

Hepatoprotective Effects of Gac (*Momordica cochinchinensis*) Aril Extract in Acetaminophen-Induced Liver Injury: Modulation of Oxidative Stress, Inflammation, and Glucose Metabolism

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Background: N-acetyl-p-aminophenol (APAP) overdose remains a leading cause of acute liver failure worldwide. The aril extracts derived from Gac (*Momordica cochinchinensis*) (MC) fruit contain diverse phytonutrients that exhibit a range of pharma-nutritional properties, including antioxidant and anti-hepatic damage properties. Despite the therapeutic potential, the molecular mechanisms underlying its hepatoprotective action, particularly in preventing drug-induced toxicity, remain unelucidated.

Purpose: This study investigated the hepatoprotective potential of MC in a mouse model of APAP-induced acute liver injury and characterized its bioactive compounds.

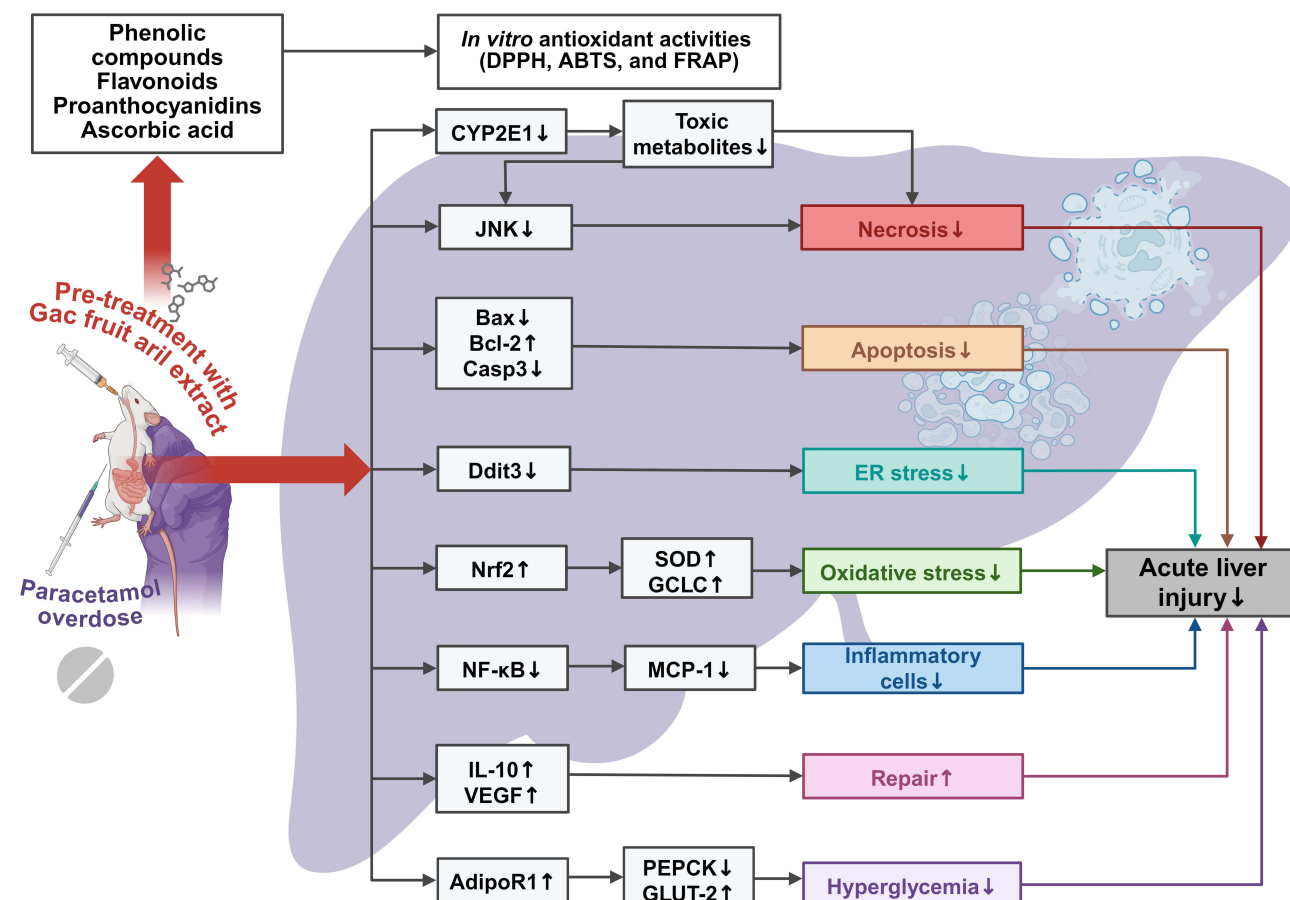
Methods: MC was prepared using aqueous extraction. Mice were pre-treated with either 500 or 1,000 mg/kg of MC for seven consecutive days prior to intraperitoneal administration of APAP 300 mg/kg to induce hepatotoxicity. Serum and liver samples were then collected to analyze biochemical changes, histopathological changes, oxidative stress markers, and/or mRNA expression. Standard chemical tests were used to identify bioactive compounds.

Results: APAP administration caused a marked rise in liver enzymes, extensive histological necrosis, suppression of hepatocyte proliferation, infiltration of neutrophils and macrophages, and significant oxidative damage ($p < 0.05$). Studies revealed the constitution of MC as phenolic compounds, flavonoids, proanthocyanidins, and ascorbic acid, significantly attenuated the hepatotoxicity ($p < 0.05$). Mechanistically, MC ameliorated hepatic mRNA expression of CYP2E1, JNK, Ddit3, Bax, Casp3, NF- κ B, and MCP-1, while increasing Bcl-2, IL-10, VEGF, Nrf2, SOD2, and GCLC mRNA ($p < 0.05$). Furthermore, MC prevented hyperglycemia by influencing the expression of AdipoR1, GLUT-2, and PEPCK ($p < 0.05$).

Conclusion: MC exhibits hepatoprotective effects against APAP-induced liver injury through multifaceted modulation of toxic metabolism (CYP2E1), cell death and ER stress (JNK, Ddit3, Bax, Casp3, and Bcl-2), antioxidant response (Nrf2 pathway), inflammation (NF- κ B pathway and IL-10), tissue repair (VEGF), and glucose metabolism (AdipoR1, GLUT-2, and PEPCK).

Keywords: acetaminophen, anti-hyperglycemia, antioxidant, immunomodulation, *Momordica cochinchinensis*

Graphical Abstract



Introduction

Although chronic diseases are a major public health concern, acute or emergency diseases, such as drug-induced liver injury, can cause rapid clinical complications and even death in patients. In the case of paracetamol, acetaminophen, or N-acetyl-p-aminophenol (APAP) overdose, a commonly used medication, it can lead to acute liver injury and fatal complications, including acute liver failure and death.^{1,2} APAP overdose is reported as the most frequent cause of acute liver failure in Western populations, accounting for approximately 50–60% of cases in the United Kingdom (UK) and the United States (USA).^{2,3} In the USA, patients with APAP overdose account for over 78,000 emergency department visits and approximately 500 deaths annually.³ Because the liver is a major xenobiotic-metabolizing organ, excessive APAP exposure leads to hepatocyte injury and death through multiple phases of pathophysiological processes.³ The first phase of hepatotoxicity is initiated by cytochrome P450 2E1 (CYP2E1) to generate toxic metabolites like N-acetyl-p-benzoquinone imine (NAPQI), which can lead to necrotic death of hepatocytes.^{2,3} These toxic metabolites can also induce oxidative stress by reducing glutathione (GSH) and suppressing nuclear factor erythroid 2-related factor 2 (Nrf2) antioxidant pathway.^{2–5} Hepatocyte death can occur through apoptosis by imbalancing pro-apoptotic molecules, B-cell lymphoma protein 2 (Bcl-2)-associated X (Bax) and caspase 3 (Casp3), and anti-apoptotic molecules, Bcl-2.² APAP-induced endoplasmic reticulum (ER) stress also initiates hepatic apoptosis through the induction of DNA damage-inducible transcript 3 protein (Ddit3) or CCAAT/enhancer binding homologous protein (CHOP).^{6,7}

During the second phase, the pro-inflammatory response is associated with the activation of nuclear factor-kappa B (NF-κB), which induces the production of monocyte chemoattractant protein-1 (MCP-1) to recruit neutrophils and

macrophages to the liver.^{2,8} Conversely, the anti-inflammatory cytokines like interleukin-10 (IL-10) are suppressed, enhancing the progression of the pro-inflammatory state.⁹ Moreover, hepatic repair is deficient due to several growth factors, including vascular endothelial growth factor (VEGF), which may be a mechanism of the failed recovery phase of hepatic abnormalities induced by APAP.^{10,11} Recent evidence has also proposed that hyperglycemia can stimulate pro-inflammatory and oxidative stress responses in the liver during APAP toxicity.¹² These responses could be linked to the dysregulation of glucose metabolism, particularly the altered regulating pathways of hepatic gluconeogenesis and glucose sensing, including adiponectin receptor (AdipoR), phosphoenolpyruvate carboxylase (PEPCK), and glucose transporter -2 (GLUT-2).^{13–16} Thus, understanding the above mechanisms can help identify targets for attenuating the severity of acute liver injury and failure caused by APAP.

Increasing pre-clinical and clinical pieces of evidence have demonstrated that several tropical fruits and their co-products contain a variety of beneficial bioactive phytochemicals and nutrients, such as phenolic chemicals, carotenoids, vitamins, and fibers, which may contribute to their health-promoting potentials, including antioxidant, immunomodulatory, hypoglycemic, hypolipidemic, hepatoprotective, and neuroprotective effects.^{17,18} A tropical vine plant Gac (scientific name: *Momordica cochinchinensis* Spreng; abbreviated as MC) is one species of the Cucurbitaceae family found in several countries for food and traditional medicinal purposes, particularly in South and Southeast Asian countries, and Northeastern Australia.^{17,19} In Thailand, it is called Fak Khao and is consumed in various forms, such as aril with pulp juice, boiled fruits, young shoots, and leaves served with chili paste or in curries.²⁰ The aril part, an outer red membrane covering the MC seed, has shown high contents of total phenolic compounds and carotenoids (lycopene and β -carotene), as well as the best antioxidant activity by the ferric reducing antioxidant power (FRAP) assay when compared to the peel, pulp, and/or seed.²⁰ This highlights the importance of further studying MC arils. Recently, the crude water extract from the aril of MC, containing polyphenolic compounds, lycopene, and β -carotene, elicited locomotor function-improvement, anti-memory impairment, anti-hyperglycemic, and anti-damage effects on male reproductive systems in animal models.^{21,22} Additionally, the lyophilized aril from MC protected against high-fat diet-induced hepatic steatosis, hepatic damage, and hyperglycemia in mice.¹⁹ Moreover, MC exhibited cytotoxic activities in cancer cell lines by influencing necrotic and apoptotic mechanisms.²³ Other parts of MC, such as seed extract and its constituents, also revealed anti-inflammatory and immunomodulatory effects in macrophages or neutrophils, as well as healing effects on gastric ulcers by enhancing VEGF and angiogenesis.^{17,24,25} The treatments with important bioactive constituents, such as polyphenolic flavonoids, proanthocyanidins, and ascorbic acid (vitamin C) were proven to exhibit multiple benefits for controlling pro-oxidant, pro-inflammatory, and cell death processes in experimental animals.^{26–28} These studies have led to the hypothesis that the hepatoprotective, antioxidant, anti-inflammatory, metabolic-regulating, and cell death-regulating properties of MC may have a beneficial preventive role in redox, immune, and metabolic dysregulations involved in various diseases through its health-promoting bioactive compounds, especially in liver injury and diseases. However, no investigation has studied the hepatoprotective effects of MC against APAP toxicity, especially its molecular mode of action. Thus, the present study mainly aimed to evaluate the protective effects of MC on hepatic cell death, ER stress, oxidative stress, inflammation, repair, and hyperglycemia in an APAP-induced liver injury mouse model, the screening pre-clinical model for investigating the pharmacological and toxic effects of novel agents and their mechanisms. This study also investigated the phenolic chemicals and ascorbic acid present in MC and its antioxidant potentials through in vitro screening experiments.

Materials and Methods

Preparation of MC

The aril portions were collected from Gac fruits (Phrae Province, Thailand). A voucher specimen (No. 2273) of the plant material used in this study, which was authenticated by Asst. Prof. Dr. Yuwalee Unpaprom, a botanist at the Center of Excellence in Agricultural Innovation for Graduate Entrepreneur, Maejo University, has been deposited at the herbarium of the same center in Chiang Mai Province, Thailand. 300 g of aril portions were blended in 1,250 mL of distilled water, as described in previous studies.^{22,29} This aril juice was filtered through Whatman qualitative filter paper No.1 using a vacuum filtration unit. Finally, the filtered solution was freeze-dried to obtain a dry aqueous extract or MC, and the MC was stored at -80°C for further experiments. The preparation process of MC is demonstrated in Figure 1A.

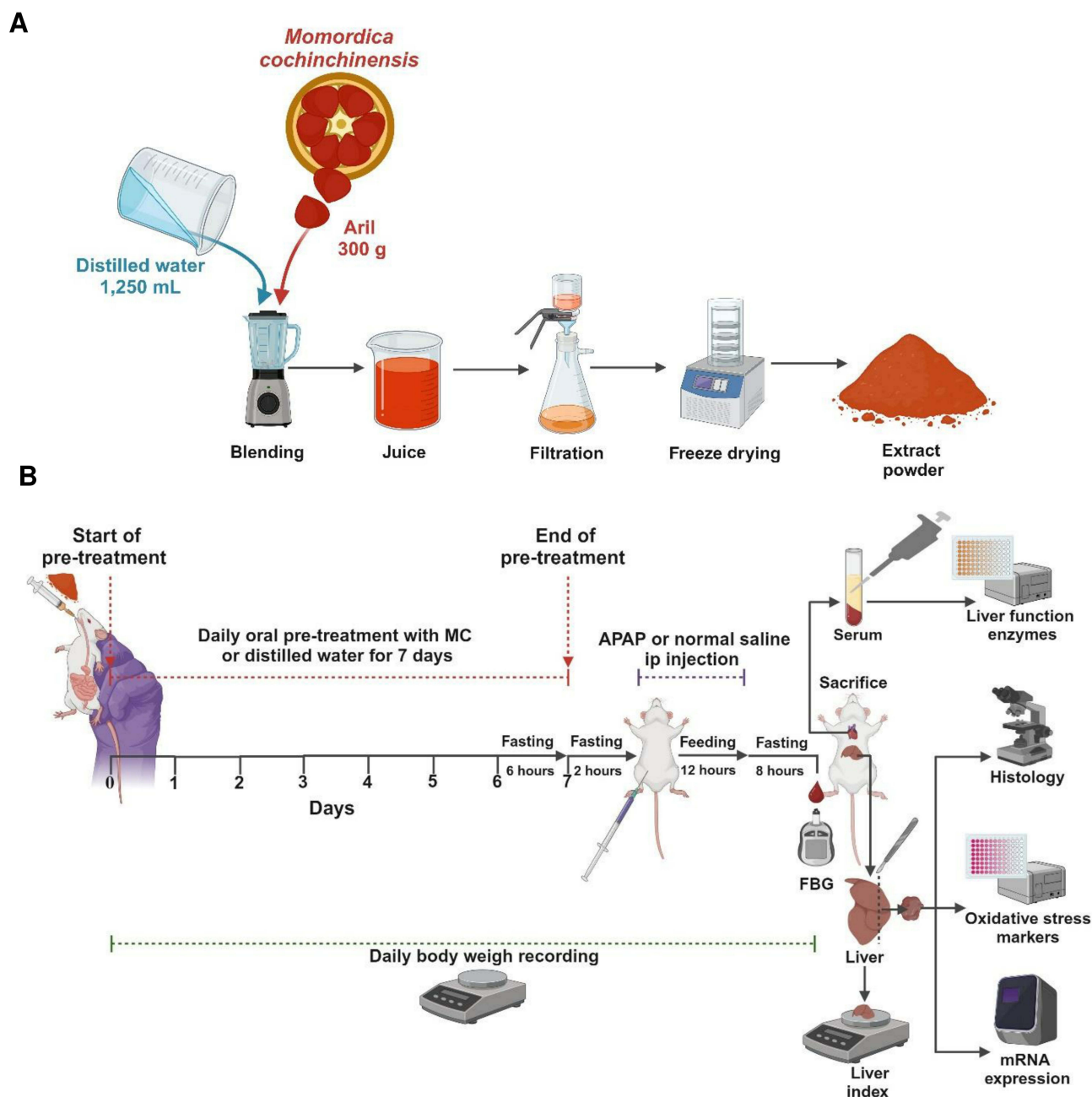


Figure 1 Schematic diagram of MC extraction protocol (A) and experimental design (B), created with BioRender.com.

Screening of Bioactive Compounds and in vitro Antioxidant Activities of MC

The screening protocols for the determination of bioactive compounds and in vitro antioxidant activities were based on previous studies^{21,30,31} with slight modifications. MC or gallic acid standard was mixed with Folin–Ciocalteu and sodium carbonate reagents (Merck KGaA, Germany) to determine their total phenolic compound contents, and these results were reported as mg gallic acid equivalent (GAE)/g of MC. In addition, MC or catechin standard was also mixed with aluminum chloride solution and vanillin (Sigma-Aldrich, Saint Louis, MO, USA) and sulfuric acid solutions (RCI Labscan Limited, Thailand) to quantify the amounts of total flavonoids and proanthocyanidins, respectively, and both amounts were reported as μg of catechin equivalent (CE)/g of MC. The ascorbic acid contents in MC were evaluated using the standard titration method with iodine (QR&C, New Zealand) and starch indicator solutions (RCI Labscan Limited). These contents were expressed as mg/100 g of MC.

The antioxidant capacities of MC were assessed by mixing MC at final concentrations of 62.5–500 µg/mL or ascorbic acid antioxidant standard at final concentrations of 0.625–10 µg/mL with free radical solutions, including stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) and diammonium 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS) (Sigma-Aldrich). The half-maximal inhibitory concentration (IC₅₀) values of each antioxidant assay were calculated to estimate the concentration of MC and standard that had the capacity to reduce the DPPH or ABTS free radicals by 50%. Based on the reducing power of agents with antioxidant properties to convert the ferric ion (Fe³⁺) to the ferrous ion (Fe²⁺), MC sample or ascorbic acid positive control solutions were combined with the FRAP solution, which included the oxidant 4,6-tri(2-pyridyl)-s-triazine (TPTZ) and iron (III) chloride hexahydrate (FeCl₃·6H₂O) in an acidic solution (Sigma-Aldrich). Using the calibration curve of iron II sulfate heptahydrate (FeSO₄·7H₂O) (Merck KGaA), the FRAP values were expressed as mg FeSO₄/g of MC.

APAP-Induced Acute Liver Injury Mouse Model

All animal testing methods were approved by the Institutional Animal Care and Use Committee of the University of Phayao, Phayao, Thailand (Approval No. 1-018-66), and conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 8th edition, 2011). Male five-week-old Institute of Cancer Research (ICR) mice (Nomura Siam International Co., Ltd., Bangkok, Thailand), weighing approximately 32 g, were housed in an environmentally controlled room (22–25°C, 60% humidity, and a normal dark-light cycle) for a seven-day acclimation period and throughout the treatment period at the Laboratory Animal Research Center, University of Phayao, Phayao, Thailand. After an 8-hour overnight fast, fasting blood glucose (FBG) was evaluated in tail-tip blood using an automatic blood glucose meter and test strips (Roche Diabetes Care GmbH, Mannheim, Germany). A total of 30 mice were further randomly divided into five groups (n = 6/group): (1) vehicle control (VC) group; (2) MC at 1 g/kg/day plus vehicle control (HM+VC) group; (3) APAP group; (4) low-dose MC at 0.5 g/kg/day plus APAP (LM+APAP) group; (5) high-dose MC at 1 g/kg/day plus APAP (HM+APAP) group. During the pre-treatment period, sterile distilled water was administered daily to mice in the VC and APAP groups by oral gavage for 6 consecutive days, while MC dissolved in sterile distilled water was administered daily to mice in the HM+VC, LM+APAP, and HM+APAP groups using the same protocol and timing. On the 7th day, the mice were fasted for 6 hours and then last administered either distilled water or MC for the next 2 hours. During the induction period, warm normal saline was injected intraperitoneally (ip) into the VC and HM+VC groups, whereas a single dose of APAP at 300 mg/kg (Sigma-Aldrich) dissolved in warm normal saline was ip injected into the APAP, LM+APAP, and HM+APAP groups for 12 hours with feeding the normal diet. The induction of acute liver injury by an APAP overdose in mouse models and the pattern of pre-treatment with the herbal extract in these models have been previously documented.^{21,32,33} FBG was evaluated after an 8-hour fast. The mice were euthanized using carbon dioxide in accordance with approved ethical guidelines. Euthanasia was confirmed by signs of unconsciousness, including the absence of pedal reflexes and movement. Following euthanasia, blood was collected via cardiac puncture, and the livers were harvested for further analyses. Daily body weight was collected, while wet liver weight was collected after euthanization. The liver index was calculated as the liver weight (g) per 100 g of final body weight (g).³⁴ Our experimental design in mice is presented in Figure 1B.

Serum Liver Injury Marker Evaluation

After clotting at room temperature, the serum samples were separated from whole-blood samples by centrifuging at 3000 rpm for 10 minutes at 4 °C. To determine liver injury, the activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT), the liver enzymes, were analyzed in the serum samples using AST and ALT assay kits from Erba Diagnostics Mannheim GmbH, Mannheim, Germany. These liver enzyme activities were calculated using equations from their standards and expressed as U/I, according to the manufacturer's instructions.

Hepatic Histological Evaluation

The hepatic tissue specimens were rinsed with normal saline, fixed in 10% neutrally buffered formalin, embedded in paraffin, microtomed into a thickness of 5 µm, and stained with a basic dye hematoxylin (nuclear purple) and an acidic dye eosin (cytoplasmic pink) (H&E), following classical tissue processing and staining techniques. Ten representative

histologic images of H&E-stained hepatic tissues at 20x magnification were captured blindly and randomly from four mice per group using an Olympus BX53-P polarizing microscope with an SC180 digital microscope camera (Olympus Corporation, Tokyo, Japan). The hepatocytes with the disappearance of the nuclear components (called karyolysis) and rupture or lysis of plasma membranes were typically used as the markers of necrosis,³⁴ and the presence of necrotic areas was measured in the total tissue area using the Olympus cellSens Standard software. Furthermore, the presence of inflammatory foci was counted from each captured image of the total ten captured images (10 fields/mouse).

Hepatic Immunohistochemical Evaluation

Liver sections were immunostained for Ki-67, a marker of proliferating cells, and cluster of differentiation 68 (CD68), a macrophage marker, using the Ultraview Universal DAB detection Kits on a Ventana automated stainer (Ventana Medical Systems, Tucson, AZ, USA). Myeloperoxidase (MPO), a marker of neutrophils, was stained using the BOND III automated stainer (Leica Biosystems, Wetzlar, Germany). All staining protocols included heat-induced epitope retrieval, primary and secondary antibody incubations, and chromogenic substrate development, followed by counterstaining. Positive cells were identified by chromogenic labeling: Ki-67 and MPO were visualized as brown staining, while CD68 was detected as red staining. For each group, randomly selected fields from four mice per group (original magnification: 40×) were captured using Olympus cellSens Standard software with an Olympus BX53-P polarizing microscope equipped with an SC180 digital camera. Quantification was performed by counting the number of positively stained cells per field.

Hepatic Oxidative Stress Evaluation

The mouse hepatic tissues were minced into very small pieces using surgical scissors and then homogenized in 0.05 M phosphate buffer with a tissue homogenizer instrument on ice. After centrifugation, the hepatic supernatants were collected and then reacted with 200 µM fluorescent 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA) solution (Sigma-Aldrich) to determine ROS levels, according to a previous protocol.³² Additionally, the supernatants were reacted with 10% thiobarbituric acid (TBA) solution (Sigma-Aldrich) and 0.67% trichloroacetic acid (TCA) solution (Merck KGaA) to determine the levels of malondialdehyde (MDA), a marker of oxidative damage, according to a previous protocol.³² Protein concentrations in the supernatant were determined using the ready-to-use Bradford reagent kit (HiMedia Laboratories Private Limited, India). The levels of ROS and MDA were reported as a percentage of the VC group and nmol/mg of protein, respectively.

Hepatic mRNA Expression Evaluation

Livers were scissor-minced and homogenized in TRIzol reagent (Ambion, Life Technologies, Carlsbad, CA, USA), and their supernatants were mixed with chloroform to collect the RNA-containing clear upper phase, following the manufacturer's protocol. The RNA-containing samples were then precipitated and washed with isopropanol and 75% ethanol, respectively. After resuspension in diethylpyrocabonate (DEPC)-treated water, total RNA levels were determined using an OPTIZEN NanoQ Microvolume Spectrophotometer (KLAB Co., Ltd., South Korea). RNA was mixed with the reaction components for complementary DNA (cDNA) synthesis using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™, Thermo Fisher Scientific Inc., Waltham, MA, USA) and amplified on a Biometra TOne Thermal Cycler (Analytik Jena GmbH +Co. KG, Germany). 50 ng of cDNA was mixed with TaqMan master mix containing fluorescein amidite (FAM) dye-labeled TaqMan Minor Groove Binder (MGB) probes specific for each target gene (Applied Biosystems™, Thermo Fisher Scientific Inc.). The TaqMan reactions were performed on a CFX96 Real-Time System (Bio-Rad Laboratories, Inc., Hercules, CA, USA), following the real-time PCR reaction program as described by the manufacturer's recommendation. The assay IDs of the FAM dye-labeled TaqMan MGB probes for the target and housekeeping genes were designed and produced by Applied Biosystems™, Thermo Fisher Scientific Inc., as shown in [Supplemental Table 1](#). The threshold cycle value of each mRNA and the $2^{-\Delta\Delta C_t}$ equation were used to quantify the relative expression levels of the target mRNA, with GAPDH mRNA used as a housekeeping gene to normalize all target mRNA expression levels. The raw data for all mRNA expression levels have been provided in [Supplementary File 2](#).

Statistical Analysis

IBM SPSS software version 26 (IBM Corp., Armonk, NY, USA) was used to calculate descriptive values and analyze statistically significant differences between the means of the experimental groups using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test. Meanwhile, the independent samples *t*-test was used to analyze statistically significant differences in vitro antioxidant activities. Values are presented as means $\pm$ standard deviation (SD). A significance level of $p < 0.05$ was used to determine the significant differences between the experimental groups and values.

Results

Bioactive Compounds and in vitro Antioxidant Activities

In phytochemical screening assays, MC contained total phenolic compounds, flavonoids, a specific subclass of poly-phenolic flavonoids, proanthocyanidins, and ascorbic acid, as shown in Table 1. In DPPH and ABTS free radicals scavenging assays, MC exhibited low potentials to directly scavenge these radicals, with its IC₅₀ values being higher than those of the antioxidant standard ascorbic acid (Table 1). The FRAP value of MC was also significantly lower than that of ascorbic acid, confirming that MC has limited effectiveness in in vitro antioxidant activities (Table 1).

In vivo Anti-Hepatic Injury and Hepatic Histology-Improving Parameters

The body weight and relative liver weight at the end of the experiment showed no significant differences between the experimental groups (Table 2). Microscopically, the livers of the VC group demonstrated a normal lining of hepatocytes with their continuous plasma membranes and presence of nuclei, incorporated with the normal sinusoidal wall (Figure 2A). However, after APAP injection, mice in the APAP group showed significantly higher serum AST and ALT activities compared to the VC group (Table 2). APAP injection markedly induced prominent histological necrosis of hepatocytes, characterized by damaged plasma membranes and loss of nuclei (Figure 2A). This was correlated with a significant increase in the percentage of necrotic area (Figure 2C), but accompanied by a low number of Ki-67-positive

Table 1 Bioactive Compound Contents and in vitro Antioxidant Activities of MC

Bioactive Compounds and in vitro Antioxidant Assays	Contents	Antioxidant Capacity Values
Total phenolic compounds (mg GAE/g)	167.97 $\pm$ 4.15	–
Total flavonoids (μ g CE/g)	551.40 $\pm$ 308.93	–
Total proanthocyanidins (μ g CE/g)	269.33 $\pm$ 4.97	–
Ascorbic acid (mg/100 mg)	2.75 $\pm$ 0.34	–
IC₅₀ values for DPPH assay (μg/mL)		
MC	–	> 500
Ascorbic acid	–	4.83 $\pm$ 0.04
IC₅₀ values for ABTS assay (μg/mL)		
MC	–	185.87 $\pm$ 2.83 ^a
Ascorbic acid	–	4.81 $\pm$ 0.07 ^b
FRAP values (mg FeSO₄/g)		
MC	–	1.42 $\pm$ 0.25 ^b
Ascorbic acid	–	5195.61 $\pm$ 839.64 ^a

Notes: Values are expressed as mean $\pm$ SD (n = 3). Different superscript letters indicate significant differences between MC and ascorbic acid ($p < 0.05$), as determined by an independent samples *t*-test.

Abbreviations: ABTS, diammonium 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate); CE, catechin equivalent; DPPH, 2-diphenyl-1-picrylhydrazyl; FRAP, ferric reducing antioxidant power; GAE, gallic acid equivalent.

Table 2 Effects of 7-Day Pre-Treatment with Low-Dose MC at 500 mg/kg/Day (LM) or High-Dose MC at 1,000 mg/kg/Day (HM) on Body Weight, Liver Index, and Liver Function Enzyme Activities in the Serum of APAP-Injected Mice or Vehicle Control Saline-Injected Mice (VC)

Groups	Body Weight (g)	Liver Index (% of Body Weight)	Serum AST Activities (U/l)	Serum ALT Activities (U/l)
VC	32.50±1.05 ^a	5.32±0.22 ^a	35.73±12.24 ^b	6.19±3.54 ^b
HM+VC	33.83±1.47 ^a	5.00±0.18 ^a	16.22±14.35 ^b	8.72±0.86 ^{ab}
APAP	33.83±1.33 ^a	5.28±0.35 ^a	111.71±64.99 ^a	36.55±36.67 ^a
LM+APAP	33.50±0.55 ^a	5.30±0.34 ^a	14.07±9.84 ^b	2.79±3.42 ^b
HM+APAP	33.20±0.45 ^a	5.30±0.33 ^a	20.87±8.14 ^b	2.83±3.54 ^b

Notes: Values are expressed as mean ± SD (n = 6/group). Different superscript letters (a,b) indicate significant differences between groups ($p < 0.05$), as determined by one-way ANOVA followed by Tukey's post hoc test; the same letter indicates no significant difference.

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase.

hepatocytes, a marker of cell proliferation (Figures 2B and D). Moreover, the APAP group exhibited pronounced infiltration of inflammatory cells, as shown by H&E staining (Figure 3A), and immunohistochemical staining for the neutrophil marker MPO (Figure 3B) and the macrophage marker CD68 (Figure 3C). This was associated with increased numbers of inflammatory foci (Figure 3D), as well as elevated counts of MPO-positive (Figure 3E) and CD68-positive cells in the liver (Figure 3F). Conversely, APAP-treated groups receiving either low-dose MC (500 mg/kg/day) or high-dose MC (1,000 mg/kg/day) showed significantly reduced serum AST and ALT activities, necrotic areas, numbers of inflammatory foci, and counts of MPO- and CD68-positive cells. These groups showed a significant, dose-dependent increase in Ki-67-positive hepatocytes and notable Ki-67-positive sinusoidal cells compared to the untreated APAP

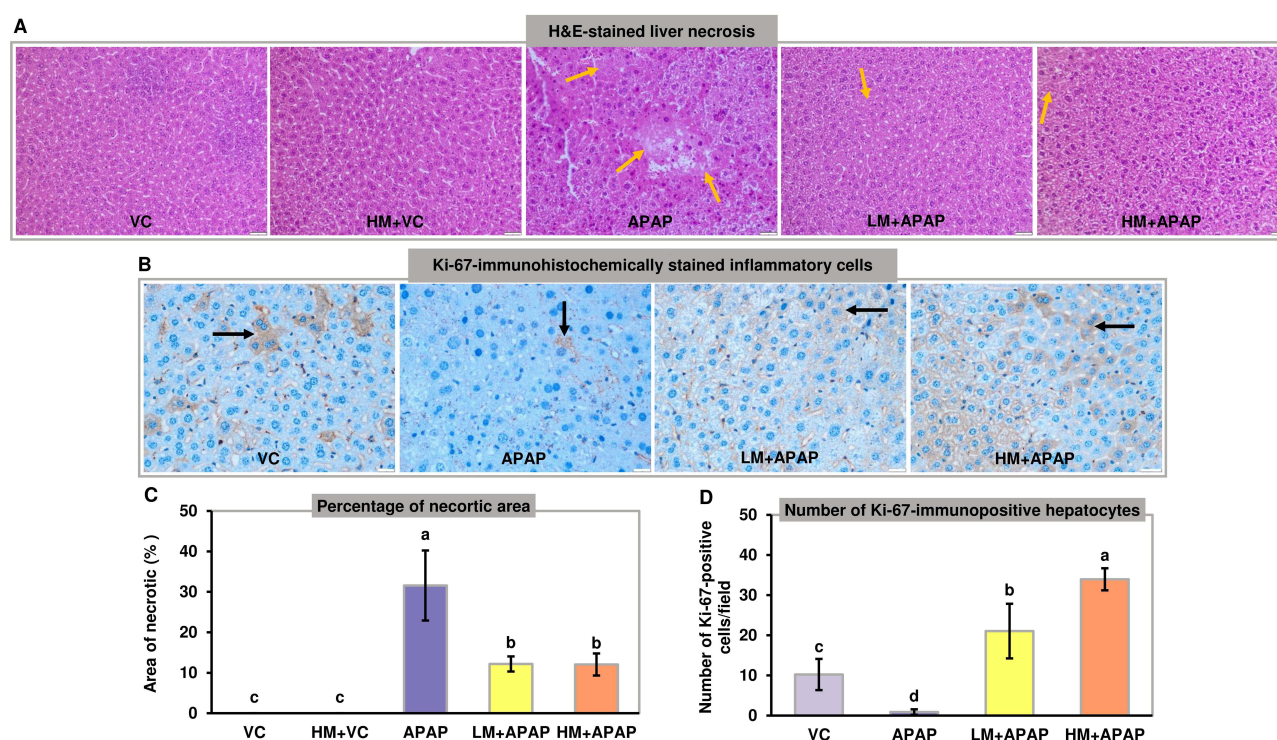


Figure 2 Effects of pre-treatment with low-dose MC at 500 mg/kg/day (LM) or high-dose MC at 1,000 mg/kg/day (HM) on hepatic histological changes, (A, orange arrows indicate necrotic areas), Ki-67-immunostained hepatocytes (B, black arrows indicate positively stained cells exhibiting brown coloration), percentage of necrotic area (C), and number of Ki-67-positive hepatocytes (D) in APAP-injected mice or vehicle control saline-injected mice (VC). H&E-stained images (A) were captured at 20× original magnification, while immunostained images (B) were captured at 40× original magnification; both include a 20-μm scale bar. Values are expressed as mean ± SD (n = 4–6/group). Different superscript letters (a,b) indicate significant differences between groups ($p < 0.05$), as determined by one-way ANOVA followed by Tukey's post hoc test; the same letter indicates no significant difference.

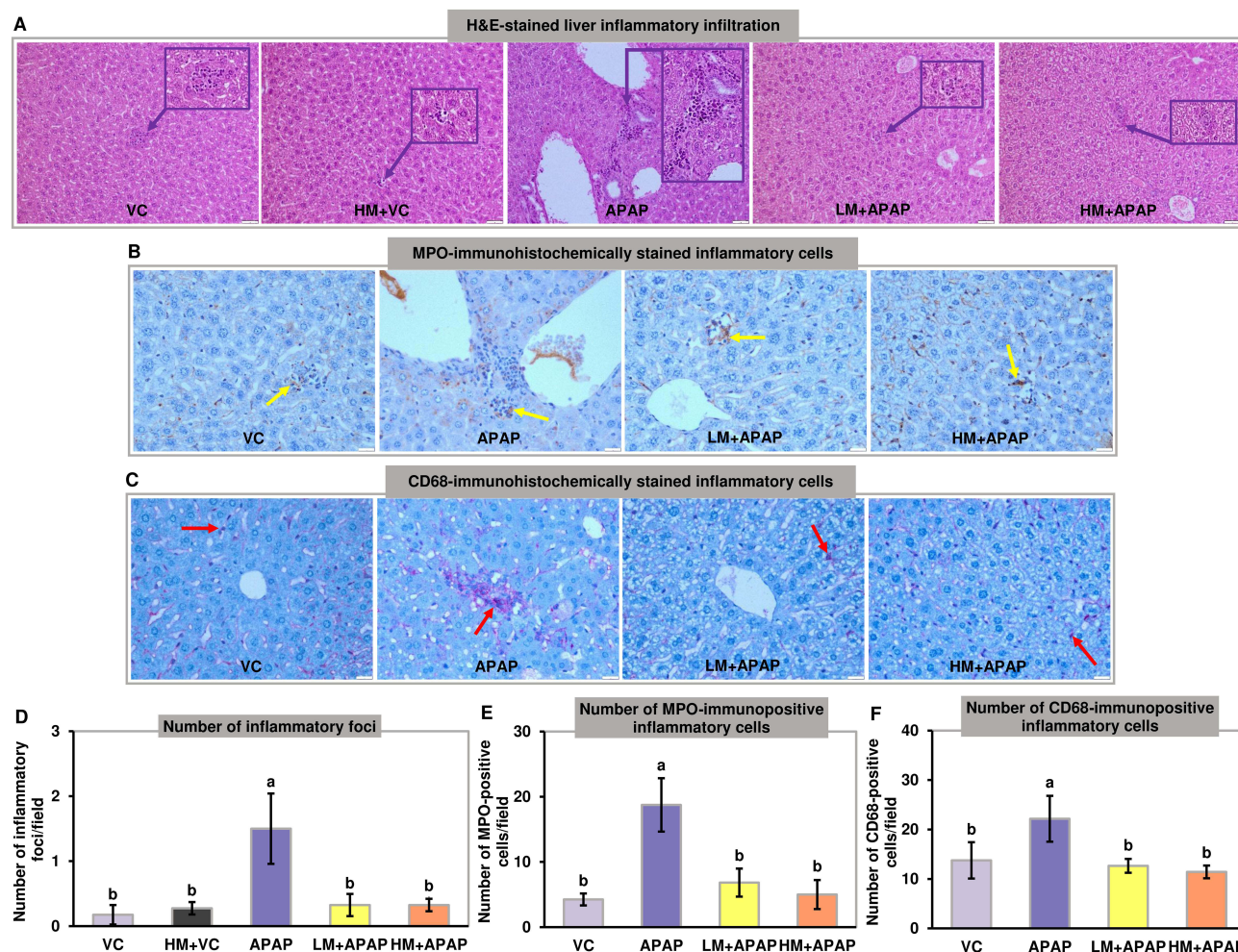


Figure 3 Effects of pre-treatment with low-dose MC at 500 mg/kg/day (LM) or high-dose MC at 1,000 mg/kg/day (HM) on hepatic inflammatory cell infiltration (**A**, purple arrows indicate inflammatory foci), neutrophil marker MPO-immunostained inflammatory cells (**B**, yellow arrows indicate positively stained cells exhibiting brown coloration), macrophage marker CD68-immunostained inflammatory cells (**C**, red arrows indicate positively stained cells exhibiting red coloration), number of hepatic inflammatory foci (**D**), number of MPO-positive inflammatory cells (**E**), and number of number of CD68-positive inflammatory cells (**F**) in APAP-injected mice or vehicle control saline-injected mice (VC). H&E-stained images (**A**) were captured at 20× original magnification, while immunostained images (**B** and **C**) were captured at 40× original magnification; both include a 20-μm scale bar. Values are expressed as mean ± SD (n = 4–6/group). Different superscript letters (a,b) indicate significant differences between groups ($p < 0.05$), as determined by one-way ANOVA followed by Tukey's post hoc test; the same letter indicates no significant difference.

group. However, these MC-treated groups had a significant percentage of necrotic area that was different from the liver of the VC group, which did not have any necrotic area. Similar to the VC group, the VC group treated with high-dose MC exhibited normal levels of general (Table 2), biochemical (Table 2), and histological parameters (Figures 2 and 3). Molecularly, real-time PCR analyses revealed significant increases in mRNA expression levels of genes encoding the APAP-metabolizing enzyme CYP2E1 (Figure 4A), APAP toxic signaling molecule JNK (Figure 4B), ER stress marker Ddit3 (Figure 4C), pro-apoptotic Bax (Figure 4D), and apoptotic enzyme Casp3 (Figure 4F), as well as significant decreases in mRNA expression levels of the anti-apoptotic Bcl-2 (Figure 4E) in the livers of APAP-injected mice, compared to the controls. In comparison with the untreated APAP-injected mice, the liver mRNA levels of CYP2E1, JNK, Ddit3, Bax, and Casp3 were significantly decreased, whereas mRNA levels of Bcl-2 were significantly increased in the APAP-injected mice treated with MC.

In vivo Immunomodulatory and Repair Parameters

Consistent with the microscopic results in Figure 3, mRNA expression levels of pro-inflammatory marker genes, NF-κB (Figure 5A), MCP-1 (Figure 5B), neutrophil marker Ly6G (Figure 5C), and macrophage marker F4/80 (Figure 5D), were

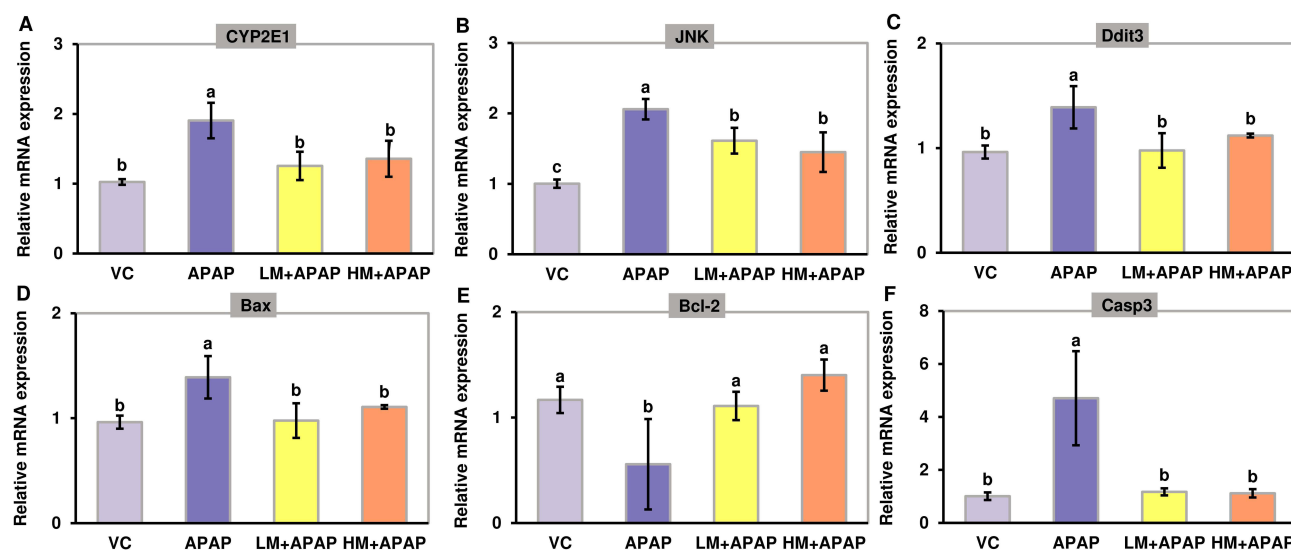


Figure 4 Effects of 7-day pre-treatment with low-dose MC at 500 mg/kg/day (LM) or high-dose MC at 1,000 mg/kg/day (HM) on mRNA levels of CYP2E1 (A), JNK (B), Ddit3 (C), Bax (D), Bcl-2 (E), and Casp3 (F) in the livers of APAP-injected mice or vehicle control saline-injected mice (VC). Different superscript letters (a,b) indicate significant differences between groups ($p < 0.05$), as determined by one-way ANOVA followed by Tukey's post hoc test; the same letter indicates no significant difference.

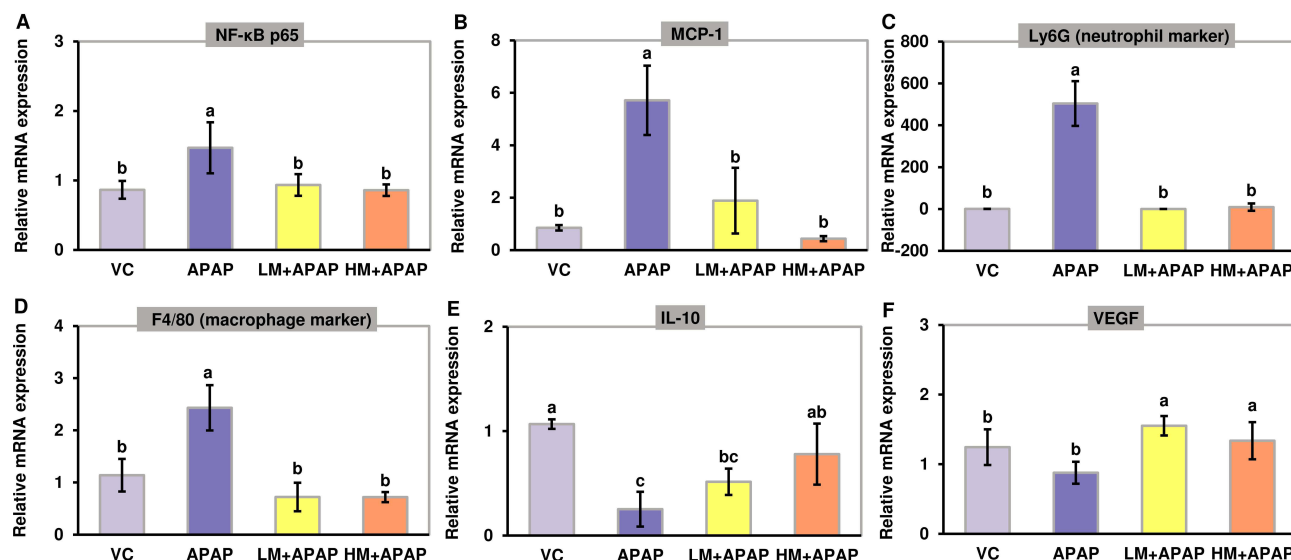


Figure 5 Effects of 7-day pre-treatment with low-dose MC at 500 mg/kg/day (LM) or high-dose MC at 1,000 mg/kg/day (HM) on mRNA levels of NF-κB p65 (A), MCP-1 (B), neutrophil marker Ly6G (C), macrophage marker F4/80 (D), IL-10 (E), and VEGF (F) in the livers of APAP-injected mice or vehicle control saline-injected mice (VC). Values are expressed as mean $\pm$ SD ($n = 4$ /group). Different superscript letters (a,b) indicate significant differences between groups ($p < 0.05$), as determined by one-way ANOVA followed by Tukey's post hoc test; the same letter indicates no significant difference.

significantly higher in the liver tissues of the untreated APAP mice than those of mice in the VC group. Nonetheless, mRNA expression levels of IL-10, an anti-inflammatory marker gene, were significantly lower (Figure 5E), while mRNA expression levels of VEGF, a tissue repair marker gene, were slightly lower in the APAP group than in the VC group (Figure 5F). Pre-treatment with MC in APAP mice significantly down-regulated the expression of NF-κB, MCP-1, Ly6G, and F4/80 but up-regulated VEGF expression, compared to APAP mice without pre-treatment with MC. Pre-treatment with high dose MC also significantly up-regulated the IL-10 expression.

In vivo Antioxidant Parameters

The untreated APAP-injected mice exhibited significant elevations in hepatic levels of ROS (Figure 6A) and MDA (Figure 6B) compared to untreated and MC-treated controls. Additionally, untreated APAP-injected mice exhibited

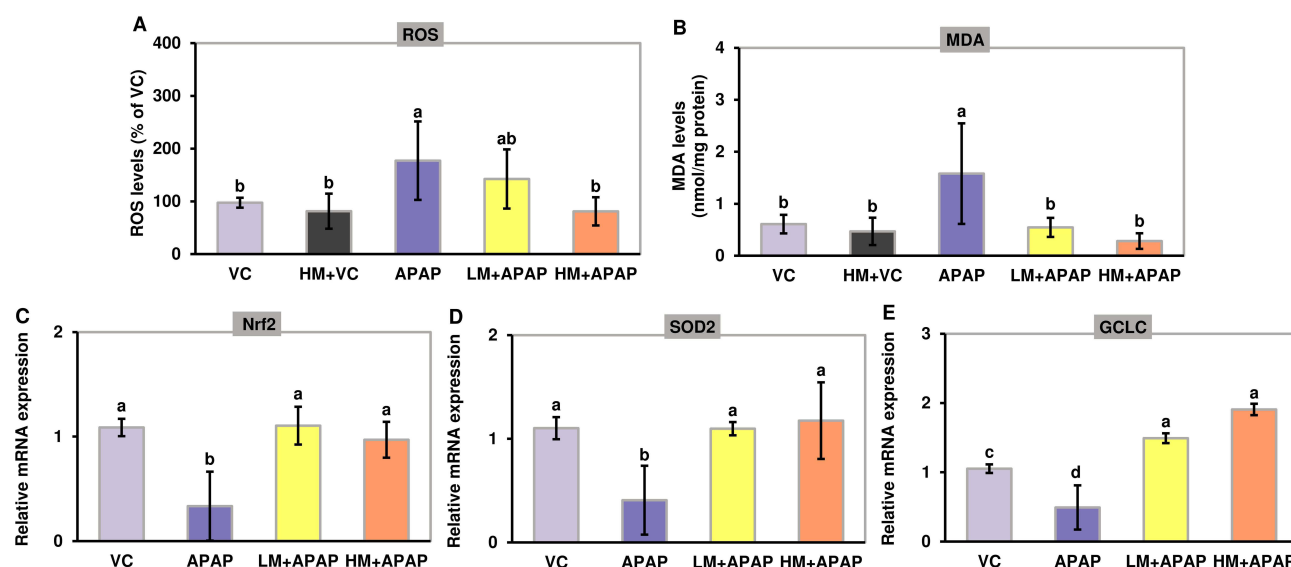


Figure 6 Effects of 7-day pre-treatment with low-dose MC at 500 mg/kg/day (LM) or high-dose MC at 1,000 mg/kg/day (HM) on ROS levels (**A**), MDA levels (**B**), and mRNA levels of Nrf2 (**C**), SOD2 (**D**), and GCLC (**E**) in the livers of APAP-injected mice or vehicle control saline-injected mice (VC). Values are expressed as mean $\pm$ SD ($n = 4-6$ /group). Different superscript letters (a,b) indicate significant differences between groups ($p < 0.05$), as determined by one-way ANOVA followed by Tukey's post hoc test; the same letter indicates no significant difference.

significant down-regulations of hepatic Nrf2 (Figure 6C), SOD (Figure 6D), and GCLC mRNAs (Figure 6E). However, the elevations of MDA levels, and down-regulations of Nrf2, SOD, and GCLC were corrected in APAP-injected mice treated with both low-dose and high-dose of MC, compared to untreated APAP-injected mice. The increased ROS levels were only statistically significantly prevented by high-dose MC when compared to the APAP group; however, ROS levels were not statistically significantly different between the low-dose and high-dose MC groups.

In vivo Anti-Hyperglycemic Parameters

The initial FBG at day 0 before APAP administration was not significantly different between the groups (Figure 7A), whereas the final FBG after APAP administration of the APAP-only group was significantly higher than those in the VC and

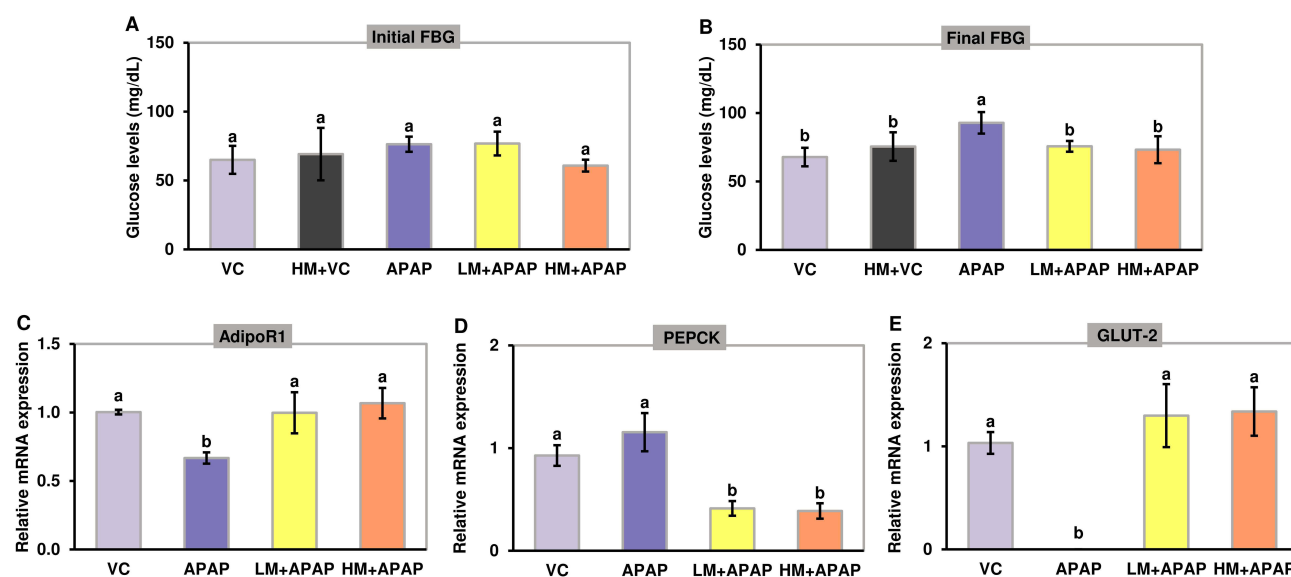


Figure 7 Effects of 7-day pre-treatment with low-dose MC at 500 mg/kg/day (LM) or high-dose MC at 1,000 mg/kg/day (HM) on FBG levels at day 0 (**A**) and day 7 (**B**), and hepatic mRNA levels of AdipoR1 (**C**), PEPCK (**D**), and GLUT-2 (**E**) in APAP-injected mice or vehicle control saline-injected mice (VC). Values are expressed as mean $\pm$ SD ($n = 4-6$ /group). Different superscript letters (a,b) indicate significant differences between groups ($p < 0.05$), as determined by one-way ANOVA followed by Tukey's post hoc test; the same letter indicates no significant difference.

HM+VC groups (Figure 7B). Additionally, after APAP administration, the expression levels of AdipoR1 (Figure 7C) and glucose-sensing gene GLUT-2 (Figure 7E) were significantly lower, while the gluconeogenic gene PEPCK (Figure 7D) showed a trend toward higher expression in the livers of APAP-administered mice than those in the VC and HM+VC groups. In contrast, MC treatment significantly attenuated FBG and PEPCK mRNA expression but up-regulated AdipoR1 and GLUT-2 mRNA expression in both LM+APAP and HM+APAP groups, compared to a group injected with APAP alone.

Discussion

In light of our results, we evaluated the primary markers of acute liver injury and death mechanisms in the serum and liver samples from APAP-induced animals pre-treated with MC (500 and 1,000 mg/kg). We found that MC, containing phenolic groups and ascorbic acid, could decrease liver injury markers and improve the expression of genes associated with the molecular mechanisms involved in the main stages of hepatocyte damage, ER stress, immune dysfunction, oxidative stress, and abnormal glucose metabolism under APAP toxicity conditions. For preliminary toxicity results in a normal condition, we discovered that MC at 1,000 mg/kg/day in control mice did not show signs of toxicity, as evidenced by normal levels of liver enzymes and normal liver histology. This is consistent with previous findings, where prolonged administration of MC at the same dose for 35 days resulted in normal blood parameters and histology in control mice.²¹ Consequently, these results suggest that MC is safe for consumption in a normal healthy condition. However, the acute and chronic safety of MC consumption needs further testing in pre-clinical animal or clinical trials.

During the initiation phase, an overdose of APAP contributes to the synthesis of NAPQI, a highly reactive toxic metabolite, by activating the main enzyme CYP2E1. NAPQI can damage hepatic components, disrupt the nucleus, and impair ATP production. This leads to the loss of nuclear components and ATP, resulting in dysfunctional and necrotic hepatocytes, and eventually the release of AST and ALT.^{2,3} In APAP-mediated hepatic apoptosis, the suppression of Bcl-2 leads to Bax mitochondrial translocation, which stimulates cytochrome c leakage and ultimately produces active Casp3.^{2,7,35,36} A pro-oxidant/antioxidant imbalance in oxidative stress plays a mechanistic role in the initiation phase of APAP hepatotoxicity.³ This imbalance is due to impaired responses of Nrf2-induced downstream antioxidant gene expression, such as SOD and GCLC, and NAPQI-induced GSH depletion. This culminates in a deficiency in antioxidant defense mechanisms, failing to counteract oxidative stress damage to hepatocytes.^{2,4,5} Pathogenic stimuli, such as ROS, can induce ER stress by causing the accumulation of unfolded proteins within the ER. ER stress signaling molecules like CHOP (Ddit3) and other signals, particularly JNK, can initiate hepatic apoptosis by affecting Bax, Bcl-2, and Casp3.^{6,7} In addition to triggering the apoptotic cascade, JNK activation by ROS in response to NAPQI is proposed as an upstream mechanism of APAP hepatotoxicity that activates multiple pathways, including mitochondrial oxidative stress, ER stress, and inflammatory cell infiltration.^{35,37,38} Similar to previous studies,^{4,6,7,39} the present study observed biochemical and histological signs of liver injury, hepatocyte necrosis, and hepatocyte apoptosis in mice exposed to acute excessive APAP. This exposure led to altered molecular hallmark pathways, including trans-activation of CYP2E1, JNK, Ddit3, Bax, and Casp3 genes, and trans-repression of the Bcl-2 gene. Additionally, APAP exposure suppressed the antioxidant defense pathway in the liver by down-regulating the Nrf2, SOD2, and GCLC genes, which may cause hepatic oxidative stress damage and cell death. Although MC showed low direct antioxidant activities in vitro chemical assays, tests in a more reliable animal model revealed that pre-treatment with MC significantly restored these hepatotoxic signs and pathways.

The pro-inflammatory state overriding the anti-inflammatory state, along with the pro-regenerative and anti-regenerative imbalance, seems to play a role in APAP hepatotoxicity.^{3,10} During the progression phase, pro-inflammatory mechanisms are evidenced by infiltrating inflammatory cells, including neutrophils and macrophages, and cytokine synthesis, induced by damage-associated molecular patterns from necrotic cells.^{2,3} These mechanisms are also mediated by the NF- κ B pathway and its target pro-inflammatory mediator gene expression, such as MCP-1.⁸ For counterbalancing mechanisms, the decline in anti-inflammatory cytokines, such as IL-10, is reported to be associated with liver pro-inflammatory responses, delayed liver recovery, and liver injury after exposure to APAP.^{9,40} In the subsequent recovery phase, mechanisms for repairing damaged hepatic tissue are driven by pro-regenerative growth factors, such as VEGF.³ Abrogation of VEGF signaling has been demonstrated to impair the restorative processes of the hepatic structure after APAP toxicity, including angiogenesis and hepatocyte proliferation, thereby sustaining hepatic necrosis.¹¹ IL-10 and VEGF treatments have been proven to inhibit APAP-induced liver necrosis in animals.^{40,41} Consistent with previous

animal studies,^{4,5,33,36,42} the present investigation provides evidence that APAP administration induced activation of the inflammatory process, reduced hepatocyte proliferation, and impaired tissue repair. These pathological changes were characterized by infiltration of neutrophils (MPO and Ly6G) and macrophages (CD68 and F4/80), elevated pro-inflammatory and chemokine gene expression of NF- κ B and MCP-1, and diminished expression of key markers involved in liver recovery, including the proliferating hepatocyte protein Ki-67, the anti-inflammatory gene IL-10, and regenerative gene VEGF. MC intervention reinstated these abnormal inflammatory and regenerative markers, suggesting that MC may prevent the progression phase and improve the recovery phase during APAP hepatotoxicity.

Hyperglycemia can exacerbate APAP-induced hepatic injury in diabetic mice by promoting a pro-inflammatory and anti-inflammatory imbalance. This imbalance is characterized by altered pro-inflammatory M1/anti-inflammatory M2 macrophage polarization, increased MCP-1 expression, decreased IL-10 expression, and enhanced ROS generation and apoptosis.¹² Adiponectin, a key adipokine regulating glucometabolism, interacts with its hepatic receptors to inhibit glucose production by suppressing PEPCK and other gluconeogenic enzymes, thereby improving insulin sensitivity.¹³ It also exhibits hepatic anti-inflammatory and antioxidant effects by targeting the NF- κ B and Nrf2 pathways.^{13,43} APAP toxicity studies have shown that adiponectin can attenuate APAP-induced hepatotoxic mechanisms, including mitochondrial damage, ER stress, inflammasome activation, oxidative stress, and necrosis.^{14,15} Glucose homeostasis involves the glucose sensor GLUT-2, a key member of the GLUT family in the liver that responds to changes in blood glucose levels. A study in mice reported that APAP impairs insulin-dependent glucose metabolism and reduces GLUT-2 expression in the liver.¹⁶ Our findings indicate that in APAP mice, hyperglycemia, down-regulation of AdipoR1 and GLUT-2 genes, and up-regulation of the PEPCK gene were observed, suggesting that these gene alterations may contribute to impaired glucose metabolism and increased susceptibility to APAP overdose's pathogenic effects. Notably, MC administration decreased hyperglycemia and restored the expression of these genes in APAP mice. This suggests that MC exerts an anti-gluconeogenic effect by enhancing the expression of AdipoR and suppressing the PEPCK pathway, as well as improving glucose-sensing by up-regulating the GLUT-2 gene. Consequently, MC contributes to the prevention of hyperglycemia and potentially alleviates hyperglycemia-induced liver damage. Additionally, the up-regulation of the AdipoR gene by MC treatment may provide further benefits for the liver, including the suppression of ER stress, inflammation, oxidative stress, and cell death.

Our experiment identified phenolic compounds, flavonoids, proanthocyanidins, and ascorbic acid in MC, in line with a previous study confirming MC as a source of these bioactive ingredients.²⁰ Animal studies have demonstrated that these phenolic phytochemicals and ascorbic acid could attenuate hepatic injury, necrosis, and apoptosis, which, in turn, might be associated with modulated expressions of CYP2E1, JNK, Bax, Bcl-2, and/or Casp3.^{26,27,44–48} Furthermore, poly-phenol extract, flavonoid extract, proanthocyanidins, as well as ascorbic acid could not only reduce hepatic ER stress by lowering CHOP expression and other ER stress markers but also inhibit hepatic oxidative stress by correcting Nrf2, SOD, or GSH pathways in animal models.^{26,27,44,45,49,50} In animal studies, well-known anti-inflammatory substances like phenolics, flavonoids, proanthocyanidins, and ascorbic acid have been reported to reduce hepatoinflammation by down-regulating NF- κ B and pro-inflammatory cytokine expressions, as well as decreasing the accumulation of inflammatory cells.^{26,44,48–50} In cell culture studies, RAW 264.7 macrophages treated with flavonoids and proanthocyanidins exhibited immunomodulatory and anti-inflammatory properties by blocking NF- κ B and MAPK or JNK signaling pathways and increasing IL-10 levels.^{28,51} Additionally, vitamin C pre-treatment has been shown to modulate the production of pro-inflammatory and anti-inflammatory cytokines in chronic myeloid leukemia cells, including an increase in IL-10 release.⁵² Prior animal studies indicate that phenolic compounds, flavonoid extracts, proanthocyanidins, and ascorbic acid have the potential to increase adiponectin levels, up-regulate AdipoR genes, and decrease insulin resistance by modulating the expression of glucometabolic factors such as PEPCK, GLUT-2, and GLUT-4.^{50,53–57} Similarly, previous research has shown that supplementation with MC aril can prevent hyperglycemia and glucose intolerance by improving insulin sensitivity, along with reducing liver lipids and damage enzymes in metabolic syndrome and diabetic mice.^{19,21} Additionally, phenolic compounds, proanthocyanidins, and ascorbic acid have been found to regulate glucose metabolism and uptake by reducing PEPCK expression and enhancing the expression of GLUT-2, GLUT-4, and other factors in HepG2 cells or their co-culture with Caco2 cells.^{58–61} Prior HPLC analyses of MC from the same batch confirmed lycopene, beta-carotene, quercetin, and rutin,^{22,29} which have demonstrated efficacy against liver injury, inflammation,

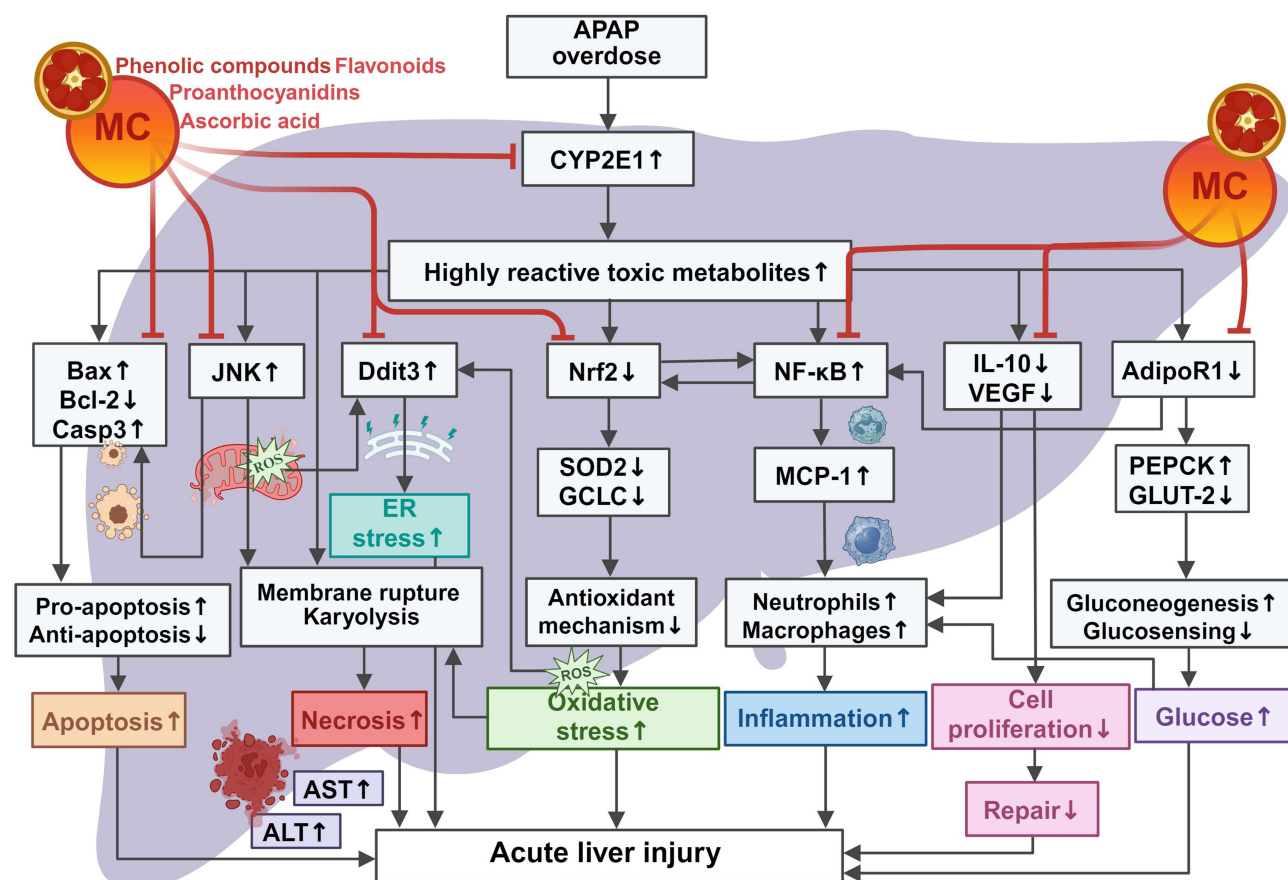


Figure 8 Schematic diagram of the proposed modes of action by MC pre-treatment in an animal model of acute hepatic injury, created using BioRender.com. Excessive APAP exposure leads to severe hepatic damage in mice through molecular mechanisms involving the formation of reactive APAP metabolites, necrosis, apoptosis, ER stress, hyperinflammation, impaired repair, oxidative stress, and hyperglycemia. In contrast, MC pre-treatment exerts protective effects by counteracting these damaging mechanisms.

and/or oxidative stress in experimental animals.^{62–66} For this reason, our study posits that MC, which contains phenolic compounds, flavonoids, proanthocyanidins, ascorbic acid, and carotenoids, protects against hepatic cell injury and death. Additionally, other phenolic subtypes and non-phenolic compounds present in MC still need to be identified.

Conclusion

Currently, MC, which contains phenolic compounds, flavonoids, proanthocyanidins, and ascorbic acid, exhibits anti-hepatic injury properties in an animal model. It does so by targeting key markers and gene expression involved in the major pathogenesis at each phase of APAP-induced liver injury, including APAP toxic metabolite production, necrosis, apoptosis, ER stress, inflammation, repair, oxidative stress, and glucose dyshomeostasis (see Figure 8). Our findings could further contribute to the development of MC and related health-promoting natural products with potential clinical applications for preventing hepatic toxicity and diseases associated with these pathogenic mechanisms. However, detailed studies are needed to identify the active phytochemicals, clarify their relationship with the liver-protective effects, and evaluate the safety and efficacy of MC in clinical settings.

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Disclosure

The authors declare no conflicts of interest in this work.

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