Chapter 3

Evaluating the efficacy of probiotic *Escherichia coli* Nissle 1917 strain containing gluconate dehydrogenase (*gad*) and pyrroloquinoline quinone (*pqq*) gene cluster in amelioration of cadmium induced toxicity in rats.

3.1. Introduction

In Chapter 2, *Ec*N-21 producing both PQQ and citric acid was found to be less effective as compared to *Ec*N-20 producing PQQ supplemented with citric acid orally due to low level of citric acid. Hence, increased levels of chelators required to be achieved. Organic acids such as 2-Ketogluconic acid can chelate Cd as well as Pb (Francis, 1990). Incorporation of *pqq* gene clusters in *E. coli* results in PQQ biosynthesis by the activity of glucose dehydrogenase enzyme which utilizes PQQ as cofactor, thus converting glucose into gluconic acid (Goosen et al., 1989; Khairnar et al., 2003). Gluconic acid further utilized by gluconate dehydrogenase enzyme encoded by *gad* operon using FAD as a cofactor converting gluconic acid into 2-ketogluconic (2-KG) acid within the periplasm (Toyama et al., 2007). Higher levels of 2-ketogluconic acid was achieved by overexpressing *gad* operon of *E. cypripedii* ATCC 29267 in *E. coli* (Yum and Lee, 1997). Therefore, the present strategy was designed to evaluate the effect of 2-ketogluconic acid and PQQ secreted by *Ec*N-23 harbouring *pqq* gene cluster and *gad* operon to chelate Cd and prevent against its toxic effects in rats. The current hypothesis is summarized in **Fig. 3.1**.

3.2. Methods and materials:

3.2.1. Animals

Free access to water and food was given to adult male Charles foster albino rats (weight 250–300g) and were maintained at relative humidity (45.5%), controlled temperature (25±1ºC), photoperiod cycle (12h light: 12h dark) as described by the Committee for the purpose of control and supervision of experiments on animals (CPCSEA) guidelines of Animal Ethical Committee (M. S. University of Baroda, India, **Reg. No. 938/A/06/CPCSEA).**

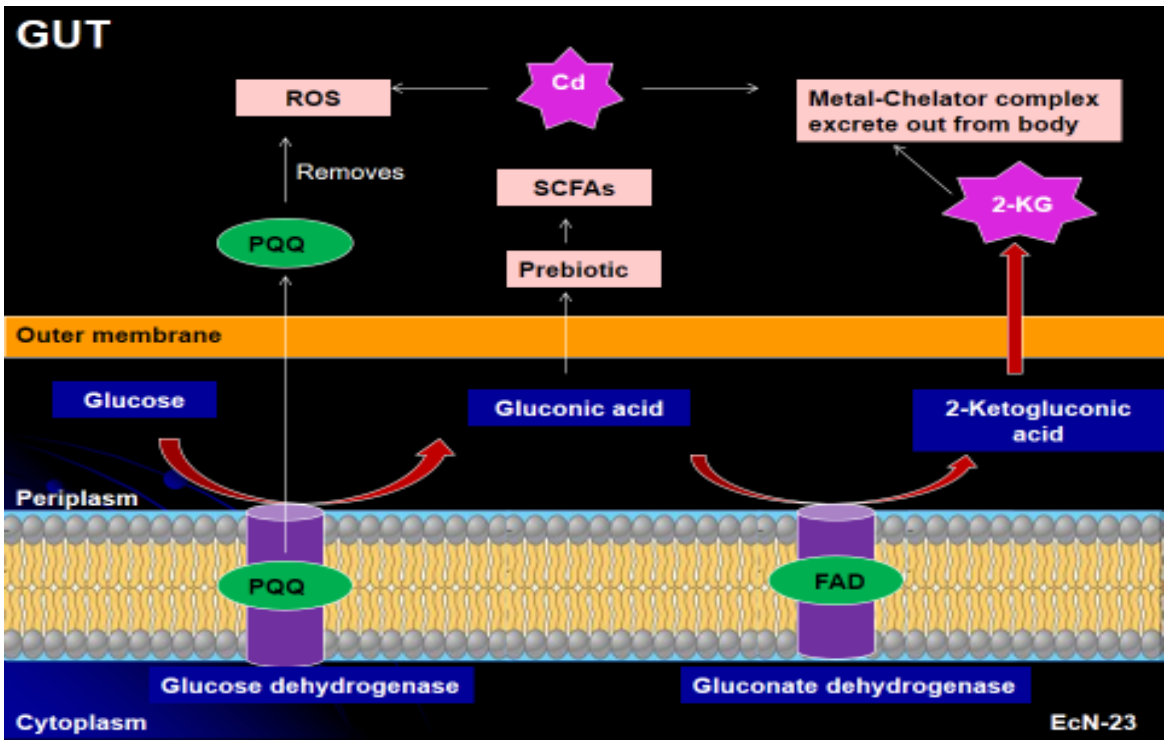


Fig. 3.1. Proposed mechanism for the detoxification of Cd using *Ec*N-22 and *Ec*N-23.

Plasmid	Characteristics	Sources/References
pUCPM18	pUC18 derived Broad-Host-Range vector; Apr (100µg/ml)	Hester et al., 2000
pJNK5	pUCPM18, Gmr (20µg/ml) with 5.1 Kb *pqq* gene cluster of *A. calcoaceticus*.	Wagh et al., 2016
pJNK6	pJNK5, Gmr (20µg/ml) with *gad* operon 3.8 Kb of *P. putida* KT 2440	Wagh et al., 2016
Strains		
***Ec*N**	*Escherichia coli* Nissle 1917 (*Ec*N) (Probiotic strain)	Sonnenborn and Schulze, 2009
***Ec*N-2**	*Ec*N strain with genomic integration of *vgb* and *gfp* genes	Singh et al., 2014
***Ec*N-22**	*Ec*N-2 harbouring pJNK5 producing PQQ and gluconic acid	This study
***Ec*N-23**	*Ec*N-2 harbouring pJNK6 producing PQQ, gluconic and 2-Ketogluconic acids	This study

Table 3.1. Plasmid and strain

3.2.2. Cloning

pJNK5 (Wagh et al. 2016) harbouring 5.1 Kb *pqq* gene cluster of *A. calcoaceticus* transformed into *Ec*N-2 to obtain *Ec*N-22 (**Table 3.1**). pJNK6 (Wagh et al. 2016) harbouring 3.8 Kb *gad* operon of *P. putida* KT2440 and 5.1 Kb *pqq* gene cluster of *A. calcoaceticus* tranformed into *Ec*N-2 to obtain *Ec*N-23. *Ec*N was acquired as a generous gift from Dr. rer. nat. Ulrich Sonnenborn, Ardeypharm GmbH, Loerfeldstrabe 20, Herdecke (Germany). *Ec*N strain was modified with integration of *vgb* and *gfp* genes in genome to produce *Ec*N-2 (Singh et al. 2014).

3.2.3. Characterization of *Ec*N transformants producing PQQ, gluconic acid and 2-Ketogluconic acid

Activity of PQQ dependent glucose dehydrogenase and FAD dependent gluconate dehydrogenase in *Ec*N-22 and *Ec*N-23 transformants was confirmed by appearance of red colour with drop in pH in Tris-buffered medium due to gluconic acid and 2-ketogluconic acid secretion (Gyaneshwar et al., 1999). Extraction of PQQ from *Ec*N transformants was performed according to Suzuki et al. (1990) and quantified using Hitachi fluorescence spectrophotometer (Hitachi High-Technologies Corporation, Tokyo, Japan) with excitation 375 nm and emission 465 nm. HPLC analysis was done according to Buch et al. (2009) for the estimation of 2-ketogluconic acid and gluconic acid excreted by *Ec*N-2 transformants.

3.2.4. Bacterial strains and culture conditions

Probiotic strains including *Ec*N-2, *Ec*N-22, *Ec*N-23 were grown in luria broth overnight at 37°C. Then reinoculated in fresh medium to achieve colony forming unit (CFU) of 10^9 cells/ml culture. One ml of the culture was pellet down, washed twice with saline, redissolved into saline and eventually tube fed to rats.

3.2.5. Experimental design

Rats were fed on normal pellet diet and divided into 6 different groups (6 rats per group): Control, Cd, *Ec*N-2+Cd, *Ec*N-2+Cd+PQQ(oral)+2-KG(oral), *Ec*N-22+Cd, *Ec*N-23+Cd. Probiotics were given (10^9 colony-forming units [CFU]/rat for 3 consecutive

days) followed by streptomycin wash (5g/l for 24h). After 7 days of treatment, colonization was confirmed by fecal count through visualizing gfp expressing colonies. Thereafter, Cd (100 ppm) was given in drinking water for 4 weeks, followed by further probiotic treatments which were given once per week till 4 weeks. Orally PQQ (10 mg/kg body weight) and 2-KG (1.2 g/kg body weight) were given once a week.

3.2.6. Preparation of tissue homogenates

Similar as described in section 2.2.6 of chapter 2.

3.2.7. Biochemical assays

Similar as described in section 2.2.7 of chapter 2.

3.2.8. ALT, AST, ALP, urea, creatinine

Similar as described in section 2.2.8 of chapter 2.

3.2.9. Histopathological changes

Similar as described in section 2.2.9 of chapter 2.

3.2.10. Statistical Analysis

Similar as described in section 2.2.11 of chapter 2.

3.3. Results

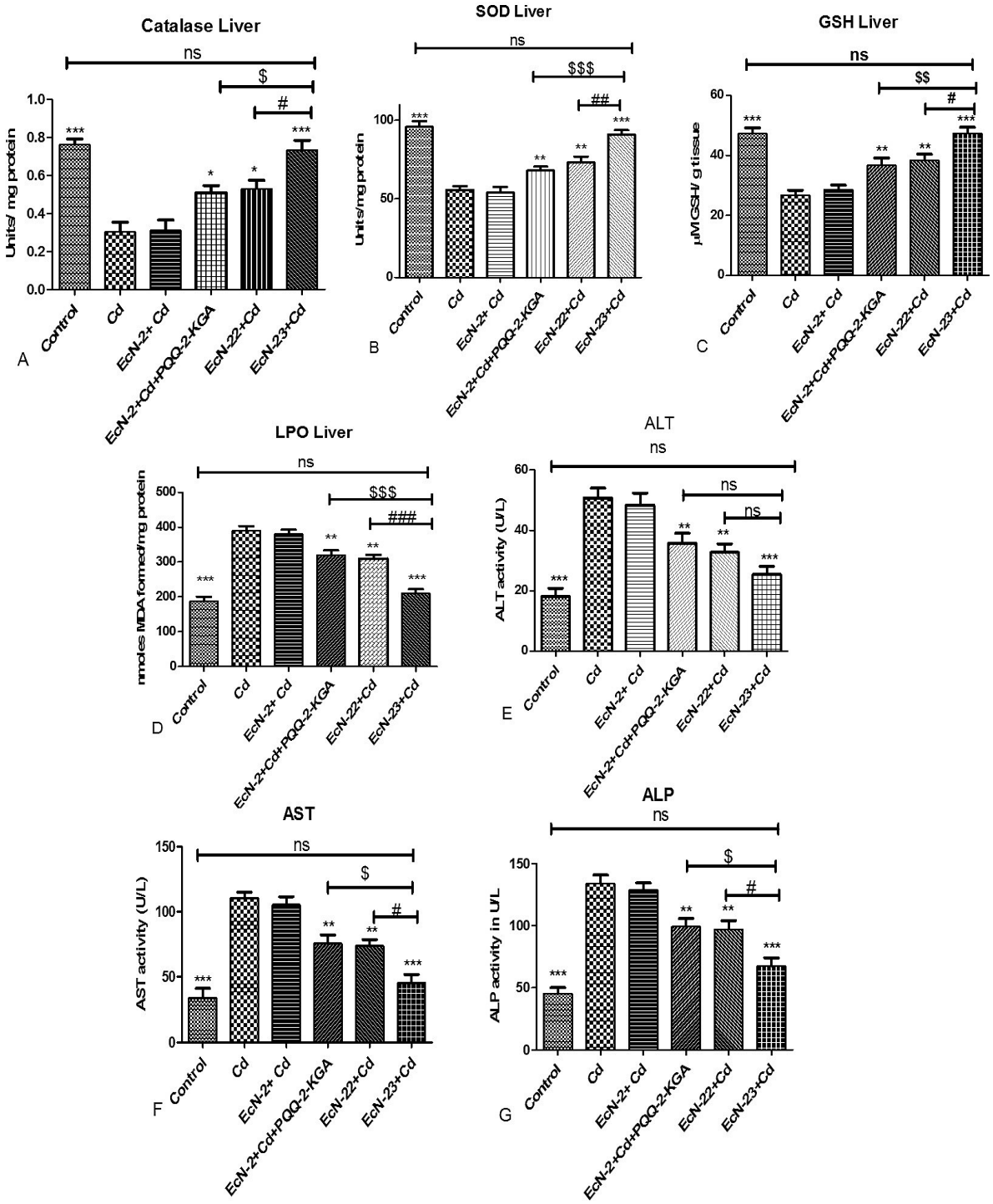
3.3.1. Characterization of PQQ, gluconic and 2-ketogluconic acid secretion by *Ec*N transformants

PQQ levels found to be 1.8±0.38 μg/ml and 1.6 ± 0.24 μg /ml of culture from *Ec*N-22 and *Ec*N-23 respectively by growing at 37°C in M9 minimal medium after 24h using glucose as carbon source. Both transformants dropped the pH of Tris-buffered medium resulting into appearance of red colour due to secretion of gluconic acid by glucose dehydrogenase using PQQ as a cofactor and 2-ketogluconic acid by gluconate dehydrogenase using FAD as a cofactor. *Ec*N-22 secreted 15.28 ± 0.63 mM gluconic acid

while *Ec*N-23 secreted 5.34 ± 0.42 mM gluconic and 8.16 ± 0.19 mM 2-ketogluconic acids.

3.3.2. Effect of *Ec*N-23 against Cd induced liver and kidney damage in rats

Exposure of Cd for one month decreased GSH levels, Catalase and SOD activities while the lipid peroxidation was significantly increased in liver and kidney as compared to control (**Fig. 3.2**). Hepatic damage marker enzymes AST, ALT and ALP activities as well as urea, creatinine levels in serum were also significantly increased compared to control after Cd exposure. Against Cd intoxication *Ec*N-23 was more effective as compared to *Ec*N-22 and *Ec*N-2 with orally given PQQ and 2-ketogluconic acid in restoring the Catalase and SOD activities, GSH levels, lipid peroxidation in liver and kidney as well as AST, ALT, ALP activities and urea, creatinine levels in serum whereas *Ec*N-2 was not effective.


Catalase Liver
Units/ mg protein
ns
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#
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*
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EcN-2+ Cd
EcN-2+Cd+PQQ-2-KGA
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Control
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EcN-2+ Cd
EcN-2+Cd+PQQ-2-KGA
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D
Control
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EcN-2+ Cd
EcN-2+Cd+PQQ-2-KGA
EcN-22+Cd
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ALT
ALT activity (U/L)
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E
Control
Cd
EcN-2+ Cd
EcN-2+Cd+PQQ-2-KGA
EcN-22+Cd
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AST
AST activity (U/L)
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F
Control
Cd
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ALP
ALP activity in U/L
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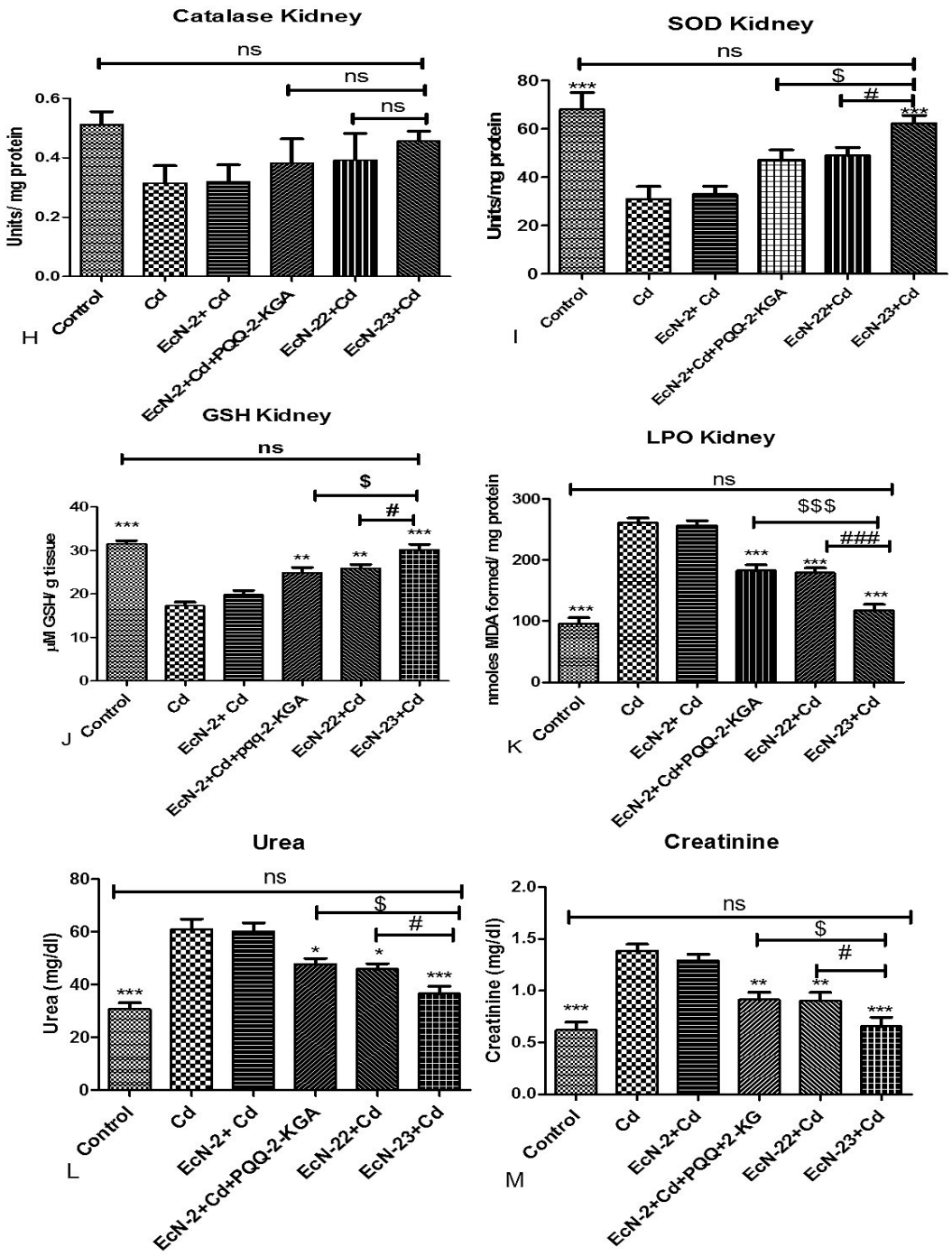

Fig. 3.2. Effect of genetically engineered probiotic *E. coli* Nissle 1917 on Cd exposure in liver: (A) Catalase, (B) SOD activity, (C) GSH levels, (D) Lipid peroxidation; (E) ALT, (F) AST, (G) ALP activity in serum and in Kidney (H) Catalase, (I) SOD activity, (J) GSH levels, (K) Lipid peroxidation; (L) Urea (M) Creatinine levels in serum.. Values are expressed as mean ± SEM (n=6 each group). *P ≤ 0.05, ** P ≤ 0.01 and ***P ≤ 0.001 compared to Cd group, $P ≤ 0.05, $$ P ≤ 0.01, $$$P ≤ 0.001compared to *Ec*N-2+PQQ+2-KG, # P ≤ 0.05 ,##P ≤ 0.01,###P ≤ 0.001compared to *Ec*N-22+Cd.

3.3.3. Histopathological damage

Histopathological studies showed the normal architecture in control group while Cd treatment induced pathological changes in liver and kidney (**Fig. 3.3 and 3.4**). In liver Cd exposure caused derangement of hepatic cords, hepatocyte vacuolation, necrosis, inflammatory cell infiltration, perinuclear halo while in the kidney it leads to necrotic renal tubules, renal tubular dilatation, distention of Bowmen's cavity, shrinkage and reducing size of glomeruli. *Ec*N-23 treatment caused near to normal appearance in liver and kidney.

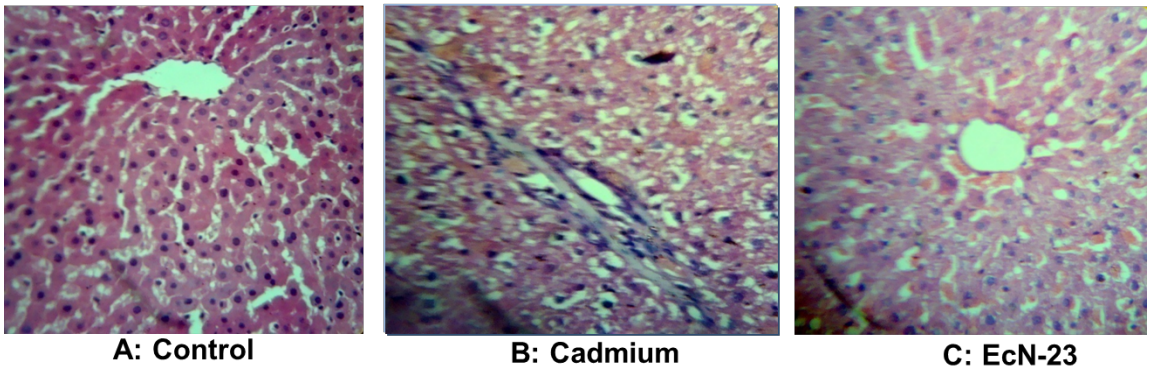

Fig. 3.3. Photomicrograph of liver stained with HE (Magnification=40x) (A) Control showing normal liver architecture, (B) Cadmium treated group showing hepatocyte vacuolation (black arrow), perinuclear halo (arrow head), inflammatory cell infiltration and necrosis (bold arrow), (C) *Ec*N-23 showing near to normal appearance of liver.

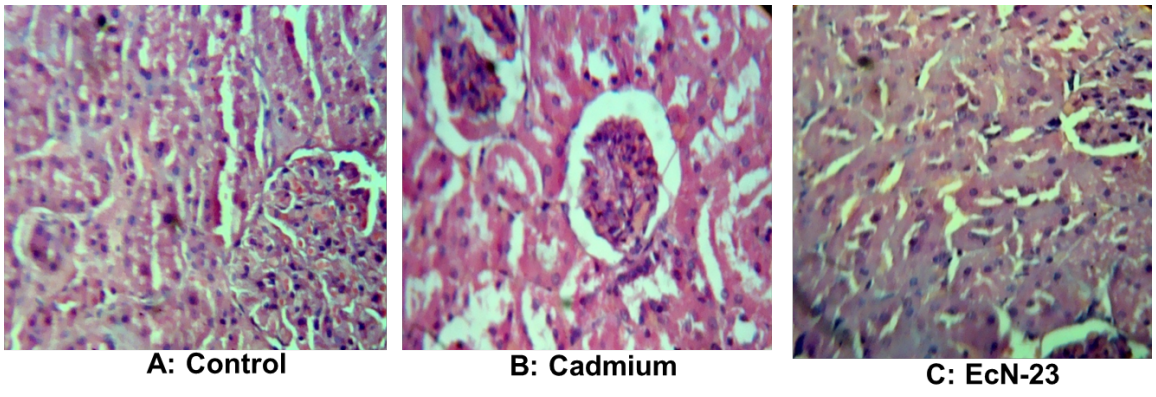

Fig. 3.4. Photomicrograph of kidney stained with HE (Magnification=40x) (A) Control showing normal kidney architecture, (B) Cadmium treated group showing renal tubular dilatation (black arrow), renal tubular necrosis (arrow head), shrinkage of glomeruli and distention of bowman's capsule (bold arrow), (C) *Ec*N-23 showing near to normal appearance of kidney.

3.4. Discussion and conclusion

The most widely accepted mechanism for heavy metal induced toxicity is the oxidative stress resulting into hepatotoxicity and nephrotoxicity caused by cellular damage involving inhibition of enzyme activities, depletion of GSH levels by binding with sulfhydryl groups and ROS mediated damage to lipid bilayer (Tchounwou et al. 2012). In the present study, in liver and kidney significant decrease in activities of catalase and SOD as well as GSH levels while increase in MDA levels were seen on Cd treatment as compared to control. Activities of hepatic damage marker enzymes like ALT, AST, ALP were also found to be high in Cd treated groups as compared to control showing hepatotoxicity. Likewise, kidney damage markers like urea and creatinine were also higher in serum after Cd treatment. Histopathological damage was also reported to be high in liver and kidney on Cd treatment as compared to control.

The rational of using antioxidants along with chelators has come forward due to oxidative stress being considered as major mechanism behind heavy metal toxicity. Several antioxidants such as *N*-acetylcysteine (NAC), lipoic acid, melatonin, and gossypin have been combined with chelators and have shown significant recoveries in animal models (Flora and Pachauri, 2010). Antioxidant potential of taurine when combined with DMSA or MiADMSA was illustrated by removal of Pb and As from blood, liver, kidney and brain. Combined administration of NAC with succimer and DMSA/MiADMSA found to be effective against arsenic and lead intoxication respectively. Likewise, vitamin C and MiDMSA together reduced the hepatotoxicity and nephrotoxicity induced by toxic metal. In present study, combined treatment with PQQ and 2-Ketogluconic acid also found to be more effective in reducing the Cd induced hepatotoxicity and nephrotoxicity as compared to *Ec*N-2. However, *Ec*N-2 was not effective against Cd induced oxidative damage in liver and kidney.

Probiotic *E. coli* CFR 16 and *Ec*N secreting PQQ were more effective then orally given PQQ against damage induced by DMH, alcohol and rotenone treatment (Pandey et al., 2014; Singh et al., 2014; Singh et al., 2015). Likewise, in present study *Ec*N-22

secreting PQQ was more effective against Cd induced damage in liver and kidney as compared to *Ec*N-2.

As described in chapter 2, *Ec*N-21 producing PQQ and citric acid was not as effective as *Ec*N-20 supplemented with citric acid orally against Cd induced liver and kidney due to low levels of secreted citric acid. However, *Ec*N-23 secreted higher levels of chelator *i.e*. 2-ketogluconic acid. Interestingly, *Ec*N-23 producing PQQ and 2-ketogluconic acid was found to be more effective in increasing the activities of catalase and SOD, GSH levels and decreasing MDA levels in liver and kidney as well as in decreasing activity of ALT, AST, ALP and urea, creatinine levels in serum as compared to *Ec*N-22 and *Ec*N-2 supplemented with orally given PQQ and 2-ketogluconic acid. The protection shown by *Ec*N-23 is due to constitutive production of PQQ and 2-ketogluconic acid, which helps in continuous scavenging of free radicals and depletion of Cd burden. As described in chapter 2, Gluconic acid produced by *Ec*N-23 acts as prebiotic and helps in secreting SCFAs, which are known to have anti-inflammatory effects against Cd toxicity.

In conclusion, present study illustrates that *Ec*N-23 producing PQQ and 2-ketogluconic acid is an effective strategy against Cd induced liver and kidney damage as compared to *Ec*N-22 and *Ec*N-2 supplemented with orally given PQQ and 2-ketogluconic acid