ORIGINAL RESEARCH PAPER | Medical Science | Volume : 6 | Issue : 8 | August 2016 | ISSN - 2249-555X | IF : 3.919 | IC Value : 74.50

Antibacterial Activity of *Moringa Oleifera* Lam Leaves on Enteric Human Pathogens

KEYWORDS: Antibacterial, enteric, pathogens, diarrhea, susceptibility

ISITUA, C. C.	IBEH, I. N.	OLAYINKA J. N.
Department of Biological Sciences, College of Sciences, Afe Babalola University, Ado Ekiti, P.M.B. 5454, Ekiti State, Nigeria	Department of Medical Laboratory Sciences, School of Basic Medical Sciences, College of Medicine, University of Benin, P.M.B. 1154, Benin City, Edo State, Nigeria.	Department of Pharmacology and Therapeutics, College of Medicine and Health Sciences, Afe Babalola University, Ado Ekiti, P.M.B. 5454, Ekiti State, Nigeria.

ABSTRACT *The rate at which bacteria are forming resistant to the commercial antibiotics meant for their prevention and cure is of great concern. In view of this, many research groups are now engaged in medicinal plant research because natural products of higher plants may give a new source of antimicrobial agents. This study was carried out to evaluate the antibacterial activity of extracts of Moringa oleifera leaves against some enteric human pathogens associated with diarrheal diseases. The susceptibility of the bacteria to M. oleifera leaf extracts was determined using agar diffusion and microbroth dilution methods with treatments arranged in a completely randomized design and replicated four times. The minimal inhibitory concentration of the leaf extracts against these bacteria were determined. The susceptibility test carried out on Staphylococcus aureus, Streptococcus pyogenes, Bacillus cereus, Escherichia coli, Pseudomonas aeruginosa, Shigella sonnei, Shigella dysenteriae, Shigella flexneri, Shigella boydii, and Proteus mirabilis showed susceptibility of these isolates to different concentrations of the plant. The ethanol extract showed a higher potency. The minimum inhibitory concentration (MIC) for aqueous extract was 64µg/ml for S. dysenteriae, while that of the ethanolic extract was 32µg/ml for S. aureus, S. dysenteriae and S. boydii. Out of the ten bacterial pathogen tested, S. dysenteriae showed highest susceptibility, while S. flexneri showed the least susceptibility. The plant extracts showed comparable zones of inhibition to the conventional antibiotic (Kanamycin) used. The results of the study showed that M. oleifera leaf extracts could inhibit diarrheagenic bacteria which suggests that its use could be effective against diarrhea of bacterial origin.*

INTRODUCTION

From the viewpoint of a bacteria looking for a place to colonize a human or animal, the digestive tract represents a very attractive environment. It provides shelter from the outside world, yet is easily accessible, provides nutrients in great number and variety, and is less likely to trigger an immune response. It is therefore not surprising that many bacteria live in the human guts, most of them harmlessly, with some even playing useful functions for their host. However, some species of bacteria can be highly pathogenic when they enter and colonize the human digestive tract. Such enteric bacterial pathogens typically cause gastroenteritis, but can also have life threatening consequences [1].

Many bacteria contaminating food and water can cause acute gastroenteritis or inflammation of the stomach and intestinal lining. When food is the source of the pathogen, the condition is often called food poisoning. Gastroenteritis can arise in two ways. The bacteria may actually produce a food-borne infection. That is, they may first colonize the gastrointestinal tract and grow within it, then either invade host tissues or secrete exotoxins [2]. Alternatively, the pathogen may secrete an exotoxin that contaminates the food and is then ingested by the host. This is sometimes referred to as food intoxication because the toxin is ingested and the presence of living bacteria is not required. Because these toxins disrupt the functioning of the intestinal mucosa they are called enterotoxins. Common symptoms of enterotoxin poisoning are nausea, vomiting and diarrhea [3].

Worldwide, diarrheal diseases are second only to the respiratory diseases as a cause of adult death; they are the leading cause of childhood death and malnutrition, and in some parts of the world they are responsible for more years of potential life lost than all other causes combined. For example, each year around 5 million children (more than 13,600 a day) die from diarrheal diseases in Asia, Africa, and South America. In the United States estimates exceed 10,000 deaths per year from diarrhea, and an average of 500 childhood deaths are reported. Bacteria associated with diarrheal diseases include; *Staphylococcus aureus, Bacillus cereus, Clostridium botulinum, Escherichia coli*, *Shigella* spp., *Salmonella* spp. amongst others [4].

Diarrhea may be defined as an increase in fluidity and frequency of stools which consequently results in fluid and electrolyte loss, and may be accompanied by griping pain. Diarrheal disease may be classified into simple and secondary types. The former includes; most common diarrhea caused by viruses which are self-limiting and lasting 2 to 3 days, diarrhea occurring in travelers visiting warm climates and poor sanitary countries, food poisoning due to dietary insult or over indulgence, while the latter includes those caused by; various gastrointestinal disorders (such as malabsorption syndrome, inflammatory bowel disease, carcinoma of the bowel, etc.), metabolic disorders (such as diabetes mellitus and thyrotoxicosis), radiotherapy, drugs, osmotic effects especially due to lactose intolerance in infants, and infections (such as respiratory tract infection and otitis in babies) [5] [6]. The World Health Organization [7] recognized three important forms of diarrhea: Acute watery diarrhea (stools are soft or liquid, but are not bloody), dysentery (stools contain blood and mucus) and persistent diarrhea (diarrhea begins acutely but lasts more than 21 days). Another clas-

Volume : 6 | Issue : 8 | August 2016 | ISSN - 2249-555X | IF : 3.919 | IC Value : 74.50

sification of diarrhea is as given by Lewis and Elvin [8]. They recognized that diarrhea could be chronic or acute. According to this classification, chronic diarrhea results from chronic intestinal infections, immunological and malabsorption syndrome while acute diarrhea results from bacterial and viral enteritis, food and toxin poisoning and gastrointestinal allergy.

All diarrheas lead to dehydration, and if untreated, progressive dehydration is fatal. It has been known for decades that the replacement of salt and fluid losses in sufficient quantity can prevent diarrheal deaths. The first important approach in the management of diarrhea is therefore rehydration to replace lost water and electrolytes by oral therapy. There are a number of formulas available today for oral rehydration solutions with varying concentrations of different salts [6]. Experiments have shown that some traditional remedies like carrot soup, coconut-water and rice-water are also effective means of oral rehydration. Starch is a polymer of glucose and studies show that it may be ideal for diarrhea treatment. When starch-based foods are given in combination with supplemental feeding the fluid is absorbed faster, the stools are less, the diarrhea stops sooner and the child may gain weight [9].

Despite the usefulness of oral rehydration therapy (ORT) in the management of diarrhea, it is important to emphasize that the solution is given to prevent dehydration, but does not actually stop the diarrhea [10]. ORT is very useful in early diarrhea and may become the only necessary treatment if the disease is due to non-pathogenic origin and self-limiting. Diarrhea of pathogenic origin must be treated with anti-infective agents; thus, antibiotics are used when there is clear clinical suggestions of invasive diarrhea (bloody stools and high fever) or cholera (in cholera endemic area), or when laboratory results become available and indicate the need for antibiotic treatment [4] [11] [12].

Diarrheal diseases caused by enteric pathogens have increased to a great extent and resistance against antibiotics becomes an ever increasing therapeutic problem [13]. Also, these antibiotics are not only expensive, but they are inaccessible and unaffordable by the rural poor. Due to this fact, many research groups are now engaged in medicinal plants research because natural products of higher plants may give a new source of antimicrobial agents [14]. Man has realized the medicinal properties and probably toxic effects of plants around him as far back as 3000 years B. C. [15] [16]. In the absence of modern medicinal remedies people relied on herbal remedies derived from herbs and spices. There are many medicinal herbs and spices, which find place in day-to-day uses, many of these are used as herbal remedies. Several plant parts either used singly or in combination with others have been utilized effectively for the treatment of virulent ailments such as arthritis, asthma, cancer, cholera, diabetes, hernia, hypertension, hemorrhoids, edema and typhoid fever [17] [18].

Traditional healers have over the years depended upon plants for the treatment of diarrheas. Most traditional healers in Nigeria employ the aqueous infusion or decoction of the various plant materials. The plant parts may be prepared alone or in combination with other parts of the same plant or with other different plants. The active constituents of the plants employed in the management of diarrhea are known to belong to a wide variety of classes ranging from alkaloids, tannins, saponins, glycosides, flavonoids amongst others [19].

This study was carried out to evaluate *Moringa oleifera* Lam. plant as one of such instruments that can function as an intervention tool in the myriad of diarrheagenic diseases that beset mankind. *M. oleifera* locally known in Nigeria as 'ewe-ile' or 'ewe-igbale' (Yoruba), 'zogalla-gandi' (Hausa), 'gawara' (Fulani), 'ikwe oyibo' (Ibo), 'isie'; 'ekie' or 'asie' (Bini) and 'drumstick' tree or 'horse-radish' tree (English) belongs to the family *Moringaceae* and is widely distributed in the Indo-Bangla subcontinent and cultivated throughout the tropical belt [20]. Different parts of this plant are used in the indigenous systems of medicine for the treatment of a variety of human ailments and are also eaten as vegetable [19]. The present study therefore aims at evaluating the antibacterial activity of *M. oleifera* leaves against clinical strains of certain microorganisms which are implicated in dysentery and diarrheas with a view to ascertaining whether any scientific basis exists for the use of this plant in antidiarrheal medicinal preparations prepared by some traditional medicine practitioners.

MATERIALS AND METHODS

Source and preparation of plant material

The plant materials used for this study are the matured leaves of *Moringa oleifera* Lam. obtained from an orchard within the premises of Abia State University, Uturu, Nigeria. The leaves were removed from the branches, washed properly with sterile distilled water and then air-dried. Upon drying, the leaves were macerated in a sterile grinder into fine powdery form to produce the medicinal preparation. The pulverized leaves of the plant (*M. oleifera*) which constituted the medicinal preparations were obtained from the National Academy for the Advancement of Science, Benin City, Nigeria. The powdered products (NAAS/09/01) packaged in sachets and bottles under high aseptic and hygienic conditions are sold commercially to people for medicinal and nutritional purposes.

Extraction of plant material

The powdered *M. oleifera* leaf was subjected to aqueous and ethanol extraction using the protocol of Fatope and Hamisu [21]. Briefly, eight (8) batches of fifty grams (50 g) each of the powdered plant materials were weighed and percolated with 500 ml and the ninth batch of twenty grams was weighed and percolated with 200 ml of 95 % ethanol in separate erlenmeyer flasks and allowed to stand for two weeks with intermittent shaking. These were filtered and concentrated using rotavapour machine at 4-10 C. They were combined, air-dried and labelled **EE** (ethanol extract). Fifty grams (50 g) of the plant material was macerated in water, heated to 50 C for eight hours and the extract recovered by percolation. The extract was concentrated *in vacuo* at 40 C to yield the aqueous extract **AE.** All extracts were kept refrigerated before use.

Test bacteria

The organisms used were clinical isolates of *Staphylococcus aureus*, *Streptococcus pyogenes, Bacillus cereus* (Gram positive), *Escherichia coli, Pseudomonas aeruginosa, Shigella dysenteriae, Shigella boydii, Shigella sonnei, Shigella flexneri and Proteus mirabilis* (Gram negative) obtained from diarrheal patients in the University of Benin Teaching Hospital, Benin City, Nigeria. These isolates were maintained on nutrient agar slants in the refrigerator prior to use.

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Standardization of inoculum
A loopful of the test isolates were picked using a sterile wire loop and emulsified in 3-4 ml of sterile physiological saline followed by proper shaking. The turbidity of the suspension was matched with that of 0.5 McFarland standards for sensitivity test as described by NCCLS [22]. The McFarland standard was prepared by mixing 0.6 ml of 1 % (w/v) dihydrate barium chloride solution with 99.4 ml of 1 % (v/v) sulphuric acid solution.

Antibacterial assay
In vitro antimicrobial screening is generally performed by disc diffusion method [23] for primary selection of the compounds as therapeutic agent. Disc diffusion method is highly effective for rapidly growing microorganisms and the activities of the test drugs are expressed by measuring the diameter of the zone of inhibition.

Sensitivity discs of 6mm diameter were punched out from Whatmann No.1 filter paper, sterilized in Bijou bottles by autoclaving at 121 C for 15 minutes. Sensitivity discs were prepared by weighing the appropriate amount of the extract and serial doubling dilution in Dimethyl-sulfoxide (DMSO) followed by placing the improvised paper discs in the solution such that each disc took up 0.01 ml to make the required disc potency. Disc potencies of 30 µg/disc and 100 µg/disc were prepared for antibacterial activity [24].

Standardized inocula of each isolates were swabbed onto the surface of Mueller-Hinton Agar in separate petri dishes. This was followed by placing the prepared discs of the extracts and standard antibiotic discs (Kanamycin 30 µg/disc) which served as control onto the surface of the inoculated media at intervals. The plates were inverted and allowed to stand for 30 minutes for the extract to diffuse into the agar after which the plates were incubated at 37 C for 18 hours. This was followed by measurement of zones of inhibition formed by the test organisms around each of the extract and standard antibiotic discs [25].

Micro-broth dilution test for minimum inhibitory concentration (MIC)
Minimum inhibitory concentration of the plant extracts were prepared by serial doubling dilutions using sterile distilled water to obtain concentrations of 16 µg/ml, 32 µg/ml, 64 µg/ml and 128 µg/ml. Equal volume of extracts and Mueller-Hinton broth (i.e. 2 ml each) were dispensed into sterilized test tubes. Specifically, 0.1 ml of standardized inocula (5.3 x10^5 CFU/ml bacteria) was added to each of the test tubes above and the tubes incubated at 37 C for 24 hours [26]. Tubes containing broth without plant extracts were inoculated and incubated alongside to serve as positive control. Uninoculated tubes containing broth and plant extracts were incubated alongside to serve as negative control. The tubes were observed after incubation to determine the minimum inhibitory concentration (MIC) as the lowest concentration that showed no evidence of growth (turbidity) or the least concentration without turbidity [25].

Determination of minimum bactericidal concentration (MBC)
The first concentration that records less than 50 colonies after subculture is considered the MBC. Mueller –Hinton agar plates were separately inoculated with sample (1 ml) from each of the test tubes that showed no turbidity and the plates were incubated at 37 C for 24 - 48 hours to determine the minimum bactericidal concentration (MBC) as the dilution from which there occurs no growth [26].

RESULTS AND DISCUSSION
The antibacterial activities of aqueous and ethanol extracts of *M. oleifera* Lam. leaf was investigated using the agar disc diffusion method against the enteric human pathogens Staphylococcus aureus, Streptococcus pyogenes, Bacillus cereus, Escherichia coli, Pseudomonas aeruginosa, Shigella dysenteriae, Shigella boydii, Shigella sonnei, Shigella flexneri and Proteus mirabilis.
The antibiogram of microorganisms exposed to extracts of *M. oleifera* leaf is shown in Table 1. All the bacterial isolates showed susceptibility to the aqueous and ethanol extract at 100 µg concentration while 06 (60%) were susceptible at the lowest concentration (30 µg) of the ethanol extract used and 04 (40%) were susceptible at the lowest concentration (30 µg) of the aqueous extract used.

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of the ethanol and aqueous extracts of M. *oleifera* leaf are shown in Tables 2 and 3. *Shigella dysenteriae, Shigella boydii* and *Staphylococcus aureus* had MIC of 32 µg / ml, while *Bacillus cereus* had MIC of 64 µg / ml for ethanol extract. *S. dysenteriae* had MIC of 64 µg / ml for aqueous extract while *B. cereus* had MIC of 128 µg / ml of the aqueous extract.

The antibiogram of microorganisms exposed to extracts of *M. oleifera* showed susceptibility of all the bacterial strains to both the ethanol and water extracts of the leaf. *Shigella dysenteriae* showed highest susceptibility because it gave the widest zone of inhibition, while *S. flexneri* gave the least susceptibility. This finding suggests that the leaf contains active principles which qualify it for medicinal use. The results obtained in this study thus provide a rationale for the use of this leaf by Asian and African native folks. In the preliminary survey, it was found that some doctors use *M. oleifera* to treat a variety of ailments ranging from all forms of microbial infections to diabetes in West Africa and high blood pressure in India [27]. If the organisms implicated in these conditions are susceptible to the antimicrobial agents in *M. oleifera*, then its application is justified.

The susceptibility of the bacterial strains to the extract *M. oleifera* may be a pointer to its potential as a drug that could be used against these susceptible bacterial strains. Furthermore, antibacterial resistance, especially, among Gram negative bacteria is an important issue that has created problems in the treatment of infectious diseases and necessitates the search for alternative drugs or natural antibacterial remedies [28]. The difference in bacterial response was possible due to the nature of the bacterial species. It is noted that the ethanol extract of *M. oleifera* leaf exhibited antimicrobial effect against both Gram positive and negative bacteria (broad spectrum activities).

The medium of extraction of *M. oleifera* leaf seem to impact on the potency of the active principles since the ethanol extract produced wider zones of inhibition and higher minimal inhibitory concentrations (Tables 1, 2 and 3). The possible reasons for the differences in the potency of leaf extracts include higher extractability of the active principle in ethanol than in water, which in turn may affect its concentration per unit volume of extract. The other factor is the synergistic effect between ethanol and the active principles of the leaf. Whatever may be the

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case, this result suggests that ethanol extraction produces better antibacterial effect of the *M. oleifera* extract [29]. Compounds like tannins and polyphenol which are found in *M. oleifera* are soluble in ethanol [30] and have been reported to possess anti-bacterial activity [28].

The performance of ethanol extract of *M. oleifera* against some highly susceptible bacterial isolates (*Staphylococcus aureus*, *Bacillus cereus*, *Shigella dysenteriae* and *Shigella boydii*) did not show significant difference when compared with established commercial antibiotic disc Kanamycin (Table 1). Noteworthy is the ability of the *M. oleifera* ethanol extract to completely inhibit the growth of *S. dysenteriae* in comparison to other bacterial strains. This suggests that *S. dysenteriae* was more sensitive to the *M. oleifera* ethanol extract and could be used as an antibiotic against diseases that are caused by *S. dysenteriae* (especially diarrhea). This result suggests the need for further studies on *M. oleifera*, although the spectrum of activity of the leaf extracts were narrower than those of Kanamycin. The plant extract may be cheaper and more readily available to the local community than the imported antimicrobial agents.

The activity of the aqueous extract against microbes investigated in this study is at variance with previous works which showed that aqueous extracts of plants generally exhibited little or no antimicrobial activities [31] [32]. The difference could be attributed to variation in the environment where the plant was collected, the season and the physiological stage of the plant when leaves were harvested [33]. This affects the chemical composition and the amount of compounds in the plant. In general, water extracts are the commonly used and are affordable to resource-limited farmers. The curative advantage is that consumers including animals tend to consume the plant material in large quantities and in high concentrations. This suggests its ability to meet the required physiological levels to inhibit the pathogen growth *in situ*. Yang *et al*. [34] reported that the inclusion of *M._oleifera* leaf meal in broiler feeds reduced the *E. coli* bacterial count in the ileum. In addition, *M. oleifera* leaf water extracts exhibited antimicrobial properties through the inhibition of the growth of *S. aureus* strains isolated from food and animal intestines. This points to the potential of *M. oleifera* as antimicrobial peptides to replace antibiotics in feeds. These findings, however, is in accordance with the study by Dahot [35] who reported that *M. oleifera* water extract had antimicrobial activity against *E. coli*, *S. aureus* and *B. subtilis.*

In this study, the *M. oleifera* ethanol and aqueous extracts had bacteriostatic and bactericidal properties against all the Gram negative organisms, which are mostly known to be multi-drug resistant [36]. The ability of the ethanol extract to inhibit and kill these organisms is noteworthy even though it was at the highest concentration (100 g) tested. Moreover, Gram negative bacteria have been reported to be resistant to antibiotics [37]. According to several authors, these bacteria are generally less sensitive to the activity of plant extracts [38] [39] [37]. Such resistance could be due to the permeability barrier provided by the cell wall or to the membrane accumulation mechanism [40]. The bactericidal and bacteriostatic activities of the *M. oleifera* extracts against these diarrheagenic organisms were established. This plant therefore, may well enrich the pool of herbs and shrubs from which modern pharmaceutical industries may rely on for raw materials.

Table 1: *In vitro* **antibacterial activity of ethanol and aqueous extracts of** *Moringa oleifera* **leaf**

Bacterial strains	Ethanol extract (30µg)	Ethanol extract (100µg)	Aqueous extract (30µg)	Aqueous extract (100µg)	Kanamycin (30µg)
	mm	mm	mm	mm	mm
(Gram positive)					
*Staphylococcus aureus**	16	18	11	15	20
Streptococcus pyogenes	00	11	00	11	20
*Bacillus cereus**	14	16	10	12	18
(Gram negative)					
Escherichia coli	00	12	00	11	21
Pseudomonas aeruginosa	11	14	00	13	25
Shigella sonnei	00	11	00	11	19
*Shigella dysenteriae**	19	20	12	16	20
Shigella flexneri	00	10	00	10	20
*Shigella boydii**	17	18	13	16	20
Proteus mirabilis	10	13	00	12	17

Mean zone of inhibition (diameter in mm); * = Highly susceptible bacteria.

Table 2: Minimum inhibitory concentrations (MIC) of *M. oleifera* **extracts based on turbidity**

	Ethanol Extract										Aqueous Extract									
	SA	SP	BC	EC	PA	SS	SD	SF	SB	PM	SA	SP	BC	EC	PA	SS	SD	SF	SB	PM
CONC. (µg ml^{-1})																				
16	+	++	+	++	+	++	+	++	+	++	++	++	++	++	++	++	+	++	+	++
32	-	++	+	+	+	++	-	++	-	+	+	++	+	++	++	++	+	++	+	++
64	-	+	-	+	+	+	-	+	-	+	+	+	+	+	+	+	-	+	+	+
128	-	+	-	+	+	+	-	+	-	+	+	+	-	+	+	+	-	+	+	+

Key: SA = *Staphylococcus aureus*, SP = *Streptococcus pyogenes*, BC = *Bacillus cereus*, EC = *Escherichia coli*, PA = *Pseudomonas aeruginosa*, SS = *Shigella sonnei*, SD = *Shigella dysenteriae*, SF = *Shigella flexneri*, SB = *Shigella boydii*, PM = *Proteus mirabilis*; CONC. = Concentration, ++ = More turbid, + = Less turbid, - = No growth.

Table 3: MIC and MBC values of the ethanol and aqueous extracts of *M. oleifera* **leaf against test organisms using microbroth dilution technique**

Extract	*Shigella dysenteriae*		*Shigella boydii*		*Bacillus cereus*		*Staphylococcus aureus*	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Ethanol extract (µg ml^{-1})	32	**	32	**	64	**	32	**
Aqueous extract (µg ml^{-1})	64	**	**	**	128	**	**	**

Key: MIC = Minimum inhibitory concentration, MBC = Minimum bactericidal concentration, ** = MIC or MBC value above 128 µg ml^{-1}

CONCLUSION

This study was conducted to demonstrate the antibacterial activity of *M. oleifera* leaves which could potentially serve to treat diarrheal diseases of bacterial infection that have become more resistant to most of the common antibiotics used for treatment. The growing incidence of resistance of microorganisms to conventional antimicrobial agents are a source of concern globally. Although the conventional antibiotic (Kanamycin) used in this study showed more effect against the pathogens in terms of inhibition zones; the plant extracts, especially the ethanol extract showed comparable zones of inhibition too. Thus, the use of this plant will be of great potential in terms of cost, affordability, availability, accessibility and health effects associated with

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the synthetic drug. The effectiveness may be improved upon, for instance, by testing different extraction methods to increase the concentration of the active chemical components. The ability of *M. oleifera* leaf extracts to inhibit diarrheagenic bacteria in this study suggests that it could be an effective agent against diarrhea of bacterial origin.

Acknowledgements
The authors wish to acknowledge the research support from DTSG Ministry of Higher Education, Delta State of Nigeria to carry out this research.

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