Chapter 5

Combinatorial Effect of Chandraprabhavati and *Ellettaria cardamom*

5.1. INTRODUCTION

Antimicrobial therapy for bacterial infections have become ineffective due to the increasing antimicrobial resistance aided by the widespread participation of biofilms by the pathogen involved leading to an increase in secondary complications and infections (Budzynska et al. 2011). This has propelled research in the quest for identifying new drug targets from varied sources such as plant parts, marine sources and food (Cushnie and Lamb, 2011). The nature is kind enough to provide us with a good source of these substances, which can possibly be an alternative to the existing treatment strategies (Alviano and Alviano, 2009).

Various phyto-compounds reported till date are known to effect diverse pharmacological effects such as antioxidant (Rozalski et al, 2013), anti-allergic, antitumorogenic, anti-inflammatory (Rosch et al, 2003), diuretic, anti-helminthic, anti-parasitic, antimicrobial (Tegos et al, 2002), etc. They act by restricting the expression of microbial virulence factors (Gauwerky et al, 2009), preventing the adhesion and aggregation of the pathogen (Kurlenda and Grinholc, 2012) or activating the host immune defence mechanisms (Kuzma et al, 2012) to combat the infections. However, the synergistic role of these target phyto-compounds as possible antibacterial agents have not been explored although they have been widely used as folklore or traditional medicine systems (Budzynska et al 2011).

The Siddha medicine, Chandraprabhavati and the Benz-c-Acridine rich extract prepared from *Elletaria cardamomum* were individually established successfully to be bacteristatic and bactericidal in nature respectively. This was due to the presence of the phytoalexins or the secondary metabolites such as flavanoids and the terpenoids, which were present in both the infusion and the compound prepared, which were capable of eliciting anti-inflammatory, anti-bacterial effect against ECRRL36. They were also capable of repairing the tissue damage caused as a result of infection in the
experimental animal models, thereby demonstrating that they could possess an extremely low risk of giving rise to resistance mechanisms.

Therefore, as these properties of the individual drugs were proven successfully, we proposed to study the synergistic effect of Chandraprabhavati and the Benz-c-Acrilide rich extract of Ellettaria cardamomum at their proven antimicrobial concentrations to use the possible enhanced combination as an alternative treatment for UTIs due to UPEC in experimentally induced mouse models of pyelonephritis.

5.2. MATERIALS AND METHODS

5.2.1. Chemicals and reagents

All chemicals and reagents used in this study were procured as mentioned earlier in 3.2.1.

5.2.2. Combined extract preparation and phytochemical analysis

The aqueous infusion of Chandraprabhavati (500 mg/ kg body weight) and the methanolic extract of the Cardamom seed (150 mg/ kg body weight) and pods (180 mg/ kg body weight) that were prepared separately were mixed at their optimum antimicrobial concentration to prepare the combined extracts.

5.2.3. In vitro antimicrobial growth kinetics study

The strains mentioned in section 3.3.5. were utilized in this study. 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).

The antibacterial activity of the phyto-compound rich fractions of CPV + C. Seed and CPV + C. Pod were assessed by tube dilution method by adding them to LB broth. To the broth containing the drug, 100 μl of McFarland matched 4h culture of standard strain or ECRRL36 were added and mixed well. The tubes were incubated at 37 °C for 24 h at static conditions and the MIC and MBC of the drug were determined
using the growth curve analysis of the obtained by estimating the OD$_{600}$ of the cultures. Amoxicillin, a synthetic analogue, which acts by inhibiting the bacterial cell wall synthesis, at 1000 mg/kg body weight to which the strain under study was susceptible was chosen as the standard drug for the study. The MBC of the extracts was used as the treatment concentration to validate the effect of the drug in vivo. The CPV + C.Seed and CPV + C.Pod extracts were spiked with varying concentrations (0 – 50 ppm) of metals – Copper, Iron and Zinc to mimic the metals present in the drug and the Cardamom fruits and to generate an metal overload model, and the antibacterial effect of the drugs were analyzed against ECRRL36 in LB broth.

5.2.4. Initiation of infection in mice, groups and treatment

Female Swiss Albino mouse (8 – 10 weeks) weighing 25 ± 2 g were infected with ECRRL36 as per the protocol outlined in 3.2.7. After inoculation, the catheter were removed and the animal was monitored regularly for any discomfort, injury or inflammation due to the procedure (Hung et al., 2009). The infected animals were separated into four groups (infection control, Amoxicillin treated animals (100 mg/kg body weight) CPV + C.Seed treated animals and CPV + C.Pod 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 animals. Bladders and kidneys were harvested from the control animals prior to the infection and the infected animals were sacrificed at 6 h, 7th day, and on the 10th day and 20th day after treatment with CP and CS extracts. The body weights of the animals were monitored at regular intervals. The harvested tissues were rinsed thoroughly using PBS (pH 7.4) and processed for biochemical studies, while a portion was stored in RNAlater for the isolation of mRNA; a portion of the rinsed tissue was stored in 10% NBF at RT for histopathological analysis.

5.2.5. Biochemical parameters

Protein (Bradford, 1976), nitrite (Guevara et al., 1998), pH were determined by indicator strips and 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 nitrite content (Guevara et al., 1998).

5.2.6. Histopathological analysis of the kidney

The 10% NBF fixed tissues were processed for paraffin embedment and 4μm tissues sections were stained with haematoxylin and eosin. 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 at 400X magnification.

5.2.7. Immunohistostaining for antimicrobial proteins and inflammatory markers

Paraffin sections (4 μm thick) 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), Ficolin (1:2500), 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 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.

5.2.8. RT PCR to analyze the m-RNA expression of antimicrobial proteins and inflammatory markers

The tissue samples stored in RNALater were blotted dry and processed as outlined in 3.2.11. The isolated mRNA was quantified and converted into cDNA using the Verso cDNA synthesis kit. The 1:4 diluted cDNA was used for analyzing the m-RNA expression of the various antimicrobial proteins and inflammatory markers as mentioned in the protocol in section 3.2.11.
5.2.10. Protein expression analysis using western blot

The protein expression analysis of the various AMP’s was carried out as mentioned in 3.2.12.

5.2.13. Statistical analysis

Data were represented as mean ± standard error of mean (SEM). 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.

5.3. RESULTS

Chandraprabhavati a polyherbal drug was proven earlier by us a possible treatment that was capable of modulating the immune system and bring about a reduction in the number of bacteria in a pyelonephritic model. Cardamom is a spice, which finds its way as a spice in various cuisines and its extracts are widely utilized for treating ailments in the traditional medicinal systems followed in India and in other countries such as China, Turkey etc. As established in our previous chapter the bactericidal and anti-inflammatory property of Benz-c-Acridine rich extracts were proven against UPEC induced pyelonephritis mouse models. This study focussed on evaluating the effect of the extracts of Chandraprabhavati when spiked with Cardamom on controlling and treating infections of the kidney using the pathogen ECRRL36.

The antimicrobial property of the two drugs were analyzed, it was noticed that CPV (500 mg/ kg body weight) spiked with C.Seed extracts (150 mg/ kg body weight) (CPV + CS) showed a better bactericidal potential when compared to that of CPV spiked with C.Pod extracts (180 mg/ kg body weight) (CPV + CP) (Fig. 5.1). Since the extracts were known to be enriched with metals, as they are prepared from various parts of the plants, the three common metals that were identified during the metal analysis of the CPV and Cardamom extracts were supplemented in varying concentrations, to enhance in their antibacterial potential. Copper at 5 ppm inhibited growth of the ECRRL36 when mixed with CPV + CS and CPV + CP (Table 5.1).
When CPV + CS and CPV + CP were mixed with varying concentrations of iron, it was noticed that iron at 20 ppm was able to effectively inhibit the growth of ECRRL36. In the zinc, overload mechanism developed CPV + C.Seed inhibited the growth of ECRRL36 at 20 ppm while CPV + C.Seed inhibited the growth of ECRRL36 at 40 ppm. The 24h supernatant from each of mentioned metal containing dilution tube was sub-cultured on LB plates at 37 °C and it was noticed that the extracts showed a bactericidal property (Fig. 5.2). A decrease in the body weight of the animals (Table 5.2) infected with ECRRL36 was evident on the 7th day post inoculation. In addition, a decrease in the pH of the urine was 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 in the body weight of the mice treated with the CPV + C.Seed were restored close to normal and CPV + C.Pod extracts there was a reduction in the body weight. The alteration in the urinary pH had marginally increased although it was not restored close to normal in the animals treated with CPV + C.Pod extracts. An increase in the urinary leukocyte and nitrite levels were noticed in the urine of the infected animals, which were
lowered to the normal levels in the case of the animals treated with CPV + C.Seed, however in the case of the animals treated with CPV + C.Pod this change was not restored to normal.

<table>
<thead>
<tr>
<th>ppm</th>
<th>Copper</th>
<th>Iron</th>
<th>Zinc</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CPV + C.Seed</td>
<td>CPV + C.Pod</td>
<td>CPV + C.Seed</td>
</tr>
<tr>
<td>0</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>15</td>
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<td>40</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>45</td>
<td>+</td>
<td>+</td>
<td>+</td>
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</tbody>
</table>

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

Protein quantification on infected and control kidney homogenates confirmed that 50 fold decrease in protein content infected mice relative to control. Upon treatment for 20 days with the specified dosage of CPV + C.Seed extracts, the protein concentration significantly increased compared to the infected mice. The increase was significant when compared to that of the control. However, in the CPV + C.Pod treated animals there was a increase in the urinary protein levels on the 20th day, after the initial decrease noticed on the 10th day. The levels of the various antioxidant enzymes catalase, nitrite, and superoxide dismutase were elevated in the infected animals when compared to the control. This indicates the probable damage inflicted on the tissue architecture of the renal epithelium because of the entry and multiplication of the pathogen and the subsequent neutrophil infiltration. Upon treatment, a considerable change was noticed in the animals treated with C.Seed extracts, however a similar effect was not noticed in the animals treated with C.Pod extracts after 20 days of treatment (Table 5.3).
Table 5.2. Urinary and biochemical parameters in the animals after treatment with combined extracts

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Infected</th>
<th>Amox</th>
<th>CPV + C.Seed 10th day</th>
<th>CPV + C.Seed 20th day</th>
<th>CPV + C.Pod 10th day</th>
<th>CPV + C.Pod 20th day</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body wt (g)</strong></td>
<td>30.5 ± 0.7</td>
<td>26.3 ± 1.2</td>
<td>29.8 ± 0.1</td>
<td>27.5 ± 0.7*</td>
<td>29.2 ± 1.3**</td>
<td>27.8 ± 1.8*</td>
<td>26.5 ± 1.3</td>
</tr>
<tr>
<td><strong>Kidney wt (g)</strong></td>
<td>1.2 ± 0.1</td>
<td>1.4 ± 0.1</td>
<td>1.1 ± 0.05</td>
<td>0.9 ± 0.0*</td>
<td>0.9 ± 0.1*</td>
<td>1.5 ± 0.0*</td>
<td>1.5 ± 0.0*</td>
</tr>
<tr>
<td><strong>pH (urine)</strong></td>
<td>5.7 ± 0.01</td>
<td>5.0 ± 0.02</td>
<td>5.7 ± 0.01</td>
<td>5.4 ± 0.1</td>
<td>5.6 ± 0.1</td>
<td>5.3 ± 0.0</td>
<td>5.5 ± 0.2</td>
</tr>
<tr>
<td><strong>No. of leukocytes</strong></td>
<td>0</td>
<td>125 ± 5</td>
<td>10 ± 2</td>
<td>25 ± 4**</td>
<td>12 ± 1***</td>
<td>45 ± 3*</td>
<td>34 ± 1**</td>
</tr>
<tr>
<td><strong>Protein (mg/g tissue)</strong></td>
<td>2.83 ± 0.2</td>
<td>1.97 ± 0.1</td>
<td>2.3 ± 0.3*</td>
<td>2.1 ± 0.03</td>
<td>2.2 ± 0.1**</td>
<td>1.6 ± 0.1</td>
<td>2.1 ± 0.5*</td>
</tr>
<tr>
<td><strong>Nitrite (µg/µL)</strong></td>
<td>0.02 ± 0.01</td>
<td>0.09 ± 0.0</td>
<td>0.04 ± 0.0*</td>
<td>0.06 ± 0.02**</td>
<td>0.06 ± 0.0*</td>
<td>0.08 ± 0.0**</td>
<td>0.05 ± 0.01*</td>
</tr>
<tr>
<td><strong>Catalase (units/ mg protein)</strong></td>
<td>0.78 ± 0.01</td>
<td>2.09 ± 0.01</td>
<td>0.74 ± 0.01</td>
<td>1.7 ± 0.1**</td>
<td>1.2 ± 0.1*</td>
<td>1.9 ± 0.1*</td>
<td>1.7 ± 0.2</td>
</tr>
<tr>
<td><strong>Super oxide Dismutase (µmol/min/mg protein)</strong></td>
<td>1.65 ±0.1</td>
<td>2.38 ±0.2</td>
<td>1.66 ± 0.2*</td>
<td>2.1 ± 0.06</td>
<td>1.8 ± 0.06</td>
<td>2.2 ± 0.1</td>
<td>2.1 ± 0.02</td>
</tr>
</tbody>
</table>

CPV (500 mg/kg body weight) + C Seed (150 mg/kg body weight); CPV (500 mg/kg body weight) + C Pod (180 mg/kg body weight). Values are expressed as mean ± standard deviation of the samples (n=6). Significant difference between the infected and the treated groups: *** p< 0.001; ** p< 0.01; * p< 0.05. ^ unit/mg of protein; Significant difference between control and treated groups: NS Not significant; * p< 0.05; ** p< 0.01; *** p< 0.001. ^ unit/mg of protein; Significant difference between control and treated groups: NS Not significant; * p< 0.05; ** p< 0.01; *** p< 0.001.

Control (0th) and the infected mouse kidney sections (H&E) 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 (Fig. 5.2). Dense infiltration by the lymphocytes and plasma cells, with disruption of the urothelium was noticed. Upon treatment with CPV + C.Seed extracts for 20 days, no evidence of pyelonephritis was noticed. The glomerular, interstitial and vascular compartments were within the normal limits as
seen in Fig. 5.2C. In the sections obtained from the animals treated with CPV + C.Pod extracts, there was a reduction in the podocyte architecture was not restored close to normal with an increase in the Bowman’s space. From the sections visible vacuolation was noticed in the proximal and distal tubular regions although the inflammation noticed in the infection tissue sections had subsided.

**Figure 5.2. H&E stained sections obtained from the mice treated with combined extracts**

A: Kidney section from control animal. B: Kidney section from infected animal. C: Kidney section from animals treated with CPV + C.Seed extracts. D: H&E stained sections of the kidney from animals treated with CPV + C.Pod extracts.

On the 7th day, the levels of the Tamm Horsfall protein were found to be drastically decreased in the proximal and distal tubules and the thick ascending loop of Henle’s, with dense lymphocytic infiltration due to the establishment of acute pyelonephritis. The expression of this protein was restored close to normal levels after 20 days of treatment with the CPV + C.Seed extract, however in the animals treated with CPV + C.Pod extract, there was a reduction in the levels of the THP expression after treatment with the C.Pod extract for 20 days, indicating that there could be possible damage to the glomerulus and the tubular region (Fig. 5.3B) and this was evident from the gene expression (Fig. 5.3D) and immunoblotting against the protein (Fig. 5.3F).

The levels of the iron-coupled protein, Transferrin, which acts as an antioxidant molecule in this case, were found to be decreased when pyelonephritis...
was established in the kidneys. Upon treatment with CPV + C.Seed the levels were restored to normal after 20 days, however due the damage due to further treatment with the CPV + C.Pod extracts, the levels of transferrin were not restored close to normal in the kidney sections (Fig. 5.4). A similar result was observed from the relative expression carried out to evaluate the mRNA encoding for the protein (Fig. 5.4D) and was substantiated by the results of the immunoblotting (Fig. 5.4F).

Due to infiltration of the neutrophil's to combat infection, the levels of the antimicrobial proteins Cathelicidin (Fig.5.5) and Lipocalin (Fig. 5.6) were also found to be increased in the sections obtained from the infection controls and were supported from the relative data relative mRNA expression and immunoblotting. Upon treatment, there was a reduction in the levels of these antimicrobial proteins in the sections obtained from the animals treated with CPV + C.Seed extracts. Such a evident change was not observed in the sections from the animals treated with CPV + C.Pod extracts. Ficolin (Fig. 5.7), a key player in the alternative pathway for the activation of cytokines and chemokines was also found to be decreased upon treatment with C.Seed extracts for a period of 20 days. However, in the animals treated with CPV + C.Pod an increase in the levels of Ficolin gene expression (Fig.5.7E) and protein expression (Fig.5.7F) were noticed

The markers of inflammation were expressed in elevated levels in the glomerular capillaries and peritubular capillaries in the case of TNF–α (Fig. 5.8) while NF–κβ (Fig.5.9) 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, while the levels remained elevated in the peritubular region, indicating that drug was effective in treating the induced pyelonephritis in the of the animals treated with CPV + C.Seed extracts. However, the reduction in the levels of the inflammatory markers was not brought to normal in the case of the animals treated with CPV + C.Pod extracts. A further increase in their levels were noticed on the 20th day when compared to the 20th day post treatment indicating that there could be inflammation either from the drug or due to recurrent infection as supported by the relative gene (Fig.5.8D & Fig.5.9D) and protein (Fig.5.8E & Fig.5.9E) expression.
Figure 5.3. Localization and Expression of Tamn Horsfall protein in kidney sections from animals treated with the combined extracts

A: IHC of CPV + C.Seed treated sections of mouse kidney treated with antibody against THP showing normal expression in the tubular and TAL regions of the kidney. B: Kidney sections obtained from mouse treated with CPV + C.Pod, showing reduced expression of THP (shown by black arrows) in the tubular and TAL regions of the kidney. C: The amplification plot of THP mRNA expression. D: The relative expression of the THP mRNA expression normalized to β–Actin levels, showing a significant increase in the levels of THP upon treatment with CPV + C.Seed and CPV + C.Pod when compared to infected samples. E: Immunoblot of THP showing reduced expression of THP in the infected sample (C: Control; I: infected; A: Amox; CPV+C.Seed.10: Combined extract treated for 10 days; CPV+C.Seed.20: treated for 20 days; CPV+C.Pod.10: treated for 10 days; CPV+C.Pod: Treated for 20 days). F: Densitometric analysis for THP.
Figure 5.4. Localization and Expression of Transferrin in kidney sections from animals treated with the combined extracts

A: IHC of CPV + C.Seed treated section of mouse kidney treated with antibody against Transferrin showing normal expression in the tubular regions of the kidney. B: The section obtained CPV + C.Pod treated mouse, showing reduced expression of Transferrin (shown by black arrows) in the tubular regions of the kidney. C: The amplification plot of Transferrin mRNA expression. D: The relative expression of the Transferrin mRNA expression normalized to β – Actin levels, showing an significant increase in the levels of Transferrin upon treatment with CPV + C.Seed and CPV + C.Pod when compared to infected samples. E: Immunoblot of Transferrin showing reduced expression of Transferrin in the infected sample (C: Control; I: infected; A: Amox; CPV+C.Seed.10: Combined extract treated for 10 days; CPV+C.Seed.20: treated for 20 days; CPV+C.Pod.10: treated for 10 days; CPV+C.Pod: Treated for 20 days). F: Densitometric analysis for Transferrin.
Figure 5.5. Localization and Expression of Cathelicidin in kidney sections from animals treated with the combined extracts

A: IHC of CPV + C.Seed treated section of mouse kidney treated with antibody against Cathelicidin showing no expression in the kidney. B: The section obtained from CPV + C.Pod treated mouse, showing increased expression of Cathelicidin (shown by black arrows) regions of the kidney. C: The amplification plot of Cathelicidin mRNA expression. D: The relative expression of the Cathelicidin mRNA expression normalized to β–Actin levels, showing a significant decrease in the levels of Cathelicidin upon treatment with CPV + C.Seed when compared to CPV + C.Pod samples. E: Immunoblot of Cathelicidin showing reduced expression of Cathelicidin in the CPV + C.Seed and CPV + C.Pod treated sample (C: Control; I: infected; A: Amox; CPV+C.Seed.10: Combined extract treated for 10 days; CPV+C.Seed.20: treated for 20 days; CPV+C.Pod.10: treated for 10 days; CPV+C.Pod: Treated for 20 days). F: Densitometric analysis for Cathelicidin.
Figure 5.6. Localization and Expression of Lipocalin in kidney sections from animals treated with the combined extracts

A: IHC of CPV + C.Seed section of mouse kidney treated with antibody against Lipocalin showing normal expression in the kidney. B: The section obtained from CPV + C.Pod showing increased expression of Lipocalin (shown by black arrows) regions of the kidney. C: The amplification plot of Lipocalin mRNA expression. D: The relative expression of the Lipocalin mRNA expression normalized to $\beta$-Actin levels, showing a significant decrease in the levels of Lipocalin upon treatment with CPV + C.Seed and CPV + C.Pod when compared to infected samples. E: Immunoblot of Lipocalin showing reduced expression of Lipocalin in the CPV + C.Seed and CPV + C.Pod treated sample (C: Control; I: infected; A: Amox; CPV+C.Seed.10: Combined extract treated for 10 days; CPV+C.Seed.20: treated for 20 days; CPV+C.Pod.10: treated for 10 days; CPV+C.Pod: Treated for 20 days). F: Densitometric analysis for Lipocalin.
Figure 5.7. Localization and Expression of Ficolin in kidney sections from animals treated with the combined extracts

A: IHC of CPV + C.Seed section of mouse kidney treated with antibody against Ficolin showing normal expression in the kidney. B: The section obtained from CPV + C.Pod showing increased expression of Ficolin (shown by black arrows) regions of the kidney. C: The amplification plot of Ficolin mRNA expression. D: The relative expression of the Ficolin mRNA expression normalized to β–Actin levels, showing a significant decrease in the levels of Ficolin upon treatment with CPV + C.Seed and CPV + C.Pod when compared to infected samples. E: Immunoblot of Ficolin showing reduced expression of Ficolin in the CPV + C.Seed and CPV + C.Pod treated sample (C: Control; I: infected; A: Amox; CPV+C.Seed.10: Combined extract treated for 10 days; CPV+C.Seed.20: treated for 20 days; CPV+C.Pod.10: treated for 10 days; CPV+C.Pod: Treated for 20 days). F: Densitometric analysis for Ficolin.
Figure 5.8. Localization and Expression of TNF - α in kidney sections from animals treated with the combined extracts

A: IHC of CPV + C.Seed treated section of mouse kidney treated with antibody against TNF - α showing normal expression in the kidney. B: Kidney sections obtained from CPV + C.Pod treated mice showing decreased levels of TNF - α. C: The amplification plot of TNF - α mRNA expression. D: The relative expression of the TNF - α mRNA expression normalized to β – Actin levels, showing an significant decrease in the levels of TNF - α upon treatment with CPV + C.Seed and CPV + C.Pod when compared to infected samples.
Figure 5.9. Localization and Expression of NF-κβ in kidney sections from animals treated with the combined extracts.

A: IHC of CPV + C.Seed treated section of mouse kidney treated with antibody against NF-κβ showing normal expression in the kidney. B: Mouse kidney sections obtained from CPV + C.Pod treated animals showing a reduction in the levels of NF-κβ in the kidney when compared to the infected samples. C: The amplification plot of NF-κβ mRNA expression. D: The relative expression of the NF-κβ mRNA expression normalized to β-Actin levels, showing a significant decrease in the levels of NF-κβ upon treatment with CPV + C.Seed and CPV + C.Pod when compared to infected samples.
5.4. DISCUSSION

Infectious diseases are the primary cause of premature deaths across the world and are responsible for approximately 50,000 deaths every day. The alarming increase in antibiotic resistant bacteria, with a high percentage of recurrence in the infection has created a bottle-neck in the treatment of infectious diseases thereby shrinking the number of drugs used against these multi-drug resistant pathogens (WHO, 2013). Gram-negative bacteria such as *E. coli, Shigella, H. Influenza* are rapidly becoming multi – drug resistant to the currently available third generation cephalosporins and other available drugs (Shioto et al, 2000), thereby increasing the pressure to identify novel drug targets from nature and traditional medicines (Yam et al, 1998). Indian traditional medicines rely heavily on plants for their medicinal properties and utilize about 2400 of the plant species available in India for the preparation of the various drugs used in Siddha, Ayurvedha and Unani forms of treatment (Chaudhary, 1996). Although the phyto-compounds have been routinely used in the treatment of infections, their functional role against the pathogen, their mechanistic approach when administered in combination and how they elicit the reaction are less explored (Palombo and Semple, 2002).

As the choices of drugs used against the urinary pathogens are shrinking, we identified CPV, a widely utilized poly herbal preparation to be an alternative treatment for UTIs. This drug was identified to be non-toxic and bacteristatic in action against the uropathogen ECRRL36 when administered to pyelonephritic mice for a period of 20 days (Christa et al, 2012). Similarly, Bezn-c-Acridence rich extracts from Ellettaria cardamomum were identified to be bactericidal against ECRRL36. When these extracts were administered in combination to pyelonephritic mice, it was noticed, that there was a significant change in the urinary, biochemical and histological parameters in the mice that were treated with the extract that was prepared with CPV + C.Seed, when compared to the mice that were treated with CPV + C.Pod, indicating that the phyto-compound rich extracts could have acted in a synergetic interaction in the CPV + C.Seed extracts (Aqil et al, 2005).

It was evident from the established animal model that there was an elevation in the urinary leukocyte level, nitrite levels, when pyelonephritis was established in
the bladder and the kidneys of the animals. It was also reflected by a decrease in the urinary pH and an elevated proteinuria in the infected animals as a result of failure in the re-absorption of protein in the tubular regions of the kidney. This was supported by the destruction in the glomerular architecture, inflammation and oedema of the tubular region of the kidney (Fig.3.7B). Biochemically, this was reflected as an increase in the levels of the antioxidant enzymes such as Catalase, Superoxide dismutase, nitrite which indicated that the levels of the ROS and RNS species could have been elevated due to the rapid multiplication, emergence and re-entry of the pathogens into the renal tissues.

The loss of kidney specific proteins from the renal milieu, which could have been lost either due to the damage in the lipid-bilayer membranes of the tubules or due to failure in reabortion in the tubular region due to their loss or due to the binding of these proteins specific to the kidney to the bacteria and thereby getting removed in the urine to prevent further damage to the renal architecture. This was demonstrated in the case of THP, a protein present exclusively in the TAL of the kidney and the tubular region. When a prominent infection was established in the kidney it was noticed that there was a reduction in the levels of the protein (Fig 5.3.C Lane 2). This was confirmed by the decreased mRNA expression of the gene encoding THP and by immunoblotting. This decrease could have been possible because this protein is of secretory nature and it could have bound with the multiplying bacteria in its secretory form, and subsequently removed from the system through the formed urine. This hypothesis was proved in the experimental mouse model generated with the help of P. mirabilis, where the presence of THP bound to the pathogen was found through co-localization technique in the urine of the infected mice. When the animals were treated with CPV + C.Seed extracts it was noticed that there was a restoration in the levels of THP in the kidney sections as seen in the relative mRNA and protein expression. However, this change was not noticed in the case of the animals treated with CPV + C.Pod.

To combat the infections, the neutrophil's and the macrophages are rapidly recruited to the site of infection. This was reflected by the increased levels of the neutrophil associated proteins, viz. Cathelicidin and Lipocalin in the infected tissues.
Upon treatment with CPV + C.Seed, a significant decrease in the levels of these proteins was noticed in the kidney of the animals and was supported by the reduced mRNA and protein levels. However due to the damage to the tubular architecture and the podocytes of the glomerulus, the levels of Cathelicidin and Lipocalin, were found to be slightly elevated, but were found to be not as high as those in the infected samples. It was also noticed that the CPV + C.Seed extract was better in altering the levels of Ficolin and the inflammatory markers.

The CPV + C.Seed extract could have probably shown a better synergistic pattern that the CPV + C.Pod extracts probably due to the interaction between the various polyphenols present in the extracts in the form of flavanoids and terpenoids (Rafi and Shahverdi, 2007), where the compounds would have enhanced the other compounds activity, and could have aided in better antibiotic penetration and thereby reducing the resistance of the bacteria to the drug molecule (Bakkali et al, 2008). Therefore the cumulative effect of phyto-compound would have occurred on the bacterial cell membrane depending on its concentration, dissolving capability, rate of diffusion, salt formation capabilities, the microbial inoculum in the tissue (McMahon et al, 2007). The lipophilicity of the compound could have also aided in its ability to cross the cell wall and the cytoplasmic membrane of the bacteria as the activity of the phyto-compound is based on its ability to permeate the membranes, leading to loss in the ion pool of the pathogen, leakage of macromolecules, reduction of potential across the bacterial membrane and its fluidity, decrease in the ATP pool and finally to osmotic lysis and eventually death of the pathogen (Bakkali et al, 2008 Alviano and Alviano, 2009; Cushnie and Lamb, 2011; Martos et al, 2013).

The variation in the antimicrobial property of the two extracts could have also been to the variation in their pro-oxidative properties, which could have lead to the accumulation of the ROS inside the renal cells causing the oxygenation of the macromolecules, followed by metabolic disturbance and finally loss of the tissue. This dual effect of the phyto-compounds was pointed out in the case of the flavanoids such as quercitin, rutin and epigallocatechin, which have been proven to be able to inhibit DNA synthesis or reduce energy metabolism, which at the same time cause a reduction in the macromolecule synthesis (Gibbons, 2008). Therefore, it could be
assumed that the phyto-compounds when administered as antimicrobial agents could vary in their site of action and nature of action and the cytotoxicity they elicit depending on their concentration and bioavailability at the site of action, which exists as a thin line, difficult to differentiate (Muller and Kramer, 2008).

5.5. CONCLUSION

Through this study we explored the possibility of enhancing the activity of the Chandraprabhavati, a Siddha drug with the Cardamom extracts. It was possible with the Cardamom seed extracts and the resultant mixture elicited a bactericidal nature by acting together in a synergistic manner. However, in the case of the Cardamom pod extracts mixed with Chandraprabhavati, a mild toxicity was observed in the animals, which could have been due to the interaction of the phyto-compounds in the extract. The future directions, would be to extract the individual compounds and study which was responsible for the bactericidal nature and study how they bring about the changes.