4. MATERIALS AND METHODS

4.1. Isolation of *E.coli*

The present study was carried out to ascertain biochemical characteristics, drug resistance, colicinogeny and virulence attributes (toxigenic genes) of *E.coli* obtained from the faecal samples of cattle, poultry and human.

4.1.a. Sample Collection

A total of 435 stool samples from different sources were used in the study. 150 stool samples from cattle (100 diarrhoeal and 50 healthy), 135 samples from human (85 human diarrhoeal stool samples and 50 healthy controls) and 150 samples from poultry (100 poultry diarrhoeal samples and 50 healthy controls) were collected from different areas in Coimbatore. The samples were transported in Stuarts transport medium in an ice cooled bag to the laboratory. Specimens were processed within 4 hours.

4.1.b. Identification of *E.coli*

For detection of *Escherichia coli*, the samples were inoculated onto sterile enrichment trypticase soy broth (Dhanashree and Mallya, 2008) and incubated at 37°C for 24 hours. After incubation, the specimens were subcultured onto Mac Conkey agar plates. Based on the colony morphology, large, irregular and lactose fermenting pink coloured colony, the suspected *Escherichia coli* isolates were streaked onto the nutrient agar medium and stored for further confirmation. *Escherichia coli* was identified as Gram negative, oxidase negative, motile, indole positive, methyl red positive, voges proskauer test negative, citrate utilisation test negative, urease test negative and catalase positive, following the standard biochemical procedures (Bergey’s manual, 2005; Murray *et al.*, 2007). Sugar fermentation in TSI agar and starch hydrolysis in starch agar were also studied. *E.coli* MTCC 730 was used as a control.
4.1.c. Bio typing of the isolates

Fermentation reaction of salicin, raffinose and sucrose was studied. One percent of each sugar in peptone water with phenol red indicator was used and the broth was heavily inoculated with the isolates. Durham’s tube was also inversely placed inside the test tube to detect the presence of gas formation. Tubes were incubated at 37°C for 24 hours (Vander et al., 1975). Based on carbohydrate fermentation of 3 sugars viz. raffinose, salicin and sucrose, all the E. coli isolates were bio typed into 7 different combinations.

4.1.d. Cultural characterization

The isolates were grown on Sorbitol MacConkey (Leung et al., 2001 and Adwan and Adwan, 2004) and Sorbitol iron agar (Rappaport and Henig, 1952) for 24 hours. Growth of pink colonies showed utilisation of sorbitol and black colour in the Sorbitol iron agar showed positive result for H₂S production. Isolates were also grown on MUG EC O157 agar and incubated. These colonies were examined for the fluorescence under UV illuminator.

4.1.e. Antimicrobial Susceptibility testing

The bacterial isolates were subjected to in vitro antibiotic susceptibility test. The antibiotic discs were obtained from Hi Media Laboratories limited, Mumbai. The isolates were tested against commonly used antibiotics like amikacin (AK-30mcg), aztreonam (AO-30mcg), cefazolin (CZ-30mcg), ceftazidime (CA-30mcg), Ampicillin (A-30mcg), ciprofloxacin (CF-5mcg), gentamicin (G-30mcg), kanamycin (K-30mcg), nalidixic acid (NA-30mcg), trimethoprim (TR-30mcg), cefixime (CFX-5mcg) and tetracycline (T-30mcg). E.coli MTCC 443 was used as a control.

Isolates were grown in nutrient broth overnight and the plates of Mueller Hinton agar were seeded by means of uniform swabbing using sterile cotton swabs. The inoculum was allowed to dry and the antibiotic discs were placed on the inoculated agar
surface. The plates were incubated at 37°C overnight and diameter of the zone of inhibition was measured. The measurement was compared with zone size interpretative chart and the zones were graded as sensitive, intermediate and resistant (CLSI, 2005).

4.1.f. Screening for ESBL activity: Double disk synergy method (DDS)

Test strains were preincubated in brain heart infusion broth (BHIB) at 37°C to an optical density matching that of 0.5 Mc Farland turbidity standard. This suspension was then used to inoculate Mueller Hinton agar (MHA) plates by swabbing them with a sterile cotton swab. 30µg discs of aztreonam, ceftazidime, ceftriaxone and cefotaxime were placed 15 mm (edge to edge) from an augmentin (amoxicillin- clavulanate; 20/10 g) disc. Inoculated plates were incubated overnight at 37°C. Enhancement of the zone of inhibition between the clavulanate disc and any one of the β lactam discs indicated the presence of an ESBL (Menon et al., 2006; Mshana et al., 2009). *K. pneumoniae* MTCC 109 (positive control) and *E. coli* MTCC 443 (negative control) were used for quality control of ESBL tests.

4.2. *IN VITRO* PATHOGENICITY TESTING

4.2.a. Congo Red Binding Assay

- Nutrient agar was supplemented with 0.003% Congo red dye and sterilized. The medium was poured onto sterile Petri plates and allowed for solidification.
- *Escherichia coli* isolates were streaked on separate plates and incubated at 37°C for 24 hours. Uninoculated plates served as controls. After 24 hours of incubation, the plates were left at room temperature for 48 hours to facilitate annotation of results.
- Virulent *Escherichia coli* were identified by their ability to uptake Congo red dye. The pathogenic isolates produced red colonies and avirulent isolates appeared colourless (Corbett et al., 1987; Sharma et al., 2006).
4.2.b. Hemolysis

- Nutrient agar medium was prepared and sterilized. 5% of defibrinated sheep blood was added to the nutrient agar medium at warm condition, mixed well and poured onto sterile Petri plates.
- *Escherichia coli* isolates were streaked and incubated at 37°C for 24 hours. After 24 hours incubation, hemolysis was observed as a clear zone of complete lysis of RBCs surrounding the colonies.

4.2.c. Serum sensitivity

- Overnight cultures of *E. coli*, grown at 37°C on blood agar, were harvested and the cells were suspended in Hank’s balanced salt solution (HBSS). (Siegfried *et al.*, 1994; Raksha *et al.*, 2003).
- The bacterial suspension (0.05 ml) was incubated with serum (0.05 ml) from healthy volunteers at 37°C for 3 hrs.
- 10μl of samples were withdrawn and spread plated on blood agar plates, which were then incubated at 37°C for 24 hours and the viable count was determined.
- Resistance of bacteria to serum bactericidal activity was expressed as the percentage of bacteria surviving after 3 hrs of incubation with serum, in relation to the original count.
- Bacteria were termed serum sensitive, if the viable count dropped to 1% of the initial value, and resistant if >90% of the organisms survived after 3 hrs.

4.2.d. Mannose resistant haemagglutination Assay

- The isolates were grown on blood agar plates overnight.
- 1-2 single colonies were picked and suspended in saline (0.85% NaCl) to give a turbid suspension equaling to 0.5 Mc Farland standard (Forbes *et al.*, 1998).
- Red blood cells suspension (3%) was prepared by washing fresh citrated blood (group O+) in phosphate buffered saline by centrifuging at 2000rpm, for 10 mins,
at 4°C. The test was performed in the presence of 0.5% (w/v) D- mannose according to Duguid et al., 1979 and Gholamhoseinian Najar et al., 2007.

- Haemagglutination was considered to be mannose resistant when it occurred in the presence of D- mannose.
- All slides were placed at 4°C, and agglutination was observed after 10 minutes.

4.2.e.Cell Surface Hydrophobicity (CSH)

- CSH was determined by the method of Salt aggregation test, according to Lindahl et al., 1981.
- Overnight culture of *Escherichia coli* cells were harvested by centrifugation at 2000rpm for 10 minutes at 4°C.
- The cell pellet was washed 3 times with Phosphate buffered saline (0.02M, pH 6.8) and the suspension was diluted with the same buffer to get turbidity.
- Different molar concentration of ammonium sulphate 0.02, 0.2, 1M, 1.4M and 2.0M were prepared. The pH of the mixture was adjusted to 6.8 with NaOH when necessary.
- On a glass depression slide, 25μl of bacterial suspension was mixed with an equal volume of salt solutions. This mixture was gently rocked for 2 minutes at room temperature and observed for aggregation.
- The highest dilution of salt (final concentration) giving visible aggregation was considered as a numerical value for bacterial surface hydrophobicity.
- All the reactions were compared to the reaction at the highest molarity of the salt (positive control) and bacterial suspension mixed with equal volume of 0.002M phosphate buffer (pH6.8) was regarded as negative control.

4.2.f.Detection of Colicinogeny- Agar overlay method

- Isolated colonies in the nutrient agar slants were stab inoculated on to the Mueller Hinton agar plates and incubated for 48 hours at 37°C.
The growth on the surface was wiped and the spot was exposed to chloroform vapours for 30 minutes at room temperature by placing a disc of whatman filter paper soaked in chloroform on the inner surface of the Petri dish lid.

To detect colicin production, 0.1 ml of broth culture of colicin sensitive strain (MTCC 728) was mixed with 5 ml of molten soft agar.

This mixture was then poured on to the inoculated Mueller Hinton agar plates.

The Petri dish was left covered on the working table for half an hour to allow the soft overlay agar to solidify. Then the plates were incubated at 37°C for 24 hours.

Colicin production was revealed by a clear halo (Arya et al., 2008, Budic et al., 2011).

4.2. f.i. Partial purification of colicin: Ammonium sulphate precipitation

- From a potent colicin positive strain, the colicin was partially purified.
- The bacterial strain was inoculated on M9 minimal medium supplemented with thiamine (1 μg/ml), lactate (0.4%, vol/vol) and Casamino Acids (0.01%, wt/vol). It was incubated at 37°C for 24 hours.
- Induction was done with mitomycin C added at 0.4 g/ml (final concentration).
- Induced cells were harvested by centrifugation (6000 rpm for 10 min) of culture. The centrifuged pellet was suspended in 200 ml washing buffer (0.01 M Tris-HCl, pH 8.0). Cells were again harvested, and supernatant was saved as colicin fraction (Curbelo and Diaz, 2000).
- To this supernatant 40% of ammonium sulphate was added and mixed thoroughly. This was incubated at 4°C for 24 hours.

4.2.f.ii. Dialysis

- The ammonium sulphate precipitated sample was centrifuged at 10,000 rpm for 15 minutes at 4°C and supernatant was separated.
- This supernatant was dialyzed against phosphate buffer for 24 hours.
- The dialysed sample was taken out of the dialysis bag carefully and lyophilised.
• 10mg of the lyophilised sample was diluted with 0.1ml of distilled water. 5µl of this reconstituted suspension was checked for colicin activity by agar well diffusion method.
• The protein content of the lyophilised sample was quantified using the Lowry et al. (1951) method, using bovine serum albumin as the standard.

4.2.f.iii. Determination of molecular weight of colicin

• The reconstituted sample was separated using 12% gel at 100volt. Medium range protein marker was used in a well.
• After electrophoresis, the gel was stained with coomasie blue R-250 dye for 8 hours & then destained. Protein bands were observed (Sambrook et al., 1989).
• The gel with the bands was soaked in 20% of isopropanol and 10% acetic acid for 2 hours to remove the SDS. This was then followed by rinsing the gel in distilled water for 4 hours.
• Then the gel was placed on soft agar seeded with the colicin sensitive strain. This was incubated for 24 hours at 37ºC. The presence of clear zone around a band indicated the colicin activity (Anthony and Peter, 1977).
• The molecular weight of the band with the zone was compared with the standard.

4.3. MOLECULAR CHARACTERIZATION OF THE ISOLATES

4.3.a. Isolation of DNA from Escherichia coli

The genomic DNA of E.coli isolates was isolated by boiling method (Bonnet et al., 2009). DNA from E. coli isolates were extracted by growing the isolates in 2 ml of Luria Bertani (LB) broth for 18 h at 37ºC. LB broth was centrifuged at 12,000 rpm for 4 minutes. The pellet was washed in 1ml saline and re-suspended in 0.5 ml distilled water and lysed by boiling for 10 min in a water bath. The suspension was centrifuged at 12,000rpm for 2 minutes and the supernatant was DNA sample. The quality of DNA was analysed in 0.8% agarose gels.
4.3.b. PCR reaction for detection of toxigenic genes

Shiga toxin producing isolates were confirmed by the presence of stx1 and stx2 genes. Additional virulence genes examined were eae (enterocyte attachment and effacement gene) by using specific primers (eae\textsubscript{1} and eae\textsubscript{2}) (Blanco \textit{et al.}, 2004). Typical and atypical EPEC differentiation was made with the help of eae gene and the bundle forming pili\textsubscript{bfp\textsubscript{A}} gene. Hly gene confirms the presence of hemolysin production. The PCR primers used, the annealing temperature and fragment size were shown in the table (Table.4.1.).

Table.4.1. Primers and PCR Conditions

<table>
<thead>
<tr>
<th>SL. No.</th>
<th>Target Gene</th>
<th>Primers FP&amp;RP</th>
<th>Oligonucleotide sequence (5’-3’)</th>
<th>Fragment Size(bp)</th>
<th>Annealing Temp.</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Stx\textsubscript{1}</td>
<td>BGRIU BGR1D</td>
<td>TCAACGAAAAATAACTTCGCT CAGTTAATGTGGTTGCGAAGG</td>
<td>316</td>
<td>61</td>
<td>Lee \textit{et al.}, 2007.</td>
</tr>
<tr>
<td>2.</td>
<td>Stx\textsubscript{2}</td>
<td>BGRD2U BGRD2D</td>
<td>ATGAAGTGTATATTATTTAAA TCAGTCATTATTTAACTGCAC</td>
<td>450</td>
<td>61</td>
<td>Lee \textit{et al.}, 2007.</td>
</tr>
<tr>
<td>3.</td>
<td>eae\textsubscript{1}</td>
<td>EAE-1 EAE-2</td>
<td>GGAACGGCAGAGGTTAATCTGCAG GGCAGCTCATCATAGTCTTTC</td>
<td>315</td>
<td>55</td>
<td>Blanco \textit{et al.}, 2004</td>
</tr>
<tr>
<td>4.</td>
<td>eae\textsubscript{2}</td>
<td>EAE-F EAE-RB</td>
<td>ATTACTGAGATTAAGGCTGAT ATTTATTTGCAGCAGCCCAT</td>
<td>350</td>
<td>55</td>
<td>Blanco \textit{et al.}, 2004</td>
</tr>
<tr>
<td>5.</td>
<td>bfp\textsubscript{A}</td>
<td>EP1 EP2</td>
<td>AATGGGTGCTTCGCTTGCTGCTG GCCGCTTTATCCAAACTGGA</td>
<td>750</td>
<td>55</td>
<td>Barman \textit{et al.}, 2008</td>
</tr>
<tr>
<td>6.</td>
<td>hly</td>
<td>hly A1 hlyA2</td>
<td>GGTGCAGCGAGAAAGTTGTA TCTCGCTGTAGTGTTGTTG</td>
<td>1551</td>
<td>60</td>
<td>Blanco \textit{et al.}, 2004</td>
</tr>
</tbody>
</table>

PCR reaction was carried out in 50\mu l of reaction mixture, containing 25\mu l of PCR master mix, 2\mu l of forward primers, 2\mu l of reverse primer, 5\mu l of DNA template and 16\mu l of nuclease free water, under the condition of initial denaturation at 94\textdegree C for 2 minutes, denaturation at 94\textdegree C for 1 min, annealing at 55\textdegree C for 1 min(differences are shown in the table.4.1.), and extension at 72\textdegree C for 1 min. This cycle was repeated at 30 times from denaturation, followed by a final extension at 72\textdegree C for 1 min.
The PCR amplified product were separated by electrophoresis with 2 percent (w/v) agarose gels in 1X TAE (0.04 M Tris acetate, 0.001M EDTA, pH-8) buffer along with 100bp DNA ladder as a molecular weight marker. After staining with ethidium bromide (0.5μg/ml in gel), the gels were viewed with the help of a gel documentation system.

4.3.c. Detection of stx2 variants by PCR

The VT2-c–VT2-d primer pair was used in a PCR protocol to detect the stx2, and stx2vh-a, and stx2vh-b genes. The VT2-cm–VT2-f primer pair was used to specifically detect the genes coding for the Stx2d variants (Stx2d-OuantandStx2d-OX3a).The Stx2e variant was detected with the VT2e-a–VT2e-b primer pair. Stx2f was detected using the128-1,128-2 primer pair. Stx2g was identified using 177U/45D primers (Table.4.2.) (Tyler et al., 1991; Pierard et al., 1998; Johnson et al., 1990; Schmidt et al., 2000).

<table>
<thead>
<tr>
<th>Target(s)</th>
<th>Primer</th>
<th>Oligonucleotide sequence (5’-3’)</th>
<th>Size of amplified product (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>VT2vh-a, VT2vh-b, VT2d-Ouant, VT2d-OX3a</td>
<td>VT2v-1 VT2v-2</td>
<td>CAT TCA GAG TAA AAG TGG CC GGG TGC CTC CCG GTG AGT TC</td>
<td>385</td>
<td>Pierard et al., 1998</td>
</tr>
<tr>
<td>VT2, VT2vh-a, VT2vh-b, VT2d-Ouant, VT2d-OX3a</td>
<td>VT2-a VT2-b</td>
<td>TTA ACC ACA CCC ACG GCA GT GCT CTG GAT GCA TCT CTG GT</td>
<td>346</td>
<td>Pierard et al., 1998</td>
</tr>
<tr>
<td>stx2, stx2vh-a, stx2vh-b</td>
<td>VT2-c VT2-d</td>
<td>AAG AAG ATG TTT ATG GCG GT CAC GAA TCA GGT TAT GCC TC</td>
<td>250</td>
<td>Tyler et al., 1991</td>
</tr>
<tr>
<td>stx2d-Ouant, stx2d-OX3a</td>
<td>VT2-cm VT2-f</td>
<td>AAG AAG ATA TTT GTA GCG G TAA ACT GCA CTT CAG CAA AT</td>
<td>256</td>
<td>Pierard et al., 1998</td>
</tr>
<tr>
<td>stx2e</td>
<td>VTe-a VTe-b</td>
<td>CCT TAA CTA AAA GGA ATA TA CTG GTG GTG TAT GAT TAA TA</td>
<td>230</td>
<td>Johnson et al., 1990</td>
</tr>
<tr>
<td>Stx2 f</td>
<td>128-1 128-2</td>
<td>AGATGGGCCGTAATTCGTTG TACTCTGGCGGCGCCCTGCTCC</td>
<td>428</td>
<td>Schmidt et al., 2000</td>
</tr>
<tr>
<td>Stx2g</td>
<td>177U 45D</td>
<td>GATGGC GGT CCA TTA TC AAC TGA CTG AATTGT GA</td>
<td>500</td>
<td>Leung et al., 2003</td>
</tr>
</tbody>
</table>
4.3. d. DNA Sequencing

Amplified products of selected isolates of \textit{eae} genes were sent to 1st BASE Laboratories Sdn Bhd, Selangor, Malaysia for DNA Sequencing and the results were recorded.

4.3. e. Phylogenetic analysis

Gene trees were then constructed by using BLAST and Clustal Omega program due to its superior performance (Vidiya \textit{et al}., 2003).

4.3.f. Statistical analysis

The Pearson chi-square statistical test was used to determine whether significant differences exist between different parameters of the present study. A chi-squared test, also referred to as chi-square test or $\chi^2$ test, is any statistical hypothesis test in which the sampling distribution of the test statistic is a chi-squared distribution when the null hypothesis is true, or any in which this is asymptotically true, meaning that the sampling distribution (if the null hypothesis is true) can be made to approximate a chi-squared distribution as closely as desired by making the sample size large enough.

Chi-square test is interpreted by
1. Determining degrees of freedom (df). Degrees of freedom can be calculated as the number of categories in the problem minus 1.
2. Determining a relative standard to serve as the basis for accepting or rejecting the hypothesis. The relative standard commonly used in biological research is $p > 0.05$. The p value is the probability that the deviation of the observed from that expected is due to chance alone (no other forces acting).
3. Referring to a chi-square distribution table. Using the appropriate degrees of freedom, locate the value closest to the calculated chi-square in the table.
4.4. SCREENING FOR BIOACTIVE PHYTOCHEMICALS AGAINST SHIGA TOXIN PRODUCING *E.coli*

4.4.a. Plant Materials selected

1. Leaf extracts of *Andrographis paniculata* (nilavembu),
2. Peels from fruits of *Punica granatum* (matulai)
3. Leaf extracts of *Prosopis juliflora* (cheemai karuvel, velikathan)
4. Leaf extracts of *Ocimum basilicum* (sada tulasi)
5. Leaf extracts of *Psidium guajava* (segappu koyyaa)

4.4.b. Preparation of Extracts

Fresh leaves of *Andrographis paniculata, Prosopis juliflora, Ocimum basilicum, and Psidium guajava* were collected from Coimbatore. The leaves were air dried in shade under natural conditions. Dried leaves were powdered and extracted by soxhlet apparatus by increasing order of polarity with petroleum ether, benzene, chloroform, ethanol and methanol for 48 hours. The various extracts obtained were concentrated and dissolved in Dimethyl sulphoxide (DMSO).

Pomegranate fruits were collected from the local markets. Peels were removed and dried in shade under natural conditions. The sample was spread out during daylight hours for 5 days until it dried to brittleness. Dried peels were powdered to get 60-mesh size using a mixer grinder. Twenty-five grams of powdered peel was extracted with 125 ml of solvents (Petroleum ether, benzene, chloroform, ethanol and methanol) with occasional shaking for 3 days at room temperature. The extracts were filtered, concentrated and dried at 50°C and the weight of each residue was recorded.

4.4.c. Antibacterial activity of extracts

The prepared extracts were tested for antibacterial activity against shiga toxin producing *E.coli* by agar well diffusion method. 1g of the prepared extract was dissolved
in 1ml of DMSO. 6mm wells were made on Mueller Hinton agar plates seeded with the test organisms and different concentrations of the extracts (10-100mg) were loaded onto the wells. A gentamicin (G-30mcg) disc was used as a standard antibiotic and DMSO was added in a separate well. The plates were incubated for 24 hours and after incubation the zone of inhibition was noted against each extract (Mahfuzul Hoque et al., 2007).

4.4.d. Minimum Bactericidal Concentration

The MBC was performed to test the antimicrobial activity of active extract using tube dilution method. The MBC was defined as lowest concentration able to kill any microbe. Dilutions of the plant extract were prepared in sterile nutrient broth to get a final concentration of 2mg, 4mg, 8mg, 16mg, 32mg and 64mg/ml respectively (Mahfuzul Hoque et al., 2007).

To each of these dilutions, a loop full of STEC culture adjusted to 0.5 Mc Farland standard, was inoculated and all the tubes were incubated at 37°C for 24 hrs. After incubation, loopful from each tube was inoculated onto nutrient agar plates. The plate without growth was recorded as MBC.

4.4.e. Phytochemical Screening

The plant with the potent antibacterial activity was used for further study. Phytochemical screening for flavonoids, alkaloids, tannins, saponins and terpenoids were done following standard methods as described by Harborne 1998, Trease and Evans, 1989 and Sofowora, 1993).

4.4.f. Thin Layer Chromatography (TLC) of Psidium guajava extracts

The dried samples were separately soxhlet extracted in 80% methanol (100 ml/ gm dry weight) on a water bath for 24 hrs (Subramanian and Nagarajan, 1969). The extract was concentrated and reconcentrated in petroleum ether (40°- 60°C) (fraction-I), ethyl ether (fraction II) and ethyl acetate (fraction-III) in succession. Each of
the steps was repeated three times to ensure complete extraction in each case. Fraction I was rejected since it was rich in fatty substances whereas fraction III was hydrolyzed by refluxing with 7% H\textsubscript{2}SO\textsubscript{4} (10 ml/gm residue) for 5 hours. The mixture was filtered and the filtrate extracted with ethyl acetate in a separating funnel. The ethyl acetate layer was washed with distilled water till neutrality and dried \textit{in vacuo} (Meena and Patni 2008). The residues were taken up in small volumes of ethanol separately and then subjected to TLC. The crude methanol extract was also subjected to TLC.

These residues were applied at 2.5 cm from the base of silica gel-G TLC plate, along with standard reference compound (quercetin). After drying, the TLC plates were developed with n-butanol: acetic acid: water (4:1:5 upper layer) as the developing solvent and were run in duplicate. One set was used as the reference chromatogram and other set was used for bioautography. The developed plates were air dried and then placed in a chamber saturated with ammonia vapours to observe the colour of spots (quercetin deep yellow) and plates were also placed in a chamber saturated with I\textsubscript{2} vapours to observe the colour of spots (yellow brown). The developed plates were sprayed with 5% ethanolic ferric chloride solution to observe the colour of the spots (quercetin -brown colour). Rf values were calculated for isolated samples and compared with standard quercetin.

4.4.4. TLC Bioautography

Chromatogram, developed as described above in TLC plates was loaded with the inoculum of Shiga toxin producing \textit{E. coli} in molten Mueller Hinton Agar, over the TLC plates. After the solidification of the medium, the TLC plate was incubated overnight at 37\textdegree{C}. Subsequently, bioautogram developed was sprayed with 1% aqueous solution of 2, 3, 5,-triphenyl tetrazolium chloride (TTC) and incubated at 37\textdegree{C} for 4 hrs. Inhibition zone indicated the presence of active compounds (Anjana \textit{et al.}, 2010).
4.4.h. TLC of *Punica granatum* methanol extracts

The TLC plates of *Punica granatum* were developed with water:acetic acid solvent system (3:2) and were run in duplicate. 5% ferric chloride reagent was sprayed and the chromatogram was observed.

The second set of chromatogram, developed as described above in TLC plates which was not subjected to chemical treatment for observing spots was loaded with the inoculum of *E. coli* in molten Mueller Hinton Agar, over the TLC plates. After the solidification of the medium, the TLC plate was incubated overnight at 37°C. Subsequently, bioautogram developed was sprayed with 1% aqueous solution of 2, 3, 5, -triphenyl tetrazolium chloride (TTC) and incubated at 37°C for 4 hrs. Ellagic acid and the antibiotic ampicillin were used as controls.

4.4.i. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

For injection in HPLC system the methanol extract and the purified ethyl acetate fractions of *Psidium guajava* were used. The HPLC was performed on YMC C18 column, 5µm, 250 × 4.6mm, using solvent mixtures – 80% hexane and 20% ethyl acetate for *Psidium guajava* extract. The flow rate was 1ml/min. For *Punica granatum* a gradient consisting of solvent A (water/formic acid, 90/10) and solvent B(methanol) was applied at a flow rate of 0.7ml/minute (Prieto et al., 2005).

4.4.j. Column chromatography and Gas Chromatography- Mass Spectrum Analysis (GC-MS)

One gram of the crude methanol extract was subjected to column chromatography using silica gel (100-200 mesh) and eluted with ethyl acetate. Antibacterial activity of the ethyl acetate fraction was confirmed by agar well diffusion method. This ethyl acetate fraction of the *Punica granatum* rind was taken for GC-MS analysis, since ethyl acetate could extract most of the bioactive compounds (Sangeetha and Vijayalakshmi, 2011).
The eluted ethyl acetate fraction was dried and dissolved in ethanol. GC-MS analysis of this extract was done using Agilent GC-MSD-5975 C (412 SLPL ES GC-MSD) system. Interpretation of mass spectrum GC-MS was done using the database of National Institute Standard and Technique (NIST 11.L).

4.5. Docking of phytochemicals against intimin and shiga toxin

The structures of Intimin and shiga toxin were retrieved from pdb protein data bank. The PDB ID of intimin was 2ZQK and the PDB ID of shiga toxin was 1R4P. The structures of the phytochemicals confirmed with the biological activity were collected from the drug bank. The ligands used and their structures are shown in table. Active site was predicted using Q site server and Docking was performed using Arguslab.

Table. 4.3. Table showing the phytochemicals and their structure used in docking studies

<table>
<thead>
<tr>
<th>Sl.No.</th>
<th>Name of the compound</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Quercetin</td>
<td><img src="image1" alt="Quercetin structure" /></td>
</tr>
<tr>
<td>2.</td>
<td>Palmitic acid</td>
<td><img src="image2" alt="Palmitic acid structure" /></td>
</tr>
<tr>
<td>3.</td>
<td>Propanedioic acid</td>
<td><img src="image3" alt="Propanedioic acid structure" /></td>
</tr>
<tr>
<td>4.</td>
<td>2,4-Dihydroxypyridine</td>
<td><img src="image4" alt="2,4-Dihydroxypyridine structure" /></td>
</tr>
<tr>
<td>5.</td>
<td>1-Propanol, 2-ethoxy-</td>
<td><img src="image5" alt="1-Propanol, 2-ethoxy- structure" /></td>
</tr>
<tr>
<td>6.</td>
<td>2,5-Furandicarboxaldehyde</td>
<td><img src="image6" alt="2,5-Furandicarboxaldehyde structure" /></td>
</tr>
<tr>
<td>7.</td>
<td>Undecane</td>
<td><img src="image7" alt="Undecane structure" /></td>
</tr>
<tr>
<td></td>
<td>Chemical Name</td>
<td>Molecular Structure</td>
</tr>
<tr>
<td>---</td>
<td>---------------</td>
<td>---------------------</td>
</tr>
<tr>
<td>8.</td>
<td>Acetamide, 2,2'-thiobis</td>
<td><img src="image" alt="Acetamide, 2,2'-thiobis" /></td>
</tr>
<tr>
<td>9.</td>
<td>4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl</td>
<td><img src="image" alt="4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl" /></td>
</tr>
<tr>
<td>10.</td>
<td>5-Hydroxymethylfurfural</td>
<td><img src="image" alt="5-Hydroxymethylfurfural" /></td>
</tr>
<tr>
<td>11.</td>
<td>1,2,3-Propanetriol, 1-acetate</td>
<td><img src="image" alt="1,2,3-Propanetriol, 1-acetate" /></td>
</tr>
<tr>
<td>12.</td>
<td>Benzene, 2-methoxy-1,3,4-trimethyl</td>
<td><img src="image" alt="Benzene, 2-methoxy-1,3,4-trimethyl" /></td>
</tr>
<tr>
<td>13.</td>
<td>Benzoic acid, 4-ethoxy-, ethyl ester</td>
<td><img src="image" alt="Benzoic acid, 4-ethoxy-, ethyl ester" /></td>
</tr>
<tr>
<td>14.</td>
<td>Dodecanoic acid</td>
<td><img src="image" alt="Dodecanoic acid" /></td>
</tr>
<tr>
<td>15.</td>
<td>Tetradecanoic acid</td>
<td><img src="image" alt="Tetradecanoic acid" /></td>
</tr>
<tr>
<td>16.</td>
<td>Hexadecanoic acid, methyl ester</td>
<td><img src="image" alt="Hexadecanoic acid, methyl ester" /></td>
</tr>
<tr>
<td>17.</td>
<td>2,4,6-Triisopropylbenzoic acid</td>
<td><img src="image" alt="2,4,6-Triisopropylbenzoic acid" /></td>
</tr>
<tr>
<td></td>
<td>Molecule</td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>--------------------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>18.</td>
<td>9,12-Octadecadienoic acid (Z,Z)-methyl ester</td>
<td></td>
</tr>
<tr>
<td>19.</td>
<td>9,12-Octadecadienoic acid (Z,Z)-</td>
<td></td>
</tr>
<tr>
<td>20.</td>
<td>3,7-Benzofurandiol, 2,3-dihydro--2,2-dimethyl</td>
<td></td>
</tr>
<tr>
<td>21.</td>
<td>Ethylene, 1,1-diphenyl-</td>
<td></td>
</tr>
<tr>
<td>22.</td>
<td>Hesperetin</td>
<td></td>
</tr>
</tbody>
</table>