CHAPTER 2

MATERIALS AND METHODS

2.1  BACTERIAL STRAINS

Strains used were EPEC wild type (WT) strain E2348/69 Nal\(^r\) (Levine and Rennels 1978), \(\Delta\)lamB made by insertional inactivation of the lamB gene of EPEC with a kanamycin expression cassette (Malladi et al 2004; Puthenedam et al 2007), and DH5\(\alpha\) Amp\(^r\) obtained from Invitrogen Life Technologies, Carlsbad, U.S.A. Clinical isolates of EPEC were a kind gift of Dr. Mary V. Jesudason, former Professor, Department of Clinical Microbiology, Christian Medical College, Vellore, India. E. coli ATCC 25922 was a kind gift of Dr. P. Rajendran, Department of Microbiology, University of Madras, Chennai. All the strains were stored at -70\(^\circ\)C as 50% (v/v) glycerol stocks.

2.1.1  Preparation of Mutant Strain \(\Delta\)lamB using a Counter-Selectable Vector System

The lamB gene was amplified from genomic DNA of WT EPEC using primers forward: 5’- AAGGAATTCCTCAGGAGATAGAATGATG-3’ and reverse: 5’-GGGCATGCTATTACCACCAGATTCC-3’), which incorporated EcoRI and SphI restriction sites. These generated a 1.4 kb PCR product that was cloned into pUC19 vector (New England Biolabs, UK). The kanamycin expression cassette was excised from pUC4K (New England Biolabs, UK) by HincII digestion and cloned into the EcoRI and SphI sites of
pRDH10. The recombinant plasmid was maintained in *E. coli* strain SM10 \(\lambda_{pir}\) and introduced by conjugation into WT EPEC (Nal\(^r\)); transconjugants were selected for chloramphenicol (20 \(\mu\)g/mL) and nalidixic acid (100 \(\mu\)g/mL) resistance on nutrient agar. Selection for loss of pRDH10 was performed by growing strains in 1% (w/v) tryptone, 0.5% (w/v) yeast extract and 5% (w/v) sucrose to mid logarithmic phase and plating suitable dilutions on tryptone-yeast extract-sucrose agar to select for sucrose and tetracycline (10 \(\mu\)g/mL) sensitivity and kanamycin (50 \(\mu\)g/mL) resistance. Both \(\text{lamB}\) and kanamycin cassette inserts were confirmed by sequencing.

2.2 BACTERIOLOGICAL MEDIA

2.2.1 Luria Bertani (LB) Broth/Agar

10g of tryptone, 5g of yeast extract and 10g of sodium chloride (NaCl) were dissolved in 1000 mL of distilled water. The pH was adjusted to 7.4 and autoclaved at 121\(^\circ\)C and 15 lbs pressure for 20 mins. For LB agar, 2% agar was finally added to the medium before autoclaving. All the constituents (tryptone, yeast extract, NaCl and agar) were purchased from HiMedia laboratories, Mumbai, India.

2.2.2 Tryptone Soya Agar

40g of Tryptone Soya Agar dehydrated base (HiMedia) was dissolved in 1000 mL of distilled water and autoclaved as above.

2.2.3 Mueller-Hinton Agar

38g of Mueller-Hinton Agar (cation adjusted) dehydrated base (HiMedia) was dissolved in 1000 mL of distilled water and autoclaved as above.
2.3 ANTIMICROBIAL SUSCEPTIBILITY DISCS

Antimicrobial susceptibility discs for assessing resistance patterns of EPEC strains were purchased from HiMedia. All the discs were stored at 0°C unless used for assays. The list of antibiotics used was as per the guidelines of Clinical and Laboratory Standards Institute [CLSI] (NCCLS 2003) for testing against Enterobacteriaceae.

**Group A (for primary reporting)**
1. Ampicillin (A\(^{10}\))
2. Cefazolin (Cz\(^{30}\))
3. Gentamicin (G\(^{10}\))

**Group B (for selective reporting)**
4. Amikacin (Ak\(^{30}\))
5. Amoxyclav (Ac\(^{30}\))
6. Ampicillin/Sulbactam (As\(^{10/10}\))
7. Piperacillin/Tazobactum (Pt\(^{100/10}\))
8. Ticarcillin/Clavulanic acid (Tc\(^{75/10}\))
9. Cefamandole (Cef\(^{30}\))
10. Cefuroxime (Cu\(^{30}\))
11. Cefepime (Cpm\(^{30}\))
12. Cefaperazone (Cs\(^{75}\))
13. Cefotaxime (Ce\(^{30}\))
14. Ciprofloxacin (Cf\(^{5}\))
15. Levofloxacin (Le\(^{5}\))
16. Imipenem (I\(^{10}\))
17. Mezlocillin (Mz\(^{75}\))
18. Piperacillin (Pc\(^{100}\))
19. Ticarcillin (Ti\(^{75}\))
Concentration of all the antibiotic discs were according to CLSI prescribed concentrations.

2.4 CELL LINES

HEp-2 (Laryngeal carcinoma cells) and J774 A.1 (Murine macrophage-like cells) cell lines were purchased from National Centre for Cell Sciences (NCCS), Pune, India.

2.5 PREPARATION OF MEDIA AND OTHER REAGENTS REQUIRED FOR CELL CULTURE

DMEM (Dulbecco’s Modified Eagle Medium) was prepared as follows:

i) The powdered media was dissolved in 900 mL of sterile glass distilled or Millipore water in an autoclaved glass conical flask under sterile conditions. The antibiotics were added with DMEM and stirred well for complete solubilisation of antibiotics.

ii) 3.7 g of Sodium bicarbonate was added to the flask and stirred until it dissolved completely. 10% FCS was added to the medium and sterilized through a 0.2 µ filter under negative pressure. The medium was immediately stored at 4°C until further use.

2.5.1 Saline: Trypsin: Versene (STV)

The following solutions were prepared: 10X Saline: NaCl 8 g, KCl 0.4 g, D-Glucose 1.0 g, NaHCO₃ (Tissue Culture Grade) 0.35 g were
dissolved in 100 mL water. 10X saline was aliquoted after filter sterilization and stored at 4°C.

i) Versene: 1 g EDTA (Tissue Culture grade) was weighed and added to 90 mL distilled water. 5N NaOH was added drop wise until it dissolved. The solution was filter sterilised and stored at 4°C.

For 100 mL of STV preparation, 100 mg of trypsin, 10 mL of 10X Saline, 2.5 mL of Versene were added and the final volume 100 mL was made up with double distilled water, which was subsequently filter sterilised, aliquoted, and stored at -20°C.

2.5.2 Passaging and Cryopreservation

HEp-2 cells showed a steady growth rate in 24 to 48 h. The cell lines that reached confluence in 2 to 3 days were passaged in order to maintain viability. Passaging was done as follows:

i) The culture medium was removed by decanting into a clean container inside the laminar flow hood, and cells were rinsed with the medium without serum.

ii) 2.5 mL of STV solution was added to the flask containing cells and incubated at 37°C for a few minutes. As soon as cells start dislodging from the surface, the solution was removed and the flask was rinsed with serum containing medium to remove all the cells.

iii) The suspensions of cells were collected in a sterile 15 mL centrifuge tube and the cells were pelleted at 1500 rpm for
3 min. The cell pellet was resuspended in fresh medium with serum and an aliquot of the cells was seeded back into the flask and the remaining cell pellet was resuspended in cryopreservative medium (10% DMSO in serum) in a cryovial and frozen at -70°C or in liquid nitrogen.

For J774 A.1 cells, differentiation was observed at 72 hours post-passage and confluency was attained in 6 days. Passaging was done as mentioned above, except that a plastic scraper was used to scrape the cells from the flask after the addition of STV. This is due to the strong adherence of the macrophages to the flask.

2.6 ADHERENCE ASSAYS USING EPEC ON HEp-2 CELLS

2.6.1 Giemsa Staining

HEp-2 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) (pH 7.4) supplemented with 10% (v/v) heat inactivated (56°C, 30 minutes) foetal calf serum (FCS; Gibco BRL, U.S.A.) and antibiotics (3 μg/mL of amphotericin B, 160 μg/mL of gentamicin, 75 μg/mL of streptomycin and 120 units/mL of penicillin). Incubation was at 37°C in 5% CO₂. Adherence assay using Giemsa stain were performed as described previously (Mathewson and Cravioto 1989) with modifications. HEp-2 cells were seeded at 0.4 × 10⁶ cells per well in 6-well tissue culture plates containing 2 mL of medium and a cover slip in each well, and incubated for 36 hours. Two hours prior to the adherence assay, the medium was removed and the cells were washed twice with 2 mL of sterile PBS, and 2 mL of fresh DMEM without serum and antibiotics was added to the wells. Strains DH5α, WT and ΔlamB were grown overnight in 3 mL of Luria–Bertani (LB) broth with vigorous shaking, and then sub-cultured at 1% into 5 mL of DMEM
without serum and antibiotics and incubated statically for 3 hours at 37°C. The culture concentration was adjusted to an OD$_{600}$ of 1 and the bacteria were added to HEp-2 cells and incubated for 3 hours at 37°C in 5% CO$_2$. After incubation, the cells were thoroughly washed six times with PBS to remove non-adherent bacteria, and then fixed in 70% methanol for 30 minutes and stained with Giemsa for 15 minutes. The cover slips were placed inverted on clean glass slides and visualised for adherence by light microscopy at 400× magnification.

2.6.2 Agar Plate Counts

Adherence assays were performed as described above; after the cells had been thoroughly washed to remove non-adherent bacteria, 1 mL of 0.1% (v/v) Triton X-100 in PBS was added to each well and incubated at 37°C for 15 minutes to lyse the HEp-2 cells. The lysates were serially diluted to $10^{-3}$ in sterile LB broth, plated on LB agar and incubated at 37°C for 24 hours for enumeration of colony forming units (CFU). Agar plate count assays were performed thrice as separate experiments.

2.6.3 Radiolabelling of Bacteria

Adherence assays with [$^3$H]thymidine–labelled bacteria were performed as described by Crane et al (Crane et al 1999) with modifications. Briefly, bacterial strains were grown overnight in 3 mL of LB containing 1% (w/v) mannose and 4 µCi of [$^3$H]thymidine (Amersham Pharmacia Biotech, U.K.) and used as a 1% inoculum in 5 mL of DMEM without serum and antibiotics. An additional 4 µCi of [$^3$H]thymidine were added and incubated statically for 3 hours at 37°C. Adherence assays were performed as described above. Triton X-100 solubilised cells were added to 96-well scintillation plates (Packard, U.S.A.). Glass fibre papers were cut into small pieces and
immersed in each of the lysates and allowed to dry. Scintillation fluid (200 µl) was added to each lysate well and radioactivity (CPM) was measured in a liquid scintillation counter (Packard, U.S.A.). Assays with thymidine-labelled bacteria were performed thrice as separate experiments.

2.7 OUTER MEMBRANE PREPARATION

Outer membrane fractions were prepared as previously described (Puthenedam et al 2007) with modifications. Bacteria were grown overnight in LB broth with appropriate antibiotics at 37°C with vigorous shaking and used as a 1% inoculum in 50 mL of DMEM without antibiotics. Cultures were incubated at 37°C till the cells reached late logarithmic phase. The cells were pelleted, washed thrice with 20 mM Tris-HCl and 10mM EDTA (TE buffer), pH 7.4, suspended in 2 mL of TE buffer and then subjected to ultrasonication (five 1-minute pulses with 2-minute pauses between) in a 240V/60Hz Labsonic sonicator (B. Braun Biotech International, Melsungen, Germany). Unbroken cells and debris were removed by centrifugation at 6000×g for 10 minutes. The supernatant fraction was subjected to further centrifugation at 45000×g for 45 minutes at 4°C (Biofuge Stratos, Heraeus, Germany). The pellet was suspended in 500 µl of 1% sodium lauryl sarcosinate (Sarkosyl) and incubated at 37°C for 2 hours. The suspension was again centrifuged at 45000×g for 45 minutes at 4°C. The pellet was suspended in 100 µl of 1% SDS and boiled for 10 minutes. Protein concentrations were estimated by the Lowry method (Lowry et al 1951).

2.8 PROTEIN ESTIMATION

Protein estimations were done according to the method of Lowry et al (1951). The following solutions were prepared: Protein standard BSA (Bovine serum albumin): a stock solution of 1 mg/mL; 1% Copper sulphate
(Solution A); 2% Sodium potassium tartarate (Solution B); 2% Sodium carbonate in 0.1 N NaOH (Solution C).

a) The solutions were mixed in the ratio of C:B:A as 98:1:1 in the same order. 4.5 mL of this mix was pipetted into clean, marked glass tubes. The standard BSA was added to the tubes at concentrations of 10 µg, 20 µg, 40 µg, 80 µg and 100 µg. 10 µL of samples were added to each marked tube and the tubes were incubated at room temperature for 10 min.

b) 0.5 mL of Folin’s reagent (1:1 solution of Folin’s Ciocalteau reagent in distilled water) were added to the tubes. The tubes were incubated in the dark for 30 min and the optical density was read at 640 nm. The readings from protein standards were used to plot a standard graph and estimate value of the samples.

2.9 SDS – POLYACRYLAMIDE GEL ELECTROPHORESIS

Outer membrane proteins of EPEC were resolved by SDS-PAGE. The various buffers used are as follows:

a) Monomer solution: 20% acrylamide and 0.8% N,N-methylene bis acrylamide in distilled water. The solution was filtered though Whatman filter paper and stored in brown bottles at 4°C.

b) Separating gel buffer: 1.5 M Tris-Cl pH 8.3

c) Stacking gel buffer: 0.5 M Tris-Cl, pH 6.8

d) Electrophoresis buffer: 0.025 M Tris-Cl, 0.192 M glycine, 0.1% SDS, pH 8.3
e) Sample solubilising buffer (5X): 10% SDS, 10% (v/v) β-mercaptoethanol, 50% sucrose, 0.025% Bromophenol blue in stacking gel buffer.

Depending on proteins to be separated, 10-15% separating gel and 5% stacking gels were used. Stacking gel was approximately 1/5 of the separating gel. Protein estimations were performed by Lowry method and equal amounts of total protein were loaded in each well. Electrophoresis was performed at room temperature with constant current of 18 mA for stacking gel and 25 mA of separating gel. The gels were stained with staining solution (0.25 g of Coomassie brilliant Blue R-250 in 45% methanol, 10% acetic acid) overnight and destained with 45% methanol, 10% acetic acid solution until a clear background was obtained. Photographs were taken using gel documentation system from Alpha Innotech, USA.

2.10 ELUTION OF THE 43 kDa LamB PROTEIN FROM SDS-POLYACRYLAMIDE GEL

An outer membrane protein fraction from a WT EPEC culture grown for adherence assay as described above was subjected to electrophoresis on a 10% (w/v) SDS-polyacrylamide gel at 22 mA for 2 hours. The gel was stained with ice-cold 1 mol l⁻¹ KCl (Bergman and Jornvall 1987) and the 43kDa band was carefully excised. The gel piece was minced, placed into a 10-cm long 32 kDa cut-off dialysis bag (Gibco BRL, U.S.A.) and subjected to dialysis against glycine buffer without SDS overnight at 4°C. The protein concentration was estimated using Lowry’s method and elution efficiency was analysed using 10% (w/v) SDS- PAGE and staining with Coomassie Brilliant Blue R250 (Laemmli 1970). Outer membrane preparation of ΔlamB served as a standard for identifying LamB of WT on the gel.
2.11 PREPARATION OF POLYCLONAL ANTISERUM AND WESTERN BLOT

Polyclonal antiserum was raised in New Zealand white rabbits according to the method of Harlow and Lane (1988). Briefly, LamB protein excised from a SDS-polyacrylamide gel was minced, homogenised with sterile PBS and injected subcutaneously; two booster doses were given at intervals of 2 weeks. Reactivity and specificity of the anti-LamB antiserum was assessed by Western blots; 20 µg each of the outer membrane preparations of DH5α, WT and ΔlamB were electrophoresed on 10% (w/v) SDS-polyacrylamide gels and electro-transferred to a nitrocellulose membrane (Amersham) at 120 mA, 40 V for 90 minutes using a semi-dry electroblotter (Amersham). The membrane was blocked with 5% (w/v) skimmed milk powder in PBS overnight at 4°C. The membrane was washed three times with PBS-0.02% (v/v) Tween20 and three times more with PBS alone. A 1:500 dilution of anti-LamB antiserum in PBS was added to the membrane and incubated at room temperature for 90 minutes. After three washes each with PBS-tween20 and PBS alone, alkaline phosphatase-conjugated mouse anti-rabbit secondary antibody (1:1000) was added and incubated at room temperature for one hour. The membrane was washed thoroughly with PBS–Tween20 thrice and with PBS alone thrice and incubated in substrate buffer (100 mM NaCl, 5 mM MgCl₂, 100 mM Tris-HCl, pH 9.5) for half an hour. Finally, nitroblue tetrazolium- (NBT-) 5-bromo, 4-chloro, 3-indolyl phosphate (BCIP) substrate (66 µl NBT, 33µl BCIP in 10 mL of substrate buffer) was added and the colour was developed. The reaction was stopped with 5 mL of PBS. Immunoblotting was performed similarly with anti-intimin antiserum (kind gift of Dr. Stuart Knutton) to check for intimin expression in the outer membranes of all three strains.
2.12 **BEAD ADHERENCE ASSAY ON HEp-2 CELLS**

Carboxylated microspheres 0.9 µm³ (CLB9, Sigma) was coated with LamB or BSA (0.5 mg of protein) according to the manufacturer’s instructions. Protein coating was verified by slide agglutination tests in which 15 µL of the beads were mixed with 3 µl of anti-LamB antiserum on a clean glass slide. Coated beads in the absence of antiserum served as controls. For bead assays, approximately $10^9$ protein-coated beads (BSA or LamB) were added to sub-confluent HEp-2 cells (0.4 million cells/mL) in DMEM with serum and antibiotics. After incubation for 3 hours, the cells were thoroughly washed with sterile PBS, fixed with 0.2% glutaraldehyde and viewed by light microscopy at 1000× magnification (Rocha-de-Souza et al 2001). To determine inhibition of adherence, beads were pre-incubated with a 1:100 dilution of anti-LamB antiserum for 1 hour at 37°C before use in bead adherence assays.

2.13 **FAS TEST WITH ΔlamB**

Fluorescence actin staining (FAS) tests was performed as previously described (Knutton et al 1989). ΔlamB was grown overnight in LB with 30 µg/mL of kanamycin. 1% of the inoculum was sub- cultured into DMEM without serum and antibiotics and grown statically at 37°C for 3 hours. After 3 hours, 10µL of the culture was used to infect sub-confluent HEp-2 cells grown on 13mm diameter glass slides in DMEM without serum and antibiotics. The infected HEp-2 cells were incubated at 37°C for 3 hours and then washed three times with 1X PBS to remove non-adherent bacteria. The cells were then fixed with 3% formalin in 1X PBS for 20 minutes. After further washing with 1X PBS for three times, the cells were permeabilised with 0.01% triton X-100 in 1X PBS for 3 minutes in a rocker at room temperature. 5 µg/mL of fluorescein isothiocyanate conjugated (FITC)
phalloidin (Sigma) was added to the cells and incubated further at room temperature for 20 minutes. The cells were washed three times with 1X PBS and viewed under a fluorescent microscope (Nikon) at 400 X magnification.

2.14 MULTIPLE SEQUENCE ALIGNMENT STUDIES

The EPEC LamB sequence was kindly provided by Dr. Nicholas R. Thomson and Dr. Julian Parkhill, Pathogen Sequencing Unit, Sanger Institute, U.K. Multiple sequence alignment of the LamB proteins of related pathogens was performed using ClustalW (www.ebi.ac.uk/clustalW/). The other pathogens were enterohaemorrhagic E. coli EDL 933 (Pubmed protein ID: NP_290670.1), Shigella flexneri 2a (Pubmed protein ID: NP_709883.2), Salmonella enterica serovar Typhi (Pubmed protein ID: CAD09215.1), Aeromonas veronii (Pubmed protein ID: AAP40342) and E. coli strains K-12 (Pubmed protein ID: NP_418460), B171 (Pubmed protein ID: ZP_00710811.1) and E22 (Pubmed protein ID: ZP_00730325.1).

2.15 EXTRACTION OF EPEC LIPOPOLYSACCHARIDE (LPS)

LPS was extracted according to the method of Hitchcock and Brown (1983) with slight modifications. Briefly, Overnight cultures of EPEC grown on Luria-Bertani Agar were suspended in ice cold PBS (pH 7) and centrifuged at 10,000 rpm for 10 minutes. The pellet was solubilized in 200μl of lysis buffer (20% glycerol, 5% 2-Mercaptoethanol, 4.6% Sodium Dodecyl Sulfate and 0.125M Tris- HCl pH 6.8) at heated at 100°C for 10 minutes. The lysate was cooled to room temperature and 40 μl of lysis buffer containing 2.5 mg/mL of proteinase K was added and the lysate was heated at 60°C for 1 hour. After boiling for 5 minutes, the lipopolysaccharide was precipitated with double the volume of 0.375M MgCl₂ in 95% ethanol at -20°C for one hour. The sample was separated on 10% Sodium Dodecyl Sulphate-Poly
Acrylamide Gel Electrophoresis (SDS-PAGE) and visualized by silver staining. 1 µg/mL of both commercial O111:B4 LPS and EPEC LPS were used for stimulating J774 A.1 cells (1×10⁶ cells/well) for a time period of 12 hours. To check the activity of the ethyl acetate extract, 20µg of extract was added to the cells 30 minutes after stimulation with EPEC LPS in a separate well. After 12 hours, the cells were harvested for RNA isolation.

2.16 EXTRACT AND PURE COMPOUND OF ALPINIA OFFICINARUM

Crude ethyl acetate extract was chosen based on bio-activity guided fractionation using non-polar to polar solvent systems (hexane, dichloromethane, ethyl acetate, methanol and water) (Subramanian et al 2008) and diarylheptanoid (pure compound) (Manjula 2005) from *Alpinia officinarum* was kindly gifted by Selvakkumar C., Tissue Culture Lab, Centre for Biotechnology, Anna University.

2.17 REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION (RT-PCR)

2.17.1 Extraction of RNA

RT-PCR was carried out as described previously (Chomczynski and Sacchi 1987). J774 A.1 cells in 6-well plates were stimulated with 1 µg/mL of LPS. The cells were co-incubated with 20 µg/mL of crude extract of *Alpinia officinarum* for 12 h before RNA extraction.

i) After the required incubation, cells were lysed in 1 mL of total RNA isolation reagent and transferred to RNAs e free 1.5 mL centrifuge tubes.
ii) 200 μL of chloroform was added and spun at 14,000 rpm for 20 min at 4°C. The aqueous phase was removed and transferred to a new 1.5 mL centrifuge tube. 500 μL of isopropyl alcohol was added and incubated in ice for 15 min. The supernatant was discarded and the RNA pellet was washed with 1 mL of 70% ethanol. The pellet was air dried and dissolved in 9 μL of deionised autoclaved DEPC treated water.

iii) Reverse transcription was carried out as follows: 200 ng/μL of Oligo d[T] was added and the total 10 μL mix was incubated in a water bath at 65°C for 10 min and snap chilled on ice.

iv) 200 units of MMLV (Moloney Murine Reverse Transcriptase), 7.5 mM of dNTP and 10 units of RNAs inhibitor were added to each 10 μL reaction mix and the mix was incubated at 42°C in a water bath for about one hour. Then the reaction mix was boiled at 90°C for 3 min to inactivate reverse transcriptase. The cDNA synthesized was further used for PCR.

2.17.2 Polymerase Chain Reaction

Primers required for Polymerase Chain Reaction (PCR) were synthesized from Sigma, India. Primer sequences of all target genes (Table 2.1) were adopted from published articles.
Table 2.1 List of primer sequences used in the current study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Predicted product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>CCA CCC ATG GCA AAT TCC ATG GCA TCT AGA CGC CAG GTC AGG TCC ACC</td>
<td>597</td>
</tr>
<tr>
<td>IL-8</td>
<td>AGG GTT GCC AGA TGC AAT AC AGC AGA CTA GGG TTG CCA GA</td>
<td>378</td>
</tr>
<tr>
<td>TNFα</td>
<td>CGG GAC GTG GAG CTG GCC GAG GAG CAC CAG CTG GTT ATC TCA CAG CTC</td>
<td>434</td>
</tr>
<tr>
<td>IL1β</td>
<td>AAA CAG ATG AAG TGC TCC TTC CAG G TGG AGA ACA CCA CTT GTT GCT CCA</td>
<td>388</td>
</tr>
<tr>
<td>TLR4</td>
<td>GCA ATG TCT CTG GCA GGT GTA CAA GGG ATA AGA ACG CTG AGA</td>
<td>406</td>
</tr>
<tr>
<td>TLR2</td>
<td>TCT GGG CAG TCT TGA ACA TTT AGA GTC AGG TGA TGG ATG TCG</td>
<td>321</td>
</tr>
</tbody>
</table>

i) For PCR reaction, 1 μL of the cDNA mixture prepared as described earlier was added to a PCR reaction mixture consisting of:
   a) 1X PCR buffer
   b) 2.5 pmol dNTPs
   c) 5 pmol of paired primers,
   d) 1.25 units of Taq polymerase (Amersham Pharmacia Biotech, U.K), and made up to 50μl with distilled water.

ii) The reaction mixture was overlaid with mineral oil and placed in a thermal cycler for the following cyclic reactions:
   a) 95°C for 5 min: Initial denaturation
b) 95°C for 1 minute: Denaturation

c) X°C for 1 min: Annealing temperature

d) 72°C for 1 minute: Extension

e) 72°C for 10 min: Final Extension

Annealing temperature (X°C) for the primers were between 50°C to 65°C

iii) The PCR products were run on agarose gels, stained with ethidium bromide and photographed.

2.18 ANTIMICROBIAL SUSCEPTIBILITY TESTS WITH EPEC CLINICAL ISOLATES

2.18.1 Agar Disc Diffusion Method

Agar disc diffusion method for testing EPEC susceptibility to standard antibiotics and the crude and pure compounds of *Alpinia officinarum* was performed according to the standards laid down by Clinical and Laboratory Standards Institute [CLSI] (NCCLS 2003). Briefly, test cultures were streaked on tryptone soya agar and incubated at 37°C for 16-18 h. After incubation, 2 or 3 isolated colonies from the plate was used to adjust the turbidity of the cultures to 0.5 McFarland (50μL of 1.175% BaCl$_2$.2H$_2$O is added to 995μL of 1% v/v H$_2$SO$_4$). This suspension corresponds to 1 to 2 × 10$^8$ CFU/mL. Within 15 minutes after adjusting the turbidity of the inoculum suspension, a sterile cotton swab was dipped into the adjusted suspension. The swab was rotated several times and pressed firmly on the inside wall of the tube above the fluid level to remove excess inoculum from the swab. The surface of Mueller-Hinton agar plates was inoculated by streaking the swab over the entire sterile agar surface. This procedure was repeated by streaking
two more times, rotating the plate approximately $60^\circ$ each time to ensure an even distribution of inoculum. As a final step, the rim of the agar was swabbed.

The lids of the petriplates are left ajar for not more than 15 minutes. After the surface dried, the battery of antimicrobial discs were placed on the agar surface and pressed gently to ensure complete contact with the medium. For the test extract and compound, respective working dilutions were made in DMSO and applied to pre-sterilised Whatman no.1 filter paper discs (6mm diameter) as volumes containing the respective concentrations of extract or compound. The plates were then incubated in an inverted position for 16-18h and observed for zone of clearance. The zones were measured to nearest millimeter using a ruler held on the back of the petriplate against an illuminated background. The sizes of the zones of inhibition are interpreted by referring to Tables 2A through 2I (Zone Diameter Interpretative Standards and equivalent Minimum Inhibitory Concentration Breakpoints) of the NCCLS M100-S12: Performance Standards for Antimicrobial Susceptibility Testing: Twelfth Informational Supplement and the organisms are reported as susceptible, intermediate, or resistant to the agents that have been tested. For the test extract and compound, zones obtained using the quality control strain *E. coli* ATCC 25922 was used as reference. Zones obtained with EPEC isolates were recorded as susceptible, intermediate, or resistant based the the size of zones being smaller or bigger than the ones obtained with the quality control strain. Susceptibility tests were performed twice as separate experiments.

2.19 **IN SILICO ANALYSIS OF DIARYLHEPTANOID BINDING TO E. COLI DNA GYRASE A USING AUTODOCK®**

The coordinates for the crystal structure of a 59-kDa fragment of gyrase A from *E. coli*, Protein Data Bank (PDB) code 1AB4, were used in the molecular docking calculations (Morais Cabral et al 1997). Diarylheptanoid
was docked with *E.coli* DNA gyrase sub-unit A using Autodock® (Morris et al 1998) version 4. The structural pocket on the enzyme covering the Quinolone Resistance Determining Region (QRDR) (Heddle and Maxwell, 2002) and some residues outside the QRDR covering Tyr122 (the residue of DNA gyrase A that interacts with the DNA during replication process) was taken up for docking studies (Ostrov et al 2007). Ciprofloxacin (pubchem ID 2764) interaction with the same pocket was used as a template. Conformations of ciprofloxacin and diarylheptanoid were placed in the selected structural pocket of gyrase and scored based on polar (H bond) interaction and binding energy.

### 2.20 STATISTICAL ANALYSIS

Data for bacterial adherence assays were expressed as mean ± standard deviations. Mean value differences were analysed by Student’s two-tailed t test and values of \( P<0.05 \) were considered to be significant. Data for antimicrobial susceptibility testing were expressed as mean ± standard deviations.