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# SCREENING OF MULTIDRUG-RESISTANT BACTERIA OF ANTIBIOTIC-ASSOCIATED DIARRHEA AND THEIR INHIBITION BY LEAF EXTRACTS OF *MORINGA OLEIFERA* LAMK

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## Abstract

Context: Emergence of multi drug resistance bacteria (MDRB) to human pathogenic infection is increasing day by day but the number of new drugs to overwhelm the problem is not sufficient. Evidences revealed that *Moringa oleifera* Lamk. has various pharmaceutical activities like antibacterial, antifungal, antispasmodic, anti-inflammatory, anticancer and diuretic. Herbal treatment may be one of the possible ways to treat diseases caused by multidrug-resistant bacteria.

Objectives: The present research was undertaken to screen of multidrug resistant bacteria (MDRB) from antibiotic-associated diarrheal samples and to evaluate the potentiality of *M. oleifera* leaf extracts on these bacteria with the view to provide scientific evidence for its application in health remedy.

Materials & Methods: Antibiotic-associated diarrheal fecal specimens were collected from pediatric ward of Rajshahi Medical College and cultured onto MacConkey agar. MDRB were determined by antibiotic susceptibility test, using disc-agar diffusion method. Biochemical tests of the MDRB were done according to Bergey's Manual of Determinative Bacteriology for identification of the species. Dried and fresh leaf of *M. oleifera* was used to prepare exaction with or without solvents such as hot water, cold water, chloroform, petroleum ether, acetone and ethanol, separately. Antibacterial assay was done by disc diffusion method and minimum inhibitory concentration of the extracts was also measured.

Results: In the present study seven isolates were screened as MDRB and the highest prevalence (42.86%) was occurred in the age group of 25-36 months and the lowest (14.28) was in the group of <1 and 1-6 months. Ethanol extract of dried leaf of *M. oleifera* Lamk. showed moderate inhibitory activity against all of the isolates while petroleum ether, chloroform and acetone extracts of dried leaf have no inhibitory effect. Fresh leaf sap powder in DMSO exhibited strong inhibitory effect against all of the test bacteria where as hot aqueous extract could not show any inhibition. The minimum inhibitory concentrations (MIC) of the potent extracts ranged 937.5 to 3750 μg/ml and 7.9 to 234.4 μg/ml in dried and fresh leaf extracts, respectively.

Conclusion: The present data indicates that *M. oleifera* leaf extract possess antimicrobial potential to control of MDRB causes infection thus it can be used as a novel drugs in future.

Key words: Antibiotic-associated diarrhea (AAD), Antibiotic susceptibility, Multidrug-resistant bacteria, *Moringa oleifera* Lamk., Minimum inhibitory concentrations (MIC).

## Introduction

Antibiotic-associated diarrhea (AAD) occurs in about 5-30% of patients either early antibiotic therapy or up to two months after the end of the treatment (Bartlett *et al.* 1978, McFarland 1998, Wiström *et al*. 2001). World Health Organization (WHO) defines antibiotic-associated diarrhea as 3 or more abnormally loose bowel movements in a 24-hr period (Tankanow *et al*. 1990, D'Souza *et al*. 2002). The disruption of the normal enteric flora caused by antibiotics may lead to overgrowth of pathogens and functional disturbances of the intestinal carbohydrate and bile acid metabolism, resulting in osmotic diarrhea (Hogenauer *et al*. 1998). The

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severity of antibiotic-associated diarrhea may range from a brief, self-limiting disease to devastating diarrhea with electrolyte disturbances, dehydration, crampy abdominal pain, pseudomembranous colitis, toxic megacolon, or even death (Bartlett 1992).

Repeated and improper uses of antibiotics are primary causes of the increase in drug-resistant bacteria. The most common culprit broad-spectrum antibiotics which include ampicillin, clindamycin, cephalosporin, sometimes erythromycins (Erythrocin), fluoroquinolones (Ciprofloxin) and tetracycline also can cause antibiotic-associated diarrhea (Antibiotic-associated diarrhea 2009). Bacteria gain antibiotic resistance genes through plasmid, transposones and integrons which result in mutations in genes responsible for antibiotic uptake. If a bacterium carries several resistance genes, it is termed as multi-resistant or super bug (Antibiotic resistance 2008) that is more dangerous and problematic to people. In the present scenario of emergence of multidrug resistance to human pathogenic infections including AAD, it has become very necessary to search for new antimicrobial substances from other sources such as plants. Herbal treatment is one possible way to treat diseases caused by multidrug-resistant bacteria (Olukoya *et al*. 1993).

The present research reports the first attempt to study the antimicrobial activity of *Moringa oleifera* Lamk. against multidrug resistant bacteria (MDRB) of AAD. Several evidences revealed that *M. oleifera* has various pharmaceutical activities such as antibacterial (Eilert *et al*. 1981, Dayrit *et al.* 1990), antifungal, antispasmodic, anti- inflammatory, anticancer and diuretic activities (Caceres *et al*. 1992). Its leaves contain 2-nitrile glycosides, niazirin and niazirinin, and 3-mustard oil glycosides, isothiocyanate, niaziminin A and B, which are reported to be responsible for hypotensive activity (Guevara *et al.* 1999) Therefore, the present attempt has been under taken to screen of MDRB from fecal specimens of AAD-patients and evaluate the potentiality of *M. oleifera* leaf extracts on these bacteria with the view to provide scientific evidence for its application in health remedy.

## Materials and Methods

*Subjects and Isolation of AAD-bacteria*: A total 50 antibiotic-associated diarrheal fecal specimens were collected in sterile screw-capped tube from pediatric ward of Rajshahi Medical College Hospital, Rajshahi, Bangladesh, during March to April, 2007. Those specimens were obtained from diarrhea affected children (1-36 months) during the first two weeks after the starting of the antibiotic treatment, because this period most likely reflects the effect of antibiotic use. The fecal specimens were then cultured following Holt *et al*. (1994) by plated onto MacConkey agar (0.001mg/ml) at 37ºC for about 24 h. After the growth of bacteria, the pink color colonies were screened as lactose fermenter.

*Antibiotic susceptibility and biochemical tests:* MDRB were determined by antibiotic susceptibility test, using the disc-agar diffusion method, as recommended by the Clinical and Laboratory Standards Institute (CLSI 2004). Commercial antimicrobial discs (Oxoid), used in this experiment were ampicillin (10 μg), amoxicillin (30 μg), cephalosporin (30 μg), erythromycin (30 μg), ciprofloxacin (5 μg) and tetracycline (30 μg). Hundred μl of 18 h old culture of standardized inoculums of each tested bacteria was spread onto nutrient agar (Difco Laboratories) plate and left for 30 min. Commercial antibiotic discs with standard concentration were carefully placed on the test organism-seeded plates and incubated at 37°C temperature for 16 h. Antibiotic susceptibility was evaluated by the diameter of inhibition zone (mm) compared with the standard manual. Biochemical tests of the MDRB were done according to Bergey's Manual of Determinative Bacteriology (Holt *et al.* 1994) using their respective standard strain for confirmation of the species. Gram staining, indole, Kligler's iron agar (KIA), methyl red (MR), Voges-Proskauer (VP), urease, citrate utilization and catalase tests were done.

*Preparation of plant extracts*: Fresh mature leaves of *M. oleifera* were collected from Rajshahi University Campus, Rajshahi, Bangladesh, during June, 2007 and authenticated by the plant taxonomist at the Department of Botany, University of Rajshahi, Bangladesh. The leaves were cleaned and dried in shade at room temperature (32-35°C) for five days (Doughari 2006) and reduced to powder with a grinder. Dried plant powder (30 g) was mixed with 300 ml of petroleum ether, chloroform, ethanol and acetone separately in a conical flask and kept on an orbital shaker for 24 h. The crude extracts were filtered, concentrated in a rotary evaporator and allowed to dry at room temperature and used in the experiment. Fresh leaves (20 g) was crushed directly without adding any solvent and leaf sap obtained was filtered and used for antibacterial activity test. Fresh leaves (100 g) was crushed directly without adding any solvent and dried at room temperature and 1.3 g dried material was then powdered and dissolved in 10 ml of dimethyl sulfoxide (DMSO). Again 20 g fresh leaves was crushed adding 50 ml of cold water, hot water, chloroform, petroleum ether, acetone and ethanol, separately. Then the leaf sap was filtered and applied for antibacterial activity test.

*Antibacterial assay and minimum inhibitory concentration (MIC)*: Antibacterial potentiality of crude extracts of *M. oleifera* leaves was evaluated by the paper disc diffusion method (Aida *et al*. 2001). Sterile filter paper discs (6 mm), previously impregnated with 10 µl of various known concentrations of extracts were carefully placed on the test organism-seeded plates and incubated at 37°C temperature for about 16 h. For each extract three replicate trials were conducted against the test organism to confirm the reproducible results. Sterile blank paper discs were impregnated with same amount of solvent separately and used as negative control and tetracycline (100 µg/ml) contained paper disc was used as positive control.

Serial dilutions of the crude extracts were made with different ranges for dried leaf extract (30-0.05 mg/ml), fresh leaf sap (29.375 -0.014 mg/ml), fresh leaf sap powder dissolved in DMSO (32.5-0.004 mg/ml), fresh leaf solvent extracts (30-0.004 mg/ml) and tetracycline (100-12.5 µg/ml). 0.5 ml of each concentration of the extract was added to 2 ml of nutrient broth and then 100 µl of standardized inoculum ($10^6$ cfu/ml) was introduced to each tube and incubated aerobically at 37°C for 24 h. Uninoculated tubes containing growth medium and extract were used as controls. The lowest concentrations of the extracts that produced no visible bacterial growth (no turbidity) compared to control was regarded as MIC.

## Results

### Enumeration and isolation of AAD-bacteria

Total numbers of colonies of different AAD-bacteria were enumerated from each sample. The highest number of colony was recorded 66 ± 0.7 cfu/plate in the age group of 25-36 months. But the lowest number of colony was recorded 21.5 ± 1.2 cfu/plate in the age group of 7-12 months (Fig.1). Initially, 25 isolates were isolated out of 50 samples screened on MacConkey agar medium. These isolates were subjected to test of antibiotic susceptibility for the determination of multidrug-resistant bacteria.

Screening of multi drug-resistant bacteria

### Antibiotic susceptibility

Only 7 out of 25 isolates were found resistant to multiple antibiotics. Among the isolates, S/11 isolate was resistant against only two antibiotics ($Ery^R$ and $Tet^R$) and other 6 isolates were resistant to three or more antibiotics. Almost all of the isolates were sensitive to ciprofloxacin and cephalosporins without S/24 and S/7 isolates (table 1). The highest resistance was observed in tetracycline (100%) followed by ampicillin (85.7%), amoxicillin (57.1 %), erythromycin (71.4 %) and ciprofloxacin (42.8 %) and the lowest was observed in cephalosporins (28.6 %) as shown in Fig 2.

The highest rate of isolation of multidrug-resistant bacteria was found 42.86% in the age group of 25-36 months and the lowest rate was found 14.28% in the age group of <1 and 1-6 months (Fig 1).

Table 1. Bacterial isolates and antibiotic resistance profiles

| Isolate | Antibiotic resistance |
|---|---|
| S/11 | Ery, Tet |
| S S/13 | Amp, Amo, Ery, Tet |
| S/9 | Amp, Ery, Tet, Cip |
| S/24 | Amp, Amo, Cep, Tet, Cip |
| S/3 | Amp, Amo, Ery, Tet |
| S/7 | Amp, Amo, Cep, Tet, Cip |
| S/19 | Amp, Ery, Tet |

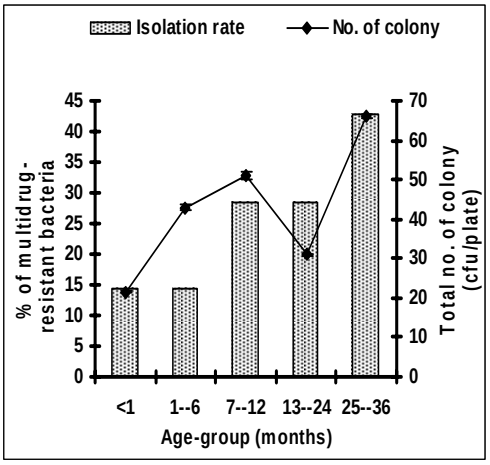


Fig 1. Total number of colony (cfu/plate) and isolation rate of multidrug-resistant bacteria in relation to age distribution of antibiotic-associated diarrheic pediatric patients

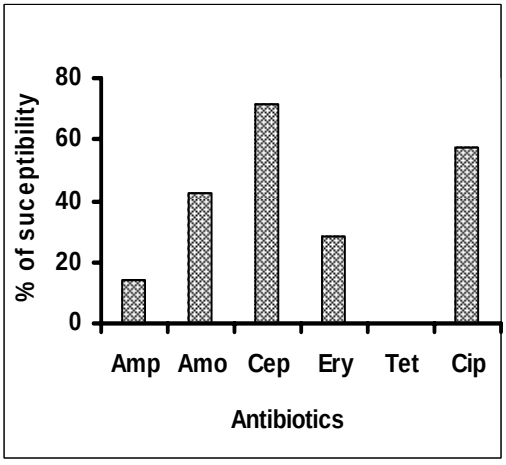


Fig 2. Antibiotic susceptibility of isolated bacteria.
Amp = Ampicillin; Amo = Amoxicillin;
Cep = Cephalosporins; Ery = Erythromycin;
Tet = Tetracycline; Cip = Ciprofloxacin

## Identification of multi drug-resistant bacteria

For identification of multidrug-resistant bacteria different biochemical tests were carried out and the results are summarized in Table 2. On the basis of growth on MacConkey agar, morphological and biochemical test, the selected multidrug-resistant bacteria were identified as *Escherichia coli*, *Shigella* sp., *Salmonella* sp., *Citrobacter* sp., *Klebsiella* sp., *Enterobacter* sp. and *Proteus* sp. In the patients with antibiotic-associated diarrhea *Escherichia coli, Salmonella* sp. and *Klebsiella* sp. observed by Hovius and Rietra (1982), Hogenauer *et al.* (1998) and Bartlett (2002).

Table 2. Morphological and biochemical characteristics of selected isolates

| Strain code | Morphological | | Biochemical | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Colony | Cell shape | Gram stain | Motility test | Growth on MacConkey agar | Indole test | KIA test | | | MR-VP test | | | UT | CT | CIT | Identified as |
| | | | | | | | Slant | Butt | $H_2S$ | Gas | MR | VP | | | | |
| S/11 | Smooth Pink | Rod | - | + | + | + | Y | Y | - | + | + | - | - | + | - | *Escherichia coli* |
| S/13 | Pale colored with 1-2mm dia. | Rod | - | - | + | - | R | Y | - | - | + | - | - | + | - | *Shigella* sp. |
| S/9 | Pale colored | Rod | - | + | + | - | R | Y | - | + | + | - | - | + | - | *Salmonella* sp. |
| S/24 | Pale colored | Rod | - | + | + | - | R | Y | - | + | + | - | - | + | + | *Citrobacter* sp. |
| S/3 | Mucoid pink | Rod | - | - | + | - | Y | Y | - | + | - | + | + | + | + | *Klebsiella* sp. |
| S/7 | Mucoid | Rod | - | + | + | - | Y | Y | - | + | - | + | - | + | + | *Enterobacter* sp. |
| S/19 | Pale colored | Rod | - | + | + | + | R | Y | + | - | + | - | + | + | - | *Proteus* sp. |

UT = Urease test; CT = Catalase test; CIT = Citrate test; - = Negative; + = Positive; Y = Yellow; R = Red

Table 3. Minimum inhibitory concentrations of different extracts of *M. oleifera* leaf against multidrug-resistant bacteria of antibiotic associated diarrhea.

| Species | Minimum inhibitory concentrations (MIC) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | DLEE | FLS | FLSP | FLCAE | FLCE | FLPE | FLAE | FLEE | TET |
| *E. coli* | 937.5 | 56 | 7.9 | 117.2 | 29.3 | 58.6 | 29.3 | 29.3 | 100 |
| *Shigella* sp. | 1875 | 14 | 15.9 | 117.2 | 117.2 | 58.6 | 29.3 | 29.3 | 100 |
| *Salmonella* sp. | 3750 | 28 | 15.9 | 234.4 | 29.3 | 29.3 | 14.6 | 14.6 | 50 |
| *Citrobacter sp.* | 3750 | 14 | 31.7 | 234.4 | 29.3 | 29.3 | 14.6 | 29.3 | 100 |
| *Klebsiella sp.* | 1875 | 28 | 63.5 | 234.4 | 117.2 | 58.6 | 29.3 | 29.3 | 100 |
| *Enterobacter* sp. | 1875 | 28 | 31.7 | 117.2 | 117.2 | 58.6 | 29.3 | 29.3 | 100 |
| *Proteus* sp. | 3750 | 14 | 63.5 | 117.2 | 117.2 | 117.2 | 117.2 | 29.3 | 50 |

DLEE = Dried leaf ethanol extract; FLS = Fresh leaf sap; FLSP = Fresh leaf sap powder in DMSO; FLCAE = Fresh leaf cold aqueous extract; FLCE = Fresh leaf chloroform extract; FLPE = Fresh leaf petroleum ether extract; FLAE = Fresh leaf acetone extract; FLEE = Fresh leaf ethanol extract; TET = Tetracycline. Minimum inhibitory concentrations at µg/ml.

## Antibacterial assay

Dried leaf extracts and fresh leaf extracts of *M. oleifera* were selected for antibacterial activity against seven different multidrug-resistant bacteria of AAD. All the extracts exhibited different degrees of antibacterial activity which were compared with the standard reference (control). Depending on the measured values of the complete inhibition diameter of the zone, the antibacterial activity can be classified as 6-9 mm: week antibacterial activity; 10-15 mm: slight antibacterial activity; 16-20 mm: moderate antibacterial activity and >20: strong antibacterial activity (Arora and Bhardwaj 1997).

In dried leaf, ethanol extract showed moderate effect against all of the isolates and the highest inhibition zone (20.07 ± 0.5 mm) was found against *E. coli* (Fig. 3) at 30 mg/ml concentration while petroleum ether, acetone and chloroform extracts did not exhibit any inhibitory activity. In another study Jabeen *et al.* (2008) reported that crude extract of *M. oleifera* had strong activity against *Fusarium solani*, *Bacillus subtillis*, and *Staphylococcus aureus.* The relatively high potency of the ethanol extract may be attributed to the dissolving power of alcohols (Majorie 1999).

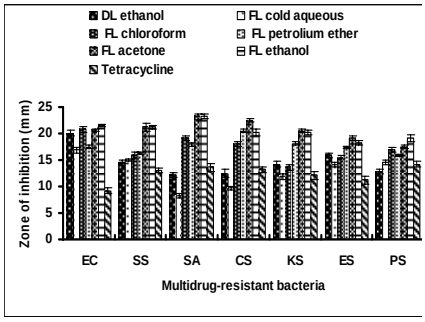


Fig 3. Inhibitory activity of solvent extracts of dried and fresh leaf of *M. oleifera* against multidrug resistant bacteria. 30 mg/ml for extracts, 100 µg/ml for tetracycline.

EC = *Escherichia coli*; SS = *Shigella* sp.; SA = *Salmonella* sp.; CS = *Citrobacter* sp.; KS = *Klebsiella* sp.; ES = *Enterobacter* sp.; PS = *Proteus* sp.; DL = Dried leaf; FL = Fresh leaf.

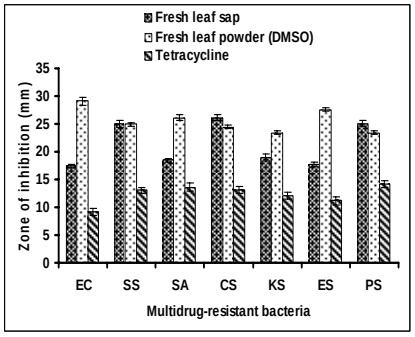


Fig 4. Inhibitory activity of fresh leaf sap of *M. oleifera* against multidrug resistant bacteria.

29.375 mg/ml for fresh leaf sap without solvent, 32.5 mg/ml for fresh leaf sap powder in DMSO and 100 µg/ml for tetracycline.

Fresh leaf extracts revealed a potential antibacterial activity against all of the isolates. Fresh leaf sap powder extract dissolved in DMSO exhibited strong inhibitory effect against all of the test bacteria with their zones of inhibition ranges 23.4±0.3 to 29.1±0.7 mm (Fig. 4) at 32.5 mg/ml. Fresh leaf sap without adding any solvent also exhibited strong zone of inhibition against *Shigella* sp. (25.07±0.6 mm), *Citrobacter* sp. (26.07±0.5 mm) and *Proteus* sp. *(*25.07±0.5 mm) at 29.375 mg/ml. Both acetone and ethanol extract of fresh leaf showed strong antibacterial effect against *E. coli, Shigella* sp., *Salmonella* sp., *Citrobacter* sp*.* and *Klebsiella* sp*.* On the other hand, cold aqueous, chloroform and petroleum ether extract displayed a moderate inhibitory effect against all of the test bacteria. Hot aqueous extract and solvents (negative control) could not show any inhibitory activity against test organism. Doughari *et al.* (2008) demonstrated that acetone extract of *Senna obtusifolia* leaf showed highest activity followed by dichloromethane extracts while water extract exhibited least activity against all the clinical isolates *Neisseria gonorrheae*, *Salmonella sp*., *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Staphylococcus aureus.*. Different solvents have various degrees of solubility for different

phyto-constituents (Majorie 1999). Results revealed that the fresh leaf sap powder extract dissolved in DMSO showed greater antibacterial activity compare to dried leaf solvents extract. This finding is interesting because in the traditional method of treating a bacterial infection, decoction of the plant parts is employed whereas in the present study organic solvent (DMSO) extract was shown to provide a better antibacterial activity. These observations may be attributed to the nature of biological active components whose activity can be enhanced in the presence of DMSO.

## MIC determination

The MIC values obtained for the extracts against the isolates varied from one extract to the other (Table 3). The MIC of the fresh leaf extracts ranged 7.9-234.4 μg/ml, with the fresh leaf sap powder (DMSO) extract demonstrating the lowest values (MIC 7.9 μg/ml) against *E. coli*, followed by fresh leaf sap against *Shigella* sp. (14 μg/ml), *Citrobacter* sp. (14 μg/ml) and *Proteus* sp. (14 μg/ml). Ethanolic dried leaf extract demonstrated comparatively higher MIC ranged 937.5-3750 μg/ml and the lowest MIC (937.5 μg/ml) exhibited against *E. coli.* Most of the MIC values were lower indicating the extracts could be bactericidal in action. Lower MIC values and higher zones of inhibition for fresh leaf extracts is the indication of high efficacy which in accordance with the results obtained by Doughari *et al*. (2008). Antimicrobial properties of substances are desirable tools in the control of undesirable microorganisms in the treatment of infections (Aboaba *et al.* 2006).

## Conclusion

The present results support the folkloric usage of the studied plant and suggest that the fresh leaf sap powder extract dissolved in DMSO posses certain constituents which can be used as antimicrobial agents to treat of infectious disease caused by multidrug-resistant bacteria. But *in vivo* studies on this medicinal plant are necessary and should seek to determine toxicity of the active constituents, their side effects, pharmacokinetic properties and diffusion in different body sites with the view to formulating novel chemotherapeutic dugs.

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