Chapter 3

Evaluating the effects of PQQ secreting *Escherichia coli* Nissle 1917 strain on rotenone induced oxidative damage in rats

3.1 Introduction

Rotenone, a potent botanical insecticide is commonly used for pest control (lice and tick control on animals) and in aquatic resource management (Fish eradication). It is one of the most efficient inhibitors of mammalian electron transport chain (ETC). It blocks the complex I activity by preventing the oxidation of the Fe-S clusters leading to following consequences (Sherer et al., 2003).

1. Increase in NADH/NAD^+ ratio, as the oxidation of NADH has been prevented.
2. Increase in CoQ pool, which means that the electron transport via the complex I has been hindered.
3. Generation of enormous amount of ROS.

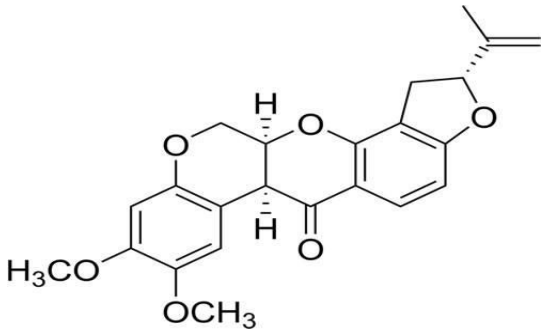

Figure 3.1 Chemical structure of Rotenone.

Rotenone is lipophilic in nature and has half-life of around 8 hours in human body (Bashkatova et al., 2003). It crosses blood brain barrier and has enormous implications in development of Parkinson's like symptoms. Sub-cutaneous injections of rotenone have been demonstrated to selectively degenerate dopaminergic neurons and α-synuclein aggregation in rats (Sherer et al., 2003). Sub-cutaneous injection of rotenone also leads to central nervous system (CNS) and systemic toxicity in animal models (Lapointe et al., 2004). Several studies have supported neurotoxic effect of rotenone, and human studies have demonstrated that exposure of rotenone is

strong risk factor for development of neurodegenerative diseases including Parkinson's disease (Spivey, 2011).

Pyrroloquinoline quinone (PQQ) is a water soluble and strong antioxidant molecule. Several studies have demonstrated antioxidant efficacy of PQQ against cellular and mitochondrial pro-oxidants (He et al., 2003; Zhu et al., 2006; Rucker et al., 2009; 2012). PQQ has been shown to prevent mitochondrial functions and oxidative injury in cardiomyocytes (Tao et al., 2007). It has also been implicated in modulating mitochondrial content and function in animals as well as humans (Stites et al., 2006; Tao et al., 2007; Chowanadisai et al., 2007; 2008; Baurley et al., 2006; 2011).

PQQ has also been shown to prevent neurons and neurological functions (Zhang et al., 2006; 2009 A; 2009 B; 2013; Hara et al., 2007). Recent study on *In Vitro* cell cultures demonstrates that PQQ prevents rotenone induced cell injury via ERK1/2 pathway . However, studies on animal models have not been done.

In the present study, we wanted to investigate the effect of genetically modified probiotic *E. coli* Nissle 1917 strain secreting PQQ on rotenone induced systemic oxidative damage in rats. We hypothesized that PQQ secreted from bacteria in the gut, after absorption and visceral extraction, prevents mitochondrial ROS and oxidative damage and eventually tissue function in rats treated with rotenone.

Intraperitonial rotenone injection causes more of systemic damage than CNS damage (Lapointe et al., 2004). Hence, in this study rotenone was given intraperitionally to animals.

PQQ gene clusters from *Gluconobacter oxydans* (3.2 Kb) have been successfully cloned and expressed in *E. coli* (Holcher and Gorisch, 2006; Yang et al., 2010). Since it is smaller in size as compared to other gene clusters we used this in the present study to reduce the plasmid load.

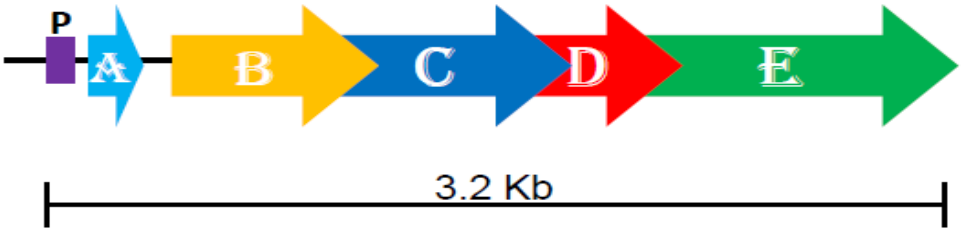

Figure 3.2 *pqqABCDE* gene clusters from *G. suboxydans* 621H.

3.2 Methods and materials

3.2.1 Bacterial strains and culture conditions

E. coli Nissle 1917 and *E. coli* DH5α strains were maintained at 37^{0}C on Luria agar and Luria broth (described in section2.2). *Gluconobacter suboxydans* 621H was obtained from MTCC, Chandigarh, India (Acc. No. 904) and maintained on MRS media at 30 ^{0}C. Different bacterial strains and their modifications have been summarized in table 3.1.

3.2.2 Plasmids and constructs

Plasmids and constructs used in this study have been summarized in table 3.1. Total genomic DNA was extracted from *G. suboxydans* by standard procedure (Sambrook et al., 2002). 3.7 Kb *pqqABCDE* gene cluster was PCR amplified using high fidelity *Taq* DNA polymerase (Thermo fisher Scientific- India) and specific primers (Holcher and Gorisch, 2006), Forward 5' GCCG GAATTCGCGGA TGTTCAGGTGTTCGC and reverse ATCGTCTA GAAGAAGATGGCCTCTCCTGGG. Gradient PCR was set to optimize the annealing temperature. The amplified PCR product was purified and ligated to pTZ57R/T vector using T4 DNA ligase (Thermo fisher Scientific- India) to generate pTPQQ-1 plasmid.

Table 3.1 Plasmids and bacterial strains

Plasmids/ Strains	Characteristics	Reference
	Plasmids	
pTZ57R/T	Cloning vector Thermo fisher Scientific ®	Thermo fisher Scientific- India ltd. Mumbai.
pTPQQ-1	pTZ57R/T vector harbouring 3.7 Kb *pqq* gene cluster from *G. oxydans*.	This Study
	Bacterial Strains	
Escherichia coli DH5α	Laboratory strain	Sambrook et al., 2002
E. coli Nissle 1917 (EcN)	Probiotic strain	Sonnenborn and Schulze, 2009
EcN-2	EcN strain with genomic	This study (Chapter 2)

	integration of *vgb* and *gfp* genes	
EcN-5	EcN-2 strain harbouring pTPQQ-1 plasmid	This study
E. asburiae PSI3	PQQ producing, phosphate solublizing bacteria isolated from pigeon pea rhizosphere	Gyaneshwar et al., 1999

3.2.3 Animals

Charles Foster male albino rats weighing 180 to 220 g and 12-15 weeks of age were selected for the present study. They were maintained in controlled conditions (temperature: 25 ± 1°C; relative humidity: 45.5 %; photoperiod cycle: 12 h light and 12 h dark) with free access to food and water as per recommendations from Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), an animal ethical committee of the institute (The Maharaja Sayajirao University of Baroda, India, Reg. No. 938/A/06/CPCSEA). All rats received regular pellet diet and were granted free access to drinking water.

3.2.4 Designing of the experiment

Rats were first divided in 2 groups (4 months old; 6 animals each group) for standardization of rotenone dose and duration. Rats received rotenone 2.5 mg/kg bw or 3.5 mg/kg bw.

For the **second experiment**, adult rats (4 months old; 6 animals each group) were used. Rotenone (2.5 mg/ kg bw) dissolved in sunflower oil was injected intra-peritionally daily for 28 days to induce systemic oxidative stress and to create accelerated aging rat model. Experimental groups and respective treatments are depicted in **Figure 3.3**. Streptomycin (5 g/l) was given for 48 hours to wash major portion of gut flora followed by probiotic treatment at 10^8 CFU (1 ml) per dose once a week. After 28 days, rats were sacrificed and samples were collected for analysis. PQQ extracted from *E asburiae* PSI3 was given in diet.

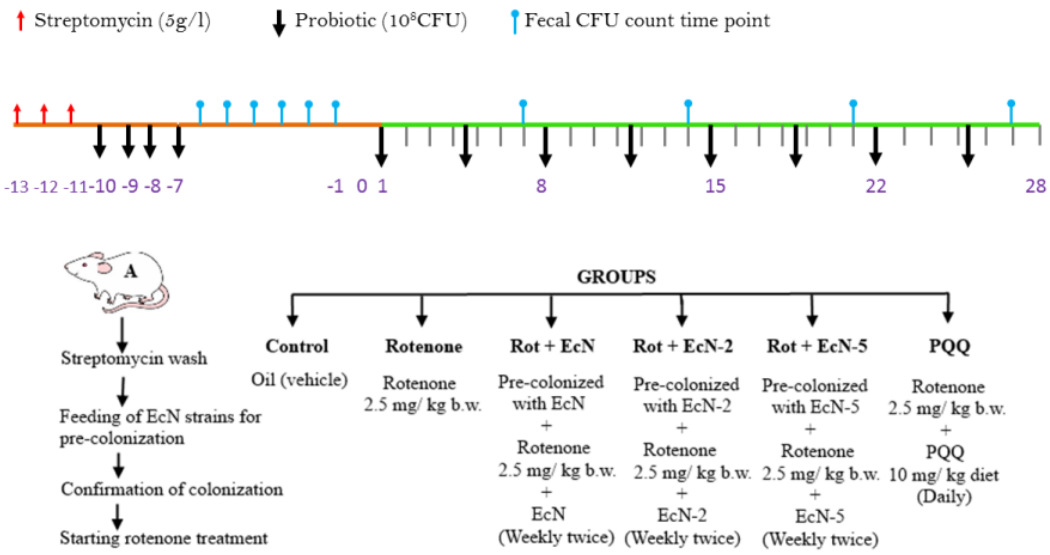

Figure 3.3 Schematic representation of strategy. Rats were randomly divided between different groups. 6 animals each group.

3.2.5 Characterization of PQQ secreting *E. coli* Nissle 1917

Probiotic *E. coli* Nissle 1917 was transformed with pTPQQ-1 plasmid harbouring *pqqABCDE* genes. Functionality of the transformants was confirmed by growing them on tris buffered medium and quantification of PQQ in M9 supernatant (see section 2.2).

3.2.6 Enzyme assays and estimations

Enzyme assays, PQQ extraction and quantification, and other biochemical estimations were performed as described in **section 2.2**.

3.2.7 mRNA expression and qRT-PCR

Total RNA was extracted from hepatic tissue using TRIzol® (Invitrogen BioServices India Pvt. Ltd., Bangalore, India) following manufacturers protocol. cDNA was generated using Reverse transcriptase kit (Applied Biosystems, Foster City, CA) following standard protocol supplied with the kit. PCR was performed using ABI QuantStudio™ 12L flex real time PCR system coupled with SYBR green technology (Applied Biosystems) following standard cycling parameters. Relative amount of liver mitochondria was determined

using real time PCR as described Sites et al. (2006). Nuclear cystic fibrosis (CF) and mitochondrial nicotinamide adenine dinucleotide dehydrogenase (ND-5) were the target genes. Primer sequences are shown in T**able 3.2.**

Table 3.2 Primer sequences used for qRT-PCR

Genes	Sequence
Proliferator-activated receptor-γ coactivator-1α (PGC-1α)	5' AATGAGCCCGCGAACATATT 3' (Forward) 5' TGAGGACCGCTAGCAAGTTTG 3' (Reverse)
B-actin	5' ACGGTCAGGTCATCACTATCG 3' (Forward) 5' GGCATAGAGGTCTTTACGGATG 3' (Reverse)
Cystic fibrosis (CF)	5' AAACTCAGGATAGCTGTCCGTTTAG 3' (Forward) 5' GCCAAATGATAGCATGGAACTCT 3' (Reverse)
Nicotinamide adenine dinucleotide dehydrogenase (ND-5)	5' GGATGATGATATGGCCTTGCA 3' (Forward) 5' CGACTCGGTTGTAGAGGATTGC 3' (Reverse)

3.2.8 Statistical analysis

Data analysis was conducted using GraphPad Prism version 5.0 (GraphPad Softwares Inc., San Diego, CA). Results were considered significant at p≤ 0.05.

3.3 Results

3.3.1 Cloning and expression of *pqqABCDE* gene cluster in EcN

Optimization of PCR conditions for amplification of pqqABCDE genes from G. oxydans genome.

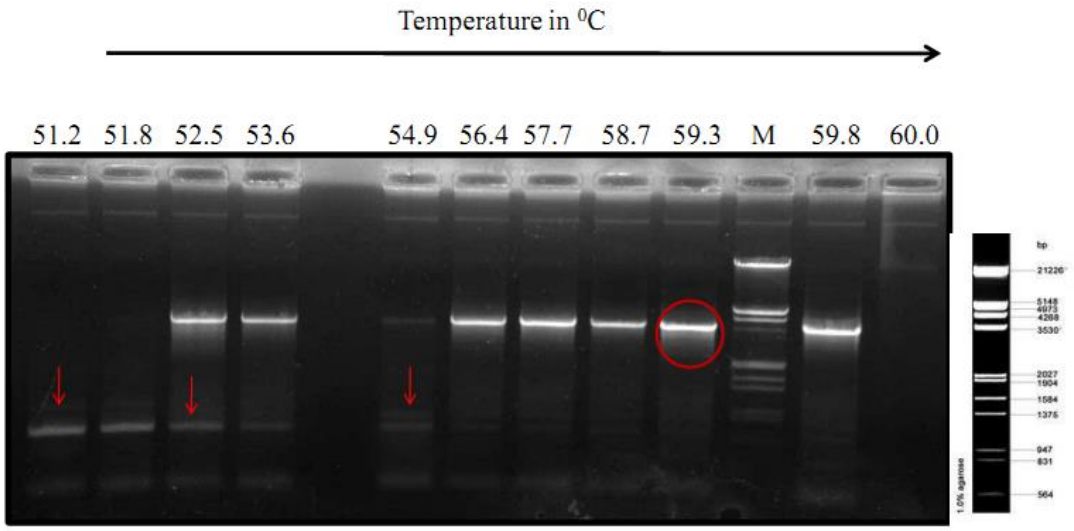

Figure 3.4 Gradient PCR and agarose gel electrophoresis of amplicons (Lambda DNA Hind III digest used as marker)

Gradient PCR of *pqqABCDE* genes using specific primers showed several non-specific bands in wells with products annealed at 58.7 ^{0}C or less. However, at 59.3 ^{0}C and 59.8 ^{0}C 3.7 Kb amplicon was obtained with minimum non-specific amplification. Hence, 59.3 ^{0}C was used as annealing temperature in all forthcoming PCR.

Cloning of 3.7 Kb pqqABCDE PCR amplicon in pTZ57R/T vector

The amplified *pqqABCDE* gene products ligated to pTZ57R/T vector using was confirmed by RE digestion depicted in **figure 3.5**. RE digestion pattern confirmed that 3.7 Kb *pqqABCDE* gene cluster has been successfully ligated in pTZ57R/T vector. As a result pTPQQ-1 vector was generated.

PQQ production In Vitro

The probiotic EcN transformed with pTPQQ-1 plasmid were grown in M9 minimal medium containing 100 mM glucose. After 72 hours of inoculation, **2.26 ± .6 µg/ ml** of PQQ was produced.

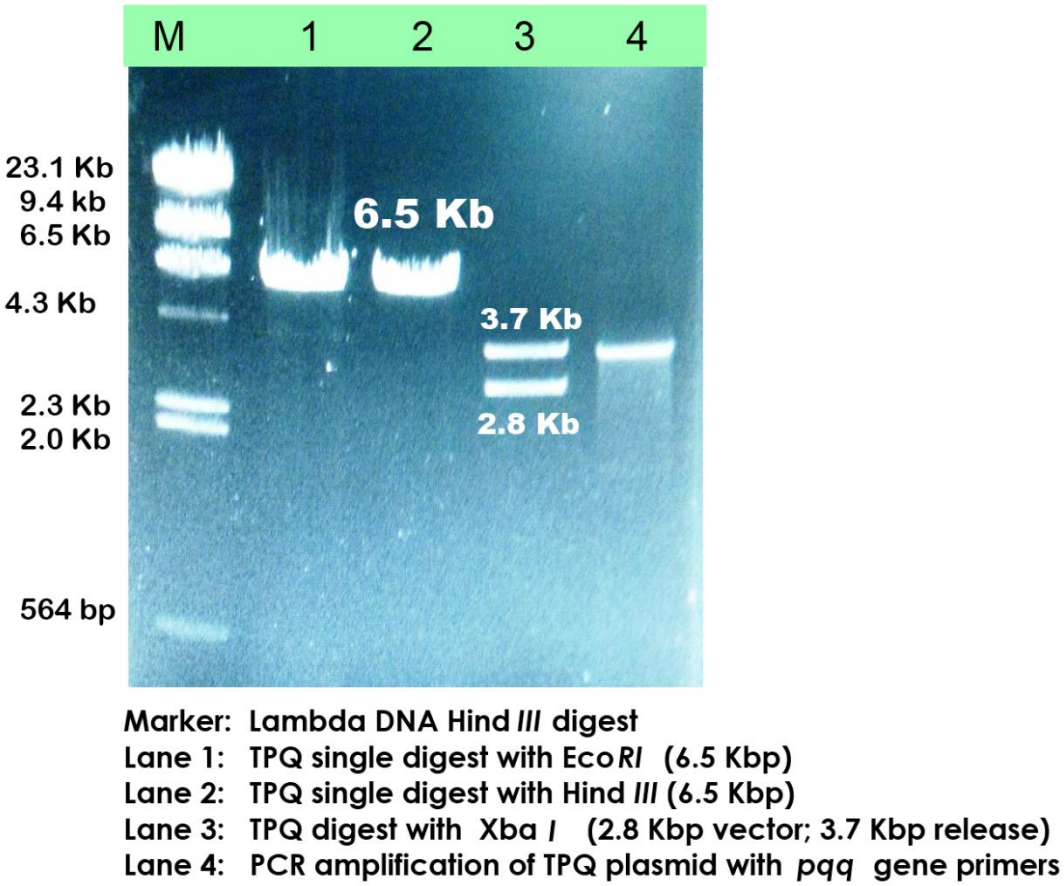

Figure 3.5 RE digestion pattern of pTPQQ-1 plasmid generated as result of ligation of *pqqABCDE* genes in pTZ57R/T vector.

3.3.2 Standardization of rotenone dose and duration for generation of systemic oxidative damage in rats

All the rats treated with rotenone 3.5 mg/kg bw died within 5 days of treatment. Rats receiving 2.5 mg/kg bw were treated till 28 days. Hepatic antioxidant parameters were performed after sacrifice **(Figure 3.6)**. Rats treated with rotenone (2.5 mg/kg bw) exhibited remarkable reduction in body weight gain. Significant increase in hepatic lipid peroxidation is also observed

in these rats as compared to vehicle control. Moreover, these rats showed drastic reduction in antioxidant enzyme activities measured in hepatic tissues.

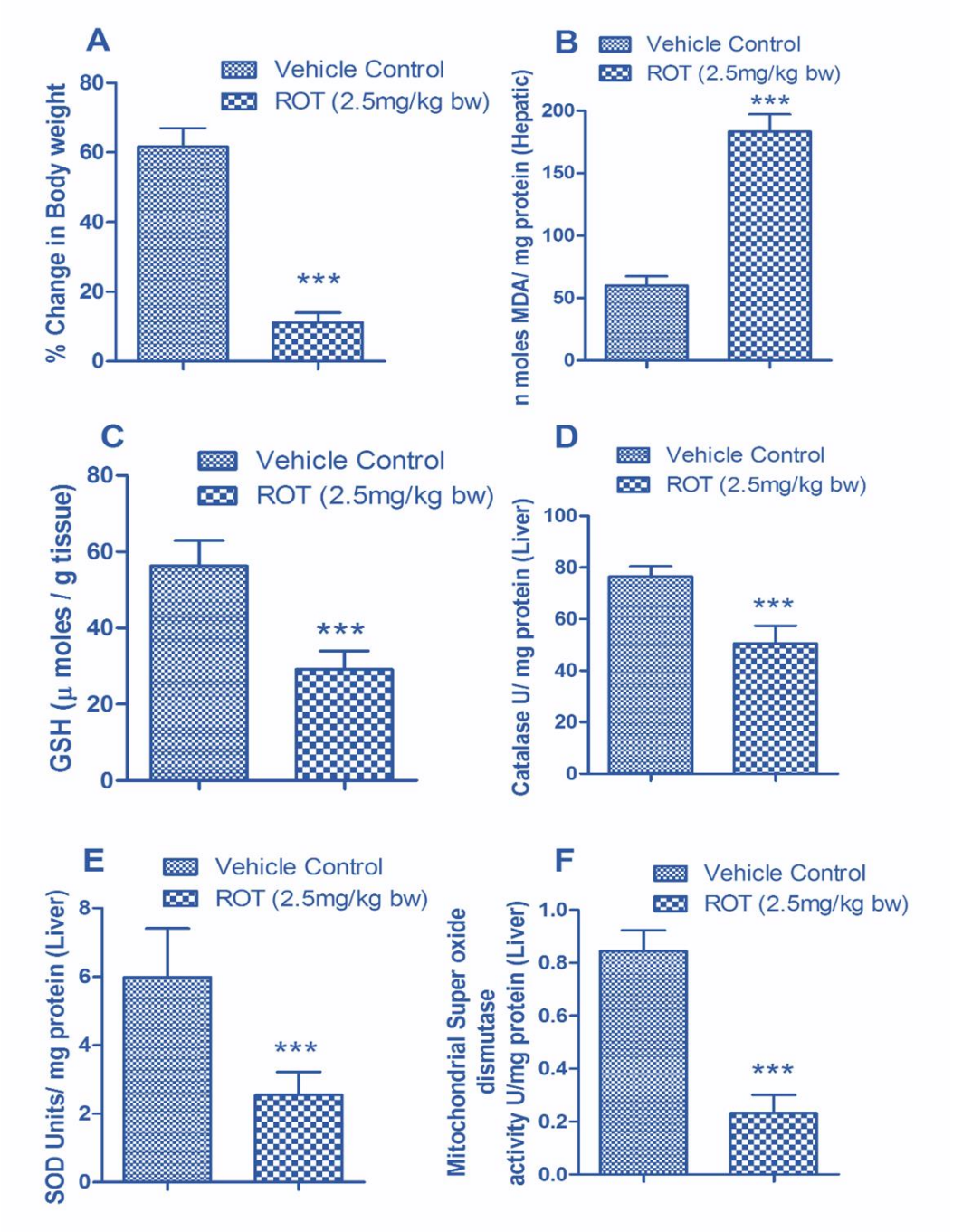

Figure 3.6 Body weight gain and antioxidant profile of rats treated with rotenone. Body weight gain (A), Hepatic lipid peroxidation (B), Hepatic GSH (C), Hepatic CAT activity (D), Hepatic SOD activity (E) and Hepatic Mit-SOD activity (F).

3.3.3 Amelioration of hepatotoxic effect of rotenone by PQQ and PQQ producing EcN-5

Body weight profile

To evaluate the protective effect of dietary PQQ and EcN-5 (PQQ producing EcN), rotenone was used to induce oxidative stress. Animals administered with rotenone exhibited severe weight loss **(Figure 3.7)**. Co-treatment of PQQ or EcN-5 significantly ameliorated the rotenone effect. However, PQQ given alone was more efficient than EcN-5.

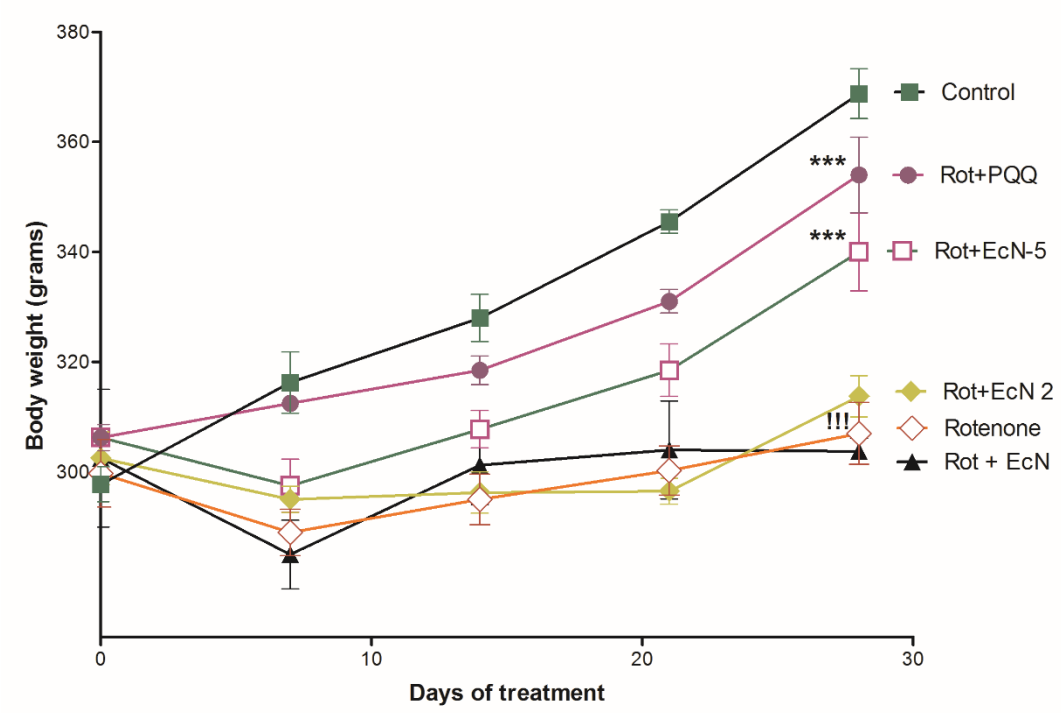

Figure 3.7 Body weight profile in rotenone induced accelerated aging model. Control group received only vehicle (sunflower oil). Rotenone group received only rotenone. Rest other groups received respective treatment along with rotenone. Experiment was carried out till 28 days.

Hepatic antioxidant status

Rotenone treated animals showed severe oxidative stress in hepatic tissues reflected by elevated lipid peroxidation, reduced enzymatic antioxidant activities (CAT and SOD) and non-enzymatic antioxidant (GSH) level (**Figure**

3.8 A-D). Animals co-treated with PQQ and EcN-5 displayed significantly lowered lipid peroxidation level along with elevated GSH level, CAT and SOD activities.

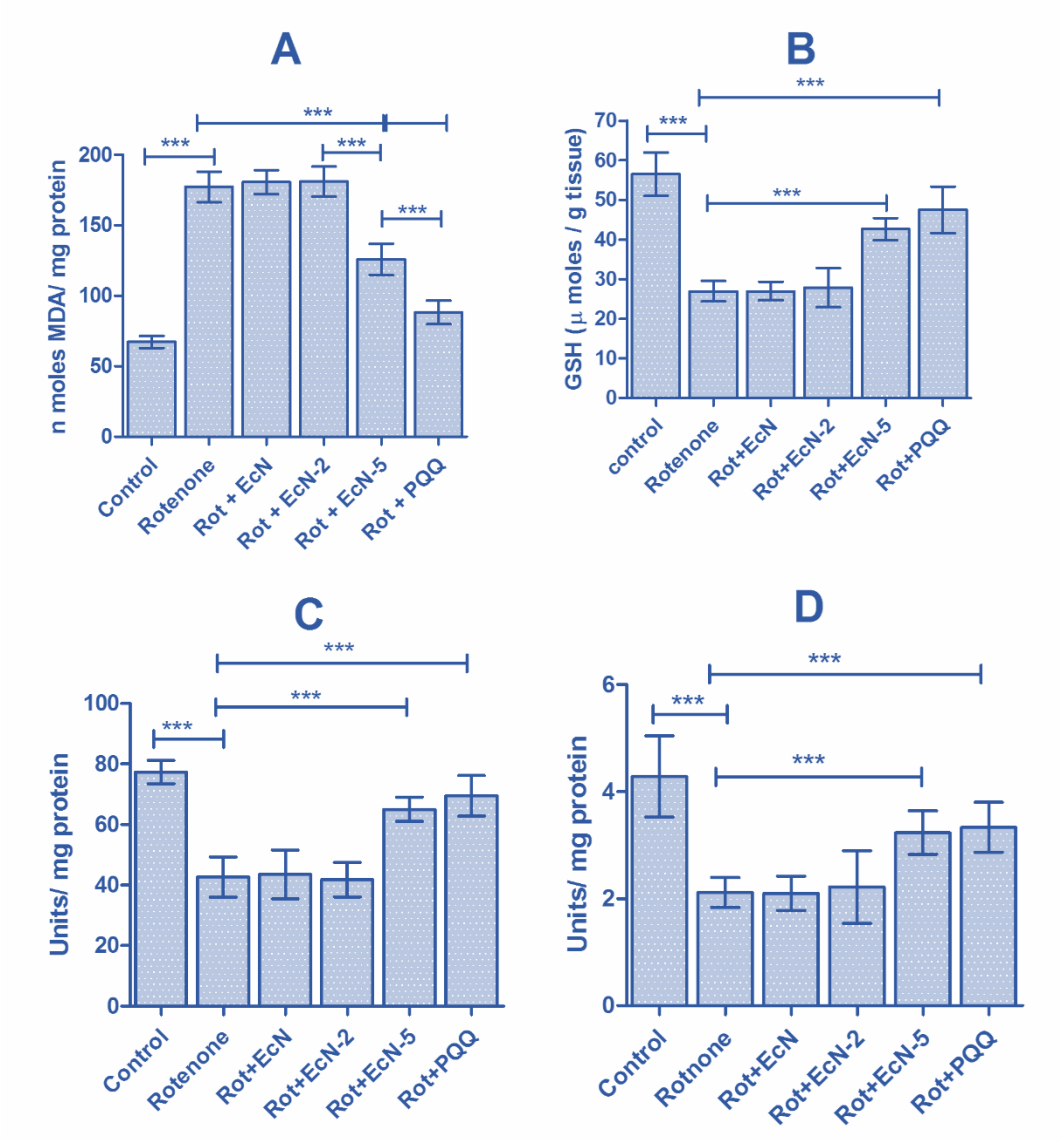

Figure 3.8 Hepatic antioxidant status of animals treated with PQQ producing EcN-5 in rotenone induced accelerated aging model. Lipid peroxidation (MDA levels) (A), GSH (B), Catalase (C) and SOD (D) were measured after 28 days of treatment. ***p<0.001 represent difference between different groups showed using horizontal bars. All values are expressed as mean ± SD (6 animals each group).

Hepatic mitochondrial metabolism and biogenesis

Hepatic mitochondrial SOD (Mit-SOD) activity was found to be significantly reduced in rotenone treated rats **(Figure 3.9)**. Co-treatment of PQQ in diet and EcN-5 blunted the rotenone effect and treated rats exhibited increase in Mit-SOD activity as compared to rotenone treated rats. Moreover, PQQ and EcN-5 treatment resulted in elevation of mitochondrial content, which was lowered by rotenone treatment. Additionally, rats treated with PQQ and EcN-5 also exhibited increase in mRNA levels of PGC-1α indicating increased mitochondrial biogenesis in these rats as compared to rotenone treated group.

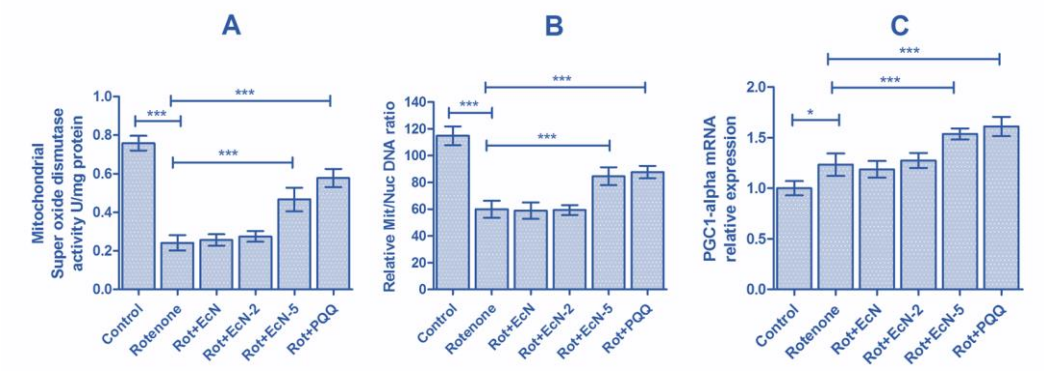

Figure 3.9 Hepatic Mitochondrial SOD activity (A), mitochondrial content (B) and PGC-1α mRNA expression of animals treated with PQQ producing EcN-5 in rotenone induced accelerated aging model. **p<0.05, **p<0.01 and ***p<0.01 represent difference between different groups showed using horizontal bars. All values in A and B are expressed as mean ± SD (6 animals each group). Values in C are expressed as mean ± SD relative to control group (6 animals each group).

3.4 Discussion

Heterologous expression of *pqqABCDE* gene cluster from *Gluconobacter suboxydans* in *Escherichia coli* Nissle 1917 was successful and the transformants could produce 2.26 ± .6 µg/ ml of PQQ after 72 h when grown in minimal medium containing 100 mM glucose as carbon source. Yang et al., (2010) has reported that when *pqqABCDE* gene cluster is expressed in *E. coli* DH-5α under T7 promoter, It could produce approximately 2 µg/ ml of PQQ after 60 h when grown in minimal medium containing glucose as carbon source. The present study suggest that *E. coli* Nissle harbouring *pqqABCDE* gene cluster from *G. oxydans* produces PQQ more or equivalent to published reports.

Free radical theory of aging proposes that ROS generated from ETS with in mitochondria during routine metabolism, in long term affects cellular efficiency and senescence (Harman, 1992). Rotenone, eukaryotic mitochondrial complex I inhibitor, has been used in the present study to create an accelerated aging rat model to evaluate the protective efficacy of PQQ and PQQ producing probiotic *E. coli* Nissle 1917 against increased mitochondrial ROS (Li et al., 2003).

Standardization experiment revealed that rotenone dose of 3.5 mg/kg body weight give intra-peritionally is toxic to rats and animals treated could not survive more than 5-7 days of daily treatment. However, lower rotenone dose of 2.5 mg/kg body weight did not exhibit any mortality and animals showed severe oxidative damage and reduced antioxidant defence. Therefore, further experiment was carried out using this dose.

Rats treated with rotenone (2.5 mg/kg body weight) daily for 28 days, exhibit severe weight loss and reduction in hepatic antioxidant status which is due to increased ROS production in the mitochondria. Co-treatment of PQQ in diet and EcN-5 (PQQ producing EcN) significantly reduced hepatic antioxidant status, which could be correlated to other tissues, and eventually restores bodyweight. PQQ is water soluble antioxidant and has been shown to permeate subcellular organelles including mitochondria and modulates its

quantity and function (Sites et al., 2006; Tao et al., 2007). In accordance with previously reported reports, the present study demonstrates that PQQ given in the diet or produced by EcN-5 in the gut, is absorbed and transported to liver, where it scavenges free radicals (cytosolic and mitochondrial) and boosts antioxidant status.

Rotenone treatment also hampers hepatic mitochondrial SOD (SOD-2) activity, biogenesis and content in treated rats. Mit-SOD activity was found to be reduced by 70-75 % in rotenone treated rats. Thus, rotenone treatment influences total mitochondrial content as well as mitochondrial antioxidant enzyme activity. PQQ is well known transcriptional regulator of genes associated with mitochondrial biogenesis and metabolism (Sites et al., 2006; Tao et al., 2007; Rucker et al., 2009). PGC-1α is transcriptional regulator of mitochondrial biogenesis. In the present study PQQ prevents cellular and mitochondrial ROS by quenching them and promotes mitochondrial biogenesis and content in the treated animals and hence blunt the effect of rotenone toxicity in rats.

In conclusion, PQQ producing probiotic *E. coli* Nissle 1917 prevents hepatic oxidative damage and improves mitochondrial antioxidant status, biogenesis and content in rotenone induced accelerated aging rat model. It would be interesting to demonstrate the efficacy of PQQ producing EcN in naturally aging rat model in a long term experiment.