

Comparative effects of Helmar-1 and Helmar-2 polyphenol extracts on mitochondrial bioenergetics, antioxidant defense, and lipid peroxidation in CCl₄-induced toxic hepatitis in rats

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ABSTRACT

Introduction: Toxic hepatitis is characterized by enhanced oxidative stress and disruption of antioxidant defense mechanisms in the liver mitochondria. In this study, we investigated the effects of polyphenolic extracts (Helmar-1 and Helmar-2) obtained from *Helichrysum maracandicum* on mitochondrial antioxidant systems in rats with experimentally induced toxic hepatitis.

Methods: Experimental toxic hepatitis was established in rats through intraperitoneal administration of carbon tetrachloride (CCl₄) diluted in olive oil (50%, 1 mL/kg), administered twice weekly over a two-week period. Liver injury was verified by elevated plasma levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST). The animals were subsequently treated with Helmar-1 and Helmar-2 extracts at a dose of 20 mg/kg per day for 10 consecutive days. The activities of key antioxidant enzymes (superoxide dismutase, catalase, and glutathione peroxidase), malondialdehyde (MDA) content, and mitochondrial respiration and oxidative phosphorylation parameters (V₂, V₃, V₄, RCR, and ADP/O ratio) were assessed in liver mitochondria.

Results: Treatment with both Helmar-1 and Helmar-2 extracts resulted in a significant enhancement of antioxidant enzyme activities, a marked reduction in MDA levels, and substantial improvement in mitochondrial respiration and oxidative phosphorylation parameters compared to untreated toxic hepatitis groups.

Conclusion: Overall, these results confirm that *Helichrysum maracandicum* polyphenol extracts improve mitochondrial respiration, oxidative phosphorylation efficiency, antioxidant defense, and membrane stability in toxic hepatitis.

Keywords: Antioxidant enzymes, Lipid peroxidation, Liver mitochondria, Oxidative stress, Polyphenols, Toxic hepatitis

Introduction

In toxic hepatitis, excessive production of reactive oxygen species (ROS) plays a central role in mitochondrial dysfunction and hepatocellular damage (1,2). We observed that polyphenol extracts from *Helichrysum maracandicum* lowered oxidative stress levels in the liver mitochondria of rats with experimentally induced toxic hepatitis. This was evidenced by a decline in lipid peroxidation levels and restoration of antioxidant enzyme activity.

The reduction in lipid peroxidation markers observed following treatment with *H. maracandicum* aligns with multiple studies indicating that plant-derived polyphenols can effectively inhibit malondialdehyde formation by scavenging free radicals and stabilizing mitochondrial membranes (3,4). Similar protective effects have been documented for polyphenol-rich extracts from *Silybum marianum* (milk thistle), which significantly mitigated lipid peroxidation and safeguarded liver mitochondria against oxidative damage in experimental models of toxic hepatitis (5).

The obtained results also indicate that polyphenols isolated from *Helichrysum maracandicum* not only reduced lipid peroxidation but also promoted the recovery of mitochondrial antioxidant defenses, particularly superoxide dismutase, catalase, and glutathione peroxidase, consistent with published data (6,7). In the present study, administration of *H. maracandicum* extract led to a significant increase in the activity of

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mitochondrial antioxidant enzymes in rat liver mitochondria. These results are consistent with earlier research that demonstrated how polyphenols can bolster endogenous antioxidant defenses by influencing redox-sensitive signaling pathways and sustaining the mitochondrial redox balance (8). Comparable enhancements in antioxidant enzyme activity have been noted for curcumin sourced from *Curcuma longa*, which improves mitochondrial antioxidant capacity while reducing oxidative harm in CCl_4 -induced liver injuries; however, its therapeutic effectiveness is often hindered by its low bioavailability (9).

Polyphenols extracted from green tea (*Camellia sinensis*), especially epigallocatechin gallate (EGCG), have been recognized for their ability to enhance mitochondrial antioxidant status and reduce oxidative stress in models of toxic liver injury (10). However, several studies have suggested that high doses of green tea polyphenols can result in pro-oxidant effects, leading to mitochondrial dysfunction (11). In contrast, the polyphenolic extracts from *H. maracandicum* demonstrated a consistent antioxidant effect without signs of mitochondrial toxicity in this study, indicating a potentially safer profile for these extracts.

Additionally, polyphenol-rich extracts from *Ginkgo biloba* and *Rosmarinus officinalis* have shown moderate hepatoprotective properties through improvements in mitochondrial respiration and enhancement of antioxidant enzyme activity; however, their impact on lipid peroxidation has been incomplete in some experimental scenarios (12,13). In contrast to these plants, the polyphenols from *H. maracandicum* exhibited a more holistic protective effect by concurrently decreasing lipid peroxidation and revitalizing mitochondrial antioxidant enzymes.

The enhanced efficacy of *H. maracandicum* may stem from the distinct composition and synergistic interactions among its polyphenolic constituents. These interactions likely improve free radical-scavenging capabilities while preserving the integrity of the mitochondrial membranes and supporting oxidative phosphorylation processes. Previous research has highlighted that the synergy among various polyphenolic compounds plays an essential role in determining their overall antioxidant and hepatoprotective effects (14). Earlier studies have revealed that polyphenols isolated from *Helichrysum maracandicum* possess pronounced antiradical activity, indicating their strong antioxidant potential (15,16).

In summary, compared to previously studied medicinal plants, polyphenol extracts derived from *Helichrysum maracandicum* display similar antioxidant efficacy but offer more stable protection for mitochondria under conditions associated with toxic hepatitis.

These findings suggest that polyphenol extracts from *Helichrysum maracandicum* have significant hepatoprotective effects that are comparable to—and in certain respects surpass—those attributed to well-established medicinal plants such as *Silybum marianum*, *Curcuma longa*, *Camellia sinensis*, and *Ginkgo biloba*. These results underscore the therapeutic potential of *H. maracandicum* polyphenols as natural agents capable of mitigating mitochondrial oxidative damage associated with toxic hepatitis.

This study aimed to investigate the effects of polyphenol extracts (Helmar-1 and Helmar-2) derived from *Helichrysum maracandicum* on antioxidant enzyme activity and lipid

peroxidation in the liver mitochondria of rats with CCl_4 -induced toxic hepatitis.

Materials and methods

Animal handling and experimental conditions

Male white outbred rats (8–10 weeks old, weighing 180–220 g) were obtained from the Animal Facility of the National University of Uzbekistan, Tashkent, Uzbekistan. Animals were maintained under standard laboratory conditions at a temperature of 20–24°C, relative humidity of 65%, and a natural light/dark cycle, with free access to standard laboratory food and water ad libitum. Before tissue collection, the animals were anesthetized with ketamine (80 mg/kg, intraperitoneally) and xylazine (10 mg/kg, intraperitoneally). Euthanasia was performed by cervical dislocation under deep anesthesia.

Ethics Statement

All experimental procedures were approved by the Local Ethics Committee of the Institute of Biophysics and Biochemistry, National University of Uzbekistan named after Mirzo Ulugbek (Protocol No. 7/BEC/IBB-NUU, dated July 4, 2022). Animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (1986).

Experimental toxic hepatitis model

Animals were housed under standard laboratory conditions, with no more than five rats per cage, and maintained on a standard laboratory diet. Food was withdrawn 24 h prior to CCl_4 intoxication.

Male rats were randomly divided into five groups ($n = 5$ per group):

- Group I – healthy control
- Group II – CCl_4 -induced toxic hepatitis (TH)
- Group III – CCl_4 + Helmar-1
- Group IV – CCl_4 + Helmar-2
- Group V – CCl_4 + silymarin

Toxic hepatitis was induced by intraperitoneal injection of carbon tetrachloride (CCl_4 ; Sigma-Aldrich, St. Louis, MO, USA) at a dose of 1 mL/kg body weight twice weekly. CCl_4 was diluted 1:1 in sterile olive oil to obtain a 50% solution. The experimental protocol was adapted from Avtandilov (17) with minor modifications.

Liver injury induction was confirmed by elevated serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels. After confirmation of toxic hepatitis, Helmar-1 and Helmar-2 were administered intraperitoneally at a dose of 20 mg/kg body weight once daily for 10 consecutive days. The reference group received silymarin according to the same schedule.

Preparation of Helmar-1 and Helmar-2 extracts

The aerial parts of *Helichrysum maracandicum* were collected, air-dried, and ground into fine powder. The plant



FIGURE 1 - Experimental timeline of toxic hepatitis induction, treatment, sample collection, and mitochondrial analyses.

material was extracted with 70% ethanol at room temperature for 48 h. The crude extract was filtered and concentrated under reduced pressure using a rotary evaporator. Fractionation of the crude extract was performed by solvent partitioning to obtain two polyphenolic fractions, designated as Helmar-1 and Helmar-2.

According to previous phytochemical studies, Helmar-1 contains isosalipurposide, cosmosiin, apigenin, luteolin, naringenin-5-O- β -D-glucopyranoside, and quercetin. Helmar-2 contains luteolin, isosalipurposide, apigenin-7-O- β -D-glucopyranoside, naringenin-5-O- β -D-glucopyranoside, and quercetin. These compounds mainly belong to the flavonoid and phenolic classes and are considered responsible for the antioxidant and hepatoprotective effects of the extracts. The phytochemical composition was interpreted according to previous reports (18,19).

Mitochondrial isolation

Mitochondria were isolated from rat liver by differential centrifugation according to the method described previously (20). Animals were anesthetized with ether and decapitated. Liver tissue was rapidly excised and homogenized using a Teflon-glass homogenizer (Wheaton Industries Inc., Millville, NJ, USA) in ice-cold isolation buffer containing 250 mM sucrose, 1 mM EDTA, and 10 mM Tris-HCl (pH 7.4).

The homogenate was centrifuged at $1,500 \times g$ for 7 min to remove nuclei and cell debris. The supernatant was then centrifuged at $6,000 \times g$ for 15 min to pellet mitochondria. The mitochondrial pellet was resuspended in a medium containing 250 mM sucrose and 10 mM Tris-HCl. All procedures were carried out at 4°C. The purity of mitochondrial fractions was evaluated based on preservation of mitochondrial integrity and minimization of cytosolic contamination during isolation.

Evaluation of lipid peroxidation

Lipid peroxidation (LPO) in liver mitochondria was assessed by measuring malondialdehyde (MDA) levels as an indicator of oxidative damage to mitochondrial membranes.

LPO was induced using an Fe²⁺/citrate-dependent system. Mitochondrial swelling kinetics were monitored spectrophotometrically at 540 nm in an open cuvette (3 mL) at 26°C under continuous stirring. Lipid peroxidation was initiated by adding 50 μ M FeSO₄ and 2 mM citrate (21).

The incubation medium contained (mM): sucrose 125, KCl 65, Tris-HCl 10 (pH 7.2). The reaction was stopped by adding 0.220 mL of 70% trichloroacetic acid, followed by centrifugation at $3,000 \times g$ for 15 min. The supernatant was mixed with thiobarbituric acid reagent and incubated in a boiling water bath for 30 min. Absorbance was measured at 532 nm, and

MDA concentration was calculated using the molar extinction coefficient ($\epsilon = 1.56 \times 10^5 \text{ M}^{-1} \cdot \text{cm}^{-1}$) (22).

Determination of antioxidant enzyme activities

The activity of antioxidant enzymes in liver mitochondria was determined spectrophotometrically.

Glutathione peroxidase (GPx) activity was measured according to the standard enzymatic method (23). The reaction mixture contained 0.05 M phosphate buffer (pH 8.0), EDTA, oxidized glutathione, mitochondrial hemolysate, and NADPH. The decrease in NADPH concentration was monitored at 340 nm after incubation at 37°C for 10 min. GPx activity was expressed as nmol/min/mg protein.

Superoxide dismutase (SOD) activity was determined according to the method of Misra and Fridovich (1972) (24), based on inhibition of epinephrine autoxidation in an alkaline medium. The change in absorbance was measured spectrophotometrically, and enzyme activity was expressed as units per mg protein.

Catalase (CAT) activity was measured by monitoring the decomposition rate of hydrogen peroxide (H₂O₂) spectrophotometrically according to the standard method. Catalase activity was expressed as μ kat/g protein (SI unit: μ mol/s/kg protein) (25).

The concentration of mitochondrial proteins in all samples was determined by the Lowry method with Peterson modification (26).

Determination of mitochondrial respiration and oxidative phosphorylation

Mitochondrial respiration and oxidative phosphorylation were evaluated polarographically using a Clark-type oxygen electrode (Strathkelvin Instruments Ltd., Glasgow, UK) according to the method described previously (27).

Oxygen consumption was measured in a thermostatically controlled closed chamber at 26°C with continuous stirring. The incubation medium contained: 120 mM KCl, 1 mM KH₂PO₄, 10 mM Tris-HCl (pH 7.2), 5 mM succinate, 1 mM MgCl₂, 1 mM EGTA, and 1 μ M rotenone.

The following respiratory parameters were measured:

V₂ (substrate respiration) – respiration rate in the presence of substrate without ADP

V₃ (active respiration) – respiration rate after ADP addition

V₄ (resting respiration) – respiration rate after ADP phosphorylation

VCCCP – maximal uncoupled respiration in the presence of 3 μ M CCCP

These parameters were used to assess mitochondrial bioenergetic function and oxidative phosphorylation efficiency.

Statistical analysis

Sample size ($n = 5$ per group) was determined based on preliminary experimental data to ensure adequate statistical power. Animals were randomly assigned to experimental groups to minimize selection bias.

All measurements were performed in triplicate, and investigators were blinded to group allocation during data analysis to reduce observer bias. No animals or data points were excluded from the analysis.

Data are presented as mean $\pm$ standard deviation (SD). Statistical analysis and graphical presentation were performed using OriginPro 8.6 software (OriginLab Corporation, Northampton, MA, USA).

Differences among groups were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test for multiple comparisons. A value of $p < 0.05$ was considered statistically significant.

Results

The successful induction of toxic hepatitis following CCl_4 administration was evidenced by marked elevations in serum liver enzyme activities relative to the intact control group. As presented in Table 1, ALT, AST, and ALP levels increased significantly in rats with toxic hepatitis, indicating substantial hepatic injury. Administration of Helmar-1 and Helmar-2 polyphenol extracts resulted in a significant reduction in these biochemical markers compared with untreated animals. Among the tested extracts, Helmar-2 exhibited a comparatively greater hepatoprotective effect, reflected by enzyme values that were closer to those observed in the silymarin-treated group.

Experimentally, changes in SOD activity in liver mitochondria during toxic hepatitis and the effects of Helmar 1 and Helmar 2 polyphenol extracts on this activity were compared with those of silymarin. The superoxide dismutase (SOD) activity in the liver mitochondria of control rats (Group I) was 4.83 ± 0.20 U/mg protein and was considered 100%. In the CCl_4 -treated group (Group II), SOD activity significantly decreased to 2.86 ± 0.10 U/mg protein, corresponding to

a reduction of 40.8% ($p < 0.05$) compared to the control (Fig. 2).

Treatment with Helmar-1 (Group III) increased SOD activity to 3.95 ± 0.18 U/mg protein, representing a 22.57% recovery relative to Group II. Similarly, Helmar-2 (Group IV) restored SOD activity to 4.18 ± 0.11 U/mg protein, corresponding to a 27.33% increase compared to the untreated toxic hepatitis group.

Thus, in toxic hepatitis, liver mitochondrial SOD activity is reduced, and the effect of Helmar 1 and Helmar 2 extracts is comparable to that of silymarin, demonstrating that the activity of this enzyme is partially restored under the influence of these extracts. In animals exposed to CCl_4 , the intensity of LPO in the liver tissue, including the mitochondrial membrane, increases sharply as a result of oxidative stress (28). An increase in LPO products in animals with toxic hepatitis leads to a sharp increase in hydrogen peroxide (H_2O_2) in liver mitochondria. Under such conditions, another important antioxidant enzyme, catalase, is activated in the mitochondria and participates in the breakdown of H_2O_2 into oxygen and water. Therefore, another experiment was conducted to determine the change in catalase activity in rats with toxic hepatitis after treatment with polyphenol extracts.

The catalase activity in the control animals was 62.33 ± 1.20 $\mu\text{kat/g}$ protein. In the CCl_4 group, catalase activity significantly decreased to 37.90 ± 1.32 $\mu\text{kat/g}$ protein (-39.2% , $p < 0.05$) (Figure 3). Administration of Helmar-1 and Helmar-2 increased catalase activity to 54.46 ± 1.67 and 58.74 ± 1.10 $\mu\text{kat/g}$ protein, respectively, corresponding to recoveries of 26.57% and 33.44% compared to Group II.

Liver mitochondrial catalase activity of group V rats corrected with silymarin was inhibited by $35.86 \pm 2.8\%$ in the pathological group, with a protein content of 60.25 ± 1.15 $\mu\text{kat/g}$. Hence, the enzyme catalase ensures the stability of the cell's antioxidant defense system by breaking down H_2O_2 , which is formed in the liver mitochondria of animals with toxic hepatitis. In addition, catalase can prevent the formation of hydroxyl radicals as a result of the Fenton reaction under pathological conditions (29).

Toxic hepatitis caused by CCl_4 increases pro-oxidant factors, which is observed as a result of the increase in the quantity of fatty acids due to membrane LPO in the liver mitochondria. Subsequently, an imbalance in the enzyme-dependent antioxidant defense system occurs in the cells

TABLE 1 - Effects of Helmar-1 and Helmar-2 polyphenol extracts on serum ALT, AST, and ALP levels in CCl_4 -induced toxic hepatitis in rats

Experimental groups	ALT (U/L)	AST (U/L)	ALP (U/L)
Group I (Healthy control)	74.0 ± 6.8	85.0 ± 4.7	704.0 ± 35.5
Group II (CCl_4 -induced TH)	$204.0 \pm 8.7^{**}$	$187.0 \pm 15.5^{**}$	$1410.0 \pm 53.4^{**}$
Group III (CCl_4 + Helmar-1)	$98.5 \pm 6.7 \#$	$101.5 \pm 7.5 \#$	$815.3 \pm 13.7 \#\#$
Group IV (CCl_4 + Helmar-2)	$90.4 \pm 4.8 \#\#$	$96.8 \pm 6.6 \#\#$	$765.0 \pm 12.9 \#\#$
Group V (CCl_4 + Silymarin)	$80.1 \pm 3.6 \#\#$	$91.0 \pm 5.3 \#\#$	$724.0 \pm 22.5 \#\#$

Values are presented as mean $\pm$ SD ($n = 5$).

** $p < 0.01$ compared with the healthy control group.

$p < 0.05$ compared with the CCl_4 -induced toxic hepatitis group.

$p < 0.01$ compared with the CCl_4 -induced toxic hepatitis group.

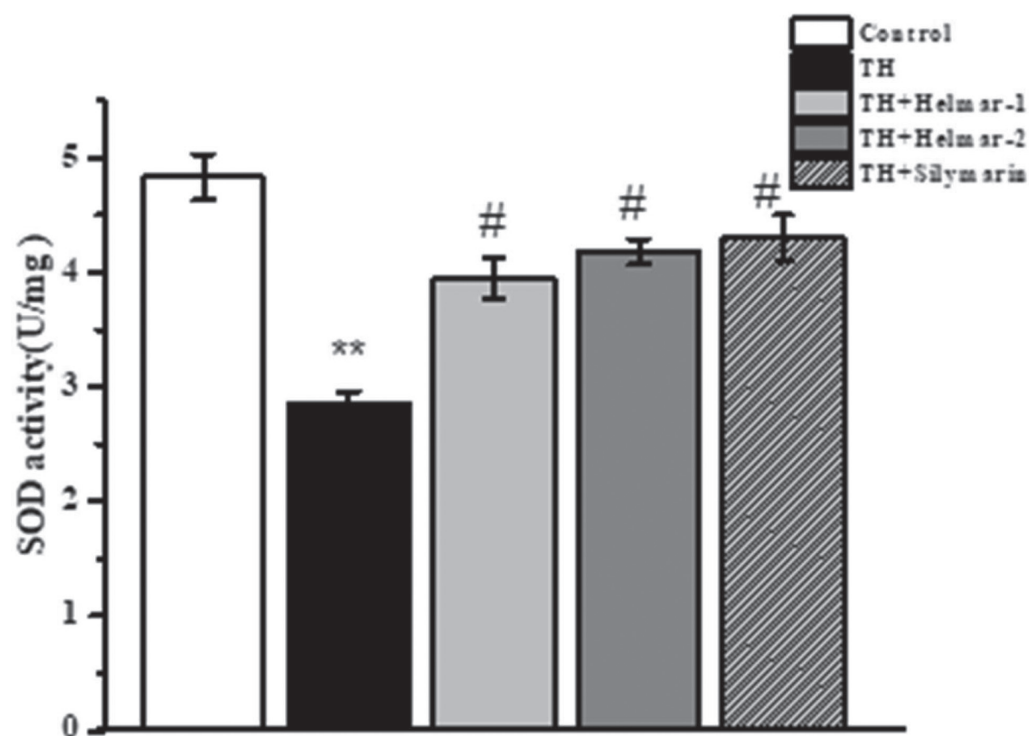


FIGURE 2 - Effect of Helmar extracts on SOD activity in liver mitochondria.

Superoxide dismutase (SOD) activity in liver mitochondria of healthy control, CCl₄-induced toxic hepatitis, and treatment groups. Toxic hepatitis significantly decreased SOD activity compared with the healthy control group. Treatment with Helmar-1, Helmar-2, and silymarin significantly improved SOD activity toward control values. Data are presented as mean $\pm$ SD (n = 5). **p < 0.01 vs healthy control; #p < 0.05 vs CCl₄-induced toxic hepatitis group.

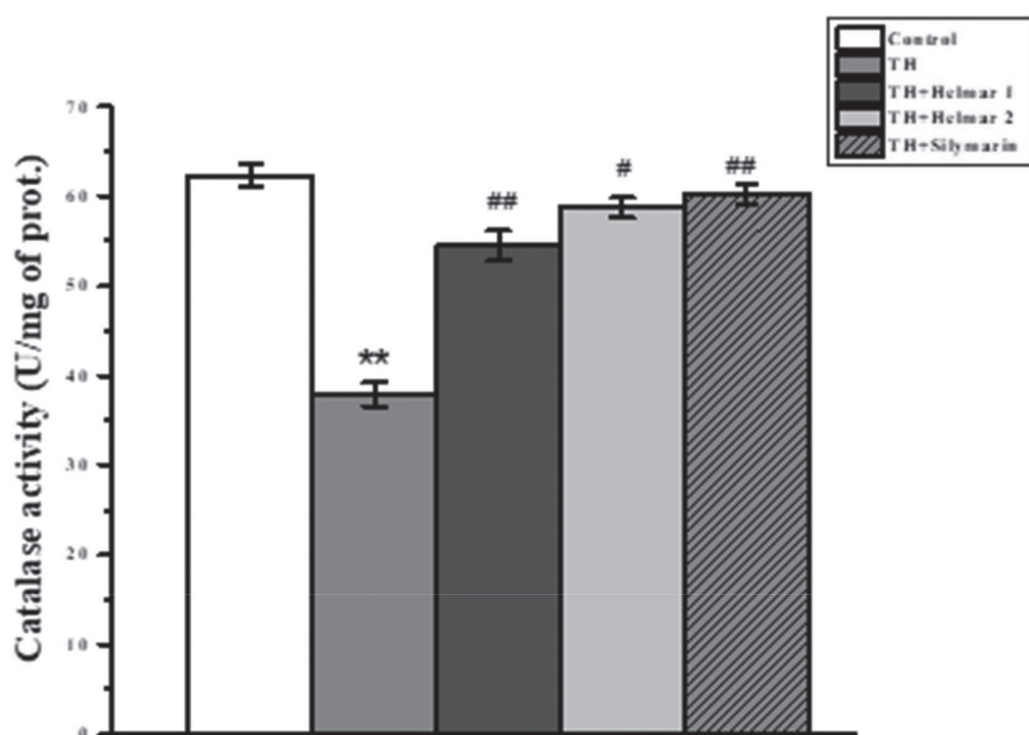


FIGURE 3 - Effect of Helmar extracts on catalase activity in liver mitochondria.

Catalase activity in liver mitochondria of healthy control, CCl₄-induced toxic hepatitis, and treatment groups. CCl₄-induced toxic hepatitis significantly reduced catalase activity compared with the healthy control group, whereas treatment with Helmar-1, Helmar-2, and silymarin significantly improved enzyme activity toward control values. Data are expressed as mean $\pm$ SD (n = 5). **p < 0.01 vs healthy control; #p < 0.05 vs CCl₄-induced toxic hepatitis group.

and mitochondria. During toxic hepatitis, the activities of the antioxidant enzymes SOD and catalase in liver tissue, as well as glutathione peroxidase activity, may be altered. There is growing interest in compounds that have the potential to protect cells and mitochondria from the detrimental effects

of free radicals under pathological conditions. Further analysis revealed that the effect of Helmar 1 and Helmar 2 polyphenol extracts on the activity of glutathione peroxidase, another antioxidant enzyme in toxic hepatitis conditions, was compared with that of silymarin.

The glutathione peroxidase (GPx) activity in the liver mitochondria of control rats (Group I) was 80.11 ± 1.75 nmol/min/mg protein. In the toxic hepatitis group (Group II), GPx activity significantly decreased to 60.88 ± 1.40 nmol/min/mg protein, representing a reduction of 17.02% ($p < 0.05$) compared to the control group (Fig. 4).

Treatment with Helmar-1 (Group III), Helmar-2 (Group IV), and silymarin (Group V) increased GPx activity to 74.51 ± 3.16 , 75.98 ± 3.56 , and 76.75 ± 1.22 nmol/min/mg protein, respectively. This indicates a recovery of 17.02%, 18.85%, and 19.82% compared to the untreated toxic hepatitis group, respectively.

The results obtained indicate that, under CCl_4 -induced toxic hepatitis conditions, polyphenol extracts Helmar-1 and Helmar-2 promote the restoration of mitochondrial enzyme activity, particularly glutathione peroxidase, with effects comparable to those of silymarin.

Disturbances in lipid metabolism observed in toxic hepatitis are closely associated with oxidative stress, characterized by excessive generation of reactive oxygen species (ROS), mitochondrial DNA damage, and impairment of cellular bioenergetic processes.

Lipid Peroxidation in Liver Mitochondria

CCl_4 -induced toxic hepatitis was accompanied by a significant increase in lipid peroxidation in liver mitochondria, as indicated by elevated malondialdehyde (MDA) levels. Increased MDA production reflects enhanced oxidative damage and impaired mitochondrial membrane stability under toxic conditions. Administration of Helmar-1 and Helmar-2

significantly reduced MDA concentrations compared with the untreated TH group, suggesting attenuation of oxidative stress and preservation of mitochondrial membrane integrity. The protective effects of both extracts were comparable to those observed in the silymarin-treated group, while Helmar-2 demonstrated a slightly stronger inhibitory effect on lipid peroxidation (Fig. 5).

In toxic hepatitis, the level of malondialdehyde (MDA) in the mitochondrial membranes increases in parallel with elevated serum ALT and AST levels, reflecting enhanced oxidative stress.

Malondialdehyde (MDA) levels in control mitochondria were significantly lower than in the toxic hepatitis group. In Group II, MDA levels increased to 3.37 ± 0.12 nmol/min/mg protein, representing a 91.5% increase compared to the control ($p < 0.05$) (Fig. 5). Treatment with Helmar-1 and Helmar-2 reduced MDA levels to 2.18 ± 0.09 and 2.09 ± 0.08 nmol/min/mg protein, respectively, while silymarin further reduced MDA levels to 1.92 ± 0.18 nmol/min/mg protein.

In rats with toxic hepatitis, LPO completely destroys the double lipid layer of the membrane, resulting in significant changes in membrane permeability, which cause cellular dysfunction and pathological processes. Thus, the increase in the amount of MDA in the liver of animals with toxic hepatitis also indicates that the process of LPO in cell membranes is accelerated. Administration of polyphenol extracts and silymarin for 10 days reduced the increase in the levels of MDA and antioxidant enzymes, thereby slowing down the LPO process. Depletion of the mitochondrial antioxidant system in rats with toxic hepatitis was also confirmed by the transition of *mPTP* to an open state (30). Increased LPO in animals

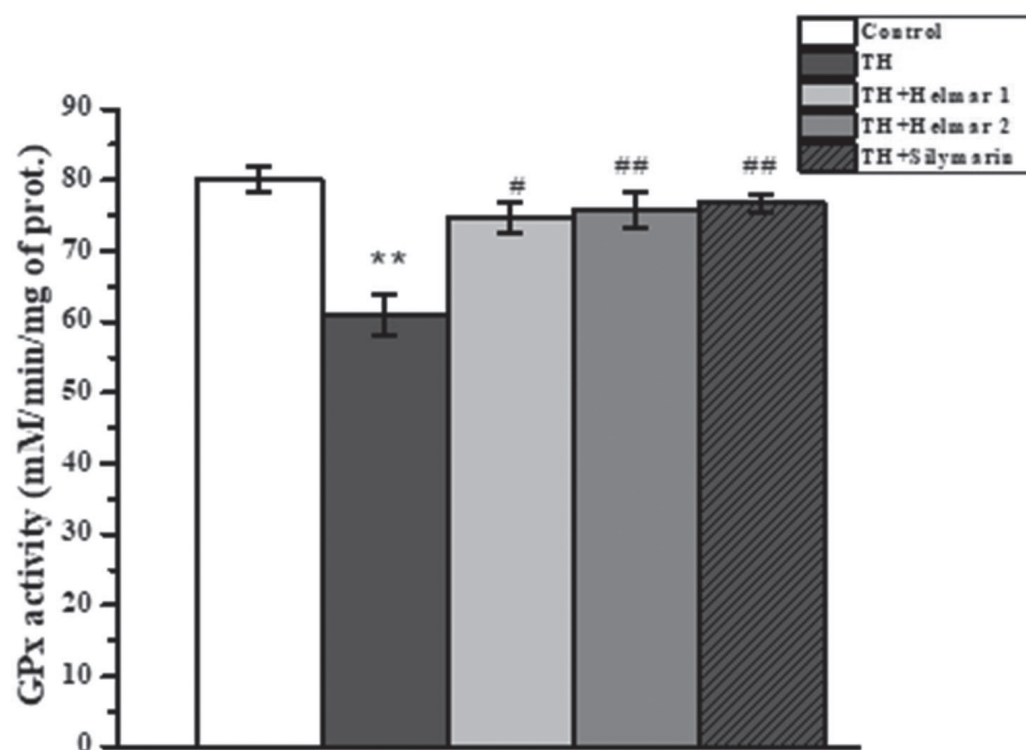


FIGURE 4 - Effect of Helmar extracts on glutathione peroxidase activity in liver mitochondria.

Glutathione peroxidase (GPx) activity in liver mitochondria of healthy control, CCl_4 -induced toxic hepatitis, and treatment groups. GPx activity was significantly reduced in the CCl_4 -induced toxic hepatitis group compared with the healthy control group and was significantly restored by treatment with Helmar-1, Helmar-2, and silymarin. Data are presented as mean $\pm$ SD ($n = 5$). ** $p < 0.01$ vs healthy control; # $p < 0.05$ vs CCl_4 -induced toxic hepatitis group.

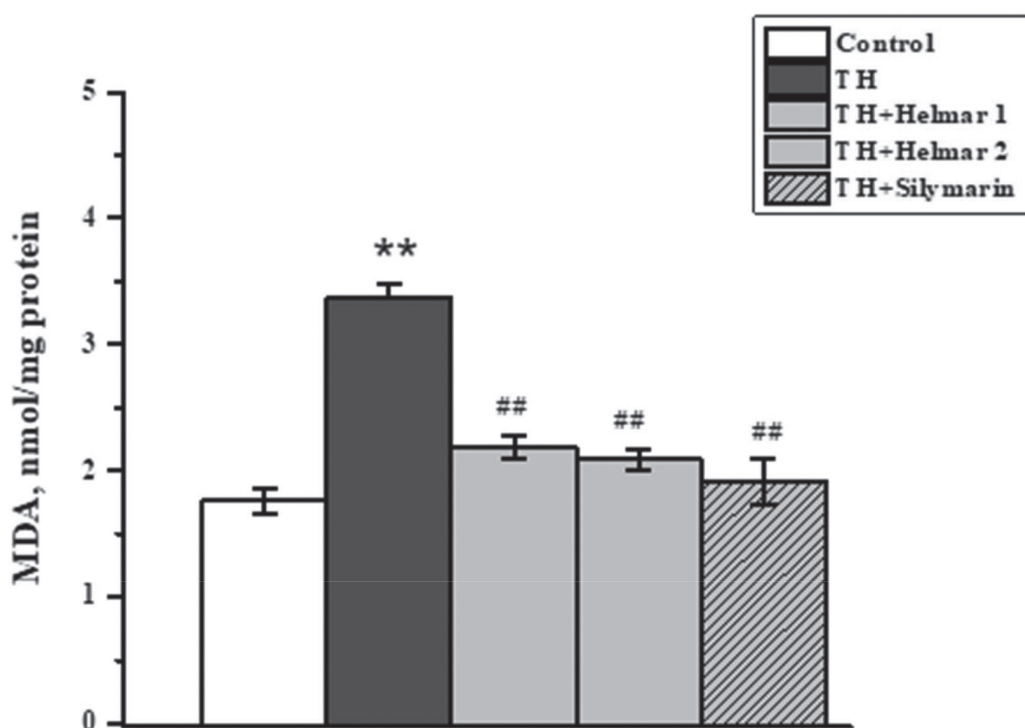


FIGURE 5 - Effect of Helmar extracts on lipid peroxidation (MDA levels) in liver mitochondria.

Malondialdehyde (MDA) levels as an indicator of lipid peroxidation in liver mitochondria of healthy control, CCl₄-induced toxic hepatitis, and treatment groups. CCl₄-induced toxic hepatitis significantly increased MDA levels compared with the healthy control group, whereas treatment with Helmar-1, Helmar-2, and silymarin significantly reduced lipid peroxidation. Data are presented as mean $\pm$ SD (n = 5). **p < 0.01 vs healthy control; #p < 0.05 vs CCl₄-induced toxic hepatitis group.

with toxic hepatitis may also cause mitochondria to dissociate from oxidative phosphorylation (OXPHOS). The polyphenol extracts selected in the study inhibited the LPO process in the liver mitochondrial membrane in rats with toxic hepatitis and returned the antioxidant enzyme activity closer to the control values.

Mitochondrial Respiration

Administration of CCl₄ induced marked alterations in mitochondrial respiration and oxidative phosphorylation parameters in rat liver mitochondria. In the TH group, the rate of substrate-dependent respiration (State V₂) was significantly reduced by 34.7% (**p < 0.001) relative to the control group. Similarly, ADP-stimulated respiration (State V₃) decreased by 28.6% (**p < 0.01), indicating impairment of mitochondrial energy metabolism.

In contrast, State V₄ respiration significantly increased by 54.6% (**p < 0.001), suggesting enhanced proton leakage and impaired coupling between oxidation and phosphorylation processes. Moreover, oxygen consumption after the addition of CCCP was lower than in the control group, indicating dysfunction of the mitochondrial respiratory chain.

Treatment with Helmar-1, Helmar-2, and silymarin for 10 days significantly improved mitochondrial respiratory function. Compared with untreated TH rats, V₂ respiration increased by 15.8%, 22.4%, and 27.5% in Helmar-1, Helmar-2, and silymarin-treated groups, respectively. A similar improvement was observed for V₃ respiration, which increased by 29.9%, 33.6%, and 36.9%, respectively.

The elevated V₄ respiration detected in TH animals was partially normalized following treatment with Helmar

extracts and silymarin. Reductions of 13.5%, 18.8%, and 25.2% were observed in Helmar-1, Helmar-2, and silymarin groups, respectively.

The respiratory control ratio (RCR) and ADP/O ratio, both of which were markedly reduced in the TH group, significantly improved after treatment. Helmar-2 produced a greater recovery effect than Helmar-1 across most mitochondrial parameters and approached the efficacy observed with silymarin treatment.

The summarized data on mitochondrial respiration and oxidative phosphorylation parameters are presented in Table 2.

Discussion

CCl₄-induced toxic hepatitis was associated with pronounced oxidative stress, as evidenced by a significant reduction in mitochondrial antioxidant enzyme activity and a marked increase in lipid peroxidation. The observed decrease in SOD, catalase, and GPx activities indicates impairment of the primary enzymatic defense system against reactive oxygen species (ROS), leading to mitochondrial dysfunction.

Excessive ROS generation promotes lipid peroxidation of mitochondrial membranes, resulting in elevated malondialdehyde (MDA) levels. This process disrupts membrane integrity, alters permeability, and impairs oxidative phosphorylation, ultimately contributing to hepatocellular damage (3,4,42).

Administration of polyphenol extracts isolated from *Helichrysum maracandicum* (Helmar-1 and Helmar-2) significantly restored antioxidant enzyme activity and reduced MDA levels. These protective effects can be attributed to the

TABLE 2 - Effects of Helmar-1 and Helmar-2 polyphenol extracts on mitochondrial respiration and oxidative phosphorylation parameters in CCl₄-induced TH

Groups	V ₂	V ₃	V ₄	RCR	ADP/O
Control (Healthy control)	32.5 ± 1.4	68.2 ± 2.1	18.3 ± 0.9	3.72 ± 0.15	2.41 ± 0.08
Group II (CCl ₄ -induced TH)	21.2 ± 1.3**	48.7 ± 1.9**	28.3 ± 1.4**	1.72 ± 0.12**	1.52 ± 0.07**
Group III (CCl ₄ + Helmar-1)	25.3 ± 1.5#	57.6 ± 2.2#	24.4 ± 1.2#	2.34 ± 0.11#	1.89 ± 0.05#
Group IV (CCl ₄ + Helmar-2)	27.8 ± 1.6##	65.1 ± 2.0##	22.5 ± 1.0##	2.91 ± 0.13##	2.18 ± 0.07##
Group V (CCl ₄ + Silymarin)	29.2 ± 1.3##	66.8 ± 2.4##	20.4 ± 0.8##	3.11 ± 0.15##	2.30 ± 0.06##

Values are expressed as mean ± SD (n = 5).

**p < 0.01 vs. healthy control group.

#p < 0.05, ##p < 0.01 vs. CCl₄-induced toxic hepatitis group.

strong free radical scavenging capacity of polyphenols and their ability to enhance endogenous antioxidant defense systems.

The antioxidant effects observed in the present study may be associated with modulation of Nrf2-related antioxidant pathways. However, this hypothesis requires further molecular validation through gene and protein expression analyses.

The decrease in MDA levels further indicates the suppression of lipid peroxidation and stabilization of the mitochondrial membranes. This suggests that Helmar extracts not only neutralize ROS but also preserve mitochondrial structural integrity and maintain the bioenergetic processes.

Notably, the effects of Helmar-1 and Helmar-2 were comparable to those of silymarin, a well-established hepatoprotective agent, highlighting their potential as natural therapeutic alternatives for liver protection. These findings are consistent with previous reports on the antioxidant and hepatoprotective properties of plant-derived polyphenols (3-6).

Overall, these results demonstrate that *Helichrysum maracandicum* polyphenols exert multifaceted protective effects by targeting key mechanisms of oxidative stress, including ROS scavenging, enhancement of antioxidant enzyme activity, and inhibition of lipid peroxidation. These findings are supported by recent studies demonstrating that oxidative stress plays a central role in the progression of liver disease and mitochondrial dysfunction (31,32). Modern research has highlighted that excessive production of reactive oxygen species leads to hepatocellular damage and impairment of cellular bioenergetics (33). Furthermore, natural polyphenols have been widely reported to exert protective effects by modulating oxidative stress and enhancing endogenous antioxidant defense systems (34). In particular, polyphenolic compounds have been shown to stabilize mitochondrial membranes and improve redox homeostasis (36).

Recent investigations have emphasized the importance of lipid peroxidation biomarkers, particularly malondialdehyde (MDA), as reliable indicators of oxidative damage in liver tissues (37,38). In addition, antioxidant-based therapeutic strategies have gained increasing attention for their role in preventing and treating hepatic disorders (39,40). The hepatoprotective effects observed in the present study are consistent with previous findings on natural compounds such as silymarin, which has been extensively studied for its efficacy in treating liver diseases (41).

Study limitations

This study has several limitations. Histopathological evaluation of liver tissue and molecular analyses of genes or proteins associated with mitochondrial bioenergetics were not performed. In addition, detailed phytochemical profiling of Helmar-1 and Helmar-2 fractions by HPLC/LC-MS was not performed in the present study, which may limit precise characterization of their active constituents. Future studies should include gene and protein expression analyses, including PGC-1α, NRF-1, TFAM, Nrf2-related antioxidant signaling pathways, and mitochondrial respiratory chain proteins, together with histopathological assessment and advanced phytochemical profiling to further clarify the underlying mechanisms.

Conclusion

The results of this study indicate that toxic hepatitis is associated with pronounced impairment of mitochondrial antioxidant defense, as reflected by reduced activities of key enzymes such as superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx), which is consistent with recent findings highlighting oxidative stress as a key mechanism of hepatocellular injury (1-3).

Our results showed that the administration of Helmar-1 and Helmar-2 polyphenol extracts (20 mg/kg) effectively restored the activity of these enzymes, confirming their strong antioxidant potential. These findings are consistent with studies indicating that plant-derived polyphenols enhance endogenous antioxidant defense and protect mitochondrial function under pathological conditions (4-6).

In addition, the observed decrease in malondialdehyde (MDA) levels suggests reduced lipid peroxidation and improved stability of the mitochondrial membranes. Similar effects of natural antioxidants on MDA reduction have been reported widely (7-9).

Importantly, the effects of Helmar-1 and Helmar-2 were comparable to those of silymarin, a well-known hepatoprotective agent, supporting previous findings on the therapeutic potential of polyphenol-rich compounds (10).

Overall, these results confirm the important role of oxidative stress in toxic hepatitis and demonstrate that *Helichrysum maracandicum* polyphenol extracts effectively restore mitochondrial function. These findings highlight their

potential as promising candidates for developing novel hepatoprotective therapies. Further investigations are needed to assess their long-term safety and to better understand the underlying molecular mechanisms of these drugs.

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