Cynodon dactylon: Antimicrobial potential of crude extract as valuable medicinal plant

A DISSERTATION SUBMITTED TO BRAC UNIVERSITY IN PARTIAL FULLFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF BACHELOR OF SCIENCE IN MICROBIOLOGY

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DECLARATION

I hereby declare that the thesis project titled "***Cynodon dactylon*: Antimicrobial potential of crude extract as valuable medicinal plant"** submitted by me has been carried out under the supervision of Dr.Zeenat Jahan, Assistant Professor, Microbiology Program, Department of Mathematics and Natural Sciences, BRAC University, Dhaka. It is further declared that the research work presented here is based on actual and original work carried out by me. Any reference to work done by any other person or institution or any material obtained from other sources have been duly cited and referenced.

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Abstract

Cynodon dactylon (Poacae) is a well known traditional plant used as a folk remedy in treatment of many symptoms and diseases like cramps, measles, tumors, wounds, warts, fever and rheumatic affections. In this study, the antimicrobial activity of the plant crude extract from three different extraction (hot and cold aqueous extraction, methanol extraction) was investigated against some of the gram positive bacteria (*Staphylococcus epidermidis, Bacillus cereus*) and gram negative bacteria (*Escherichia coli, Pseudomonas aeroginosa, Salmonella typhi, Shigella dysenteriae*) using disc diffusion method. Amoxicillin and Gentamicin were taken as positive control. The diameter of the clear zone of inhibition surrounding the disc was measured. The aqueous extract of *Cynodon dactylon* had antimicrobial activity against all the test organisms indicating broad spectrum activity of the extract for both gram positive and gram negative bacteria. No clear zone formed with methanol extract. It can be concluded that aqueous extract of whole plant of *Cynodon dactylon* may be considered as an antibacterial agent and can be used to source antibiotic substances for possible treatment of bacterial infections.

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LIST OF ABBREVIATIONS

BPA	Baird-Parker Agar
CNS	Central Nervous System
EMB	Eosin Methylene Blue Agar
ICDDR,B	International Center for Diarrheal Disease Research, Bangladesh
IMViC	Indole, Methyl red, Voges-Proskauer, Citrate
LB	Luria Bertani
MAC	MacConkey Agar
MR	Methyl Red
MSA	Mannitol Salt Agar
MYP	Mannitol Egg Yolk Polymyxin Agar
NA	Nutrient Agar
TSI	Triple Sugar Iron
TSB	Trypticase Soy Broth
UTIs	Urinary Tract Infections
XLD	Xylose Lysine Deoxycholate

Chapter 1: Introduction

1. Introduction

Herbal products were being the effectual source of both traditional and modern medicines which are used widely to treat several medical problems. It is evident that the plant kingdom contains enormous and inexhaustible source of active ingredients vital in the management of many diseases. Use of plants as to cure health related problems in the traditional way is very popular and important for 80% population of the world's population in African, Asian, Latin America and Middle Eastern countries. Use of such plants has minimal side effects. In recent years, pharmaceutical companies spent substantial amount of time and money in developing therapeutic products which is based upon natural products extracted from plants [Ben Sassi *et al.,* 2007 and Coruh *et al.,* 2007]. Whole plant of the *Cynodon dactylon* is traditionally used to treat painful and inflammatory condition.

1.1 Green medicine Vs synthetic medicine:

In the present time multiple drug resistance in microbial pathogens become a serious health problem to humankind worldwide [Peng *et al.,* 2006]. It is aroused due to indiscriminate and repetitive use of antimicrobial drugs [Shariff., 2001]. Synthetic drugs are not only expensive and inadequate for the treatment of diseases but also often associated with adulterations and side effects. Therefore, there is need to search new infection fighting strategies to control microbial infections. However, the past record of rapid, widespread emergence of resistance to newly introduced antimicrobial agents indicates that even new families of antimicrobial agents will have a short life expectancy while there are some advantages of using medicinal plants, such as often fewer side effects, better patient tolerance, relatively less expensive, acceptance due to long history of use and being renewable in nature. For these reasons, researchers are increasingly turning their attention to herbal products looking for new leads to develop better drugs against multiple drug resistant microbial strains [Benkeblia., 2004]. Plant materials continue to play an important role in the maintenance of human health since antiquity. Over 50% of all modern chemical drugs originated from natural plant sources. These plant products are the major source of drug development in pharmaceutical industry [Burton, G.W., 1983]. Several plants are now being used in part or as a whole to treat many diseases. Active components of these plants are now being investigated, extracted and developed into drugs with little or no negative effects or contra-indication [Oluyemi, K. A., 2007]. Rural dwellers in most parts of the world do not depend on the orthodox medicine for the cure of diseases and aliments. As a result of this, a larger section has resulted to the use of

traditional medicines, which are believed to be less expensive, and of little or no side effects. One of such plant considered of great importance is *Cynodon dactylon* a creeping grass found in warm climates all over the world between 450 south and north altitude. The *Cynodon dactylon* is available throughout the year; the material is used by the domestic animals as food.

1.2 General Characteristics of *Cynodon dactylon* –

The Grass *Cynodon dactylon* sp. is also known as the Bermuda grass or the Doob grass is a creeping grass, light green in color, very tough and has a rough texture. It taxonomically belongs to the family Graminae/Poaceae. It consists of three parts i.e root, stem and leaves. It is fast growing and its root grows where ever a node touches the ground, forming a thick mat. It is drought resistant, but it is not very shade tolerant and appears in short cylindrical pieces about 3 to 20 mm long & 2 to 3 or sometimes 4 mm in diameter. The stems are slightly flattened, and are blossoming purple in color. Its Surface is hard, smooth, uncovered, longitudinally furrowed and yellow to yellowish brown in color and does show the presence of node on some pieces. It has a sweet gelatinous taste. The grass has different vernacular names. Bengali, it is commonly known a**s-** Durva, Dub, Dubla, Durba, Doorva, Neel Doorva. [Asthan *et al*., 2012] In English,it is called as the Creeping panic grass, Couch grass, Bahama grass, Bermuda grass, Dun grass, Devil's grass, Doab grass, Doorwa, Dog's teeth grass and in In Hindi, it is known by the following names such as- Doob, Dub, Dubra, Khabbal, Kaligas, Neelee Doob.

1.3 Scientific Classification

C. dactylon was described on the basis of its taxonomic position [Kumar *et al.*, 2011]

- Kingdom: Plantae- Plants.
- Subkingdom: Tracheobionta- Vascular plants.
- Super division: Spermatophyta- Seed plants.
- Division: Magnoliophyta- Flowering plants.
- Class: Liliopsida- Monocotyledons.
- Subclass: Commelinidae.
- Order: Cyperales.
- Family: Poaceae- Grass family.

- Genus: Cynodon Rich.- Bermuda grass.
- Species:*Cynodon dactylon*

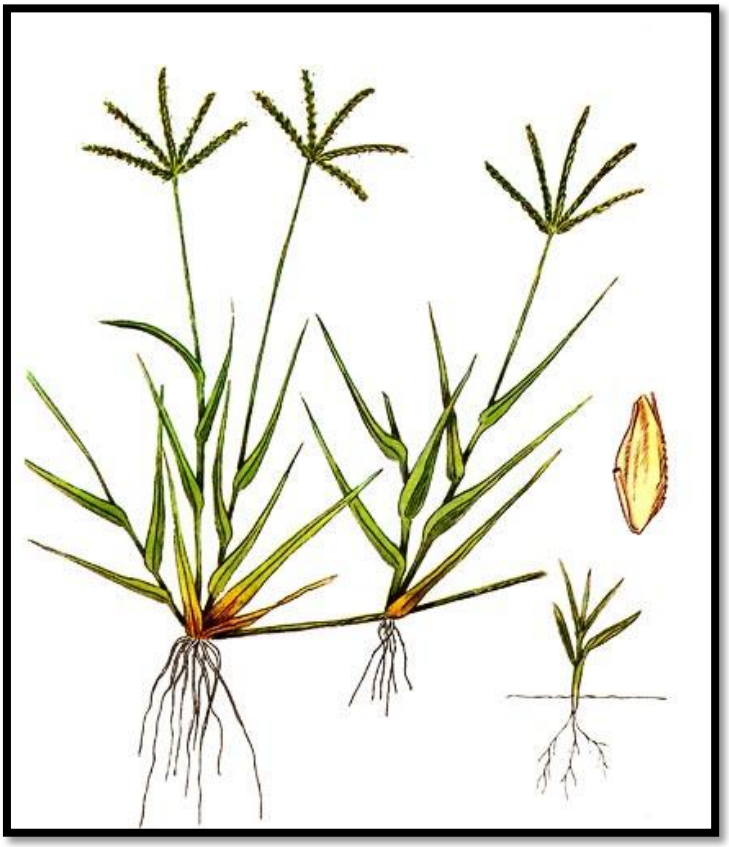


Figure1: *Cynodon dactylon*

1.4 Botanical Description of *Cynodon dactylon*:

A perpetual creeping herb, stem (culms) lean and wiry. Leaves are 2-10 cm x 1.25-3 mm, narrowly linear or non-subdivided, acute and soft. It contains spikes 2-6, diverging from slender ascending peduncle, green or purplish. Grains are 1.05 mm long. Flowering and Fruiting time is August- October (also throughout the year). Other characteristics are stated bellow,

Root – Fibrous, cylindrical, up to 4 mm thick, minute hair like roots arise from the main roots; cream coloured.

Stem – willowy, horizontal, up to 1 mm thick, jointed, leafy, very smooth, yellowish-green in colour.

Leaf – 2 to 10 cm long and 1.25 to 3 mm wide, narrowly linear or unsubdivided, finely acute more or less opaque, usually conspicuously opaque in the barren shoots and at the base of the stem; covered light, sometimes bearded, ligule a very fine ciliate rim. [Asthan *et al*., 2012]

1.5 Chemical Constituents

The chemical constituents present in *Cynodon dactylon* are – β- sitosterol, β- carotene, vitamin C, palmitic acid, triterpenoids, arundoin, friedelin, selenium, alkaloids- ergonovine ergonovinine, ferulic, syringic, p- coumaric, vanilic, p hydroxybenzoic and o-hyroxyphenyl acetic acids, cyanogenic hyperoside, cyanogenic glucoside- triglochinin, furfural, furfural alcohol, phenyl acetaldehyde, acetic acid, phytol, β- ionone; mono and oligosaccharides, lignin (wholeplant); hydrocarbons (tritriacontane) esters, eicosanoic and docosanoic acids,[14-18] freealcohol, free aldehydes (hexadecanal) and free acids (hexadecanoic acid) (surface cuticularwax); flavone – apigenin, luteolin, flavone glycosides – orientin (8-C-β-D-glycosyl luteolin),vitexin (8-C-β-D-glycosyl apigenin), iso –orientin (6-C-β-D-glycosyl luteolin) and iso- vitexin (6-C-β-D-glycosyl apigenin) (aerial parts). [Ashthan *et al*., 2012]

1.6 **Pharmacological Activity**

The grass has various pharmacological activities. The dried extracts of aerial parts of *Cynodon dactylon* was examined for CNS activities in mice. Antidiabetic, antiulcer, analgesic and anti-pyretic, diuretic and antimicrobial activity are some of the various essential functions of it. *Cynodon dactylon* is very effective in snakebite therapy and the anti snake venom from the plant extract is very effective to treat patients who are bitten by a snake. The grass is used as a traditional folk medicine in India and many other places for the treatment for various diseases and disorders. Other prominent activity includes anti-inflammatory and antioxidant activity. [Kumar *et al*., 2011]

Table 1. Traditional uses of medicinal plant used in the present study [Hema *et al*., 2013]

Botanical name	Family	Traditional uses
Cynodon dactylon	Poaceae	Urinary tract infections,syphilis, tooth ache, dysentery, prostatitis

1.7 Antimicrobial Activity

Cynodon dactylon is a type of perennial grass that possesses great medicinal values. In this study, the antimicrobial activity of the grass, taking the three different extracts i.e, Cold aqueous, Hot aqueous and Methanol extract which was investigated against some pathogens (*Bacillus cereus, Escherichia coli K12 and LTST, Pseudomonas aeruginosa, Staphylococcus aureus, Shigella dysenteriae and Salmonella typhi*) using disc diffusion method. These organisms were tested against the antibiotics namely, Amoxillin and Gentamicin and was taken as standard to compare against the antimicrobial activity of the grass [Abdullah *et al*., 2013]

Table 2. Profile of Clinical pathogens used in this study [*Hema et al.*, 2013]

S.No	Test pathogens	Infections
1.	*Escherichia coli*	causes urinary tract and wound infections, also problems after surgery
2.	*Pseudomonas aeruginosa*	Causes severe infections in burn victims, cancer patients, and cystic fibrosis.
3.	*Salmonella typhi*	causes food poisoning and typhoid
4.	*Shigella dysentery*	causes dysentery
5.	*Bacillus cereus*	causes skin infetion, food poisoning, food-borne intoxications and also causes ocular infections
6.	*Staphylococcus epidermidis*	Catheter infections along with catheter-induced UTIs lead to serious inflammation and pus secretion, Septicemia and endocarditis

1.8 Objectives

In this study, the three different extracts of the *Cynodon dactylon* (hot aqueous, cold aqueous and methanol extract) was investigated for their antibacterial activity against the gram positive bacteria (*Staphylococcus epidermidis, Bacillus cereus*) and gram negative bacteria (*Escherichia coli, Pseudomonas aeroginosa, Salmonella typhi, Shigella dysenteriae*). Although pharmaceutical industries have produced a number of new antibiotics in the last few decades but simultaneously the resistance to these drugs by the microorganisms have also increased. Due to such increasing resistance in microbes and synthetic antibiotic side effects, such medicinal plants are increasing importance for the treatment of bacterial infections.

On the basis of above context, the objectives of the present study are:

- To determine the antimicrobial activity of different extract of *Cynodon dactylon* against several pathogenic bacteria.
- To compare the extract with standard antibiotic against both gram negative and gram positive bacteria.

Chapter 2: Materials and Methods

2.1 Working Place

Overall research was performed in the Microbiology Specialized Research Laboratory, Department of Mathematics and Natural Sciences, BRAC University.

2.2 Bacterial strains

In this study, several bacterial species were used such as *Escherichia coli* LTHT, *Escherichia coli* Non Pathogenic strain K12, *Bacillus cereus*, *Salmonella typhi*, *Shigella dysenteriae*, *Pseudomonas aeroginosa* and *Staphylococcus epidermis.* All these species were obtained from ICDDR,B (International Center for Diarrheal Disease Research, Bangladesh)**.**

2.2.1 Preparation for reviving the bacteria

1. Nutrient agar was prepared for each of the seven microorganisms to be revived.

2. The media was prepared and autoclaved at 121 °C for 15 minutes (SAARC)

3. After incubation, each of the organisms from their respective glycerol stock culture was streaked on nutrient agar plate.

4. The plates were incubated at 37 °C for 24 hours.

2.2.2 Confirmation of plating bacteria

The revived bacteria from the nutrient agar plate were confirmed by streaking in the respective selective media. The media used for the respected bacteria were as follows:

- *E.coli* LTST and Non pathogenic strain K12: MacConkey agar and Eosin-Methylene blue (EMB) agar
- *Pseudomonas aeroginosa*: Cetrimide agar
- *Salmonella typhi:* Xylose Lysine Deoxycholate agar (XLD) agar
- *Shigella dysenteriae:* MacConkey (MAC) and Xylose Lysine Deoxycholate (XLD) agar
- *S. epidermidis:* Mannitol Salt agar (MSA)
- *Bacillus cereus:* Mannitol-Egg Yolk-Polymyxin Agar (MYP)

2.2.3 Preparation of stock sample

For short-term preservation, 3 ml of T_1N_1 agar butt in a vial was inoculated by stabbing bacterial growth of each isolate from nutrient agar plate. Then the vial was kept at 4^{o}C for an hour to gelatinize. After an hour, the surface of the medium was covered with sterile paraffin oil and the vial was stored at room temperature and at -20 ^{O}C as well.

Long-term preservation

For long-term preservation, 500 µl of bacterial culture grown in Trypticase Soy Broth (Oxoid, England) at 37^{o}C for 6 hours was taken in a sterile cryovial. Then 500 µl of sterile glycerol was added to the broth culture and the cryovial was stored at -20 ^{o}C.

2.2.4 Biochemical Identification

Biochemical tests were performed according to the methods described in Microbiology Laboratory Manual [Cappuccino *et al*., 2005]. The biochemical tests carried out were Citrate utilization, Indole production, Methyl-red, Voges- Proskauer, Triple sugar iron, Oxidase, Catalase, Carbohydrate fermentation test.

Citrate utilization Test

- Colorless bacterial colonies were picked from the Nutrient agar plate by a straight wire and inoculated into the slope of Simmon's citrate agar (Oxoid ltd, England) and incubated overnight at 37 ^{o}C.
- If the organism had the ability to utilize citrate, the medium changed its color from green to prussian blue; a negative slant would have no growth of bacteria and would remain green.

Indole production Test

- Colorless bacterial colonies were picked from the Nutrient agar plate and were inoculated in peptone water which contains amino acid tryptophan and incubated overnight at 37^{o}C.
- Following incubation a few drops of Kovac's reagent were added.
- Detection of positive result would give a red layer at the top or a negative result had a yellow or brown layer.

Methyl red (MR) Test

- The bacterium to be tested was inoculated into potassium phosphate broth (MR-VP broth), which contained dextrose, peptone and potassium phosphate and incubated at 37 ^{o}C for 24 hours.
- Over the 24 hours the mixed-acid producing organism might produce sufficient acid to overcome the phosphate buffer and remained acidic.
- The pH of the medium was tested by the addition of five drops of MR reagent. Development of red color was taken as positive. MR negative organism produced yellow color.

Voges-Proskauer Test

- Bacterium to be tested was inoculated into potassium phosphate broth (MR-VP broth) and incubated for 24 hours.
- Barritt's reagent A was added to the test broth and shaken.
- Barrit's reagent B was added and the tube was allowed to stand for 15 min.
- Appearance of red color was taken as a positive test, negative tube might be held for an hour after addition of reagents.

Triple Sugar Iron (TSI) Test

- To inoculate, colorless isolated colony from the Nutrient agar plate was picked with a cool, sterile needle, stabbed into the TSI, (Himedia, India) containing dextrose, lactose and sucrose butt.
- Incubated with caps loosened at 37°C for overnight and examined after 24 hours for carbohydrate fermentation, CO_2 and H_2S production.
- A yellow (acidic) color in the butt indicated that the organism being tested capable of fermenting all the three sugars, whereas red (alkaline) color in the slant and butt indicated that the organism being tested is a non fermenter.

- Detection of H_2S production identified by black precipitation in the butt of the tube.
- CO_2 Gas production was indicated by splitting and cracking of the medium.

Oxidase Test

- A loopful of bacteria from the Nutrient agar plate was streaked onto a piece of filter paper (Whatman, 1MM).
- A few drops of oxidase reagent (*N,N,N′,N′*-tetramethyl-*p*-phenylenediamine) were added onto the streaked bacteria on the filter paper. Positive reactions turned the bacteria from violet to purple within 1 to 30 seconds. Delayed reactions should be ignored.

Catalase Test

- A small amount of bacterial colony was transferred from the Nutrient agar plate to a surface of clean, dry glass slide using a clean toothpick.
- A drop of the catalase reagent (Hydrogen Peroxide) was placed on to the slide and mixed.
- A positive result gave a rapid evolution of oxygen within 5-10 seconds and was evidenced by bubbling reaction.
- A negative result showed no bubbles or only a few scattered bubbles.

Carbohydrate fermentation Test

- The Durham tubes should be inserted in an inverted position into all the tubes, fully filled with broth(Lactose, Dextrose and Sucrose)
- Each labeled carbohydrate broth (Lactose, Dextrose and Sucrose) was inoculated aseptically with each of the seven bacterial cultures.
- After inoculation into a particular sugar, the loop was sterilized in order to avoid cross contamination of the tube with other sugars.
- The tubes were incubated for 24 hours at 37 ^{o}C.

- Following incubation, the tubes showed either of the results: Acid production, acid and gas production or no fermentation at all.
- The presence of acid and gas changes the medium into a yellow color indicating a positive result. Gas production can be detected by the presence of small bubbles in the inverted durham tubes. The broth retaining the red color is an indication of the absence of fermentation.

2.3 Antibiotic susceptibility test

2.3.1 Preparation of antibiotic solution

- 4 capsules, each of Maxacil 500 (amoxicillin 500 mg) of Square; DAR No: 298-45-60 and Gentamicin (500MG) of Oxoid was cracked open and measured.
- 1.2 gm of total powdered antibiotic were taken in a vial and suspended with 2 ml Phosphate Buffer Saline (PBS). The suspension was shaken to mix it thoroughly.

2.3.2 Preparation of inoculum

- Using a sterile inoculating loop two or three isolated colony of the organism to be tested was taken from the subculture Nutrient agar plate.
- The test organisms were suspended in 5 ml of nutrient broth.
- The broth containing the test organism was vortexed to create a smooth suspension.
- The broth was kept at 37 °C for overnight incubation in an incubator.
- The cultures were used after 24 hours of preparation.

2.3.3 Inoculation on the nutrient agar (NA) plate

- A sterile swab was dipped into the inoculum tube and the test organisms were suspended in 5 ml of nutrient broth.
- The swab was rotated against the side of the tube using firm pressure, to remove excess fluid, but the swab was not dripped wet.

- The dried surface of the Nutrient agar plate was inoculated by streaking the swab three times over the entire agar surface; the plate was rotated approximately 60 degrees each time to ensure an even distribution of the inoculum .The plate was rimmed with the swab to pick up any excess liquid .
- Leaving the lid slightly ajar, the plate was allowed to sit at room temperature at least 3 to 5 minutes for the surface of the agar plate to dry before proceeding to the next step.

2.3.4 Placement of the antibiotic disks

- Two sterile disks were placed on the surface of an agar plate, using a forcep. The forcep was sterilized by immersing the forceps in alcohol then igniting it.
- The disks were gently pressed with the forcep to ensure complete contact with the agar surface. The disks were placed away from the edge of the plates so that it is easily measured.
- Once all disks are in place, the plates were inverted, and placed them in a 37 °C incubator for 24 hours

2.3.5 Measuring zone sizes

- Following incubation, the zone sizes were measured precisely using a ruler.
- All measurements were made while viewing the back of the petri dish.
- The zone size was recorded on the recording sheet.

2.4 Plant Maintenance

The grass was collected during the month of March, 2014.It was about 1.2 kg and was obtained from the BRACnursery (BRAC kanon) from Gulshan, Dhaka. The grass was air dried, away from the sunlight and grinded to fine powder after a month with the aid of a mixer grinder. The powdered material was used for the preparation of the extract. Three types of extract were prepared namely, cold aqueous extract, hot aqueous extract and methanol extract.

Figure2: air dried *Cynodon dactylon*

2.5 Extract preparation of Durva Grass (*Cynodon dactylon*)

Cynodon dactylon, commonly known as the Durva grass was obtained from the BRAC Nursery. The raw herb was separated from its roots and was carefully trimmed with the help of a sterilized scissor. The grass was then soaked in 70% ethanol.

2.5.1 Cold Aqueous Extract

- 50 gram of the powder was dissolved in 200 ml of distilled water in a conical flask.
- The flask was closed tight with a rubber cork and kept at room temperature for 24 hours.
- Filtration was done using Whatman, 1MM filter paper in a sterile conical flask.
- After filtration, the extract was allowed to evaporate using the water bath which was set at 100 °C.
- Finally, the extract was stored at 4 °C

2.5.2 Hot Aqueous Extract

- 50 gram of the powder was dissolved in 200ml of distilled water in a conical flask. The suspension was boiled for 30 minutes and was left undisturbed for 24 hours.
- Filtration was done using Whatman, 1MM filter paper in a sterile conical flask.
- After filtration, the extract was allowed to evaporate using the water bath which was set at 100 °C.
- Finally, the extract was stored at 4 °C

2.5.3 Methanol Extract

- 50 gram of the powder was dissolved in 400 ml of methanol in a Duram bottle.
- The solution was placed in a shaking incubator (Daihan Scientific, Korea) at 25 °C at 50 rpm.
- Filtration was done using Whatman, 1MM filter paper in a sterile conical flask.
- After filtration, the extract was allowed to dry at room temperatureFinally, the extract was stored at 4 °C

Figure3: The three extracts prepared from Cynodon dactylon; Hot Aqueous (top left), Cold aqueous and Methanol extract.

2.6 Antimicrobial activity test:

The plant extract disc was prepared from Whatman, 1MM filter paper by punching with a hole punch of 6 mm diameter. The disc was autoclaved at 121 °C for 15 minutes. At first, each of the nutrient agar plates prepared was lawned with the respective bacterial strains and incubated at 37 °C for 24 hours. 300 µg and 600 µg of each of the extract were diluted with 1ml of Phosphate Buffer Saline (PBS) to make the final concentration of the extract 100 mg/ml. The autoclaved discs were placed in the nutrient agar plate. 50 µl (300µg) and 100 µl (600µg) of each of the final concentration were taken and dropped in the disc placed on each of the plate. A 5 µl of Phosphate Buffer Saline (PBS) was place in the disc which served as a control. All the plates were incubated at 37 °C for 24 hours. Following incubation, the plates were observed for its antimicrobial activity

2.7 Measuring Activity Index

Following formula was used to measure Activity Index, Activity Index = (Zone of inhibition of extract/Zone of inhibition of antibiotic). Zone of inhibition of stocks against each bacterial species and similarly zone of inhibition of Antibiotic (Gentamicin and Amoxicillin) were measured.

Chapter 3: Results

3.1 Confirmation of clinical strains

Clinical strain of the seven bacterial species *i.e Escherichia coli* LTST, *Escherichia coli* Non Pathogenic strain K12, *Bacillus cereus*, *Salmonella typhi*, *Shigella dysenteriae*, *Pseudomonas aeroginosa* and *Staphylococcus epidermis* obtained from ICDDR,B (International Center for Diarrheal Disease Research) was confirmed by streaking in the respective selective media. Selective medium types are formulated to support the growth of one group of organisms, but inhibit the growth of another. These media contain antimicrobials, dyes, or alcohol to inhibit the growth of the organisms not targeted for study.

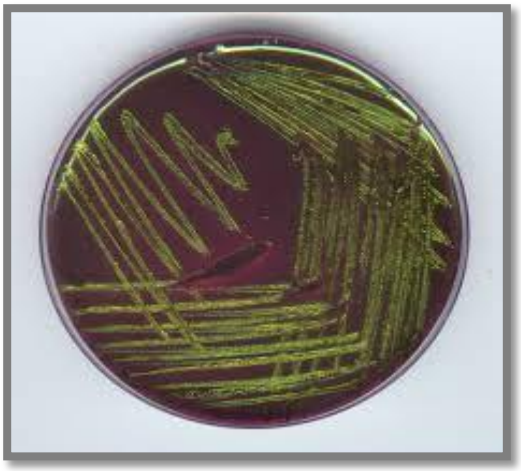
Table 3: Cultural characteristics of clinical strains on respective selective media

Isolates/ Presumptive organism	Cultural characteristics						
	Medium	**Size**	**Margin**	**Elevation**	**Form**	**Pigment**	**Consistency**
B. cereus	MYP agar	Large (4-5mm)	Undulate	Raised	Circular	Bright pink colonies with egg yolk precipitation.	Creamy,Smooth
E. coli K12	MAC	Moderate (1-2 mm)	Entire	Raised	Circular	Pink	Rough
	EMB	Large (2-3 mm)	Entire	Slightly raised	Circular	Blue-black colonies with metallic green sheen	Shiny,Smooth
E. coli LTST	MAC	Moderate (1-2 mm)	Entire	Raised	Circular	Pink	Smooth
	EMB	Large (2-3 mm)	Entire	Raised	Circular	Blue-black colonies with metallic green sheen	Smooth
P. aeruginosa	Cetrimide agar	Small	Undulate	Raised	Circular	Green colonies that turns the media greenish	Mucoid
S. typhi	XLD	Moderate (2-4mm)	Entire	Raised	Convex	Red colonies with a black centre	Smooth

S. dysenteriae	XLD	Moderate(1-2mm)	Entire	Convex	Convex	Pinkish to red colonies	Smooth
S. epidermidis	MSA	Moderate(2-3mm)	Entire	Slightly raised	Convex	Slight pink	Smooth
	BPA	Pinpoint	Entire	Raised	Convex	Black	Smooth

a)

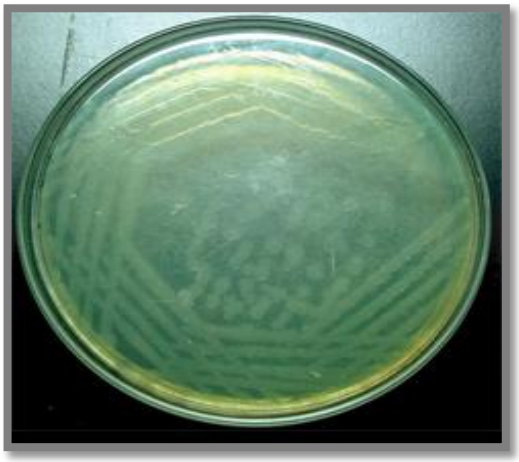
b)



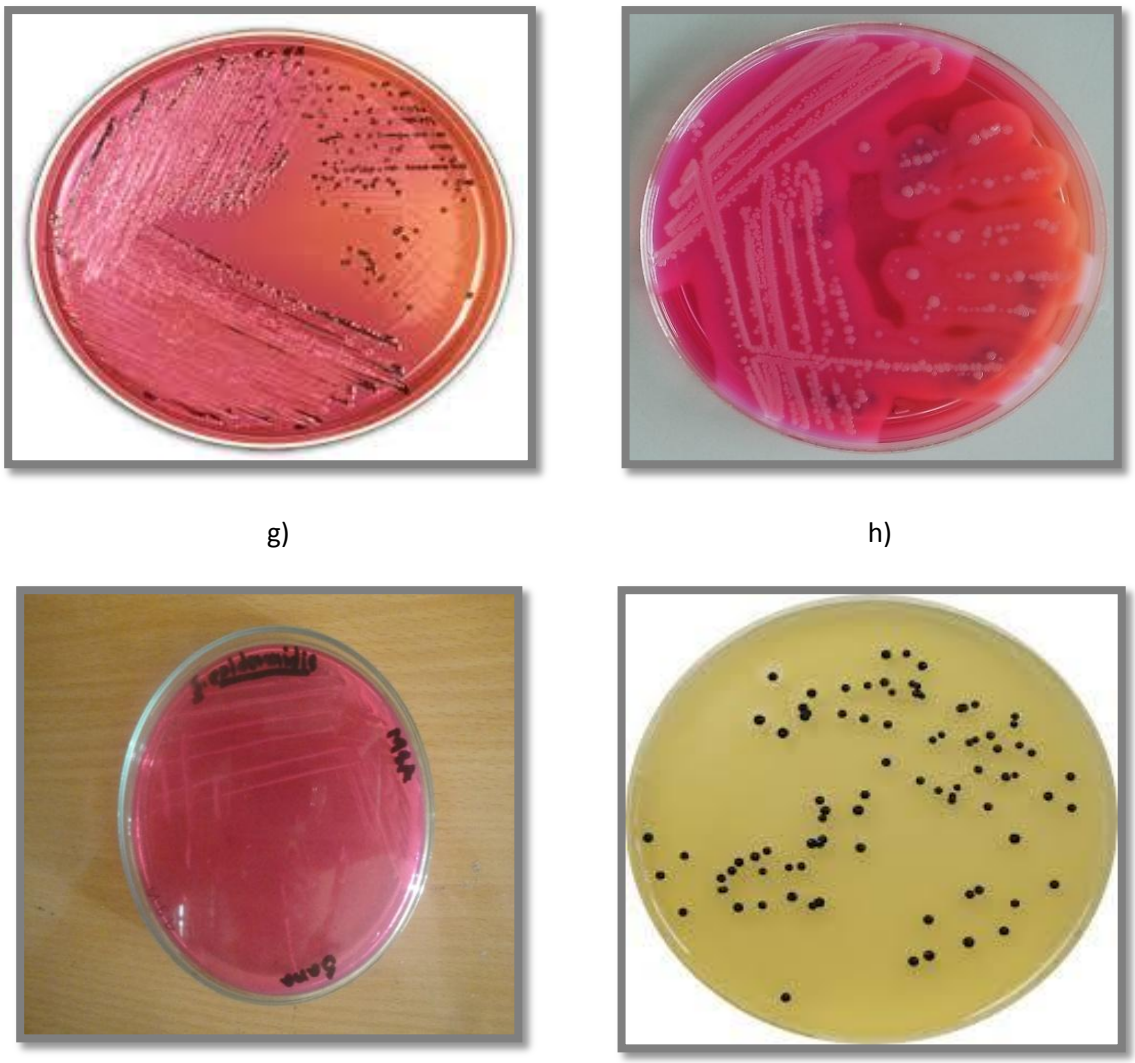
c)

d)

e)



f)



g) h)

i) j)

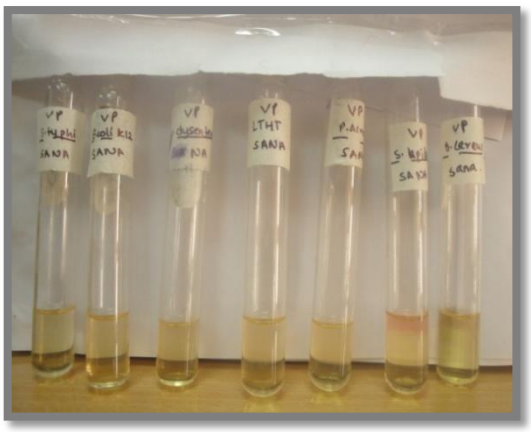
Figure 4: Cultural characteristics of clinical strains on respective selective media a) *Bacillus cereus* in MYP agar b) *Escherichia coli* K12 in MacConkey agar c) *Escherichia coli* K12 in Eosin Methylene blue agar d) *Escherichia coli* LTST in MacConkey agar e) *Escherichia coli* LTST in Eosin Methylene blue agar f) *Pseudomonas aeroginosa* in Cetrimide agar g) *Salmonella typhi* in Xylose Lysine Deoxycholate agar h) *Shigella dysenteriae* in Xylose Lysine Deoxycholate agar i) *Staphylococcus epidermidis* in Mannitol Salt agar and j) *Staphylococcus epidermidis* in Baird parker agar.

The results of each biochemical tests are mentioned below:

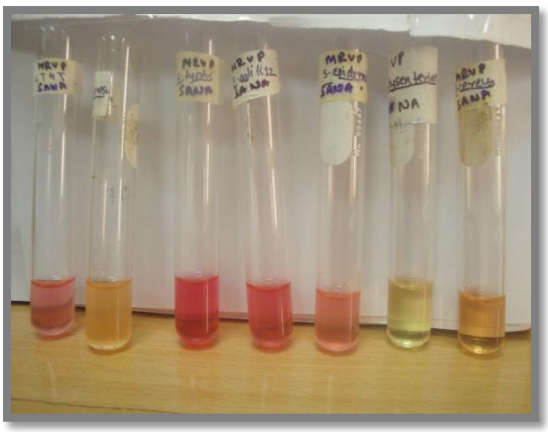
Table 4: Biochemical test results

Isolates/ Presumptive organism	Biochemical tests												
	Indole production test	Methyl red reaction test	Voges Proskeur's reaction test	Citrate utilization test	TSI fermentation				Fermentation test			Catalase activity test	Oxidase activity test
					Slant	Butt	CO_2	H_2S	Lactose	Sucrose	Dextrose		
B. cereus	-	-	-	-	A	A	-	-	-	A	A	+	+
E. coli K12	+	+	-	-	A	A	+	-	AG	A	AG	+	-
E. coli LTST	+	+	-	-	A	A	-	-	AG	A	AG	+	-
P. aeroginosa	-	-	-	+	K	K	-	-	-	-	-	+	+
S. typhi	-	+	-	-	K	A	-	+	-	-	AG	+	-
S. dysenteriae	+	+	-	-	A	A	-	-	-	-	AG	+	-
S. epidermidis	-	+	+	-	A	A	-	-	A	A	A	+	-

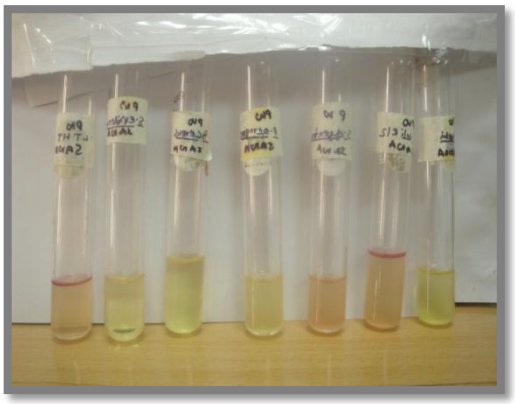
KEY: A= acidic condition, K= alkaline condition, + = positive, - = negative, AG= both acid & gas production.



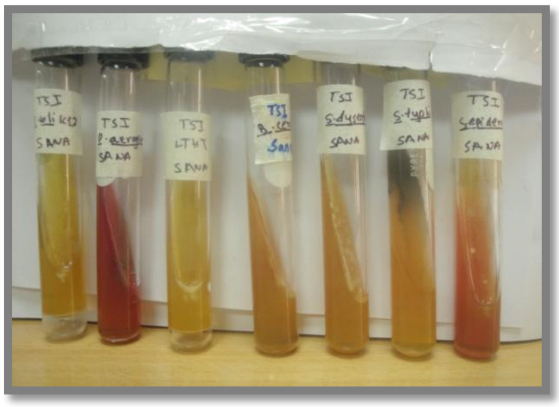
a)



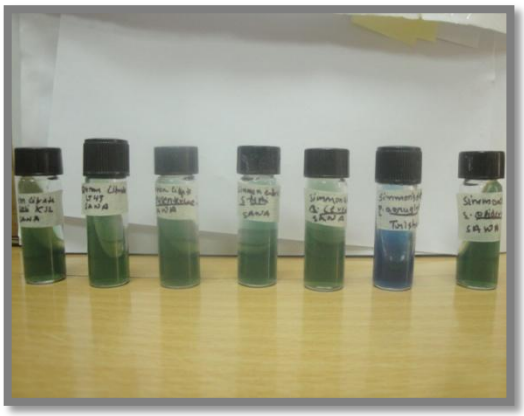
b)



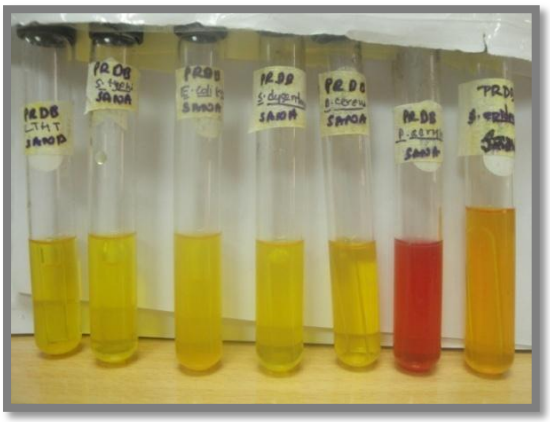
c)



d)

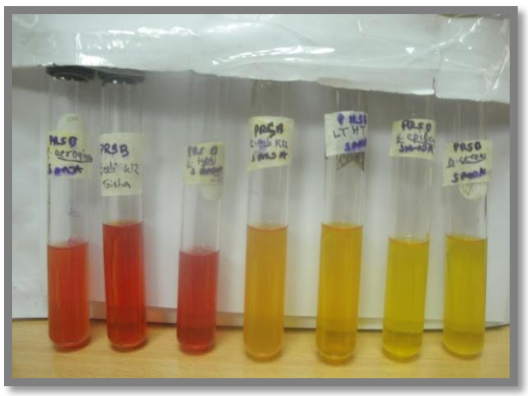


e)



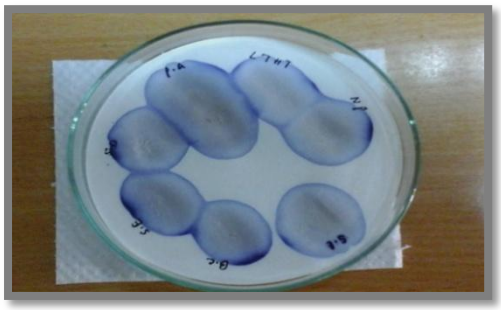
f)

g)



h)

i)



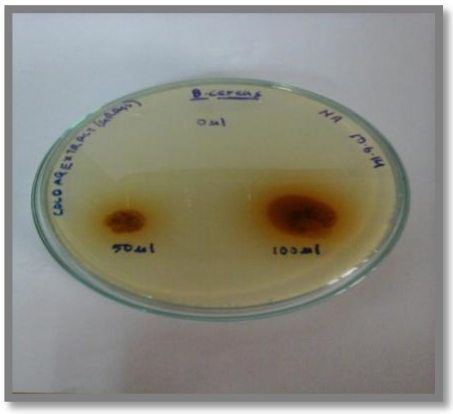
j)

Figure 5: Biochemical test results of each clinical strain. a)Voges-Proskauer test b)Methyl red test c) Indole production test d) Triple Sugar Iron test e)Citrate utilization test f) Dextrose utilization test g)Lactose utilization test h)Sucrose utilization test i) Catalase test and j)Oxidase test

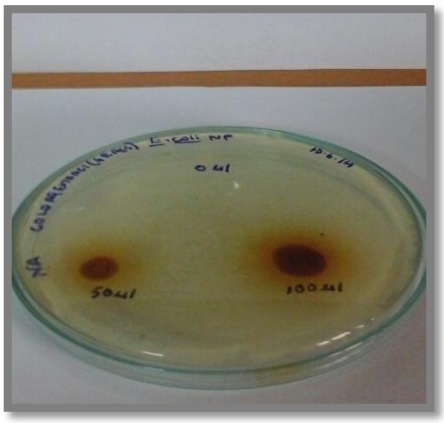
3.2 Antimicrobial activity Test: Antimicrobial activity of the cold aqueous, hot aqueous and methanol extract of Cynodon dactylon was tested and the zone of inhibition was observed and the diameter (mm) was calculated as follows:

Table 5: Antimicrobial activity of Cold Aqueous Extract

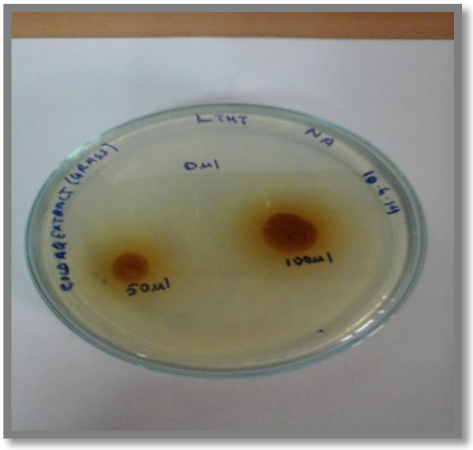
	Diameter (mm)		
Organism	**0 µl**	**50 µl (300 µg)**	**100 µl (600 µg)**
Bacillus cereus	0	8	16
Escherichia coli K12	0	10	12
Escherichia coli LTST	0	11	16
Pseudomonas aeruginosa	0	7	10.5
Salmonella typhi	0	10	11
Shigella dysentriae	0	9	11
Staphylococcus epidermidis	0	11	12.5


B.cereus
0ml
NA
50ml
100ml

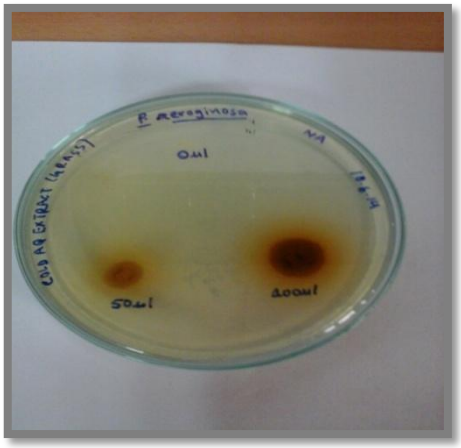
a)


L.cell NP
0 ml
NA
50ml
100ml

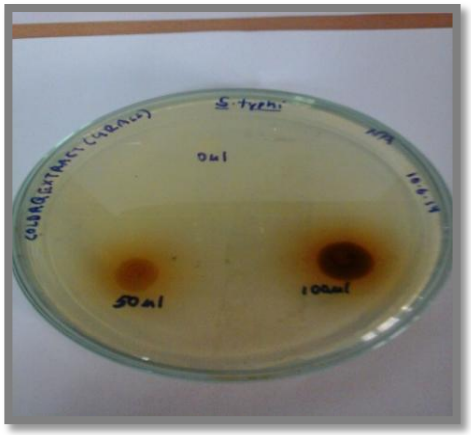
b)


L.INT
NA
0ml
50ml
100ml

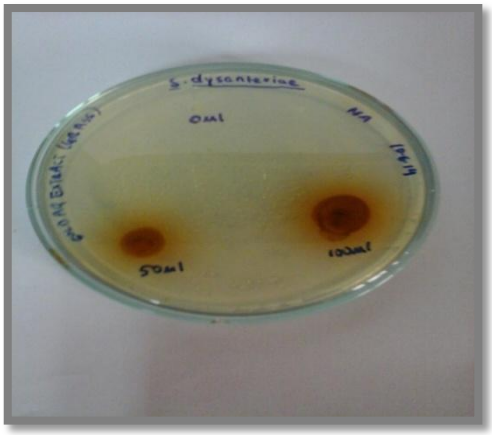
c)


P.aeroginosa
NA
0ml
50ml
100ml

d)


S.typhi
0ml
50ml
100ml

e)


S.dysenteriae
0ml
NA
50ml
100ml

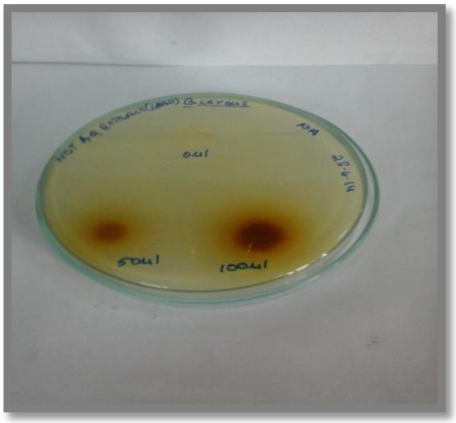
f)

g)

Figure 6: Antimicrobial activity of Cold Aqueous Extract of *Cynodon dactylon* on a) *B.cereus* b) *E.coli* K12 c) *E.coli* LTST d) *P.aeroginosa* e) *S.typhi* f) *S.dysenteriae* g) *S.epidermidis*

Table 6: Antimicrobial activity of Hot Aqueous Extract

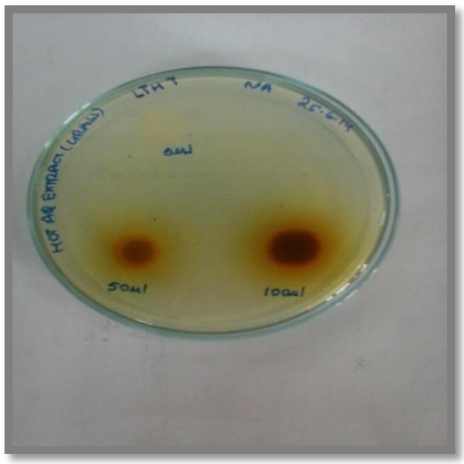
Diameter (mm)			
Organism	**0 µl**	**50 µl (300 µg)**	**100 µl (600 µg)**
Bacillus cereus	0	11	14
Escherichia coli K12	0	12	16
Escherichia coli LTST	0	13	17
Pseudomonas aeruginosa	0	11	12
Salmonella typhi	0	10	13
Shigella dysentriae	0	10	13
Staphylococcus epidermidis	0	15	18


0ul
50ul
100ul

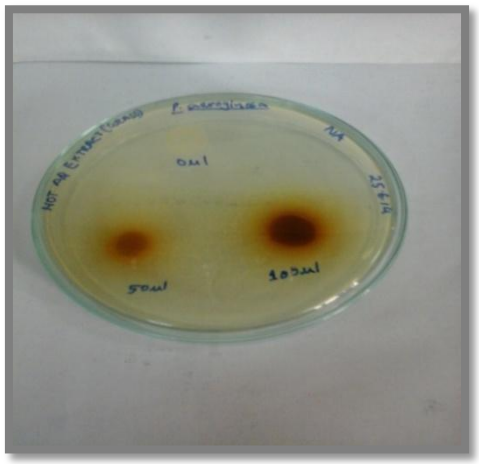
a)


0ul
50ul
100ul

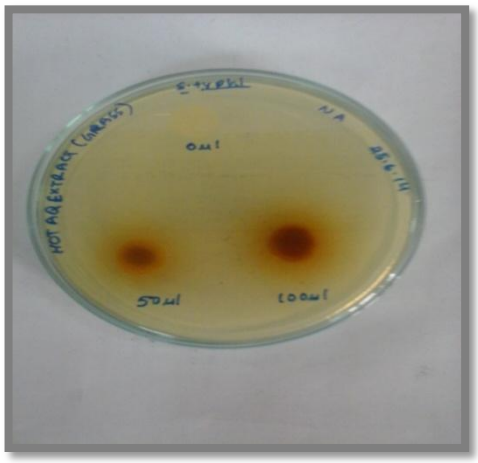
b)


0ul
50ul
100ul

c)


0ul
50ul

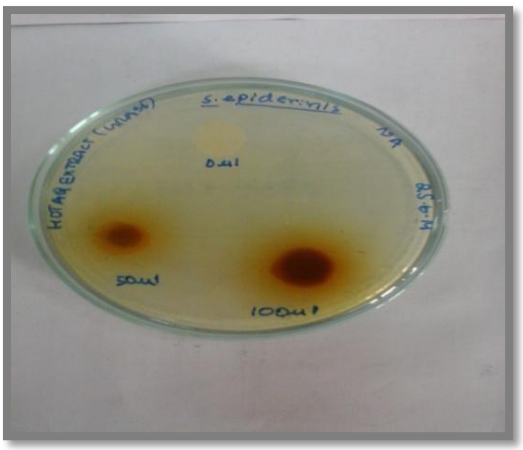
d)


0ul
50ul
100ul

e)


0ul
50ul
100ul

f)

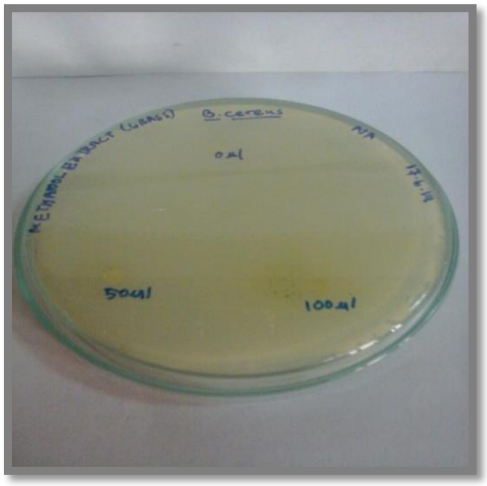


g)

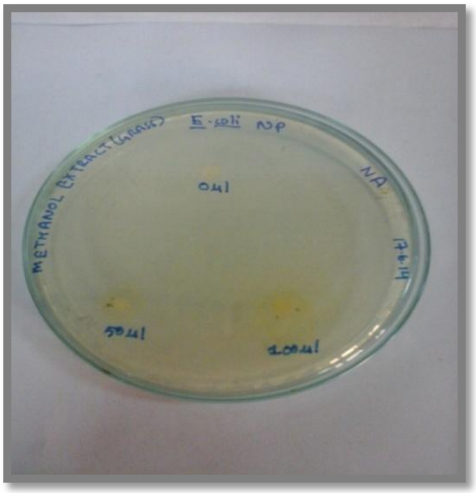
Figure 7: Antimicrobial activity of Hot Aqueous Extract of *Cynodon dactylon* on a)*B.cereus* b)*E.coli* K12 c)*E.coli* LTST d)*P.aeroginosa* e) *S.typhi* f)*S.dysenteriae* g) *S.epidermidis*

Table 7: Antimicrobial activity of Methanol Extract

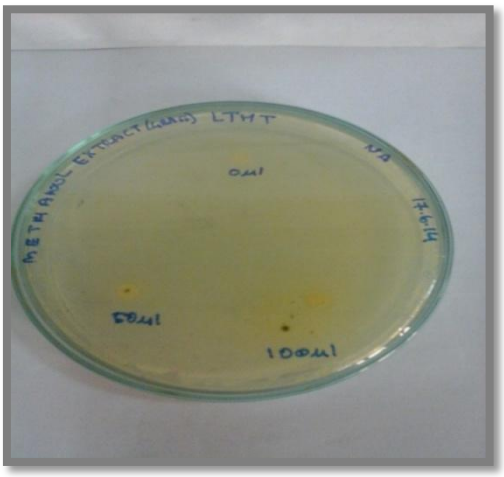
Diameter (mm)			
Organism	**0 µl**	**50 µl (300µg)**	**100 µl (600 µg)**
Bacillus cereus	0	0	0
Escherichia coli K12	0	0	0
Escherichia coli LTST	0	0	0
Pseudomonas aeruginosa	0	0	0
Salmonella typhi	0	0	0
Shigella dysentriae	0	0	0
Staphylococcus epidermidis	0	0	0


METHANOL EXTRACT (LEAVES) B. cereus
NA
17.6.14
0 µl
50 µl
100 µl

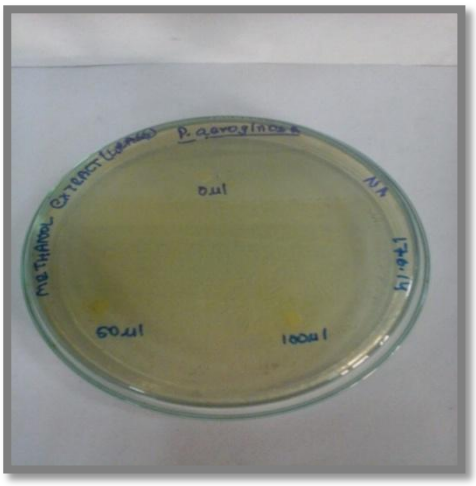
a)


METHANOL EXTRACT (LEAVES) E. coli NP
NA
17.6.14
0 µl
50 µl
100 µl

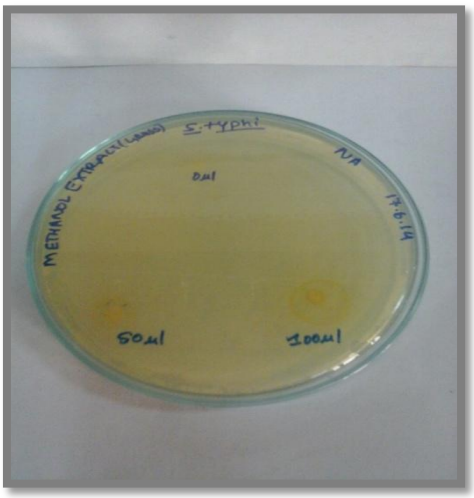
b)


METHANOL EXTRACT (LEAVES) LTHT
NA
17.6.14
0 µl
50 µl
100 µl

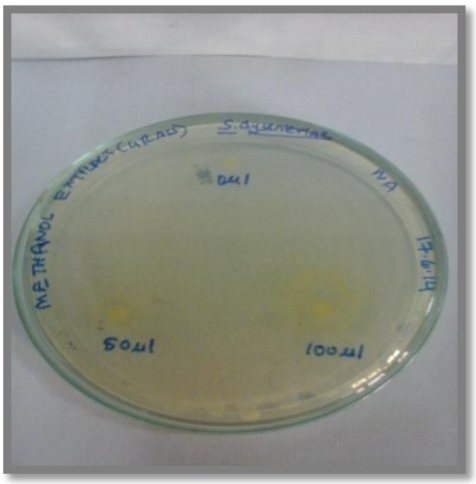
c)


METHANOL EXTRACT (LEAVES) P. aeruginosa
NA
17.6.14
0 µl
50 µl
100 µl

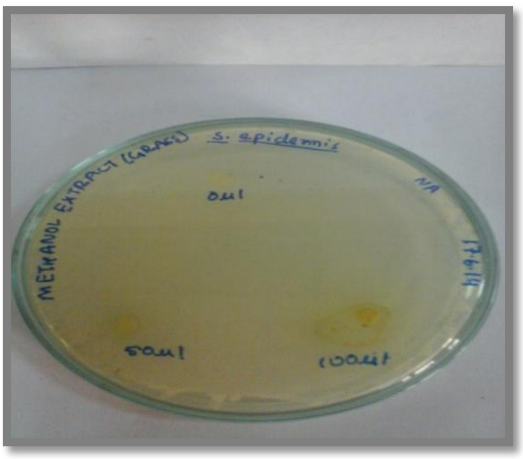
d)


METHANOL EXTRACT (LEAVES) S. typhi
NA
17.6.14
0 µl
50 µl
100 µl

e)


METHANOL EXTRACT (LEAVES) S. aureus
NA
17.6.14
0 µl
50 µl
100 µl

f)



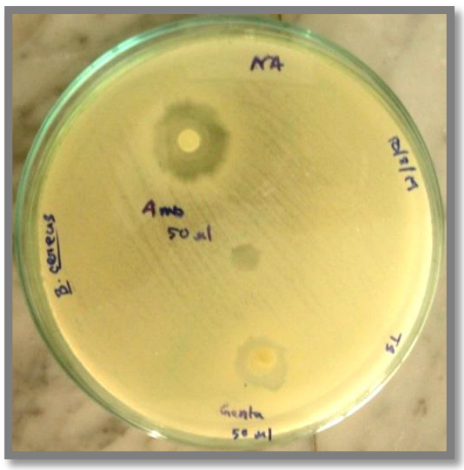
g)

Figure 8: Antimicrobial activity of Methanol Extract of *Cynodon dactylon* on a) *B.cereus* b) *E.coli* K12 c) *E.coli* LTST d) *P.aeroginosa* e) *S.typhi* f) *S.dysenteriae* g) *S.epidermidis*

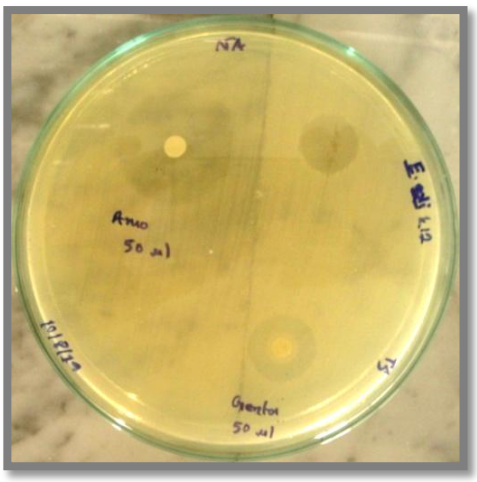
3.3 Antibacterial activity of test organisms against Amoxicillin & Gentamicin: Antimicrobial activity of the seven test organisms was tested against the two antibiotics, amoxicillin and gentamicin and was taken as standard to compare it with the antimicrobial susceptibility tests of the crude extract of *Cynodon dactylon*

Table 8: Antibacterial activity of test organisms against Amoxicillin & Gentamicin

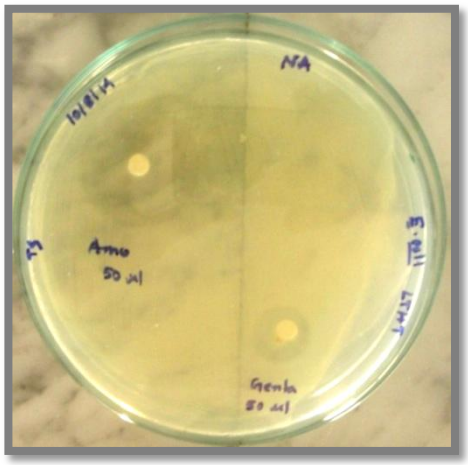
Name of Organism	**Antibiotics**			
	Amoxicillin (diameter in mm)		**Gentamicin (diameter in mm)**	
	50 µl (300µg)	100 µl (600µg)	50 µl (300µg)	100 µl (600µg)
Bacillus cereus	8	10	6	8
Escherichia coli K12	14	19	9	13
Escherichia coli LTST	16	19	7	10
Pseudomonas aeruginosa	0	0	0	0
Salmonella typhi	26	28	11	16
Shigella dysentriae	0	2	0	0
Staphylococcus epidermidis	27	30	17	15


NA
Amo
50 µl
Genta
50 µl
TS

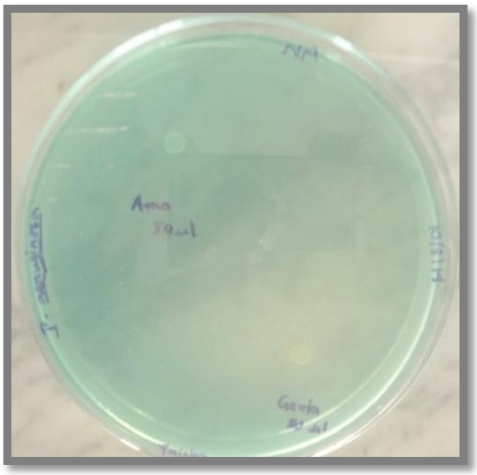
a)


NA
Amo
50 µl
Gentax
50 µl
TS

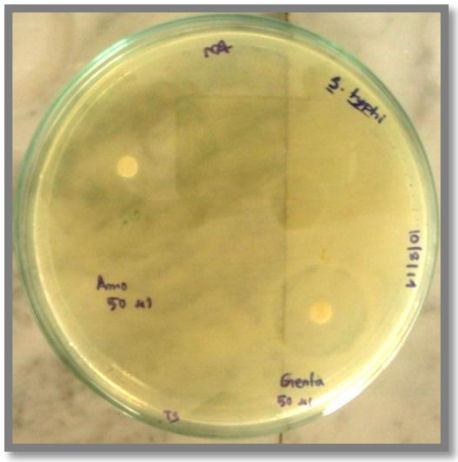
b)


NA
Amo
50 µl
Genta
50 µl
TS

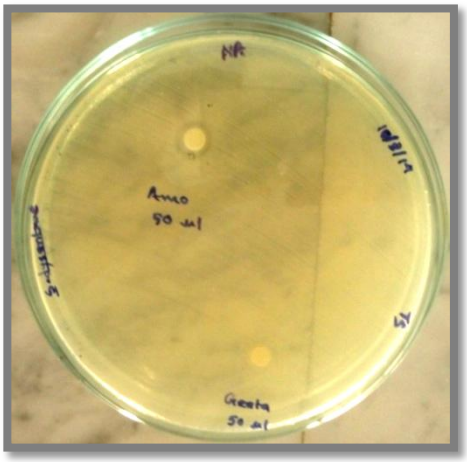
c)


NA
Amo
50 µl
Genta

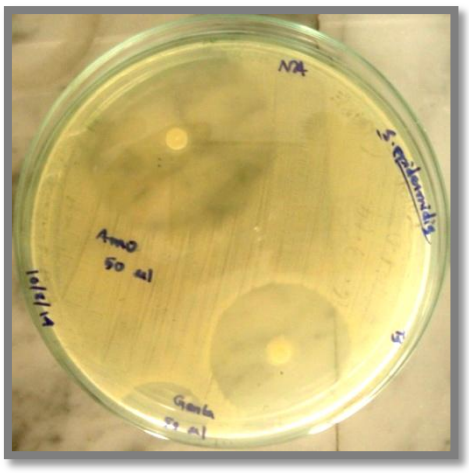
d)


NA
Amo
50 µl
Genta
50 µl
TS

e)

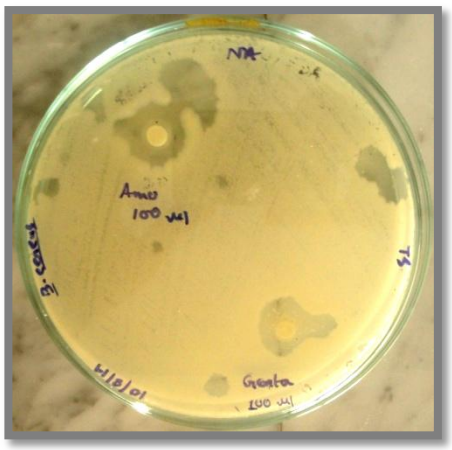

NA
Amo
50 µl
Genta
50 µl
TS

f)

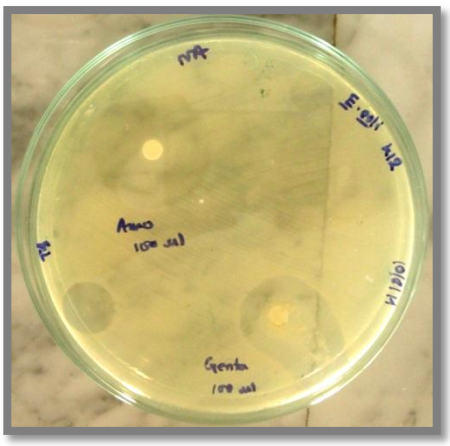


g)

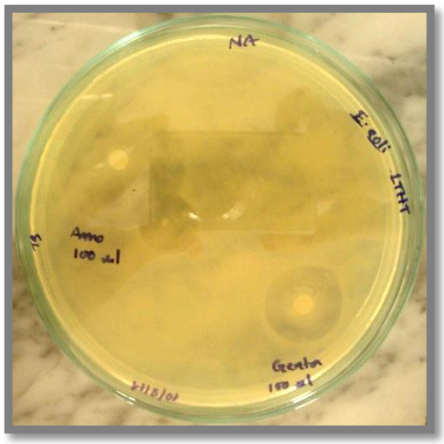
Figure 9: Antimicrobial susceptibility of a) *B.cereus* b) *E.coli* K12 c) *E.coli* LTST d) *P.aeroginosa* e) *S.typhi* f) *S.dysenteriae* g) *S.epidermidis* against amoxicillin and gentamicin (300 µg antibiotic solution per disk).


NA
Amo
100 µl
Genta
100 µl

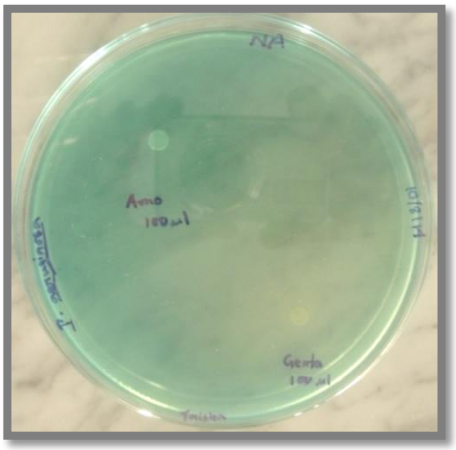
a)


NA
Amo
100 µl
Genta
100 µl

b)


NA
E. coli
Amo
100 µl
Genta
100 µl

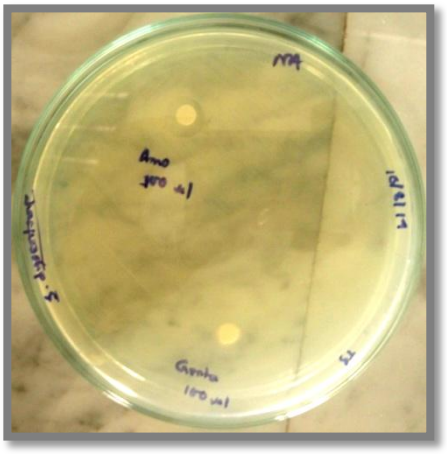
c)


NA
Amo
100 µl
Genta
100 µl

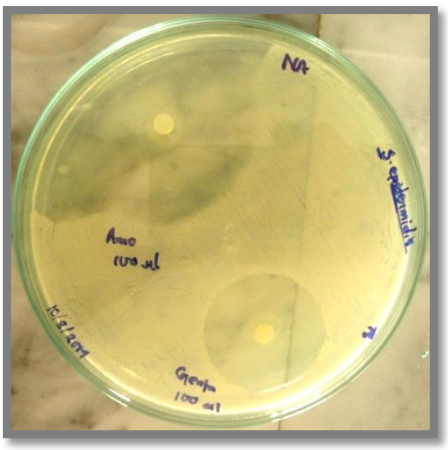
d)


NA
Amo
100 µl
Genta
100 µl

e)


NA
Amo
100 µl
Genta
100 µl

f)



g)

Figure 10: Antimicrobial susceptibility of a) *B.cereus* b) *E.coli* K12 c) *E.coli* LTST d) *P.aeroginosa* e) *S.typhi* f) *S.dysenteriae* g) *S.epidermidis* against amoxicillin and gentamicin (600 µg antibiotic solution per disk).

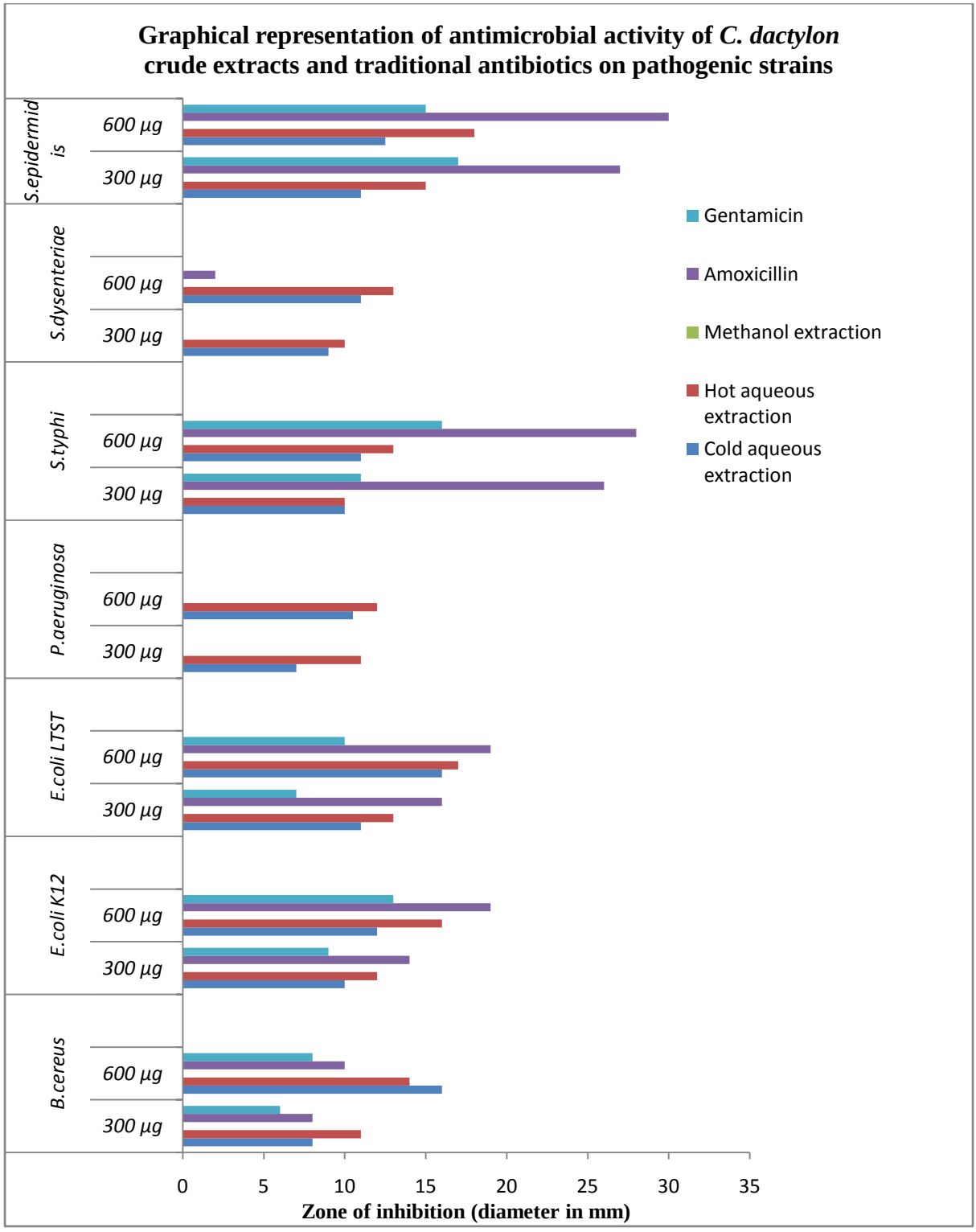


Figure 11: Graphical representation of antimicrobial activity of *C.dactylon* crude extracts and traditional antibiotics on pathogenic strains

3.4 Activity Index of *Cynodon dactylon* extracts:

From the data of average zone of inhibition for *Cynodon dactylon* whole plant extract, average zone of inhibition for Amoxicillin and Gentamicin (300 µg and 600 µg) and using this activity index of *Cynodon dactylon* whole plant extract for 2 gram positive and 5 gram negative isolated bacterial strain were calculated (Table 9). For gram positive bacterial strains maximum antibacterial effect was evident in *Bacillus cereus* according to the activity Index to the test antibiotics Amoxicillin and gentamicin for both hot (with amoxicillin activity index=1.4 and with gentamicin activity index=1.8) and cold aqueous (with amoxicillin activity index=1.6 and with gentamicin activity index=2) extract. Whereas minimum antibacterial effect was observed for *Salmonella typhi* for both hot with amoxicillin activity index=0.46 and with gentamicin activity index=0.91 and cold aqueous (with amoxicillin activity index= 0.393and with gentamicin activity index=0.91) extract. Other test organisms showed significant antimicrobial activity according to activity index in compare to *Bacillus cereus. O*nly for two bacterial species *Pseudomonas aeroginosa* and *Shigella dysent*eriae it was not possible to calculate activity index as both of these bacteria are resistant to the respective antibiotics though they showed a significant zone of inhibition for the aqueous extract indicating their antimicrobial activity.

Table 9: Activity Index of Cynodon dactylon extracts

Name of Organism	Zone of inhibition (diameter in mm)								Activity Index = (Zone of inhibition of extract/Zone of inhibition of antibiotic)							
	Cynodon dactylon whole extracts				Antibiotics				with Amoxicillin				with Gentamicin			
	Cold aqueous		Hot aqueous		Amoxicillin		Gentamicin		Cold aqueous		Hot aqueous		Cold aqueous		Hot aqueous	
	50 µl (300 µg)	100 µl (600 µg)	50 µl (300 µg)	100 µl (600 µg)	50 µl (300 µg)	100 µl (600 µg)	50 µl (300 µg)	100 µl (600 µg)	50 µl (300 µg)	100 µl (600 µg)	50 µl (300 µg)	100 µl (600 µg)	50 µl (300 µg)	100 µl (600 µg)	50 µl (300 µg)	100 µl (600 µg)
Bacillus cereus	8	16	11	14	8	10	6	8	1	1.6	1.375	1.4	1.33	2	1.83	1.75
Escherichia coli K12	10	12	12	16	14	19	9	13	0.714	0.632	0.857	0.842	1.11	0.923	1.33	1.23
Escherichia coli LTST	11	16	13	17	16	19	7	10	.687	0.842	0.812	0.895	1.57	1.6	1.85	1.7
Pseudomonas aeruginosa	7	10.5	11	12	resistant	resistant	resistant	resistant	--	--	--	--	--	--	--	--
Salmonella Typhi	10	11	10	13	26	28	11	16	.0385	0.393	0.385	0.464	0.91	0.688	0.909	0.812
Shigella dysentriae	9	11	10	13	resistant	2	resistant	resistant	--	--	--	--	--	--	--	--
Staphylococcus epidermidis	11	12.5	15	18	27	30	17	15	0.407	0.412	0.556	0.6	0.647	0.833	0.88	1.2

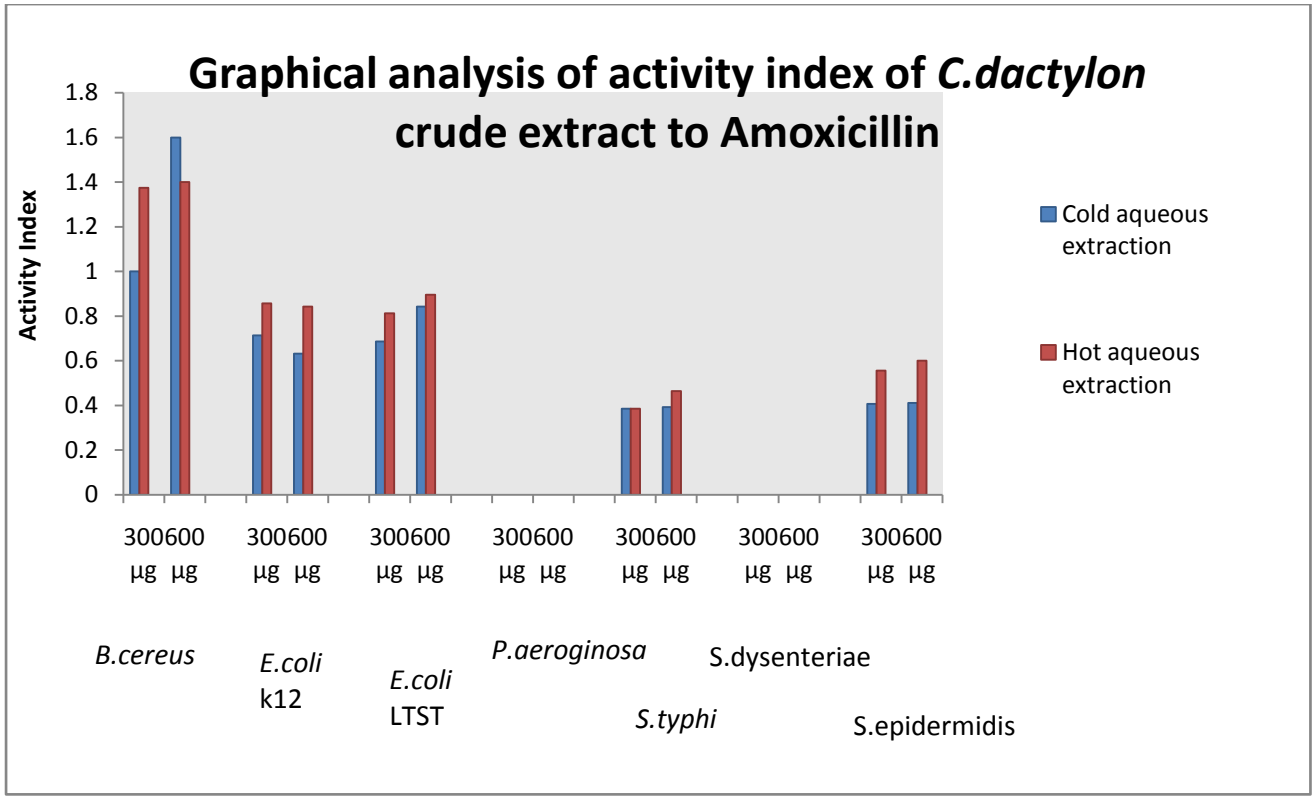


Figure 12: Graphical analysis of activity index of *C.dactylon* crude extract to Amoxicillin

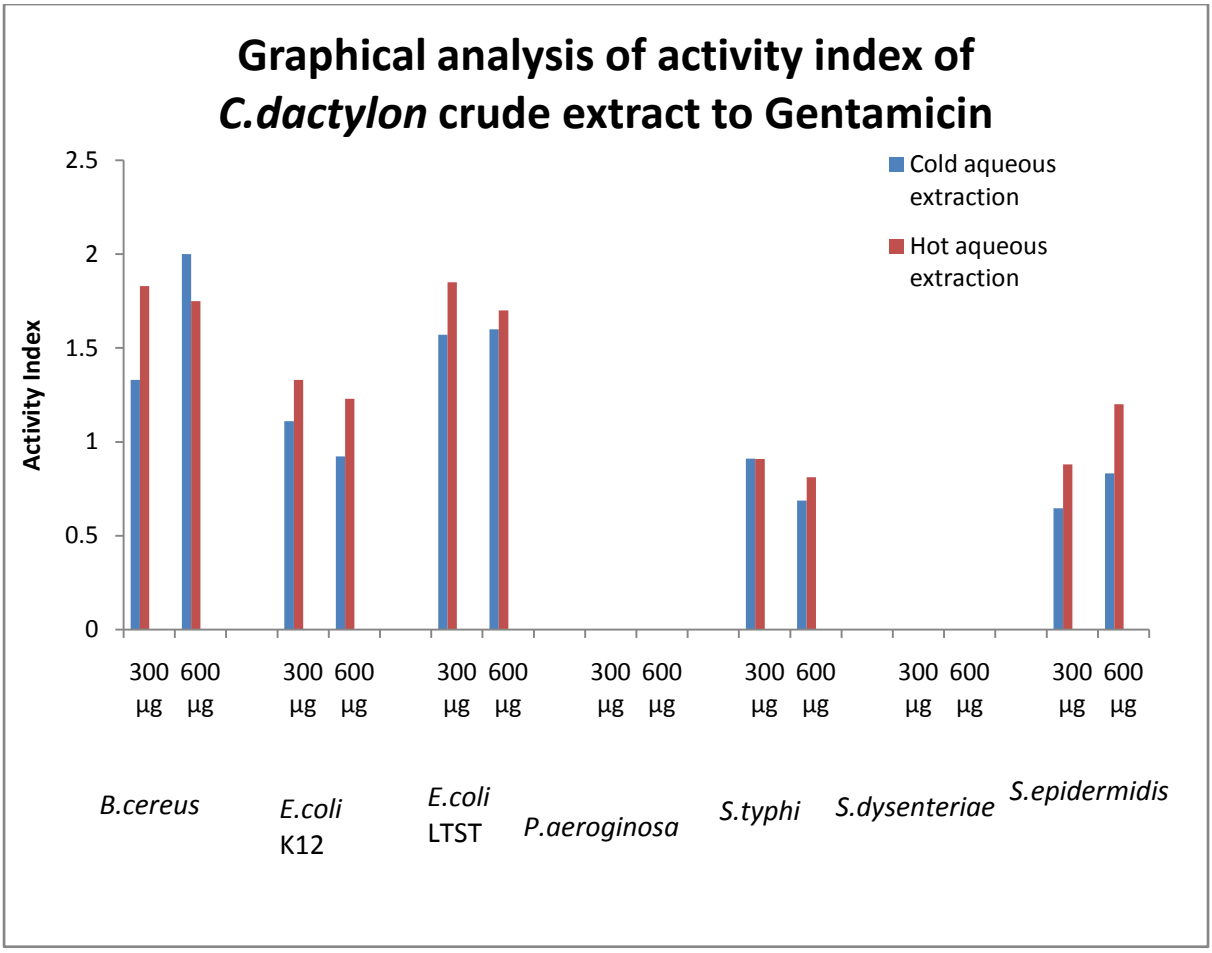


Figure 13: Graphical analysis of activity index of *C.dactylon* crude extract to Gentamicin

Chapter 4: Discussion

4. Discussion

Infectious diseases are a major cause of morbidity and mortality in developing country like Bangladesh. The number of multiple drug resistant strains and the appearance of the strains with reduced susceptibility to antibiotics are continuously increasing. This situation provided the incentive to the search for new antimicrobial substances from various sources like medicinal plants. One of such plants considered of great importance is *Cynodon dactylon.* It is a perennial grass of poaceae family, has variety of medicinal properties like alterative, antiseptic, aperients, astringent, cyanogenetic, demulcent, depurative, diuretic, emollient, sudorific, and vulnerary in traditional medicine. It is folk remedy for anasarca, calculus, cancer, carbuncles, convulsions, cough, cramps, cystitis, diarrhea, dropsy, dysentery, epilepsy, headache, hemorrhage, hypertension, hysteria, insanity, laxative, measles, rubella, snakebite, sore stones, tumors, urogenital disorders, warts, and wounds [Harbone, J.R. 1984]. It is found to be cultivated throughout the topics and subtopics regions of the world. Therefore it is important to investigate scientifically this herb which has been used in traditional medicines as potential source of novel antimicrobial compounds. The first step towards this goal is the *in vitro* anti bacterial activity assay.

This study is reporting the antimicrobial activity of *C. dactylon* against some common pathogens such as *S. dysenteriae, P. aeroginosa, B. cereus, S. typhi, E. coli* LTST, *E.Coli* K12, *S. epidermidis* which are highly associated with nosocomial infection.

The result obtained showed antimicrobial activity through disc diffusion method for both hot and cold aqueous extract, which is evident by the formation of clear zone for seven clinical strain used in this study. Amoxicillin and Gentamicin were taken as positive control. For hot aqueous extraction, in terms of zone diameter measurement *S. epidermidis* showed maximum clear zone formation both with 50 µl (300 µg/ µl) and 100 µl (600 µg/ µl) of extract concentration indicating their greater sensitivity to this specific extract. Along with this *E coli* K12 and *E. coli* LTST also showed greater zone diameter while other species like *S. dysenteriae, P. aeroginosa, B. cereus, S. typhi* showed zone diameter which is closely related. On the other hand for cold

aqueous extraction, maximum diameter of zone is observed in case of *E. coli K12*, *B. cereus* and *E. coli* LTST. Other organisms showed similar type of zone diameter. The organic extraction such as methanol extraction showed no zone of inhibition in table 7.

For both test antibiotics Amoxicillin and Gentamicin, an activity index was calculated to determine the significant activity of *C. dactylon* crude extract. It is clearly observed that *Bacillus cereus* has the maximum activity for both test antibiotics with hot and cold aqueous extract and minimum for *Salmonella typhi* according to activity index data (Table 9). As *Pseudomonas aeroginosa* and *Shigella dysenteriae* showed resistant pattern against traditional antibiotics, use of crude extracts of *Cynodon dactylon* gave promising result to control their infectious activity. All of the extracts were found to be effective against all the test organisms with significant activity index.

The result of anti-bacterial activity of the crude extract of *C. dactylon* by agar disc diffusion method shown in table 5 and 6, it was revealed that both the hot and cold aqueous extract of *C. dactylon* possesses an effective and equipotent antibacterial activity against both gram positive and gram negative bacteria. The demonstration of activity against both gram-negative and gram-positive bacteria is an indication that the plant can be a source of bioactive substances that could be of broad spectrum of activity. The fact that the plant was active against both clinical and laboratory isolates is also an indication that it can be a source of very potent antibiotic substances that can be used against drug resistant microorganisms prevalent in hospital environments. In the present study the aqueous extract proved to be a potent antibacterial agent more probably due to the presence of various phytotoconstituents included Saponins, Tannins, steroids and Flavonoids [*Bonjar et al*., 2004]

Further studies are required to confirm this antibacterial activity and to separate the active constituents and evaluate their antibacterial activity.

Chapter 5: Conclusion

5. Conclusion

Phytochemical analysis of aqueous extract showed the presence of tannins, steroids, flavonoids, saponins, and absence of alkaloids and phlobatanins. Phytochemical constituents are secondary metabolites of plants that serve a defense mechanism against prediction by many microorganisms, insects and other herbivores [*Bonjar et al*., 2004]. The primary phytochemical analysis revealed that the extracts contained some phytoconstituents such as saponins, steroids, tannins flavonoids, which could be responsible for the observed antimicrobial property. These bioactive compounds are known to act by different mechanism and exert antimicrobial action.

Aqueous extract demonstrated a broad-spectrum of activity against both gram-positive and gram-negative bacteria. The broad-spectrum antibacterial activities of the plant extract, possibly due to the identified phytochemical constituents, further confirm its use as a health remedy in folklore medicine. Bioactive substances from this plant can therefore be employed in the formulation of antimicrobial agents for the treatment of various bacteria including gonorrhea, pneumonia, eye infections. Isolation, identification and purification of these phytoconstituents and determination of their respective antibacterial potencies and toxicological evaluation with the view to formulating novel chemotherapeutic agents should be the future direction for investigation. From the present study we can draw a conclusion that the traditional use of plant *Cynodon dactylon* for the infectious disease is promising, mainly against bacteria.

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APPENDIX-I

Media composition

The composition of the media used in the present study has been given below. Unless otherwise mentioned, all the media were autoclaved at 121^{o}C for 15 min.

1. Nutrient Agar (Himedia,India)

Ingredients	Amounts (g/L)
Peptic digest of animal tissue	5.0
Beef extract	1.50
Sodium chloride	5.0
Yeast extract	1.50
Agar	15.0

2. Nutrient Broth (Oxoid, England)

Ingredients	Amount (g/L)
Lab-lemco powder	1.0
Yeast extract	2.0
Peptone	5.0
Sodium chloride	5.0

3. Cetrimide agar (Merck, India)

Ingredients	Amount (g/L)
Pancreatic digest of gelatin	20.0
Magnesium chloride hexahydrate	1.4
Potassium sulfate anhydrous	10.0
Cetrimide	0.3
Agar-Agar	13.0

4. **T_1N_1 soft agar**

Ingredients	Amount (g/L)
Tryptone	0.6 g
Sodium chloride	0.3g
Agar	0.42 g

5. **Tryptone soy broth, (Oxoid, England)**

Ingredients	Amount (g/L)
Pancreatic digest of Casein	17.0
Papaic digest of soybean meal	3.0
Sodium chloride	5.0
Di-basic potassium phosphate	2.5
Glucose	2.5

6. **MacConkey agar (Oxoid, England)**

Ingredients	Amount (g/L)
Peptone	20.0
Lactose	10.0
Bile salts	5.0
Sodium chloride	5.0
Neutral red	0.075
Agar	12.0

7. **Simmon's citrate agar (Oxoid, England)**

Ingredients	Amount (g/L)
Magnesium sulfate	0.2
Ammonium dihydrogen phosphate	0.2
Ammonium phosphate	0.8
Sodium citrate	2.0
Sodium chloride	5.0
Agar	15.0
Bacto brom thymol blue	0.08

8. **Peptone Water**

Ingredients	Amount (g/L)
Peptone	10.0
Sodium chloride	5.0

9. MR-VP broth

Ingredients	Amount (g/L)
Peptone	7 g
Dextrose	5 g
Potassium phosphate	5 g

10. Triple sugar iron agar (Himedia, India)

Ingredients	Amount (g/L)
Peptic digest of animal tissue	10.0
Sodium chloride	5.0
Lactose	10.0
Sucrose	10.0
Dextrose	1.0
Ferrous sulfate	0.20
Sodium thiosulfate	0.30
Casein enzymatic hydrolysate	10.0
Yeast extract	3.0
Beef extract	3.0

11. Eosine methylene blue agar (Oxoid, England)

Ingredients	Amount (g/L)
Peptone	10.0
Sucrose	5.0
Lactose	5.0
Di-potassium phosphate	2.0
Eosin Y	0.14
Methylene blue	0.065
Agar	13.50

12. Mannitol Salt agar (Oxoid, England)

Ingredients	Amount (g/L)
Peptone	10.0
Manitol	10.0
Lab-lemco powder	1.0
Sodium chloride	75.0
Phenol red	0.025
Agar	15.0

13. Thiosulfate Citrate Bile Salts Sucrose agar (Difco, USA)

Ingredients	Amount (g/L)
Proteose peptone	10.0
Sodium thiosulfate	10.0
Sodium citrate	10.0
Yeast extract	5.0
Oxgall	8.0
Sucrose	20.0
Sodium chloride	10.0
Ferric citrate	1.0
Bromothymol blue	0.04
Thymol blue	0.04
Agar	15.0

14. Xylose Lysine Deoxycholate agar (Himedia, India)

Ingredients	Amount (g/L)
L- lysine	5.0
Lactose	7.50
Sucrose	7.50
Xylose	3.50
Sodium chloride	5.0
Sodium deoxycholate	2.50
Yeast extract	3.0

15. Phenol red (Lactose, Dextrose, Sucrose) Broth

Ingredients	Amount (g/L)
Trypticase	0.4
Lactose	0.2
Sucrose	0.2
Dextrose	0.2
Sodium chloride	0.2
Phenol red	0.00072
Final pH	7.3

APPENDIX-II

Buffers and reagents

1. Phosphate buffered saline (PBS)

PBS was prepared by dissolving 8.0 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4 and 2.0 g of KH_2PO_4 in 800 ml of distilled water. The pH was adjusted to 7.4 with HCl. The final volume was adjusted to 1 liter by distilled water. The solution was sterilized by autoclaving and was stored at room temperature.

2. Kovac's reagent

5 g of para-dimethylaminobenzaldehyde was dissolved in 75 ml of amyl alcohol. Then concentrated HCl was added to make the final volume 25 ml. This reagent was covered with aluminum foil and stored at 4^oC.

3. Methyl red reagent

0.1 g of methyl red was dissolved in 300 ml of 95% ethyl alcohol. Then distilled water was added to make the final volume 500 ml. This reagent was covered with aluminum foil and stored at 4^oC.

4. Barritt's reagent

Solution A

5 g of alpha-naphthol was dissolved in 95% ethanol. This solution was covered with aluminum foil and stored at 4^oC.

Solution B

40 g of KOH was dissolved in distilled water. The solution became warm. After cooling to room temperature, creatine was dissolved by stirring. Distilled water was added. This solution was covered with aluminum foil and stored at

5. Oxidase reagent

100 mg of N,N,N^1,N^1-tetramethyl-p-phenyldiamine-dihydrochloride was dissolved in 10 ml of distilled water and covered with aluminum foil. Then the solution was stored at 4^oC.

APPENDIX-III

Instruments

The important equipments used through the study are listed below:

Autoclave	SAARC
Freeze (-20°C)	Siemens
Incubator	SAARC
Micropipette (10-100µl)	Eppendorf, Germany
Micropipette (20-200µl)	Eppendorf, Germany
Oven, Model:MH6548SR	LG, China
pH meter, Model: E-201-C	Shanghai Ruosuaa Technology company, China
Refrigerator (4^{o}C), Model: 0636	Samsung
Safety cabinet Class II Microbiological	SAARC
Shaking Incubator, Model: WIS-20R	Daihan Scientific, Korea
Vortex Mixture	VWR International
Water bath	Korea
Weighing balance	ADAM EQUIPMENT™, United Kingdom