Chapter 5

5. RESULTS

This section presents the outcomes of both *in-vitro* and *in-vivo* studies that evaluated the impact of chloramphenicol on liver toxicity and the hepatoprotective efficacy of Astaxanthin and Quercetin.

5.1 In-Vitro Results (Mitochondrial Toxicity Assessment by ATP Assay)

Long-term exposure (6 days) of HepG2 cells to chloramphenicol (3–3000 μM) led to significant ATP depletion. The IC values were 797.39 μM in glucose medium and 130.61 μM in galactose medium. The fold change (6.11) indicated pronounced mitochondrial toxicity (threshold >1.5). (Table 5 and Graph A-Fig.6)

- Co-treatment with Astaxanthin (5–15 μM) restored ATP levels to 32.32% in glucose and 30.55% in galactose media. IC fold change reduced to 0.787, indicating mitigation of toxicity. **(Table 6 and Graph B-Fig.6)**
- Co-treatment with Quercetin (10–30 μM) resulted in an ATP fold change of 1.38, demonstrating moderate protection. **(Table 7 and Graph C-Fig.6)**
- These results indicate that when cells were exposed to chloramphenicol in the presence of Astaxanthin or Quercetin, the toxicological impact of chloramphenicol was significantly reduced. To support these findings, IC50 values were calculated using non-linear regression in GraphPad Prism, and group comparisons of ATP levels were analyzed using one-way ANOVA followed by Dunnett's multiple comparisons test ($p < 0.05$ was considered statistically significant).

Assessment of cellular Adenosine Triphosphate (ATP)

Fold change of Adenosine Triphosphate (ATP) amount ratio of test was calculated by diving observed IC50 Glucose Vs IC50 Galactose. IC50 was calculated using Graph Pad Prism software (significance at $p < 0.05$). If Fold change of IC50 is > 1.5 drug shall be considered toxic.

5.1.1 Assessment of impact of antioxidants on Mitochondrial toxicant by ROS

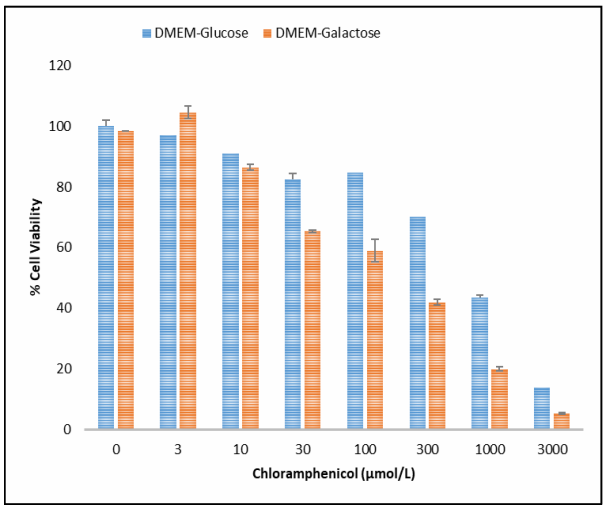
For comparison of ROS production in presence of Chloramphenicol alone and Chloramphenicol with antioxidants, cells were maintained similarly as done for respiration

studies and the assay procedure was followed as suggested in Promega ROS-Glo™ H2O2 assay kit protocol.

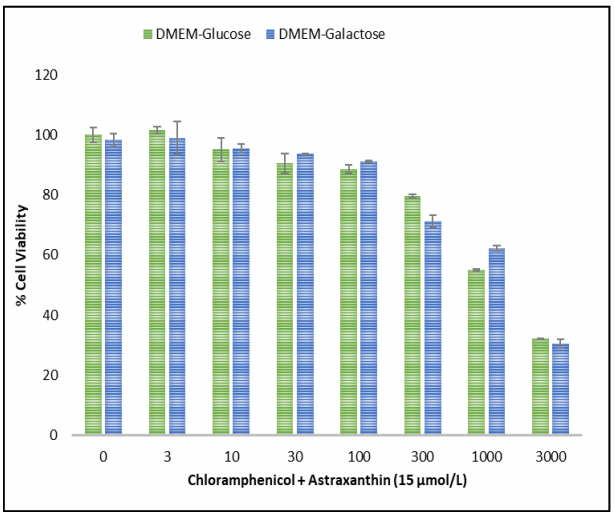
- Chloramphenicol (3000 µM) exposure induced a ~10.4-fold increase in ROS levels compared to control (4585905 vs. 442084 RLU).
- Astaxanthin (10 µM) reduced ROS to 1565008 RLU.
- Quercetin (25 µM) reduced ROS further to 1034323 RLU.
- Positive control (Rotenone) induced ROS at 5269391 RLU.

The antioxidant effect was statistically significant ($p < 0.005$, ANOVA with Dunnett's test).

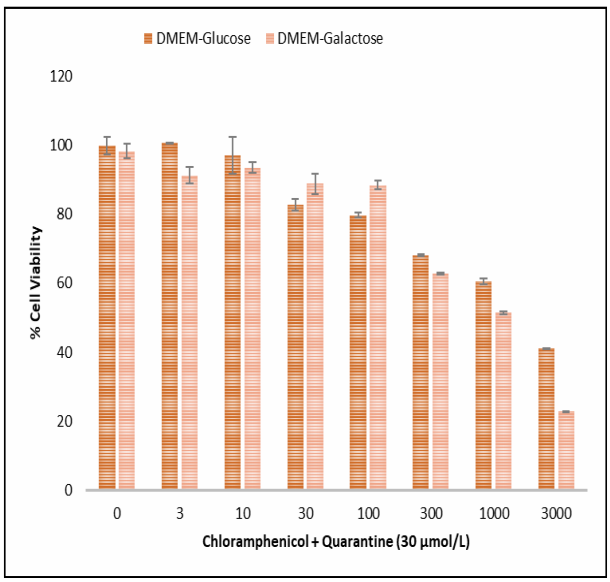
(Table-8 and Graph D-Fig.6)



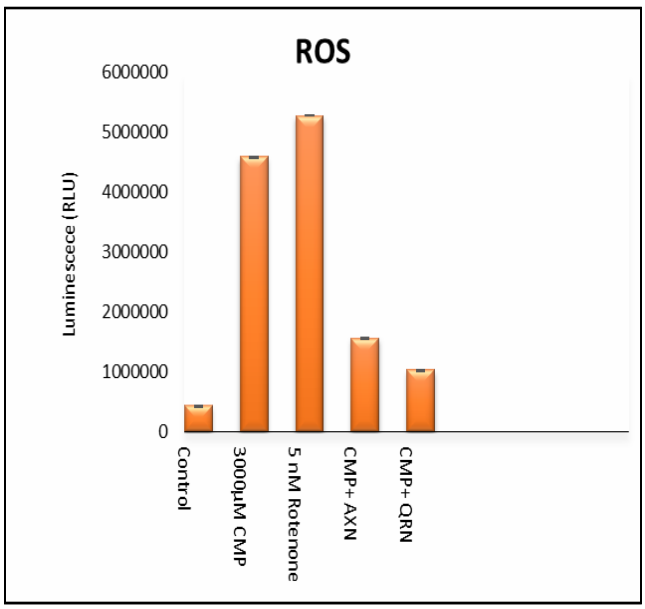
Graph: A



Graph: B



Graph: C



Graph: D

Figure 6: Graphs A–C show ATP-based cytotoxicity and Graph D compares ROS levels across treatment groups

Note: Graphs A–C show ATP-based cytotoxicity in HepG2 cells exposed to chloramphenicol alone (A) or co-treated with Astaxanthin (B) or Quercetin (C). IC values were calculated using non-linear regression. Graph D compares ROS levels across treatment groups, including untreated (negative control) and Rotenone-treated (positive control) cells. Co-treatment with Astaxanthin (AXN) or Quercetin (QRN) significantly reduced ROS production compared to chloramphenicol (CMP) alone. Data are presented as mean ± SEM (n = 3). Statistical comparisons were performed using one-way ANOVA with Dunnett's post-test ($p < 0.005$ vs. CMP).

Test substance	Concentration (µmol/L)	DMEM-Glucose Medium		DMEM-Galactose Medium		Fold change
		ATP amount ratio (%)	IC50 (µmol/L)	ATP amount ratio (%)	IC50 (µmol/L)	
Chloramphenicol	0	100.00	797.39	98.30	130.61	6.11
	3	97.09		104.58		
	10	91.11		86.39		
	30	82.38		65.32		
	100	84.60		58.84		
	300	70.19		41.92		
	1000	43.88		19.85		
	3000	13.78		5.23		

Table 5: Long-term exposure of chloramphenicol

Test substance	Concentration (µmol/L)	DMEM-Glucose Medium		DMEM-Galactose Medium		Fold change
		ATP amount ratio (%)	IC50 (µmol/L)	ATP amount ratio (%)	IC50 (µmol/L)	
Chloramphenicol + Astaxanthin	0	100.00	1255.88	98.30	1596.16	0.787
	3	101.56		98.89		
	10	95.12		95.46		
	30	90.43		93.63		
	100	88.56		91.14		
	300	79.59		71.23		
	1000	55.04		62.27		
	3000	32.32		30.55		

Table 6: Long-term exposure of chloramphenicol+ Astaxanthin

Test substance	Concentration (µmol/L)	DMEM-Glucose Medium		DMEM-Galactose Medium		Fold change
		ATP amount ratio (%)	IC50 (µmol/L)	ATP amount ratio (%)	IC50 (µmol/L)	
Chloramphenicol + Quercetin	0	100.00	2090.71	98.30	1510.42	1.38
	10	100.68		91.31		
	30	97.22		93.54		
	100	82.73		88.85		
	300	79.75		88.53		
	1000	68.15		72.79		
	3000	60.55		61.39		
	6000	41.07		22.85		

Table 7: Long-term exposure of chloramphenicol+ Quercetin

Sr. No.	Treatment	Mean Response (RLU)
1	Control	442084
2	Chloramphenicol (CMP)	4585905
3	Rotenone	5269391
4	CMP+ AXN	1565008
5	CMP+ QRN	1034323

Table 8: Comparison of ROS production

5.1.2 Assessment of Mitochondrial etiology status through Gene expression study

1. Gene expression:

Quantitative PCR (qPCR) analysis was performed on a panel of genes significantly implicated in liver toxicity. This panel included SURF1, SOD2, NRF1, TFAM and UCP2. We used housekeeping genes RPLP as a reference. Real-time PCR was employed to evaluate changes in the gene expression profiles after 6 days of drug exposure to HepG2 cells. Concentration of Chloramphenicol and antioxidant astaxanthin and Quercetin were chosen based on the cytotoxicity and ROS experiments outcome. Each mRNA/sample assigned a CT value calculated from the 7500 Fast Real Time PCR instrument software. The difference in threshold cycle between the targeted gene and the reference/housekeeping gene was used to represent ΔCT. The difference in ΔCT between treatment group and vehicle control group was expressed as ΔΔCT and fold changes were calculated with 2 -ΔΔCT compared to control vs CMP and CMP vs AXN or QRN. Calculated 2 -ΔΔCT value indicates that after long term exposure of chloramphenicol, gene expression of SURF1 and TFAM is showing downregulation, however, for NRF1, SOD2 and UCP2 upregulation is observed. Interestingly, in the presence of antioxidants (Astaxanthin and Quercetin), the expression levels were significantly reversed compared to observed in the presence of Chloramphenicol alone. Statistical analysis of gene expression data was conducted using one-way ANOVA followed by Bonferroni's multiple comparisons test to evaluate differences among treatment groups. Statistical significance was considered at $p < 0.05$. (Fig.7).

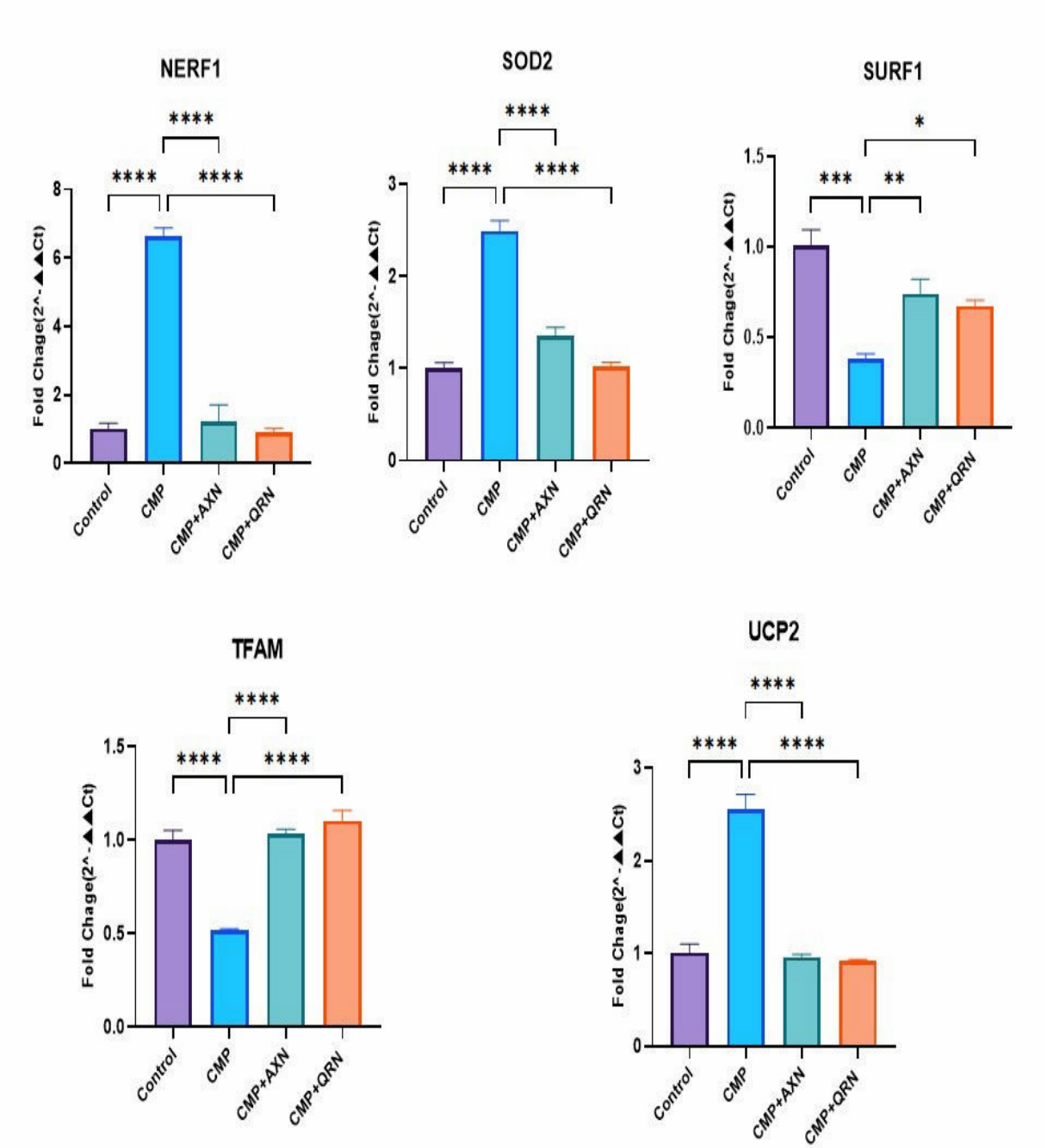


Figure 7: Graphs demonstrating the fold change representing relative gene expression for selected genes using RT-qPCR in HepG2 cells.

Note: Graphs demonstrating the fold change representing relative gene expression for selected genes using RT-qPCR in HepG2 cells. The gene expression was checked for controls, in response to chloramphenicol alone, or in combination with astaxanthin or quercetin. Results are presented as means ± SEM, where n=3. Significance levels are indicated as ***P value < 0.001 vs control and ***P value < 0.003 Chloramphenicol Vs Antioxidants (one-way ANOVA followed by Bonferroni's post-test).

5.2 *In-Vivo* Results

Both antioxidants, Astaxanthin and Quercetin, significantly mitigated the oxidative stress and toxicity induced by chloramphenicol, which is evident by observed recovery of GSH levels and reduction in NO levels. This demonstrates that both the antioxidants have hepatoprotective effects against Chloramphenicol-induced toxicity.

1. **Glutathione (GSH) Levels**
   - Control group: 8.5 ± 0.6 nmol/mg
   - Chloramphenicol group: 4.1 ± 0.2 nmol/mg ($p < 0.0001$ vs. control)
   - Astaxanthin group: 7.2 ± 0.2 nmol/mg ($p = 0.0305$ vs. CMP)
   - Quercetin group: 7.8 ± 0.1 nmol/mg (not significant vs. control; $p < 0.0001$ vs. CMP)

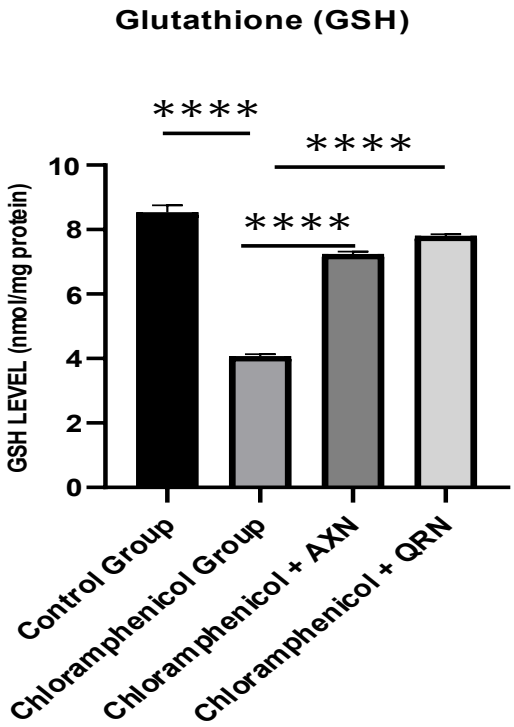
   Quercetin demonstrated slightly superior efficacy in restoring GSH.

2. **Nitric Oxide (NO) Levels**
   - Control group: 15.6 ± 0.7 µM
   - Chloramphenicol group: 25.4 ± 0.6 µM ($p < 0.0001$ vs. control)
   - Astaxanthin group: 17.1 ± 0.3 µM ($p = 0.0323$ vs. control)
   - Quercetin group: 16.4 ± 0.2 µM ($p = 0.0349$ vs. control)

Both antioxidants effectively attenuated NO elevation caused by chloramphenicol.

Observed GSH and NO levels in Rat Blood		
Group	**Mean GSH Levels (nmol/mg)**	**Mean NO Levels (µM)**
Control Group	8.5, 8.0, 9.2, 7.8, 8.7, 9.0	15.6, 14.8, 15.2, 16.3, 14.7, 16.2
Chloramphenicol Group	4.1, 3.8, 4.3, 4.0, 4.2, 4.0	25.4, 26.0, 24.8, 25.7, 25.1, 26.3
Chloramphenicol + Astaxanthin	7.2, 7.5, 6.9, 7.3, 7.1, 7.4	17.1, 16.8, 17.5, 16.9, 17.2, 16.7
Chloramphenicol + Quercetin	7.8, 7.6, 7.9, 7.7, 8.0, 7.8	16.4, 16.2, 16.7, 16.5, 16.3, 16.8

Table 9:Observed GSH and NO levels in Rat Blood



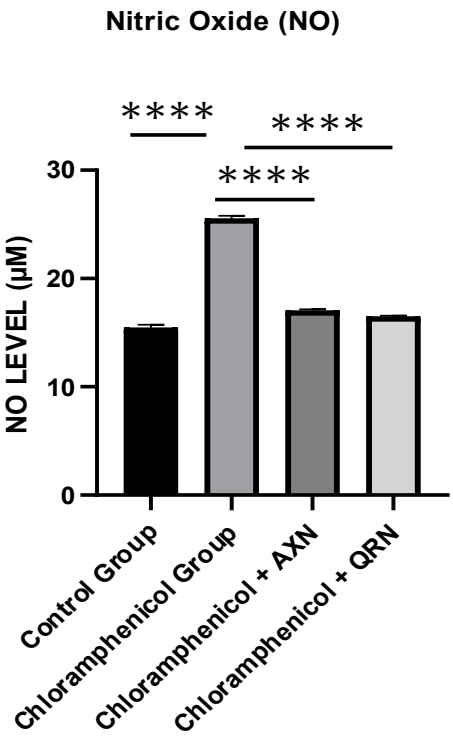


Fig: 8 **Fig:9**

Figure 8&9: Effect of antioxidants on GSH and NO levels

Note: Data expressed as mean ± standard deviation (SD). Statistical comparisons between groups were conducted using one-way analysis of variance (ANOVA) followed by Sidak's multiple comparisons test. Significance level set at $p < 0.05$.

3. Summary of Key Findings

Study Model	Parameter	Chloramphenicol Effect	Astaxanthin Effect	Quercetin Effect
In-Vitro	**ATP Levels**	↓↓↓ (Mitotoxicity)	↑↑↑ (protective)	↑↑ (moderate)
	ROS Levels	↑↑↑	↓↓	↓↓↓
	Gene Expression	TFAM↓, SURF1↓; SOD2, NRF1, UCP2 ↑	Reversal of changes	Reversal of changes
In-Vivo	**GSH Levels (*in-vivo*)**	↓↓	↑↑	↑↑↑
	NO Levels (*in-vivo*)	↑↑	↓↓	↓↓↓

Table 10: Summary of key findings