Growth inhibitory effect of ethanolic neem leaves extract on *Klebsiella*, *Salmonella* and *Staphylococcus aureus*
Introduction

Since prehistoric times, neem (Azadirachta indica) has been used by humankind for medicinal purpose. It has been extensively used in Ayurveda, Unani and Homeopathic medicine (Rajasekaran et al., 2008; Girish and Bhat, 2008). Besides versatile medicinal uses it is as well used in agriculture (Mishra et al., 2013; Maragathavalli et al., 2012). Different parts of neem contain more than 135 compounds having vast arrays of biological activity (Yerima et al., 2012). Neem (leaves, flowers, seeds, fruits, roots, bark) found its utility since ancient days and have been used to treat infections, inflammation, fever, skin diseases and dental disorders (Helmy et al., 2007; Mosaddek and Rashid, 2008). Neem leaf has been the mostly used part of the tree and possesses immunomodulatory, anti-inflammatory, anti-hyperglycemic, antiulcer, antimalarial, antifungal, antibacterial, anti-oxidant, anti-mutagenic and anticarcinogenic properties (Reddy et al., 2013; Girish and Bhat, 2008). It also possesses a wide spectrum of antibacterial action against Gram-positive and Gram-negative microorganisms (Mamman et al., 2013; Sarmiento et al., 2011; Jahan et al., 2007).

Although a number of antibiotics are available for the treatment of infectious diseases, they have certain limitations like adverse effects, high cost and development of antimicrobial resistance (Kumar et al., 2013). Indiscriminate use of antibiotics is very common in Bangladesh and other developing countries being a major cause for development of antimicrobial resistance (Faiz and Basher, 2011; Rahman and Huda, 2014; Shamsuzzaman et al., 2007).

Considering all these, we tried to find an effective antimicrobial agent from plant source against a few common human pathogens like - Klebsiella sp., Salmonella sp. and Staphylococcus aureus. We conducted this study to determine the antimicrobial effect of ethanolic neem leaf extract on these commonly occurring bacteria to determine if it has any prospect as future antibiotic.

Materials and Methods

This experimental study was conducted from January 2010 to December 2010.

Preparation of neem extract: Fresh, mature deep green leaves of neem tree were collected from the medicinal plant garden of BCSIR, Dhaka. After identification and authentication by Dhaka Laboratories, BCSIR and these...
were cleaned and washed with plain tap water and air dried in shade at room temperature by spreading in large stainless steel trays for 24 hours. The dried leaves were crushed well. Five hundred grams of crushed leaves were then suspended in 3 L of petroleum ether and kept in refrigerator overnight for removing all the fatty substances. After this, the supernatant was discarded and the residue was dried at room temperature. Then the residue material were suspended into 5 liters of 95% ethanol in sterile conical flask and kept at 4°C for 72 hours. The supernatant was filtered through filter paper (Whatman No. 1) and the filtrate was put into rotary vacuum evaporator to get concentrated neem extract. The filtrate thus obtained was further purified by filtration through Whatman No. 1 filter paper. This stock solution of extract was sterilized by filtration through Millipore membrane filter of 0.45 mm pore size. Then the concentrated extract was Freeze dried and stored in sterile capped vials of 0.45 mm pore size. Then the concentrated extract was Freeze dried and stored in sterile capped vials. Suitable amount of ethanol was mixed to dilute the extract to get a solution of 100 mg/mL. Then it was further diluted using double fold serial dilution to obtain 50, 25, 12.5, 6.25 and 3.125 mg/mL concentrations of extract. This was then incubated at 37°C for 24 hours.

**Collection of microorganism**

*Klebsiella sp. ATCC strain, Salmonella sp. ATCC strain and Staphylococcus aureus ATCC 25923* was obtained from the Department of Microbiology, BSMMU, Dhaka.

**Preparation of culture media, McFarland standard and bacterial cell suspension**

Nutrient agar and McFarland stand-dard was prepared by standard method (Hudzicki, 2009). For preparation and standardization of bacterial cell suspension 5 colonies each of *Klebsiella sp.*, *Salmo-nella sp.* and *S aureus* were picked and put into separate sterile test tube containing nutrient broth then incubated at 37°C for 24 hours. The turbidity produced by the organism was adjusted and used to match with the turbidity of 0.5 McFarland standard. If the suspension was too light more organisms was added or if it was too heavy it was diluted by sterile saline (Hudzicki, 2009).

**Determination of minimum inhibitory concentration (MIC) on the test organisms**

The MIC of the extract was determined by broth dilution method.

**Preparation of different concentrations of extract**

For preparation of different concentrations of extract, 10 g of freeze dried extract was taken and was diluted in 100 mL of ethanol. Thus, the initial concentration was 100 mg/mL. Then it was further diluted using double fold serial dilution to obtain 50, 25, 12.5, 6.25 and 3.125 mg/mL concentrations of extract.

**Inoculation of bacteria into extract**

In 3 sets of test tubes containing 5 test tubes each, 0.1 mL of *Klebsiella sp.*, *Salmonella sp.* and *S. aureus* inoculums were added. A negative control was set up by 5 mL sterile extract and 5 mL sterile nutrient broth and positive control by 5 mL of sterile nutrient broth and 0.1 mL of bacterial inoculum.

**Examination of growth after overnight incubation**

Test tubes were incubated at 37°C for 24 hours. The growth of the test organism in each concentration of extract was examined and compared against the controls by matching their turbidity. The growth of the bacterial inoculum in the broth was indicated by turbidity or cloudiness of the broth. The test tube containing the lowest concentration of extract and showing no visible sign of growth or turbidity was considered as the MIC. The mean MICs were recorded.

**Detection of bacterial susceptibility**

Bacterial susceptibility was determined by disc diffusion method in nutrient agar medium. The nutrient agar plate was inoculated by streaking a swab stick dipped into an inoculum tube containing standardized bacterial cell suspension three times over the entire agar surface. Then it was kept at room temperature for 3 to 5 min for drying. Whatman No. 1 filter paper discs of 6 mm diameter were made with the help of a punching machine and these discs were sterilized by hot air oven. Then 50 µL of different concentrations of extract were applied to soak the sterile discs and were placed on the inoculated agar plate. A disc was soaked by 50 µL of ethanol and was also placed on the agar plate which served as control. The plates were allowed to stand for one hour for pre-diffusion of the extracts then incubated at 37°C for 24 hours. Diameters of zone of inhibition were measured in millimeter.

**Results**

The lowest concentration of extract showing an inhibitory effect on the growth of *Klebsiella* and *Salmonella* was 12.5 and 6.25 mg/mL respectively. Growth of *S aureus* was failed to be inhibited with the highest concentration used (50 mg/mL). So, the MIC of extract against *Klebsiella sp.* and *Salmonella sp.* was 12.5 and 6.25 mg/mL respectively. *S. aureus* being resistant to the effects of extract (Table I).

Antibacterial susceptibility of *Klebsiella, Salmonella* and *S aureus* subculture on to nutrient agar was performed on MHA and diameters of zone of inhibition for extract was measured after overnight incubation at 37°C, aerobically. Average diameter of zone of inhibition against *Klebsiella* was 18 mm at 12.5 mg/mL and *Salmonella* 20 mm at 6.25 mg/mL. *S aureus* did not show any zone of inhibition with the highest concentration (50.0 mg/mL) of extract (Table II).
Discussion

The World Health Organizations (WHO) has recently pronounced a warning saying that the world is entering a ‘post-antibiotic era’ when most of the commonly available antibiotics will become ineffective (Reardon, 2014). Antimicrobial resistance is a threat to all branches of medical and public health practice (Kumar et al., 2013). It jeopardizes progress in health sectors by increasing morbidity and mortality and imposes huge economic burden (WHO, 2014; Karmakar and Sattar, 2012; Cosgrove and Carmeli, 2003). Moreover, most of the new antibiotics are costly and possesses threat of adverse drug reaction. Quest is going on for safe and effective alternatives of modern antibiotics from plant sources (Victor and Igeleke, 2012; Huttner et al., 2013). Studies suggest that plants can be source of drugs against infective organisms (Rajasekaran et al., 2008; Reddy et al., 2013; Rozarina, 2013).

In the present study the extract was subjected to a preliminary screening for antimicrobial activity against Klebsiella, Salmonella and S. aureus. The MIC of extract inhibiting the growth of Klebsiella was 12.5 and 6.25 mg/mL for Salmonella. Growth of S. aureus was not inhibited even with the highest concentration used (50 mg/mL). This suggests that extract possesses antimicrobial activity against Klebsiella and Salmonella. Mamman et al. (2013) in their study found aqueous and methanolic neem leaf extract inhibiting Salmonella spp. The finding was not consistent with that of Helmy et al. (2007) where native extracts from the neem leaves (20 µg/disk) were inhibitory to S. aureus, E. coli and some fungi.

To see the bacterial susceptibility of extract, the test organisms were subcultured. The results obtained in the agar diffusion plates followed the same trend with what was obtained in the MIC tests. With extract, the size of zone of inhibition was 18 mm for Klebsiella, 20 mm for Salmonella with a disk potency of 12.5 and 6.25 mg/mL respectively. This finding is similar to findings of previous studies done by Odunbaku and Ilusanya (2008) where ethanolic extract of neem showed significant antimicrobial activity against E. coli, Klebsiella pneumonia, S. aureus, Proteus mirabilis and few other bacteria. Similar observations were also made by other investigators (Chaturvedi et al., 2011; Irshad et al., 2011). Jahan et al. (2007) in her study found growth inhibitory effect of neem oil on Salmonella typhi, S. aureus and E coli. Mamman et al. (2013) in their study showed that crude methanolic and aqueous extract of neem leaves was inhibitory to S. aureus, Salmonella spp and E. coli.

In our study even at 50 mg/mL concentration of extract failed to suppress the growth of S. aureus. This finding is not supported by most of the previous studies where neem leaf extract at different concentrations inhibited the growth of S. aureus (Reddy et al., 2013; Mamman et al., 2013; Sarmiento et al., 2011; Mishra et al., 2013; Prashar et al., 2012). Khan et al. (2010) in their study found that chloroform extract of neem leaves inhibited the growth of S. aureus, which was just comparable to streptomycin. In another study Maragathavalli et al. (2012) found comparative inhibitory effect of extract

<table>
<thead>
<tr>
<th>Serial of test tubes</th>
<th>Concentration of extract (mg/mL)</th>
<th>Klebsiella sp.</th>
<th>Salmonella sp.</th>
<th>S. aureus</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50</td>
<td>Growth completely inhibited</td>
<td>Growth completely inhibited</td>
<td>Growth not inhibited</td>
</tr>
<tr>
<td>2</td>
<td>25</td>
<td>Growth inhibited</td>
<td>Growth inhibited</td>
<td>Growth not inhibited</td>
</tr>
<tr>
<td>3</td>
<td>12.5</td>
<td>Growth inhibited</td>
<td>Growth inhibited</td>
<td>Growth not inhibited</td>
</tr>
<tr>
<td>4</td>
<td>6.25</td>
<td>Growth not inhibited</td>
<td>Growth inhibited</td>
<td>Growth not inhibited</td>
</tr>
<tr>
<td>5</td>
<td>3.125</td>
<td>Growth not inhibited</td>
<td>Growth not inhibited</td>
<td>Growth not inhibited</td>
</tr>
<tr>
<td>Positive control</td>
<td>-</td>
<td>Huge growth</td>
<td>Huge growth</td>
<td>Huge growth</td>
</tr>
<tr>
<td>Negative control</td>
<td>5</td>
<td>Growth inhibited</td>
<td>Growth inhibited</td>
<td>Growth not inhibited</td>
</tr>
</tbody>
</table>

Table I

**Inhibitory effect of neem extract on Klebsiella sp., Salmonella sp. and S. aureus**

<table>
<thead>
<tr>
<th>Name of bacteria with average diameter of zone of inhibition</th>
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<tr>
<td>Neem extract</td>
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<tr>
<td>Hole potency</td>
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<tr>
<td>12.5 mg/mL</td>
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<tr>
<td>6.25 mg/mL</td>
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<td>50.0 mg/mL</td>
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</tbody>
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and gentamicin against *S. aureus*. The methanolic extract of neem leaves inhibitory effect was much better than that of gentamicin.

**Conclusion**
The ethanolic neem leaves extract shows variable antibacterial effect on *Salmonella spp* and *Klebsiella spp*, and failed to inhibit *S. aureus* which maybe due to the lower concentration used.

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**Conflict of Interest**
Authors declare no conflict of interest

**References**


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