antimicrobial susceptibility of test organisms

The test organisms used in this study was obtained from the stock culture in the microbiological laboratory of Kogi State University and revived in a broth to detect the viability of the organisms. Preliminary identification tests were done to further identify the organisms. Antimicrobial sensitivity of the test organisms was carried out using the appropriate antibiotic disks. The test organisms were streaked on the prepared media plates and the Gram negative disk was placed on the Gram negative bacteria while the Gram positive disk was placed on Gram positive bacteria. The plates were incubated at 37^oC for 24hours. After incubation, the zone of inhibition around the Beta lactam antibiotic was observed.

Extract processing

The stored extracted *Moringaoleifera* seed was recovered from the refrigerator and left on a sterile bench to acclimatize to room temperature as described by Cheesbrough (2008).0.02g of the chelated extract was dissolved in 10ml of sterile distilled water to a concentration of 2000µg/ml in a sterile conical flask. The extract was diluted to varying concentration by using two fold dilution method as described by Kizitoand Nwankwo (2015). This was done by adding 10ml of sterile distilled water in 2000µg/ml concentration to get 1000µg/ml concentration. This was repeated consecutively by 10ml of distilled water to 1000µg/ml to get 500µg/ml, 250µg/ml, 125µg/ml and 62.5µg/ml. A cork borer was used to bore a well on the prepared media agar and 1ml of the extract was placed differently at the bottom of each well on the plate. The extract was allowed to diffuse for 15 minutes into the medium before incubation. The plates were incubated at 37^oc for 24hours. After incubation, the diameter of the clear zone of inhibition around the wells containing the plant extracts in the different agar plates was measured. The greater the zone of inhibition, the greater the activity of the test material against the test organism. Ciprofloxacin 2mg/ml was used as standard antibiotic.

Brine shrimp cytotoxicity assay

Brine shrimp lethality bioassay was carried out to investigate the cytotoxicity of *Moringaoleifera* seed. This was done using hatched brine shrimp (Artemiasalina) larvae by the method of Meyer *et al* (1982) modified by Arogba (2014). This was carried out by hatching Artemiasalina (brine shrimp) in a vessel filled with stimulated sterile artificial sea water for hatching.

Artificial sea water compoundment

Artificial sea water (brine solution) was compounded by dissolving 32g of Sodium Chloride analyte in 1000ml of distilled water as reported by Arogba (2014) at a pH of 8.0.

Hatching of brine shrimp eggs

Some 100µg of brine shrimp eggs was added to 100ml of artificial sea water and incubated under bright light connected to an air voltage pump to aerate the mixture. It was sealed and kept under this condition for 24 hours to hatch. At the end of the incubation, 10 brine shrimp larva was counted and transferred to different vial using Pasteur pipette and volume of artificial sea water was added to make up to 100ml of brine shrimp solution (Arogba and Omede., 2012).

Cytotoxicity test

Constant volume of 100ml of each varied concentration (1000, 500, 250 and 125µg/ml) of the test *Moringaoleifera* seed secondary metabolite was added to vials containing 10 shrimps. This was also repeated by adding 100µL of Potassium dichromate on vials containing 10 shrimps. After 6 hours interval, the dead brine

shrimp larvae was counted for the determination of the percentage lethality. This was done in triplicates (Arogba and Omede., 2012).

III. RESULTS

Antimicrobial susceptibility of test organisms

The preliminary screening of the test organisms for Beta lactam resistance (Table 3) revealed that *Shigellaspp*and *Salmonella spp*were resistant to Amoxil and Ampiclox whereas *Escherichia coli* and *Staphyococcusaureus* were resistant to Ampicillin and Ceporex. The multiple resistance spectrum shown by the test isolates to Beta lactam antibiotics was suitable for their application in this study.

Antimicrobial assay of *Moringaoleifera*seed extract

The antimicrobial activity of *Moringaoleifera* seed extract against Salmonella spp at the varied concentrations indicated in table 4 showed high zone of inhibition ranging from 28mm to 21mm. *Moringaoleifera* seed extract activity against *Shigellaspp*showed lower zone of inhibiton ranging from 16mm to 11mm. Also, *Moringaoleifera* seed extract showed higher zone of inhibition against *Stapylococcusaureus* and *Escherichia coli*ranging from 25mm to 18mm and 28mm to 22mm respectively. The positive control (Ciprofloaxacin) showed highest zone of inhibition ranging from 36mm to 29mm. The negative control (water) showed no zone of inhibition against the *Moringaoleifera* seed extract (Table 4).

Table 1: Antimicrobial susceptibility of test organisms

Test Isolate	Multiple resistance antibiotic
Shigella spp	Amoxil, Ampiclox
Escherichia coli	Ampicillin, Ceporex
Salmonella spp	Amoxil, Ampiclox
Staphylococcus aureus	Ampicillin, Ceporex

Table 2: Antimicrobial assay of *Moringaoleifera*seed extract

Concentration (µg/ml)	Zone of inhibition (mm) Test organisms			
	Salmonella	*Shigella*	*S. aureus*	*E. coli*
1000	28	16	25	28
500	27	15	22	25
250	24	12	20	24
125	21	11	18	22
Positive control (Ciprofloxacin)	30	29	36	35
Negative control (Water)	0	0	0	0

Percentage lethality of m*oringa*seed extract after 6 hours
At the 6th hour interval, *Moringaoleifera* seed extract at a concentration of 62.5µg/ml had the highest mortality of 20% while a concentration of 250µg/ml had the lowest mortality of 6.7% (Table 5).

Percentage lethality of m*oringa*seed extract after 12 hours
At the 12th hour interval, *Moringaoleifera* seed extract at a concentration of 500µg/ml had the highest mortality of 40% while a concentration of 62.5µg/ml had the lowest mortality of 20% (Table 6).

Percentage lethality of m*oringa*seed extract after 18 hours
At the 18th hour interval, *Moringaoleifera* seed extract at a concentration of 1000µg/ml had the highest mortality of 73% while a concentration of 125 and 62.5µg/ml had the lowest mortality of 60% (Table 7).

Percentage lethality of m*oringa*seed extract after 24 hours
At the 24th hour interval, *Moringaoleifera* seed extract at a concentration of 1000µg/ml had the highest mortality of 100% while a concentration of 500µg/ml had the lowest mortality of 97% (Table 8).

Table 3: Percentage lethality of m*oringa*seed extract after 6 hours

Concentration (µg/ml)	Number of test organisms	Number of dead (Triplicate)			Mean dead	Percentage mortality (%)
1000	10	2	2	0	1.3	13
500	10	0	0	4	1.3	13
250	10	0	2	0	0.67	67
125	10	2	2	0	1.3	13
62.5	10	0	0	6	2.0	20

Table 4: Percentage lethality of m*oringa*seed extract after 12 hours

Concentration (µg/ml)	Number of test organism	Number of dead			Mean dead (Triplicate)	Percentage mortality (%)
1000	10	3	2	6	3.67	37
500	10	1	4	7	4.0	40
250	10	2	3	6	3.67	37
125	10	2	4	3	3.0	30
62.5	10	3	2	1	2.0	20

Table 5: Percentage lethality of m*oringa*seed extract after 18 hours

Concentration (µg/ml)	Number of test organisms	Number of dead			Mean dead (Triplicate)	Percentage mortality (%)
1000	10	5	7	10	7.33	73
500	10	8	7	7	7.33	73
250	10	5	7	10	7.3	73
125	10	6	6	6	6.0	60
62.5	10	9	5	4	6.0	60

Table 6:Percentage lethality of m*oringa*seed extract after 24 hours

Concentration (µg/ml)	Number of test organisms	Number of dead			Mean dead (Triplicate)	Percentage mortality (%)
1000	10	10	10	10	10	100
500	10	10	9	10	9.67	97
250	10	10	10	10	10	100
125	10	10	10	10	10	100
62.5	10	10	10	10	10	100

Percentage lethality of potassium dichromate (positive control) after 6 hours

At the 6th hour interval, potassium dichromate at a concentration of 1000µg/ml had the highest mortality of 10% while a concentration of 62.5µg/ml had the lowest mortality of 20% (Table 9).

Percentage lethality of potassium dichromate (positive control) after 12 hours

At the 12th hour interval, potassium dichromate at a concentration of 1000µg/ml had the highest mortality of 57% while a concentration of 125 and 62.5µg/ml had the lowest mortality of 23% (Table 10).

Percentage lethality of potassium dichromate (positive control) after 18 hours

At the 18th hour interval, potassium dichromate at a concentration of 1000µg/ml had the highest mortality of 83% while a concentration of 250µg/ml had the lowest mortality of 50% (Table 11).

4.10 Percentage lethality of potassium dichromate (positive control) after 24 hours

At the 24th hour interval, potassium dichromate at a concentration of 1000, 500,250 and 62.5µg/ml had the highest mortality of 97% while a concentration of 125µg/ml had the lowest mortality of 90% (Table 12).

Table 7: Percentage lethality of potassium dichromate (positive control) after 6 hours

Concentration (µg/ml)	Number of test organisms	Number of dead (Triplicate)			Mean dead	Percentage mortality (%)
1000	10	2	1	0	1.0	10
500	10	2	0	2	1.3	13
250	10	2	2	0	1.3	13
125	10	0	0	0	0	0
62.5	10	0	0	0	0	0

Table 8: Percentage lethality of potassium dichromate (positive control) after 12 hours

Concentration (µg/ml)	Number of test organisms	Number of dead (Triplicate)			Mean dead	Percentage mortality (%)
1000	10	6	6	5	5.70	57
500	10	4	4	3	3.70	37
250	10	3	4	4	3.70	37
125	10	2	2	3	2.30	23
62.5	10	3	4	4	2.30	23

Table 9: Percentage lethality of potassium dichromate (positive control) after 18 hours

Concentration (µg/ml)	Number of test organisms	Number of dead (Triplicate)			Mean dead	Percentage mortality (%)
1000	10	10	8	7	8.33	83
500	10	6	6	7	6.33	63
250	10	6	5	6	5.7	57
125	10	5	4	6	5.0	50
62.5	10	9	7	7	7.7	77

Table 10: Percentage lethality of potassium dichromate (positive control) after 24 hours

Concentration (µg/ml)	Number of test organisms	Number of dead			Mean dead (Triplicate)	Percentage mortality (%)
1000	10	10	10	10	9.67	97
500	10	10	10	9	9.67	97
250	10	10	10	9	9.67	97
125	10	8	9	10	9.0	90
62.5	10	10	9	10	9.67	97

4.11 Percentage lethality of artificial sea water (negative control)

At the 6th hour interval, artificial sea water had the lowest mortality of 0% while at the 24th hour interval the highest mortality of 87% was observed (Table 13).

Table 11: Percentage lethality of artificial sea water (negative control)

Time Interval	Number of test organisms	Number of dead			Mean dead (Triplicate)	Percentage mortality (%)
6 hours	10	0	0	0	0	0
12 hours	10	3	4	5	4.0	40
18 hours	10	6	4	6	5.3	53
24 hours	10	8	8	10	8.67	87

IV. DISCUSSION

Antimicrobial susceptibility of test organisms

The pre-screening of the test isolates for β-lactam resistant bacteria showed multiple resistance spectrum to the derivative of penicillin and other β-lactam group as illustrated in table 3. The test isolates (*Shigella spp., Escherichia coli, Salmonella spp., Staphylococcusaureus)* were resistant to derivative of Penicillin and other β-lactam group such as Amoxicillin, Ampicillin and Ceporex. Amoxicillin and Ampicillin are semi synthetic derivatives of Penicillin while Ceporex is a derivative of Cephalosporins family.

Antimicrobial assay of *Moringa seed extract*

The results of the antimicrobial assay reveal that the *Moringa seed* extract showed antibacterial properties in various degrees on test organisms. The Clinical Laboratory Standards (2018) for antimicrobial activity states that zone of inhibition between 10-12mm indicates moderate activity of the extract, zone of inhibition greater than or equal to 14mm indicates high activity of the extract, zone of inhibition less than or equal to 10mm indicates no activity of the extract.

From table 4, the *Moringa*seed extract was highly active according to the Clinical laboratory standards (2018) against the growth of *Salmonella* with the 1000µg/ml concentration giving the highest measurement of zone of inhibition at28mm. It was observed that concentrations of 500µg/ml, 250µg/ml, 125µg/ml of *Moringa seed extract* was highly active with zone of inhibition at 27mm, 24mm, 21mm respectively. The findings agrees

with the work of other researchers, Suarez *et al., 2003;* Kawo,20007 who independently reported a high antimicrobial activity of *Moringaoleifera* seed on some test organisms. The possible reason for this high level of antimicrobial activity could be attributed to the bioactive chemical constituent of *Moringaoleifera* as it is in agreement with the report of Guevera and others (1999).

Moringa seed extract was highly active against the growth of *Shigella*with the 1000 µg/ml and 500 µg/ml concentration giving low measurement of zone of inhibition at 16mm and 15mm. The possible reason for this high activity against gram negative test organism is not known, but it can be attributed to the proposed mode of mechanism of action of the test extract which in the previous report of Olsen and others presented that they attack the cell wall of bacteria. This report also agrees with the report of Silvestre *et al. (2000).* It was observed that the 250 µg/ml and 125 µg/ml was moderately active against the test organisms giving zone of inhibition at 12mm and 11mm.

*Moringa*seed extract was highly active against the growth of *Staphylococcus aureus*with the 1000µg/ml, 500µg/ml, 250µg/ml, 125µg/ml concentration giving a high measurement of zone of inhibition at 25mm, 22mm, 20mm, 18mm respectively.

Moringa seed extract was highly active against the growth of *E.coli*with the 1000µg/ml, 500µg/ml, 250µg/ml, 125µg/ml concentration giving a high measurement of zone of inhibition at 28mm, 25mm, 24mm, 22mm respectively.

However, antibiotic Ciprofloaxacin (positive control) showed the inhibition zone of 30mm for *Salmonella*, 29mm for *Shigella*, 36mm for *S.aureus*, and 35mm for *E.coli.* The positive control express a high level of antimicrobial activity against the test extracts which agrees with the assertion that Ciprofloxacin is a broad spectrum antibiotic.

Distilled water (negative control) showed no zone of inhibition against the growth of *Salmonella, Shigella, S.aureus and E.coli.* The obsolete antimicrobial activity observed with the negative control also complement the assertion that water contain no antimicrobial properties and is in total agreement with the report of Madsen *et al* (1987) where he reported that water has no bioactive activity against some test bacteria.

The antibacterial activity of *Moringaoleifera* seed has been highlighted by many authors (Olsen *et al.,*1987; Madsen *et al.,*1987; Kawo, 2007). The antimicrobial activity of *M. oleifera* seed is due to the presence of an array of phytochemicals, but most importantly due to the activity of a short polypeptide named 4 (ά–L –rhamnosyloxy) benzyl-isothiocyanate (Eilert*et al.,*1981; Guevara *et al.,* 1999). The peptide may act directly on microorganisms and result in growth inhibition by disrupting cell membrane synthesis or synthesis of essential enzymes (Silvestre *et al.,* 2000; Suarez *et al.,* 2003).

Brine shrimp lethality bioassay

Brine shrimp lethality assay is known to be an efficient, rapid and inexpensive test. It is an important tool for the preliminary cytotoxicity assay of plant extract and others based on the ability to kill a laboratory cultured larvae (nauplii). The nauplii was exposed to different concentrations (1000µg/ml, 500µg/ml, 250µg/ml, 125µg/ml, 62.5 µg/ml) of plant extract for 24hours. The number of motile was calculated for the effectiveness of the extract. Potassium dichromate ($K_2Cr_2O_7$) was used as positive control while artificial sea water was used as negative control. Varied concentrations of Moringa seed extract was introduced into the test tubes containing 10 brine shrimp larvae. The lethality of the *Moringa*seed extract against the brine shrimp was observed, after 6 hours interval till the 24 hours was achieved.

At the 6^{th} hour, it was observed that the variation in concentration did not affect the toxicity of the *Moringa*seed extract (Table 5). At 1000 µg/ml concentration, the extract was toxic to 13% of the brine shrimp larvae. An increased toxicity of 20% was observed at the least concentration (62.5 µg/ml) this might be as a result of gradual inhibition of the cell's activities by the Moringa seed extract leading to increased mortality. At the 12^{th} hour, the mortality gets higher but towards the middle (250 µg/ml) there is stability in the mortality due to the cell adapting (Table 6). The mortality then starts to reduce at 125 µg/ml and 62.5 µg/ml concentration as the cell shows signs of acclimatization. At the 18^{th} hour, the cell can no longer accommodate the toxicity of the extract, so it causes more lethal effects leading to high mortality (Table 7). At the 24^{th} hour, the varied concentrations showed the highest level of mortality (Table 8). This could be due to the effect of longer time activity of the extract resulting in a very high level of mortality. Also, could be as result of environmental conditions like temperature and feeding conditions. The positive control, Potassium dichromate is a known toxic substance to humans, the cytotoxic test between Potassium dichromate and brine shrimps showed high level of mortality at varied concentrations of the *Moringa*seed extract (Table 9-12). The negative control, artificial sea water, also showed a little level of mortality this could be due to the fact that people still die based on natural causes . The mortality could also be due to environmental and feeding conditions.

V. CONCLUSION

The antimicrobial activity of the Moringa seed extract was evaluated based on the inhibition zone using the well diffusion assay. The bacterial strains *Salmonella sp, Staphylococcus aureus, Shigellasp and Escherichia coli* was identified to be resistant against Beta-lactam antibiotics and used against the extracts of M. oleifera to check the antimicrobial activity. The above strains were collected from Kogi state University Microbiology Laboratory. In this study, we found that the methanolic extract of seeds of *M. oleifera* was active against all the bacteria. The zone of inhibition showed highly active against *Salmonella sp, Staphylococcus aureus, and Escherichia coli*. Moringa seed extract was moderately active *Shigella sp.* Therefore, *M. oleifera* seed could be a promising antibacterial agent with potential applications in pharmaceutical industry for controlling the pathogenic bacteria.

Brine shrimp lethality assay was used to evaluate the cytotoxicity of Moringa seed extract based on the ability to kill a laboratory cultured larvae (nauplii). On exposure of the nauplii to different concentrations of the Moringa seed extract for 24 hours, it was revealed that the Moringa seed extract exhibited high brine shrimp toxicity level between the 18^{th} and 24^{th} hour which could be as a result of environmental conditions like pH, temperature and feeding conditions. As such, the Moringa seed extract may be explored for the development of useful plant- based pharmaceuticals, considering the threat of multi drug resistance. Furthermore, evaluation of Moringa seed extract through in vivo based research is highly recommended to achieve low cost, less side effect treatment and prevent recurrent infection.

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