Chapter 3

Modulatory effect of Chandraprabhavati

3.1 BACKGROUND:

The misuse of various antibiotics (WHO, 2000) and their synthetic analogues has increased by 40% in the span of four years from 2005-2009 (Ganguly et al., 2011) leading to the emergence of broad-spectrum resistant pathogens (Dimitri and James, 2008). This has in turn lead to an alarming and exponential use of non-specific antibiotics and the high cost of prescribed medications for the management of the infective microbes (WHO, 2011), raising the need for alternate and safe medication (Holloway et al., 2009). To prevent generation of antibiotic resistant microbes, World Health Organization (WHO), recommended the search for safer, green and alternative medication for infectious diseases in humans (WHO, 2000). In India, two forms of alternative medications have found widespread usage from time immemorial: Siddha and Ayurvedha. Siddha, drugs and formulations offer an array of chemicals, with synergistic interactions (Barnes, 1999) for treating ailments leading to revitalization of plant products as sources of new drugs (Astin, 1998).

In vitro and/or in vivo evidences that support synergism between constituents in herbal extracts, lack of valid documentations relating to usage, toxicity regime, and pharmacovigilance limits their wide-spread usage (Aronson, 2009) and widens the uncertainty pertaining to the extent in which the active principle acts (Fontanarosa et al., 2003). Siddha offers a wide range of herbo-mineral drugs for the management of UTIs like Nagaparpam, Silasthuparpam, and the most widely used Chandraprabhavati (CPV) (AYUSH, 2003). CPV is a poly-herbal preparation from Cyperus rotundus, Phyllanthus emblica, Piper longum, Zingiber officinale, Coriandrum sativum, Curcuma longa, etc. CPV has been prescribed for various urinary problems, anaemia, renal calculi, to strengthen the kidney and for general malaise (AYUSH, 2003) in India. The exact mechanism and principles responsible for its activity remain a mystery although it serves as an effective alternative medication. Our objective through this study was to explore the effect of CPV as an effective medication for the
management of urinary tract infected mice and analyse its role in modulating the levels inflammatory markers and AMP expression upon administration.

3.2. MATERIALS AND METHODS

3.2.1. Chemicals and reagents

DPPH and pH indicator strips were procured from Sigma Aldrich (Bangalore, India). Chemicals, solvents and reagents used were purchased from Sisco Research Laboratories (Mumbai, India) and the microbiological media from Himedia Pvt. Ltd (Mumbai, India). The antibodies against Cathelicidin, Lipocalin, and TNF –α raised in goats, and NF –κβ raised in rabbits were procured from Santa Cruz Biotechnology, Inc., CA, USA , antibodies against Ficolin were received as a gift from Dr. Preetham Elumalai, and the polyclonal antibody against normal Tamm Horsfall Protein (THP) and Transferrin (Sigma Aldrich, India) was raised in-house in New Zealand white rabbits and standardized for ELISA, Western blotting and Immuno-histochemistry (IHC).

3.2.2. Chandraprabhavati: Solubility, extract preparation and composition analysis

Chandraprabhavati (CPV) was procured from M/s Indian Medical Practitioners Co-operative pharmacy and Stores (IMCOPS) Ltd., Chennai, India. The solubility of the drug to prepare for supplementation in various solvents was analyzed. For oral administration the aqueous infusion of the drug (aq.CPV) was prepared by mixing 10 g of the drug in 100 ml of water at 60 °C (Luthje et al., 2011). This aq.CPV was used to analyze the heavy metals (Hung et al., 2009) present in the drug using Atomic Absorption Spectroscopy (AAS) (VARIAN, AA240).

3.2.3. Phytochemical analysis of aqueous CPV

The aq.CPV was subjected to preliminary phytochemical analysis. Based on the results, the phyto-groups were separated on a silica column of mesh size 300 – 400, with a pore size of 60Å that was prepared into slurry using distilled water. The various solvents such as water (distilled), methanol, ethanol, ethyl acetate, butanol, acetone, chloroform and dichloromethane in the increasing order of their polarity were used to separate the various phyto-groups present in the aq.CPV. The fractions
containing terpenoids and flavanoids were identified by carrying out the phytochemical analysis on the collected fractions and confirmed using thin layer chromatography.

The reverse phase (RP) - HPLC was performed on the fractions that tested positive for the phyto-compounds like flavanoids and terpenoids on a Waters HPLC (Germany) with a 20 μl rheodyne injector, on a RP C-18 column (5mm 4.6 x 150 mm), using a Waters 1525 binary HPLC pump and a Dual λ Absorbance Detector. To identify the various terpenoids present, the fractions were separated using MetOH/ H₂O/ Phosphoric acid (85:15:1 v/v/v) as the mobile phase in isocratic conditions with a flow rate of 2 ml/min. In order to identify the flavanoids present in the fractions, the mobile phase comprising of Solvent A: H₂O/ Acetic acid (99:1, v/v) and Solvent B: MeCN under gradient conditions i.e., 18% B to 32% in 15 min and finally to 50% in 40 min with a flow rate of 1 ml/min was utilized.

3.2.4. Microbial strains used in the study

E.coli 25922 (MTCC No. 443, IMTECH Chandigarh, India), a wild non-pathogenic strain was utilized as the standard and the clinical isolate Escherichia coli RRL - 36 (ECRRL36) (Genbank accession number: JQ398845.1), served as the pathogenic strain for in vitro and in vivo antimicrobial studies.

A single colony of ECRRL36 was inoculated into 10 ml of LB broth and statically incubated overnight at 37 °C to ensure pilation. The bacterial culture was spun down at 5,000 g for 5 min at 4 °C and the pellet was re-suspended in sterile PBS (pH 7.4). The ten-fold diluted bacterial suspension was used for further analysis after determining cfu/ml (Turck et al., 1963).

3.2.5. In vitro antimicrobial growth kinetics study

The antibacterial activity of the aq.CPV was assessed by tube dilution method using varying concentrations (0 – 1000 mg/ml) and were added to LB broth. To the broth containing the drug, 100 μl of McFarland matched 4 h culture of standard strain or ECRRL36 was added and mixed well. The tubes were incubated at 37 °C for 24 h at static conditions and the minimum inhibitory concentration (MIC) and minimum
The bactericidal concentration (MBC) of the drug were determined using the growth curve analysis by estimating the OD_{600} of the cultures. Amoxicillin, a synthetic analogue, which acts by inhibiting the bacterial cell wall synthesis, at 100 mg/ kg body weight to which the strain under study was susceptible was chosen as the standard drug for the study.

3.2.6. Initiation of infection in mice

Female Swiss Albino mice (8 – 10 weeks) weighing 25 ± 2 g were housed in polypropylene cages (6 animals per cage) lined with paddy-husk bedding. An ambient temperature of 24 ± 1° C, with a 12 h light/dark light cycle and 65 ± 2% relative humidity was maintained. The animals were fed with food and water ad libitum. The Institutional Animal Ethical Committee (IAEC), VIT University approved the study design and protocols (Approval number: VIT/ SBST/ IAEC/III/2011/19).

Each single anesthetized mouse was infected with 2.5x10^8 cfu of ECRRL36/(30μl of sterile PBS), trans-urethrally using lubricated catheters delivering ~10 μl of inoculum into the bladder. After inoculation, the catheter was removed and the animal was monitored regularly for any discomfort, injury or inflammation due to the procedure. The procedure was repeated on 18 mice, which were separated into three groups (infection control, Amoxicillin treated animals and aq.CPV treated animals) upon confirmation of urinary tract infection on the 7th day post infection. Six animals that were not infected using ECRRL36 served as the experimental control.

3.2.7. Groups and treatments

Upon confirmation of pyelonephritis, the mice were separated into two groups of 6 animals each and scheduled for treatment protocol for 20 days orally. The group 1 animals were administered with 100 mg/kg bodyweight of Amoxicillin (standard drug) and the group 2 animals were supplemented with the aq.CPV extract (500 mg/kg body weight derived after the growth kinetics studies using the drug on ECRRL36). The body weights of the animals were monitored at 24 h regular intervals. The bladders and kidneys were harvested from the control animals prior to the infection and the infected animals were sacrificed at 6 h, 7th day, 10th day and 20th day after treatment with aq.CPV. The harvested tissues were rinsed thoroughly using
PBS (pH 7.4) and processed for biochemical studies; a portion of the rinsed tissue was stored in 10% NBF at RT for histopathological analysis. A portion of the kidney was blotted dry to remove all traces of the PBS and stored in an equal volume of RNAlater (Sigma Aldrich, India) and mixed well. The tissue samples stored in RNAlater were maintained at -20 °C until utilized for the analysis of mRNA expression of the various antimicrobial proteins and inflammatory markers.

3.2.8. Biochemical parameters

Protein (Bradford, 1976), nitrite (Guevara et al., 1998), and pH of the urine sample were determined. The leucocytes enumeration on a Haemocytometer (Neubauer, HBG, Germany) were carried out to determine their levels in the urine of the control, the infected and treated groups. The tissue homogenate was analyzed for markers of oxidative stress such as superoxide dismutase (Kakkar et al., 1984), catalase (Goth, 1991), and total nitrite content (Guevara et al., 1998).

3.2.9. Histopathological analysis of the kidney

The 10% NBF fixed tissues were processed for paraffin embedment and 4 μm tissues sections were stained with H&E. The histological structures of the kidney and bladder were studied for evidence of UTI such as inflammation, inflammatory cell infiltrates, destruction of renal epithelium and urothelium, changes in the architecture of the glomeruli, tubules, interstitium and vessels, resolved and persistent bacteriuria and the degree of nephritis using a light microscope.

3.2.10. Immunohistostaining for antimicrobial proteins

Paraffin sections (4 μm) were mounted on silanized slides, dewaxed in xylene and rehydrated. Endogenous peroxidase activity was blocked by incubation with 3% H₂O₂ for 15 min. After washing with PBS containing 0.1% Polysorbate 20, the slides were incubated overnight with the primary antibody for THP (1: 200), Transferrin (1: 200), Lipocalin (1: 2500), Cathelicidin (1: 2500), and Ficolin (1: 2500) at 4 °C. Immuno-detection was performed by incubating with horseradish peroxidase conjugated goat-anti-rabbit IgG antibody at a dilution of 1:5000 for 30 min at RT and 0.05% of 3, 3’-diaminobenzidine (Dako) served as the chromogen. Slides were
counterstained with hematoxylin, rinsed in tap water, dehydrated, placed in xylene, mounted and read at 400X magnification.

3.2.11. Immunostaining for inflammatory markers

As mentioned in section 3.2.10., paraffin sections were utilized for inflammatory marker studies. Kidney sections were incubated with the primary antibody for TNF –α (1: 200), NF –κβ, (1: 200) at 4°C. Immuno-detection was performed by incubating with horseradish peroxidase conjugated goat-anti-rabbit IgG antibody (1:5000) for 30 min at RT and 3, 3’-diaminobenzidine (Dako) served as the chromogen. Slides were counterstained with hematoxylin, rinsed in tap water, dehydrated, placed in xylene, mounted and read at 400X magnification.

3.2.12. m-RNA expression of antimicrobial proteins and inflammatory markers using qPCR

3.2.12.1. Extraction of RNA

Isolation of total RNA is a crucial step in analysing the gene expression level in a tissue. The RNA was isolated using TRIzol reagent (Sigma Aldrich, India), which is also known as Guanidium-thiocyanate-phenol chloroform mixture (Chomczynski et al., 1987), has the ability to disrupt and break down the cells and its components. It also maintains the integrity of RNA during tissue homogenisation. During centrifugation, chloroform separates the solution into aqueous and organic phases. The RNA remains in the aqueous phase, leaving the DNA and proteins in the organic phase. When isopropyl alcohol was added to the aqueous phase, RNA is precipitated and recovered. The total RNA extracted using TRIzol reagent is free from proteins and DNA contamination.

The kidneys were removed from RNAlater and blotted dry to remove all traces of the solution. It was washed with phosphate buffered saline and equal amount of tissue slices (0.2 g) from different groups were taken. The tissues slices were placed in sterile mortar and pestle and were snap frozen using liquid nitrogen. The snap frozen tissue was homogenised with 0.5 ml of TRIzol reagent and was transferred to a
sterile eppendorf and vortexed. After complete mixing, it was centrifuged at 10500 g for 5 min at 4 °C and the tissue chunks were removed.

To a fresh microfuge tube the supernatant was transferred and 400 µl of chloroform was added to it and mixed well. It was centrifuged at 10500 g for 15 min at 4 °C and the mixture was separated into three phases; the aqueous phase contains the RNA, inter-phase contains DNA and the organic phase contains protein. The aqueous phase was transferred to a fresh tube and 500 µl of isopropanol was added to it, mixed and allowed to stand for 10 min at RT. Further, the contents were centrifuged at 10500 g for 10 min at 4 °C and RNA was formed as a white pellet at the bottom. After discarding the supernatant, the pellet was washed with 75% ethanol and centrifuged at 8500 g at 4 °C for 5 min. The pellet was air-dried and was re-suspended in 50 µl of Diethylpyrocarbonate (DEPC) water

3.2.12.2. Quantification of RNA

The RNA samples were diluted 200 times using sterile distilled water and the absorbance of the RNA samples was checked at 260 nm and 280 nm. The absorbance ratio was calculated as follows:

$$\text{Absorbance ratio} = \frac{\text{OD}_{260}}{\text{OD}_{280}}$$

RNA sample was considered pure, when the absorbance ratio was found to be around 1.8. The amount of RNA in the sample was analyzed as follows:

$$\text{Quantity of RNA (µg/ml)} = \text{OD}_{260} \times \text{Dilution factor} \times 40$$

3.2.12.3. Synthesis of cDNA

Complementary DNA is synthesised from m-RNA in reverse transcriptase PCR (RT-PCR) using reverse transcriptase enzyme. It is the most commonly used technique used in molecular biology to detect RNA expression level (Freeman et al., 1999). The gene expression level can be qualitatively detected using qPCR by synthesising cDNA transcripts from RNA. Apart from qualitative study of gene
expression, RT-PCR can also be used for quantification of RNA, in both relative and absolute terms, by incorporating qPCR with it (Joyce et al., 2002).

Verso cDNA (Merck Millipore, India) synthesis kit was used for the synthesis of cDNA. Verso enzyme mix includes reverse transcriptase enzyme, RNase inhibitor and RT enhancer. RT is active at high temperatures, highly sensitive and can generate long cDNA strands. RNase inhibitor protects the RNA template from degradation, by inhibiting the RNase enzyme. RT Enhancer eliminates the need for DNase I treatment, by removing the contaminating DNA.

The reaction mixture was prepared in PCR tubes as mentioned in Table 3.1. provided below and was placed on ice till the start of the experiment. The amount of RNA to be added for maximum cDNA synthesis was optimised to be 5 µg.

**Table 3.1. Master mix preparation for cDNA synthesis**

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 5X cDNA synthesis buffer</td>
<td>4 µl</td>
</tr>
<tr>
<td>2 dNTP mix</td>
<td>2 µl</td>
</tr>
<tr>
<td>3 RNA primer (random hexamer/oligo dT)</td>
<td>1 µl</td>
</tr>
<tr>
<td>4 RT enhancer</td>
<td>1 µl</td>
</tr>
<tr>
<td>5 Verso enzyme mix</td>
<td>1 µl</td>
</tr>
<tr>
<td>6 Water (PCR grade)</td>
<td>Variable</td>
</tr>
<tr>
<td>7 Template RNA</td>
<td>1 - 5 µl</td>
</tr>
<tr>
<td><strong>TOTAL VOLUME</strong></td>
<td>20 µl</td>
</tr>
</tbody>
</table>

The tubes containing the prepared reaction mixture were placed in PCR machine and the cycling programming mentioned in Table 3.2. was set, and the instrument was allowed to run. The synthesized cDNA was stored at -20 °C for further analyses.

**Table 3.2. Reaction conditions for synthesis of cDNA**

<table>
<thead>
<tr>
<th></th>
<th>Temperature</th>
<th>Time</th>
<th>No. of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>cDNA synthesis</td>
<td>42 °C</td>
<td>30 min</td>
<td>1 cycle</td>
</tr>
<tr>
<td>Inactivation</td>
<td>95 °C</td>
<td>2 min</td>
<td>1 cycle</td>
</tr>
</tbody>
</table>
3.2.12.4. Relative quantification of AMP and inflammatory marker genes

The gene expression was quantified using qPCR. The target gene was expressed and amplified using respective forward and reverse primers for each gene as mentioned in Table 3.3 with cDNA as the template. qPCR can quantify nucleic acids by relative quantification (Dhanasekaran et al., 2010).

**Table 3.3. Primer sequence for the various genes**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Sequence</th>
<th>Reverse Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Actin</td>
<td>5' CCACCATGTACCAGGCATT 3'</td>
<td>5' AGGGGTGAACACGAGCTCA 3'</td>
</tr>
<tr>
<td>THP</td>
<td>5' GTCCTGGGCTAGGCAGTCA 3'</td>
<td>5' AGGAGCACTTAACGAGCCCA 3'</td>
</tr>
<tr>
<td>Transferrin</td>
<td>5' TGTCCAGTTTCTTCTCGGCG 3'</td>
<td>5' TGATGACTCCACTGACAC 3'</td>
</tr>
<tr>
<td>Cathelicidin</td>
<td>5' ATCAGCTGAACGAGCTGG 3'</td>
<td>5' AGGCACTACTCTGGCTCA 3'</td>
</tr>
<tr>
<td>Lipocalin</td>
<td>5' CACACGGAATCAACCAGTTCGC 3'</td>
<td>5' TCAGTGTCAATGCATGGTCGTG 3'</td>
</tr>
<tr>
<td>Ficolin</td>
<td>5' GAAAGTTTTCTGGTGCTGIAATCGA 3'</td>
<td>5' TGAGCAITCGIAAGTTCACCA 3'</td>
</tr>
<tr>
<td>TNF-α</td>
<td>5' ACAGAAAGCATGATCCGCGA 3'</td>
<td>5' CCTCCACTTGGTGTTCCTA 3'</td>
</tr>
<tr>
<td>NF-κβ</td>
<td>5' GCAGCATAACTTTCTGCC 3'</td>
<td>5' GATTAGGGGCATCAGCTGC 3'</td>
</tr>
</tbody>
</table>

The reaction mixture was prepared as mentioned in Table 3.4 in qPCR tubes (Eppendorf, UK) and placed in the machine and the lid temperature was set at 105 °C and the final hold temperature was set at 4 °C. PCR was performed using the programme mentioned in Table 3.5. Threshold cycle value (Ct) was obtained for all the samples and the expression ratio was calculated based on Livak method (Livak and Schmittgen, 2001).
Table 3.4. Master mix Preparation for qPCR of the housekeeping and target genes

<table>
<thead>
<tr>
<th></th>
<th>Reagents</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SYBR green master mix (2X)</td>
<td>10 µl</td>
</tr>
<tr>
<td>2</td>
<td>Forward primer</td>
<td>2 µl</td>
</tr>
<tr>
<td>3</td>
<td>Reverse primer</td>
<td>2 µl</td>
</tr>
<tr>
<td>4</td>
<td>cDNA template</td>
<td>4 µl</td>
</tr>
<tr>
<td>5</td>
<td>Nuclease free water</td>
<td>2 µl</td>
</tr>
<tr>
<td></td>
<td>TOTAL VOLUME</td>
<td>20 µl</td>
</tr>
</tbody>
</table>

Table 3.5. Reaction conditions for qPCR of the target genes

<table>
<thead>
<tr>
<th></th>
<th>Temperature</th>
<th>Time</th>
<th>No. of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme activation/hold</td>
<td>95 °C</td>
<td>7 min</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95 °C</td>
<td>30 sec</td>
<td>40</td>
</tr>
<tr>
<td>Annealing/ Elongation</td>
<td>58 °C</td>
<td>1 min</td>
<td>40</td>
</tr>
</tbody>
</table>

3.2.13. Protein expression analysis using western blot

The glass plates were clamped along with the spacers and checked for the leakage. 10 ml of 10% separating gel mixture was prepared, mixed well and poured between the glass plates. The solution was overlaid with butanol and the gel was allowed to polymerize. Once the resolving gel was polymerized, butanol was removed completely. 5 ml of 5% stacking gel mixture was prepared and added over the separating gel. Immediately after adding the solution, comb was placed carefully without any air bubble and the gel was allowed to polymerize. Once polymerized, the comb was removed and the gel along with the glass plates was placed in the electrophoresis tank, which was filled sufficiently with running buffer.
The concentration of protein in each sample was estimated and equal amount of protein was loaded in each well based in the protein/creatinine ratio. To the samples, equal volume of sample buffer was added and heated in boiling water bath for 10 min for effective denaturation of proteins. The samples were loaded into the wells with a protein ladder in one well. The electrophoresis apparatus (Medox, India) was connected to power supply at 50 v for 20 min, till the sample reaches the separating gel. The voltage was increased to 100 v for approximately 90 min, till the dye front reaches the bottom of the gel. Once it reaches the bottom, the power supply was switched off and the glass plates were taken out. The plates were separated without damaging the gel and the gel was placed in a beaker containing transfer buffer for 30 min.

The scotch pads along with filter papers were soaked in transfer buffer for 30 min. Nitrocellulose membrane (NC) was pre-treated with transfer buffer for 5 min. The scotch pads were assembled and both the gel and NC membrane were sandwiched between them without any air bubble. After assembling the setup, it was placed inside the electrophoresis tank such that the gel was facing towards the negative electrode. The apparatus was connected to power supply and the experiment was performed at 100 V for 2 h. After the transfer, the membrane was carefully taken out and the protein transfer was confirmed by staining in Ponceau S for 15 min. After observing the bands and confirming the transfer, the membrane was washed with distilled water for few minutes to remove the stain.

The membrane was blocked by incubating with 5% skimmed milk prepared in TBS for 1 h to avoid non-specific binding of antibodies. The membrane was incubated with specific antibodies and incubated overnight at 4 °C. The membrane was washed three times with TBST for 10 min, with change of buffer at every wash. It was then incubated with anti-rabbit IgG HRP for 1 h at RT. The membrane was washed three times with TBST for 10 min each, with change of buffer after every wash. Followed by this, the membranes were then washes thrice with TBS for 10 min, with the change of buffer after each wash. After washing, the membrane were blotted dry of the buffer and an equal volume of ECL solution A and solution B that were mixed well were added on to the blot. Once the ECL solution was treated on the surface, the solution was drained and the blot was placed on a cassette inside a plastic
wrap. On top of the ECL treated membrane, the X-ray sheets (Thermo Scientific, India) were placed and allowed for exposure. After exposure the X-ray sheets were processed in the developer & fixer (Kodak Carestream, Sigma Aldrich, India) and rinsed thoroughly in distilled. The X-ray film was scanned and the intensity of the bands was observed and the relative protein expression was calculated using densitometric analysis.

3.2.14. Statistical analysis

Data were represented as mean ± standard deviation of mean (SD). Mean difference between the groups were analyzed by one way ANOVA and p value ≤ 0.05 was considered significant. Statistical analysis was performed using SPSS v16.

3.3. RESULTS

The Siddha drug, Chandraprabhavati, is prepared as a mixture of various parts of 21 herbs and is commonly administered for the management of various kidney malaises. In our study, we evaluated this drug for its effect on controlling and treating infections of the kidney using the pathogen ECRL36. The solubility profile of the drug was analyzed using various solvents. It was found that the drug was insoluble in solvents such as methanol, ethanol, chloroform, and was sparingly soluble in water (maintained at room temperature) and coconut oil. The drug was highly soluble in warm water (50 °C) and in 5% DMSO. As the percentage of DMSO was very high to be used for any biological assay, hence the drug dissolved in warm water (aq.CPV) was used for further analyses.

The preliminary phytochemical analysis of aq.CPV revealed that the drug was a rich source of various phyto-compounds (Table 3.6) such as terpenoids, flavanoids, anthroquinone etc. that could act as an active principle in managing UTIs. Our metal analysis revealed the presence of 6.1 ± 0.3 mg of Zinc and 0.2 ± 0.1 mg of Iron per litre of aq.CPV.

The methanolic fractions of aq.CPV collected after silica separation was found to be concentrated with 9 different flavanoids that were identified based on their retention time to be as Shikimic acid (R_t 4.133 min), Gallic acid (R_t 7.045 min), Cathechin (R_t 8.921 min), Caffeic acid (R_t 14.3 min), Syringic acid (R_t 20.874 min),
Quercitin (Rt 29.2 min) and Ferulic acid (Rt 33.873 min) were identified to be present while the acetone fraction with five terpenoids (Fig. 3.1A), while the terpenoids from the chromatogram were identified to be α-Terpiene (Rt 2.776 min) and tocopherol (Rt 16.066 min) (Fig. 3.1B).

Table 3.6. Phyto – compound analysis of the aq. infusion of CPV

<table>
<thead>
<tr>
<th>Phyto – compound</th>
<th>aq.CPV</th>
<th>Phyto - compound</th>
<th>aq.CPV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tannins</td>
<td>+</td>
<td>Alkaloids</td>
<td>+</td>
</tr>
<tr>
<td>Saponins</td>
<td>+</td>
<td>Coumarins</td>
<td>–</td>
</tr>
<tr>
<td>Flavanoids</td>
<td>+</td>
<td>Anthraquinone</td>
<td>+</td>
</tr>
<tr>
<td>Steroids</td>
<td>+</td>
<td>Reducing sugar</td>
<td>+</td>
</tr>
<tr>
<td>Glycosides</td>
<td>+</td>
<td>Pholobotamines</td>
<td>+</td>
</tr>
<tr>
<td>Terpenoids</td>
<td>+</td>
<td>Lipids / fats</td>
<td>+</td>
</tr>
</tbody>
</table>

The antioxidant profile of aq. CPV was carried out up to 0.5 mg/ml concentration. CPV scavenged 60% of the free radicals produced at 0.1 mg/ml, 100% of the generated superoxide radicals at 0.15 mg/ml concentration, 50% hydroxyl radical scavenging activity at 0.1 mg/ml, an increasing total antioxidant activity and reducing power activity (Table 3.7).

The 24 h supernatant from each of the macro-dilution tube was sub-cultured on LB plates at 37 °C in the absence of drug. Amoxicillin showed complete inhibition at 100 mg, whereas in CPV, a reduction in the number of cfu was observed after 500 mg. There was no complete inhibition in the subsequent concentrations of the drug, indicating its bacteriostatic nature (Fig. 3.3) and 500 mg of CPV was set as the concentration for further elucidation of its in vitro and in vivo properties. The growth kinetics of ECRRL36 in the presence of Amoxicillin (100 mg) and 500 mg of CPV are shown in Fig 3.2.
A decrease in the body weight of the animals (Fig. 3.4) infected with ECRRL36 was evident on the 7th day post inoculation. In addition, a decrease in the pH of the urine (Fig. 3.5) was also observed, indicating that due to the utilization of the nutrients in the urine and growth of the bacterium, there was an acidic shift noticed in the urine. Upon treatment this change, however not significant when compared to the control, the body weight and pH of the urine were restored close to normal.

An increase in the weight of the infected kidney was also evident due to fluid accumulation to combat the rapidly multiplying pathogen in the kidneys when compared with the normal. This increase was not noticed in the kidneys excised from the animals treated with aq.CPV (Fig. 3.6). In the infected animal an increase in the leukocyte content (Fig. 3.7) and nitrite content (Fig. 3.8) were noticed. This increase in the leukocyte and nitrite content was brought down close to normal in the animals treated with the aq. infusion of CPV after a period of 20 days.
Table 3.7. Antioxidant profile of aq. Infusion of CPV

<table>
<thead>
<tr>
<th>Assay</th>
<th>100 μg/ml</th>
<th>200 μg/ml</th>
<th>300 μg/ml</th>
<th>400 μg/ml</th>
<th>500 μg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPPH free radical (% inhibition)</td>
<td>34.4 ± 0.3</td>
<td>66.6 ± 0.7</td>
<td>52.3 ± 0.25</td>
<td>51.2 ± 0.71</td>
<td>46.7 ± 0.5</td>
</tr>
<tr>
<td>Reducing power (BHT equivalents)</td>
<td>16.5 ± 0.01</td>
<td>19.5 ± 0.03</td>
<td>22.6 ± 0.02</td>
<td>26.9 ± 0.01</td>
<td>30.1 ± 0.01</td>
</tr>
<tr>
<td>Hydroxyl radical (% inhibition)</td>
<td>31.6 ± 0.1</td>
<td>0 ± 0.0</td>
<td>0 ± 0.0</td>
<td>0 ± 0.0</td>
<td>0 ± 0.0</td>
</tr>
<tr>
<td>Total Antioxidant (% inhibition)</td>
<td>16.5 ± 0.01</td>
<td>19.5 ± 0.3</td>
<td>22.6 ± 0.02</td>
<td>26.9 ± 0.01</td>
<td>30.1 ± 0.02</td>
</tr>
<tr>
<td>Lipid peroxidation (% inhibition)</td>
<td>0 ± 0.0</td>
<td>17.41 ± 0.2</td>
<td>21.6 ± 0.06</td>
<td>29.0 ± 0.03</td>
<td>15.4 ± 0.01</td>
</tr>
<tr>
<td>Superoxide scavenging (% inhibition)</td>
<td>6.8 ± 0.01</td>
<td>12.0 ± 0.03</td>
<td>14.7 ± 0.1</td>
<td>23.4 ± 0.02</td>
<td>83.4 ± 0.02</td>
</tr>
</tbody>
</table>

The values are expressed as mean ± standard deviation of the sample size (n = 18)

Figure 3.2. Effect of the aq. infusion of CPV on the growth kinetics of ECRRL36
Figure 3.3. Macro-dilution technique indicating the MBC of the aq. infusion of CPV

A: *E. coli* ATCC 25922; B: *E. coli* RRL-36; 1: CPV: 200 mg/kg body weight; 2: CPV 300 mg/kg body weight; 3: CPV 400 mg/kg body weight; 4: CPV 500 mg/kg body weight  
C1: ECRRL36 Amox 100 mg/kg body weight.

Figure 3.4. Body weight of the animals treated with the aq. infusion of CPV

The values are expressed as mean ± standard deviation of the sample size (n = 6).
Figure 3.5. Weight of the kidneys excised from the animals treated with aq. infusion of CPV

![Graph showing weight of kidneys with error bars]

The values are expressed as mean ± standard deviation of the sample size (n = 6).

Figure 3.6. pH of the urine of the animals treated with the aq. infusion of CPV

![Graph showing pH levels with error bars]

The values are expressed as mean ± standard deviation of the sample size (n = 6).

Figure 3.7. Leukocytes in the urine of the animals treated with the aq. infusion of CPV

![Graph showing leukocytes count with error bars]

The values are expressed as mean ± standard deviation of the sample size (n = 6).
Figure 3.8. Nitrite content in the urine of the animals treated with the aq. infusion of CPV

![Graph showing nitrite content in urine over days for different groups.]

The values are expressed as mean ± standard deviation of the sample size (n = 6).

Figure 3.9. H&E stained kidney sections from the animals treated with the aq. infusion of CPV

![H&E stained kidney sections. A: Experimental control; B: Infection Control; C: aq.CPV (500 mg/ kg body weight) of the animal for 20 days; D: Amoxicillin (100 mg/ kg body weight) of the animal for 20 days.]

The above figure shows the H&E stained kidney sections obtained from the mice treated with aq.CPV for 20 days. A: Experimental control; B: Infection Control; C: aq.CPV (500 mg/ kg body weight) of the animal for 20 days; D: Amoxicillin (100 mg/ kg body weight) of the animal for 20 days.
Protein quantification on the infected and control kidney homogenates confirmed a 50 fold decrease in the protein content of the infected mice relative to control. Upon treatment for 20 days with the specified dosage of aq.CPV, the protein concentration in the kidney of the treated mice significantly increased compared to the infected mice. The increase was also significant when compared to that of the control. The levels of the various antioxidant enzymes catalase, nitrite, and superoxide dismutase (Table 3.8) were also found to be elevated in a significant manner the infected animals when compared to the control mice. This indicated that the decrease could be due to the damage inflicted on the tissue architecture of the renal epithelium because of the entry and multiplication of the pathogen and the subsequent neutrophil infiltration into the tissues to combat the infection (Table 3.8).

The infected mouse kidney sections (H&E) as seen in Fig. 3.9B obtained on the 7th day revealed that acute pyelonephritis was established in the model with neutrophilic infiltration in the ureters and pelvi-ureteric junction with the sparing of the renal parenchyma when compared to the control (Fig. 3.9A). Dense infiltration by the lymphocytes and plasma cells, with disruption of the urothelium was also noticed in the infected renal tissues. Upon treatment with aq.CPV for 20 days at 500 mg/kg body weight, no evidence of pyelonephritis was noticed. The glomerular, interstitial and vascular compartments were within the normal limits as seen in Fig. 3.9D. A similar observation was also noticed in the sections obtained from the animals treated with Amoxicillin at 100 mg.kg body weight (Fig. 3.9C)

Table 3.8. Biochemical parameters in the kidney after treatment with aq.CPV

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Protein (mg/g tissue)</th>
<th>Catalase (units/ mg protein)</th>
<th>Nitrite (μg/μl)</th>
<th>SOD (μmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.33 ± 0.2</td>
<td>0.68 ± 0.01</td>
<td>0.06 ± 0.01</td>
<td>1.65 ± 0.1</td>
</tr>
<tr>
<td>Infected (7th day)</td>
<td>1.47 ± 0.1</td>
<td>2.09 ± 0.01</td>
<td>0.09 ± 0.001</td>
<td>1.98 ± 0.2</td>
</tr>
<tr>
<td>Treated (20th day)</td>
<td>aq.CPV 2.17 ± 0.1***</td>
<td>0.87 ± 0.01**</td>
<td>0.06 ± 0.01**</td>
<td>1.71 ± 0.02*</td>
</tr>
<tr>
<td></td>
<td>Amox 2.25 ± 0.3*</td>
<td>0.74 ± 0.1</td>
<td>0.04 ± 0.01**</td>
<td>1.66 ± 0.4***</td>
</tr>
</tbody>
</table>

Significant difference between control and treated groups: **NS Not significant; * p< 0.05; ** p< 0.01; *** p< 0.001.
The expression of THP was found to be normal in the tubular and thick ascending limb (TAL) of Henle as seen in Fig. 3.10A. On the 7th day, the levels of the THP were found to be drastically decreased in the proximal and distal tubules with dense lymphocytic infiltration due to the establishment of acute pyelonephritis. The normal expression of THP was found to be reduced in the sections obtained from the mice that were trans-urethrally infected with ECRRL36, which had evident pyelonephritis in the kidneys at the end of 7th day. This reduced expression of the protein was confirmed by the qPCR, which revealed that the $C_T$ variation (Fig. 3.10E) in the expression of the mRNA coding for THP was very minimal when compared to that of the control (Fig. 3.10H). This result also coincided with the immunoblot carried out to check for the expression of the protein in the kidney tissues, where a significant reduction in the levels of the protein were observed in the tissues obtained from the infected mice. The expression of this protein was restored to normal levels in the Amoxicillin treated animals (Fig. 3.10C) and to near-normal levels after 20 days of treatment with aq.CPV (Fig. 3.10D). This result also coincided with that of the qPCR and immunoblotting, where an increased expression in the mRNA and protein levels were observed.

The levels of the iron-coupled protein, Transferrin, which not only acts as an anti-oxidant molecule but also as the chief source of iron for the rapidly multiplying bacteria in the host were found to be expressed in the proximal tubular and distal tubular region and also in the podocytes of the glomerulus (Fig. 3.11A). The expression of this protein was found to be decreased when pyelonephritis was established in the kidneys (Fig 3.11B) when compared to the sections obtained from the control animals (Fig.3.11A). This reduced localization of the protein was confirmed by the decreased mRNA expression (Fig 3.11E) and protein expression (Fig. 3.11G) in the infected samples. Upon treatment with aq.CPV, the levels were restored close to normal in both the amoxicillin treated animals (Fig. 3.11C) and CPV treated animals (Fig. 3.11D). This was confirmed by the results obtained from the relative mRNA expression and the relative intensity of the protein expression, where the levels of the protein were found to be significantly increased upon treatment with aq.CPV for a period of 20 days.
Due to infiltration of the neutrophil's to combat infection into the tubular regions of the kidney, the levels of the neutrophil associated AMP’s Cathelicidin (Fig. 3.12B) and Lipocalin (Fig. 3.13B) were found to be increased in the sections obtained from the infected animals when compared to the respective controls. A 18 fold increase in the expression levels of the mRNA (Fig.3.12F) encoding for Cathelicidin, which correlates to its protein expression through immunobloting (Fig. 3. 12H) was noticed. Similarly, a 5-fold increase in the mRNA levels of Lipocalin (Fig.3.13F), the other AMP associated with neutrophil infiltration also coincided with the increased protein expression (Fig.3.13.H) in the infected samples. Upon treatment with aq.CPV for a period of 20 days, a reduction in the elevated levels of these proteins was brought close to normal levels (Fig. 3. 12D & Fig. 3.13D) in a significant manner.

Ficolin, a key player in the alternative pathway for the activation of cytokines and chemokines was also found to be elevated upon infection with ECRRL36 in the as noticed in the sections obtained from the infected mouse kidney as noticed in Fig.3.14B. This result was also confirmed by the elevated levels of the mRNA (Fig.3.14F) encoding for Ficolin and its corresponding protein expression (Fig.3.14H). When the infected mice were treated for a period of 20 days with aq.CPV a significant decrease in the levels of the mRNA expression and protein levels were observed as noticed by the reduction in the relative mRNA and protein expression, which was similar to the levels in the animals treated with amoxicillin.

The markers of inflammation were expressed in elevated levels in the glomerular capillaries and peritubular capillaries in the case of TNF–α (Fig. 3.15B) while NF–κβ (Fig. 3.16B) was expressed in the peritubular capillaries. Dense lymphocyte infiltration of the interstitium was a common feature noticed in both the sections as shown. The elevated levels of TNF–α were completely restored to normal in the glomerular capillaries and supported by the reduction in the mRNA levels obtained from the mouse treated with aq.CPV (Fig. 3.15F), while the levels remained elevated in the peritubular region, indicating that drug was effective in treating the induced the pyelonephritis. The levels of NF –κβ were reduced and the sections appeared normal after 20 days of treatment and were observed as a reduction in the mRNA expression of the levels (Fig.3.16F)
Figure 3.10. Localization and Expression of THP in kidney sections from animals treated with the aq. infusion of CPV

A: IHC of control section of mouse kidney treated with antibody against THP showing normal expression in the tubular and TAL regions of the kidney. B: The section obtained from ECRRL36 infected mouse, showing reduced expression of THP (shown by black arrows) in the tubular and TAL regions of the kidney. C: Kidney sections obtained from Amoxcillin (100 mg/kg body weight) treated mice showing restored levels of THP. D: Mouse kidney sections obtained from CPV (500 mg/kg body weight) treated animals showing a near normal restoration in the levels of THP in the kidney. E: The amplification plot of THP mRNA expression. F: The relative expression of the THP mRNA expression normalized to β-Actin, showing an significant increase in the levels of THP upon treatment with CPV when compared to infected samples. G: Immunoblot of THP showing reduced expression of THP in the infected sample. (C: Control; I: infected; A: Amox treated); CPV.10: 10 days after treatment with aq.CPV; CPV.20: 20 days after treatment with aq.CPV); H: Densitometric analysis for THP.
Figure 3.11. Localization and Expression of Transferrin in kidney sections from animals treated with the aq. infusion of CPV

A: IHC of control section of mouse kidney treated with antibody against Transferrin showing normal expression in the tubular regions of the kidney. B: The section obtained from ECRRL36 infected mouse, showing reduced expression of Transferrin (shown by black arrows) in the tubular regions of the kidney. C: Kidney sections obtained from Amoxillin (100 mg/ kg body weight) treated mice showing restored levels of Transferrin. D: Mouse kidney sections obtained from CPV (500 mg/ kg body weight) treated animals showing a near normal restoration in the levels of Transferrin in the kidney. E: The amplification plot of Transferrin mRNA expression. F: The relative expression of the Transferrin mRNA expression normalized to β–Actin, showing an significant increase in the levels of Transferrin upon treatment with CPV when compared to infected samples. G: Immunoblot of Transferrin showing reduced expression of Transferrin in the infected sample (C: Control; I: infected; A: Amox treated); CPV.10: 10days after treatment with aq.CPV; CPV.20: 20days after treatment with aq.CPV). H: Densitometric analysis for Transferrin.
Figure 3.12. Localization and Expression of Cathelicidin in kidney sections from animals treated with the aq. infusion of CPV

A: IHC of control section of mouse kidney treated with antibody against Cathelicidin showing no expression in the kidney. B: The section obtained from ECRRL36 infected mouse, showing increased expression of Cathelicidin (shown by black arrows) regions in the kidney. C: Kidney sections obtained from Amoxicillin (100 mg/ kg body weight) treated mice showing reduced levels of Cathelicidin. D: Mouse kidney sections obtained from CPV (500 mg/ kg body weight) treated animals showing a reduction in the levels of Cathelicidin in the kidney. E: The amplification plot of Cathelicidin mRNA expression. F: The relative expression of the Cathelicidin mRNA expression normalized to β – Actin, showing an significant decrease in the levels of Cathelicidin upon treatment with CPV when compared to infected samples. G: Immunoblot of Cathelicidin showing reduced expression of Cathelicidin in the CPV treated sample. C:Control; I: infected; A: Amox treated; CPV.10: 10days after treatment with aq.CPV; CPV.20: 20days after treatment with aq.CPV). H: Densitometric analysis for Cathelicidin.
Figure 3.13. Localization and Expression of Lipocalin in kidney sections from animals treated with the aq. infusion of CPV

A: IHC of control section of mouse kidney treated with antibody against Lipocalin showing no expression in the kidney. B: The section obtained from ECRRL36 infected mouse, showing increased expression of Lipocalin (shown by black arrows) regions in the kidney. C: Kidney sections obtained from Amoxcillin (100 mg/ kg body weight) treated mice showing reduced levels of Lipocalin. D: Mouse kidney sections obtained from CPV (500 mg/ kg body weight) treated animals showing a reduction in the levels of Lipocalin in the kidney. E: The amplification plot of Lipocalin mRNA expression. F: The relative expression of the Lipocalin mRNA expression normalized to β–Actin, showing an significant decrease in the levels of Lipocalin upon treatment with CPV when compared to infected samples. G: Immunoblot of Lipocalin showing reduced expression of Lipocalin in the CPV treated sample C:Control; I: infected; A: Amox treated); CPV.10: 10days after treatment with aq.CPV; CPV.20: 20days after treatment with aq.CPV). H: Densitometric analysis for Lipocalin.
Figure 3.14. Localization and Expression of Ficolin in kidney sections from animals treated with
the aq. infusion of CPV

A: IHC of control section of mouse kidney treated with antibody against Ficolin showing normal
expression in the kidney. B: The section obtained from ECRRL36 infected mouse, showing
decreased expression of Ficolin (shown by black arrows) regions in the kidney. C: Kidney sections
obtained from Amoxcillin (100 mg/ kg body weight) treated mice showing decreased levels of
Ficolin. D: Mouse kidney sections obtained from CPV (500 mg/ kg body weight) treated animals
showing a reduction in the levels of Ficolin in the kidney when compared to the infected samples.
E: The amplification plot of Ficolin mRNA expression. F: The relative expression of the Ficolin
mRNA expression normalized to β – Actin, showing an significant decrease in the levels of Ficolin
upon treatment with CPV when compared to infected samples. G: Immunoblot of Ficolin showing
reduced expression of Ficolin in the CPV treated sample C:Control; I: infected; A: Amox treated;
CPV.10: 10days after treatment with aq.CPV; CPV.20: 20days after treatment with aq.CPV). H:
Densitometric analysis for Ficolin.
Figure 3.15. Localization and Expression of TNF-α in kidney sections from animals treated with the aq. infusion of CPV

A: IHC of control section of mouse kidney treated with antibody against TNF-α, showing normal expression in the kidney. B: The section obtained from ECRRL36 infected mouse, showing increased expression of TNF-α (shown by black arrows) regions of the kidney. C: Kidney sections obtained from Amoxcillin (100 mg/kg body weight) treated mice showing decreased levels of TNF-α. D: Mouse kidney sections obtained from CPV (500 mg/kg body weight) treated animals showing a reduction in the elevated levels of TNF-α in the kidney when compared to the infected samples. E: The amplification plot of TNF-α mRNA expression. F: The relative expression of the TNF-α mRNA expression normalized to β-Actin levels, showing a significant decrease in the levels of TNF-α upon treatment with CPV when compared to infected samples.
Figure 3.16. Localization and Expression of NF-κβ in kidney sections from animals treated with the aq. infusion of CPV

A: IHC of control section of mouse kidney treated with antibody against NF-κβ showing normal expression in the kidney. B: The section obtained from ECRRL36 infected mouse, showing increased expression of NF-κβ (shown by black arrows) regions of the kidney. C: Kidney sections obtained from Amoxicillin (100 mg/kg body weight) treated mice showing decreased levels of NF-κβ. D: Mouse kidney sections obtained from CPV (500 mg/kg body weight) treated animals showing a reduction in the elevated levels of NF-κβ in the kidney when compared to the infected samples. E: The amplification plot of NF-κβmRNA expression. F: The relative expression of the NF-κβ mRNA expression normalized to β-Actin, showing an significant decrease in the levels of NF-κβ upon treatment with CPV when compared to infected samples.
3.4. DISCUSSION

In our study we attempted to study the effect of administering an alternative medicine Chandraprabhavati, a poly-herbal drug, commonly used in the treatment of various infections of the kidney in Siddha as a medication for UTI. Drugs made from medicinal plants contain ingredients such as dietary fibers, vitamins, minerals, antioxidants, oligosaccharides, essential fatty acids and lignins, that make them a functional food used for treating infectious diseases (Dixit et al., 2005; Kulkarni et al., 2006). The metal analysis of the drug revealed the presence of Zinc and Iron in CPV. Zinc is required in optimum concentration (0.16mM per $1.8 \times 10^8$ cfu/ml) for the growth of micro-organisms, but at higher concentrations it is cytotoxic to the bacteria. During the establishment of UTI in vivo, the bacteria scavenge the Zinc available in the host, thus causing a depletion in the environment rapidly, due to which the progression of the infection was not inhibited (Zalewaski et al., 2006). The external supplementation of Zinc, as in CPV, not only restores the depleted zinc pools in the body fluids but also acts as a cytotoxic agent to the bacteria (Magneson et al., 1987). The supplementation of iron in CPV could be utilized by the bacteria for their growth and thus prevents iron scavenging from the chelated proteins (Finkelstein et al., 1983), maintain its levels in the serum and thereby prevents anaemia in the UTI patients during pyelonephritis (Turnowsky et al., 1983; Nies, 1999). These nutrient limitations of zinc and iron are known to have effects on the composition of the bacterial envelope (Brown, 1977), changes in the antibiotic sensitivity pattern (Anwar et al., 1983), host defence mechanisms (Ombaka et al., 1983), and the bacterial virulence factor production (Bielaszewakaa et al., 2007).

Majority of the ascending UTI are due to the UPEC (Connell et al., 1996). Fimbriation of UPEC aids in the adhesion of bacterium to the bladder epithelium, and colonization of the sterile urinary tract (Schilling et al., 2002). The bacteria internalize by penetrating the lipid rafts of the urothelium and reside before they produce an infection (Duncan et al., 2004) and thereby silently evade the innate immunity of the host (Mulvey et al., 2001). At this stage the pathogens are resistant to the antibiotic regimen thus paving way for recurrent UTI in the bladder and the kidneys (Mates et al., 1999).
Rapid multiplication of UPEC in the bladder and kidney leads to lymph accumulation and inflammation. The entry and re-emergence of the UPEC from the urothelium, leads to the increased levels of ROS and RNS in the body; it also results in inflammation due to the activation of NF-κβ (Brown and London, 2000). The ROS such as superoxide, hydrogen peroxide and hydroxyl radicals act as effectors of cellular responses to kill the pathogens and in the regulation of the immune response (Bedard and Krause, 2007). In renal cell culture models, in the cells that were in contact with UPEC, an increase in the extracellular oxygen concentration coincides with the superoxide and hydrogen peroxide decomposition. It also reports an elevation in the ROS in the endoplasmic reticulum present close to the cell nuclei and mitochondria, where the physiological process such as lipid and protein synthesis occurs, when compared to the cell plasma. Assumptions also portray that variations in atmospheric CO$_2$ and pH could be a possible reason for the increase in the ROS (Zhao et al, 2010). This results in the damage of the surrounding macromolecules (Rahmann et al., 2008) and the sloughing of the infected bladder and renal epithelial cells resulting in the loss of the various glycoproteins lining the epithelial surface such as Caveolin-1 (Hawthorn and Reid, 1990), Tamm Horsfall protein (THP), Transferrin, and Lipocalin etc.

The innate immune response impedes the invasion of the UPEC in the urinary tract within minutes, by recruiting neutrophil’s and macrophages to the site of action. During inflammation, the phagocytes and macrophages work towards the elimination of the pathogen through the secretion of ROS, RNS, and electrophilic compounds (Nathan and Shiloh, 2000). These oxidants and radicals not only damage the pathogen but act as a double-edged sword damaging the surrounding host tissue, leading to DNA adduct formation and somatic mutations, that leads to apoptosis of the cell (Grisham et al., 2000).

The adhesion of LPS to the TLR on the urothelial cell surface triggers an inflammatory response (Schilling et al., 2003) that in turn activates the pro-inflammatory signal cascade leading to the activation of the transcription factor NF-κβ (Glorie et al., 2006). This in turn produces ROS through the NADPH oxidase involvement that generates superoxide, which transforms into hydrogen peroxide and
oxygen, which leach out of the cell (Bedard and Krause, 2007), leading to increase in extracellular ROS increase. The presence of P fimbriae and T1F further attenuates the production of ROS in vivo (Zhao et al, 2010).

In a healthy individual, the generation of ROS and RNS are under check by the antioxidant defence that offer different levels of protection such as prevention, interception and repair (Knight, 2000). However, an alteration in the physiochemical environmental or pathological agents shifts this delicately balanced system in favour of the pro-oxidants leading to oxidative stress (Sies, 1996). Cellular damage implicated by the oxidative stress is a hallmark feature of infectious diseases (Devasagayam et al., 2004).

This increased neutrophil presence was noticed in the pelvi-uretic section of the mice. This increased neutrophil elevation was reflected by the elevated levels of the antimicrobial proteins, Cathelicidin and Lipocalin, which act as pore formers, leading to the expulsion of the cytoplasmic contents in the bacterial cells. The increase in the levels of the Ficolin in the tissue sections during infection suggests that this protein, an activator of the alternative pathway of complement action suggests the induction of cytokines and chemokines to combat infection. Invasion of the uropathogen increases cytokine levels, due to which inflammatory receptors such as TNF-α and NF – κβ are expressed at the cell surface and lead to urothelial inflammation. This increase in the ROS and RNS has to be prevented through the administration of antioxidants.

The terpenoids present in CPV combat UTI by bringing down the inflammation. This was demonstrated by the immunostaining of the histological sections with TNF-α and NF-κβ, obtained on 10th and 20th day of treatment with the aq.CPV. The terpenoids from plant sources have been reported in earlier cases, to have the capacity to act against the E.coli responsible for oral infections (Saemann et al., 2005). This would be the first time, where the terpenoids from varied plant sources synergistically have been responsible for the reduction of infection and inflammation due to UPEC, as the number of bacteria have been shown to be reduced considerably in the sections obtained after 20 days of treatment with the aq.CPV.
The flavanoids present in aq.CPV act by quenching the ROS and RNS levels, which were increased in the urinary system upon infection and thereby reducing their damage to the lipid bi-layer and the various bio-molecules, present on the luminal wall of the tubules in the kidney. This potential antioxidant nature of the drug is evident from the increase in the THP expression, an abundant protein in the normal urine, produced and secreted by the proximal convoluted tubules. This protein has a reduced expression during the infection as indicated by the immunostaining of the kidney sections on the 7th day. Upon treatment, the levels of this protein were increased, indicating that this could be due to the antioxidant activity of the drug.

Studies in THP knockout mice for the establishment of UTI have revealed the presence of numerous bacteria in the urine, larger and swollen bladders, frequent discoloration of the kidneys with abscess formation necessitate THP for the prevention of UTI. Also bacteria have been shown to bind to renal cells through the extraneous THP, thus preventing the adherence of the type 1 pilated UPEC. This study also indicated that the binding of THP does not reduce all competitive uropathogenic adhesion, and therefore aid in bacterial colonization (Raffi et al., 2009). A third study showed bound THP with E.coli in the case of renal calculi (Vaisanen et al., 1981).

These insights suggest the probable role of THP as an AMP in preventing the UPEC from adhering to the kidney and bladder epithelia (Bates et al., 2004). This can be brought about when fimbriated UPEC binds with THP (in abundance) as they gain entry into the kidney and are removed before they can establish pyelonephritis. In our study, the infected mice were allowed to develop pyelonephritis and the sections were stained with anti-THP antibodies. THP levels were remarkably reduced in the kidney on the 7th day post infection, in corroboration with Raffi et al. (2009) study. In addition, the presence of mannose in the media effectively reduced the colonization of bacteria in the in vitro systems. This implies that THP prevents the progression of UTI by UPEC and thereby serves as a host defence molecule, due to which its levels have decreased in the renal tubular sections as shown in Fig. 3. 10B (Bates et al., 2004). This is supported by the earlier study involving the role of THP in UTI due to Proteus mirabilis, where this protein was found to be bound to the pathogen in the
immunostained sections and in the urine of the infected animals. The levels of THP were increased upon treatment for 20 days with Amoxicillin and aq.CPV (Fig. 3. 10C and Fig.3. 10D) and the levels were subsequently close to normal in the immunostained sections. This could be correlated to the protein levels present in the kidney and in the excreted urine. On the 7th day post infection, ~50% reduction in the protein levels in the infected kidney were noticed which corresponds to the increased protein levels in the urine collected from the animals. This increased protein levels returned to normal levels upon treatment, indicating that the decreased protein concentration in the kidney could be due to the increased excretion rate of THP, which calls in for an entirely new study which are out of the aims of the current study being discussed here. Also, it has to be noted here that THP cannot only be the sole protein that could be responsible for the elevation of the protein content in the urine. The other candidate proteins which are excreted out in an elevated condition have to be identified to portray the exact mechanism by which the AMP’s present in the kidney ward-off an UPEC mediated infection. Also, more studies are warranted to reveal the exact mechanism of how THP interacts with the bacteria and thus prevents the progression of infection.

In addition, the use of this drug as an alternative to the existing chemical analogues is recommended based on the fact that this drug is capable of increasing, the in vivo antioxidant enzyme levels such as superoxide dismutase and catalase levels, in order to reduce the inflammation caused due to the presence of microbes. Also the generated nitrite radicals and decreased protein content of the kidney, due to infection were brought to normal upon treatment with the drug.

4.5. CONCLUSION

Our study explored the possible role of THP as an antimicrobial protein and the use of aq.CPV as a potential source of antioxidants and antimicrobial agent that can be effectively used to remove the invading and persisting bacterial population in the bladder and kidney of the experimental animal models. The reduction in the inflammatory markers TNF-α and NF-κβ shows that CPV is a potential anti-inflammatory drug. Besides, the restoration of the THP to its normal level concludes that the drug is capable of reducing the pathological changes caused by UTI. From the
findings of the present study, we can thus conclude that CPV could effectively serve as an alternative treatment to combat UTI when compared to the costly synthetic analogues. The exact mechanism of action for the drug against UTI and the role of THP during UTI have to be elucidated.