Medicinal Plants: An Alternative Source for Antimicrobials against Emerging Pathogens

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Bachelor in Science in Microbiology

Submitted by

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Abstract

Tagetes erecta, an extensively used medicinal plant is a small shrub which grows up to 1-2 mm. The present study was aimed to investigate the antibacterial activity of this locally available plant. Antimicrobial activity of three different crude extracts (cold aqueous, hot aqueous and methanol extracts) of *Tagetes erecta* leaves and flowers was evaluated by disk diffusion method against seven different pathogenic gram positive and gram negative bacteria. The leaf and flower of this plant part found to show a broad spectrum of antibacterial activity. All the extracts were found effective though the hot aqueous extract showed maximum inhibition against the test microorganisms followed by cold aqueous and methanol extracts. Current results will provide a background for further endeavor in development of antimicrobial agents from this important medicinal plant.

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❖ BPA	Baird-Parker Agar
❖ CFU	Colony Forming Unit
❖ EMB	Eosin Methylene Blue Agar
❖ ESBLs	Extended Spectrum β Lactamases
❖ 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
❖ MRSA	Methicillin-resistant *Staphylococcus aureus*
❖ MSA	Mannitol Salt Agar
❖ MYP	Mannitol Egg Yolk Polymyxin Agar
❖ NA	Nutrient Agar
❖ PBS	Phosphate Buffer Saline
❖ TSI	Triple Sugar Iron
❖ TSB	Trypticase Soy Broth
❖ UTIs	Urinary Tract Infections
❖ XLD	Xylose Lysine Deoxycholate

1.1 Overview

The increased prevalence of antibiotic-resistant superbugs due to the extensive use of antibiotics may render the current antimicrobial agents inefficient to control some bacterial diseases. Herbal medicine is recurrently a part of a larger therapeutic system such as traditional and folk medicine. It is necessary to appraise the scientific basis and the potential use of folk medicine for the treatment of infectious diseases produced by common pathogens. Medicinal plants might represent an alternative treatment of infectious diseases and can also be a possible source for new potent antibiotics to which pathogen strains are not resistant. These plants represent a rich source from which antimicrobial agents may be obtained. Plants are used medicinally in different countries and are a source of many potent and powerful drugs [1]. Unlike pharmaceutical medicines, herbal medicines have complex biological constituents having thousands of active compounds those work together synergistically. Several comprehensive study of the pure extract of medicinal plants has been found to be more effective and less toxic in both *in vivo* and *in vitro* the system. Uses of an herb's 'active component' are more reliable and safer than administration of the herb itself. Many herbs are now in use whose therapeutic properties and active principle are as yet not well understood [2].

Resistance to antimicrobial agents has resulted in morbidity and mortality from treatment failures and increased health care costs. Although defining the precise public health risk and estimating the increase in costs is not a simple undertaking, there is little doubt that emergent antibiotic resistance is a serious global problem. For this reason, conventional plant based remedies are back and find increasing interest as source of potential therapeutic agents. Substantial scientific data have been generated worldwide in this direction and there are spurt of study on natural product study [3]. The present study has been designed to compile and provide data on potential antibacterial properties, action and uses of a renowned medicinal plant *Tagetes erecta*, in order to contribute material leading to the discovery of new drugs of natural origin.

1.2 About *Tagetes erecta*

Tagetes erecta is an aromatic annual herb originally belongs to the family Asteraceae which has been used for medicinal purpose in many countries from ancient time.

Table 1: Scientific classification of *Tagetes erecta*

Kingdom	Plantae
Order	AsteTrales
Family	Asteraceae
Genus	*Tagetes*
Species	*erecta*

Figure 1: *T. erecta* leaves and flower

Tagetes erecta, the Mexican marigold, also called Aztec marigold, is a species of the genus *Tagetes* native to Mexico and Central America. Despite its being native to the Americas, it is often called African marigold. Marigold is a common garden plant which is rather coarse, erect, branched and grows to about one meter high. There are short or dwarf varieties as well. The leaves are very deeply incised and sharply toothed; flower heads are solitary, long-stalked, and thickening upward. The flowers are bright yellow, brownish-yellow or orange [4]. Marigold is widely used as an ornamental herb. In south Asia, it is used in offerings, weddings, funeral and other ceremonies. Marigold petals are also used to produce a yellow dye for food coloring [5].

1.3 Medical importance of *Tagetes erecta*

T. erecta is now being studied for its uses in medicine as well for it is proved to contain medicinal value like antibacterial activity and anti-inflammatory activity. Different parts of this plants are used in folk medicine to cure various diseases viz., colic's, diarrhea, vomiting, fever, skin diseases and hepatic disorders [6] .The leaves are reported to be effective against piles, kidney troubles, muscular pain, ulcers, and wounds. The pounded leaves are used as an external application to boils and carbuncles. Flowers are especially used in fevers, epileptic fits (Ayurveda), astringent, carminative, stomachic, scabies and liver complaints and are also employed in diseases of the eyes. They are said to purify blood and flower juice is given as a remedy for bleeding piles. They are also used in rheumatism, colds and bronchitis [7].

Tagetes erecta exhibits significant potency against human bacterial pathogens. Study of various extracts evaluated the antimicrobial activity of T. erecta leaves and flowers. The alcoholic extracts of *T. erecta* L. leaves showed promising antimicrobial activity against *Klebsiella pneumonia, staphylococcus aureus, Salmonella typhi, Proteus vulgaris, Pseudomonas aeruginosa* and *Escherichia coli* [8]. Methanol extract of T. erecta was found to have better inhibitory activity than cold and hot aqueous extracts [9].

1.4 History of bacterial resistance to antibiotics

In the past 60 years, antibiotics have been critical in the fight against infectious disease caused by bacteria and other microbes. Antimicrobial chemotherapy has been a leading cause for the dramatic rise of average life expectancy in the Twentieth Century. However, due to indiscriminate use of commercial antimicrobial drugs commonly used in the treatment of infectious diseases, multiple drug resistance in human pathogenic microorganisms has been developed. The development of antibiotic resistance is multifactorial, including the specific nature of the relationship of bacteria to antibiotics, the usage of antibacterial agent, host characteristics and environmental factors [10]. Nowadays, about 70 percent of the bacteria that cause infections in hospitals are resistant to at least one of the drugs most commonly used for treatment. Some organisms are resistant to all approved antibiotics and can only be treated with

experimental and potentially toxic drugs. An alarming increase in resistance of bacteria that cause community acquired infections has also been documented, especially in the staphylococci and pneumococci (*Streptococcus pneumoniae)*, which are prevalent causes of disease and mortality [11]. From these microbes resistant to antibiotics, Methicillin-resistant *Staphylococcus aureus* (MRSA) and multidrug-resistant *P. aeruginosa* strains are major cause of nosocomial infections [12].

Multidrug-resistant Enterobacteriaceae, mostly Escherichia coli, produces extended-spectrum β lactamases (ESBLs) such as the CTX-M enzymes. These enzymes were named for their greater activity against cefotaxime than other oxyimino-beta-lactam substrates such as ceftazidime, ceftriaxone, or cefepime have emerged within the community setting as an important cause of urinary tract infections (UTIs). Recent reports have also described ESBL-producing *E. coli* as a cause of bloodstream infections associated with these community-onsets of UTI [13].

1.5 Screening for alternative antibiotics against emerging pathogens

No doubt that antibiotics are miracle drugs. They stand against various infectious diseases for decades and saved millions of lives. However, the recent failure of antibiotics due to the dramatic emergence of multidrug resistant superbugs and the rapid spread of the new infections urge the health organizations and pharmaceutical industries all over the world to change their strategy and stop going on with the slow growing production of more synthetic antibiotics against the fast growing antibiotics-resistant microorganisms, while there are considerable alternative sources of natural antimicrobials from plants with different mode of actions, some of which are already employed in traditional medicine for centuries and was found to have competitive effects compared to some commercial antibiotics [14].

In this present study aqueous and organic fractions of the leaves and flowers of *Tagetes erecta* were subjected to antimicrobial testing by the disc diffusion method against seven clinical isolates of bacteria. The profile of the clinical pathogens used in this study is given in table 2.

Table 2: Profile of Clinical pathogens used in this study

Test organisms	Infections	Antibiotic susceptibility
Bacillus cereus	causes food-borne illnesses and diarrhea, also known to cause chronic skin infections	Vancomycin, gentamicin, chloramphenicol, and erythromycin
Escherichia coli K12	causes urinary tract and wound infections	Cephaloridine
Escherichia coli (LTST)	cause of diarrhea in children in the developing world, as well as the most common cause of traveler's diarrhoea	Fluoroquinolones
Pseudomonas aeruginosa	causes severe infections in burn victims, cancer patients as well as cystic fibrosis and neutropenic patients	Gentamicin, ciprofloxacin, ceftazidime, polymyxin B
Salmonella typhi	causes food poisoning and typhoid	Azithromycin, ciprofloxacin
Shigella dysenteriae	causes dysentery; associated with the development of hemolytic uremic syndrome	Ampicillin, ciprofloxacin, tetracycline
Staphylococcus epidermidis	patients with compromised immune systems are at risk of developing nosocomial infection; particular concern for people with catheters or other surgical implants, also causes endocarditis, most often in patients with defective heart valves	Vancomycin

1.6 Aims and objectives

The world wide increase of pathogenic bacteria resistant to antibiotics makes it an imperative to exploit alternate strategies to combat this threat. The therapeutic application of safe and controlled use of plant extracts may turn out to be a valuable approach. This study concerns the exploitation of plant extracts as biocontrol agents to eliminate medically important pathogens. Therefore the objectives of this research were set to:

1. Extract the leaf and flower of *Tagetes erecta* plant by standard protocols.
2. Analyze the therapeutic effect of specific dose of leaf and flower extracts on target microbial species.
3. Study the impact of regular conventional antibiotics on given pathogenic microorganisms.

2.1 Working laboratory

All project works were performed in the Microbiology Research Laboratory, Department of Mathematics & Natural Sciences, BRAC University.

2.2 Reference bacterial strains

In this study, we had worked with seven standard clinical strain of *Bacillus cereus, Staphylococcus epidermidis, E.coli K12, E. coli* LTST*, Salmonella typhi, Shigella dysenteriae* and *Psedomonas aeruginosa* obtained from ICDDR,B (International Center for Diarrheal Disease Research).

2.2.1 Preparation of plating bacteria

1. A single bacterial colony of each clinical isolates was inoculated into a screw cap test tube containing 5 ml Luria Bertani (LB) broth.
2. The tube was incubated at 37^{o}C in a shaking incubator (Daihan Scientific, Korea) at 120 rotations per min (rpm) for 3 hours.
3. After incubation 1 ml culture from the test tube was taken in an eppendorf and centrifuged (Eppendorf, Germany;) at 12,000 rpm for 10 min and the supernatant was discarded.
4. Bacterial pellet was suspended in 1 ml of 10mM $MgSO_4$ properly to make it homogenous.
5. The suspension was stored at 4^{o}C until used.
6. The suspension were then diluted 1:100 in TSB broth to obtain 10^6 CFU/ml before use.

2.2.2 Confirmation of the reference strains

Reference bacterial strains were identified routinely to distinguish one organism by subculturing on recommended selective media. The cultural properties of each organism were inspected and recorded.

1. For *B. cereus, E. coli*, *S. epidermidis* and *P. aeruginosa* respectively mannitol egg yolk polymyxin (MYP) agar, MacConkey agar (MAC), mannitol salt agar (MSA) and cetrimide agar was used.
2. Xylose lysine deoxycholate (XLD) agar was used for both *S. typhi* and *S. dysentrae*.

2.2.3 Biochemical confirmation of the clinical strains

Subsequently each bacterial strains were employed for morphological and biochemical confirmation.

1. Recommended biochemical tests like indole test, methyl red test, voges proskeur's test, citrate utilization test, oxidase test, catalase test, TSI agar were performed for all the strains.
2. The colonies were confirmed by some carbohydrate fermentation test as well; methyl red lactose broth, methyl red dextrose broth and methyl red sucrose broth were used to observe acid and CO_2 gas formation by the organisms.
3. In order to confirm *E. coli* strains the pink colonies from MacConkey agar were streaked onto eosin methylene blue (EMB) agar plates. Similarly, pink colonies from MSA plates were streaked onto Baird-Parker agar (BPA) for further confirmation.

2.2.4 Biochemical identification

Biochemical tests were performed with specific standard isolates developed in specific media according to the methods described in Microbiology Laboratory Manual [15]. Before proceeding to any biochemical identification test all bacterial cultures were grown on nutrient agar plates in incubator at 37^{o}C.

Indole production test

- Bacterial colonies were picked up from each nutrient agar plate to inoculated in 3 ml peptone water (A-I) which contains amino acid tryptophan and incubated overnight at 37^{o}C.
- Following overnight incubation five drops of Kovac's reagent were added.
- Detection of positive result would form a rose red ring 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 3 ml dextrose phosphate broth (MR-VP broth), which contained dextrose and a phosphate buffer 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's test

- Bacterium to be tested was inoculated into 3 ml dextrose phosphate broth (MR-VP broth) and incubated for at least 24 hours.
- To the aliquots of each broth cultures 10 drops of Barritt's reagent A was added and the cultures were shaken. Immediately, 10 drops of Barritt's reagent B was added and the cultures were reshaken. Cultures were kept aside for 15 minutes for the reaction to be occurred.
- After 15 minutes the colors of the cultures were examined and the results were recorded. Appearance of red color was taken as a positive test.

Citrate utilization test

- A single bacterial colony was picked up from each nutrient agar plates by a needle and inoculated into the slope of Simmon's citrate agar (Difco, USA) and incubated overnight at 37^{o}C.
- If the organism had the ability to utilize citrate, the medium changed its color from green to prussion blue; a negative slant would have no growth of bacteria and would remain green.

Triple Sugar Iron (TSI) Test

- To inoculate, isolated colony from nutrient agar plate was picked with a cool, sterile needle, stabbed into the TSI containing dextrose, lactose and sucrose butt.
- Incubated with caps loosened at 35°C for overnight and examined after 18-24 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 was 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 nutrient agar plate was streaked onto a piece of filter paper (Whatman, 1MM).
- Two drops of oxidase reagent (*p*- Aminodimethylaniline oxalate) 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

- One drop of catalase test reagent (hydrogen peroxide) was placed on a sterile glass slide. An isolate from a nutrient agar plate was picked up with a sterile toothpick and placed on to the reagent drop.
- Immediate bubble formation indicates positive result.

Lactose, sucrose, dextrose fermentation test:

- Isolates from nutrient agar was inoculated in durham tubes carrying 6 ml phenol red lactose broth, phenol red sucrose broth and phenol red dextrose broth each of which contained an inverted inner vial.
- Carbohydrate that has been fermented with the production of acidic wastes causes the phenol red to turn yellow, thereby indicating a positive reaction. Cultures that were not capable of fermenting a sugar did not change the indicator and the tubes appeared oranage.
- Evolution of CO_2 gas was visible as a bubble in the inverted tube.absence of bubble indicated negative result.

2.2.5 Preparation of stock sample

Short-term preservation

3 ml of T_1N_1 agar butt in each vial was inoculated by stabbing isolates from nutrient agar plates. Then the vials were incubated at 37^{o}C (SAARC) for 6 hours. After incubation, the surface of the medium was covered with 200 µl sterile paraffin oil and the vials were stored at room temperature appropriately labeled.

Long-term preservation

For long-term preservation, 500 µl of bacterial culture grown in Trypticase Soy Broth 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.

2.3 Plant maintenance

During the month of March, 3.5 kg of fresh *Tagetes erecta* plants were collected from BRAC nursery (BRAC KANON), Gulshan, Dhaka.

Figure 2: *Tagetes erecta* plant species used to prepare leaf and flower extracts

Botanical Description of *Tagetes erecta*:

The marigold plant was hardy, annual; about 0.4 meter tall, erect and branched. Leaves were pinnately divided and leaflets were lanceolate and serrated. Flower was single to fully double with large globular heads. The florets were either 2-lipped or quilled. Flower color was lemon yellow.

Leaves were 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.

2.3.1 Plant pre-treatment protocol

Green colored fresh leaves of medium age were selected. Leaves and flowers were separated from stem and washed with tap water and then with 70% w/w isopropyl alcohol followed by distilled water to remove all soil and other contaminants. All plant parts were allowed to air dried at 28±2°C for about 25 days in properly shaded area. The dried plant leaves and flowers were then separately crushed with the aid of a mechanical grinder to powdered form. This powder was stored in an air sealed polythene bags before extraction.

Figure 3: *Tagetes erecta* leaves and flowers after cleaning

2.3.2 Preparation of plant extracts

2.3.2.1 Cold aqueous extract

1. Twenty grams (20g) of the leaf powder was weighed out and soaked in 80 ml of distilled water in a 250ml conical flask, covered with aluminium foil and left for 24 hours.
2. The soaked material was filtered using a sterile Whattman no. 1 filter paper into a sterile conical flask.
3. Filtrate was then heated with a bunsen burner in low flame to get a thicker consistency.

4. The concentrated filtrate was transferred to a falcon tube and subjected to water-bath evaporation at about 80°C to obtain a gelatinous extract.
5. The standard extracts obtained were stored in a refrigerator at 4°C until required for use (Samsung, Model: 0636).
6. For flower cold extraction process similar protocol was maintained using 35 gm of flower powder.

2.3.2.2 Hot aqueous extract

1. Twenty grams (20g) of the dried leaf powder was soaked in 280 ml of water and boiled for thirty minutes in a beaker.
2. Flask was left undisturbed for 24hrs and then filtered using sterile filter paper.
3. The filtrate was allowed to heat with a bunsen burner and evaporated to dryness at 80°C.
4. The standard extracts obtained were stored in a refrigerator at 4°C until required for use.
5. Similarly 35 gm of flower powder was used for hot aqueous extraction.

2.3.2.3 Methanol extract

1. Twenty grams (20g) of dried leaf powder was soaked in 160 ml methanol in 500 ml air tight Schott Duran bottle at room temperature for 24 h with shaking in orbital shaker at 50 rpm. For flower extraction, 35 gm powder was added to 280 ml methanol.
2. The extract obtained was protected from sunlight by wrapping bottle with aluminium foil paper.
3. The content was filtered with Whatman No.1 filter paper.
4. The filtrate was allowed to dry at room temperature until dry methanol extract was obtained and were stored in a refrigerator at 4°C until required for further use.

2.4 Disc diffusion for antibacterial susceptibility testing

In disc diffusion assays, the response of each seven employed growing population of microorganism to the plant leaf and flower extract was investigated to record the potential antibacterial activity.

1. Bacterial lawn of each given clinical strain was prepared on nutrient media respectively.
2. Sterile discs (Oxoid) were soaked separately with 50 µl and 100 µl of each of the extract prepared by hot and cold aqueous extraction and organic solvent like methanol at a concentration of 100 mg/ml and 200 mg/ml respectively and then dried.
3. These discs were placed on nutrient agar plates, previously swabbed with the target bacterial isolate at a concentration of 10^6 CFU/ml.
4. In one disc, the PBS was added as negative control to determine possible inhibitory activity of the target extracts.
5. After overnight incubation for a period of 24 h at 30^oC, formation of any zone of growth inhibition was monitored.
6. Antibacterial activity was defined as the diameter (mm) of the clear inhibitory zone formed around the discs.

2.5 Antibiotic sensitivity test

The test microorganisms were also tested for their sensitivity against two common antibiotic- Amoxicillin 500 mg for Gram-negative bacteria and Gentamycin 500 mg for Gram-positive bacteria.

2.5.1 Preparation of antibiotic solution

1. 4 capsules of Maxacil 500 (amoxicillin 500 mg) of Square; DAR No: 298-45-60 and Gentamycin (500 mg) of Oxoid was cracked open and measured.
2. 1.2 gm of total powdered antibiotic was taken in a vial and suspended with 2 ml PBS. The suspension was shaked to mix it thoroughly.

2.5.2 Preparation of inoculums

1. Using a sterile inoculating loop four or five isolated colony of the organism to be tested was touched from the subculture plate.
2. The organism was suspended in 5 ml of nutrient broth.
3. The broth containing test tube was vortexed to create a smooth suspension.
4. The broth was kept at 37°C for overnight incubation in an incubator.
5. The cultures were used after 24 hours of preparation.

2.5.3 Inoculation of the nutrient agar (NA) plate

1. A sterile swab was dipped into the inoculum tube. The swab was rotated against the side of the tube (above the fluid level) using firm pressure, to remove excess fluid, but the swab was not dripped wet.
2. The dried surface of a NA 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 .
3. The plate was rimmed with the swab to pick up any excess liquid.
4. 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.

2.5.4 Placement of the antibiotic disks

1. 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.
2. The disks were gently pressed with the forcep to ensure complete contact with the agar surface. Placing disks close to the edge of the plates was avoided as the zones will not be fully round and can be difficult to measure.
3. Once all disks are in place, the plates were inverted, and placed them in a 37°C air incubator for 24 hours.

2.5.5 Measuring zone sizes

1. Following incubation, the zone sizes were measured to the nearest millimeter using a ruler.
2. All measurements are made with the unaided eye while viewing the back of the petri dish.
3. The zone size was recorded on the recording sheet.

2.6 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 extracts against each bacterial species and similarly zone of inhibition of antibiotic (amoxicillin and gentamicin) were measured.

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 was confirmed by their cultural properties (table 3) upon streaking on the respective selective media (figure 4). 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.

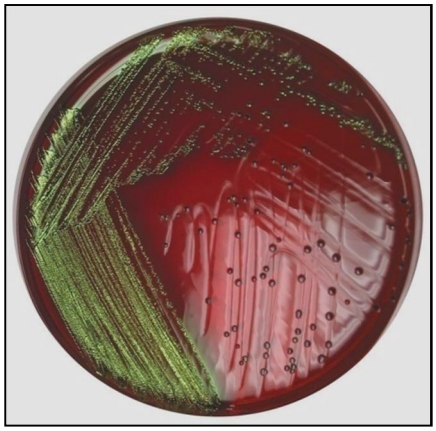
Table 3: Cultural characteristics of clinical strains on respective selective media

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	Smooth
	EMB	Large (2-3 mm)	Entire	Slightly raised	Circular	Blue-black colonies with metallic	Shiny, Smooth

						green sheen	
E. coli LTST	MAC	Moderate (1-2 mm)	Entire	Raised	Circular	Pink	Smooth
	EMB	Large (2-3 mm)	Entire	Slightly raised	Circular	Blue-black colonies with metallic green sheen	Shiny, Smooth
P. aeruginosa	Cetrimide agar	Moderate (1-2 mm)	Undulate	Raised	Circular	Greenish colonies colour change of the medium to greenish	Mucoid
S. typhi	XLD	Moderate (2-3 mm)	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	Pinpointed	Entire	Raised	Convex	Black	Smooth

(i)

(ii)



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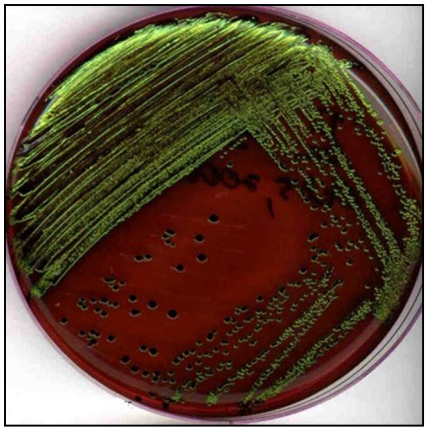

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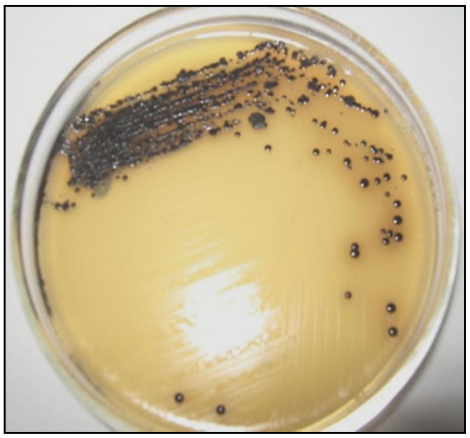
(v)



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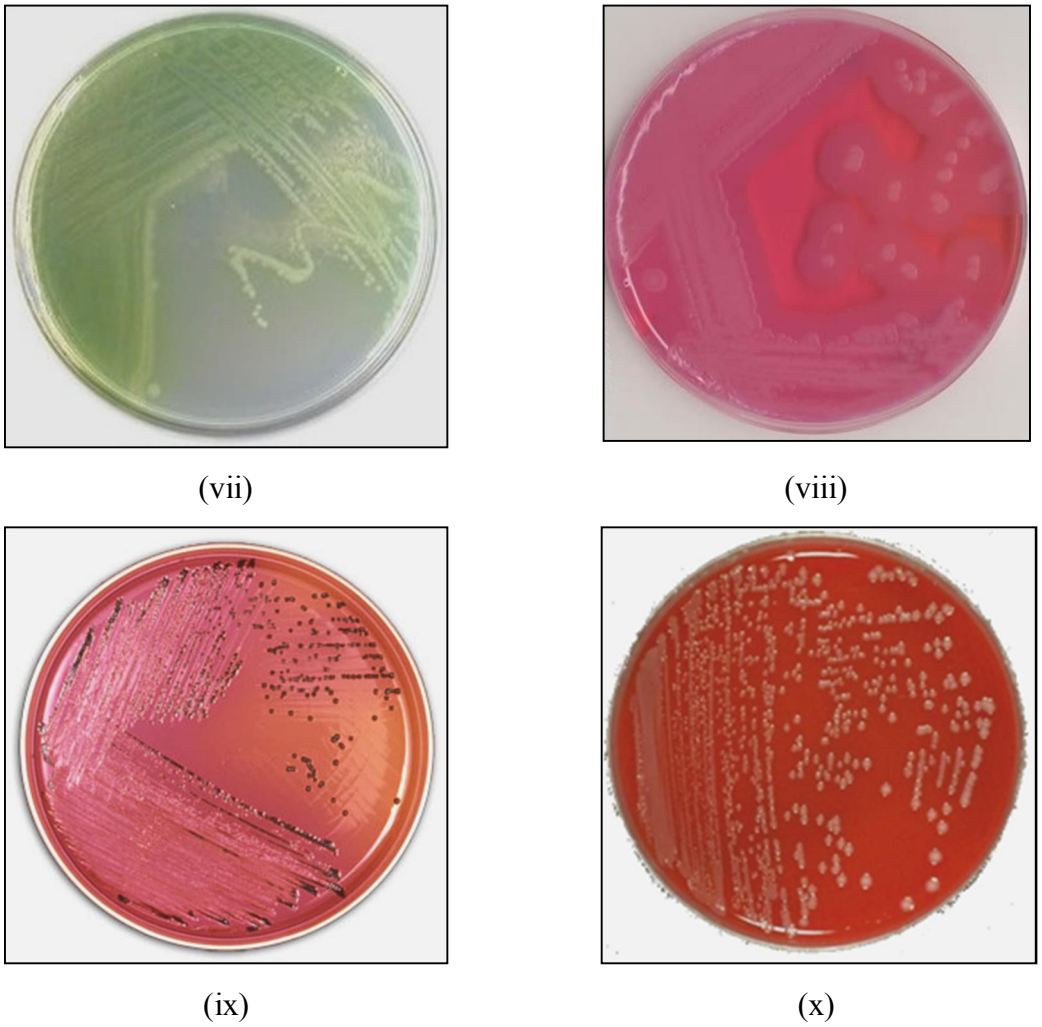


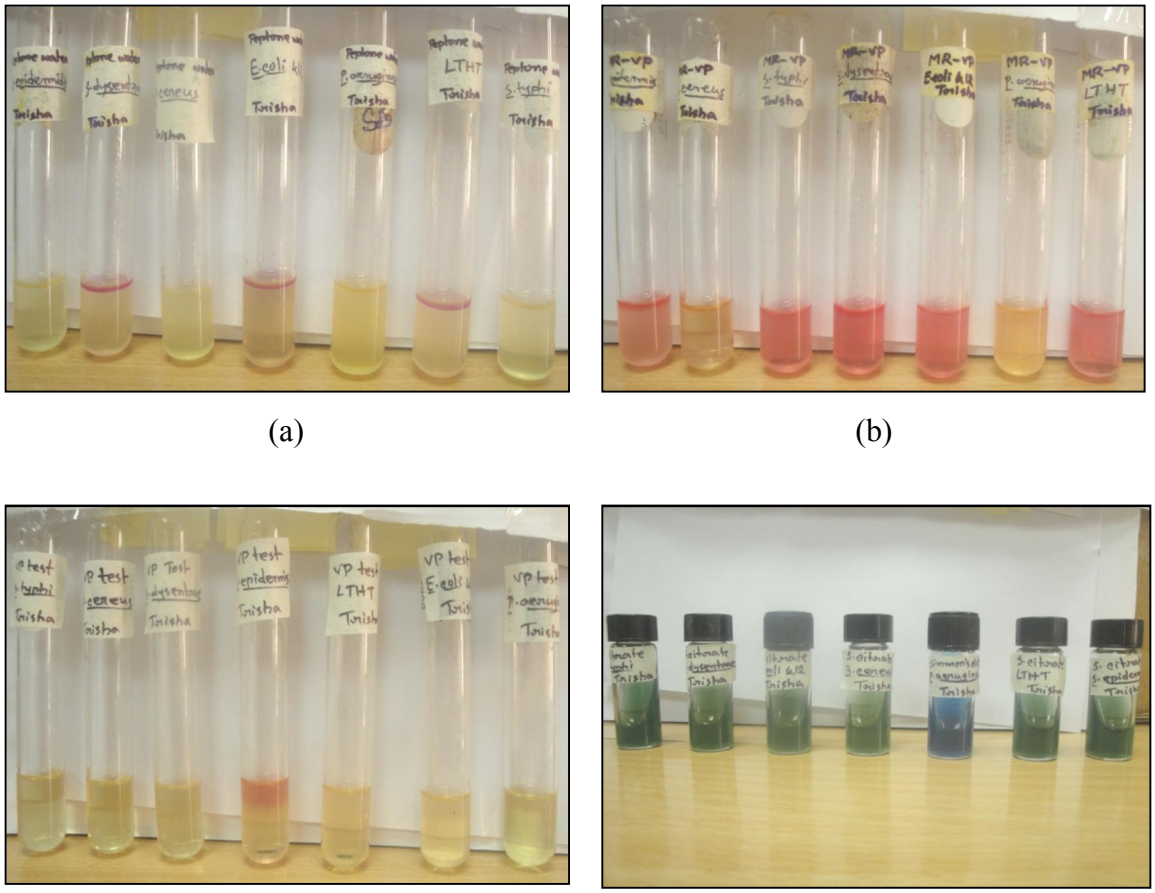
Figure 4: Growth of (i) *E. coli* K12 on MacConkey agar (ii) *E. coli* LTST on MacConkey agar (iii) *E. coli* K12 on EMB agar (iv) *E. coli* LTST on EMB agar plates (v) *S. epidermidis* on MSA agar (vi) *S. epidermidis* on BPA agar (vii) *P. aeruginosa* on cetrimide agar (viii) *B. cereus* on MYP agar (ix) *S. typhi* on XLD agar (x) *S. dysenteriae* on XLD agar plate

Isolates showed (figure 4-9) pattern of biochemical reactions typical for each bacteria according to Cappucino et al are mentioned in table 4 showing the results of biochemical tests of the clinical isolates.

Table 4: Standard results of biochemical tests of target strains

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

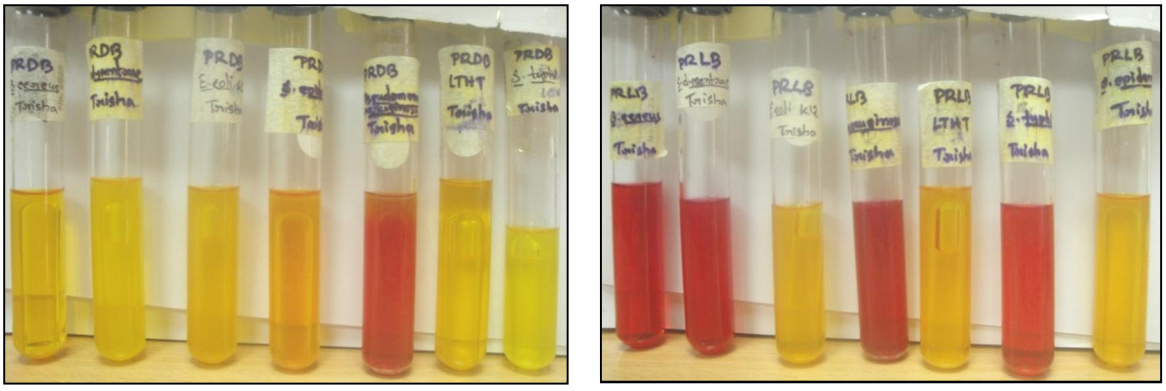
KEY: A= acidic condition, K= alkaline condition, + = positive, - = negative, AG= both acid & gas production.



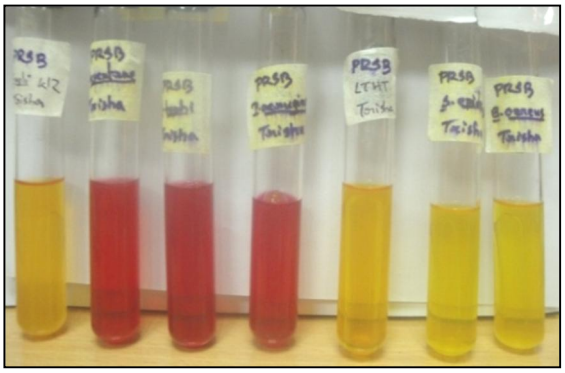
(a) (b)

(c) (d)

Figure 5: IMViC test results; (a) Indole test, (b) Methyl red test, (c) Voges Proskeur's test, (d) Citrate utilization test



(a) (b)



(d)

Figure 6: Carbohydrate fermentation tests; (a) dextrose, (b) lactose, (c) sucrose

Figure 7: Triple sugar iron (TSI) test

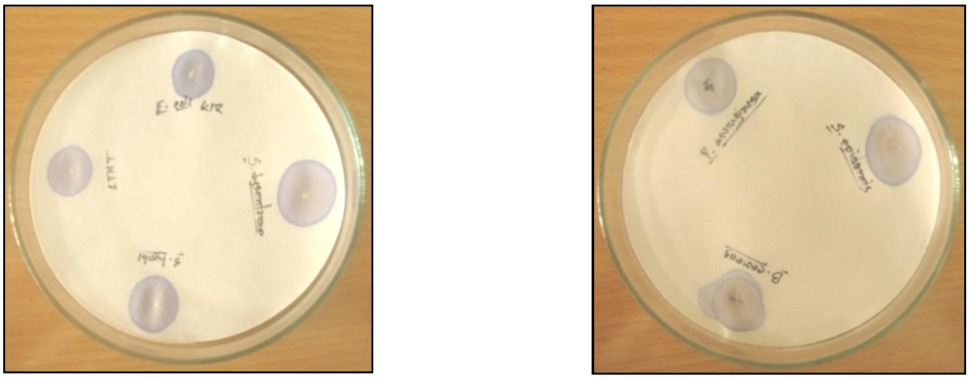


Figure 8: Oxidase test

Figure 9: Catalase test

3.2 Antimicrobial susceptibility test

Diffusion method has been employed to study the antimicrobial activity of the plant. A number of modifications have been made in the technique in order to obtain better results.

Both extracts of *T. erecta* leaves showed varying degree of antibacterial activity against the test organisms. The result of the susceptibility of the test organisms is shown in table 5 against leaf extracts. In cold aqueous extract, all the test organisms gave good susceptibility with zone of inhibition ranging from 10 mm to 14 mm per 300 µg and 12 mm to 16 mm per 600 µg; *Staphylococcus epidermidis* was the most susceptible (16 mm zone of inhibition in 600 µg) and *Pseudomonas aeruginosa* was the least susceptible (12 mm zone of inhibition per 600 µg *extract)* to the extract (figure 16). In comparison to the cold aqueous extract, the hot aqueous extracts of leaves were less effective. The zone of inhibition was between the ranges of 7 mm to 10 mm per 300 µl extract and 12 mm to 14 mm per 600 µg extract. Hot aqueous extract gave maximum inhibition with *Staphylococcus epidermidis* (11 mm and 14 mm respectively for 300 and 600 µg extract) and minimum inhibition with *Bacillus cereus* (7 mm and 12 mm respectively for 300 and 600 µg extract) showed in figure 10. However the effectiveness of methanol leaf extract was not found to be impressive.

Compared to leaf extract, flower extracts of *T. erecta* were less inhibitory against test pathogens (table 5). The cold aqueous extract showed some inhibition against *Staphylococcus epidermidis* and both the pathogenic and non-pathogenic strains of *E. coli* only when used in amount of 300 µg. Surprisingly, the hot aqueous extract showed no bacteristatic activity. However, unlike the leaf extract, the methanol extract of flower was found to be effective against some of the test organisms visualized in figure 11. Among them, non-pathogenic *E. coli* K12 was the most susceptible showing a zone of inhibition of 11 mm for 300 µg extract and 13 mm for 600 µg extract, while the pathogenic *E. coli* LTST was found to be the least susceptible showing a zone of inhibition of 8 mm for 300 µg extract and 12 mm for 600 µg extract.

Table 5: Antimicrobial activity of various leaf & flower extracts of *Tagetes erecta* against Gram positive & Gram negative pathogenic bacteria

Name of Organism	**Zone of inhibition (diameter in mm)**											
	***Tagetes erecta* leaf extracts**						***Tagetes erecta* flower extracts**					
	Cold aqueous		**Hot aqueous**		**Methanol**		**Cold aqueous**		**Hot aqueous**		**Methanol**	
	300 µg	600 µg	300 µg	600 µg	300 µg	600 µg	300 µg	600 µg	300 µg	600 µg	300 µg	600 µg
Bacillus cereus	12	14	7	12	—	—	—	—	—	—	—	—
Escherichia coli K12	12	15	8	13	—	—	—	10	—	—	11	13
Escherichia coli LTST	12	15	10	14	—	—	—	9	—	—	8	12
Pseudomonas aeruginosa	10	12	9	12	—	—	—	—	—	—	—	—
Salmonella typhi	10	13	10	13	—	—	—	—	—	—	10	12
Shigella dysentrae	14	16	10	14	—	—	—	—	—	—	—	—

Staphylococcus epidermidis	13	16	11	14	—	—	—	10	—	—	—	—

Table 6: Antibacterial activity of test organisms against amoxicillin & gentamycin

Name of Organism	**Antibiotics**			
	Amoxicillin (diameter in mm)		**Gentamycin (diameter in mm)**	
	300 µg	600 µg	300 µg	600 µg
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 dysentrae	0	2	0	0
Staphylococcus epidermidis	27	30	17	15

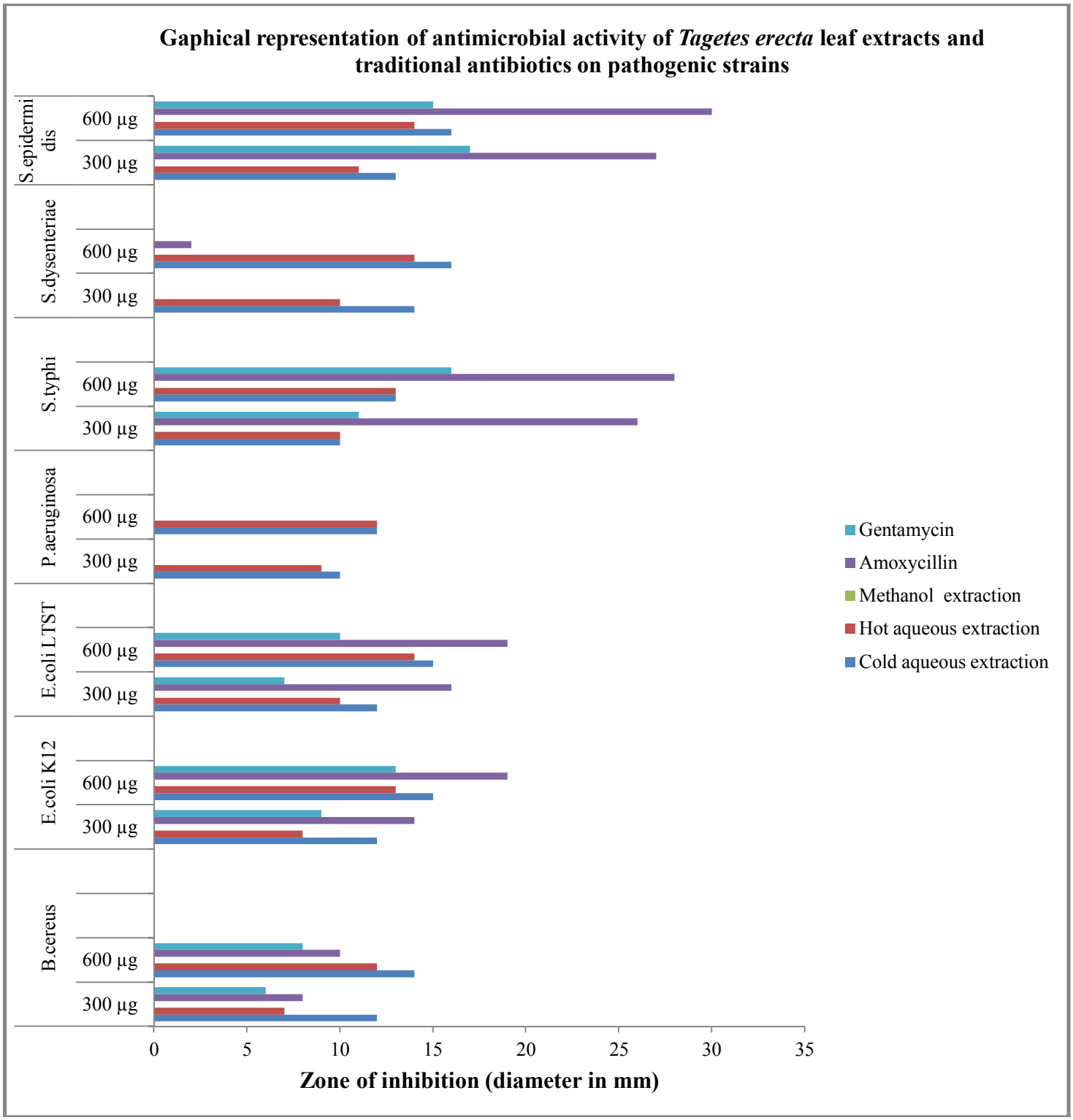


Figure 10: Graphical representation of antimicrobial activity of *T. erecta* leaf extracts and traditional antibiotics against test microorganisms

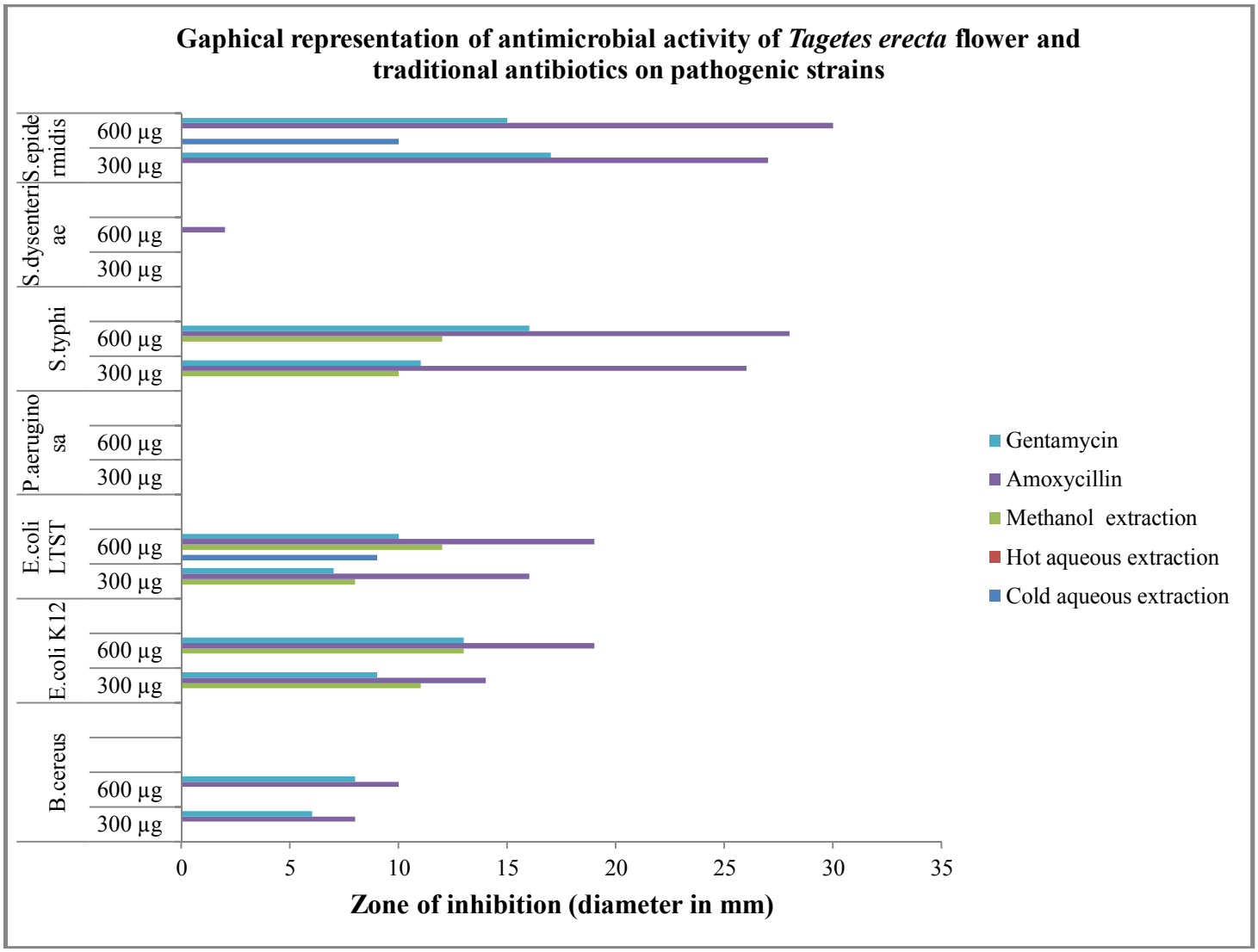


Figure 11: Graphical representation of antimicrobial activity of *T. erecta* flower extracts and traditional antibiotics against test microorganisms

3.4 Activity Index of Mexican marigold (*Tagetes erecta*) leaf and flower extract

The activity index were calculated from the obtained readings of zone of inhibition for marigold leaf and flower extract for all concentration and zone of inhibition for Gentamycin and Amoxicillin and enlisted in (table 7, 8).

Table 7: Average activity index of *Tagetes erecta* leaf extracts for test bacterial strains

Name of Organism	Zone of inhibition (diameter in mm)								Activity Index = (Zone of inhibition of extract/Zone of inhibition of antibiotic)							
	Tagetes erecta leaf extracts				Antibiotics				with Amoxicillin				with Gentamycin			
	Cold aqueous		Hot aqueous		Amoxicillin		Gentamycin		Cold aqueous		Hot aqueous		Cold aqueous		Hot aqueous	
	300 µg	600 µg	300 µg	600 µg	300 µg	600 µg	300 µg	600 µg	300 µg	600 µg	300 µg	600 µg	300 µg	600 µg	300 µg	600 µg
Bacillus cereus	12	14	7	12	8	10	6	8	1.5	1.4	0.875	1.2	2	1.75	1.16	1.5
Escherichia coli K12	12	15	8	13	14	19	9	13	0.875	0.789	0.571	0.684	1.33	1.15	0.89	1.0
Escherichia coli LTST	12	15	10	14	16	19	7	10	.75	0.789	0.625	0.737	1.71	1.5	1.43	1.4
Pseudomonas aeruginosa	10	12	9	12	resistant	resistant	resistant	resistant	--	--	--	--	--	--	--	--
Salmonella typhi	10	13	10	13	26	28	11	16	.038	0.464	0.384	0.464	0.91	0.812	0.91	0.812
Shigella dysentrae	14	16	10	14	resistant	2	resistant	resistant	--	--	--	--	--	--	--	--
Staphyloccus epidermidis	13	16	11	14	27	30	17	15	0.48	0.533	0.407	0.47	0.76	1.06	0.65	0.93

Table 8: Average activity index of *Tagetes erecta* flower extracts for test bacterial strains

Name of Organism	Zone of inhibition (diameter in mm)								Activity Index = (Zone of inhibition of extract/Zone of inhibition of antibiotic)							
	Tagetes erecta flower extracts				Antibiotics				with Amoxicillin				with Gentamycin			
	Cold aqueous		Methanol		Amoxicillin		Gentamycin		Cold aqueous		Methanol		Cold aqueous		Methanol	
	300 µg	600 µg	300 µg	600 µg	300 µg	600 µg	300 µg	600 µg	300 µg	600 µg	300 µg	600 µg	300 µg	600 µg	300 µg	600 µg
Bacillus cereus	0	0	0	0	8	10	6	8	0	0	0	0	0	0	0	0
Escherichia coli K12	0	10	11	13	14	19	9	13	0	0.53	0.79	0.68	0	0.77	1.22	1.0
Escherichia coli LTST	0	9	8	12	16	19	7	10	0	0.47	0.5	0.63	0	0.9	1.14	1.2
Pseudomonas aeruginosa	0	0	0	0	resistant	resistant	resistant	resistant	--	--	--	--	--	--	--	--
Salmonella typhi	0	0	10	12	26	28	11	16	0	0	0.38	0.42	0	0	0.91	0.75
Shigella dysentriae	0	0	0	0	resistant	2	resistant	resistant	--	0	--	0	--	--	--	--
Staphylococcus epidermidis	0	10	0	0	27	30	17	15	0	0.33	0	0	0	0.67	0	0

For cold and hot aqueous leaf extraction a remarkable activity index was found in comparison to flower extract but in case of methanol extract the result was opposite. For leaf aqueous extract, maximum antibacterial effect was found in *Bacillus cereus* for both Amoxicillin and Gentamycin antibiotics whereas minimum for *Staphylococcus epidermidis (*figure 12, 13*)*. However marigold flower had very less antibacterial effect on test organisms for hot and cold aqueous extraction but gave a promising result in activity index of methanol extraction visualized in figure 14, 15. Though for the antibiotic resistant bacterial strains like *Pseudomonas aeruginosa* and *Shigella dysenteriae* no activity index were calculated but the efficacy of plant extract in controlling their growth process were clearly represented in table 7, 8.

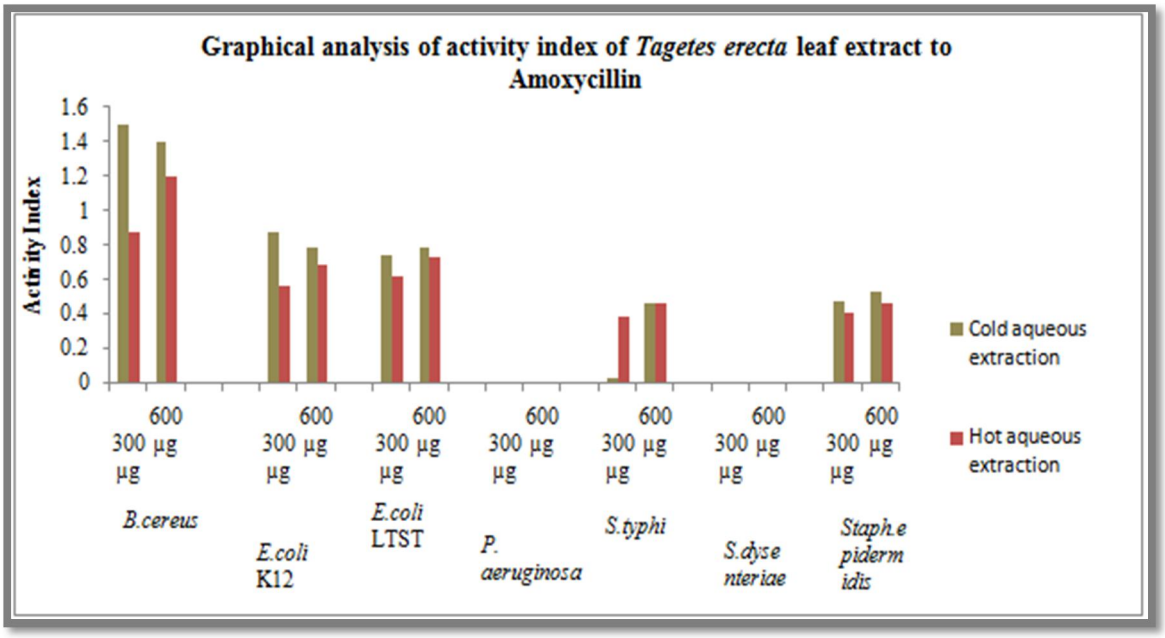


Figure 12: Graphical analysis of activity index of *T. erecta* leaf extract to amoxicillin

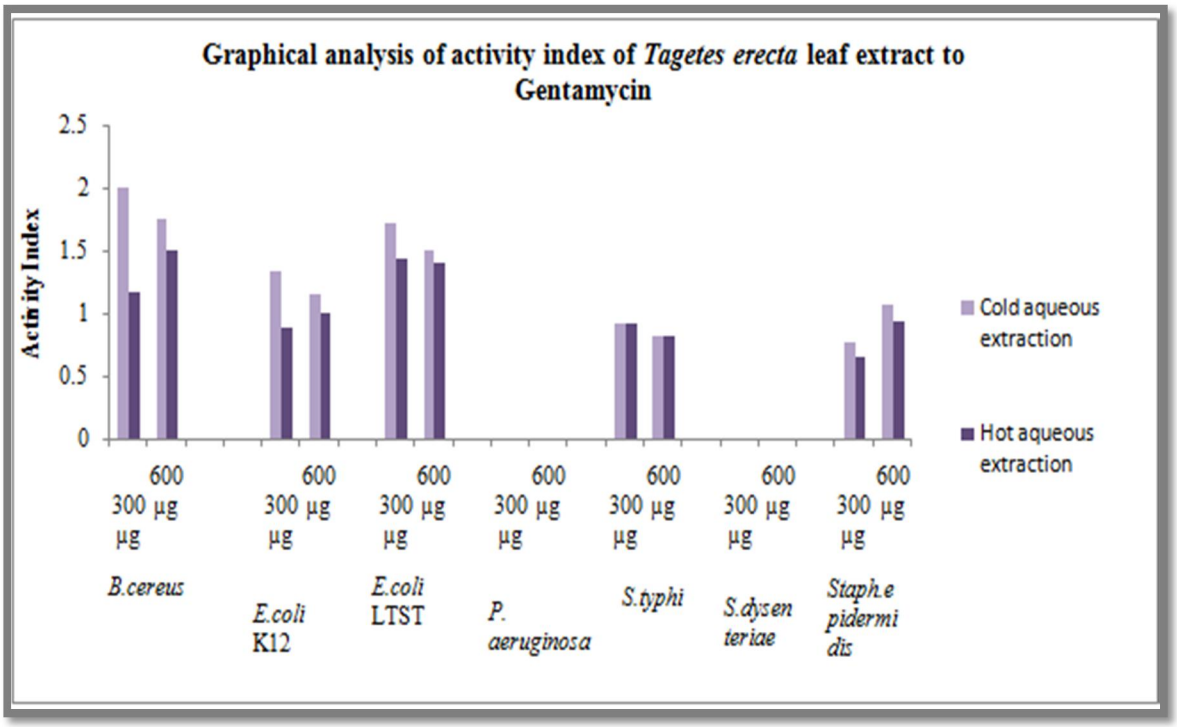


Figure 13: Graphical analysis of activity index of *T. erecta* leaf extract to gentamicin

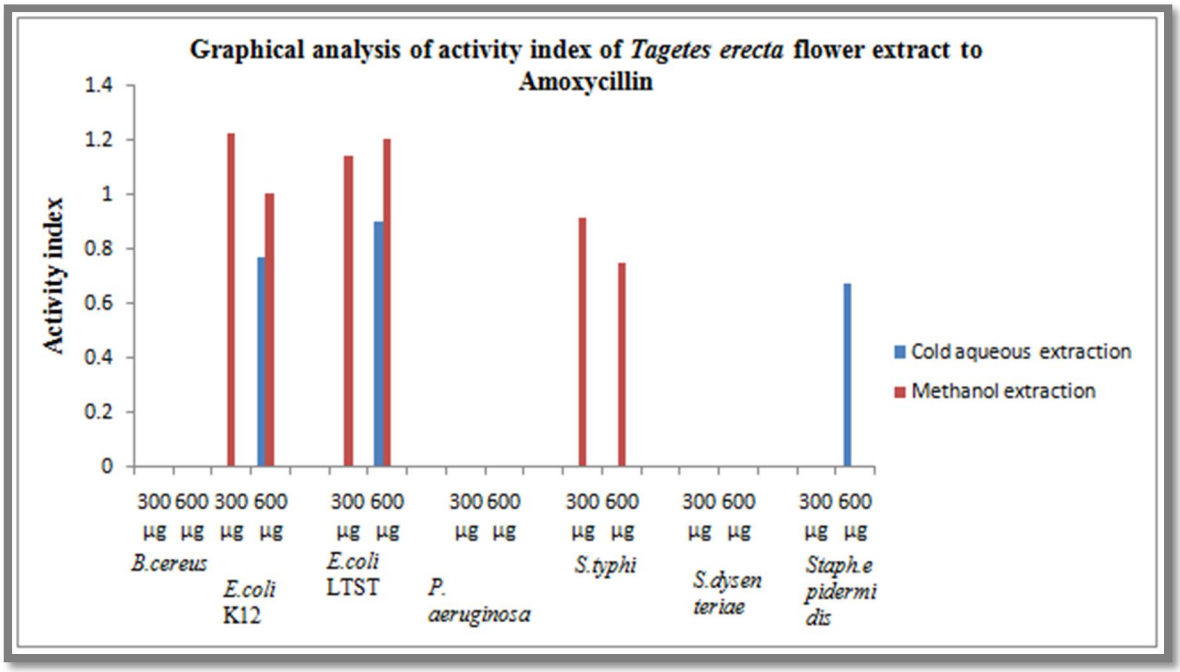


Figure 14: Graphical analysis of activity index of *T. erecta* flower extract to amoxicillin

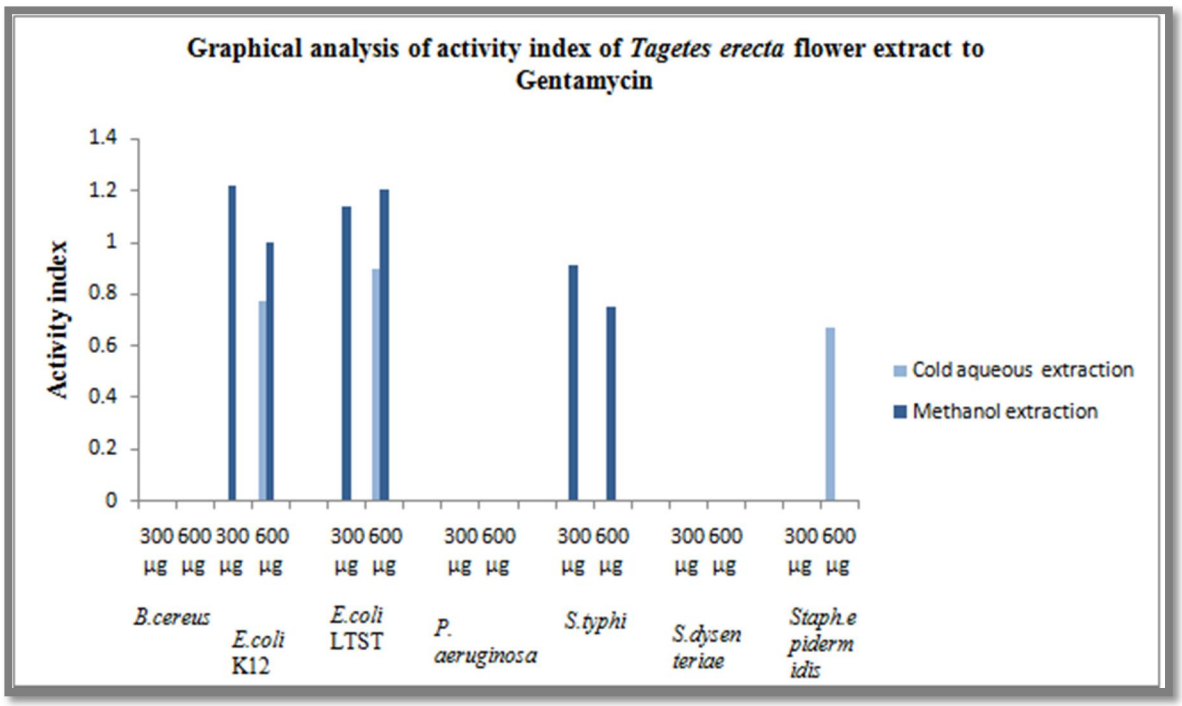
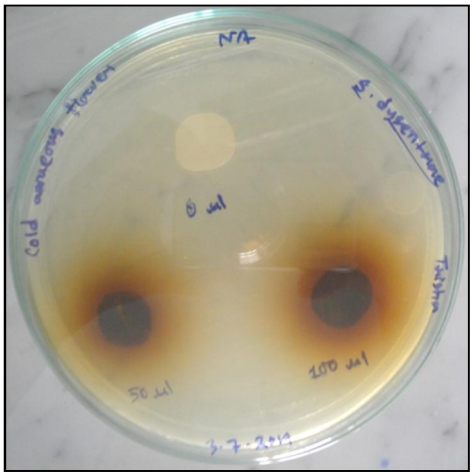


Figure 15: Graphical analysis of activity index of *T. erecta* flower extract to gentamicin



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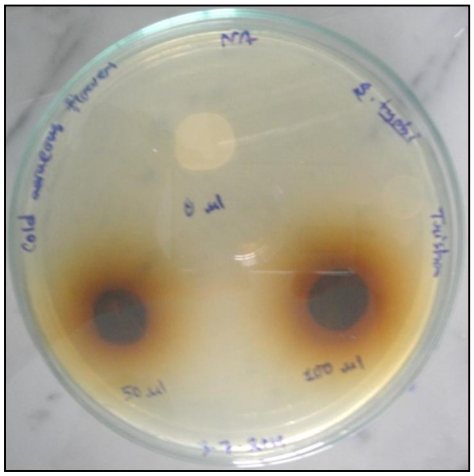

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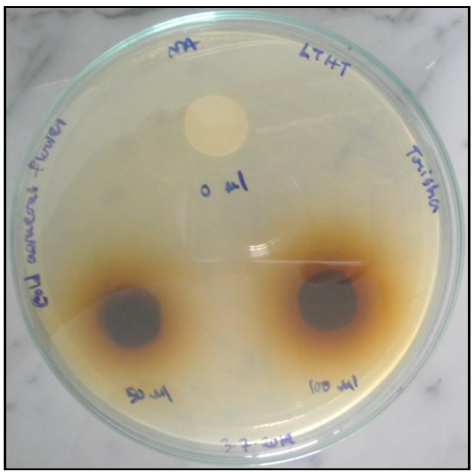

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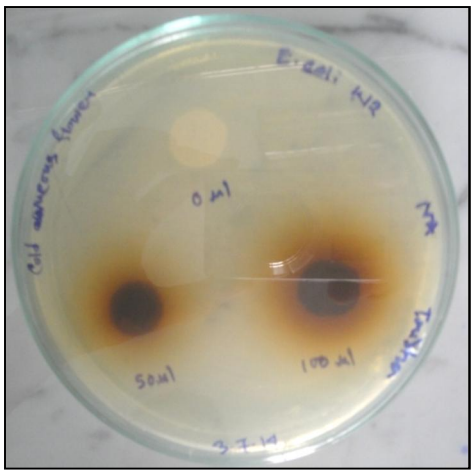

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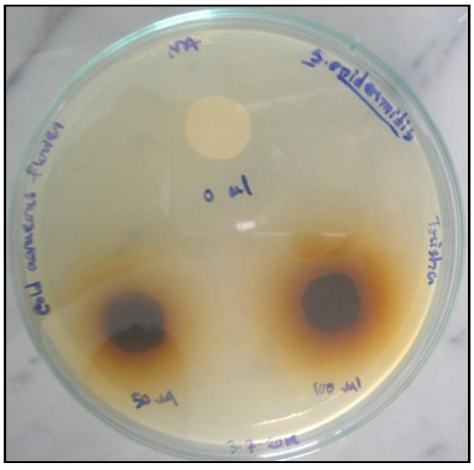

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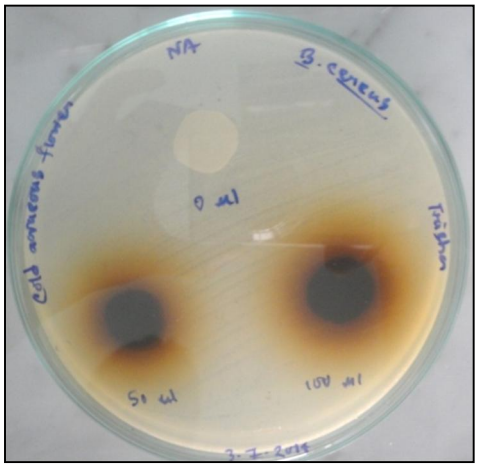

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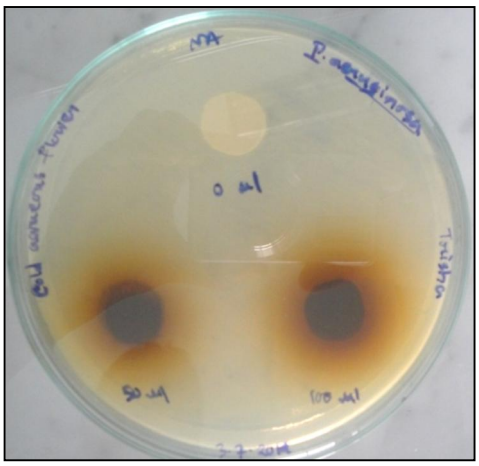
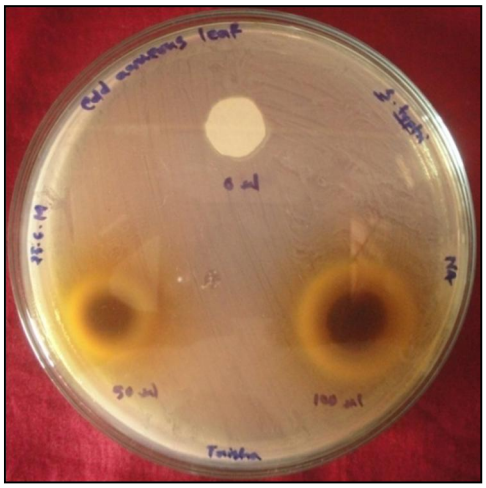


(vii)

Figure 16: Antimicrobial activity of cold aqueous extract of *T. erecta* flower on (i) *S. dysentriae* , (ii) *S. typhi* , (iii) *E. coli* LTST, (iv) *E. coli* K12, (v) *S. epidermidis* , (vi) *B. cereus* , (vii) *P. aeruginosa*

(i)



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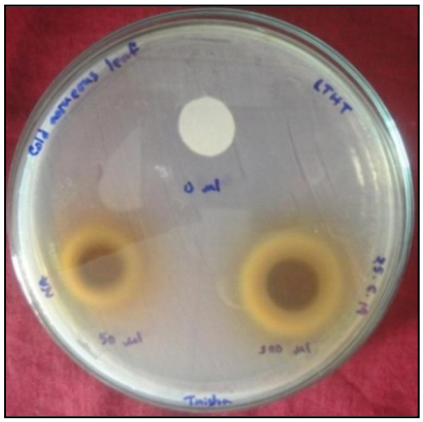

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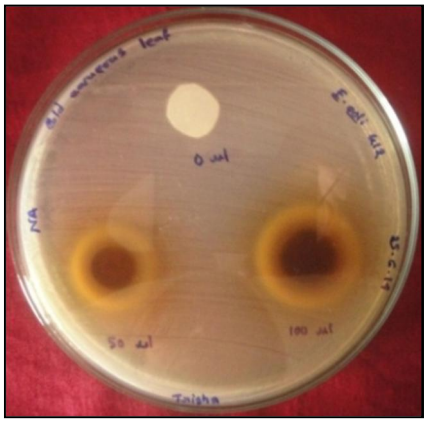

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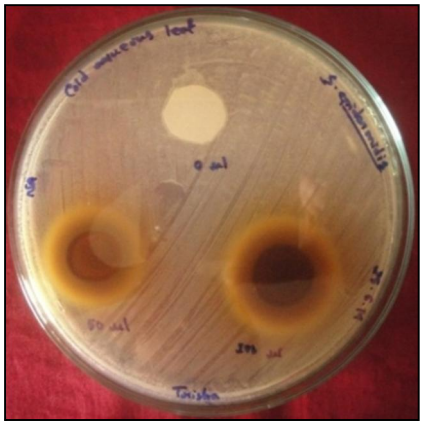

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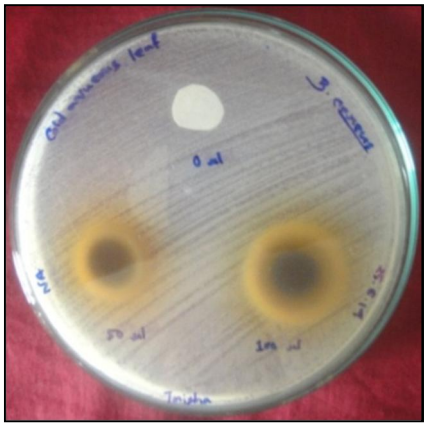

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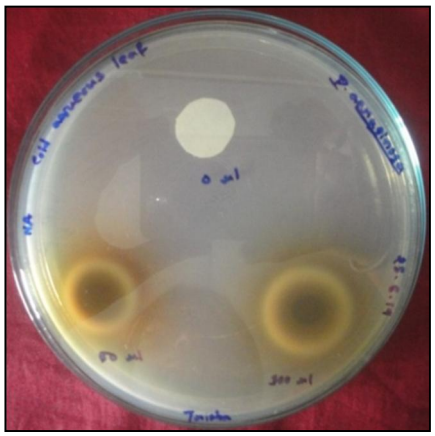
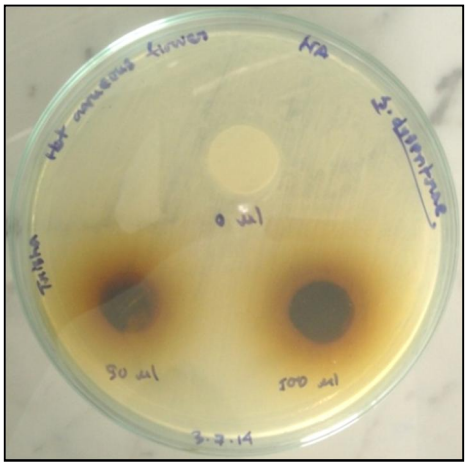


(vii)

Figure 17: Antimicrobial activity of cold aqueous extract of *T. erecta* leaf on (i) *S. dysentriae* , (ii) *S. typhi* , (iii) *E. coli* LTST, (iv) *E. coli* K12, (v) *S. epidermidis* , (vi) *B. cereus* , (vii) *P. aeruginosa*



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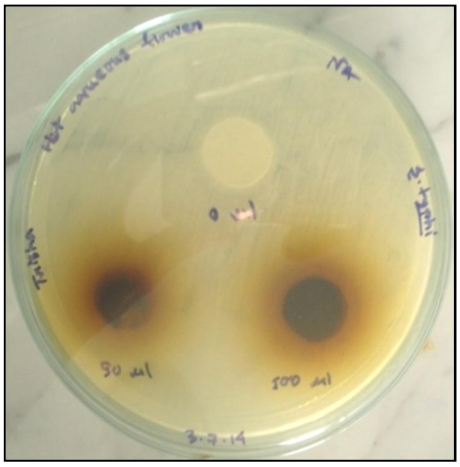

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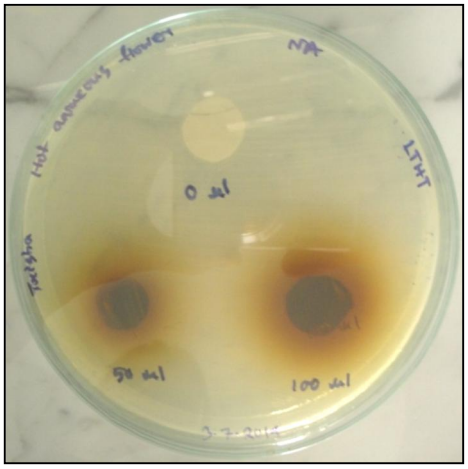

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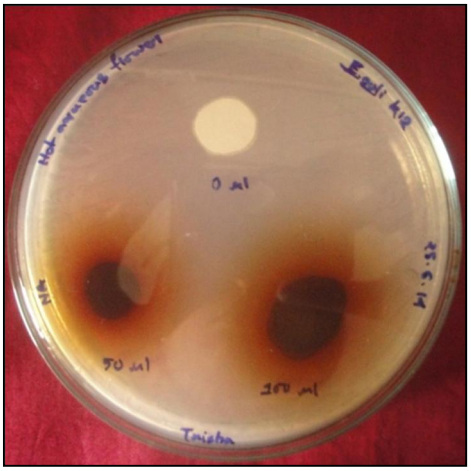

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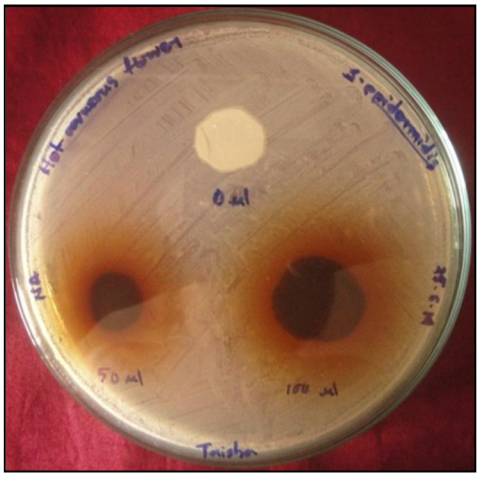

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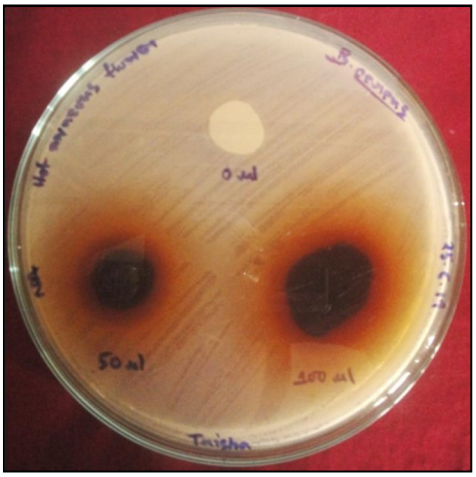

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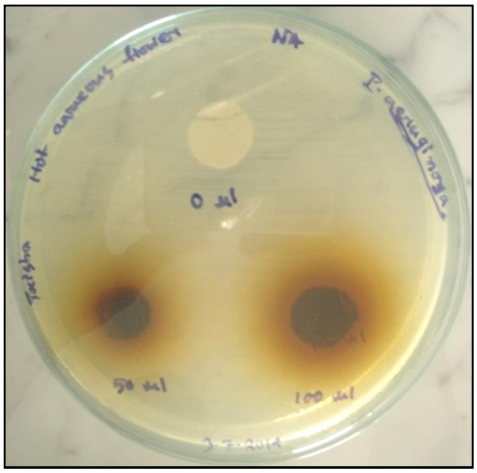

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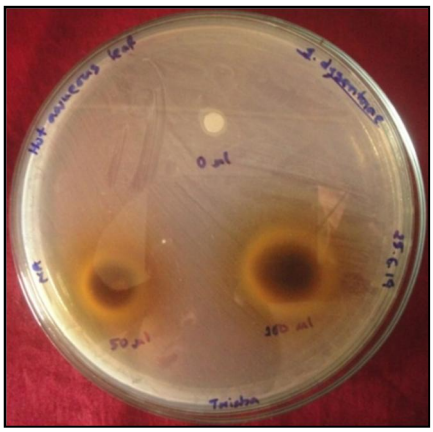
Figure 18: Antimicrobial activity of hot aqueous extract of *T. erecta* flower on (i) *S. dysentriae* , (ii) *S. typhi* , (iii) *E. coli* LTST, (iv) *E. coli* K12, (v) *S. epidermidis* , (vi) *B. cereus* , (vii) *P. aeruginosa*



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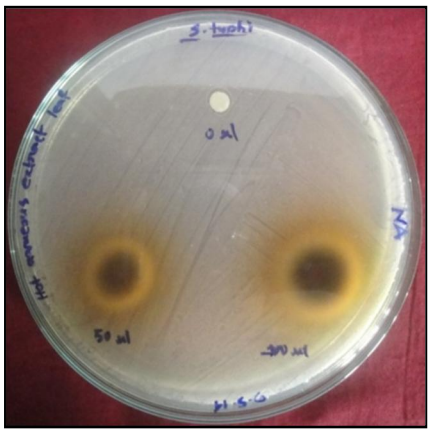

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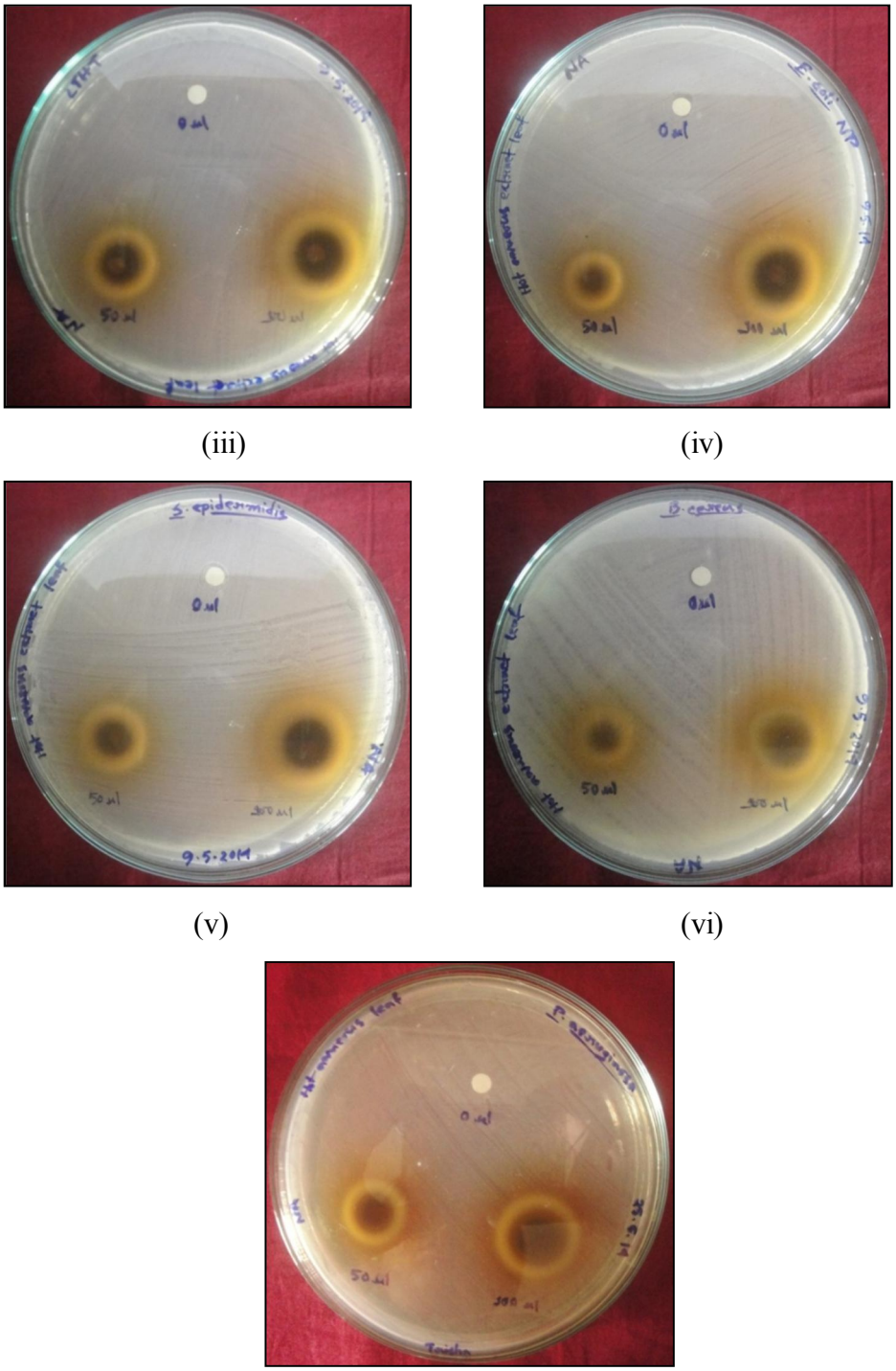
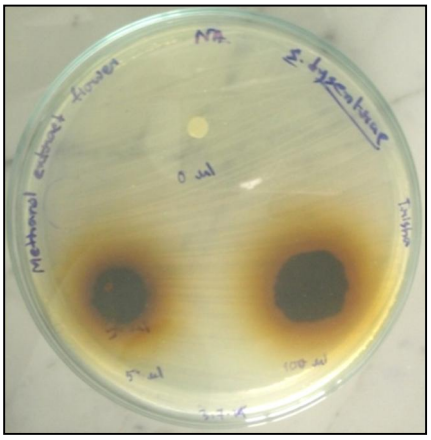


(iii) (iv)

(v) (vi)

(vii)

Figure 19: Antimicrobial activity of hot aqueous extract of *T. erecta* leaf on (i) *S. dysentriae* , (ii) *S. typhi* , (iii) *E. coli* LTST, (iv) *E. coli* K12, (v) *S. epidermidis* , (vi) *B. cereus* , (vii) *P. aeruginosa*



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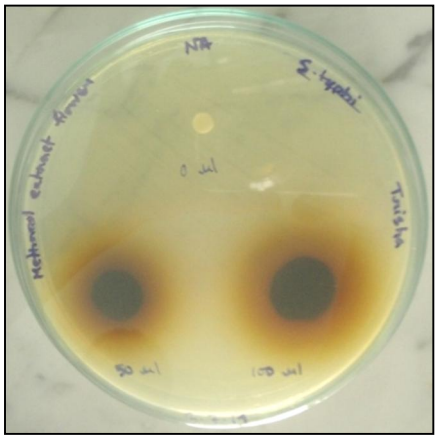

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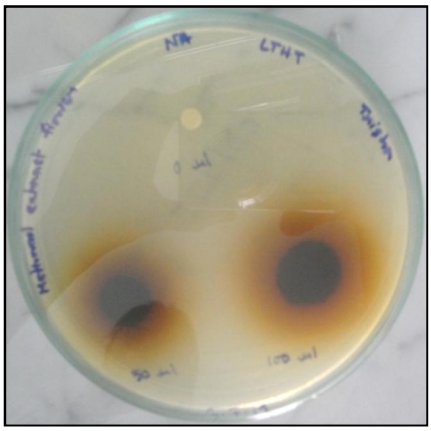

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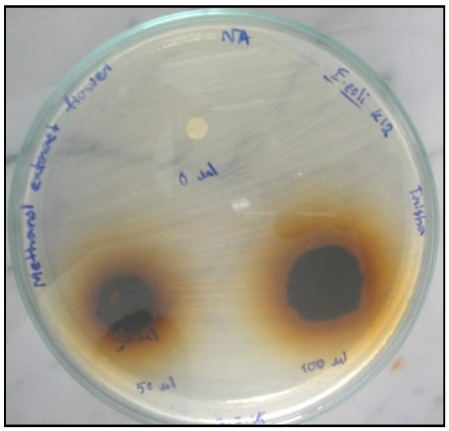

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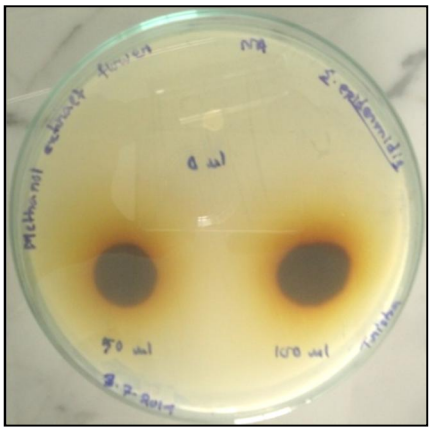

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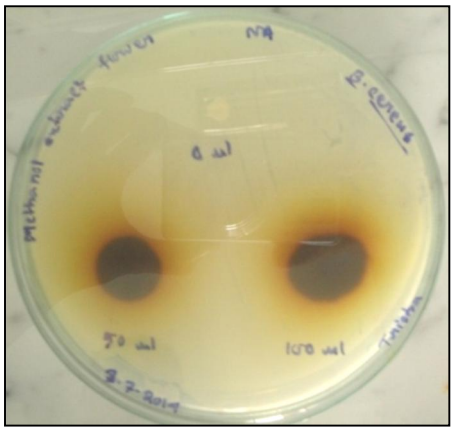

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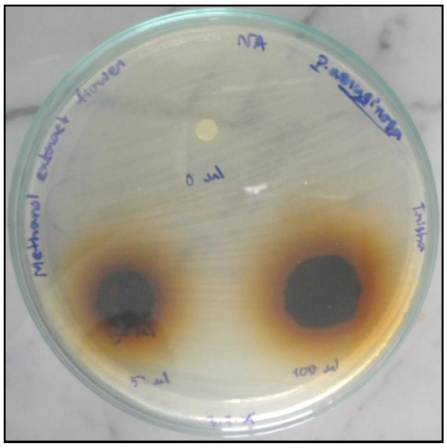

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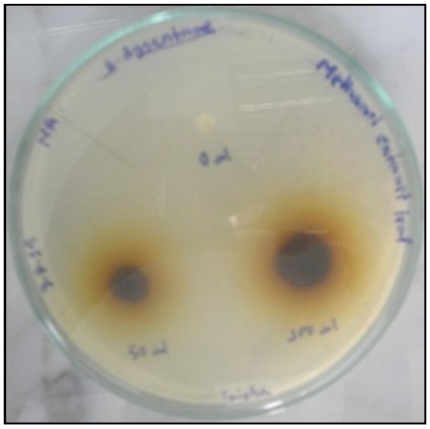
Figure 20: Antimicrobial activity of methanol extract of *T. erecta* flower on (i) *S. dysentriae* , (ii) *S. typhi* , (iii) *E. coli* LTST, (iv) *E. coli* K12, (v) *S. epidermidis* , (vi) *B. cereus* , (vii) *P. aeruginosa*



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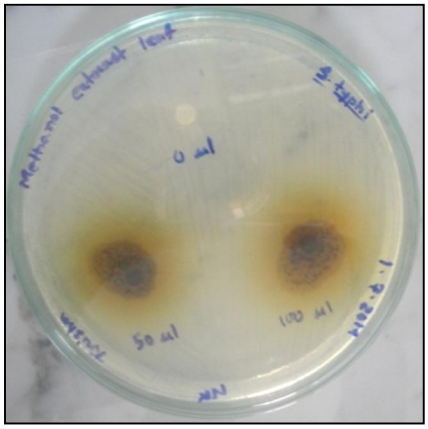

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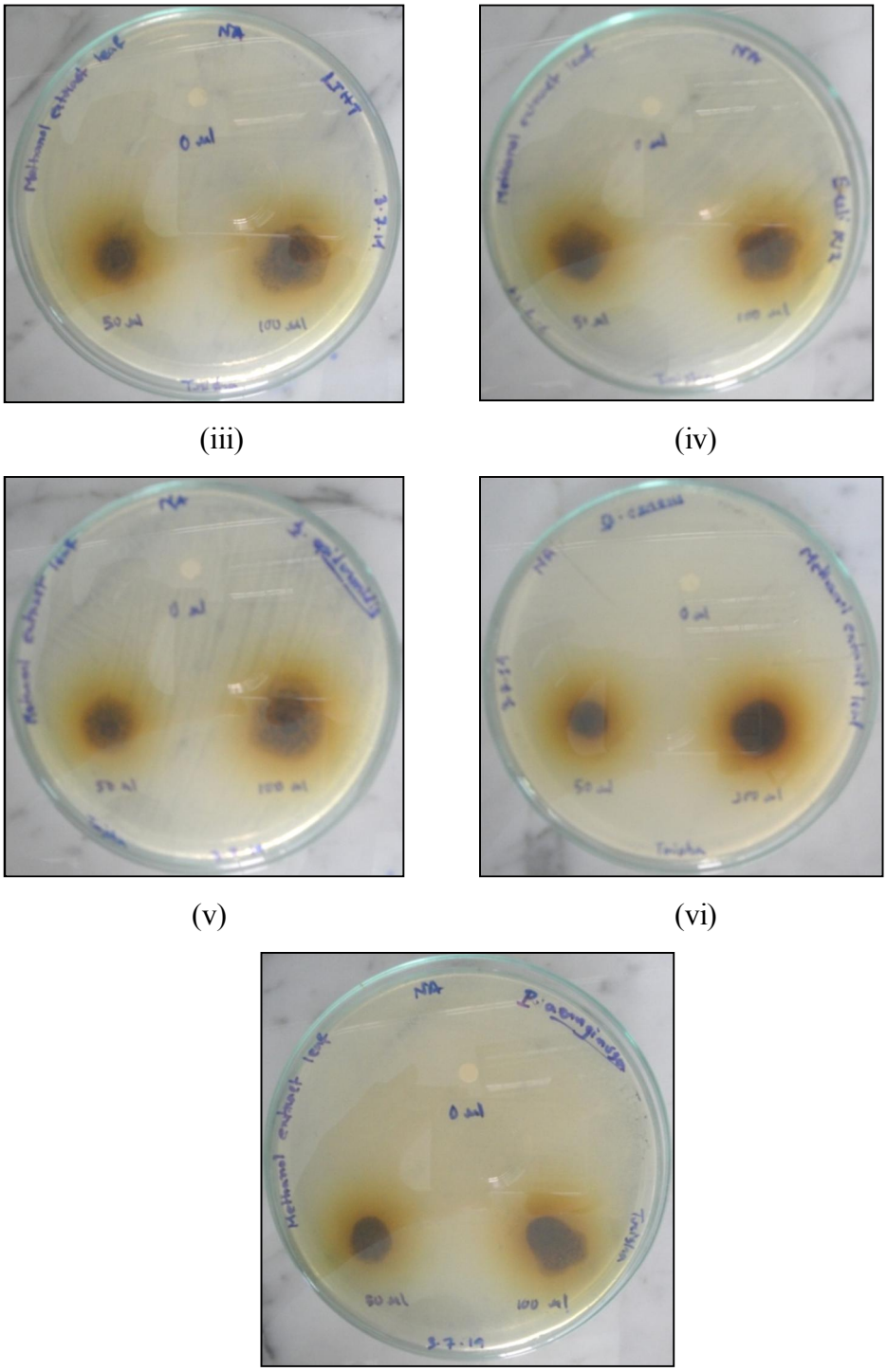
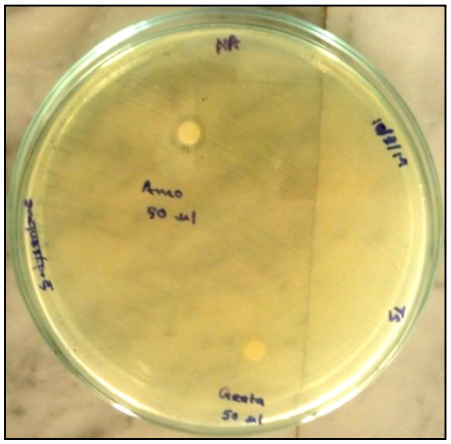


(iii) (iv)

(v) (vi)

(vii)

Figure 21: Antimicrobial activity of methanol extract of *T. erecta* leaf on (i) *S. dysentriae* , (ii) *S. typhi* , (iii) *E. coli* LTST, (iv) *E. coli* K12, (v) *S. epidermidis* , (vi) *B. cereus* , (vii) *P. aeruginosa*



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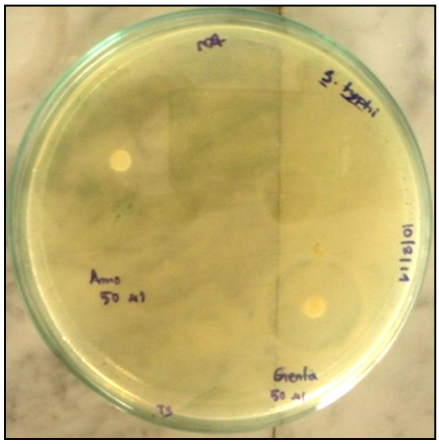

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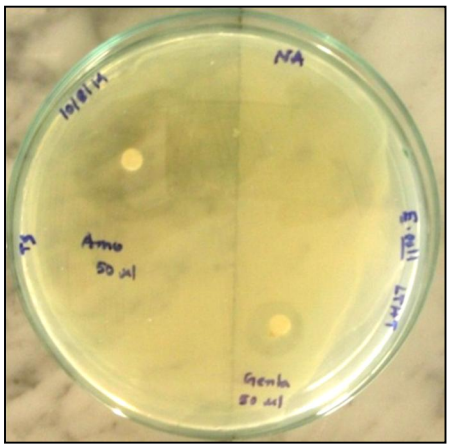

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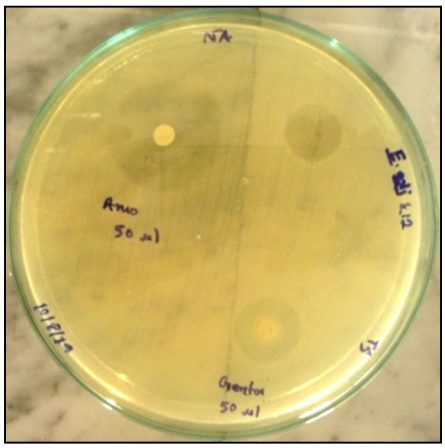

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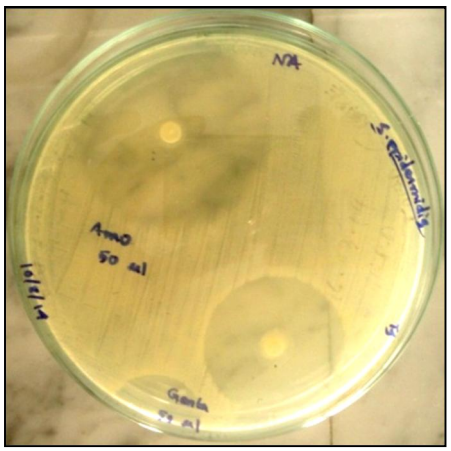

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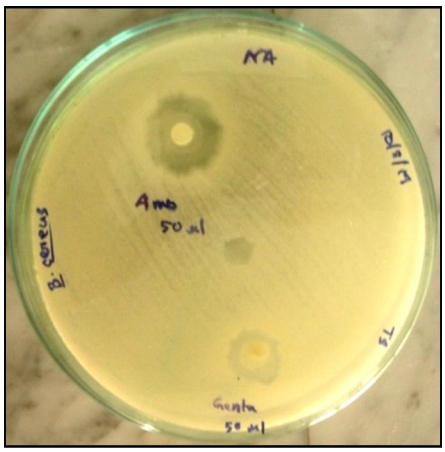

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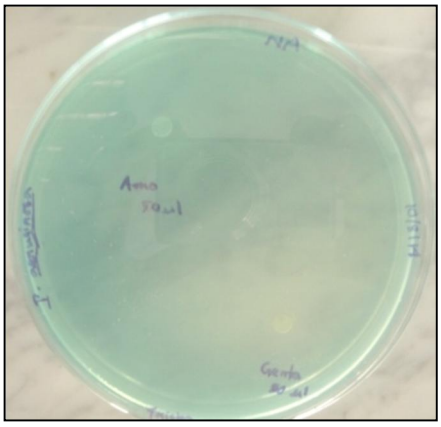
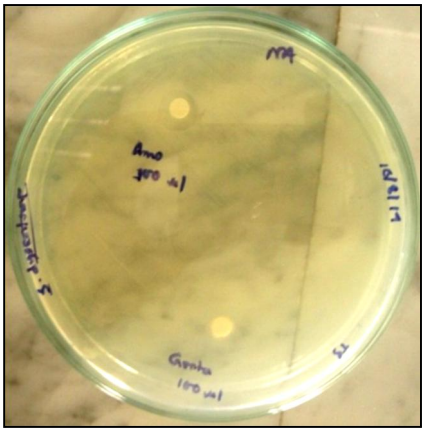
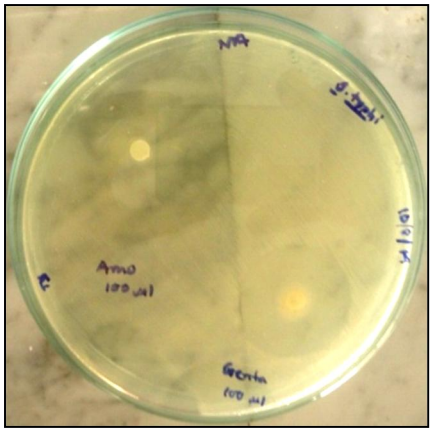


(vii)

Figure 22: Antibiotic susceptibility of (i) *S. dysentriae* , (ii) *S. typhi* , (iii) *E. coli* LTST, (iv) *E. coli* K12, (v) *S. epidermidis* , (vi) *B. cereus* , (vii) *P. aeruginosa* against amoxicillin and gentamicin (300 ug antibiotic solution per disk)



(i)



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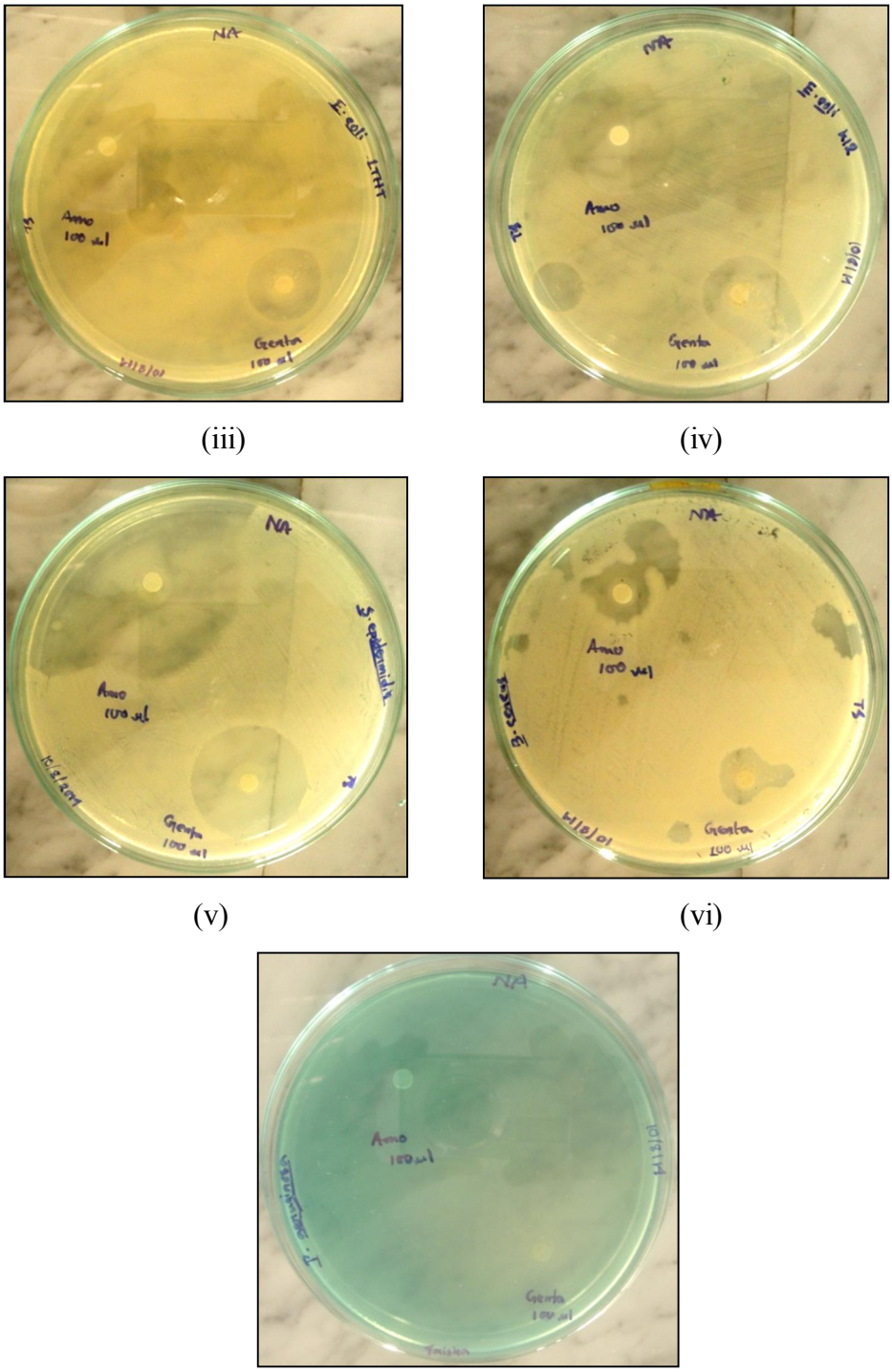

(iii) (iv)

(v) (vi)

(vii)

Figure 23: Antibiotic susceptibility of (i) *S. dysentriae* , (ii) *S. typhi* , (iii) *E. coli* LTST, (iv) *E. coli* K12, (v) *S. epidermidis* , (vi) *B. cereus* , (vii) *P. aeruginosa* against amoxicillin and gentamicin (600 µg antibiotic solution per disk).

Increased concerns about reliance on antibiotics as the primary treatment targeting many bacterial infections have led to a reassessment of other existing treatments as well as a search for novel alternatives. The need of alternatives to antibiotics is heightened because of surging numbers of antibiotic-resistant strains. Natural treatments such as probiotics remain important for prevention and treatments of symptoms of bacterial infections. The World Health Organization estimates that, about 80 percent of the world's population relies on herbs for its primary healthcare needs. More than 35,000 plant species are being used around the world for the medicinal purposes in traditional and ethnomedicinal practices.

Tagetes erecta has been used in ayurvedic medicine since ancient times, with various biological applications. It is reported to have antioxidant, antimycotic, analgesic activity and 18 active compounds are identified by GC-MS, many of them are terpenoids. Although the crude extract and whole fresh plant has numerous medicinal applications as well as clinical applications but academic medicinal plant researchers remained rather skeptical about the medical use of *Tagetes erecta* which can be made only after extensive research on its bioactivity, mechanism of action, pharmaco-therapeutics and extensive safety studies. However persistent research is going on and it would be easier to develop new drugs on basis of mechanism of action and pharmacological effects of its crude extracts.

The anti-bacterial activity of *T. erecta* flowers in different solvents were reported against *Alcaligens faecalis, Bacillus cereus, Campylobacter coli, Escherichia coli, Klebsiella pneumoniae, Proteus vulgaris, Pseudomonas aeruginosa, Streptococcus pyogens* showing maximum zone of inhibition for *K. pneumonia* [16]. Along with 18 other Colombian traditional medicinal plants used for cutaneous infection, the antimicrobial activity of *T. erecta* was screened against *Neisseria gonorrhoeae* (NG) by disc susceptibility assay. The *T. erecta* flower parts showed maximum inhibitory action against NG strain [17].

The present literature survey paints an optimistic picture for the prospect of use of leaf and flower extracts of *Tagetes erecta* against various pathogenic strains showed in figure 16-21. Both the cold and hot aqueous leaf extracts of *T. erecta* leaves showed varying degree of antibacterial activity against the test organisms (table 5). However the methanol extract of *T. erecta* leaves was not found to be effective. In cold aqueous extract, all the test organisms gave good susceptibility with potential zone of inhibition. *Staphylococcus epidermidis* was the most susceptible while *Pseudomonas aeruginosa* was the least susceptible to the extract. In comparison to the cold aqueous extract, the hot aqueous extracts of leaves were less effective. Hot aqueous extract gave maximum inhibition with *Staphylococcus epidermidis* and minimum inhibition with *Bacillus cereus*.

Compared to leaf extract, flower extracts of *T. erecta* were less inhibitory against test pathogens. The cold aqueous extract showed maximum inhibition against *E. coli* K12 while startlingly, the hot aqueous extract showed no bacteristatic activity. However, unlike the leaf extract, the methanol extract of flower was found to be effective against some of the test organisms. Among them, non-pathogenic *E. coli* K12 was the most susceptible while the pathogenic *E. coli* LTST was found to be the least susceptible.

The antibacterial efficacy of our target plant extracts could be defined in terms of the activity index where the inhibition zone of flower and leaf extracts were compared with the zone of inhibition of traditional antibiotics . The maximum activity index of marigold leaf extract was obtained for *Baillus cereus* for both Amoxicillin and Gentamycin test antibiotics with cold aqueous leaf extract and minimum was for *Salmonella typhi* (figure 12, 13). As *Pseudomonas aeruginosa* and *Shigella dysenteriae* showed resistant pattern against traditional antibiotics, use of leaf extracts gave promising result to control their infectious activity. On the other hand, the flower extract activity index gave clear profile that in comparison to leaf extract, organisms showed minimum sensitivity pattern which is shown in figure 14, 15. Nevertheless all the extracts were found effective yet the hot aqueous extract showed maximum inhibition against the test microorganisms followed by cold aqueous and methanol extracts.

The results suggested us that species of marigold i.e. *Tagetes erecta* has antibacterial effect against food and water borne disease caused by gram positive and gram negative bactera. Bioactive substances from this plant can therefore be employed in the formulation of antimicrobial agents for the treatment of various emerging pathogens.

The time clock is ticking down and the pressure is on for researcher clinicians, and public health officials to come across doable alternatives to antibiotics and to effectively implement these alternatives in clinical settings.

Aqueous extract of *T. erecta* 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 therapy in traditional medicine.

However, there is adequate body of data and a desperate enough need to discover alternative treatment modalities against fast emerging organisms to merit further research in the field of development of herbal antibiotics particularly because of the concomitant increase in immunosuppressed population worldwide. The present investigation evidenced that the traditional medicinal herb, *Tagetes erecta* might provide a better therapeutic alternatives for treating bacterial infections.

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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^{o}C.

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',N'-tetramethyl-p-phenyldiamine-dihydrochloride was dissolved in 10 ml of distilled water and covered with aluminum foil. Then the solution was stored at 4^{o}C.

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