ANTIBACTERIAL ACTIVITY OF MORINGA OLEIFERA LEAF EXTRACTS AGAINST GRAM-NEGATIVE BACTERIA

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ABSTRACT

About 80% of the populations in developing countries prefer the use of herbal extracts and their active components in traditional medicine therapy. Moringaoleifera is largely safe for human and animal consumption. Hence, it is use in the treatment of various ailments. This study was carried out to determine the antibacterial activity of Moringaoleifera on Gram-negative bacteria. Moringaoleifera leaves were obtained and identified in Plant Science and Biotechnology Laboratory of Benue State University Makurdi. The leaves were washed, air dried and pulverized, Aqueous and Methanol extract were prepared. The antibacterial effects of aqueous and methanol extracts of the leaves were determined using Agar well diffusion method. The minimum Inhibitory Concentration (MIC) and the minimum Bactericidal Concentration (MBC) of the extracts on both aqueous and methanol extracts were carried out at concentrations of 100mg/ml, 50mg/ml, 25mg/ml. Phytochemical screening o the extracts revealed the presence of bioactive constituents such as Tannins, Flavonoids, Saponins and Alkaloid. Higher concentrations of Methanol extract (100mg/mL) were more effective against E. coli (17.83 ± 40.92), K. pneumonia (17.67 ± 3.33), S. typhi (17.67 ± 4.63), P. mirabilis (20.00 ± 3.58). The methanol extract of Moringaoleifera contributed considerably to its efficiency in inhibiting the bacteria than aqueous extract. Higher concentration of methanol extracts should be used as an alternative for the treatment of Gram-negative bacteria.

Keyword: *Antibacterial, Gram negative bacteria, Leaf extract, Moringaoleifera*

1.0 INTRODUCTION

Moringaoleifera (MO) belongs to the monogeneric family, Moringaceae, and is widely cultivated in many tropical countries, e.g. Africa, India, Sri Lanka, Ceylon, Thailand, Burma, Mexico, Malaysia and the Philipines. It has been used for treating bacterial infection, fungal infection. Sexually transmitted diseases, malnutrition and diarrhea (Farooq*et al.,* 2012).*Moringaoleifera* has been used worldwide in medicine due to its medicinal compounds and pharmacological properties. It is commonly called drumstick tree has many active components such as alkaloids, tannins, flavonoids and saponins with a potential for antihelmintic activity and antibacterial effect. *M. oleifera* is a rich source of various phytochemical compounds including glucosinolates and is known to have antibacterial activity. According to the World Health Organization (WHO), about 80% of the populations in developing countries prefer to use herbal extracts and their active components as traditional medicine therapy (Alhakmani*et al.,* 2013).
M. oleifera is largely safe for human and animal consumption; hence it is used in the treatment of bacteria. The increasingly high numbers of bacteria that are developing resistance to classical antibiotics drive much of the current interest on natural antimicrobial molecules in hope that they may provide useful leads into anti-infective drug candidates. Several antimicrobial agents were isolated from plant including secondary metabolites as essential oil and terenoids, amongst which can be cited xanthones, benzophenones, coumarins, and flavonoids. These new chemical subtances can also serve as templates for producing more effective drugs through semi-synthetic and total synthetic procedure. About 74% of 119 plant-derived pharmaceutical medicines or biotechnology medicines are used in modern medicine in ways that correlate directly with their traditional uses.

Antibacterial activity of Moringa been shown in different studies. Using the Disc agar diffusion technique, researchers have evaluated the antibacterial activity of *Moringaoleifera* leaf and seed chloroform and ethanol extracts. One Gram-positive bacterium (*Staphylococcus aureus)* and six Gram-negative bacteria (*Escherichia coli, Salmonella typhi, Enterobacteraerogenes, Satyphimurium, Shigella*spp and *S. aeruginosa*) were used to test the antibacterial activities of *Moringaoleifera.*

In traditional Indian medicine various parts of the tree are used therapeutically for treatment of venomous bites, ascites and rheumatism and helps in lowering blood pressure. The root and bark of young trees are considered rubefacient, stomachic caminative, vesicant and abortifacient. The flowers and roots contain an antibiotic that is highly effective in the treatment of cholera. Moringa leaves have been reported to contain more vitamin A than carrots, more calcium than milk, more iron than spinach, more vitamin C than oranges, and more potassium than bananas, and the protein quality of Moringa leaves rivals that of milk and eggs. The nutritional properties of Moringa are now so well known that there seems to be little doubt of the substantial health benefit to be realized by consumption of Moringa leaf powder in situation where starvation is imminent (Dodiya and Amin, 2015).

In addition, certain antibiotics present undesirable side effects such as nausea, depression of bone marrow, thrombocytopenic purpura and agranulocytosis leading to the emergence of previously uncommon diseases. This has encouraged scientists to carry out more research on newer and alternative microbial compounds from medicinal plants. Besides, the high cost of conventional drugs, particularly in resource limited communities has led to the increase use of plants as an alternative for treatment of infectious diseases. Plant extracts and phytochemicals with antimicrobial properties are of great significance in therapeutic treatments. Their antimicrobial properties are due to compounds synthesized in the secondary metabolism of the plant. The screening of plant extracts and plant products for antimicrobial activity has shown that plants represent a potential source of novel antibiotic protypes (Moyo*et al*., 2012). The aim is to determine the antibacterial activity of aqueous extracts of *Moringaoleifera* against Gram negative bacteria.

3.0 MATERIALS AND METHODS

3.1 STUDY AREA

This study was carried out at CharisRhema Laboratories, High level, Makurdi, Benue State. The city is located in central Nigeria along the Benue River. It is located on latitude 7.73˚N and longitude 8.53˚E and situated 104 meters above sea level. The annual rainfall ranges from 150mm to 180mm, and the mean temperature ranges from 27^{o}C to 38^{o}C, along the banks of the Benue River which is a major tributary to the Niger River. It is an agricultural catchment area and has a variety of potentials in human capital and material resource.

3.2 SAMPLE COLLECTION

The leaves of Moringa plant were collected in a polythene bag from staff quarters, Benue State University, Makurdi and authenticated at Plant Science and Biotechnology laboratory,Benue State University, Makurdi. They were later washed with distilled water, shade dried, and pulverized to obtain fine powder.

3.3IDENTIFICATION OF BACTERIAL ISOLATES

The bacteria include; *Escherichia coli, Klebsiella pneumoniae, Salmonella typhi and Proteus mirabilis* which were identified morphologically and biochemically (Sahay*et al.,* 2017). The bacteria were maintained in nutrient agar slant and stored in the refrigerator at 4^{o}C. The bacteria were sub-cultured onto a fresh media at regular interval until required for the test.

3.4BIOCHEMICAL IDENTIFICATION OF ISOLATES

3.4.1Gram Staining

A smear of suspension was prepared on a clean slide with loopful of the bacteria sample.Crystal violet was used as a primary stain to stain the bacteria for one minute, Iodine was then applied for one minute as a mordant and the sample was washed with water, Ethanol was applied rapidly to decolourize the sample, finally, Safranin was used as a counter stain. Slides were observed under x100 magnification.The Gram negative organisms took on red colour from the safranin (Cheesbrough, 2006).

3.4.2 Catalase Test

The bacterial sample was added to the hydrogen peroxide on a slide, the principle of the test is based on the enzymatic breakdown of hydrogen peroxide into water and oxygen which was indicated by production of bubbles of air and it was used to identify *Staphylococcus* from other Gram-positive cocci. Hydrogen peroxide forms an oxidative end product of aerobic carbohydrate metabolism. Staphylococci produce catalase enzyme which reacted with hydrogen peroxide thereby producing bubbles of oxygen (Cheesbrough, 2006).

3.4.3 Indole Test

This test is used for indole production in differentiation of Gram negative bacilli. The enterobacteria produce aromatic amino acid tryptophan which is present in the medium. A bright red ring with formation indicating that bacteria had broken the amino group and indole was produced. After overnight growth Kovac's reagent was added to the broth culture which reacted with indole to form a bright red ring colour at the surface of the bijou bottle which was indicative of a positive test. The test was negative when Kovac's reagent was added and no colour change occurred. Indole test was used to differentiate between *E.coli* from *Klebsiella pneumoniae* (Cheesbrough, 2006).

3.4.4 Citrate Test

The citrate agar (green) slants and butt was streaked with test organisms containing citric acid, which was a tricarboxylic acid and Bram Cresol agar. The citrate was metabolized to acetoin and carbon dioxide. The isolates with citrate permease allowed intake of citric acid, causing alkaline end products that change pH indicator from green to blue. The isolate was identified by the ability to utilize citrate as the source of carbon, and ammonium as its source of nitrogen. Those organisms turned slant green were negative. The test was used in identification of enterobacteria such *E.coli*and *Klebsiella* (Cheesbrough, 2006).

3.5 PREPARATION OF AQUEOUS AND METHANOL EXTRACTS OF *Moringaoleifera*

The cold maceration extraction was the extraction method used. One hundred grams± (100g) of the powder obtained after homogenizing the Moringa leaves were separately soaked in 700ml of methanol, and distilled water respectively.The reagent bottles containing the samples were shaked four times at 30 minutes intervals to aid proper mixing of the solution, and allowed to stand for 14days to permit the full extraction of the bioactive compound. The fluids were then filtered using Whatman no1 filter paper. The extracts were rotary dried to obtain the concentrate. 1g/ml of each extract were reconstituted with 10ml of DMSO (Dimethyl sulfoxide) and fractionated into 50, 25, 12.5, 6.25, 3.13 and 1.56µg/ml needed for the bioassay (Abdlkadir*et al*., 2015).

3.6QUALITATIVE METHOD OF ANALYSIS (PHYTOCHEMICAL ANALYSIS)

The phytochemical analysis was done to screen the extracts for qualitative detection of bio active ingredients such as, Tannins, Saponins, alkaloids, flavonoids,phenol. The *Moringaoleifera* leaves filtrate was prepared by soaking 20grams ofthe powder into methanol and distilled water respectively. The methods as described by Ajayi and Fadeyi (2015) were adapted in the qualitative phytochemistry determination.

3.6.1 Test for alkaloids
Alkaloid was determined based on the modified method of Trease and Evans, about 0.2g of *Moringaoleifera* sample was warmed with 2% of H2SO4 for 2 minutes.It was filtered and few drops of Dragendoffs reagent were added. Orange red precipitate indicates the presence of alkaloids.

3.6.2 Test for Tannins
The test was performed by following a standard procedure of Maxson and Rooney. 1ml of the filtrate was mixed with 2mls of FeCl. A dark green colour indicates a positive test for the tannins.

3.6.3 Test for Saponins
1ml of *Moringaoleifera* leave filtrate was diluted with 2mls of distilled water, the mixtures were vigorously shaked and left to stand for 10minutes during which time, the development of foam on the surface of the mixtures lasting for more than 10minutes indicates the presence of Saponins.

3.6.4 Test for Flavonoids
1ml of *Moringaoleifera* leaves filtrate was mixed with 2ml of diluted NaOH, a golden yellow colour indicates the presence of flavonoids.

3.7PREPARATION OF ANTIBACTERIAL ASSAY OF *Moringaoleifera*

This test was performed using Agar Well diffusion method. In this technique organisms isolated were inoculated in normal saline with the help of sterile wire loop. Briefly, colonies were taken from 24 hours culture plates into nutrient broth. The turbidity formed was adjusted to an equivalent of 0.5 McFarland. The test organism was streaked over the surface of Mueller Hinton agar plates using sterile cotton swabs. A sterile cork borer of 6mm in diameter was used to create three (3) wells on the agar plates .Two (2) wells were filled with 20µL of the reconstituted extracts which is equal to 0.2ml.Control experiment was carried out where the third well was filled with 0.2ml of Dimethyl sulfoxide (DMSO) and antibiotic disc (Imipenen) were placed on plates firmly by means of sterile forceps aseptically. (Imipenen =positive control and DMSO=negative control). Each well was labeled representing a particular concentration. . The process was carried out for each extract and the plates were left for few minutes for extracts to diffuse into the agar. The plates were incubated at 37°C in an upright position for 24hours, after which the zones of inhibition were measured where obtainable using a meter rule (Abdlkadir*et al*., 2015).

3.8 DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION (MIC)

The minimum inhibitory concentration was determined against bacteria after the antimicrobial test have been performed. This shows the lowest concentration of an antimicrobial agent that will inhibit the visible growth of a microorganism after overnight incubation.
Macro dilution method was used for this purpose. Two fold dilution was made to obtain six (6) different concentrations of the extracts.Standardized suspensions of the test organisms were inoculated into sterile tubes of peptone water containing dilutions (50, 25, 12.5, 6.25,3,13 and 1.56µg/ml) of the extracts and incubated at 37°C for 24hours.

The MICs were read as least concentration that inhibited any visible growth (absence of turbidity) of the test organisms (Abdlkadir*et al*., 2015).

3.9DETERMINATION OF MINIMUM BACTERICIDAL CONCENTRATION

A loopful of the broth from each of the test tubes without visible growth (no turbidity) in the MIC determination was subcultured onto fresh extract free nutrient plates and incubated at 37°C for 24hours. The least concentration with no visible growth was read as the MBC (Abdlkadir*et al*., 2015).

The data gotten during this laboratory study were subjected to analysis of variance (ANOVA), and the Fisher's Least Significant Different (FLSD) was used to ascertain the significance between the different treatment means at 5% level of probability

4.0 RESULTS

The phytochemical screening of aqueous and methanol extracts of *Moringaoleifera*is presented in Table 1. Alkaloids, Tannin, Saponin, Flavonoids and Phenol were identified from the extracts. The biochemical characteristics of the isolates are presented in Table 2.

Table 3 showsthe main effects of the treatments and concentration of the aqueous and methanol leaf extracts of *M. oleifera* on some test organisms.The aqueous and methanol treatments had no significant effect on*E. coli* ($F(1,7) =0.101$, $p=0.759$). The main effect of the concentration on the other hand was significant ($F (2, 7) =26.047$, $p=0.001$).Thehighest effect *E. coli* was observed at 100mg/ml which was considered significant when compared with the effect at 25mg/ml ($p=0.001$) and 50mg/mL($p=0.003$) with no significant difference with the control ($p=1.000$).

The main effect of the aqueous and methanol extracts on *K. pneumoniae* was not statistically significant ($F (1,7)=3.727$, $p=0.095$), the methanol extract performed slightly better than the aqueousextract however, no significant difference was observed between them ($p>0.05$). The main effect of the concentration on *K.pneumoniae* was also not significant ($F(2,7)=3.789$, $p=0.077$). The control preformed slightly better than the treatments followed by 100mg/mL treatment and 50mg/mL however, no significant difference was observed between them ($p>0.05$).
The main effect of the treatment on *S.typhi* was significant ($F(1,7)=16.219$, $p=0.005$). The methanol extract was observed to perform significantly better than the aqueous extract ($p=0.012$). The main effect of the concentration was also observed to be significant on *S. typhi*($F(2,7)=5.377$, $p=0.038$). The highest effect was observed at 100mg/ml but with no significant difference with the effect at 50mg/ml and 25mg/ml respectively ($p>0.05$) compared with the control, the control performed slightly the better than all the treatments with a significant difference with only the effect at 25mg/ml ($p=0.018$).

The main effect of the treatments on *P.mirabilis* was significant ($F (1,7) =7.631$, $p=0.028$) with the methanol extract performing significantly better than the aqueous extract.

The main effect of the concentration was also statistically significant ($p<0.05$) against the test organism than the control ($p=0.045$) and the 25mg/ml treatment ($p=0.017$).

Table 4.4shows the interaction effect between the treatment and concentration on the test organisms. The interaction effect on*E. Coli* was not statistically significant ($F(2,7)=2.460$, $p=0.155$) with the methanol extract performing slightly better than the aqueous extract at all concentrations. A significant difference was however observed between them ($p<0.05$). The interaction effect of *K.pneumonia*e was also statistically not significant ($F(2,7)=0.072$, $p=0.931$). The same observation was also made on *P.mirabilis* respectively. The interaction effect on *S.typhi* on the other hand showed the methanol extract to perform significantly better at 50mg/ml at ($p=0.021$) respectively.

Table 1: Phytochemical Constituents of Aqueous and Methanol Leaf Extracts of *Moringaoleifera*Leaves

Phytochemical constituent	Aqueous	Methanol
Alkaloid	+	+
Tannins	+	+
Saponins	-	+
Flavonoids	+	-
Phenol	+	+

Key
+ = Positive
- = Negative

Table 2: Biochemical Characteristics of the Gram-negative bacteria Isolates

CODE	GR	MOT	IND	CIT	UR	LAC	SUC	GLU	GAS	H2S	SLOPE	BUTT	ISOLATES IDENTIFIED
A	-	+	+	-	-	+	+	+	+	-	A	A	*Escherichia coli*
B	-	-	-	+	+	+	+	+	+	-	A	A	*Klebsiella pneumoniae*
C	-	+	-	-	-	-	-	+	-	+	K	A	*Salmonella typhi*
D	-	+	-	+	+	+	-	-	+	+	K	A	*Proteus* spp

KEY
GR=Gram reaction,MOT= Motility, IND = Indole, CIT = Citrate, UR = Urease, LAC = Lactose, SUC = Sucrose, GLU = Glucose, H2S= Hydrogen Sulphide, A =Acid reaction, K=Alkaline reaction

Table 3: The main effect of the aqueous and methanol extracts of *Moringaoleifera* on some selected Gram-negative organisms

Treatments	**Zones of Inhibition (mm)**			
Treatments	*E. coli*	*K.pneumonia*	*S. typhi*	*P.mirabilis*
Aqueous extract	16.83±4.17	14.50±3.56	10.50±3.73	16.33±3.67
Methanol extract	17.83±4092	17.67±3.33	17.67±4.63	20.00±3.58
FLSD (0.05)	NS	NS	7.17	NS
Concentration(mg/L)				
100	22.00±1.41	19.00±2.94	18.00±4.32	21.75+2.99
50	17.25±4.91	15.75±2.99	13.25±5.85	17.75±2.63
25	12.75±0.00	13.50±303.42	11.00±4.83	15.00±3.27
Control	22.00+0.00	20.00±0000	22.00±0.00	15.00±0.00
FLSD(0.05)	9.250	NS	11.00	6.75

*values are mean +standard deviation in 2 replications. Mean values with similar alphabets in a column are significant. NS=No significant difference. FLSD = Fisher's Least Significant Difference

Key:

E. coli – Escherichia coli

K. pneumoniae – Klebsiella pneumoniae

S. typi – Salmonella typhi

P. mirabilis – Proteus mirabilis

Table 4. The interaction Effect between *Moringaoleifera* aqueous and methanol extracts and concentration on some selected Gram-negative organisms

	Zones of Inhibition (mm)				
Concentration	Treatments	*E. coli*	*K. pneumonia*	*S. typhi*	*P.mirabilis*
100mg1mL	Aqueous	21.00±1.41	17.00±2.83	15.00±1.41	19.50±2.12
	Methanol	23.00±2.83	21.00±1.41	21.00±4.24	24.00±1.41
50mg1mL	Aqueous	17.00±2.83	14.50±3.54	8.50±2.12[a]	16.50±3.53
	Methanol	17.50±2.12	17.00±2.12	18.00±1.41[a]	19.00±1.41
25mg1ml	Aqueous	12.50±2.12	12.50±2.12	8.00±1.41	13.00±2.83
	Methanol	13.00±2.83	13.00±2.83	14.00±5.66	17.00±2.83
FLSD(0.05)		NS	NS	9.50	NS

*Values are mean +standard deviation in 2 replications. Mena values with similar alphabets are significant. NS =No significant Difference. FLSD=Fisher's Least significant difference

5.1 DISCUSSION

The phytochemical screening of *Moringaoleifera*leaf extracts (methanol and aqueous) have been known to confer the inhibitory effects exhibited by *Moringaoleifera*on several bacteria (Laurie, 2019). The presence of these phytochemical compounds in this study may be responsible for the inhibitory properties observed. This is because they have been shown by the reports of Laurie (2019) to inhibit the growth of bacteria. Indeed, the inhibition of the isolates in this study could also be due to the presence of these compounds.

The higher efficacy of methanol extracts of *Moringaoleifera*reported in this study at higher concentrations align with the work of Bhumika and Bijal(2015). The study however contrasts the finding of Peter *et al*. (2011) who reported lower efficacy of methanol extracts of *M. oleifera*at higher concentrations. Methanol extracts of *M. oleifera*performed better than aqueous extracts due to the differences in polarity of the extraction solvent. The methanol solvent is highly polar and as such a better extraction yield was observed, significantly affecting the bioactive compounds of the extract.

The effect observed by the aqueous and methanol extracts on*E. coli, K. pneumoniae S. typhi* and *P. mirabilis* aligns with the work of Lar*et al*. (2011), Bhumika and Bijal (2015, and Peter *et al*. (2011) who reported the efficacy of the extracts on several organisms. The efficacy observed in this study could be as a result of the phytochemicals reported. Since these phytochemicals are known to have inhibitory characteristics on these organisms, their use an alternatives for antibiotics is plausible.

Although there was no significant relationship, the higher efficacy reported at higher concentrations for both aqueous and methanol extracts suggests that higher concentrations of the extracts are potent in the inhibition of these bacteria. The findings in this study hence agree with the work of Patel and Mohan (2018), who although used cold water extracts of *Moringaoleifera*, posited that the phytochemicals identified in the extracts played a vital role in its inhibitory characteristics.

Consequently, the zones of inhibition reported in this study is a reflection of the potency of the extracts of *Moringaoleifera*and its use in the treatment of infections caused by these bacteria. *Escherichia coli*, *S. typhi, K. pneumoniae,* and *P. mirabilis* have been shown to cause severe bacterial infections which if left untreated may cause death to the host. Hence, reports of antibiotic resistance to modern drugs point to the use of higher concentrations of *Moringaoleifera*as potent cures for the inhibition of these bacteria (Bukar*et al*., 2010).

5.2 CONCLUSION

The findings in this study suggest that higher concentrations of *Moringaoleifera* are effective in the inhibition of all bacteria isolated in this study. However, Methanol extracts of the plant were more effective in comparison with the aqueous extracts, although they were all effective but with varying effects at varying concentrations.

5.3 RECOMMENDATIONS

From the findings in this study, it is recommended that:

i. The extracts of *Moringaoleifera*should be used as alternatives for the treatments of infections caused by these Gram negative bacteria.
ii. Higher concentrations of methanol extracts of the plant extract should be used as they have shown more promising effects on the bacteria used.
iii. More research should be carried out on the efficacy of the extracts in the treatment of other bacterial infections.
iv. Side effects from consumption of these treatment extracts should be studied for effective health management.

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