Chapter 4

Bactericidal activity of *Elletaria cardamom*

4.1. INTRODUCTION

Cardamom or *Ellettaria cardamomum*, popularly known as the ‘queen of spices’ is a herbaceous plant belonging to the Zingerberaceae family (Kapoor, 2000). This natural resource finds wide utilization as a culinary ingredient and as a natural drug for the treatment of peptic ulcer due to its gastro-protective effect, cures stomach aches, cardiovascular and neuronal disorders (Ravindran, 2002). The fruits have a warm, slightly pungent and highly aromatic flavour due to which they find wide uses as an oil, in the making of perfumes, soaps, and detergents for its soothing properties (Dorman, 2000). It finds use in food industry as a preservative, as a flavourant in liquor and pharmaceutical industry. The phenolic compounds present in the hydro-distilled extract of this plant act as a potent insecticide (Zheng and Wang, 2001). As a spice, cardamom finds an array of uses in the preparation of curry, coffee and desserts (Ravindran, 2002).

In medicine, it finds varied uses due to the presence of volatile oils, tannins, alkaloids and lipids in the extracts, which render its antimicrobial characteristics against various pathogens, its powerful aromatic, antiseptic, stimulant, carminative, stomachic, expectorant, anti-spasmodic, diuretic properties, and finds widespread use due to its toxic, mutagenic and carcinogenic properties (Ferrira and Ferrao Vargas, 1999). In the traditional medicine, the fruits are used as a digestive aid and for the treatment of dental caries, periodontal disease associated with oral bacteria due to its antibacterial properties against most gram-negative pathogens (Mahady et al., 2005).

The aim of this work was to determine the mode of the antibacterial effect of *E. cardamomum* on UPEC as a potential antibacterial drug, and to study the impact of the active principles of this drug on the metabolic, inflammatory markers, and biochemical parameters, and to check regulation of antimicrobial proteins during the management of pyelonephritis in *Swiss albino* mice.
4.2. MATERIALS AND METHODS

4.2.1. Chemicals and reagents

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

4.2.2. Cardamom: extract preparation and phytochemical analysis

Cardamom fruits were procured from the local market and was validated to be *Elletaria cardamomum*. The fruits were separated into pod and seeds. The methanolic and acetone extract of the pods and seeds were prepared individually using 50 g of each to analyse the flavanoid and terpenoid rich fractions in the extracts. The fractions were confirmed using HP-TLC and further analyzed using RP-HPLC following the protocol mentioned in 3.2.4. In addition, the GC-MS analysis of the extracts was carried out to identify the major phyto - compound present in the extracts.

4.2.3. Metal analysis in the cardamom extracts

The methanolic extract of cardamom pod (C.Pod) and cardamom seed (C.Seed) was used to analyze the heavy metals present in the extracts using Atomic Absorption Spectroscopy (VARIAN, AA240).

4.2.4. 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 terpenoid rich fractions of C.Pod and C.Seed were assessed by tube dilution method using varying concentrations of the extracts (0 – 1000 mg/ml) in LB broth. To the broth containing the drug, 100 μl of McFarland matched 4 h 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 OD600 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. The MBC of the extracts was used as the treatment concentration to validate the effect of the drug in vivo.

4.2.5. Study design

Each 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 was removed and the animal was monitored regularly for any discomfort, injury or inflammation due to the procedure (Hung et al., 2009). After successful infection, the mouse were separated into four groups (infection control, Amoxicillin treated animals (100 mg/kg body weight), C.Pod treated animals (150 mg/kg body weight) and C.Seed treated animals (180 mg/kg body weight)) 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. Urinary bladder 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 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; a portion was stored in RNAlater for the isolation of mRNA and a portion of the rinsed tissue was stored in 10% NBF at RT for histopathological analysis.

4.2.6. 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).
4.2.7. 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.

4.2.8. Immunohistostaining for antimicrobial proteins

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), 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.

4.2.9. Immunostaining for 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 thrice with PBS containing 0.1% polysorbate 20, the slides were blotted dry of moisture and incubated overnight with the primary antibody for TNF –α (1:200) and 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.
4.2.10. qPCR 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 AMP and inflammatory markers as mentioned in the protocol in section 3.2.11.

4.2.11. Protein expression analysis using western blot

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

4.2.13. 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.

4.3. RESULTS

Cardamom is a wonder spice widely utilized as a part of various cuisines and in the traditional medicinal systems followed in India and in other countries such as China, Turkey etc. Our study focussed on evaluating the effect of the extracts of Cardamom on controlling and treating infections of the kidney using the pathogen ECRRL36. The methanolic extract of the drug revealed the presence of various flavanoids while acetone extract contained terpenoid rich fractions as revealed by preliminary phytochemical analysis followed by confirmation using HP-TLC on the C.Seed (Fig. 4.1A) and C.Pod (Fig. 4.1B). The RP – HPLC analysis was carried out to confirm the various flavanoids (Fig. 4.2) and terpenoids (Fig. 4.3) present in the seed and pod extracts of Cardamom. From the GC – MS data, the major component present in the C.Seed and C.Pod extracts was identified to be Benz-c-Acridine (Fig. 3, 4).
The antioxidant profile of C.Pod and C.Seed was carried out up to 0.25 mg/ml concentration. C.Pod scavenged 89% of the free radicals produced at 0.15 mg/ml, while C.Seed 27% of the free radicals at 0.15 mg/ml concentration. The reducing power activity was appreciable in the C.Pod extract at 0.15 mg/ml concentration. C.Pod extract showed 93% lipid peroxide inhibition while at 0.05 mg/ml concentration C.Seed showed a 89% of the generated radicals. The hydroxyl radical scavenging activity of the extracts was noticed at appreciable levels at 0.05 mg/ml concentration (Table 4.1).

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/ kg body weight, whereas in the Cardamom extracts, a complete reduction in the number of cfu was observed after 150 mg/ kg body weight in the case of C.Seed and at 180 mg/ kg body weight in the case of C.Pod (Fig 4.4).
Figure 4.2. RP-HPLC chromatogram of the various flavanoids present in the Cardamom seed and pod extracts

Figure 4.3. RP-HPLC chromatogram of the various terpenoids present in the Cardamom seed and pod extracts

A: C. Seed extract; B: C. Pod extract.
Figure 4.4. GC – MS chromatogram indicating the presence of Benz-c-Acridine in the Cardamom seed and pod extracts

Figure 4.5. Effect of the Cardamom seed and pod extracts on the growth kinetics of ECRRL36
A decrease in the body weight of the animals (Fig. 4.6) infected with ECRRL36 was evident on the 7th day post inoculation. In addition, a decrease in the pH of the urine (Fig. 4.7) 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 in the body weight of the mice treated with the cardamom seed and pod extracts were restored close to normal, although they were not significant when compared to the control animals. The alteration in the urinary pH had marginally increased although it was not restored close to normal in the treated animals. An increase in the urinary leukocyte (Fig. 4.8) and nitrite levels (Fig. 4.9) were noticed in the urine of the infected animals, which

### Table 4.1. Antioxidant profile of Cardamom seed and pod extracts

<table>
<thead>
<tr>
<th>Assay</th>
<th>Concentration (μg/ml)</th>
<th>50</th>
<th>100</th>
<th>150</th>
<th>200</th>
<th>250</th>
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<td></td>
<td></td>
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<tr>
<td>DPPH free radical (%) inhibition</td>
<td>C.Seed</td>
<td>18 ± 0.4</td>
<td>22.5 ± 0.1</td>
<td>26.7 ± 0.3</td>
<td>24.8 ± 0.1</td>
<td>20.4 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>C.Pod</td>
<td>10.1 ± 0.1</td>
<td>19.4 ± 0.1</td>
<td>89.2 ± 0.4</td>
<td>48.4 ± 0.3</td>
<td>20.8 ± 0.5</td>
</tr>
<tr>
<td>Reducing power (BHT equivalents)</td>
<td>C.Seed</td>
<td>3.1 ± 0.02</td>
<td>2.5 ± 0.04</td>
<td>2.7 ± 0.04</td>
<td>2.5 ± 0.03</td>
<td>2.6 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>C.Pod</td>
<td>36 ± 0.02</td>
<td>37 ± 0.5</td>
<td>39.1 ± 0.1</td>
<td>38 ± 0.4</td>
<td>37.3 ± 0.1</td>
</tr>
<tr>
<td>Hydroxyl radical (%) inhibition</td>
<td>C.Seed</td>
<td>22.5 ± 0.1</td>
<td>0 ± 00</td>
<td>0 ± 00</td>
<td>0 ± 00</td>
<td>0 ± 00</td>
</tr>
<tr>
<td></td>
<td>C.Pod</td>
<td>26.7 ± 0.1</td>
<td>17 ± 0.2</td>
<td>0 ± 00</td>
<td>0 ± 00</td>
<td>0 ± 00</td>
</tr>
<tr>
<td>Lipid peroxidation (%) inhibition</td>
<td>C.Seed</td>
<td>88.6 ± 0.6</td>
<td>87 ± 0.2</td>
<td>66 ± 0.2</td>
<td>54.9 ± 0.2</td>
<td>40.4 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>C.Pod</td>
<td>74.2 ± 0.3</td>
<td>92.1 ± 0.1</td>
<td>93.3 ± 0.1</td>
<td>92.5 ± 0.1</td>
<td>89.7 ± 0.1</td>
</tr>
</tbody>
</table>
were lowered to the normal levels in the case of the animals treated with C.Seed, however in the case of the animals treated with C.Pod this change was not evident.

Figure 4.6. Body weight of animals treated with Cardamom seed and pod extracts

![Graph showing body weight of animals treated with different extracts](image)

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

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 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 C.Pod treated animals there was a increase in the urinary protein levels on the 20\textsuperscript{th} day, after the initial decrease noticed on the 10\textsuperscript{th} 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 4.2).

Figure 4.7. pH of urine from the animals treated with Cardamom seed and pod extracts

![Graph showing pH levels over time](image)

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

Figure 4.8. Leukocyte enumeration in the urine of mice treated with Cardamom seed and pod extracts

![Graph showing leukocyte enumeration over time](image)

The values are expressed as mean ± standard deviation of the sample size (n = 6)
Figure 4.9. Nitrite estimation in the urine of the mice treated with Cardamom seed and pod extracts

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

Figure 4.10. Haematoxylin and Eosin stained sections from the animals treated with the Cardamom seed and pod extracts

The above figure shows the H&E stained kidney sections obtained from the mice treated with Cardamom extracts for 20 days. A: Experimental control; B: Infection control C: Cardamom seed extract treated mice (150 mg/kg body weight of the animal); D: Cardamom Pod extract treated mice (180 mg/kg body weight of the animal).
**Table 4.2. Biochemical parameters in the kidney after treatment with Cardamom extracts**

<table>
<thead>
<tr>
<th>Tissue parameters</th>
<th>Protein (mg/g tissue)</th>
<th>Nitrite (µg/µl)</th>
<th>Catalase (units/mg protein)</th>
<th>Super oxide Dismutase (µmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CS</td>
<td>CP</td>
<td>CS</td>
<td>CP</td>
</tr>
<tr>
<td>Control</td>
<td>2.33 ± 0.2</td>
<td>0.02 ± 0.01</td>
<td>0.68 ± 0.01</td>
<td>1.65 ±0.1</td>
</tr>
<tr>
<td>Infected</td>
<td>1.47 ± 0.1</td>
<td>0.09 ± 0.001</td>
<td>2.09 ± 0.01</td>
<td>2.38 ±0.2</td>
</tr>
<tr>
<td>10th day post treatme nt</td>
<td>1.4 ± 0.1</td>
<td>1.6 ± 0.1</td>
<td>.1 ± 0.01*</td>
<td>0.1 ± 0.03**</td>
</tr>
<tr>
<td>20th day post treatme nt</td>
<td>1.9 ± 0.1**</td>
<td>1.8 ± 0.5*</td>
<td>.04 ± 0.01*</td>
<td>.06 ± 0.01*</td>
</tr>
</tbody>
</table>

Significant difference between control and treated groups: NS Not significant; * p˂ 0.05; ** p˂ 0.01; *** p˂ 0.001.

Control (0th) (Fig. 4.10A) and the infected mouse kidney sections (Fig. 4.10B) (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. Dense infiltration by the lymphocytes and plasma cells, with disruption of the urothelium was noticed. Upon treatment with Cardamom seed extracts for 20 days at 150 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. 4.10C. In the sections obtained from the animals treated with 180 mg/kg body weight of C.Pod extracts (Fig. 4.10D), although the podocyte architecture was restored close to normal, the Bowman’s space was not restored close to normal. It was also evident from the sections that visible vacuolation was noticed in the proximal and distal tubular regions although the inflammation noticed in the infection tissue sections had subsided.
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 C.Seed extract (Fig.4.11A.), however in the animals treated with 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. 4.11B). The results obtained correlated with the results generated from the qPCR experiment where the relative mRNA expression of THP (Fig. 4.11D) was measured and with the protein expression of the same carried out through immunoblotting (Fig.4.11E).

The levels of the iron-coupled protein, Transferrin, which was also found to act as an antioxidant molecule in this case, were found to be decreased when pyelonephritis was established in the kidneys. Upon treatment with C.Seed the levels were restored to normal after 20 days (Fig.4.12A), however due to the damage in tubular region of the mice treated further with the C.Pod extracts, the levels of transferrin were not restored close to normal in the kidney sections (Fig. 4.12B). These results correlated with the elevation in the relative mRNA expression of transferrin in the C.Seed treated samples as seen in Fig. 4.12D. and also with the elevated protein expression of the same as demonstrated through immunoblotting (Fig.4.12E).

Due to infiltration of the neutrophil's to combat infection, the levels of the antimicrobial proteins Cathelicidin and Lipocalin were also found to be increased in the sections obtained from the infected animals. Upon treatment, there was a reduction in the levels of these antimicrobial proteins in the sections obtained from the animals treated with C.Seed extracts (Fig.4.13A, Fig.4.14A) and were supported by the data obtained through the relative mRNA expression from the qPCR studies (Fig. 4.13D.) and the protein expression through immunoblotting as seen in Fig.4.14D. Such an evident change was not observed in the sections from the animals treated with C.Pod extracts (Fig. 4.13B, Fig.4.14B). This Ficolin, a key player in the alternative pathway for the activation of cytokines and chemokines was also found to
Figure 4.11. Localization and Expression of Tamm Horsfall protein in kidney sections from animals treated with the Cardamom extracts

A: IHC of 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 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, showing a significant increase in the levels of THP upon treatment with C.Seed and C.Pod when compared to infected samples. E: Immunoblot of THP showing reduced expression of THP in the infected sample. F: Densitometric analysis for THP
Figure 4.12. Localization and Expression of Transferrin in kidney sections from animals treated with the Cardamom extracts

A: IHC of 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 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 C.Seed and C.Pod when compared to infected samples. E: Immunoblot of Transferrin showing reduced expression of Transferrin in the infected sample. F: Densitometric analysis for Transferrin.
Figure 4.4. Localization and Expression of Cathelicidin in kidney sections from animals treated with the Cardamom extracts

A: IHC of C.Seed treated section of mouse kidney treated with antibody against Cathelicidin showing no expression in the kidney. B: The section obtained from 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 an significant decrease in the levels of Cathelicidin upon treatment with CPV when compared to infected samples. E: Immunoblot of Cathelicidin showing reduced expression of Cathelicidin in the C.Seed and C.Pod treated sample. F: Densitometric analysis for Cathelicidin.
Figure 4.14. Localization and Expression of Lipocalin in kidney sections from animals treated with the Cardamom extracts

A: IHC of C.Seed section of mouse kidney treated with antibody against Lipocalin showing normal expression in the kidney. B: The section obtained from 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 β – Actin levels, showing an significant decrease in the levels of Lipocalin upon treatment with C.Seed and C.Pod when compared to infected samples. E: Immunoblot of Lipocalin showing reduced expression of Lipocalin in the C.Seed and C.Pod treated sample. F: Densitometric analysis for Lipocalin.
Figure 4.15. Localization and Expression of Ficolin in kidney sections from animals treated with the Cardamom extracts

A: IHC of C.Seed section of mouse kidney treated with antibody against Ficolin showing normal expression in the kidney. B: The section obtained from 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 C.Seed and C.Pod when compared to infected samples. E: Immunoblot of Ficolin showing reduced expression of Ficolin in the C.Seed and C.Pod treated sample. F: Densitometric analysis for Ficolin.
Figure 4.5. Localization and Expression of TNF - α in kidney sections from animals treated with the cardamom extracts

A: IHC of C.Seed treated section of mouse kidney treated with antibody against TNF - α showing normal expression in the kidney. B: Kidney sections obtained from 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 C.Seed and C.Pod when compared to infected samples.
Figure 4.6. Localization and Expression of NF-κβ in kidney sections from animals treated with the Cardamom extracts

A: IHC of 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 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 C.Seed and C.Pod when compared to infected samples.
be decreased upon treatment with C.Seed extracts for a period of 20 days (Fig. 4.15A). The results obtained correlated with the results generated from the qPCR experiment where the relative mRNA expression of Ficolin (Fig. 4.15D) was measured and with the protein expression of the same carried out through immunoblotting (Fig.4.15E).

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 C.seed was effective in treating the induced pyelonephritis (Fig.4.16A., Fig.4.17A). These results correlated with the reduction in the relative mRNA expression of TNF–α and NF-κβ in the C.Seed treated samples as seen in Fig. 4.16D. and Fig.4.17D) and also with the elevated protein expression of the same as demonstrated through immunoblotting (Fig.4.16E and Fig.4.17E). However, this change in the levels of the inflammatory markers was noticed in the animals treated with C.Pod extracts probably due to the inherent toxicity of the extracts.

4.4. DISCUSSION

This study explored the antibacterial effect of cardamom on pyelonephritis-induced mice. The adhesion of UPEC, its rapid multiplication, the generation of ROS and RNS, the alteration in the levels of the antimicrobial proteins and inflammatory markers play a key role during pyelonephritis. The therapeutic interactions administered to treat pyelonephritis of one or both the kidneys, should have a remarkable effect on restoring the levels of the AMP’s and the inflammatory markers to normal and at the same time, remove the uropathogen from the urinary milieu. Since cardamom was found to have a bactericidal effect on the oral pathogens and gram-negative bacteria in general, this study was focused on ascertaining the effect and mode of action of its methanolic extract, which contained the terpenoid and flavanoid fractions in treating pyelonephritis.

From the study, it was evident that the terpenoid and the flavanoid contents were higher in the C.Seed when compared to the C.Pod. However, the number of terpenoids and flavanoids reported were higher in the C.Pod extracts. This could have been to the reason that in the C.Pod extracts the number of alkaloids was probably
less because it was exposed to the environment where it could have broken down in to its secondary metabolites due to the action of the environmental factors and due to the release of volatile compounds. In the case of the C.Seed the alkaloids content was found to be higher as they were shielded from environmental factors, which resulted in their subsequent preservation.

The spectrum of biological effects of cardamom such as antioxidant, antimicrobial, anti-cancer, anti-septic, anti-spasmodic and diuretic properties are due to the wide array of phyto-chemicals present in it (Takemura et al., 1995). The major active principle (>60%) in our study was identified to be Benz – c – acridine (BcAcr), through the GC-MS studies carried out by us. Acridines are polycyclic, aromatic hydrocarbons that are a large, widespread and well-characterized group of compounds formed during the incomplete of combustion of organic matter (Dipple, 1976). Nitrogen is incorporated into their aromatic ring to form nitrogen heterocycles under appropriate conditions. Chemically acridine is an alkaloid from the anthracene and has an irritating odour, and causes allergic reactions on the skin. The other sources from where BcAcr have been identified are are coal-derived fuels (Shultz et al., 1977), urban air particulates (Sawicki, 1967; Stanley et al., 1968), and aquatic sediments (Wakeham, 1979). Several of these acridines, when they are found beyond their threshold level have been reported to be carcinogens (Lacassaggne et al., 1956). Naturally, these acridine alkaloids have been identified in the plants belonging to the family Rutaceae (Ryan et al., 1980), which are known for the anti-cancer phyto-compounds.

Acridine was identified for its antimicrobial property by Ehrlich and Benda in 1917 and later as a possible anti-malarial drug during world war II; the works of Adrien Albert, a renowned Australian chemist, portrayed the idea that the structure of acridine, could be essential for its activity relationship (Wainwright, 2001). Through his work, he rationalized that the cationic ionization, high levels of ionization at neutral pH and the planar molecular surface area ≥ 38 Å, were the features in an acridine molecule that portrayed it as an effective anti-bacterial compound (Wainwright, 2001). Further research have identified that this alkaloid has anti-inflammmatory, anticancer (Srivastava and Nizamuddin, 2004), antihelminthic
(Elslager and Worth, 1969), insecticidal, rodenticidal (Mayer et al., 1970), fungicidal (Pathak and Bahel, 1980) and anti-tumour and anti-cancer activities (Takemura et al., 1995), surface anaesthetic for eye, peripheral nerve blocker and spinal anaesthetic, and as a bacteriostatic agent against gram-positive bacteria (Harikrishnan and Kapoor, 2005). The recent inflation in the levels of antimicrobial resistance towards the various drugs have re-vitalized the drug discovery pipeline to identify newer drugs, thereby spurring researchers to rework on this compound whether it could be an alternative and promising antibacterial drug (Kumar et al., 2012) to the existing β-lactams.

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The methanolic extract of C.Pod and C.Seed containing BcAcr, were tested for its antimicrobial effect in vitro against ECRRL36, a gram-negative pathogen. The mode of inhibition was identified to be bactericidal corresponding to the earlier reports against oral pathogens and its use as an insecticide in the pest management in cotton flowers (Josephrajkumar et al., 2007). This probed us to analyze the effect of the methanolic extracts against the pyelonephritis induced mouse models using the same strain at its bactericidal concentration.

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The initiation of infection in the kidneys due to the rapid multiplication of the bacteria brings about an alteration in the delicate homeostasis that exists between the levels of the antioxidant molecules and the ROS and RNS species. This resultant imbalance in the levels of the antioxidant enzymes was reflected by a significant increase in the tissue antioxidant enzymes such as SOD, Catalase and total nitrite and nitrate content, which were observed through the direct measurements of these parameters. The increase in the damage to the resultant tissues also lead to acute tubular necrosis, resulting in damage to the tubular regions of the kidney and also complete destruction in the glomerular architecture (Fig.4.10B). This damage to the kidneys was reflected as proteinuria in the infected animals leading to a significant loss of body weight in the infected animals when compared to the control animals due to the failure in the reabsorption of the proteins in the kidney (Fig.4.6). A shift in the urinary pH towards the acidic side (Fig.4.7) as a result of rapid multiplication of the bacteria within the urinary bladder was also observed due to the utilization of the nutrients present there.
This imbalance results in an increase in the damage to the surrounding macromolecules such as DNA and proteins. In order to prevent the multiplication of the bacteria and to remove them from the system, the polymorphonuclear leukocytes such as neutrophil's and macrophages were recruited into the surrounding tissues. This also led to a remarkable tissue oedema in the kidney tissues (Fig.4.10B), to aid in the removal of the dead tissues and to hasten the self-renewal process within the tissues. This infiltration of the neutrophil’s lead to an increase in the levels of the inflammatory markers such as TNF – α, which are present at the surface of the cell. This was reflected by the increase in the expression levels of TNF – α in the tissue excised from the infected on the 7th day post-infection (Fig.4.16B). Due to the subsequent increase in the levels of TNF – α, a membrane bound inflammatory marker, further activation of the signalling molecules, lead to the increase in the levels of the nuclear inflammatory marker, NF – κβ (Fig.4.17B).

The administration of C.Seed (150 mg/kg body weight) and C.Pod (180 mg/kg body weight) extracts brought about a significant change in the levels of the antioxidant enzymes, which were altered due to the progression of infection. The observed changes were more pronounced in the animals that were treated with C.Seed when compared to those treated with C.Pod on the 10th day post treatment with the extracts. But on the 30th day post-treatment it was noticed in the animals that were treated with C.Pod, there was an increase in the levels of the antioxidant enzymes, followed by an decrease in the total protein levels in the kidney (Table 4.2). In the sections obtained from the animals there was evidence of acute tubular damage. This tubular damage could have been as result of the prolonged usage of the secondary metabolites present in the extract, however, the exact secondary metabolite could not be pointed out at this moment, as the individual metabolite has to be analyzed for its toxicity profile and the damage it causes to the renal tissues upon prolonged usage.

The administration of the Cardamom extracts brought about a significant reduction in the levels of the inflammatory markers thereby suggesting that it could be the synergistic activity of the various alkaloids such as terpenoids and flavanoids present in it. The terpenoids could have effectively acted as the bactericidal agents that prevented the multiplication of the bacteria within the renal milieu, while the flavanoids, renowned for their antioxidant capabilities as demonstrated by the in vitro
antioxidant systems, were known to intervene and bring about an alternation in the imbalance created due to infection.

The C.Seed extracts had a considerable effect in restoring the levels of the Cathelicidin (Fig.4.13E) and lipocalin (Fig.4.14E) close to normal, upon administration for a period of 30 days. The levels of these AMP’s were elevated in the infected tissues as noticed in the IHC slides, due to increased infiltration of the neutrophil's into the infection prone regions of the kidney to combat it. However, in the C.Pod treated animals there was a slight increase in the levels of the antimicrobial proteins, as a result of tissue damage. Ficolin, the mannose bound lectin, was also found to have a reduced level of expression in the animals treated with C.Seed extracts.

When a pronounced infection had set in, a drastic reduction in the expression levels of the iron bound protein transferrin was noticed. This could have been due to the siderophore activity of the uropathogen. The siderophores released by the uropathogens generally act by coupling to the host iron bound proteins and scavenging them to utilize the free iron for their growth, thereby resulting in a loss in the host free iron pool and drastically altering the iron homeostasis in the host. When the cardamom extracts were administered, it was noticed that there was a significant increase in the expression of transferrin in the tubular region (Fig.4.12D).

Tamm-Horsfall protein, an AMP, found to have been drastically reduced during the establishment of pyelonephritis in the kidneys as reported by our previous studies and by the other independent studies, was also found to be reduced when a prominent infection was established in the kidneys. This further confirms the hypothesis that this protein could have a definite role in the progression of infection. However, a reduction in the expression levels of this protein when the animals were treated with C.Pod for prolonged time were observed, which could be due to the damage to the tubular architecture (Fig.4.11B). In the sections obtained from the C.Seed treated animals it was noticed that there was significant restoration in the levels of this protein in the tubular region close to normal, thereby suggesting that the C.Seed extract could definitely have a pronounced renoprotective effect on the kidney when compared to the C.Pod extracts. This renoprotective effect of the C.Seed could
have been due to the synergistic action of the terpenoids and the flavanoids present in it.

4.5. CONCLUSION

Our study explored the possible role of *Ellettaria cardamomum* as an bactericidal agent the uropathogenic *Escherichia coli* (ECRRL36) and its possible regulatory role against the antimicrobial proteins and inflammatory markers to effectively 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 Cardamom extracts have a potential anti-inflammatory as confirmed earlier in induced arthritis models. 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 C.Seed extract when compared to the C.Pod extract could effectively serve as an alternative bactericidal treatment with renoprotective effect to combat UTI.