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Protective Effect of *Emblica officinalis* in *Cyprinus carpio* against Hepatotoxicity Induced by Malachite Green: Ultrastructural and Molecular Analysis

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Abstract: Malachite green (MG) dye, besides coloring is used as an effective aquaculture therapeutic. The present study assesses the mitigating ability of *Emblica officinalis* (EO) fruit extract against the dye induced chronic (60 days) cyto-toxicity in *Cyprinus carpio*. For this, four experimental groups were maintained: group I—control, group II—MG, group III—EO (positive control), group IV—MG + EO. The study was made at three tiers: detailing structural anomalies using a light microscope and transmission electron microscope (TEM), biochemical estimation of antioxidant enzymes, and lipid peroxidation and molecular analysis of expression patterns of HSP70, and CYP1A genes. MG intoxication resulted in necrosis, cytoplasmic vacuolation, glycogen depletion, abundant macrophages, loss of cell integrity and prominent nuclear alterations. Significant ($p < 0.05$) inhibition in the activities of catalase (CAT), superoxide dismutase (SOD), glutathione-S-transferase (GST) and reduced glutathione (GSH), along with an elevation in malondialdehyde (MDA) levels, occurred after 60 days of MG exposure. CYP1A and HSP70 genes presented a significant change in their expression in MG treated fish. Whereas oral supplementation with EO significantly restored the histo-architecture, normalized the altered enzymatic activity, reduced the oxidative stress level and regulated the expression of HSP70 and CYP1A genes. Thus, it can be concluded that EO acted as an effective ameliorant against malachite green induced cyto-toxicity in *Cyprinus carpio*.

Keywords: amelioration; *Cyprinus carpio*; liver; malachite green; qRT-PCR; toxicity; oxidative stress

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1. Introduction

Malachite green (MG) is a dye belonging to the tri-aryl methane family [1]. It is vividly used in dyeing textiles, leather, paper, fur, and confectionaries [2–5]. The unrestricted release of dye effluents in water poses non-targeted toxicity to the fish and aquatic organisms, affecting their vision, physiology, genetic constitution, and histology [6–9]. Despite its reported toxicity, it is still being used illegally in many Asian countries (India and Bangladesh) as an aquaculture therapeutant in Saprolegniasis and *Ichthyophthirius multifiliis* infection [10–14]. MG has been reported to severely affect the aquatic biota due to its non-biodegradable nature and tendency to bio accumulate [15,16]. MG metabolization in the fish body produces free radicals, developing oxidative stress and consequent structural lesions [17].

The study of structural deformations in response to toxicants elucidates their impact on tissues [18–21]. Histopathological studies serve as reliable biomarkers for toxicity assessment [22]. Transmission electron microscopy details the damage incurred at the cellular level, illustrating the affected organelles. Antioxidant enzymes (catalase and

super-oxide dismutase) are first line of defense against oxidative stress and neutralize the reactive oxygen species (ROS) produced by toxicants. The development of stress induces modification in enzyme activity and genetic expression. Heat shock proteins (70 kDa) are molecular chaperones, performing de novo protein folding, the degradation of miss-folded proteins and other regulatory processes [23]. CYP1A, belonging to the cytochrome family, constitutes enzymatic oxidants that are actively involved in the bio-elimination of xenobiotics [24]. Considering MG's impact on genes, the expression of heat shock proteins (HSP70) and cytochrome 450A (CYP1A) was observed for the present investigation.

Emblica officinalis (EO) fruit, commonly known as Indian gooseberry, is a cheap, non-toxic and bio-compatible herbal protector [25,26]. It is reported to possess nephroprotective, anti-oxidative, hepato-protective, and anti-cancerous properties [27,28]. EO fruit is a prominent source of ascorbic acid (vitamin C), tannins, alkaloids, phenols, and flavonoids [29–31]. The supplementation of herbal extracts has been shown to augment heavy metal and pesticide induced oxidative stress in organisms [32–34]. Studies have demonstrated that the supplementation of ascorbic acid, a major component of EO, improves the immunity and health of fish [35,36]. Therefore, an attempt has been made to evaluate the risk associated with the chronic exposure of malachite green, and to assess the reparative effect of dietary supplementation of *Emblica officinalis* in *Cyprinus carpio* at the ultra-structural and molecular level. The present investigation holds importance by augmenting MG toxicity and aiding in the detoxification of dye from the fish, making it suitable for human consumption.

2. Materials and Methods

2.1. Toxicant Used, Procurement of Fish and Experimental Setup

Malachite green oxalate of analytical grade was purchased from Central Drug House (CDH, New Delhi, India). Stock solution (1 mg/L) was made with distilled water and stored in a dark container to prevent photolysis. Healthy fingerlings (12 ± 1 cm, 18 ± 1 gm) of *Cyprinus carpio* were procured from the Sultan Fish Seed Farm, Karnal, Haryana. These were brought in oxygen filled air bags and acclimated (15 days) in a glass aquarium (145 × 43 × 32 cm) fitted with aerators and filters. Fish (n = 120) were equally distributed in 4 experimental groups in triplicate. Semi-static assay was carried out in 65 L tanks of dimension 60 × 30 × 30 cm. The physico-chemical characteristics maintained for water were pH 7.2 ± 0.1, dissolved oxygen 8.0 ± 0.3 mg/L, temperature 25 ± 2 °C, total alkalinity 175 ± 10 mg/L and total hardness 18 ± 0.5 mg/L. Additionally, 96 h LC50 of MG was found to be 0.438 mg/L using Probit analysis [37]. Chronic bioassay with sub-lethal concentration, 0.146 mg/L (1/3rd of LC50 of MG), was maintained following OECD guidelines [38]. The measured concentration of MG has been found to be 0.100 mg/L against a nominal value of 0.146 mg/L using Max Signal MG/LMG ELISA Test Kit (Bioo Scientific, Austin, TX, USA) with a detection limit of 0.07 ng/g or ppb for fresh water. Experimental groups were maintained as group I—control, group II—0.146 mg/L MG exposed, group III—positive control fed with *Emblica officinalis*, and group IV—0.146 mg/L malachite green + *E. officinalis* co-supplementation for 60 days. Fish were fed according to their body weight (2% BW), twice a day (at 9 and 17 h), and feeding was stopped 24 h prior to sampling. For toxicity assessment, fish were sampled at 15, 30 and 60 days of experiment and were euthanized through cervical dislocation following the guidelines of the Institutional Animal Ethical Committee (IAEC), Panjab University, Chandigarh with Ref. no. PU/IAEC/S/14/150 dated 22 January 2014.

2.2. Feed Preparation

Emblica officinalis fruit was procured from the university campus, identified, and verified (Voucher No. 20619) from a taxonomist of the department of Botany, Panjab University, Chandigarh. The fruits were shade dried, ground, extracted in ethanol (absolute) using Soxhlet extraction method, lyophilized (MAC Lyophilizer, Mumbai, India) and evaporated (Roteva Equitron, Mumbai, India) [39]. EO extract was analyzed for phytochemical components using RP-HPLC (Reverse Phase High performance liquid

chromatography) (Shimadzu, Kyoto, Japan), equipped with two LC-10 ATVP pumps, an SPD-10AVP UV–vis detector, a C18 column (4.5 × 250 mm), and a Rheodyne injector with a 20 µL loop at 275 UV detector wavelength and with a detection limit of 0.008 µg/mL. The results obtained were processed with Shimadzu LC-solution software (6.42). Standards had regression values R^2 = 0.998 and 0.997, and line equation y = 2884x + 12,822 and y = 23,616x − 15,280 for ascorbic acid and gallic acid, respectively. Observed phytochemical components in EO extract were gallic acid (640.1 ± 5.440 mg/g, RT—3.139 min) and ascorbic acid (197.7 ± 4.378 mg/g, RT—2.668 min). Diet supplement was prepared by adding 1000 mg of the ethanolic extract of *E. officinalis* (EO) per kg of the fish feed, as described previously [9]. The ingredients were combined to make dough, pelleted to about 3 ± 1 mm granules and were fed to the fish twice a day.

2.3. Histopathological Studies

Cyprinus carpio were euthanized using cervical dislocation and liver tissue was extracted in cold saline and processed following Baker [40] and Pearse [41]. 3 µ thick sections were obtained and stained using Hematoxylin and Eosin. Sections were viewed under light microscope (Leica DC 100, PC 1 Interface Digital Camera, Washington, DC, USA). Histo-pathological alteration index (HAI) was carried out for semi-quantitative analysis and determination of the degree of change induced in the sample [22]. A total of 50 random sections from each experimental group were studied. Scoring (S: 0–6) was assigned based on response, and importance factor (I: 1–3) based on the intensity of the particular response affecting fish health. The index for each histopathological alteration was given by:

$$\text{Index for each histopathological alteration Ialt} = \sum (\text{Sorg alt} \times \text{Iorg alt})$$

$$\text{Total pathological index of the organ by Iorg} = \sum\sum (\text{Sorg alt} \times \text{Iorg alt}).$$

where "org" is the organ (liver); "S org alt" is the score value for a specific alteration of the organ; and "I org alt" is the importance factor for a specific alteration of an organ.

2.4. Ultra-Structural Studies

Extracted liver tissue was sectioned (>1 mm), fixed in 2% glutaraldehyde-paraformaldehyde overnight and further processed following Reynolds [42]. Dehydrated in acetone in ascending concentration (50% to 100% for 10 min each), polymerized by thermal curing at 45 °C for 1–2 h and raised to 80 °C for 48 h. Sections of 1 µ were stained with 1% toluidine blue, made into copper grids, and viewed under Leo Morgagni268d, Netherlands, TEM at AIIMS, New Delhi.

2.5. Oxidative Stress

For the determination of oxidative stress, the activity of enzymatic and non-enzymatic antioxidants (catalase (CAT), superoxide dismutase (SOD), glutathione-s-transferase (GST), and reduced glutathione, (GSH)) and lipid peroxidation (LPO) markers were measured [43–47]. Post-mitochondrial supernatant was obtained by homogenizing and centrifuging (10,000 rpm) the extracted liver in cold phosphate buffer (0.1 M). Homogenate suspensions were retained for LPO and GSH analysis and were spectrophotometrically analyzed on a UV/VIS spectrophotometer, UV 3000+ (Lab India Analytical).

2.6. RNA Extraction, cDNA Synthesis and qRT-PCR

To obtain total RNA, liver tissue was crushed and processed following the protocol of the Thermo-fischer RNA extraction kit, and further analyzed for the purity and concentration on Nano drop (Nano Spectrophotometer BSNA-101).

cDNA was synthesized using the Thermo-fischer cDNA synthesis kit in a thermocycler following incubation of the 2 µL RNA and primer mixture at 65 °C for 15 min, snap-chill for 5 min and incubation at 42 °C for 1 h. cDNA formed was stored at −80 °C till further use. Primers were designed using NCBI Blast. The criteria used for primer

selection were: Tm (58–60 °C), length (18–24 nt), GC content (50–60%) and amplicon size (100–200 bp). Primers were checked for secondary structure, primer-dimer and spanning intron–exon junction. Gene sequences used for mRNA expression studies were HSP70 (F-GCTGGAGAAAGTCTGCAATCC, R-TCCCTGGACCATTCATTCCT, Accession No.-XM_019074376.1) and CYP1A (F-AGTGGCCTACCCTGAGATCC, R-TCCATTGAGCGAC GTGTCTT, Accession No. XM_019064218.1). 18S (F-GAATTGACGGAAGGGCACCACC, R-AACCAGACAAATCGCTCCACCAAC, [48]) was considered as normalizer.

For quantitative differential expression, cDNA samples from the control and treated group were run on Roche Light Cycler® 480 (Roche Life Science, Basel, Switzerland). PCR reaction mix was comprised of 5 µL of Q-PCR Master mix (Kapa Sybr® Fast), forward and reverse primers (1 µL each), 1 µL of cDNA and 2 µL of nuclease free water. PCR conditions followed initial denaturation (94 °C for 2 min), 35 cycles of denaturation (94 °C), annealing (59–61 °C), and extension (72 °C) for 30 s each. Relative fold expression of genes was determined with the $2^{-\Delta\Delta CT}$ method [49].

2.7. Bioaccumulation

The bio-concentration of MG was measured in hepatic tissue of the fish through competitive colorimetric Enzyme Linked Immune Sorbent Assay (ELISA). For this, samples were prepared following the instructions of the Max Signal MG/LMG ELISA test kit (Bioo Scientific, Austin, TX, USA) and processed in MG-antibody coated wells with a detection limit of 0.1 ppb in the fish sample.

2.8. Statistical Analysis

The data were statistically analyzed with two-way ANOVA followed by post hoc Tukey's test at the significance level $p < 0.05$ using SPSS (version-18), and represented as mean ± SE. Principal component analysis (PCA) was performed to analyze the variance in the entire data set comprising of various assessment parameters such as LPO, CAT, SOD, GST, GSH, and HAI. A principal component (PC) with an Eigen value greater than "1" was retained and observed in the scree plot.

3. Results

3.1. Histopathology and Ultra-Structural Studies

The histo-architecture of the *C. carpio* liver displayed hepatocytes with central nucleus and interspersed blood spaces (sinusoids) under light microscope (Figure 1A). Further, TEM of the liver detailed the central nucleolus in the nucleus, enclosed by the nuclear membrane extending into RER, and the marked presence of rod to ovoid shaped mitochondria (Figure 1B). Space of Disse, space amid the hepatocyte and sinusoids were evident with investing microvilli from the hepatocyte (Figure 1C). Bile canaliculi (BC) were lined with dense microvilli and apparent GC cisterns in its vicinity. Ample glycogen content was also found (Figure 1D).

MG exposed *C. carpio* evidenced erythrocytes infiltration, pyknotic and hypertrophied cells under light microscope (Figure 2A). Concomitantly, TEM studies displayed MG-induced karyological alterations such as pyknosis and organelle deformations including the loss of mitochondrion cristae and RER fragmentation (Figure 2B). Bile canaliculi showed membrane whorls and phagosomes in the vicinity (Figure 2C), indicating hampered cellular morphology and physiology. Instead, the amelioration (MG + EO) assay of 15 days histologically revealed the presence of pyknotic nuclei (Figure 2F). EO-induced defense system was evident with ultra-structural findings of the marked presence of macrophage cell and digested organelles in phago-lysosomes in the hepatocytes (Figure 2G,H). Histo-pathological study made in fish of group III, EO supplemented, showed a hepatocyte structure similar to the control (Figure 2E); however, the electron-micrograph also documented proliferated RER in the hepatocytes (Figure 2D).

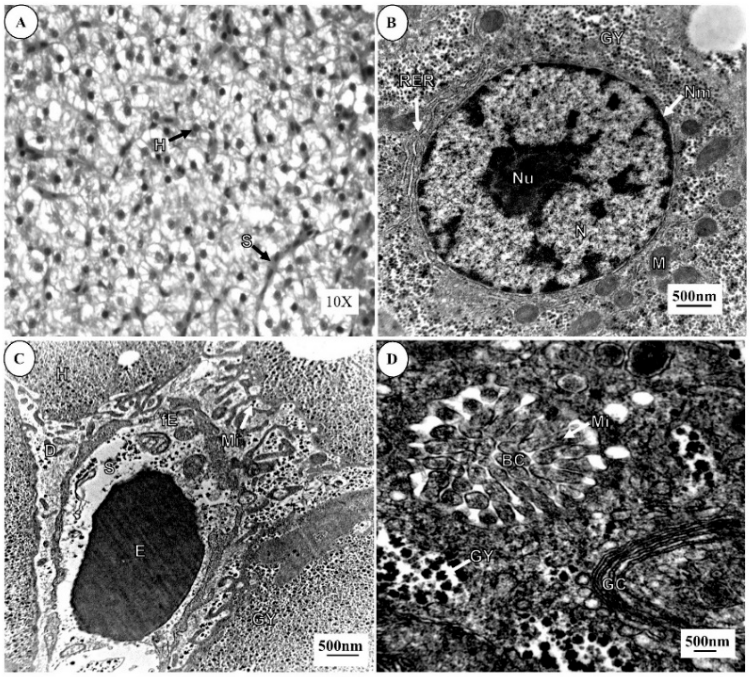


Figure 1. Hepato-architecture of control *C. carpio*. (**A**) (10×): Liver with sinusoid (S). (**B**): Hepatocyte (H) showing central nucleolus (Nu), spherical nucleus (N) enclosed in nuclear membrane (Nm) and extended into RER, spherical to oval mitochondria (M) and abundant glycogen (GY) molecules. (**C**): Space of Disse (D) present between hepatocytes (H) and sinusoid (S), bearing erythrocyte (E) enclosed within fenestrated endothelium (fE). Microvilli (Mi) radiate in the space between. (**D**): Bile canaliculi (BC) with irradiating microvilli (Mi) and cisterns of Golgi complex (GC).

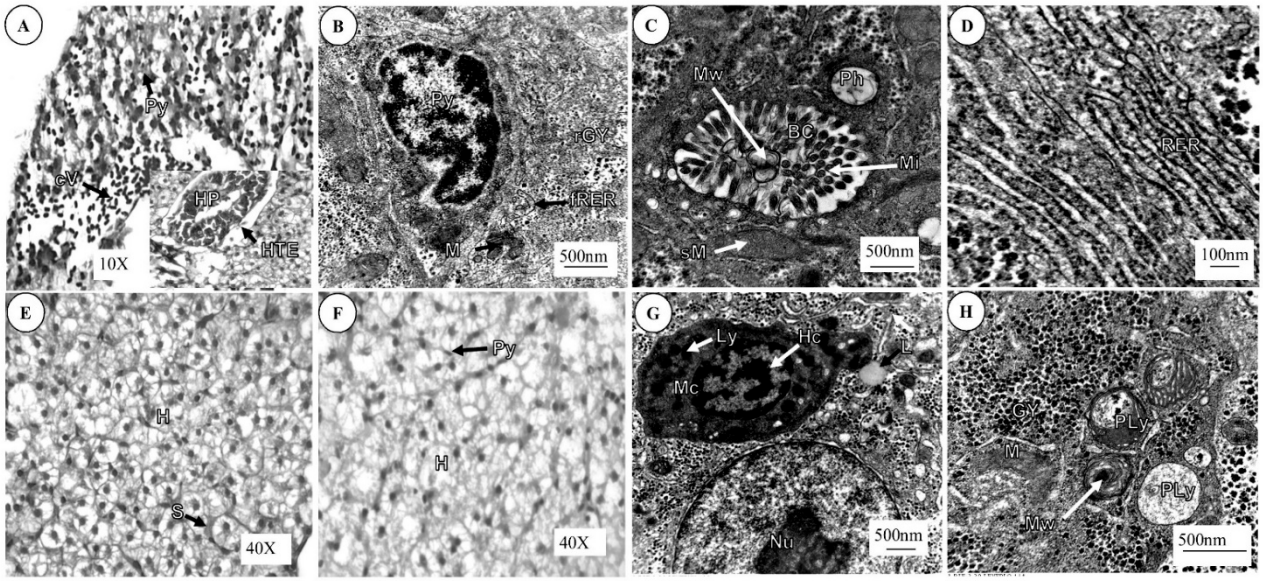


Figure 2. Alterations in hepato-architecture of *C. carpio* after exposure to malachite green and *E. officinalis* for 15 days. MG: (**A**) (10×)—Liver with congested vessel (cV), pyknotic nucleus (Py), Hepatopancreas (HP) and hypertrophied erythrocytes (HTE). (**B**): Pyknotic nuclei (Py), fragmented RER (fRER), rGY—reduced glycogen content. (**C**): Swollen mitochondrion (sM), phagosomes (Ph) and membrane whorls (Mw) in Bile canaliculi. *E. officinalis*: (**D**): Parallel arrangement of copious RER. (**E**) (40×)—Hepatocytes (H) with interspersed sinusoids (S). MG + *E. officinalis*: (**F**) (40×)—Pyknotic nuclei (Py) in polygonal hepatocytes (H). (**G**): Macrophage (Mc) with lysosomes (Ly) and heterochromatin (Hc) nucleus, central nucleolus (Nu) in spherical nucleus and presence of lipid inclusion (L); (**H**): numerous phagocytic vesicles, phagolysosomes (pLy), mitochondria and glycogen content.

Chronic (30 days) exposure of MG in fish resulted in the appearance of prominent nuclear alterations and hypertrophied cells with congested vessels in hepatic tissue (Figure 3A). Cytological study made through TEM detailed distorted nuclear shape, lipid inclusions and conspicuous macrophage cells with numerous phagosome bodies (Figure 3C). Bile canaliculi exhibited the occurrence of membrane whorls and a reduced number of microvilli (Figure 3B). Significant depletion of the liver glycogen content was observed in MG intoxicated fish. The amelioration of MG toxicity with *E. officinalis* resulted in inhibited alterations, and furthermore displayed erythrocyte infiltration (Figure 3F). Ultra-structurally, hepatocytes showed clumped mitochondria and numerous RER strands in the cytoplasm (Figure 3G). Prominent glycogen content was also evident (Figure 3H). The positive control (EO) exhibited structures similar to that of the control, demonstrating mitochondrion cristae and abundant RER (Figure 3D,E).

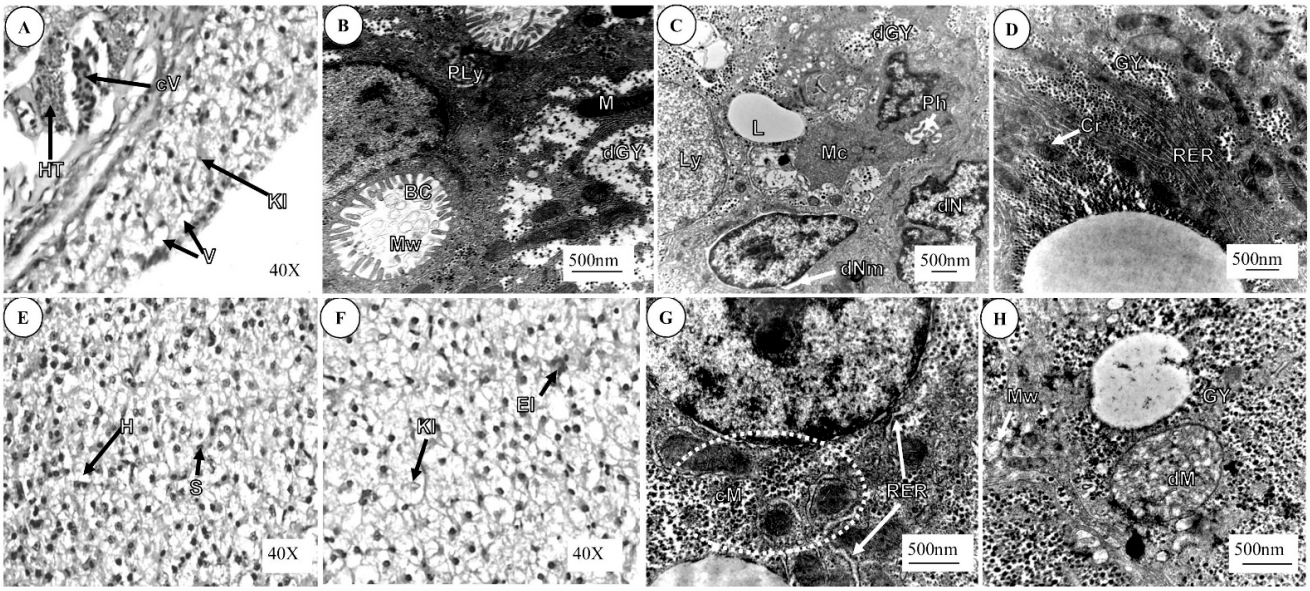


Figure 3. Alterations in hepato-architecture of *C. carpio* after exposure to malachite green and *E. officinalis* for 30 days. MG: (**A**) (40×)—Liver with nuclear alteration such as karyolysis (KL), hypertrophied cells (HT) and congested vessel (cV) and cytoplasmic vacuolation (V). (**B**): Bile canaliculi displayed decreased Microvilli (Mi) count and abundant membrane whorls, phagolysosomes (PLy) digesting various organelles, scarcity of glycogen content (dGY) and mitochondria (M). (**C**): Lipid deposits (L), lysosomes (Ly), macrophage (Mc), phagosomes (Ph), deformed nucleus (dN) and its membrane (dNm) in hepatocytes. *E. officinalis* alone: (**D**): regular hepato-architecture, mitochondrion with cristae (Cr), glycogen (GY) and large number of RER strands. (**E**) (40×)—Hepatocytes (H) with interspersed sinusoids (S). MG + *E. officinalis*: (**F**) (40×)—Karyolysis (Kl) and erythrocyte infiltration (EI). (**G**): Clumped mitochondria (cM), proliferated RER. (**H**): Dissolving mitochondrion cristae (dM), membrane whorls (Mw) in space of Disse and restored glycogen content (GY).

Light microscopy revealed conspicuous deformities in the nucleus, along with the presence of a large number of cytoplasmic vacuolation and necrotic bodies in hepatocytes after 60 days of MG treatment (Figure 4A,B). At cellular level, extensive vacuolization and diminished glycogen content occurred in hepatocytes. Cellular organelles underwent modifications in the form of RER vesiculation and fragmentation of the Golgi complex (Figure 4C,D). Hepatocytes demonstrated lost integrity of tight junctions (Figure 4E). Mitochondria swelling and the abundance of lysosomes and autophago-lysosomes indicated debilitated organelles upon MG exposure (Figure 4C–E). The inclusion of *E. officinalis* diet in MG intoxicated fish might have protected liver architecture and maintained glycogen content (Figure 4J–L). Ultra-structural examination demonstrated revived microvilli structure and number in Bile canaliculi (Figure 4L). Space of Disse showed the existence of the phagosome in consort with lysosomes functioning for the removal of waste (Figure 4J,K). Plant extract supplementation seemed to possess no negative impact (Figure 4F–H), depicting

regular hepatocytes (H) with interspersed sinusoids (S). Moreover, TEM study sustained the histological observations by the appearance of euchromatic nucleus, intact RER (Figure 4G); sinusoids bearing macrophage cells and nucleated erythrocytes (Figure 4H). Statistical semi-quantitative analysis of the histopathological alteration index (HAI) was made by scoring alterations as reversible, irreversible, and circulatory following [22], and this is presented in Figure 5F.

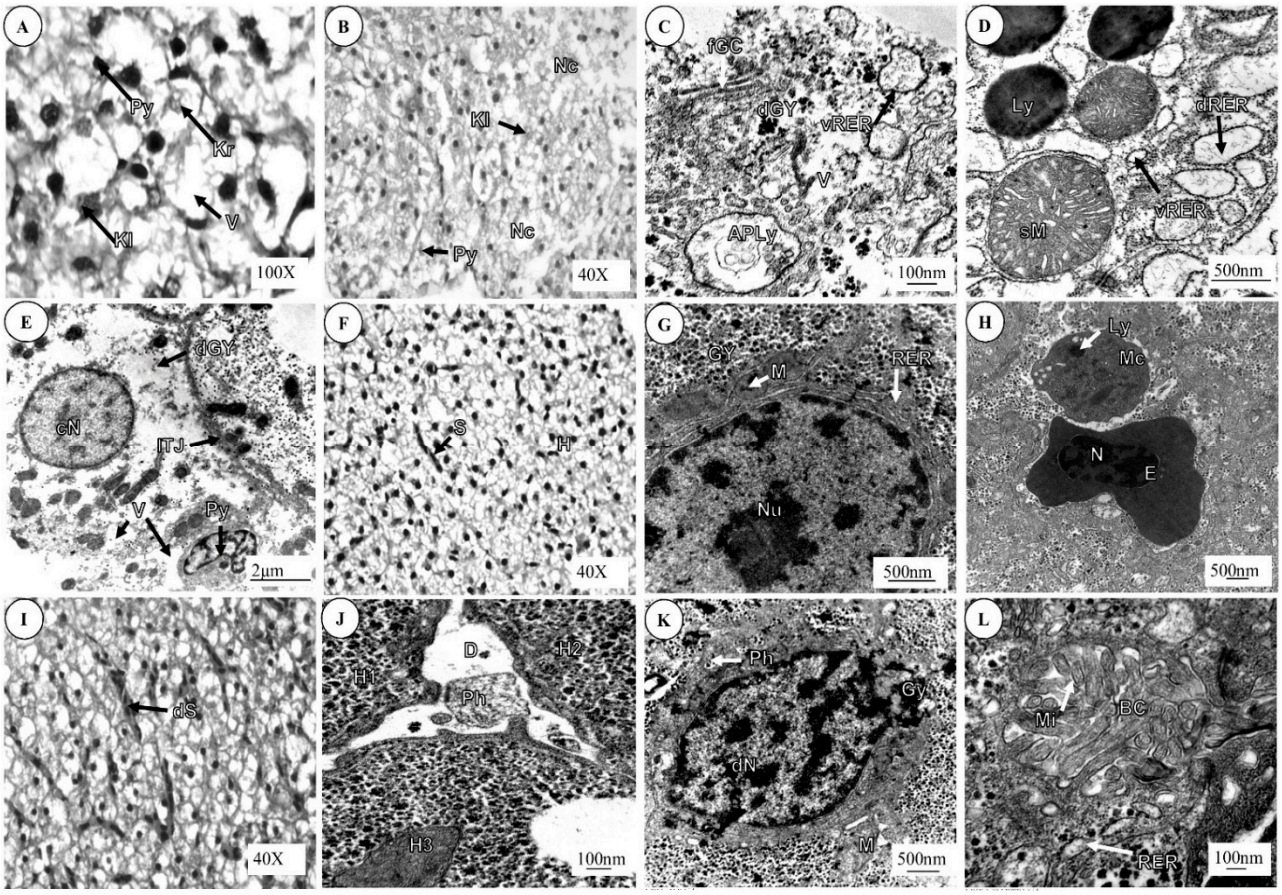


Figure 4. Alterations in *C. carpio* hepato-architecture after exposure to malachite green and *E. officinalis* for 60 days. MG: (**A**) (100×) and (**B**) (40×)—Prominent nuclear alterations such as pyknosis (Py), karyolysis (Kl) and cytoplasmic vacuolation (V) and necrotic hepatocytes (Nc). (**C**): vesiculated RER (vRER), vacuolation (V), auto-phagolysosome (APLy), fragmented GC (fGC) and depleted glycogen content (dGY). (**D**): RER transformation into dilated RER (dRER) and vesiculated RER (vRER), prominent lysosomes (Ly) and highly swollen mitochondria (sM). (**E**): Compact nucleus (cN), lost tight junction integrity (lTJ), sparse distribution of glycogen (dGY), vacuolation (V) and pyknosis (Py). *E. officinalis*: (**F**) (40×)—Hepatocytes (H) with interspersed sinusoids (S). (**G**): Regular hepatocyte architecture comprising nucleolus (Nu) in nucleus (N), RER extension from nuclear membrane, mitochondria (M) in vicinity and glycogen abundance (GY). (**H**): Sinusoid (S) showing macrophage with lysosomes (Ly) and erythrocyte (E) with central nucleus (N). MG + *E. officinalis*: (**I**) (40×)—Protected liver showing fewer nuclear alterations and dilated sinusoid (dS). (**J**): Phagosome (Ph) in space of Disse (D) formed by surrounding the hepatocytes (H1–H3). (**K**): Deformed nucleus (dN), phagosomes (Ph), mitochondria (M) and restored glycogen content (GY). (**L**): Bile canaliculi (BC) with regular microvilli (Mi) and interspersed RER.

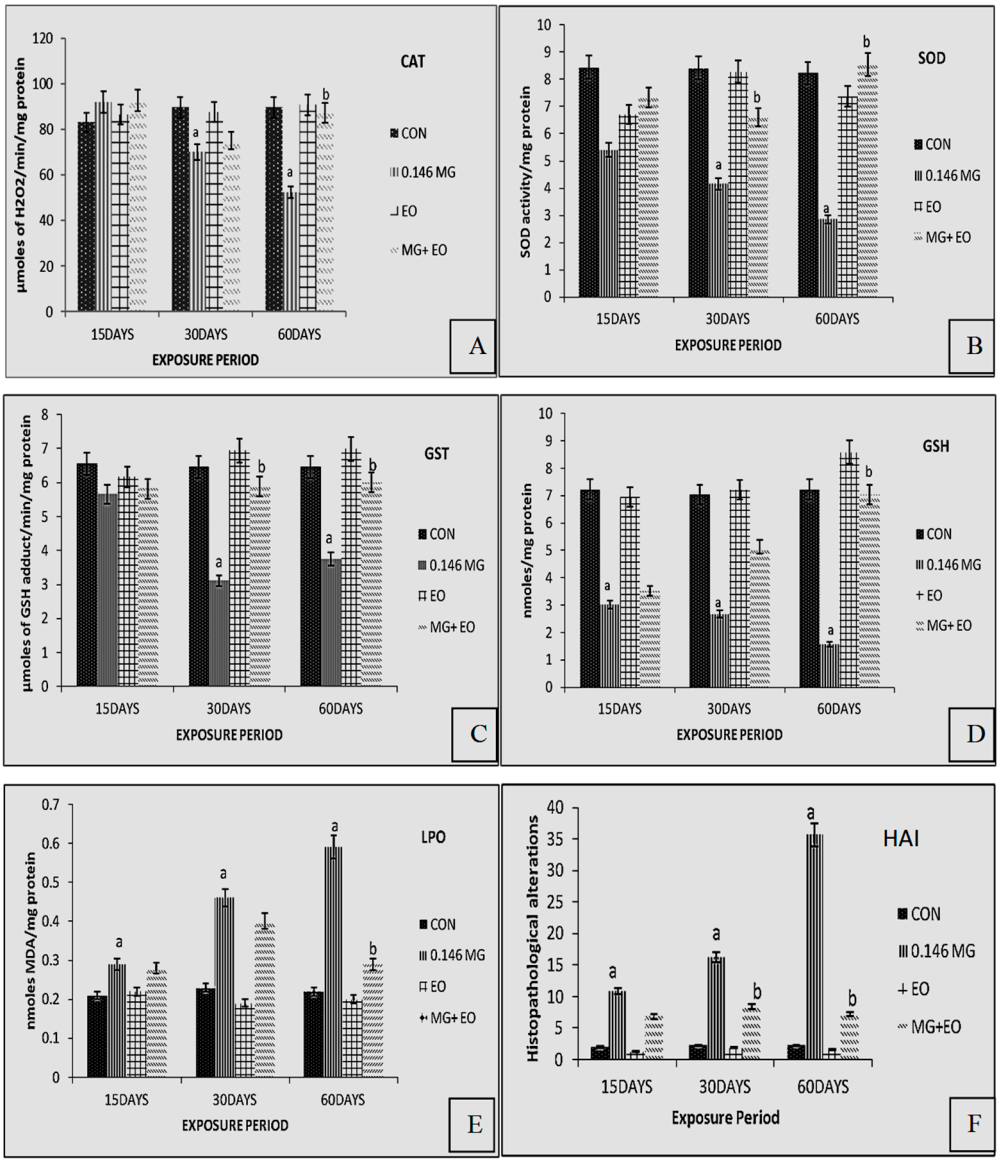


Figure 5. Variation in the anti-oxidant enzyme activity and histopathological index (HAI) in liver of *C. carpio* exposed to malachite green and *E. officinalis* for 15, 30 and 60 days. (**A**)—Catalase; (**B**)—Superoxide dismutase; (**C**)—Glutathione-S-Transferase; (**D**)—Reduced Glutathione; (**E**)—Lipid peroxidation; (**F**)—Histopathological Alteration Index. Abbreviations: CON: control; 0.146 MG: 0.146 mg/L MG; EO: *Emblica officinalis*; MG + EO: 0.146 mg/L MG + *E. officinalis*. "a" indicates significant ($p < 0.05$) change with respect to control in MG treated fish, and "b" indicates significant amelioration (MG + EO) with respect to MG treatment ($n = 9$).

3.2. Antioxidant Activity

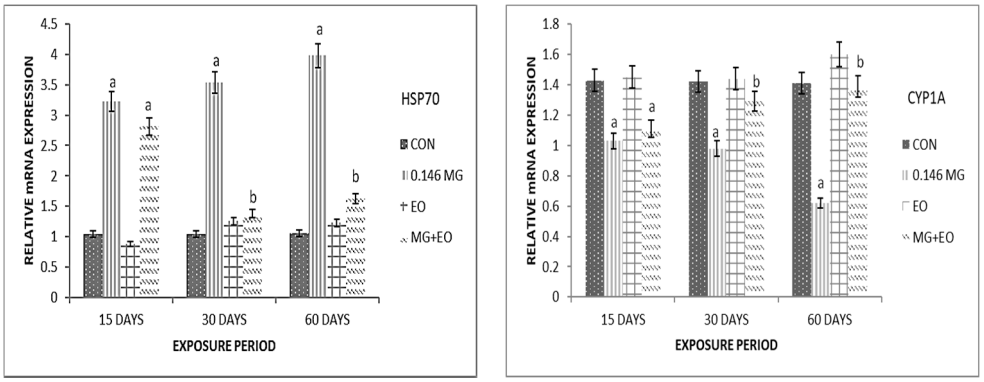
The initial 15 days of MG exposure elicited CAT activity. However, prolonged (30 and 60 days) MG exposure exhibited significantly ($p < 0.05$) depressed CAT, SOD and GST activity (Figure 5A–C), while amelioration assay showed the attenuation of dye toxicity by elevating CAT, SOD and GST levels. Concordantly, altered GSH level has been noticed in the MG influenced liver of *C. carpio* (Figure 5D). EO co-administration along with MG induced significant modulator impact in fish after 60 days. Values near to the control occurred in the EO fed group.

3.3. Lipid Peroxidation (LPO)

MG exposure led to a significant increase in malondialdehyde, MDA formation compared to control *C. carpio* indicating higher levels of lipid peroxidation (Figure 5E). The MDA formation were significantly inhibited by *E. officinalis* extract supplementation.

3.4. Genotoxicity Assessment

mRNA obtained from liver tissue was reverse transcribed into cDNA and primed at temperature (18S—61 °C, HSP70—59 °C and CYP1A—59 °C). RT-PCR was run with 18S, HSP70 and CYP1A primer. A negative control without a complementary DNA (cDNA) template and no-RT control RNA sample without reverse transcriptase were included to determine the specificity of target cDNA amplification and to control genomic DNA contamination. The PCR reaction efficiency was 2 for the reaction. A significant ($p < 0.05$) alteration in gene expression was evident, with upregulated HSP70 and downregulated CYP1A gene in response to MG treatment (Figure 6), whereas amelioration (MG + EO) assay depicted modulated expression with values near to the control after 30 days and 60 days.



(**A**) (**B**)

Figure 6. Relative expression of HSP70 and CYP1A in relation to 18S in *C. carpio* exposed to malachite green and *E. officinalis* for 15, 30 and 60 days. (**A**)—HSP70 gene expression; (**B**)—CYP1A gene expression. Abbreviations: CON: control; 0.146 MG: 0.146 mg/L MG; EO: *Emblica officinalis*; MG + EO: 0.146 mg/L MG + *E. officinalis*. "a" indicates significant ($p < 0.05$) change in groups (II, III, IV) compared to control group, while "b" indicates significant change compared to the MG treatment (group II) in the amelioration group (group IV) ($n = 9$).

3.5. Bioaccumulation of MG

Both control and plant extract fed carp exhibited not detectable (ND) MG concentration. However, in MG exposed *C. carpio*, there was a subsequent accumulation of 70% (30 days) and 91% (60 days) as compared to 15 days. EO supplementation assay demonstrated significantly ($p < 0.05$) inhibited MG deposition by 29% (30 days) and 60% (60 days) in *C. carpio* (Table 1).

Table 1. Bioaccumulation of MG in liver of *C. carpio* exposed for 15, 30 and 60 days.

Exposure	Control	MG	EO	MG + EO
15 Days	ND	7.97 ± 0.014	ND	7.14 ± 0.001
30 Days	ND	13.51 ± 0.020 [a]	ND	9.29 ± 0.044 [b]
60 Days	ND	15.22 ± 0.063 [a]	ND	6.54 ± 0.008 [b c]

Values are expressed mean ±SE ($n = 9$). ND: Not detectable concentration. "a" indicates significant ($p < 0.05$) difference compared to MG (15 days); "b" indicates significant ($p < 0.05$) difference in amelioration group compared to respective MG treatment; "c" indicates significant ($p < 0.05$) difference compared to MG (30 days).

3.6. Integrated Biomarker Response through Multivariate Analysis

Principal component analysis (PCA) was performed to analyze the components with maximum variance and demonstrated two principal components (PCs). PC1 comprised GST, LPO, GSH, SOD and HAI, while PC2 comprised CAT, having an Eigen value greater than 1, and 90.98% total variance. PCA study concluded the major impact of GSH and GST in MG induced toxicity (Table 2).

Table 2. Statistical parameters generated by PCA on various biomarkers in liver of *C. carpio* on 60th day of the experiment.

Variable		Rotated Component Matrix		Eigen Value	Variance Percentage	Cumulative Percentage
		1	2			
PC1	GST	0.990	0.108	4.03	67.17	67.17
	LPO	0.971	−0.139			
	GSH	0.965	0.203			
	SOD	0.959	0.251			
	HAI	0.958	−0.893			
PC2	CAT	0.325	0.796	1.42	23.81	90.98

Extraction method: principal component analysis. Kaiser–Meyer–Olkin measure of sampling adequacy = 0.741. Bartlett's test of sphericity: $p < 0.001$ (df = 15). Approx. Chi-square: 287.772.

4. Discussion

Malachite green exposure has been documented to negatively influence the fish metabolism despite its therapeutic potential [5]. In the present study, the appearance of lysosomes and auto-phagosomes indicated the induced defense system of the hepatocytes, functioning for the elimination of the unnecessary and debilitated organelles [50–52]. The extensive RER proliferation observed could be accredited to active protein production and accelerated detoxification activity by the liver [53]. The presence of membrane whorls in bile canaliculi can be associated with the cholestasis [54]. Nuclear transformations indicated the affected DNA. Concordantly, degenerated hepatocytes were also reported in MG intoxicated *Oncorhynchus mykiss* and *Heteropneustes fossilis* [55,56], while Seel-Audom et al. [57] reported a similar loss of sinusoidal space, vacuolation, pyknotic nuclei and cell enlargement in leuco-MG treated Nile tilapia. Furthermore, they reported mitochondria as the target organelle of MG toxicity, which is in conformity to the findings of the present study, evidenced by cristolysis and mitochondrial swelling.

MG, due to the presence of imine structure, acts as a strong electron accepting compound and thus produces ROS [58]. The initial rise in the catalase activity noticed on subjection to MG for 15 days could be accredited to the cell's ability to cope the stress [15], while the decline in the activity of the antioxidant enzymes (CAT and SOD), GST and GSH is associated with the exhaustion of enzymes due to the burden of oxidative stress or the inhibition of enzymes [17,59]. MG exposure has induced severe lipid peroxidation (LPO) in the hepatic tissue and hampered the cell membrane integrity, as evidenced by TEM and histopathological assay [8]. With the subsequent ROS accumulation, cell metabolism and genetic expression are affected. This was evidenced by the altered expression of CYP1A and HSP70 genes. MG exposure developed oxidative stress (observed in the present study), which led to upregulated expression of the HSP70 gene [60]. Similar increased HSP70 expression has been reported in oyster upon MG treatment [61]. Significantly ($p < 0.05$) downregulated CYP1A mRNA expression was noticed, which is in conformity to biochemical results of other xenobiotic metabolizing enzyme (XME) enzymes (GST) of the present study. The depressed CYP1A expression can be related to toxicant mediated inhibition of the gene activity [12]. Later, Dhamgaye et al. [62] have reported MG as a substrate for CYP1A and credited the xenobiotic metabolism of MG into Leuco-MG (LMG) [63,64]. This LMG is accumulated in the tissues in conformity to present findings, illustrating the enhanced and prolonged MG toxicity. Moreover, LMG poses a half-life of more than ten months, raising a concern upon its contamination in fish [14,65]. Correspondingly,

MG has been reported in higher concentration in the liver and gall bladder of exposed *Oncorhynchus mykiss* [66]. The hepatotoxicity mechanism of MG can be inferred due to mitochondria targeted toxicity, evidenced by ultrastructural studies with further development of oxidative stress, demonstrated by biochemical and molecular analysis. Adding to this toxicity mechanism could be the role of LMG, by triggering redox reactions even after the removal of the source [67].

EO dietary co-administration in MG intoxicated fish for 60 days imparted effective modulation against MG-induced genotoxicity and cytotoxicity. It enhanced the activity of anti-oxidant enzymes (CAT and SOD) in the fish. In accordance with the present findings, Mirghaed et al. [32] reported the mitigatory effect of *Artemisia annua* against ammonia exposure in common carp, eventually by enhancing the CAT enzyme activity and gene expression. In 1994, Meister [68] elucidated GSH function in the regulation of metabolism by the donation of a reducing equivalent to free radicals, which might have been aided by the supplementation of *Emblica officinalis*, as evidenced by its elevated levels in amelioration assay [69]. The inhibited MDA formation observed may be accredited to the presence of ascorbic acid in EO that acts by initiating the cascade of scavenging the O^{2-} and OH^{-} and breaking down the OH^{-} by the ascorbate peroxidase reaction [69–72]. Another major phyto-constituent of EO is gallic acid that acts by donating electrons to free radicals [73]. Collectively, these cause the inhibition of cellular damage evident by TEM studies [74,75]. Similarly, modulated oxidative stress has been reported in MG exposed *Saccharomyces cerevisiae* upon supplementation of *Terminalia chebula*, *Clitoria ternatea* and *Boerhaavia diffusa* [76].

EO supplementation facilitated MG removal from the body, probably by activating various detoxification enzymes, inducing multiple inhibition mechanisms and restricting the production of electrophilic metabolites [77,78]. Other phyto-constituents of EO (tannins, alkaloids, and phenols) have been documented to synergistically impact the cell by preventing protein degradation and supporting the elimination of MG mediated by GST and GSH enzymes. The activated detoxification system and enhanced ROS scavenging activity might have resulted in the restored expression level of CYP1A and HSP70 [68,69,79,80].

Factor analysis was executed on biomarkers of oxidative stress (LPO, CAT, SOD, GST and GSH) and the histological alterations index (HAI) to assess the inter-relationship between them. It revealed the major role of GST and GSH, elucidating the importance of the detoxification system in MG toxicity.

5. Conclusions

The present study inferred that malachite green, even at sub-lethal concentration, could induce hepatotoxicity in *Cyprinus carpio*, which was evident through histopathological and ultra-structural lesions, accompanied by impaired antioxidant enzyme activity. MG also caused altered expression of the genes, and its prolonged toxicity may be accredited to the bioaccumulation ability of the dye. TEM has elaborated the MG targeted mitochondrial toxicity and development of oxidative stress in the fish. However, *Emblica officinalis* feed supplementation prevented architectural defects, boosted antioxidant enzyme activity and normalized altered gene expression, citing the effectiveness of *E. officinalis* fruit extract as a nutraceutical against MG toxicity in *C. carpio*.

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Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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