

# Research Article

Malays. j. med. biol. res.



## Antibacterial effect of Aqueous Neem (*Azadirachta indica*) leaf extract, crude neem leaf paste, and Ceftriaxone against *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*

Shamima Sultana<sup>1</sup>, A.S.M. Shahidullah<sup>2</sup>, Md. Mahbulul Islam<sup>3\*</sup>, A.F.S.A. Wasey<sup>4</sup>, Shamsun Nahar<sup>5</sup><sup>1</sup>Associate Professor, Department of Pharmacology, Mymensingh Medical College, Mymensingh, BANGLADESH<sup>2</sup>Assistant Professor, Department of Community Medicine, SSNIMC, Kishoregonj, BANGLADESH<sup>3</sup>Chief Scientific Officer, Agronomy Division, Bangladesh Jute Research Institute, Manikmia Avenue, Dhaka, BANGLADESH<sup>4</sup>Consultant, Department of Microbiology/Clinical Pathology, United Hospital, Dhaka, BANGLADESH<sup>5</sup>Professor Pharmacology, Department of Pharmacology, Mymensingh Medical College, Mymensingh, BANGLADESH

\*Correspondence (Email):

mahbulul\_agronomy@yahoo.com

### ABSTRACT

The study was conducted during the period of July 2004 to June 2005 in the Department of Pharmacology & Therapeutics in collaboration of Department of Microbiology, Mymensingh Medical College, Mymensingh to determine the profile of antibacterial effect of crude neem leaf paste (CNLP), aqueous neem leaf extract (ANLE), and standard antibiotic Ceftriaxone against *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*. Five separate experiments were done e.g. I) Determination of inhibitory effect of crude neem leaf paste (CNLP) by incorporation into nutrient agar media (NA), against *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*. II) Determination of minimum inhibitory concentration (MIC) of aqueous neem leaf extract (ANLE) against that three test organisms by broth dilution technique, III) Determination of minimum inhibitory concentration (MIC) of standard antibiotic ceftriaxone against test organisms by broth dilution technique as well as making a comparison with MIC of ANLE and IV) Subculture study of materials from effective CNLP, ANLE, NLEE and Ceftriaxone in nutrient agar medium for confirmation of respective results of different experiments conducted. Results revealed that inhibitory effects were observed against the growth of *Staph. aureus*, *Esch. coli* and *Ps. aeruginosa* at 15%, 20% and 25% respectively of CNLP incorporated into NA media. The broth dilution technique was followed to determine the MICs of ANLE and Ceftriaxone. The MIC of ANLE was 714 µg/ml against *S. aureus* and that against *E. coli* and *P. aeruginosa* was 1428 µg/ml. The MIC of Ceftriaxone was 10µg/ml against *S. aureus* and that against *E. coli* and *P. aeruginosa* was 25 µg/ml. The MIC of Ceftriaxone was the lowest in comparison to MICs of ANLE. The subculture study showed similar results with that of previous experiments in terms of inhibitory effects of CNLP and MICs of ANLE, Ceftriaxone against all of the organisms studied.

**Key words:** Antibacterial effect, crude neem leaf paste (CNLP), Aqueous neem leaf extract (ANLE), Ceftriaxone (antibiotic), Minimum inhibitory concentration (MIC), *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*

6/30/2016

Source of Support: Nil, No Conflict of Interest: Declared

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### INTRODUCTION

Bangladesh is enriched for many kinds of medicinal plants. The sources of many of the modern medicine are these medicinal plants e.g. the cardiac glycosides, morphine, atropine, castor oil, acetyl salicylic acid (aspirin) Quinine, etc. Gani (2003) stated that approximately 25% of the people of Bangladesh get modern medical treatment and 75%, remain deprive of it and they depend on depend on traditional medicine to maintain their sound health. Not only in

Bangladesh, but in many other countries of the world, use of traditional medicine is increasing. In USA the use of herbal medicine is increased 38% within 1990-1997 and are used as non prescription drugs. In Germany 70% of the people are fond of herbal medicine in their primary treatment (Gani, 2002). In India herbal medicinal treatment is very much popular. The people of Bangladesh have been using herbal medicine for decades.

Gani (2003) showed that the herbal medicine shares side by side with modern medicine. The budget of health care system in Bangladesh is poor. The traditional medicine are cost effective with low socio-economic condition of poor people in Bangladesh. But all of its constituents are not scientifically evaluated. Dahanukar (2000) reported evidence of immense potential of medicinal plant used in various traditional systems.

Tewari (1992) stated that Neem is a versatile multipurpose tree of tropics. Its pharmacological properties are played the role of "village dispensary" in India. Every part of the tree finding use in one way or other, the Neem is a very important tree due to its medicinal value. Natarajan *et al.* (2003) had stated in his paper that the medicinal properties of *A. indica* (*neem*) were studied by several workers. The antipyretic effect, antimalarial effect, antitumor effect, antiulcer effect, antidiabetic effect, antifertility effect, effect on central nervous system and cardiovascular effect were some of the studies of the earlier workers. Antimicrobial properties of *A. indica* were studied by several authors. Roa *et al* reported the antimicrobial activity of the neem seed oil against a variety of pathogens. The people of Bangladesh use Neem leaves traditionally for different skin diseases, fungal and microbial infections. We planned the present study to find out the antibacterial effect of Neem leaf extract against *S. aureus*, *E. coli*, and *P. aeruginosa*. If we find any antibacterial effects of Neem leaf extract, it will definitely reduce the primary health care cost and random use of antibiotics.

Neem Tree Farms Company (2005) stated about mechanism of action of Neem that while scientists have not yet determined how Neem works. It kills or slows the growth of many disease-causing organisms such as bacteria, virus and fungi. Upadhyay (1998). Investigations on the immunomodulatory properties of Neem with a view to developing neem based products for human use. The results show that neem oil components activate macrophages and stimulate "T" cell response leading to enhanced cell mediated immunity (CMI). Intrauterine neem treatment (IUNT) may also protect against sexually transmitted diseases (STDs). Kabra and Upadhyay (2000) stated that neem does not kill insects- at least not immediately instead it both repels and disrupts their growth and reproduction. Crude neem extracts absorb the viruses, effectively preventing them from entering unaffected cells. About toxic effects, Neem Tree Farms Company (2005) stated that neem is generally considered to be one of the safest of available medicinal herbs. In neem leaf the following active ingredients are present e.g. Nimbin, Nimbidin, Nimbidol, Gedonin, Sodium nimbinat, Quercetin, Salannin, Azadirachtin etc.

Therefore the study was undertaken to determine the inhibitory effect of CNLP incorporating into solid media against *S. aureus*, *E. coli* and *P. aeruginosa*, to investigate the antibacterial effect of ANLE against the organisms by determining its MIC and to assess the MICs of standard antibiotic Ceftriaxone against test organisms and comparison of MICs of ANLE with Ceftriaxone.

## MATERIALS AND METHODS

The study was carried out in the Department of Pharmacology & Therapeutics in collaboration of Department of Microbiology, Mymensingh Medical College, Mymensingh, during the period from July 2004 to June 2005. The present study was designed as a prospective observational one. Recently picked up fresh mature deep green leaves of Jati Neem commonly found in Trishal Upazilla, Mymensingh, Bangladesh. *Ceftriaxone*: A standard antibiotic of third generation of Cephalosporin (Injection form), 500mg/vial was bought from local market, manufactured by Square Pharmaceuticals LTD., Bangladesh. Test organisms were standard reference strains of *Staphylococcus aureus*, ATCC 25923; *Escherichia coli*, ATCC 25922 and *Pseudomonas aeruginosa*, ATCC 27853. The all of the test organisms were collected from the Department of Microbiology, Mymensingh Medical College, Mymensingh. Four separate experiments were done viz., i) Determination of inhibitory effects of crude neem leaf paste (CNLP) by incorporation into nutrient agar media (NA) against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, ii) Determination of minimum inhibitory concentration (MIC) of aqueous neem leaf extract (ANLE), against test organisms by broth dilution technique, iii) Determination of MIC of standard antibiotic Ceftriaxone against test organisms by broth dilution technique as well as making a comparison between MICs of ANLE and Ceftriaxone, and iv) Subculture study of materials from effective CNLP, ANLE, and Ceftriaxone in nutrient agar medium for confirmation of respective results of previous experiments I, II, III.

*Procedure of Experiment -I: Inhibitory effects of CNLP against test organisms*

*Technique: By incorporation of CNLP into Nutrient agar media*

*Preparation of CNLP: Collected Neem leaves were cleaned and washed with plain tap water and dried in shade spreading them on three large trays overnight at normal room temperature in air. The dried Neem leaves were crushed well in mortar under pestle, thus the CNLP was prepared. By weighing with electric balance 500 grams of CNLP were taken for the experiment – I. This CNLP was considered as 100% crude neem leaf paste.*

**Sterilization:** Hot air oven: Glass made materials e.g petri dishes, beaker, flat bottomed flasks, measuring cylinder and test tubes, used in the experiment were sterilized by hot air oven, placing them into hot air oven at 160°C for one hour. Autoclave: The flask containing the CNLP incorporated into nutrient agar medium used in the experiment - I was sterilized by autoclave.

**Preparation of NA media:**

Composition (Oxoid)	grams per litre
Lab lemco powder	1.0
Yeast extract	2.0
Peptone	5.0
Sodium chloride	5.0
Agar	15.0

According to manufacturers instructions 28gms of dehydrated nutrient agar (Oxoid) were measured by an electric balance and taken in a flask. This powder was to be suspended in 1 litre or 1000ml DW. Now the preparation was brought to boil to dissolve the medium completely. The medium was sterilized by autoclaving under 15lbs pressure at 121°C temperature for 15minutes.

After sterilization the flask containing the media was allowed to cool in a water bath at 50 – 55°C. Then the NA media was dispensed in sterile petri dishes (approximately 10ml in 90 mm petri dishes). Prepared media plates may be stored up to one week at 4°C in a refrigerator. Their surfaces should be dried before use.

*Preparation of CNLP incorporated into Nutrient agar (NA) media set I:*

Set – I: To prepare 0.5% CNLP incorporated into NA media, 0.5grams of CNLP was taken in a measuring cylinder. Then NA medium (dissolved) was added with CNLP contained in the measuring flask up to 100ml and this solution was poured in flat bottomed flask and autoclaved for sterilization. After autoclaving the CNLP incorporated into NA media was allowed to cool in water bath around the temperature of 50 – 55°C. Then the media was dispensed in previously sterilized petri dishes (approximately 10ml in each of 90mm petri dishes).

Similarly 1gm, 2gm, 5gm, 10gm, 15gm, 20gm, 25gm, 30gm and 35gms of CNLP were added with dissolved NA media separately to make the volume of each solution up to 100ml and thus 1%, 2%, 5%, 10%, 15%, 20%, 25%, 30%, and 35% respectively CNLP incorporated NA media were prepared & were designed as set – I to set – X. The control NA media were also prepared without adding any CNLP with NA media in set – XI (Table 1).

Table 1: Composition of different percentages of CNLP incorporated into NA media

No. of Sets	CNLP gm	Solution containing CNLP in NA media (dissolved) ml	Percentage of CNLP incorporated into NA media %	Test organisms
Set – I	0.5	100	0.5	One loop full *
Set – II	1	100	1	One loop full
Set – III	2	100	2	One loop full
Sets – IV	5	100	5	One loop full
Set – V	10	100	10	One loop full
Set – VI	15	100	15	One loop full
Set – VII	20	100	20	One loop full
Set – VIII	25	100	25	One loop full
Set – IX	30	100	30	One loop full
Set – X	35	100	35	One loop full
Control				
Set – XI	-	100	-	One loop full

\*One loop full = 20 µl

*Preparation of inoculums or bacterial suspension:* For each of the test organisms the separate inoculums were prepared in the following ways. 3 – 5 similar colonies from 18 – 24 hours old agar plates were picked up with sterile wire loop and mixed with sterile saline. The turbidity was adjusted with 0.05 McFarland standards by adding saline or colony to the suspension. The turbidity of 0.05 (one half of) McFarland standard corresponds to  $1.5 \times 10^8$  organisms/ml

*Inoculation:* A sterile cotton swab was dipped in the bacterial suspension and pressed against the wall of the test tube to remove excess fluid. The entire surface of the CNLP incorporated NA media was inoculated by streaking with the swab. It was repeated more than two times by rotating the plates approximately at 60° angle. Finally the rim of the plate was inoculated. Bacterial suspensions were also inoculated in control plates.

The plates were inverted and their strength were marked with black marker pen and placed in the incubator at 37°C for 18-24 hours. After incubation at 37°C the growth of test organisms were examined by naked eyes. Control plates without incorporated CNLP were also examined for growth of test organisms (Table – II)

Procedure of Experiment – II: Determination of MICs of ANLE against *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginos* by broth dilution.

Syringe filter: ANLE (35.7 mg/ml) was sterilized by membrane filtration using a 20ml syringe filter. Prior to filtration, the membrane filter along with the apparatus was autoclaved. ANLE was poured in the syringe and pressure from the back was applied by piston to quicken filtration in a flat bottomed flask & by hot air oven & autoclave as before.

*Preparation of ANLE:* Six hundred grams of Neem leaves after cleaning, washing and drying were crushed well in mortar under pestle. Two hundred and eighty (280)gms of crushed Neem leaves were measured in an electric balance and was taken in a beaker and dissolved in 560ml of sterile DW and mixed well by stirring and placing the beaker on the laboratory rotator for one hour. Then this preparation was kept at 4°C for 24 hours. Then the preparation was filtered by using a funnel with Whatman filter paper no. – I and collected in a measuring flask. The weight of waste neem leaves after filtration was again measured by the electric balance and was weighed about 260gms. So, (280gm – 260gm) 20gms neem leaf extract was found in 560ml of DW. With ANLE of concentrations 35.7mg/ml thus prepared, 2 ml of ethanol was mixed (as preservative) to prevent fungal growth. This ANLE was preserved at 4°C to determine its MIC against test organisms (Calculations were attached in this chapter in page no.34). ANLE was sterilized by membrane filter before use (Mrs. Monowara Begum, Director, Plant Extraction Section BCSIR, Dhaka, Bangladesh. Personal communication)

Preparation of Nutrient broth medium

Composition (Oxoid)	grams per litre
Lab lemco powder	1.0
Yeast extract	2.0
Peptone	5.0
Sodium chloride	5.0

*Method of preparation:* According to manufacturers instructions 13gm of dehydrated nutrient broth medium was measured by an electric balance and taken in a flask. This was then suspended in 1 litre or 1000ml DW. Now the preparation was brought to boil to dissolve the medium completely. The medium was sterilized by autoclaving under 15lbs pressure at 121°C temperature for 15minutes. After sterilization the flask containing the media was allowed to cool at room temperature. Then the media was dispensed in measured amounts in 15ml test tubes for the use in different experiments.

*Preparations of stock ANLE:*

*Calculations:*

Crushed neem leaves in gms – waste neem leaves after filtration in gms = NLE in gms

280gm – 260gm = 20gm of neem leaf extract.

560ml of DW contained 20gm of NLE

1 ml DW contained 0.0357gm = 35.7 mg of NLE

10ml of DW contained 357mg of NLE

10ml of sterile ANLE of concentration 35.7mg/ml was mixed with 90ml of sterile DW to make 100ml of stock solution in a sterile beaker.

Now, 100ml of the solution contained 357mg of NLE

1ml of the solution contained 3.57mg of NLE

Now, 1ml of stock ANLE solution contained 3.57mg/ml NLE.

This solution was used as stock solution in the measurement of minimum inhibitory concentration (MIC) of the aqueous neem leaf extract (ANLE). From this stock ANLE different dilutions and concentrations were made.

*Method of preparations of different dilutions and concentrations of stock ANLE:*

Set – I of 1:1.25 dilution of stock ANLE was made by mixing 8ml of stock ANLE by a measuring pipette with 2ml of nutrient broth medium (10ml) in a (15ml) test tubes. Now the concentration of this dilution was 2856µg/ml.

1ml of stock ANLE contained 3.57mg of NLE

So, 8ml of stock ANLE contained  $3.57 \times 8 \text{mg} = 28.56 \text{mg} \times 1000 = 28560 \text{µg/ml}$  of NLE.

Now 10ml of the preparations contained 28560 µg/ml of NLE

1ml of the preparations contained 2856 µg/ml of NLE

So, Set – I of 1:1.25 dilution of stock ANLE contained 2856µg/ml of NLE

Similarly, Set - II. of 1:2 dilution of stock ANLE solution was made by mixing 5ml of stock ANLE with 5ml of nutrient broth medium in test tubes. Now the concentration of this dilutions was 1785µgm/ml.

Then Sets – III, IV,V,VI,VII,VIII and IX of 1:2.5dil., 1:5dil. 1:10 dil., 1:20dil., 1:40dil., 1:80dil., and 1:160 dilutions respectively of stock ANLE were made in different test tubes by mixing of measured amount of stock ANLE with measured amount of nutrient broth medium. The concentrations of these sets were 1428 µg/ml, 714 µg/ml, 357 µg/ml, 178.5 µg/ml, 89.25 µg/ml, 44.62 µg/ml and 22.31µg/ml respectively (Table 6.2). Three types of controls were also made. Controls –1 were made each with 10ml of stock ANLE (to be inoculated with bacterial suspension) in test tubes. Controls 2 – were made with 10ml of nutrient broth medium (to be inoculated with bacterial suspension) in test tubes. Controls – 3 were made with 10ml of nutrient broth medium (did not inoculated with bacterial suspension) in test tubes (Table 2).

Table 2: Composition of different dilutions, concentrations of ANLE and the controls

No. of sets	Dilutions of stock ANLE	ANLE ml	Nutrient broth Medium ml	Total ml	Concentration of ANLE µg/ml	Test Organisms µl
Set – I	1:1.25	8	2	10	2856	20
Set – II	1: 2.	5	5	10	1785	20
Set – III	1: 2.5	4	6	10	1428	20
Set – IV	1: 5	2	8	10	714	20
Set – V	1:10	1	9	10	357	20
Set – VI	1:20	0.5	9.5	10	178.5	20
Set – VII	1:40	0.25	9.75	10	89.25	20
Set – VIII	1:80	0.125	9.875	10	44.62	20
Set – IX	1:160	0.625	9.375	10	22.31	20
Controls						
Set – X	Control:1	10	-	10	3570	20
Set – XI	Control:2	-	10	10	-	20
Set – XII	Control:3	-	10	10	-	-

With each 10ml preparation except control 3 (set XII) 20 µl bacterial suspensions were added after matching its opacity with that of McFarland Standard.

#### McFarland Standard Tube No. 1

*Composition and preparation:* It is a turbidity standard. 1% (V/V) solution of chemically pure (0.36 N) Sulphuric acid and 1.175% (W/V) solution of chemically pure (0.048 M) barium chloride was prepared in nutrient broth in two separate sterile flasks. Then 9.9 ml of sulphuric acid and 0.1 ml of barium chloride were added to the clean screw capped test tube and sealed. The barium sulphate suspension corresponds approximately to McFarland standard tube No. I with corresponding cell density of  $3 \times 10^8$  organisms/ml.

To make the turbidity standard of cell density to one half of the McFarland standard tube No. 1 which corresponds to cell density of  $1.5 \times 10^8$  organisms/ml for determination of antibiotic sensitivity by kirby -Baur inoculated technique. Barium chloride ( $\text{BaCl}_2, 2\text{H}_2\text{O}$ ) 0.5 ml of 1.75% (W/V) was added to 99.5 ml of 1% (V/V) sulphuric acid (0.36 N), mixed well and 5-10 ml was distributed in sterile screw capped test tubes and sealed. Preparation of inoculums was same as previously mentioned in experiment I.

*Inoculation of bacterial suspension to different dilutions and concentrations of stock ANLE with nutrient broth media in test tubes:* After matching the turbidity of bacterial suspension with the McFarland standard 20µl or one drop (0.02ml) of bacterial suspension of *Staph. aureus*, *Esch. coli* and *Ps. aeruginosa* were separately added with preparations of each dilutions and concentrations of stock ANLE in separate test tubes by sterilized micropipettes. These inoculums were also added with the controls (1 and 2) except Controls-3. The test tubes were marked set wise with black marker pen. Then these preparations were placed in the incubator at 37°C for 18-24 hours.

*Examination of growth of test organisms in different concentrations of ANLE preparations:* After 18 – 24 hours of incubation at 37°C, the growth of test organisms in each preparations of ANLE were examined and compared against that of controls by matching their turbidity. The clear preparations were considered as no growth of bacteria and turbid

ones, as growth of bacteria. The MIC was reported as lowest concentration of ANLE required to prevent the visible growth of test organisms (Table 3).

*Procedure of experiment-III:* Determination of MIC of Ceftriaxone against test organisms by broth dilution. All the materials were the same as experiment II except stock Ceftriaxone solution and sterile DW instead of ANLE. Sterilization was as experiment II.

*Preparation of nutrient broth medium:* After sterilization by autoclave nutrient broth media was allowed to cool below 40°C to be poured in the test tubes in measured amount to mix with the measured amount of stock solution of Ceftriaxone to prepare its different dilutions and concentrations. Then for stock solution of Ceftriaxone five hundred (500) mg of Ceftriaxone powder in the vial was mixed well with 5ml of supplied sterile DW by supplied syringe. The prepared Ceftriaxone injection had the concentration of 500mg in 5ml. So, 100mg of Ceftriaxone contained in 1ml of sterile DW. Then 1ml of solution was mixed with 9ml of sterile DW. This 1:10 dilution of stock Ceftriaxone solution had the concentration was 10mg/ml. 1ml of this preparation was mixed with 9ml of sterile DW. The concentration of prepared solution of Ceftriaxone was 1mg/ml, which was used as its stock solution for the determination of its MIC.

*Calculations:*

Ceftriaxone 500 mg in 5ml  
500 mg Ceftriaxone in 5 ml.  
100 mg Ceftriaxone in 1 ml.  
↓ 1: 10 dilutions.

Concentration: 10 mg / ml

↓ 1: 10 dilutions

Concentration: 1 mg / ml

1 mg / ml preparation was used as the stock solution of Ceftriaxone for the determination its MIC by broth dilution technique. Different dilutions of this stock Ceftriaxone solution were made to have different concentrations.

*Step – IV: Method of preparation of different dilutions and concentrations of stock Ceftriaxone solution:*

Set – I. of 1:10 dilution of stock Ceftriaxone solution was made by adding 1ml of Ceftriaxone stock solution with 9ml of nutrient broth medium. The concentration of Ceftriaxone in this dilution was 100 µg/ml.

*Calculations: Set I:*

1:10 dil. of stock Ceftriaxone solution contained 1ml of stock Ceftriaxone solution mixed with 9ml of NB medium (10ml).

1ml of stock Ceftriaxone solution contained 1mg or 1000 µg of Ceftriaxone .

Then 10ml of set I preparation contained 1000 µg of Ceftriaxone .

Then 1ml of set I preparation contained 100 µg of Ceftriaxone /ml

Set-II of 1:20 dilution of stock Ceftriaxone solution was made by adding 0.5ml of Ceftriaxone stock solution with 9.5ml of nutrient broth medium. The concentration of Ceftriaxone in this dilution was 50µg/ml.

*Calculations: Set II:*

1:20 dil. of stock Ceftriaxone solution contained 0.5ml of stock Ceftriaxone solution mixed with 9.5ml of NB medium (10ml).

1ml of stock Ceftriaxone solution contained 1mg or 1000 µg of Ceftriaxone.

0.5ml of stock Ceftriaxone solution contained 500 µg of Ceftriaxone .

Then 10ml of set II preparation contained 500 µg of Ceftriaxone .

Then 1ml of set I preparation contained 50 µg of Ceftriaxone /ml

Similarly, Set – III, IV,V and VI of 1:40dil.,1:100 dil.,1:200 dil. and 1:1000 dilutions respectively were made by adding measured amount of Ceftriaxone stock solution with measured amount of broth medium. The concentrations of Ceftriaxone in these dilutions were 25µg/ml, 10µg/ml, 5µg/ml and 1µg/ml, respectively (Table 3). Controls 1- were made with 10ml of nutrient broth medium (to be inoculated with bacterial suspension) in test tubes. Controls-2 was made with 10ml of nutrient broth medium (no to be inoculated with bacterial suspension) in test tubes (Table 3).

Table 3: Composition of different dilutions, concentrations of Ceftriaxone and the controls

No. of sets	Dilutions of stock Ceftriaxone	Ceftriaxone ml	Nutrient broth Medium ml	Total ml	Concentration of Ceftriaxone µg/ml	Test organisms µl
Set – I	1:10	1	9	10	100	20
Set – II	1:20	0.5	9.5	10	50	20
Set – III	1: 40	0.25	9.75	10	25	20
Set – IV	1: 100	0.1	9.9	10	10	20
Set – V	1:200	0.05	9.95	10	5	20
Set – VI	1:1000	0.01	9.99	10	1	20
Controls						
Set – VII	Control : 1	-	10	10	-	20
Set – VIII	Control :2	-	10	10	-	-

With each 10ml preparation except control 2 (set VIII) 20 µl bacterial suspensions were added after matching its opacity with that of McFarland Standard.

Preparation of inoculums was same as in experiment-II.

*Inoculation of bacterial suspension to different dilutions and concentrations of stock Ceftriaxone in test tubes:* Same as previous experiment II. Incubation was same as previous experiments to II.

*Examinations of test organisms in different dilutions and concentrations of Ceftriaxone:* After 18–24 hours of incubation at 37°C, the growth of test organisms in each preparations of Ceftriaxone were examined and compared against that of controls by matching their turbidity. The clear preparations were considered as no growth of bacteria and turbid ones, as growth of bacteria. The MIC was reported as lowest concentration of Ceftriaxone required to prevent the visible growth of test organisms.

Experiment – IV: Subculture study of materials from effective CNLP, ANLE, and Ceftriaxone preparations for confirmation of respective results of experiments I, II & III: Subculture of CNLP materials in NA media: The materials from last two sets of growth and all sets of no growth of CNLP incorporated into NA media were subcultured in the pure NA (solid) media plates (without any incorporation of CNLP). After 18-24 hours of incubation at 37°C, the growth of test organisms were examined.

*Subculture of materials from effective dilutions of ANLE, and Ceftriaxone in NA media:* The materials from last two sets of growth and all sets of no growth of *S. aureus*, *E. coli* and *P. aeruginosa* from dilutions of ANLE, and Ceftriaxone preparations were sub cultured in the pure NA (solid) media plates (without any NLE and antibiotic mixed with the media). After 18 – 24 hours of incubation at 37°C, the growth of test organisms were examined.

## RESULTS AND DISCUSSIONS

The MIC or Minimum Inhibitory Concentration was the smallest concentration of a drug or an antibiotic in the series that prevents the development of visible growth of the test organisms. There was no inhibition of growth of *Staph. aureus*, *Esch. coli* and *Ps. aeruginosa* at .5%, 1% and 2% crude neem (*A. indica*) leaf paste incorporated solid media (Figure 1). The growth of *staph. aureus* was started to be inhibited from 5% and complete inhibition of growth occurred at 15% and above of CNLP incorporated into NA media. There was no inhibition of growth of *Esch. coli* from 0.5% to 5% CNLP incorporated medium. The growth of *Esch. coli* started to be inhibited from 10% CNLP incorporated media and their growths were completely inhibited at 20% and above. The growth of *Ps. aeruginosa* started to be inhibited from 15% and completely inhibited at 25% and above of CNLP incorporated into NA media. Controls without any CNLP show huge growth of all of the test organisms. Crude neem (*A. indica*) leaf paste (CNLP) incorporated into nutrient agar media had a definite inhibitory effects against growth of *Staph. aureus*, *Esch. coli* and *Ps. aeruginosa* at 15% (Set VI), 20%(Set VII), 25%(Set VIII) respectively.

Table 4: Inhibitory effect of CNLP incorporated into Nutrient agar medium against growth of *S. aureus*, *E. coli* and *P. aeruginosa*.

NO. of Sets	Percentages of CNLP incorporated into NA media	Observations		
		<i>Staphylococcus aureus</i>	<i>Escherichia coli</i>	<i>Pseudomonas aeruginosa</i>
Set – I	0.5%	Growth not inhibited	Growth not inhibited	Growth not inhibited
Set – II	1%	Growth not inhibited	Growth not inhibited	Growth not inhibited
Set – III	2%	Growth not inhibited	Growth not inhibited	Growth not inhibited
Set – IV	5%	Medium growth	Growth not inhibited	Growth not inhibited
Set – V	10%	Just visible (scanty) growth	Medium growth	Growth not inhibited
Set – VI	15%*	Growth completely inhibited *	Just visible (scanty) growth	Medium growth
Set – VII	20%*	Growth completely inhibited	Growth completely inhibited *	Just visible (scanty) growth
Set – VIII	25% *	Growth completely inhibited	Growth completely inhibited	Growth completely inhibited *
Set – IX	30%	–	Growth completely inhibited	Growth completely inhibited
Set – X	35%	–	–	Growth completely inhibited
Control				
Set – XI	Media without any CNLP	Huge growth	Huge growth	Huge growth

\* indicates the lowest percentages of CNLP incorporated into nutrient agar media for complete inhibition of growth of test organisms.

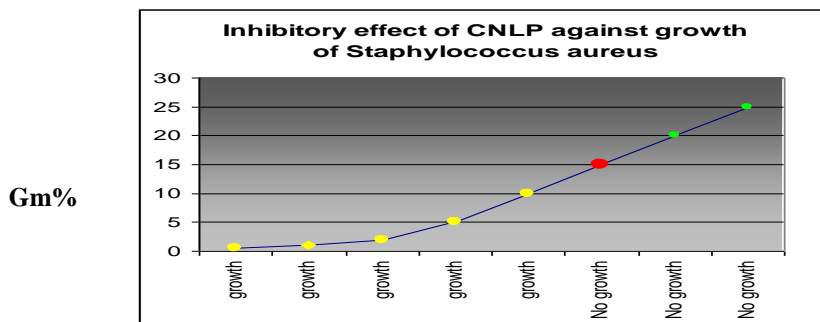


Fig 1: The line graph representing by yellow markings the percentages of CNLP (0.5%-10%) of visible growth and by red and green markings, (15%-25%) of no growth of *S. aureus*. The red marking indicates the lowest percentage of CNLP (15%) showing no growth or complete inhibition of growth of *S. aureus*.

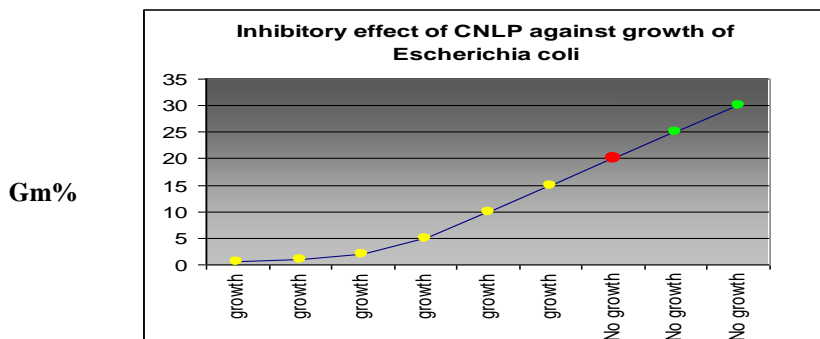
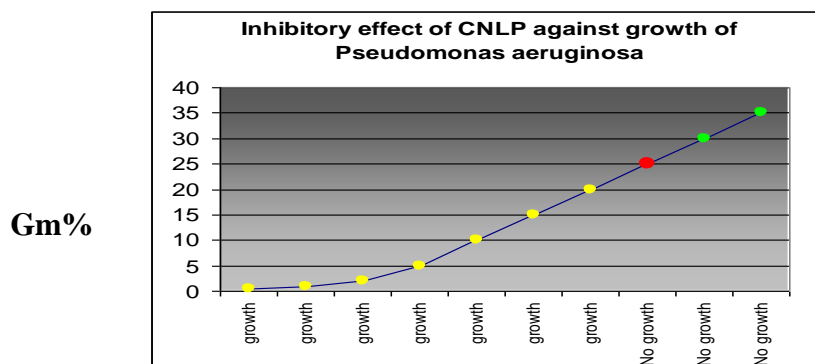


Fig 2: The line graph representing by yellow markings the percentages of CNLP (0.5% - 15%) of visible growth and by red and green markings, (20% - 30%) of no growth of *Esch. coli*. The red marking indicates the lowest percentage of CNLP (20%) showing no growth or complete inhibition of growth of *Esch. coli*.





**Fig 3:** The line graph representing by yellow markings the percentages of CNLP(0.5% - 20%) of visible growth and by red and green markings, (25% - 35%) of no growth of *Ps. aeruginosa*.. The red marking indicates the lowest percentage of CNLP (25%) showing no growth or complete inhibition of growth of *Ps. aeruginosa*.

The full part of neem leaves were used in CNLP incorporated in NA media. The CNLP incorporated into nutrient agar (NA) media of different percentages e.g. 0.5%, 1%, 2%, 5%, 10%, 15%, 20%, 25%, 30% and 35% of were made. *S. aureus* was inoculated in 0.5% to 25%; *E. coli*, in 0.5% to 30% and *P. aeruginosa* in 0.5% to 35% of CNLP incorporated into NA media. The overnight incubated culture plates showed visible growth of *Staph. aureus* in 0.5% to 10% and that failed to grow in 15% to 25%. The lowest percentage at which *S. aureus* failed to grow was 15% CNLP incorporated into NA media. The inoculation of *E. coli* showed visible growth in 0.5% to 15% and no growth in 20%–30%. The 20% CNLP incorporated into NA media was the lowest percentage showing inhibitory effects against *E. coli*. The inoculation of *P. aeruginosa* showed its visible growth in 0.5% to 20% and its no growth from 25% to 35%. The lowest percentage with no growth of *P. aeruginosa* was 25% CNLP incorporated into NA media. Churasia and Jain (1978) investigated inhibitory effects of essential oils of *A. indica* (by disk diffusion method) found that *A. indica* have inhibitory activity against all of the eleven test organisms including *Staph. aureus* and *Esch. coli*. He also stated that similar inhibitory effects were reported by Jain and Agrawal (1976) who found that the leaf extract of "*Azadirachta indica*" possessed an anti-microbial property against the (-) strain of "*Nannizzia gypsea*" and Fries (1937) has reported the essential oils contain sterols and terpenes and these components are the main active ingredients responsible for their anti-microbial activity.

The MIC of ANLE against *S. aureus* was 714 µg/ml of 1:5 dilutions of Set IV and that against *Esch. coli* and *Ps. aeruginosa* were similar and was 1428 µg/ml in 1:2.5 dilutions of Set III Figure 2. Visible growth of *S. aureus* were observed in 1:10 dil. to 1:160 dilutions and the organisms failed to grow in 1:5 dil. to 1:1.25dilutions. So the minimum inhibitory concentration (MIC) of ANLE against of *Staphy. aureus* was in 1:5 dilution of concentration 714 µg/ml. The visible growth of *Esch. coli* and *Ps. aeruginosa* were shown in 1:5 dil. to 1:160 dilutions. When their growth were not visible in 1:2.5 dil to 1:1.25 dilutions. So the MIC of ANLE against *Esch. coli* and *Ps. aeruginosa* was the similar and found in 1:2.5 dilution of concentration 1428 µg/ml (Figure 2). The test organisms failed to grow in controls - 1: containing pure stock solution of ANLE with bacterial inoculations. Controls - 2: containing nutrient broth medium with inoculation of bacteria showed their visible growth and Controls - 3: containing nutrient broth medium without any bacterial inoculation showing no visible growth of test organisms (Table 5).

#### Calculations:

Thus Set - III of 1: 2.5 dilution of stock ANLE being composed of 4ml stock ANLE mixing with 6ml of nutrient broth medium (10ml).

1ml stock ANLE contained 3.57mg of NLE

4ml stock ANLE contained 3.57mg × 4 = 14.28mg of NLE

Now, 10ml of preparation contained 14.28mg of NLE

1ml of preparation contained 1.428mg of NLE × 1000 = 1428 µg/ml.

So, the concentration of 1:2.5 dilutions of stock ANLE was 1428 µg/ml.

Set IV of 1:5 dilution of stock ANLE was composed of 2ml of stock ANLE diluted with 8ml of nutrient broth medium (10ml)

1ml stock ANLE contained 3.57mg of NLE

2ml stock ANLE contained 3.57mg × 2 mg of NLE = 7.14mg of NLE

Now, 10ml of preparation contained 7.14mg of NLE

1ml of preparation contained 0.714mg × 1000 = 714 µg/ml of NLE

So, the concentration of 1:5 dilutions of stock ANLE was 714 µg/ml.

Table 5: MIC of ANLE against of *S. aureus*, *E. coli* and *P. aeruginosa*

No. of Sets	Dilutions of stock ANLE	Concentrations $\mu\text{g/ml}$	Observations		
			<i>Staph. aureus</i>	<i>Esch. coli</i>	<i>Ps. Aeruginosa</i>
Set – I	1:1.25	2856	No growth	No growth	No growth
Set – II	1:2	1785	No growth	No growth	No growth
Set – III	1: 2.5	1428 *	No growth	No growth	No growth
Set – IV	1: 5	714 *	No growth	growth	growth
Set – V	1:10	357	Growth	Growth	Growth
Set – VI	1:20	178.5	Growth	Growth	Growth
Set – VII	1:40	89.25	Growth	Growth	Growth
Set – VIII	1:80	44.62	Growth	Growth	Growth
Set – IX	1:160	22.31	Growth	Growth	Growth
Controls					
Set – X	Control - 1	Pure stock ANLE + Bacterial inoculation	No growth	No growth	No growth
Set – XI	Control 2:	NB Media+ Bacterial inoculation	Growth	Growth	Growth
Set – XIII	Control 3:	NB Media+ No bacterial inoculation	No growth	No growth	No growth

\*= MIC of Aqueous neem leaf extract

ANLE was fully a water soluble extract. The water soluble ingredients of neem leaves were present in ANLE. Using broth dilution technique, the stock solution of ANLE of conc. 3.57 mg/ml was diluted to 1:1.25 dil, 1:2 dil, 1:2.5 dil, 1:5 dil, 1:10 dil, 1:20 dil, 1:40 dil, 1:80 dil. and 1:160 dilutions by mixing different amounts of stock ANLE with different amounts of nutrient broth (NB) medium to prepare different concentrations of solutions such as 2865 $\mu\text{g/ml}$ , 1785 $\mu\text{g/ml}$ , 1428 $\mu\text{g/ml}$ , 714 $\mu\text{g/ml}$ , 357 $\mu\text{g/ml}$ , 178.5 $\mu\text{g/ml}$ , 89.25 $\mu\text{g/ml}$ , 44.62 $\mu\text{g/ml}$  and 22.31  $\mu\text{g/ml}$  respectively. The inoculation of *Staph. aureus* showed visible growth of *Staph. aureus* in 1:10 dil. to 1:160 dil. and that failed to grow in 1:5 dil. to 1:1.25 dilutions. The minimum inhibitory concentration (MIC) of ANLE against *Staph. aureus* was in 1:5 dilution of concentration 714  $\mu\text{g/ml}$ . In case of *Esch. coli* and *Ps. aeruginosa* visible growth was found in 1:5 dilution to 1:160 dilutions and no visible growth, in 1:2.5 to 1:1.25 dilutions of ANLE. So, the MIC of ANLE against both of the organisms was in 1:2.5 dilution of concentration 1428  $\mu\text{g/ml}$ . Rao *et al.* (1986) stated that two hundred clinical isolates of bacteria subjected to antibacterial effects of Neem oil showed 92% susceptibility, the minimum inhibitory concentration (MIC) varying between  $\frac{1}{4}$  to  $\frac{1}{64}$  dil.

The MIC of Neem oil for *Ps. aeruginosa*, ranged from  $\frac{1}{8}$  dil. for 50% of strains to  $\frac{1}{32}$  dil for 40% of strains. For *Staph. aureus* it varied between  $\frac{1}{8}$  dil. for 40% strains to  $\frac{1}{64}$  for 20% strains and for *Esch. coli* it was from  $\frac{1}{32}$  dil. for 70% strains and  $\frac{1}{64}$  dil. for 20% strains. Natarajan *et al.* (2003) used clinical isolates of dermatophytes (*Trichophyton rubrum*, *Trichophyton mentagrophytes* and *Microsporum nanum*) were treated with extracts of leaves and seeds of the plant *Azadirachta indica*, (Neem) for antifungal activity *in vitro* by tube dilution technique and found the MIC of neem seed extracts was 31 mg/ml. for all the dermatophytes tested. Das *et al.* (1999) and Parida *et al.* (2002) reported that Aquaneem, an emulsified product prepared from the neem (*A. indica*) kernel was tested against four pathogenic bacteria of fish (i.e. *Aeromonas hydrophila*, *Pseudomonas fluorescens*, *Escherichia coli* and *Myxobacteria* spp.) to test its efficacy as an antibacterial agent. *A. hydrophila*, *P. fluorescens* and *Myxobacteria* spp. exhibited maximum sensitivity to Aquaneem in terms of percentage reduction of bacterial cell population in comparison to *E. coli*.

The MIC of Ceftriaxone against *S. aureus* was 10  $\mu\text{g/ml}$  in 1:100 dilution of set IV and that against *E. coli* and *P. aeruginosa* was the same and was 25  $\mu\text{g/ml}$  in 1: 40 dilution of set III (Table 6). Visible growth of *S. aureus* was observed in 1:200 dil. and 1:1000 dilutions. But the organisms failed to grow in 1:100 dil to 1:10 dilutions. So the minimum inhibitory concentration (MIC) of Ceftriaxone against *S. aureus* was in 1:100 dilution of concentration 10 $\mu\text{g/ml}$ . Figure 4 also showed visible growth of *E. coli* and *P. aeruginosa* in 1:100 dil. to 1:1000 dilutions. But the organisms failed to grow in 1:40 dil. to 1:10 dilutions. So the MIC of Ceftriaxone against *E. coli* and *P. aeruginosa* was in 1:40 dilution of concentration 25  $\mu\text{g/ml}$  (Figure 4). Controls-1: containing nutrient broth medium without any bacterial inoculation had no visible growth and Controls-2: containing nutrient broth medium with bacterial inoculation observed their visible growth (Table 6).

Set III of 1: 40dilution of Ceftriaxone was composed of 0.25ml of stock Ceftriaxone solution mixing with 9.75ml (10ml) of nutrient broth medium.

1ml stock Ceftriaxone solution contained 1mg of Ceftriaxone

So, 0.25ml stock Ceftriaxone solution contained  $1 \times 0.25\text{mg} = 0.25\text{mg}$  of Ceftriaxone

Now, 10ml of 1:40 dilution contained 0.25mg of Ceftriaxone

So, 1ml of 1:40 dilutions contained 0.025mg of Ceftriaxone =  $0.025 \times 1000 \mu\text{g} = 25 \mu\text{g}$  of Ceftriaxone.

Set IV of 1:100dilution of Ceftriaxone was composed of 0.1ml of stock Ceftriaxone solution mixing with 9.9ml of nutrient broth medium (10ml).

1ml stock Ceftriaxone solution contained 1mg of Ceftriaxone

So, 0.1ml stock Ceftriaxone solution contained  $1 \times 0.1\text{mg} = 0.1\text{mg}$  of Ceftriaxone

Now, 10ml of stock Ceftriaxone with NB media of 1:100 dilution contained 0.1mg of Ceftriaxone.

So, 1ml of 1:100 dilutions contained 0.01mg of Ceftriaxone =  $0.01 \times 1000 = 10\mu\text{g}$  of Ceftriaxone.

Table 6: Ceftriaxone against of *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*

No. of Sets	Dilutions of stock Ceftriaxone	Concentrations µg/ml	Observations		
			<i>Staphylococcus aureus</i>	<i>Escherichia coli</i>	<i>Pseudomonas aeruginosa</i>
Set – I	1:10	100	No growth	No growth	No growth
Set – II	1:20	50	No growth	No growth	No growth
Set – III	1: 40	25 *	No growth	No growth	No growth
Set – IV	1: 100	10 *	No growth	growth	growth
Set – V	1:200	5	Growth	Growth	Growth
Set – VI	1:1000	1	Growth	Growth	Growth
Controls					
Set – VII	Control -1	Nutrient broth medium + no bacterial inoculation	No growth	No growth	No growth
Set – VIII	Control -2	Nutrient broth medium + bacterial inoculation	Growth	Growth	Growth

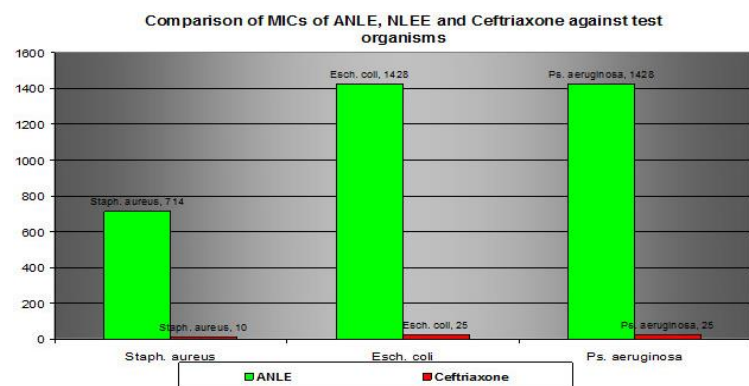
\* = MIC of Ceftriaxone

The standard antibiotic, Ceftriaxone was sensitive to all of the test organisms. So far less concentrations of Ceftriaxone than that of crude ANLE was required to inhibit the growth of all of the test organisms by broth dilution technique. Also the known MIC of Ceftriaxone(30µg/ml) was determined by the same broth dilution technique to assess the method that was used to determine the MIC of ANLE. Using broth dilution technique, stock solution of Ceftriaxone of conc. of 1 mg/ml was diluted to 1:10 dil., 1:20 dil., 1:40 dil., 1:100 dil., 1:200 and 1:1000 dilutions by mixing different amounts of stock Ceftriaxone solution with different amounts of nutrient broth (NB) medium to make the concentrations of the solution 100 µg/ml, 50 µg/ml, 25 µg/ml, 10 µg/ml, 5 µg/ml and 1 µg/ml respectively. The culture of *Staph. aureus* showed visible growth in 1:200 dil and 1:1000 dilution and that failed to grow 1:100 dil to 1:10 dilutions. The minimum inhibitory concentration (MIC) of Ceftriaxone was found in 1.100 dilution of concentration 10 µg /ml against *Staph. aureus*. Against *Esch. coli* and *Ps. aeruginosa* visible growth was found in 1:100 dil to 1:1000 dilutions and that failed to grow in 1:40 to 1:10 dilutions. So, the MIC of Ceftriaxone against both of the organisms was in 1:40 dilution of concentration 25 µg/ml.

Results of Comparative study of MICs of ANLE, and Ceftriaxone: MICs of ANLE and Ceftriaxone against *S. Aureus* were 714µg/ml, and 10 µg/ml respectively against *E. coli* and *P. aeruginosa* MICs of ANLE Ceftriaxone were 1428 µg/ml, and 25 µg/ml respectively. So it was observed that MIC of Ceftriaxone was far less than that of ANLE and against all of the test organisms. Against *E. coli* and *P. aeruginosa* MICs of ANLE and Ceftriaxone by broth dilution technique were the similar (Table 7). The antibacterial effects of CNLP, ANLE Ceftriaxone on test organisms were bactericidal (i.e killing effects). Otherwise results of subculture study did not similar with that of respective results of experiment I and II. If bacteriostatic action, the visible growth of test organisms might be found in the subculture plates in concentrations at which their inhibitory effects were found in respective culture plates in previous experiments. The materials from last two sets of growth and all sets of no growth of CNLP incorporated into NA media were subcultured in the pure nutrient agar (solid) medium (without any incorporation of CNLP). The results of subculture plates coincided with the respective results of experiments I. The materials from last two sets of growth and all sets of no growth of ANLE and Ceftriaxone preparations were subcultured in the pure nutrient agar (solid) medium (without any NLE or antibiotic). The results of subculture plates coincided with the respective results of experiment II. Natarajan *et al.* (2003) and Chaturvedi *et al.* (1978) reported that the MFC was regarded as the lowest concentration of the extract of *A. indica* that prevented the growth of any fungal colony on subculture plates in the solid medium and the MICs and MFCs of ethanol extract, ethyl acetate extract, extract in hexane of neem leaves and seed extract of neem coincided with each other.

Table 7: Comparative study of MICs of ANLE and Ceftriaxone against *S. aureus*, *E. coli* and *P. aeruginosa*

Sl. no	MICs of	Test organisms		
		<i>Staphylococcus aureus</i>	<i>Escherichia Coli</i>	<i>Pseudomonas aeruginosa</i>
I	ANLE	714µg/ml	1428 µg/ml	1428 µg/ml
II	Ceftriaxone	10 µg/ml	25 µg/ml	25 µg/ml



Multiple bar diagram represents the minimum inhibitory concentrations (MICs) of ANLE, NLEE and Ceftriaxone by green, yellow and red bars respectively. The MICs (concentrations: 714 µg/ml, 666 µg/ml and 10 µg/ml) of ANLE, NLEE and Ceftriaxone respectively are against *S. aureus*. The MICs of ANLE, NLEE and Ceftriaxone having the concentrations of 1428 µg/ml, 1332 µg/ml and 25µg/ml respectively are against *E. coli*. The MICs (concentrations: 1428 µg/ml, 1332 µg/ml and 25 µg/ml) of ANLE, NLEE and Ceftriaxone respectively are against *P. aeruginosa*.

Table 8: Subculture study of materials from effective CNLP, ANLE and Ceftriaxone in nutrient agar medium for confirmation of respective results of previous experiments

Test organisms	CNLP		ANLE		CEFTRIAZONE	
	Inhibitory effects against growth	No growth in subculture plates	MIC	No growth in subculture plates	MIC	No growth in subculture plates
	gm/100ml		µg /ml		µg/ml	
<i>Staphylococcus aureous</i>	15%	15%	714	714	10	10
<i>Escherichia coli</i>	20%	20%	1428	1428	25	25
<i>Pseudomonas aeruginosa</i>	25%	25%	1428	1428	25	25

Results of subculture study of materials from effective CNLP and Ceftriaxone in NA media for confirmation of respective results of previous experiments: The lowest percentages of CNLP showing complete inhibition of growth of *S. aureous*, *E. coli* and *P. aeruginosa* in subculture plates were coincided with previous lowest percentages of CNLP incorporated into NA media showing inhibitory effects against all of the test organisms, Similar results were observed in experiment-I. The minimum inhibitory concentrations (MICs) of ANLE and Ceftriaxone were also coincided with results of their subculture in NA media against all of the test organisms. Similar results were observed in experiment-II (Table 8).

## CONCLUSION

From the study it is clearly observed that there is definite antibacterial effects of aqueous neem leaf extract (ANLE) against *Staphylococcus aureus*, *Escherichia coli* and, *Pseudomonus aeruginosa*. The crude neem leaf paste (CNLP) also have its definite inhibitory effects against all of the organisms studied. But the antibacterial effect of neem leaf extract as covered in the study does not fulfilled the criteria for its therapeutic use, only local application can be advised. Further study is required to isolate the active ingredients responsible for antibacterial effects of neem leaves and also to know their efficacy, mechanism of action, toxicological aspect and safety margin for its therapeutic use.

## ACKNOWLEDGEMENT

The authors expressed their cordial thanks and gratitude to Prof. AKM Shamsuzzaman, Prof. of Microbiology, CDC, Dghs, Dhaka, who helped too much in different affairs of the research work and writing this scientific article.

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