MATERIALS AND METHODS

"All we have yet discovered is a brief in comparison of what still lies in the great treasury of nature" – ***Antony Van Leewenhoek***

Diarrhoea is a propulsive passing of three or more watery stool within 24 hours. The epidemiological significance of gastroenteritis varies with geographical area and environmental factors like temperature, relative humidity, rainfall and others. Socio economic status, poor sanitation and overcrowding also have an impact on it (Iruka *et al.*, 2000).

4.1. Materials

4.1.1. For epidemiological study

A total of 1259 stool samples were collected from clinically diagnosed cases of acute gastroenteritis (AGE). Stool samples were also collected from 250 age and sex matched, apparently healthy normal people (control group). All these samples formed the study materials for epidemiological study of diarrhoeal pathogens.

4.1.2. For Antimicrobial activity

4.1.2.1. Plants chosen

Seed kernel of *Mangifera indica* and fruit rind of *Punica granatum* were the plant materials chosen for antibacterial activity studies.

4.1.2.2. Microbes chosen

4.1.2.2.a. Clinical isolates

Escherichia coli, Salmonella sp. and *Shigella sp.* isolated from the diarrhoeal patients were used as test organism.

4.1.2.2.b. Referral standards

Standard referral *Escherichia coli* strain MTCC 46, *Salmonella typhimurium* MTCC 1235 and *Shigella flexneri* MTCC 1537 obtained from Microbial Type Culture Collection Centre, Chandigarh were also used for reference purpose.

4.1.3. For antibiotic sensitivity - Standard antibiotics

Both broad spectrum and narrow spectrum antibiotics were used to assess sensitivity pattern of the clinical isolates i.e., *Escherichia coli*, *Salmonella sp.* and *Shigella sp.*. Antibiotics like Amikacin (30µg), Azithromycin (15µg), Azlocillin (75µg), Aztreonam (30µg), Carbenicillin (100µg), Cefdinir (5µg), Cefepime (30µg), Cefixime (5µg), Cefpodoxime (10µg), Cefprozil (30µg), Chloramphenicol (30µg), Ciprofloacxin (5µg), Doxycycline (30µg), Fosfomycin (200µg), Levoflaxacin (5µg), Meropenem (10µg), Methicilline (5µg), Minocycline (30µg), Nalidixicacid (30µg), Novobiocin (30µg), Ofloxacin (5µg), Rifamycin (5µg), Sparfloxacin (5µg), Ticarcillin (75µg), Vancomycin (30µg), Kanamycin (30µg), Clarithromycin (15µg), Trimethoprim (5µg), Gentamycin (10µg), Spectinomycin (100µg), Amoxycillin (30µg), and Oxytetracycline (30µg), were used to see the sensitivity pattern of enteric pathogens.

4.2. Categories of clinical samples

Stool samples were collected from children below 15 years of age. The samples were categorized based on age, sex and clinical conditions. In addition to clinical conditions, duration of illness was also considered for categorization of the samples. Based on clinical symptoms samples were collected from diarrhoea, diarrhoea with vomiting, dysentery and diarrhoea with dysentery (Table – 4.1 and 4.2). For comparitive analysis, age and sex matched stool samples were also collected from apparently healthy normal children.

4.3. Source of samples

Stool samples were collected from clinically diagnosed acute gastroenteritis (AGE) patients admitted in Annal Ghandhi Memorial Government Hospital, Tiruchirappalli; Kinsley Hospital, Tiruchirappalli and Maruthi Hospital, Tiruchirappalli. Age and sex matched control stool samples were collected from apparently healthy normal individuals of Tiruchirappalli city and children of Srirangam Government School.

4.4. SOURCE OF PLANT MATERIAL

Good quality fruit rind of pomegranate and seed kernel of mango were collected from the fruits purchased from the fruit stalls of Srirangam, Tiruchirappalli, Tamilnadu, India.

4.5. Parameters Analysed

4.5.1. Clinical sample collection

4.5.2. Grouping of clinical samples

4.5.3. Processing of clinical samples

4.5.4. Examination of clinical samples.

4.5.4.1. Gross examination

4.5.4.2. Macroscopic examination

4.5.4.3. Microscopic examination - Direct smear

4.5.4.3.1. Iodine wet mount

4.5.4.3.2. Saline wet mount

4.5.5. Culture of clinical samples for bacterial pathogens

4.5.5.1. Isolation and identification of *Escherichia coli*

4.5.5.2. Isolation and identification of *Salmonella sp.*

4.5.5.3. Isolation and identification of *Shigella sp.*

4.5.5.4. Isolation and identification of *Vibrio cholerae*

4.5.5.5. Isolation and identification of *Aeromonas sp.*

4.5.5.6. Isolation and identification of *Campylobacter sp.*

4.5.5.7. Isolation and identification of *Yersinia enterocolitica*

4.5.5.8. Isolation and identification of *Enterobacter sp.*

4.5.5.9. Isolation and identification of *Citrobacter sp.*

4.5.6. Identification of bacterial isolates

4.5.6.1. Macroscopic examination

4.5.6.2. Microscopic examination

4.5.6.3. Biochemical tests.

4.5.7. Environmental factors

4.5.8. Antibiotic sensitivity test

4.5.9. Serogrouping of *Escherichia coli*

4.5.10. Plant materials selection, collection and extraction

4.5.10.1. Plant material selection and collection

4.5.10.2. Preparation of extract

4.5.11. Antibacterial activity test

4.5.11.1. Test microorganisms – preparation and maintenance

4.5.11.2. Disc preparation

4.5.11.3. Inoculum preparation

4.5.11.4. Determination of anti - bacterial activity

4.5.11.5. Determination of Minimum Inhibitory Concentration

4.5.12. Phytochemical analysis

4.5.12.1 Analysis of macromolecules

4.5.12.1.1. Qualitative analysis of macromolecules

4.5.12.1.1 a. Analysis of carbohydrates

4.5.12.1.1 b. Analysis of protein

4.5.12.1.1 c. Analysis of amino acids

4.5.12.1.1 d. Analysis of lipid

4.5.12.1.2. Quantitative analysis of macromolecules

4.5.12.1.2 a. Quantification of carbohydrates

4.5.12.1.2 b. Quantification of reducing sugar

4.5.12.1.2 c. Quantification of protein

4.5.12.1.2 d. Quantification of amino acids

4.5.12.1.2 e. Quantification of lipids

4.5.12.2. Qualitative analysis of secondary metabolites

4.5.12.2.1. Flavonoids

4.5.12.2.2. Alkaloids

4.5.12.2.3. Steroids

4.5.12.2.4. Glycosides

4.5.12.2.5. Triterpenoids

4.5.12.2.6. Saponins

4.5.12.2.7. Tannins

4.5.12.2.8. Anthroquinones

4.5.13. Analysis of phytochemical compounds by Thin Layer Chromatography (TLC).

4.5.14. Analysis of phytochemical compounds by High Performance Thin Layer Chromatography (HPTLC)

4.5.15. Statistical analysis

4.5. Methods

Various standard methods were adopted to analyse the epidemiology of acute gastroenteritis (Henry, 1994 and Koneman *et al.*, 1994). Standard techniques were followed to study the anti microbial activity of the selected plant material on clinical isolates of diarrhoeal pathogens (Bauer *et al.*, 1966 and NCCLS, 1993). Phytochemical analysis of the plant material was done by making use of standard methods (Harboune, 1976).

4.5.1. Clinical sample collection

A part of body fluid or any other material, which represent actual infection of an individual are called clinical sample or specimen. In this study, stool happened to be the clinical sample or specimen. Stool samples were collected in a wide mouthed container (Hi-media) from clinically diagnosed patients and apparently healthy control groups. After collection of samples, the containers were closed tightly to avoid any leakage during transportation. Fresh samples are essential for the recovery of motile trophozoites and so they were brought to the laboratory within an hour of collection. The samples were not refrigerated, as some of the enteric pathogens are highly sensitive to temperature. While collecting the sample, care was taken to label the sample. The label had the name of the patient, date of collection, age and sex of the patients and clinical condition along with hospital name, ward name, ward number and physicians name (Koneman *et al.*, 1994).

4.5.2. Grouping of clinical samples

Details about the patients were collected initially by making use of patients history card. The physicians of the pediatric ward and enteric wards of respective hospitals grouped the patients clinically, based on their signs and symptoms. Stool samples from 1259 patients were collected for this study. Clinical samples were from the patients

with diarrhoea, patients with vomiting and diarrhoea, patients with dysentery and patients with diarrhoea and dysentery (Table – 4.1).

4.5.3. Processing of clinical samples

Various types of processing were done to find out the correct etiological agent of the disease. Processing varies in accordance with the aim of the study and the pathogens looked for. Stool samples were emulsified with saline and lugols iodine to look for protozoans and nematodes. Stool samples were enriched with different enrichment media to recover all kinds of pathogenic bacteria available in the sample.

4.5.3.1. Gross examination of clinical samples

Clinical samples were subjected to gross examination to check for the presence of segmented adult worms of intestinal helminthes. Samples were also looked for the presence of helminthic larvae.

4.5.3.2. Macroscopy of clinical sample

Fresh stool samples were looked for their physical nature like colour, odour, consistency and other such morphological features. This is to look for the types of organisms present. Based on consistency, faecal samples were categorized as formed, semiformed, soft and watery. In addition to nature, colour, odour and consistency, samples were also looked for the presence of mucous and blood. These findings would be of great help in identifying the nature of infection, which would facilitate in finding the type of infection (Table – 4.3).

Table – 4.3

Macroscopic nature of stool sample and probable pathogens (Monica, 1987)

S.No	Nature of sample	Clinical condition	Probable pathogen
1	Unformed stool with pus, mucous and blood	Shigellosis and / or Gastroenteritis	*Campylobacter sp.* *Shigella sp.*
2	Unformed stool with blood and mucous and acid pH	Amoebic dysentery	*Entamoeba histolytica*
3	Unformed or semi formed stool often with blood and mucous	Schistosomiasis	*Schistosoma sp.*
4	Watery stool	Gastroenteritis and / Toxigenic colitis	*Entero toxigenic E. coli* *Rotavirus*
5	Rice water stool	Cholera	*Vibrio cholerae*
6	Unformed or watery stool and sometimes with blood, mucous and pus	Salmonellosis	*Salmonella sp.*
7	Unformed, pale coloured, frothy, unpleasant smelling stool that float on water	Giardiasis	*Giardia sp.*
8	Unformed or semi formed black stool	Hook worm infection or Iron therapy or Melaena.	Hook worm, Chemicals, Antibiotics.

4.5.3.3. Microscopy of clinical sample

Microscopic examination of stool sample was done to get an idea about the nature of infection, inflammatory response and type of microbial infection and its association.

4.5.3.3.1. For protozoan and other worms

Direct smear and concentration methods were used to detect trophozoites, cysts and eggs of worm.

4.5.3.3.1.1. Direct smear

Direct smear was prepared to look for the presence of protozoan and worm infection. The smear was examined to assess the type of infection i.e., bacterial or protozoan or worm.

It was done by wet mount method by making use of saline and lugols iodine (Koneman *et al.,* 1994). Negative samples were subjected for concentration by sedimentation using formal ether method (Koneman *et al.,* 1994).

4.5.3.3.1.1a. Saline wet mount

Worm burden of an individual was screened by saline wet mount method. Saline wet mount was performed to observe the motile nature of trophozoites and cysts of protozoans and eggs of nematodes. Saline contains 0.85% Sodium chloride. It prevents osmolysis of protozoan cysts and worm eggs.

Saline wet mount was made by emulsifying a small portion of stool sample in a drop of saline on a microscopic slide and overlaying the mixture with a cover slip. Then it was observed under microscope (10X objective and 40X objective). Morphology of helminthes and protozoans were identified under the microscope for their nature particularly size, shape, motility and other features.

4.5.3.3.1.1b. Iodine wet mount

Iodine wet mount was done to look for the nature of protozoans and nematodes. Definite identification of trophozoites or cysts of protozoans and worms were made by this method. This method arrests the motility of protozoans. Iodine stains the nuclei and intra cytoplasmic organelles.

A small quantity of stool sample was mixed with a drop of iodine on a microscopic slide. An even suspension formed was covered with a

cover slip and the mount was examined directly under low power (10X) and high power objective (40X) of light microscope (Henry, 1994).

4.5.5. Culture of the sample to look for bacterial etiology

Culturing the organism in enriched, selective cum differential medium helps to isolate and identify the bacterial etiology of stool samples. **Enrichment medium** is used to increase the number of bacterial isolates by making use of added ingredients. Particular group of microorganisms were selected by adding **selective** ingredients to the medium, which allows the growth of only specific microorganisms. Generally bile salts and deoxycholate are used to inhibit gram positive microorganisms. **Differentiation** was done by adding indicator to the medium. The indicator will differentiate the microbial group based on its physiological characters.

Table – 4.4 gives a clear picture about the nature, purpose, principle and results of various enriched, selective and differential media used to isolate various diarrhoeal pathogens.

Table – 4.4
Characteristic features of diarrhoeal pathogens on various media

S. No	Name of the Media	Nature and Purpose	Principle	Result
1	Selenite F broth	Enrichment broth for *Salmonella sp.*	Sodium selenite inhibits *Escherichia coli*, *Shigella sp.* and other coliforms	Development of turbidity within 4-5 hours
2	Gram Negative broth	Enrichment broth for *Shigella sp.*, *Salmonella sp.* and *Escherichia coli*	Deoxycholate inhibits gram positive bacteria. High concentration of mannitol limits the growth of *Proteus* and other coliforms.	Development of turbidity within 4-5 hours

S. No	Name of the Media	Nature and Purpose	Principle	Result
3	Mac Conkey agar	Differential media to look for lactose fermentation	Bile salts and crystal violet inhibits gram positive bacteria and fastidious gram negative bacteria. Lactose is the sole carbon source in this medium	Lactose fermenting bacteria produce pink colour colony. *Escherichia coli, Klebsiella sp., Enterobacter sp., Citrobacter sp., Serratia sp. and Hafnia sp.* are lactose fermenters
4	Eosin Methylene Blue agar	Differential media to differentiate Enterobacteriaceae members	Eosin and methylene blue inhibits gram positive bacteria and fastidious gram negative bacteria.	Mixed acid fermenters produce strong acid which precipitates dyes available in the medium. Produce metallic sheen colonies. *Escherichia coli* produce metallic sheen colonies
5	*Salmonella Shigella* Agar	Highly selective cum differential media for *Salmonella sp., Shigella sp.* and *Escherichia coli*	High bile salt concentration inhibits the growth of gram positive bacteria and gram negative bacteria except *Salmonella sp., Shigella sp.* and *Escherichia coli*. Neutral red is an indicator. Sodium thiosulphate is the source of sulphur	Any bacterium that produce hydrogen sulphide gas are detected by black precipitate formed with ferric citrate. Lactose fermenters produce red coloured colonies. *Salmonella* produce colourless colony with black dot in the center. *Escherichia coli* produce red colonies and *Shigella* produce colourless colonies

S. No	Name of the Media	Nature and Purpose	Principle	Result
6	Hektoein Enteric agar	Selective cum differential media for *Salmonella sp., Shigella sp.* and *Escherichia coli*	High bile salt concentration inhibits the growth of gram positive bacteria and inhibits the growth of many coliforms. Acid produced from carbohydrates reacts with acid fuschin and thymol blue to form yellow , orange or salmon colour. Sodium thiosulphate is the source of sulphur	Any bacterium that produce hydrogen sulphide gas are detected by black precipitate formed with ferric ammonium citrate. Mixed acid producers produce salmon coloured colonies. *Salmonella* produce colourless colony with black dot in the center. *Escherichia coli* produce salmon coloured colonies and *Shigella* produce colourless colonies
7	Xylose Lysine Dextrose agar	Less selective cum differential media for *Salmonella sp., Shigella sp.* and *Escherichia coli*	Three carbohydrates (xylose, dextrose and sucrose) are available to detect fermentation. Phenol red is an indicator.	Lysine positive colonies may produce initial yellow colonies due to xylose utilization and later red colour due to lysine decarboxylation. *Salmonella sp.* produce colourless colony with black dot in the center. *Escherichia coli* produce yellow coloured colonies and *Shigella sp.* produce colourless colonies

S. No	Name of the Media	Nature and Purpose	Principle	Result
8	Raj Hans medium	Highly selective cum differential media for *Salmonella sp.*, *Shigella sp.* and *Escherichia coli*	Production of acid from propylene glycol is a novel characteristic of *Salmonella sp.* Sodium deoxycholate inhibits gram positive bacteria rendering more selective media for enteric microorganisms. BC indicator turns pink in the presence of acid from propylene glycol.	Lactose fermenting bacteria yield blue coloured compound that gives blue colour to the colonies. Other enteric group produce colourless colonies. *Salmonella sp.* produce red coloured colonies. *Escherichia coli* produce bluish green colonies and *Shigella sp.* produce colourless colonies.
9	Yersinia Selective Medium	Selective cum differential media. It is a modified media of CIN (Cefsulodin - Irgasan – Novobiosin) media	Deoxycholate and crystal violet inhibits gram positive bacteria. This medium differentiates mannitol fermenters from non fermentors. Addition of antibiotics (Cefsulodin- Irgasan – Novobiosin) selects *Yersinia sp.*	*Yersinia enterocolitica* forms dark red colonies resembling bulls eye.
10	Alkaline Peptone Water	Enrichment media for *Vibrio sp.*	Higher salt and pH only allow the growth of alkalophilic and halophilic bacteria. *Vibrio sp.* survive in high salt and pH concentration. pH 8 and high salt concentration selectively enrich *Vibrio sp.*	Development of turbidity.

S. No	Name of the Media	Nature and Purpose	Principle	Result
11	Trisodium Citrate Bile salt Sucrose agar	Selective and differential media for *Vibrio sp.*	Oxgall and sodium citrate inhibits gram positive bacteria. Sodium thiosulphate acts as a sulphur source. Bromothymol blue and thymol blue are the pH indicators and detects sucrose fermentation. High salinity and alkaline pH enhances the growth of halophiles and alkalophiles.	*Vibrio sp.* tolerate high salt and pH concentration. *Vibrio cholerae* produce circular yellow colour colonies.
12	Inositol Brilliant green Bile agar	Selective media for *Plesiomonas sp.* and *Aeromonas sp.* from stool.	Bile salt and brilliant green inhibits gram positive and most other gram negative bacteria. It differentiates the bacteria based on inositol fermentation.	*Plesiomonas shigelloides* produce pink colour colonies. *Aeromonas hydrophila* produce colourless colonies.
13	Campylo Thioglycollate medium	Selective media for the isolation of *Campylobacter sp.* from stool.	The medium contains nutrients that support the growth of *Campylobacter sp.* Other gram positive and gram negative bacteria were inhibited by adding antibiotics like vancomycin, amphotericin B, polymyxin B and trimethoprim. Cephalothin also is added to inhibit the growth of *Campylobacter fetus.* Thioglycollate acts as a reducing agent of molecular oxygen.	Growth was observed and *Campylobacter sp.* was grown as pink coloured colony.

S. No	Name of the Media	Nature and Purpose	Principle	Result
14	Bismuth Sulphite Agar	Selective media for the isolation of *Salmonella sp.*	Bismuth sulphite inhibits the intestinal gram positive and gram negative bacteria. *Salmonella typhi, S .typhimurium* and *S. enteritidis* grow with black metallic sheen resulting from H_2S production and reduction of sulphite to black ferric sulphide.	*Salmonella typhi, S .typhimurium* and *S. enteritidis* produce jet black colonies.

4.5.5.1. Isolation and identification *Escherichia coli*

Escherichia coli is one of the gram negative bacteria. It belongs to the family Enteriobacteriaceae. Enrichment, differential and selective media were used to isolate *E.coli.*

4.5.5.1a. Enrichment

Small portion of the stool sample was inoculated on gram negative broth (GN) and was incubated at 37°C for 3 to 5 hours to enrich Gram Negative enteric pathogens. After the incubation period it was looked for visible growth of microorganisms.

4.5.5.1b. Recovery

A loop full of culture from GN broth was taken and inoculated on Hektoein Enteric Agar (HE) and was incubated at 37°C for 24 hours under aerobic condition. Salmon colour colony was selected from HE medium and was inoculated on other selective cum differential media like Mac Conkey agar, EMB agar, XLD medium, Raj Hans medium and SS agar. All of them were incubated aerobically at 37°C for 24 hours and were looked for specific colony morphology, which would confirm

the isolation of *Escherichia coli*. *E.coli* growth was observed in selective and differential medium and the results were tabulated.

4.5.5.1c. Identification of *Escherichia coli*

Escherichia coli was identified by making use of biochemical tests in addition to its growth characters on nutrient agar and microscopic analysis (Plate – I and II).

4.5.5.1d. Differentiation of Hemorrhagic *Escherichia coli*

Chromogenic agar was used to differentiate haemorrhagic *Escherichia coli* from other pathogenic groups. It is based on sorbitol fermentation. Sorbitol positive organisms produce acid and hence produce pink colour colonies. *Escherichia coli* was inoculated on Sorbitol - Mac Conkey agar and it was incubated for 24 hours at 37°C and observed for sorbitol non fermenting colonies (Roger, 2004).

4.5.5.2. Isolation and identification of *Salmonella sp.*

Salmonella was shown to be a pathogenic bacterium in human intestine. Proper recovery of this strains need enrichment and selective cum differential medium. Selenite F broth was used to enrich *Salmonella sp.*. A loop full of stool sample was taken and inoculated on Selenite F broth and was incubated at 37°C for 8 to12 hours and the broth was looked for visible growth. A loop full of culture from enrichment broth was inoculated on selective and differential media like Hektoein enteric agar, XLD agar, Bismuth Sulphite agar, Rajhans medium and SS agar. Bismuth sulphite agar was used for the differentiation of *Salmonella enteritidis* from other *Salmonella sp.* Microscopy, macroscopy and biochemical tests were also performed to identify *Salmonella sp.*(Plate – III).

4.5.5.3. Isolation and identification of *Shigella sp.*

Mucoid or bloody portion of the stool sample was used to recover *Shigella sp.* Recovery of *Shigella sp.* was not an easy job as it was highly sensitive to altered environmental conditions. Gram negative broth (GN) was used to enrich *Shigella sp.* GN broth allows the growth of *Shigella* while inhibiting the growth of most of the nonpathogens or normal flora. Mucoid or bloody portion of the stool was inoculated on GN broth.

Mac Conkey, XLD, Hektoein enteric agar, SS agar and Deoxycholate citrate agar were the common selective cum differential media used for the recovery of *Shigella.* A loopful of inoculum from GN broth was inoculated on these medium and incubated at 37°C for 24 to 48 hours and were examined for suspicious colonies. *Shigella sp.* will appear as colourless to slight pink colonies on Mac Conkey, XLD, SS agar and Deoxycholate agar and as green on Hekotein enteric agar. Specific colonies were inoculated into nutrient agar slants for storage and further use.

To identify *Shigella* biochemical tests were done in addition to macroscopic and microscopic examinations(Plate – IV).

4.5.5.4. Isolation of *Vibrio cholerae*

Alkaline peptone water (APW) was used as an enrichment medium for *Vibrio cholerae.* Enriched stool sample was inoculated on Trisodium Citrate Bile salt Sucrose agar (TCBS agar). It was incubated at 37°C for 24 hours under aerobic condition. *Vibrio cholerae* was grown as circular yellow coloured colony and was subjected to further characterization by microscopy and biochemical testing.

4.5.5.5. Isolation of *Aeromonas sp.*

To isolate *Aeromonas sp.* stool samples were enriched with alkaline peptone water at 37° C for 18 hours. The enriched inoculum

from APW was streaked on ampicillin blood agar and inositol brilliant green bile agar, followed by incubation at 37°C for 24 hours. Isolated colonies were subjected to oxidase test. Oxidase positive colonies were further confirmed by biochemical test in addition to macroscopy and microscopy (Kannan *et al.*, 2001).

4.5.5.6. Isolation of *Campylobacter sp.*

To isolate *Campylobacter sp.* stool samples were inoculated on Campy Thioglycollate agar plates (Hi-media, Mumbai) and incubated under microaerophilic condition for 2 days. Colonies were subjected to biochemical characterization test in addition to macroscopy and microscopy.

4.5.5.7. Isolation of *Yersinia enterocolitica*

For *Yersinia* isolation stool samples were directly streaked on Cefsulodin – Irgasan - Novobiocin (CIN) plates and were incubated at 25°C for 24 hours. The presumptive *Yersinia* isolates, which show bulls eye colour colony on CIN agar were subjected for four biochemical tests, viz. urease, TSI, motility and VP. The isolates that showed positive results to the above tests were subjected for further biochemical characterization (Singh *et al.*, 2003).

4.5.5.8. Isolation of *Enterobacter sp.*

For *Enterobacter sp.* isolation, a loopful of stool sample was streaked on Mac Conkey agar and was incubated at 37°C for 24 hours. Lactose fermenting, large and mucoid colonies were selected for further biochemical tests. It was inoculated on XLD and other enteric media for confirmation. Identification was done by biochemical tests.

4.5.5.9. Isolation of *Citrobacter sp.*

For *Citrobacter sp.* isolation a loopful of stool sample was streaked on Mac Conkey agar and was incubated at 37°C for 24 hours. Lactose

fermenting, large and mucoid colonies were selected for further biochemical tests. It was inoculated on XLD and other enteric isolation media for confirmation. Confirmed isolates were subjected for further identification by biochemical tests (Koneman, 1994).

4.5.6. Identification of clinical isolates

Selected colonies from selective and differential media were subjected to macroscopy, microscopy and biochemical tests for identification.

4.5.6.1. Macroscopic observation

Colony morphology on agar surface aids to identify the bacterial isolate. Each and every individual species of microorganism form colonies of characteristic shape, size and appearance (Presscott *et al.*, 1999). Characteristic features of the organism were observed by macroscopic observations.

A loopful of culture from overnight grown broth was streaked on the surface of nutrient agar and were incubated at 37°C for 24 hours. Colony morphology, size, shape, colour, odour and appearance were observed and tabulated.

Table – 4.5

Identification features of the pathogens on nutrient agar

S. No	Test organism	Form	Elevation	Margin	Colour	Consistency
1	*Escherichia coli*	Circular	Raised	Entire	Colourless	Translucent
2	*Salmonella sp.*	Circular	Raised	Entire	Colourless	Translucent
3	*Shigella sp.*	Circular	Flat	Entire	Colourless	Translucent

S. No	Test organism	Form	Elevation	Margin	Colour	Consistency
4	*Aeromonas sp.*	Circular	Convex	Entire	Colourless	Viscous
5	*Citrobacter sp.*	Circular	Raised	Entire	Colourless	Translucent
6	*Yersinia enterocolitica*	Circular	Convex	Entire	Colourless	Translucent
7	*Enterobacter sp.*	Circular	Raised	Entire	Colourless	Translucent

4.5.6.2. Microscopic observations

Microscopic observations like size, shape and motility reveal the availability of different morphological characters among microorganisms. Simple staining, gram staining and hanging drop methods were done to look for their shape, grams nature and motility of the isolate respectively (Henry, 1994).

4.5.6.2a. Shape of the organism

Shape of an isolate was identified by making use of **simple staining** procedure followed by its observation under light microscope. Bacterial smear was stained with methylene blue dye and examined under bright field microscope (Nikon). Microbial cells were observed for their shapes like rod, cocci or spiral.

4.5.6.2b. Grams nature

Gram staining was performed to look for the grams nature of the isolate. A purple coloured cell retains grams crystal violet and were called gram positive bacterium. Pink coloured cells lost primary stain and picked up safranin colour and were called as gram negative bacterium.

4.5.6.2c. Motility

Bacteria were motile by their flagella. The number and location of which vary among different species. Motility can be observed directly by hanging drop technique i.e., by placing a drop of culture on a microscopic slide and looked under microscope by keeping them inverted.

Table – 4.6

Microscopic and grams nature of the pathogens

S. No	Test organism	Shape	Grams nature	Motility
1	*Escherichia coli*	Rod	Negative	Motile
2	*Salmonella sp.*	Rod	Negative	Motile
3	*Shigella sp.*	Rod	Negative	Non motile
4	*Aeromonas sp.*	Rod	Negative	Motile
5	*Citrobacter sp.*	Rod	Negative	Motile
6	*Yersinia enterocolitica*	Rod	Negative	Motile
7	*Enterobacter sp.*	Rod	Negative	Motile

4.5.6.3. Biochemical tests

Physiological and metabolic characteristics of the microorganisms were assessed through biochemical tests. These characteristics are very useful because they are directly related to the nature and activity of microbial enzymes and transport proteins. Proteins are gene products. Analysis of these characteristics provides an indirect comparison of microbial genomes. The following tests were done to identify the

isolates. They are Indole test (I), Methyl red test (MR), Voges Proskauer test (VP), Citrate utilization test (C), Urease production test (U), Nitrate reduction test (N), Decarboxylation of lysine, ornithine and arginine, Phenylalanine deaminase test, Oxidase test, Catalase test, TSI agar test and Carbohydrate fermentation test (Table – 4.7).

Table – 4.7
Biochemical characters of enteric isolates

S. No	Biochemical character	1	2	3	4	5	6	7	8
1	Indole	+	-	-	-	-	-	+	-
2	Methyl red	+	-	-	+	-	-	-	-
3	Voges Proskauer	-	-	-	-	+	+	-	-
4	Citrate utilization	-	+	+	-	+	+	+	+
5	Triple Sugar Iron agar	A/A	K/A	K/A	K/K	K/A	A/A	A/A	K/K
	Gas production	+	+	+	-	-	+	+	-
	H_2S production	-	+	-	-	-	-	+	-
6	Urease	-	-	-	+	+	+	+	-
7	Nitrate	+	+	-	-	-	+	+	-
8	Catalase	+	+	+	+	+	+	+	+
9	Oxidase	-	-	-	+	-	-	-	-
10	Phenylalanine deaminase	-	-	-	-	+	-	-	-
11	Lysine decarboxylation	+	+	-	-	-	-	-	-
12	Arginine decarboxylation	-	+	-	-	-	+	-	-
13	Ornithine decarboxylation	+	+	+	-	-	-	-	-
14	Esculin hydrolysis	-	-	-	-	-	-	-	+

1. Escherichia coli *2. Salmonella sp.* *3.Shigella sp.* *4. Aeromonas sp.*
5. Yersinia sp. *6. Enterobacter sp* *7. Citrobacter sp.*
8. Campylobacter

A/A - Acid slant acid butt
K/A – Alkaline slant acid butt
K/K – Alkaline slant alkaline butt.
\+ - Positive
\- - Negative

4.5.7. Environmental Factors

As environmental factors like temperature, rainfall and humidity plays a major role in the incidence of diarrhoea, an attempt was made to correlate the level of environmental factors in relation to prevalence of diarrhoea. Hence data on temperature, rainfall and relative humidity were collected during the study period from Agriculture department, Tiruchirappalli and Tamilnadu agricultural university, Coimbatore and were used for analysis (Figure – 5.13 a; 5.13 b and 5.13 c).

4.5.8. Antibiotic sensitivity assay

Diarrhoeal symptoms like fever, vomiting, loose motion, dehydration are treated symptomatic and supportive therapies. Children with high fever are treated with an antipyretic agent (eg. Paracetamol). Toxins of microbes are inactivated with adsorbents like koalin, attapulgite, smegtite, cholestyrame. Intestinal motility is reduced with anti - motility drugs (Loperamide hydrochloride, tincture of opium, codeine etc. Bismuth salicylate decreases the number of stool per day. Antiemetics like perchlorperazine, chlorpromazine and ondansetron are used to stop vomiting. To reduce the burden of dehydration, supportive therapy is given. It is given in two forms. They are oral dehydration therapy and intravenous fluid rehydration therapy.

In addition to that anti-microbial agents were also given to reduce the burden of microbial agents. To study the effectiveness of antibiotics, antibiotic sensitivity assay is necessary and was performed by making use of disc diffusion method (Bauer *et al.,* 1966) and by using commercial antibiotic disc.

4.5.8 .1. Materials

4.5.8 .1a. Test organism

Escherichia coli, Salmonella sp. and *Shigella sp.* were the prevalent organism among the pathogens isolated from diarrhoeal

patients. Based on the prevalence - pattern, these three organisms were selected as test organism. Standard referral strains obtained from Microbial Type Culture Collection Center, Chandigarh were also used. Referral standards used were *Escherichia coli* strain MTCC 46, *Salmonella typhimurium* MTCC 1235 and *Shigella flexneri* MTCC 1537. A total of 368 *Escherichia coli*, 186 *Salmonella sp.* and 126 *Shigella sp.* were subjected to antibiotic sensitivity assay.

4.5.8.1b. Test antibiotics

Both broad spectrum and narrow spectrum antibiotics were used to assess sensitivity pattern of the clinical isolates i.e., *Escherichia coli*, *Salmonella* sp. and *Shigella* sp.. Antibiotics like Amikacin (30µg), Azithromycin (15µg), Azlocilin (75µg), Aztreonam (30µg), Carbenicillin (100µg), Cefdinir (5µg), Cefepime (30µg), Cefixime (5µg), Cefpodoxime (10µg), Cefprozil (30µg), Chloramphenicol (30µg), Ciprofloxacin (5µg), Doxycycline (30µg), Fosfomycin (200µg), Levoflaxacin (5µg), Meropenem (10µg), Methicilline (5µg), Minocycline (30µg), Nalidixicacid (30µg), Novobiocin (30µg), Ofloxacin (5µg), Rifamycin (5µg), Sparfloxacin (5µg), Ticarcillin (75µg), Vancomycin (30µg), Kanamycin (30µg), Clarithromycin (15µg), Trimethoprim (5µg), Gentamycin (10µg), Spectinomycin (100µg), Amoxycillin (30µg), and Oxytetracycline (30µg), were used to see the sensitivity pattern of enteric pathogens.

4.5.8.2. Preparation of inoculum

Clinical isolates and referral strains were inoculated in nutrient broth and incubated at 37° C for 4 hours in an incubatory shaker (Orbitek). This 4 hours culture was used for anti - bacterial activity test.

4.5.8 .3. Determination of Antibacterial activity

Disc diffusion method was followed (Bauer *et al.*, 1966) to look for anti - bacterial activity. Petri plates containing 20 ml of Mueller Hinton agar were seeded with 4 hours fresh culture of clinical isolate and

referral standard individually. By making use of template drawn commercial antibiotic discs were dispensed on the solidified Mueller Hinton agar. This was incubated at 37° C for 24 hours in an incubator (Rands SBC) and were looked for the development of clearance / inhibition zones around the antibiotic disc. The zone of inhibition was measured by making use of antibiotic zone scale (Hi-Media) and the results were recorded.

4.5.9. Serotyping of *Escherichia coli*

4.5.9.1. Materials

4.5.9.1.1. Serotyping kit

Serotyping was performed to look for O antigen of *E. coli* by slide agglutination test. Serotyping of *E. coli* was done by making use of the commercial kit supplied by M/s Denka Seiken Co., Ltd, Tokyo, Japan.

4.5.9.2. Serotyping of *Escherichia coli*

4.5.9.2.1. Antigen preparation

Various *E. coli* isolates were inoculated on Trypticase Soya Agar (TSA) and incubated for 24 hours at 37°C. The culture was suspended in two tubes containing normal saline and the bacterial load was adjusted to 1.8 X 10^9 bacterium per ml (approximately). One tube was used for agglutination reaction in live condition and other one was heated in a boiling water bath for one hour and was used for agglutination reaction in dead condition. Both the tubes were subjected to antigen antibody reaction with all antisera supplied in the kit.

4.5.9.2.2. Slide Agglutination

Both live and killed bacteria were subjected to serotyping. Based on slide agglutination (Patrica and Kenneth, 1973) reaction of live and killed bacteria with specific antiserum, the organism was categorized into various pathogenic *E. coli* belonging to different serotypes like

Enteropathogenic *Escherichia coli* (EPEC) , Enterotoxigenic *Escherichia coli* (ETEC), Enteroinvasive *Escherichia coli* (EIEC), Enteroaggregative *Escherichia coli* (EaggEC) and Enterohaemorrhagic *Escherichia coli* (EHEC).

4.5.10. Medicinal plants and their antimicrobial activity

4.5.10.1. Plant material – collection and process

Based on ancient and recent literature, traditional use and Siddha and Ayurvedha system of medicine fruit rind of pomegranate and seed kernel of mango were choosen to look for their anti - bacterial activity.

Good quality pomegranate fruit was purchased from the local market of Srirangam, Tiruchirapalli, Tamilnadu. Rind was collected from the fruit, shade dried and powdered by making use of mechanical blender (Smith mixie). The powder was stored in an airtight container and was used for extraction.

Mango fruit was purchased from Srirangam and Tiruchirappalli of Tamil Nadu. Seed was collected from the fruits and the kernel of the seed was removed, dried in sun and powdered by making use of mechanical blender (Smith mixie). Dried powder was stored in an air tight container and was used for extraction.

4.5.10.2. Extraction

Known quantity of *Punica granatum* and *Mangifera indica* coarse powder were taken and the crude extract was made by cold extraction with water and 100 % ethyl alcohol. Cold extraction was repeated 3 or 4 times and the crude aqueous extracts were collected. The extracts were dried in a vacuum disicator and were stored in a sterile container for further use (Kelmanson *et al.*, 2000). Coding was given to the crude aqueous extracts.

Known quantity of *Punica granatum* and *Mangifera indica* coarse powder were successfully extracted with various solvents like hexane, benzene, chloroform, ethyl acetate, methanol and water. Different fractions collected were filtered, evaporated in vacuo. Coding was given to various fractions and were stored till use.

4.5.11. Antimicrobial activity test

4.5.11.1 Test microorganisms

Escherichia coli, Salmonella sp. and *Shigella sp.* isolated from the diarrhoeal patients were used as test organism. Standard referral *Escherichia coli* strain MTCC 46, *Salmonella typhimurium* MTCC 1235 and *Shigella flexneri* MTCC 1537 obtained from Microbial Type Culture Collection Centre, Chandigarh were also used for reference purpose.

4.5.11.2. Preparation of disc

Known quantity of extracts or fractions were dissolved of DMSO : Methanol of 1:1 ratio. This in turn was diluted with equal volume of phosphate buffered saline (PBS pH 7). It was then filter sterilized by making use of sortorious syringe filter of pore size 0.22µm. Sterile discs of 6 mm diameter (Hi-Media) were loaded with various concentrations of extracts and fractions and were dried. Dried discs were stored in sterile containers till use. Solvent loaded discs were also prepared and used as negative control. Oxytetracycline loaded Hi-Media discs were used as positive control.

4.5.11.3. Preparation of inoculum

Clinical isolates and referral strains were inoculated in nutrient broth and incubated at 37^{0}C for 4 hours in a shaker (Orbitech, Scigenics, India) and was used for anti - bacterial activity test and to look for the MIC of various extracts and fractions.

4.5.11.4. Determination of Antibacterial activity

Disc diffusion method was followed (Bauer *et al.*, 1966) to determine the anti - bacterial activity of various extracts and fractions. Petriplates containing 20 ml of Mueller Hinton agar were seeded with 4 hours old fresh culture of clinical isolates and referral strains. By making use of template drawn extracts and fractions loaded discs were dispensed on the solidified Mueller Hinton agar with test organisms. Oxytetracycline antibiotic disc obtained from M/s Hi-Media laboratories Ltd, Mumbai was used as a positive control and solvent loaded discs were used as a negative control. This was incubated at 37°C for 24 hours in an incubator (Rands SBC). The test was performed in triplicates. The zone of inhibition was measured by making use of Antibiotic zone scale (Hi - media).

4.5.11.5. Determination of Minimum Inhibitory Concentration

Agar dilution method was used to find out Minimal Inhibitory Concentration (NCCLS, 1993). Stock concentration of various plant extract was prepared by making use of DMSO : Methanol, in the ratio of 1:1 which in turn was diluted with equal volume of phosphate buffered saline, pH 7. Mueller Hinton agar was prepared, sterilized and kept ready in molten condition. 20ml of the molten media was taken and was mixed with known concentration of different extracts / fractions and were added in different tubes. This mixture was swirled carefully for complete mixing of extract and media and poured on to the plate. After getting solidified it was inoculated with the test organism and standard organism. The plates were incubated at 37°C for 24 hours. MIC was recorded based on the growth of the organisms.

4.5.12. Phytochemistry

Medicinal plants were used for the treatment of infectious diseases. The activity of a medicinal plant may be antiviral,

antibacterial, antifungal and antihelminthic. Phytoconstituents are responsible for these activities. Knowledge of pharmacologically active compound is necessary to develop good and effective medicine from plants. Medicinal plants are the good source of macromolecules and secondary metabolites. To know the phytoconstituents, particularly for their nutritive value and medicinal value of *Mangifera indica* seed kernel and *Punica granatum* fruit rind the extracts and fractions were subjected to the analysis of macromolecules and secondary metabolites by using standard methods (Harboune, 1976).

4.5.12.1. Analysis of Macromolecules

Plants contain macro and micro molecules. These compounds are generally considered as major elements and are used for their nutritive value. Secondary metabolites of plants are considered to be the active constituents which are used as a medicine. In home made remedies *M. indica* seed kernel and *P.granatum* fruit rind were used to cure diarrhoea. Even today some siddha doctors use these two plants as remedy for AGE. To have an understanding about their phytoconstituents primary metabolites like carbohydrates, protein, lipids and secondary metabolites were assessed. To know the active principle and nutritive value of *Mangifera indica* seed kernel and *Punica granatum* fruit rind, the following simple methods were done. Qualitative analysis of macromolecules was done for carbohydrates, proteins and lipids by the following methods.

4.5.12.1.1. Qualitative analysis of macromolecules

4.5.12.1.1a. Qualitative analysis of carbohydrates (Benedict's test)

Various extracts and fractions were tested for the presence of carbohydrates by benedicts test (Sadasivam and Manikam, 1996).

4.5.12.1.1b. Qualitative analysis of protein (Biuret method)

Various plant extracts and fractions were tested for the presence of protein by Biuret method (Sadasivam and Manikam, 1996).

4.5.12.1.1c. Qualitative analysis of lipid

Presence of lipid content of the plant extracts were analyzed by making use of commercial diagnostic kit supplied by M/s Span Diagnostic Ltd, Mumbai.

4.5.12.1.2. Quantification of macromolecules

After knowing the presence of macromolecules in the extracts it was subjected for quantification of carbohydrates, protein, amino acids and lipids.

4.5.12.1.2a. Quantification of carbohydrate

Carbohydrates available in the plant extracts were estimated by Anthrone method (Sadasivam and Manikam, 1996).

4.5.12.1.2b. Quantification of reducing sugars

Reducing sugars were quantified by dinitro salicylic acid (DNS) method (Sadasivam and Manikam, 1996).

4.5.12.1.2c. Quantification of protein (Lowry *et al.*, 1951)

Total Protein content of the extract was estimated by Lowry *et al.*, (1951) method.

4.5.12.1.2d. Quantification of Amino acids by ninhydrin method

Amino acid content of the plant extract was estimated by using ninhydrin method (Sadasivam and Manikam, 1996).

4.5.12.2. Phytochemical analysis of secondary metabolites

The extracts were subjected for screening secondary metabolites like alkaloids, flavonoids, glycosides and steroids by standard methods (Harboune, 1976).

4.5.12.2.1. Qualitative analysis of flavonoids

1ml of the extract was taken along with 1ml of sodium hydroxide in a test tube. Ammonia vapour was passed to that and looked for the colour development and the results were recorded. Development of yellow colour indicates the presence of flavonoids.

4.5.12.2.2. Qualitative analysis of alkaloids.

1ml of the extract was taken and 1ml of Mayer's reagent was added to that in a test tube and this mixture was allowed to stand for some time to develop colour and results were recorded. Development of cream colour indicates the presence of alkaloids.

4.5.12.2.3. Qualitative analysis of steroids

To 1ml of the extract, 1ml of sulphuric acid was added in a tube and it was allowed to stand for some time. The colour developed was recorded. Development of reddish brown colour indicates the presence of steroids.

4.5.12.2.4. Qualitative analysis of glycosides

To 1ml of the extract, 1ml of the α – napthol was added to which chloroform was added along the sides and it was looked for the development of colour and the result was recorded. Development of violet colour indicates the presence of glycosides.

4.5.12.2.5. Qualitative analysis of triterpenoids

To 1ml of the warm extract 1ml each of tin and thionyl chloride were added. It was allowed to develop colour and the colour was noted. Development of pink colour shows the presence of triterpenoids.

4.5.12.2.6. Qualitative analysis of saponins

1 ml of the extract was taken with 1 ml of water, shaked and was allowed to stand for some time to develop froth. Formation of stable froth indicates the presence of saponin.

4.5.11.2.7. Qualitative analysis of tannins

To 1 ml of the extract, 1 ml of 1% lead acetate was added and was allowed to stand for some time. Formation of white precipitate shows the presence of tannins.

4.5.12.2.8. Qualitative analysis of anthraquinone

To 1 ml of the extract, 1 ml of dilute sulphuric acid was added and was allowed to stand for some time to develop colour. Development of pink colour shows the presence of anthraquinone

4.5.13. Qualitative analysis of secondary metabolites by TLC (Annonymous, 1998a)

TLC was performed by making use of ready made silica coated aluminium plate supplied by M/s Qualigens chemicals Pvt Ltd, Mumbai. The thickness of the TLC plate is about 0.25mm. TLC plate was activated by heating at 110°C for 10 minutes in a hot air oven and allowed to cool.

4.5.13.1. Application of sample

20x20cm size TLC plate was taken. Starting line was drawn 15 mm above the lower edge using marking pencil. Plant extract was applied on the starting line as spot by making use of capillary tube. All the extracts and fractions of the single plant were applied in a single plate with 15 mm space in between. Spot was made up to 4mm diameter and was allowed to cool at room temperature.

4.5.13.2. Development of chromatogram

TLC chamber was saturated with solvent mixture which contains chloroform: ethyl acetate: formic acid in the ratio of 5:4:1. The TLC plate was placed in the saturated chamber and the chromatogram was allowed to run. The chamber was closed and the chromatogram was developed at room temperature by allowing the solvent to asent the specified distance. TLC plate was removed from the chamber and position of the solvent front was marked. Solvent available in the plate was allowed to evaporate at room temperature.

4.5.13.3. Observation

TLC plate was observed in daylight initially. Sulphuric acid reagent was sprayed on the plate. Plate was placed in a hot air oven and heated at 60°C for 10 minutes, the coloured spot developed was observed. The distance of each spot to the point of application was recorded. Rf value was calculated by making use the formula

$$Rf = \frac{\text{Distance of the spot}}{\text{Distance of the solvent traveled}}$$

4.5.14 HPTLC

HPTLC was performed by making use of a Camag - HPTLC unit of M/s Asthagiri Herbal foundation, Chennai. Plant extracts and fractions were dissolved in respective solvents. Sample was applied on 0.22mm

thick silica gel plate by making use of Camag automatic TLC sampler. Sample was applied as band and is not spot. Chromatogram was developed with active stationary, mobile and vapour phases. Stationary phase plate was put into the mobile phase containing organic solvents like hexane: ethyl acetate: methanol in the ratio of 1:2:1. Stepwise automatic procedure was followed at room temperature to run the column. Automatic developing chamber was used to develop the chromatogram. Chemical compounds are quantitatively and evaluated through spectral scanner. Scanning was controlled by Camag software © 1998 available in the instrument. Computerised scanning HPTLC report provided the informations like Rf value, λ max and % of chemical constituents present in the sample. The results were recorded and interpretated.

4.5.15. Statistical analysis

Statistical analysis was done by students "t" test and one-way ANOVA using Origin 6.0 software for epidemiological studies. Antimicrobial activity results were assessed by calculating mean ± SD. p values were noted and the significance level was made.