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, antiviral, antioxidant, antimutagenic 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 Bashar, 2011; Rahman and Huda, 2014; Shamsuzzaman et al., 2007).

Considering all these, we tried to find an effective anti-microbial agent from plant source against a few common human pathogens like Klebsiella, Salmonella sp. and Staphylococcus aureus.

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 liters 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 conical flask and refrigerated at 4°C. Suitable amount of ethanol was mixed to dilute the extract to get a solution of different strength. The extract was tested for sterility by introducing 2 mL of extract into 10 mL of sterile nutrient broth. 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 standard was prepared by standard method (Hudzicki, 2009). For preparation and standardization of bacterial cell suspension 5 colonies each of Klebsiella sp., Salmonella 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 prediffusion 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 mg/mL 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 mg/mL 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
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 mg/mL 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.

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

| Serial of test tubes | Concentration of extract (mg/mL) | *Klebsiella* sp. | *Salmonella* sp. | *S. aureus* |
|----------------------|----------------------------------|-----------------|-----------------|------------|
| 1                    | 50                               | Growth completely inhibited | Growth completely inhibited | Growth not inhibited |
| 2                    | 25                               | Growth inhibited | Growth inhibited | Growth not inhibited |
| 3                    | 12.5                             | Growth inhibited | Growth inhibited | Growth not inhibited |
| 4                    | 6.25                             | Growth not inhibited | Growth inhibited | Growth not inhibited |
| 5                    | 3.125                            | Growth not inhibited | Growth not inhibited | Growth not inhibited |
| Positive control      | -                                | Huge growth      | Huge growth      | Huge growth |
| Negative control      | 5                                | Growth inhibited | Growth inhibited | Growth not inhibited |

Table II: Diameter of zone of inhibition of neem extract and ciprofloxacin disk against *E. coli*

| Neem extract | Hole potency | Zone of inhibition | *Klebsiella* sp. | Hole potency | Zone of inhibition | *Salmonella* sp. | Hole potency | Zone of inhibition | *Staphylococcus aureus* | Hole potency | Zone of inhibition |
|--------------|--------------|-------------------|-----------------|--------------|-------------------|-----------------|--------------|-------------------|--------------------------|--------------|-------------------|
|              | 12.5 mg/mL   | 18 mm             |                 |              |                   |                 |              |                   |                          |              |                   |
|              | 6.25 mg/mL   | 20 mm             |                 |              |                   |                 |              |                   |                          |              |                   |

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; 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 and gentamicin against *S. aureus*. Here an interesting finding was that methanolic extract of neem leaves inhibitory effect was much better than that of gentamicin.

In this context we can conclude by saying that 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.
References

Chaturvedi P, Bag A, Rawat V, Jyala NS, Satyavali V, Jha PK. Antibacterial effects of Azadirachta indica leaf and bark extracts in clinical isolates of diabetic patients. NJIRM. 2011; 2: 5-9.

Cosgrove SE and Carmeli Y. The impact of antimicrobial resistance on health and economic outcomes. Clinical Inf Dis. 2003; 36: 1433-37.

Faiz MA, Bashir A. Antimicrobial resistance: Bangladesh experience. Regional Health Forum. 2011; 15: 1-8.

Girish K, Shankara Bhat S. Neem: A green treasure. Electronic J of Biol. 2008; 4: 102-11.

Mamman PH, Mshelia WP, Susbaturu SC, Sambo KW. Antibacterial effects of crude extract of Azadirachta indica against Escherichia coli, Salmonella spp and Staphylococcus aureus. Int J Med Med Sci. 2013; 5: 14-18.

Helmy WA, Amer H, EL-Shaye NMA. Biological and antimicrobial activities of aqueous extracts from neem tree (Azadirachta indica A Juss, Meliaceae). J Appl Sci Res. 2007; 3: 1050-55.

Hudzicki J. Kirby-Bauer disk diffusion susceptibility test protocol. MicrobeLibrary.org. 2009.

Huttner A, Harbarth S, Carlet J, Cosgrove S, Goossens H, Huttner A, Jarlier V, Voss A, Pittet D. Antimicrobial resistance: A global view from the 2013 World Healthcare-Associated Infections Forum. Antimicrobial Res Inf Cont. 2013; 2: 31.

Irshad S, Butt M, Younus H. In-vitro antibacterial activity of two medicinal plants neem (Azadirachta indica) and peppermint. Int R J Pharmaceut. 2011; 1: 9-14.

Jahan T, Begum ZA, Sultana S. Effect of neem oil on some pathogenic bacteria. Bangladesh J Pharmacol. 2007; 2: 71-72.

Karmakar P, Sattar MM. Antibiotic prescribing pattern in Bangladesh. B J Prog Sci Tech. 2012; 10: 13-16.

Khan I, Sriakoluppu SR, Darsipudi S, Gotteti SD, Amaranadh CH. Phytochemical studies and screening of leaf extracts of Azadirachta indica for its anti-microbial activity against dental pathogens. Archives of App Sci Res. 2010; 2: 246-50.

Kumar BU, Bhurbaneswari A, Tejasri MVV, Radhakrishna P and Amritha KDK. Comparative antimicrobial activities of the combined crude leaf extract of Bixa Orellana, Azadirachta indica and Ocimum sanctum. Int Res J Pharm. 2013; 4(4): 189-93.

Maragathavalli S, Brindha S, Kaviyarasi NS, B. Anandurai B, Gangwar SK. Antimicrobial activity in leaf extract of neem (Azadirachta indica Linn.). IJSN. 2012; 3: 110-13.

Mosaddek ASM, Rshid MMU. A comparative study of the anti-inflammatory effect of aqueous extract of neem leaf and dexamethasone. Bangladesh J Pharmacol. 2008; 3: 44-47.

Mishra A, Mamta, Neema, Niketa, Poonam, Pranjal and Priyanka. Antibacterial effects of crude extract of Azadirachta indica against Escherichia coli and Staphylococcus aureus. Int J Sci Environ Tech. 2013; 2: 989-93.

Odunbaku OA, Illusany OA. Antibacterial activity of the ethanolic and methanolic leaf extracts of some tropical plants on some human pathogenic microbes. Res J Agric Biol Sci. 2008; 4: 373-76.

Prashar P, Pruthi H, Akhlaq A. In vitro antimicrobial activity of Azadirachta indica against pathogenic bacteria. J Pharm Res. 2012; 5: 363-64.

Rahman MS, Huda S. Antimicrobial resistance and related issues: An overview of Bangladesh situation. Bangladesh J Pharmacol. 2014; 9: 218-24.

Rajasekaran C, Meignanan E, Vijayakumar V, Kalaivani T, Ramya S, Premkumar N, Siva R, Jayakumararaj R. Investigations on antibacterial activity of leaf extracts of Azadirachta indica A. Juss (Meliaceae); A traditional medicinal plant of India. Ethnobotanical Leaflets. 2008; 12: 1213-17.

Reardon S. Antibiotic resistance sweeping developing world. Nature 2014; 509: 141-42.

Reddy YRR, Kumari CK, Lokanatha O, Mamatha S, Reddy CD. Antimicrobial activity of Azadirachta indica (neem) leaf, bark and seed extracts. Int J Phytochem Pharmacol. 2013; 3: 1-4.

Rozarina NJA. Antimicrobial potentials of the methanolic extracts of plants. IJSRR. 2013; 2: 89-95.

Sarmiento WC, Maramba CC, Gonzales MLM. An in vitro study on the antibacterial effect of neem (Azadirachta indica) leaf extract on methicillin-sensitive and methicillin-resistant Staphylococcus aureus. PIDSJP. 2011; 12: 40-45.

Shamsuzzaman AKM, Paul SK, Mahmud MC, Musa AKM, Hossain MA. Emerging antimicrobial resistance amongst common bacterial pathogens in Myensingsh Medical College Hospital, Bangladesh J Med Microbiol 2007; 1: 4-9.

Victor IU, Igeleke CL. Antimicrobial properties of the extracts of locally sold garlic and neem leaf in Benin City, Nigeria. Int J Biosci. 2012; 2: 21-27.

WHO. Antimicrobial resistance: Global report on surveillance 2014.

Yerima MB, Jodi SM, Oyinbo K, Maishanu HM, Farouq AA, Junaidu AU, Al-Mustapha MN, Shinkafi AL. Effect of neem extracts (Azadirachta indica) on bacteria isolated from adult mouth. Nigerian J Basic App Sci. 2012; 20: 64-67.