Original article

In Vitro comparison in the antimicrobial effect between Ciprofloxacin and Neem leaf extract (*Azadirachta indica*) on *Eschericia coli* Growth

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Abstract:

Objective: Medicinal plants remain in vogue to treat some diseases in lower socio-economic communities, despite the availability of antimicrobials, often. Majority of rural Bangladeshi and tribal people being grossly illiterate and ignorant, use various herbs to treat a wide range of diseases. Of several medicinal-plants, neem is reported to have enormous impact in treating inflammation and infections. We, therefore, compared the antimicrobial effect of ethanolic neem leave extract (ENLE) on *Escherichia coli* (*E. coli*), with that of Ciprofloxacin. ***Materials & Methods***: This experimental study compared the in vitro antimicrobial activity between ENLE and Ciprofloxacin on *E. coli* carried out in Department of Pharmacology and Therapeutics of SS-Medical College, Dhaka, Bangladesh. Antimicrobial efficacy of ENLE and ciprofloxacin (5µg; Oxoid, UK) was determined against *E. coli* following minimum inhibitory concentration. By filtration and evaporation of Neem leaves ENLE was prepared. Antibiotic Sensitivity Test was performed on Muller-Hinton agar using a twofold serial dilution. ***Results***: ENLE showed an inhibitory effect on the growth of *E. coli* at the concentration of 3.125 mg/ml. Antibacterial susceptibility of *E. coli* was performed on MHA and diameters of zone of inhibition by both ENLE and Ciprofloxacin were measured after overnight aerobic incubation at 37°C. Diameter of zone of inhibition against *E. coli* was 28 ± 0.16 mm with ENLE, 36 ± 0.07 mm with Ciprofloxacin (5µg/disk) ($p<0.000$). ***Conclusion***: Findings of this preliminary in-vitro experiment though suggests that, ENLE against *E. coli* showed limited efficacy, better efficacy of Ciprofloxacin cannot be ruled out unless further in depth studies elucidates stronger evidences to support it.

Keywords: *Azadirachta indica*; *Escherichia coli*; Minimum Inhibitory Concentration (MIC); Ciprofloxacin

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Introduction:

Amongst a wide range of human diseases, infectious diseases remains the major cause of morbidity and mortality worldwide[1] including Bangladesh[2] having detrimental effect on the overall economy, particularly in the underdeveloped countries [1, 2]. Uses of traditional medicinal plants in primary care have steadily been increased worldwide [3, 4]. Despite widely available antibiotics; medicinal herbs are still used in several communities. It is reported in India that scientists are in search of new phytochemical that could be developed as useful anti-microbial for treatment of infectious diseases [5-7].

Out of many medicinal plants available, Neem has been extensively used in ayurveda, Unani and homeopathic medicine since prehistoric times and has been declared as the "Tree of the 21st century" by the United Nations [5-8]. Neem tree possesses a wide spectrum of antibacterial action against gram-negative and gram-positive microorganisms and

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fungus [9-13]. It also shows immune-modulatory, anti-inflammatory, anti-hyperglycemic, anti-ulcer, anti-malarial, anti-fungal, antiviral, antioxidant, anti-mutagenic & anti-carcinogenic properties [5-8].

For the treatment of various infectious diseases although a number of modern antibiotics are available, they have certain limitations like serious side effects, cost and development of resistance [14]**.** Like other developing countries, indiscriminate use of antibiotics being common in Bangladesh, often leads to developing antimicrobial resistance [2, 15, 16].

In view of the above facts and figures, we compared the antimicrobial effect of ENLE with ciprofloxacin-a very commonly prescribed antibiotic [2, 14] in Bangladesh on *E. coli*- the commonest pathogenic bacteria among the communities of Bangladesh [15,16] alike other countries.

Materials and methods:

This experimental study was conducted during January to December 2010 at the Department of Pharmacology and Therapeutics, Sir Salimullah Medical College, Dhaka, Bangladesh in collaboration with Bangabandhu Sheikh Mujib Medical University (BSMMU) and the Bangladesh Council of Scientific and Industrial Research (BCSIR). This project was approved by the ethical committee of Sir Salimullah Medical College.

Preparation of ENLE

Fresh, mature deep green leaves of neem tree were collected from the medicinal plant garden of BCSIR (identified and authenticated by BCSIR Laboratory, officially), Dhaka, Bangladesh. These were cleaned and washed with distilled water and air dried in shade at room temperature overnight on a large pre-sterile stainless steel tray under a safety hood. Next morning the dried leaves were crushed well, and 500 gm. of crushed-leaves were suspended in 3 liter of petroleum ether and kept overnight in refrigerator at 4°Cto remove all fatty substances.

Next day, the supernatant was discarded and the residue was dried at room temperature under a safety hood. These were then suspended into 5 liter of 95% Ethanol in a sterile conical flask and kept at 4°C for the next 72 hrs. The supernatant was then filtered using filter paper (Whatman No. 1) and put into rotary vacuum evaporator to get the concentrated ethanolic neem leaf extract. The filtrate thus obtained was further purified by re-filtration to make a "stock solution" (extract) which was sterilized by filtration through Millipore membrane filter of 0.45 mm pore size.

Then this extract was freeze dried and stored in sterile cotton capped conical flask at 4° C for further use. Suitable amount of ethanol was mixed to dilute the extract for making ENLE solutions of different strengths. The extract was subjected for sterility testing by introducing 2ml of extract into 10 ml of sterile nutrient broth and incubated overnight at 37° C, to prove sterility having no growth.

Collection of microorganism

E. coli (ATCC strain # 25922) was obtained from the Department of Microbiology, BSMMU, Dhaka, Bangladesh.

Preparation of culture media, McFarland standard and Bacterial Cell suspension

Nutrient agar and McFarland standard was prepared by standard Kirby-Bauer method[17]. For preparing and standardizing bacterial cell suspension, 5 colonies of *E. coli* were mixed into sterile test tubes containing nutrient broth and then incubated at 37° C for 24 hours. The turbidity produced by the organism was adjusted to match with the turbidity (Optical Density) of 0.5 McFarland standard. More organisms were added if suspension got too light and it was diluted by adding sterile saline if it got too heavy [17].

Determination of Minimum Inhibitory Concentration (MIC) on the test organisms

The MIC of the extract was determined by broth dilution method following standard bacteriological procedure.

Preparation of different concentrations of ENLE:

For preparing different concentrations of ENLE, 10 gm. of freeze dried extract was taken and was diluted in 100ml of Ethanol. Thus, the initial concentration was 100mg /ml. Then, it was further diluted using ×2 folded serial dilutions to obtain 50, 25, 12.5, 6.25 and 3.125mg/ml. concentrations of ENLE, for subsequent antimicrobial sensitivity testing.

Inoculation of bacteria into ENLE

In five test tubes containing different concentrations of ENLE, 0.1ml of *E. coli* aliquot was added to each. A negative control was prepared by adding 5 ml sterile ENLE and 5 ml sterile nutrient broth showing no growth, contrary to a positive control using 0.1 ml of bacterial inoculum in 5 ml of sterile nutrient broth, showing bacterial growth.

Examination of growth after overnight incubation

Test tubes were incubated at 37° C for 24 hours. The growth of the test organism in each ENLE concentration was examined to compare against the controls that matches their turbidity. The growth of the bacterial inoculum was indicated by

the turbidity (cloudy) 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, which was finally recorded in a lab register or log book.

Detection of bacterial susceptibility

Bacterial susceptibility was determined by disc diffusion (Kirby-Bauer) method (17) in MHA medium.

Inoculation of the test plate

The nutrient agar plate was lawned using a sterile swab stick dipped into an inoculum tube containing standardized bacterial cell suspension three times over the entire agar surface for getting a homogenous bacterial spread. Then, these plates were kept at room temperature for 3 to 5 minutes for drying under a biosafety hood.

Application of extract and antibiotic disks

Whatman no.1 filter paper discs of 6mm 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 ENLE 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 antibiotic disc of Ciprofloxacin having disc potency of 5 μgm. /disc was placed on the inoculated agar plate which served as standard 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 using a transparent plastic ruler against a standardized chart to read inhibition zone as produced by respective antibiotics and/or ENLE.

Interpretation and reporting of the results

The diameter of zone of inhibition denotes the relative susceptibility to a particular antimicrobial agent. For Ciprofloxacin, any zone of inhibition ≥21mm was considered as sensitive (S), between 16-20mm as intermediate (I) and ≤15 mm as resistant (R) [18]. For ENLE, the diameters of zone of inhibition for respective concentrations were recorded in the lab register &/or log book, accordingly.

Results:

Determination of MIC

Of 5 different concentrations (3.125, 6.25, 12.5, 25 and 50mg/ml), ENLE showed the highest inhibitory effect on growth of *E. coli* at 3.125 mg/ml (3125μg/ml). So, the MIC of ENLE against *E. coli* was read as 3.125 mg/ml (3125μg/ml), (Table-1).

Detection of bacterial susceptibility

The antimicrobial activity of the ENLE at the MIC and that of ciprofloxacin on *E. coli* was determined by respective zone of inhibitions. The zone of inhibition with ENLE was 27.96 ± 0.16 mm and

Table 1. Inhibitory effect of ENLE (n = 5) on *E. coli*.

Serial of test tubes	Concentration of ENLE (mg/ml)	*E. coli*
1	50 mg/ml	Growth inhibited
2	25 mg/ml	Growth inhibited
3	12.5 mg/ml	Growth inhibited
4	6.25 mg/ml	Growth inhibited
5	3.125 mg/ml	Growth inhibited
Positive control*	-	Growth observed
Negative control†	5 ml	Growth Inhibited

*Positive control = 5 ml of sterile nutrient broth and 0.1 ml of bacterial inoculums.
†Negative control = 5 ml sterile plant extract (undiluted) and 5 ml sterile nutrient broth.

Table 2. Diameter of zone of inhibition of ENLE and Ciprofloxacin disk against E. coli

ENLE and Antimicrobial disk	Average diameter of zone of inhibition on *E. coli*	
	Disk potency	Zone of inhibition (mean ± SD) mm
ENLE (n = 6)	156.26 mg/disk†	27.96 ± 0.16
Ciprofloxacin (n = 6)	5μg/disk	35.98 ± 0.07*

* Significant ($P < 0.001$); showing better efficacy of ciprofloxacin
†Using 50μl of 3125 μg/ml ENLE/disk

with ciprofloxacin 35.98 ± 0.07 mm (Table 2) and, also depicted (Figure-1).

Discussion:

In treating diverse human diseases, medicinal plants/herbal medicine remain in vogue in several lower socio-economic communities since long [1,2,19] as primary care [3,4], despite of having number of choices of antibiotics- which reportedly remains costly and emerged as multidrug resistance (MDR) [20].

The continuous quest towards newer and safer medicinal plants having AB property has been a long standing effort to treat bacterial infections in human and plants. [9, 21] Alike other parts of the world, majority of rural people of Bangladesh often use various herbs/plants in treating infections. [5-8, 22] Neem (*Azadirachta indica or A. Indica or A. Juss*) has been reported to have enormous impact on cure and heal. It is extensively used in traditional medicine, too [5-8, 23] since it possesses a large scale of antimicrobial action against both bacteria and fungus [9-13].

E. coli shows its increasing trend of resistance to ciprofloxacin [15], found as the second most commonly prescribed antibiotic (21.24%) in Bangladesh [14].

The World Health Day, 2011 was focused on "Antimicrobial Resistance" so as to draw global attention to growing public health threat [24] which jeopardizes progress in health care sectors with increasing morbidity/mortality imposing huge societal economic burden [14, 25] with some threat of adverse drug reactions [26].

Exploration for safe and effective alternatives of modern antibiotics from plant sources are on the move [19, 26] against a wide range of infective organisms and fungus [10-13]. Hence, we studied the ENLE, subjected to a preliminary screening for antimicrobial activity against *E. coli* (ATCC 25922).

In our *in-vitro* experiment, ciprofloxacin disks were standardize prior to compare that with ENLE. The results obtained in the agar diffusion plates followed the same trend with what was obtained in the MIC tests. With ENLE, the size of zone of inhibition was 27.96 ± 0.16 mm for *E. coli* with a disk potency of 3.125 mg/ml. Antimicrobial activity of alcoholic extracts of neem on E coli was also reported in previous studies [27-28].

The lowest MIC of ENLE to inhibit the growth of *E. coli* was 3.125 mg/ml or 3125μg/ml suggest it antimicrobial effect- a finding that is consistent with another study [29] who reported native extracts from neem leaves (20μg/disk) were inhibitory to *E. coli, S. aureus* and fungi. Further, another study [30] reporting ethanolic neem extract showed good inhibitory effect on some pathogenic bacteria with low MIC (75-250μg/ml) does not remain comparable to that of ours.

Similarly, Chaturvedi *et al.,* (2011) [31] in their study found that ethanolic extract (300-500mg/ml) of *A. indica* inhibited growth of *E. coli*. This finding is similar to a study carried out in Bangladesh in 2007 [23], evidenced antimicrobial efficacy of neem oil on *E. coli* where the zone of inhibition was 19.5 mm. All the aforesaid facts affirm that, ethanolic neem leaf-extract do demonstrates an antibacterial effect on *E. coli* though less effectively than ciprofloxacin. However, findings in this issue varied among findings of various studies carried out in various countries. ENLE could be a potential antimicrobial agent used as mono-therapy and/ or an adjunct with commonly used antibiotics to overcome the development of microbial resistance. Moreover, this can allow minimization of adverse effects caused by those antibiotics when used as combination agents by reducing the doses of both.

To sum up, findings of our study do not readily allow us to conclude for sure that ENLE has the similar or better antibacterial effect on *E. coli* , *in vitro*, than that of ciprofloxacin. We strongly recommend that larger studies on ENLE on some more pathogenic bacteria, extra to *E. coli* should be carried out in diverse geographic conditions prior to refute or agree to our findings.

Conclusion:

With the increasing concentration of ENLE showed increasing growth inhibition of *E. coli*. This preliminary *in-vitro* experiment suggests ENLEs has antimicrobial efficacy against *E. coli* although not as potent as ciprofloxacin.

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