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POTENTIAL *IN VITRO* INHIBITORY EFFECTS OF *MORINGA OLEIFERA* LEAF EXTRACTS ON EXTENDED-SPECTRUM β-LACTAMASE-PRODUCING BACTERIA

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ABSTRACT

Extended-spectrum β-lactamase (ESβL) enzymes are produced by certain bacteria as a mechanism of resistance to β-lactam and extended-spectrum β-lactam antibiotics. Previous investigations have confirmed that *M. oleifera* contains several bioactive compounds. This study aimed at evaluating the *in vitro* antibacterial effects of ethanolic and methanolic leaf extracts of *M. oleifera* on extended-spectrum β-lactamase producing bacteria. The leaf extracts of the plant were prepared, screened for bioactive compounds and evaluated for *in vitro* inhibitory effects against the ESβL-producing bacteria using agar well diffusion method. Different phytochemicals were detected from the extracts. Both methanolic and ethanolic leaf extracts showed a high inhibitory activity, which increased with an increase in concentration, from lowest to highest based on the zones of growth inhibition produced. Amongst the test organisms, *Klebsiella pneumoniae* was the most sensitive isolate to methanolic extract at 200, 100, 50 and 25 mg/mL followed by *Escherichia coli*, and then *Proteus mirabilis*. However, in terms of the ethanolic extract, using the same concentrations as those of methanol extracts, *E. coli* was found to be the most sensitive isolate followed by *K. pneumoniae* and then *P. mirabilis*.

Keywords: *Moringa oleifera*, antibacterial effect, phytochemicals, ESβL-producing bacteria, antimicrobial resistance.

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1. INTRODUCTION

The emergence and re-emergence of drug-resistant organisms have been on a global increase, jeopardising the effectiveness of promising antibiotics which have metamorphosed medicine to save zillions of lives [1-2]. Following several decades of successful use of antibiotics to treat first patients, menace due to bacterial infections resurfaced. Antibiotics resistance has been linked to misuse and overuse of drugs and inadequate development of new antibiotics by the pharmaceutical industries probably due to regulatory challenges and economic reasons [3-6]. According to Centres for Disease Control and Prevention (CDC), several bacteria are known to present pressing, stern, and concerning intimidations largely due to antibiotic resistance, majority of which have been responsible for a great clinical and financial liability on the government healthcare systems, patients, as well as their families [6].

The extended-spectrum β-lactamase (ESβL) enzymes have been produced by bacteria as a strategy to develop resistance to β-lactam and extended-spectrum beta lactam antibiotics such as penicillins and cephalosporins, respectively. The enzymes symbolize the key source of multidrug resistance among Gram negative bacteria, especially members of the family enterobacteriaceae such as *Escherichia coli*, *Klebsiella pneumoniae*, and *Morganella morganii* [7]. The discovery of innovative antimicrobial agents is very crucial for the control of pathogenic microorganisms and the management of infections caused by drug-resistant organisms [8]. The increased number of multidrug-resistant microorganisms poses a public health concern. However, several research works suggest that medicinal plants could serve as alternative means of treating infectious diseases as well as a possible source of novel and cheap antibiotic that could be effective against resistant strains of pathogenic microorganisms [9].

Moringa oleifera appears to grow abundantly as a woody tree both in tropical and subtropical regions like Africa, Middle East and Asia. The tree is nutritionally rich, having several potential uses or medicinal values. Different parts of *M. oleifera* including the roots, stem bark, flowers, seeds, fruits, and immature pods have proven medicinal applications such as antiulcer, antihypertensive, anti-inflammatory, antispasmodic, antioxidant, antidiabetic, antipyretic, antiepileptic, antitumor, hepatoprotective, lowering cholesterol, antibacterial,

antifungal, as well as cardiac and circulatory stimulants [8,10]. Several studies have confirmed *M. oleifera* to constitute numerous bioactive substances such as polyphenols, phenolic acids, carotenoids, flavonoids, vitamins as well as essential amino acids. Moreover, many reports have confirmed the successful antimicrobial activity of crude extracts prepared from various parts of *M. oleifera* against a wide number of microorganisms [8]. However, reports on the antibacterial activity of *M. oleifera* against drug-resistant ESβL-producing bacteria have been very scarce. Therefore, this study aimed at evaluating the *in vitro* antibacterial effects of ethanolic and methanolic leaf extracts of *M. oleifera* on extended spectrum β-lactamase producing bacteria.

2. RESULTS AND DISCUSSION

2.1 Physical properties and Physicochemical Constituents of Extracts

Qualitative phytochemical analysis is very crucial as it provides the preliminary knowledge on the key families of secondary metabolites present in the plant materials [11-12]. The *M. oleifera* leaves were extracted using percolation method with methanol and ethanol organic solvents. The ethanolic extract revealed a lower percentage yield of 35% compared to that of methanol extract with 40% yield. The two extracts were observed as dark green and gummy in colour and texture, respectively (Table 1). Phytochemical screening of the extracts established the presence of Tannins, flavonoids, alkaloids and Carbohydrate. However, steroids were observed only in ethanolic extract while saponins were detected only in methanolic extract but phenolic compounds were not detected in both extracts (Table 2).

Table 1: Physical Properties of *Moringa oleifera* Leaf Extracts

S/No	Parameter	Solvent/Extraction Yield	
		Methanol	**Ethanol**
1	Mass of extract (g)	7	8
2	Colour	Dark green	Dark green
3	Texture	Gummy	Gummy

Table 2: Phytochemical constituent of *Moringa oleifera* leaf extracts

S/No	Phytochemical	Type of Extract	
		Methanolic	**Ethanolic**
1	Tannins	+	+
2	Saponnins	_	+
3	Flavonoids	+	+
4	Alkaloid	+	+
5	Steroids	+	_
6	Phenolic compound	_	_
7	Carbohydrate	+	+

Key: + = Detected**, -** = Not detected

2.2 *In Vitro* Inhibitory Effects of *Moringa oleifera* leaf extracts

The *in vitro* Inhibitory effects of the leaf extracts of *Moringa oleifera* prepared from methanol and ethanol were evaluated on the ESβL-producing *Escherichia coli, Klebsiella pneumoniae* and *Proteus mirabilis* using agar well diffusion method as described in the methodology section. Four different concentrations (200, 100, 50 and 25 mg/ml) of each extract were tested on these bacteria. Both extracts showed strong inhibitory effects, which increased with an increase in concentration, from lowest to highest as observed from the zones of growth inhibition. However, the results of sensitivity tests of the methonolic extract indicated higher inhibitory effects (Table 3) over the ethanolic extract (Table 4) against the test bacterial isolates. Figure 1 shows some representative sensitivity plates of methonolic extract against the test bacterial isolates.

Table 3: Sensitivity patterns of test bacteria to Methanolic leaf extract of *Moringa oleifera*

Methanolic extract (mg/ml)	**Test Bacterial Isolates**		
	E. coli	***K. pneumoniae***	***P. mirabilis***
	Zone of growth inhibition (mm)		
200	22	27	15
100	16	21	12
50	12	17	11
25	10	12	10
Augmentin (30µg)	27	26	25

Key: mg = Milligram, mm = Millimeter

Table 4: Sensitivity patterns of test bacteria to ethanolic leaf extract of *Moringa oleifera*

Ethanolic extract (mg/ml)	**Test Bacterial Isolates**		
	E. coli	***K. pneumoniae***	***P. mirabilis***
	Zone of growth inhibition (mm)		
200	20	12	11
100	17	11	10
50	13	10	9
25	10	8	8
Augmentin (30µg)	27	26	25

Key: mg = Milligram, mm = Millimetre

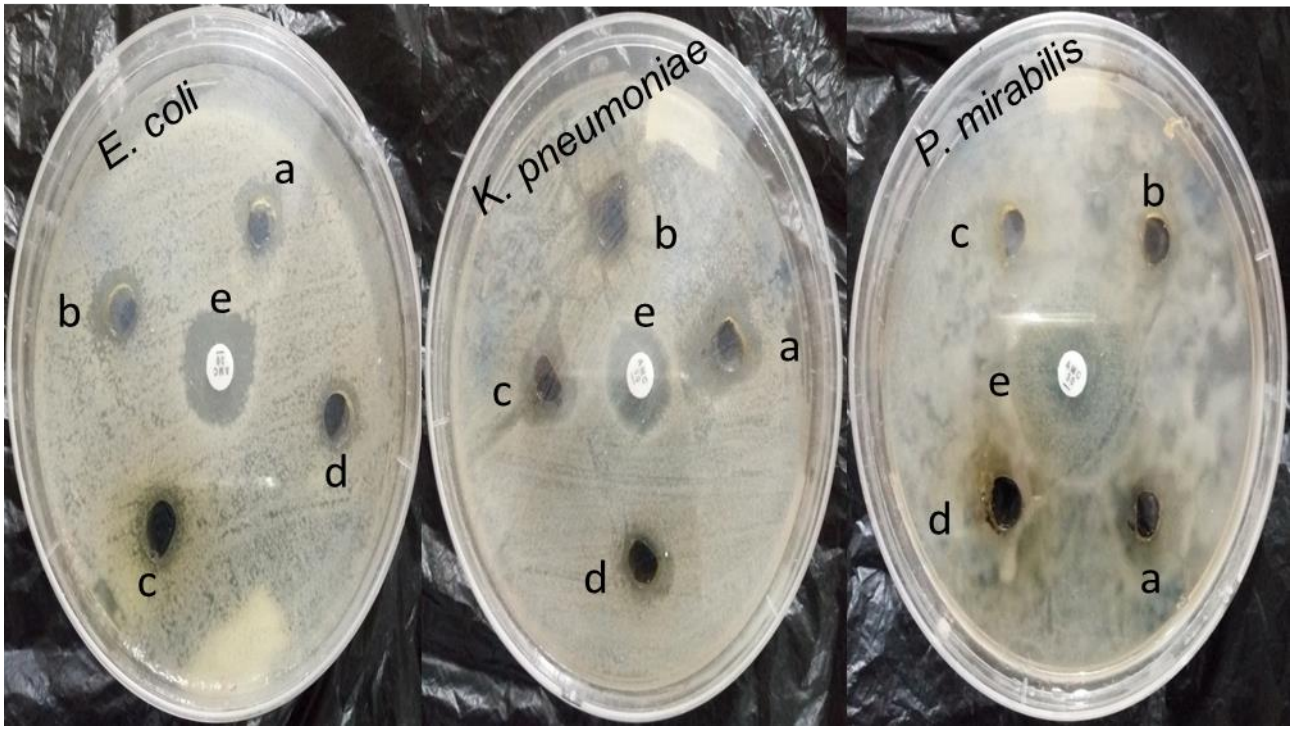

Fig.1. Susceptibility patterns of ESβL-producing bacteria to the leaf extract of *Moringa oleifera*. Standardized inocula of the bacteria were cultured on Mueller Hinton Agar plates and incubated for 24 hours at 37 ^{o}C. Zones of growth inhibition produced by various concentrations of 200, 100, 50 and 25 mg/ml were represented by letters a, b, c, and d, respectively while letter 'e' represents Augmentin which served as control antibiotic

2.3 Minimum Inhibitory Concentrations and Minimum Bactericidal Concentrations

Table 5 shows the results of Minimum Inhibitory Concentrations (MIC) and Minimum Bactericidal Concentrations (MBC) of both methanolic and ethanolic extracts of the *M. oleifera* leaves investigated on the ESβL-producing bacteria, *E. coli*, *K. pneumoniae* and *P. mirabilis* using broth dilution method. As the lowest concentration to inhibit the growth of test organisms, the MICs of the methanolic and ethanolic extracts for each test bacterium were determined at 12.5 and 25 mg/ml, respectively from the tubes that showed no evidence of growth (broth culture without turbidity) after incubation. The MBCs of the methanolic and ethanolic extracts obtained from the lowest concentrations of the broth tubes without turbidity following incubation and showed no growth after subculture on solid media correspond to 25 and 50 mg/ml, respectively for *E. coli*, *K. pneumoniae* and *P. mirabilis*. Despite the differences in extraction solvents, test organisms and other germane factors, the MIC and MBC results

obtained in this study are comparable to many earlier reports [13-15].

Table 5: MIC and MBC of *Moringa oleifera* leaf Extracts

Organisms	Methanolic extract (mg/ml)		Ethanolic extract (mg/ml)	
	MIC	**MBC**	**MIC**	**MBC**
E. coli	12.5	25	25	50
K. pneumoniae	12.5	25	25	50
P. mirabilis	12.5	25	25	50

Key: MIC= Minimum inhibitory concentration; MBC= Minimum bactericidal concentration

The exploration of alternative therapy for infectious diseases becomes necessary due largely to the emerging and re-emerging antibiotic-resistant organisms [9,16-18]. To solve the problems of antibiotic resistance, several plant materials have been investigated for their potential antimicrobial properties. In this study, *Moringa oleifera* leaf extracts were prepared from methanol and ethanol. The extracts have revealed the presence of some phytochemicals and established strong *in vitro* inhibitory effects against three confirmed ESβL-producing bacteria including *E. coli*, *K. pneumoniae* and *P. mirabilis*. The *K. pneumoniae* was found to be more sensitive to methanolic extract followed by *E. coli* and then *P. mirabilis* whereas *E. coli* was more sensitive to ethanolic extract followed by *K. pneumoniae* and then *P. mirabilis* as depicted in Tables 3 and 4. Moreover, it was generally observed that methanolic extract demonstrated more inhibitory effect against all the test bacteria compared to ethanolic extract. This could be attributed to higher polarity of methanol over the ethanol as the higher the polarity of an organic solvent the more its extractability of plant active compound which could be justified by the total extracts recovered following extraction as presented in Table 1. It is interesting that *E. coli* and *K. pneumoniae* were actively inhibited by the leaf extract of *M. oleifera*. These bacteria have been reported as the most prevalent ESβL-producing Gram negative organisms in both community and healthcare settings, causing serious treatment

failure globally [19-22].

The results of this study generally corroborate with previous works reported on the antimicrobial properties of *M. oleifera*. For instance, Othman and Ahmed, 2017 [9] evaluated the inhibitory effect of ethanolic extract of *M. oleifera* leaves against multidrug resistant Gram-positive and Gram-negative bacteria. These bacteria were confirmed to be sensitive to the extract within the MIC and MCB range values of 10-20 and 30-40 mg/ml, respectively. According to Sayeed *et al*., 2012 [10], methanolic extract of *M. oleifera* fruit showed broad-spectrum antibacterial and antifungal activity. Higher zones of growth inhibition were observed with *Pseudomonas aeruginosa* and *Colletotrichum* Sp at the concentration of 200 µg/disc but moderate activity was recorded with *Staphylococcus aureus, Bacillus subtilis, Vibrio cholerae, Bacillus cereus, Salmonella typhi, Shigella dysenteriae, Pseudomonas aeruginosa, Klebsiella species* and *Proteus species* at the same concentration. In contrast, Otter *et al*., 2013[23] investigated the *in vitro* antibacterial effect of aqueous and ethanolic Moringa leaf extracts against several Gram-positive and Gram-negative bacteria including *Escherichia coli, Staphylococcus aureus, Vibrio parahaemolyticus, Enterococcus faecalis, Pseudomonas aeruginosa, Salmonella enteritidis and Aeromonas caviae* using disk diffusion method after soaking the disks in 100, 200, 300 and 400 µL of the extracts. However, most of these bacteria were shown to be resistant except *S. aureus, V. parahaemolyticus*, *E. faecalis* and *A. caviae* which were found to be sensitive at 400 µL.

3. MATERIALS AND METHODS

3.1 Collection and authentication of *M. oleifera* leaves

The leaves of *M. oleifera* were obtained from the Botanical Garden of the Department of Biological Sciences, Gombe State University. The leaves were authenticated at the herbarium unit of the same department and obtained a voucher number, 083.

3.2 Preparation of leaf extracts of *M. oleifera*

Extracts of *M. oleifera* leaves were prepared using percolation method at room temperature as demonstrated by Yusha'u *et al*., 2008[24]. The leaves were dried in shade for about two (2) weeks and made into powder form using laboratory motor and pestle. About 40g of the

powder was dissolved in 400 mL of methanol or ethanol contained in a conical flask for the extraction. The mixture was kept for two (2) weeks with regular shaking on an orbital shaker followed by filtration using a Whatman filter paper after which the solvents were allowed to evaporate on a rotary evaporator at 40 ^{0}C.

3.3 Phytochemical analysis of *M. oleifera*

The *M. oleifera* leaf extracts were investigated for the existence of different phytochemicals by adopting the qualitative methods of Harborne, (1973) [25], demonstrated by Garba *et al*., 2019[22] as described below:

Test for flavonoid

About 1 mL of concentrated sodium hydroxide (NaOH) was mixed with 2 ml of the extracts. A few drops of diluted hydrochloric acid were then added to the mixture and check for flavonoids.

Test for alkaloid

Aliquot of Dragendroff"s reagent (2-4 drops) was mixed with 5 ml of each extract. Presence of alkaloids was confirmed following a colour change.

Test for steroid

Aliquot of 2-3 drops of chloroform were mixed with 2 ml of each extract. Some drops of concentrated sulphuric acid (H_2SO_4) were then added and observed.

Test for tannin

To 2 ml of each extract, 2-3 drops of ferric chloride were added and observed for the presence of tannin.

Test for reducing sugar

To 1ml of each extract, 2 ml of distilled water were added, shaken and mixed with fehling's solution. The mixture was kept at 40 ºC and observed for the presence of reducing sugar [26].

3.4 Preparation of extract concentrations

About 0.4 g of each extract was weighed and dissolved in 2 ml of dimethyl sulfoxide (DMSO) to arrive at a concentration of 200 mg/ml. Subsequently, three (3) more concentrations of 100, 50, and 25 mg/ml were derived using 2-fold serial dilution.

3.5 Extended Spectrum β-Lactamse producing bacteria

Isolates of ESβL-producing bacteria comprising of *Escherichia coli, Klebsiella pneumoniae* and *Proteus mirabilis* were obtained from Gombe State Specialist Hospital. The isolates were confirmed for ESβL-production using Double Disc Synergy (DDS) test with Ceftadizime (CAZ 30 µg, Oxoid England), Cefotaxime (CTX 30 µg, Oxoid England) and Augmentin (30µg, Oxoid England) discs reported previously [27] according Jarlia *et al*., 1988 [28].

3.6 Preparation of standard inocula

Inoculum of each isolate was standardized by emulsifying a loopful of its colonies from an overnight culture into 10 ml of sterile normal saline solutions in a test tube to match 0.5 McFarland turbidity standards [29].

3.7 Evaluation of *in vitro* inhibitory effect of leaf extracts of *M. oleifera*

All media preparations were done according to manufacturers' instructions. The standardized inocula of the test bacteria in test tubes were absorbed using sterile swab sticks. The sticks were pressed at the walls of the tubes to remove excess fluid and directly swabbed onto prepared Mueller Hinton Agar (MHA) plates. Wells of 6 mm in diameter were made in each plate using a sterile cork borer. A 100 µl of each extract concentration was placed into the wells using a micropipette. Augmentin (30µg, Oxoid England) discs were used alongside to serve as positive control. The plates were left to stand at room temperature for 30 minutes to enable the extracts to diffuse into the agar followed by incubation for 24 hours at 37 ºC. After the incubation, the inhibitory effects of the extracts were determined by measuring the diameter of growth inhibition zones in millimetre against the test bacterial isolates [22].

3.8 Determination of Minimum Inhibitory Concentration (MIC)

The Minimum inhibitory concentrations (MICs) of the extracts were determined from the lowest concentration that showed activity on the plate. This was performed by preparing four different concentrations, 12.50, 6.25, 3.13, and 1.56 mg/ml of each extract in 2 ml of nutrient broth contained in test tubes. The tubes were inoculated with the bacterial isolates and incubated for 24 hours at 37 ºC. Alongside each experiment, two control tubes were prepared by mixing the extracts and growth medium without the standardized inocula in test tubes (positive control) and the tube containing the growth medium and the inocula (organism

control).

3.9 Determination of Minimum Bactericidal Concentration (MBC)

The Minimum Bactericidal Concentration (MBC) was determined by sub-culturing all tubes that showed no visible bacterial growth from the MIC on fresh solid media and incubated for 24 hours at 37 ^{o}C.

4. CONCLUSION

The results of this study revealed the presence of different phytochemicals from both methanolic and ethanolic extracts. *In vitro* antibacterial evaluation of the extracts showed varying inhibitory effects against all the ESβL-producing bacteria tested. Moreover, the results support the traditional claim of *Moringa oleifera* leaves in the treatment of various ailments.

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