Journal of Ethnopharmacology and Toxicology
Research Article

Traditional indigenous polyherbal preparation Liv52 protects against acetaminophen-induced hepatotoxicity in mice by suppressing MAPK and caspases while augmenting Nrf2-mediated responses

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Volume: 4, Issue: 1, Pages: 19-40
DOI: https://doi.org/10.37446/jet/rsa/4.1.2026.19-40
Received: 30 August 2025 / Accepted: 25 January 2026 / Published: 18 March 2026

Acetaminophen (paracetamol/APAP) overdose is a major cause of drug-induced liver damage, linked to mitochondrial dysfunction, hepatocyte apoptosis, and oxidative stress. Liv52, an indigenous herbal formulation comprising components from 18 plants, has been suggested to offer liver protection, although its protective mechanisms are unclear. This study demonstrates Liv52's efficacy in reducing APAP-induced hepatotoxicity and oxidative stress in Wistar albino mice. Mice pre-treated with Liv52 exhibited significant decreased mortality, reduced liver injury and oxidative stress markers, as well as less adverse alterations in both their histological and ultra-structural characteristics after being exposed to APAP. Immunohistochemistry and western blot analyses indicated that Liv52 mediates protection against APAP hepatotoxicity through mechanisms involving MAPK, Nrf2/Keap-1, and caspases, thereby mitigating oxidative stress, hepatocyte apoptosis, and mitochondrial dysfunction. These findings elucidate the mechanisms by which Liv52 combats APAP-induced liver damage.

Keywords: *acetaminophen, Liv52, hepatotoxicity, oxidative stress, hepatocyte apoptosis, mitochondrial dysfunction*

Introduction

Acetaminophen (APAP; N-acetyl-para-aminophenol) poisoning is the primary cause of liver toxicity and liver transplantation in the United States and the second-leading cause globally (Agrawal & Khazaeni, 2023; Castaldo & Chari, 2006; Mindikoglu et al., 2009). APAP toxicity is common because APAP is widely available and considered safe. Moreover, it's available without a prescription (also known as paracetamol or Tylenol) and is frequently used in combination with other drugs (Bertolini et al., 2006; Lancaster et al., 2015; Rotundo & Pyrsopoulos, 2020; Yoon et al., 2016). For adults, the maximum recommended dose is 4 g/day (350–650 mg every 4–6 h) (Dart & Bailey, 2007). Liver toxicity can result from taking APAP frequently or excessively or from ingesting an acute overdose (Hodgman & Garrard, 2012; Lancaster et al., 2015; McCrae et al., 2018; Rotundo & Pyrsopoulos, 2020; Yoon et al., 2016). After an APAP overdose, extensive formation of NAPQI (N-acetyl-p-benzoquinone imine) and depletion of glutathione often occur

(James et al., 2003; Ramachandran & Jaeschke, 2017), which results in oxidative stress (Indumathi et al., 2024; Das et al., 2010) and mitochondrial dysfunction (Jaeschke et al., 2018; Jaeschke et al., 2021). The antioxidant N-acetyl cysteine (NAC) has been used to treat APAP-associated hepatotoxicity caused by NAPQI (within 8 hours of an APAP overdose) (Licata et al., 2022; Zyoud et al., 2015). However, the therapeutic efficiency of NAC alone is limited, and liver transplantation is the only choice for those in advanced stages of hepatotoxicity to increase survival chances (Licata et al., 2022). Therefore, identifying more effective hepatoprotective drugs that can shield the liver from APAP-induced damage is crucial. Several herbal components appear to have therapeutic benefits similar to NAC (Chang et al., 2020). About 80% of the world's population use traditional preparations for various indications (Parveen et al., 2015). India, Africa, China, and Japan are the countries in which herbal preparations are most often used (Arumugam & Watanabe, 2017; Chang et al., 2020; Shakya, 2020; Singh & Mani, 2015; Stickel & Schuppan, 2007; Vaidya & Devasagayam, 2007; Wang et al., 2014). In India, more than 15,000 medicinal plants have been described, of which 7000–7500 are used by the native population to treat diseases (Parasuraman et al., 2014). About 700 distinct plant species are identified by the Ayurvedic medical systems (Indian Pharmacopoeia) as having therapeutic value for various diseases (Parasuraman et al., 2014), including hepatotoxicity, nephrotoxicity, and cancer (Girish & Pradhan, 2012; Saggar et al., 2022; Panda et al., 2017; Sen & Chakraborty, 2017). Because of their potent therapeutic benefits and the lack of known adverse effects, Ayurvedic polyherbal formulations have gained importance in recent years for treating ailments. However, many of these formulations are used without an understanding of their underlying mechanisms. A wide variety of indigenous commercial formulations are available in the market. Liv52, the most popular product sold for strengthening the liver and addressing liver damage, is marketed by a Himalayan drug company (since the 1950s) located in Bengaluru, Karnataka, India. The name Liv52 derives from the extensive efficacy testing (52 trials) of the ingredients in developing the ideal supplement (Leslie, 1989; Rabiul et al., 2011; Sahni, 2017). Liv52 is available in multiple formulations, including tablets, syrups, and capsules (Chauhan & Kulkarni, 1991; Ganesh et al., 2022; Kantharia et al., 2023), and is used in both human and veterinary medicine.

Table 1. Major components of Liv52 pet liquid/10ml

SL. No	Scientific name	Sanskrit name	Common name	Dosage	Plant part used in the formulation	Examples of hepatoprotective action
1	*Capparis spinosa* L.	Himsra	Caper bush	68mg	Root	P-methoxy benzoic acid, which is extracted from *Capparis spinosa*, has been shown to have potent anti-inflammatory, antioxidant, and protective effects against hepatotoxicity brought on by carbon tetrachloride (al-Said et al., 1988; Gadgoli & Mishra, 1999; Germano et al., 2002).
2	*Cichorium intybus* L.	Kasani	Wild chicory	68mg	Seeds	*Cichorium intybus* protects the liver against the damaging effects of alcohol and exhibits strong antioxidant properties (Amirghofran et al., 2000; Gazzani et al., 2000; Kim et al., 2002).
3	*Solanum nigrum* L.	Kakamachi	Black night shade	32mg	Whole plant	*Solanum nigrum* can prevent oxidative stress and liver damage induced by carbon tetrachloride (Raju et al., 2003).
4	*Terminalia arjuna* (Roxb.) Wight & Arn.	Arjuna	Arjun	32mg	Bark	*Terminalia arjuna* reduces cholesterol levels and provides relief from liver problems. It also exhibits high antiviral, antibacterial, and antioxidant properties (Ali et al., 2003; Cheng et al., 2002; Manna et al., 2006; Munasinghe et al., 2001).
5	*Cassia occidentalis* L.	Kasamarda	Negro coffee	16mg	Seeds	*Cassia occidentalis* exhibits notable hepatoprotective effects in liver injury generated by chemicals, ethyl alcohol, and paracetamol. It also exhibits anti-inflammatory and anti-oxidant properties (Jafri et al., 1999; Yadav et al., 2010).
6	*Tamarix gallica* Auct., Dyer in part, non-L	Jhavuka	Tamarisk	16mg	Whole plant	*Tamarix gallica* has hepatoprotective properties against rifampicin and isoniazid-induced liver injury, stimulates liver detoxification, and improves gastrointestinal digestion (Urfi et al., 2018).
7	*Achillea millefolium* L.	Biranjasipha	Yarrow	16mg	Aerial parts	*Achillea millefolium* has anti-hepatoma activities and can be beneficial in treating chronic hepatitis (Candan et al., 2003; Lin et al., 2002; Yaeesh et al., 2006).
Additionally, Liv52 pet liquid contains 11 minor components that are essential for its hepatoprotective properties: *Eclipta alba* L. (whole plant), *Phyllanthus amarus* L. (root), *Tinospora cordifora* Hook F and Thoms (stem), *Boerhaavia diffusa* L.(root), *Berberis aristata* DC (root), *Raphanus sativus* L (root), *Emblica offiinalis* Gaertn. (fruit), *Plumbago zeylanica* L.(root), *Embelia ribes* Burm. f (fruit), *Terminalia chebula* Retz. (fruit and root), and *Fumaria officinalis* L. (whole plant).						

In this study, we used Liv52 pet liquid in our mouse experiments. The herbal formulation Liv52 pet liquid comprises seven main plant-based components and 11 minor plant components (Table 1). Liv52 components were chosen based on their claimed historical applications (Ganesh et al., 2022; Kantharia et al., 2023). A broad range of hepatoprotective actions are documented for these components (Kantharia et al., 2023). Previous studies indicate that taking Liv52 2-3 times a day can protect the liver from a variety of illnesses, including cirrhosis, viral hepatitis, alcoholic liver disease, non-alcoholic steatohepatitis and fatty liver disease, and jaundice (Ganesh et al., 2022; Siregar et al., 2021). Additionally, Liv52 has been used for long-term illness and convalescence in conjunction with hepatotoxic drugs (statins, antiretrovirals, and chemotherapeutic agents). Liv52 has been reported to enhance both animal and human appetite and digestion (Ganesh et al., 2022). The high level of phenolic compounds, particularly polyphenols, may be the reason for these beneficial effects (Vidyashankar & Patki, 2010). Examination of Liv52 on high-powered thin-liquid chromatography revealed the presence of caffeic acid, rutin, and mangiferin (Ramasamy et al., 2018). Research indicates that the Liv52 polyherbal formulation is well tolerated because no notable or medication-related adverse effects have been observed (Ganesh et al., 2022; Kantharia et al., 2023). Previously, we showed that the single micronutrient selenium ameliorated hepatotoxicity-induced in mice by a non-lethal dose of APAP (Indumathi et al., 2024). Our main objective was to evaluate whether an easily accessible, extensively marketed indigenous multicomponent herbal medication was effective in treating liver damage at the toxic level. Thus, in the present study, we found that Liv52 significantly protected mice from APAP-induced mortality and hepatotoxicity as evidenced by lower levels of liver injury biomarkers and oxidative stress markers. Furthermore, we observed that Liv52 significantly protected the liver by successfully reversing the detrimental morphological, histological, and ultra-structural changes caused by APAP. We showed that Liv52 increased the expression of nuclear factor erythroid 2-related factor 2 (Nrf2), heme oxygenase-1 (HO-1), and B-cell lymphoma 2 protein (BCl2) and decreased the expression of cleaved caspase-3, cleaved caspase-9, Keap1, and phosphorylated MAPKs. These results suggest that Liv52 may help to reduce oxidative stress, hepatocyte apoptosis, and mitochondrial dysfunction. This study offers experimental support for the beneficial effects of Liv52 against APAP-induced hepatotoxicity.

Materials and Methods

Materials

APAP, protease inhibitor cocktail (Roche-04693116001), hexadecyltrimethyl ammonium bromide, direct red 80 (sirius red-365548), and phosphatase inhibitor (04906845001) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Commercially available Liv52 was bought from medical supply stores in Mysore (Himalaya drug company, Bengaluru, KA, India), SGOT, SGPT, LDH, and ALP biomarker kits were obtained from Agappe Diagnostics Ltd (Ernakulam, KL, India). Glutathione reduced (GSH), glutathione oxidized (GSSG), Nicotinamide adenine dinucleotide phosphate reduced (NADPH), glutathione reductase (GR), bovine serum albumin (BSA), ethylenediaminetetra acetic acid, phenylmethylsulfonyl fluoride, guanidine hydrochloride, quercetin and 1-chloro-2,4-dinitrobenzene (DNB), were purchased from Sisco Research Laboratory Pvt. Ltd (Mumbai, MH, India). Hematoxylin was procured from Merck (Kenilworth, NJ, USA). Eosin and O-dianisidine were purchased from SD Fine Chemicals (Mumbai, MH, India). We bought 2,4-thiobarbituric acid and dinitrophenylhydrazine from Loba Chemie Pvt. Ltd. (Mumbai, MH, India). Transmission electron microscopy (TEM) was conducted in the National Institute of Mental Health and Neuro-Sciences (Transmission Electron Microscopy Laboratory, Bengaluru, India), and reagents used for TEM were from TAAB Laboratories (Aldermaston, Berks, RG7 8NA, England). Cytosolic and nuclear protein extraction kits were obtained from Boster Biological Technology (Pleasanton, CA, USA). Primary antibodies BCl2 (ab196495), Nrf2 (ab92946) and HO-1 (ab223349) were purchased from Abcam (Boston, MA, USA). P-p38 (#4511), P-SAPK/JNK (#4668), (P)-ERK (#9101), total (T)-ERK (#9102), Keap1 (#7705), T-p38 (sc-7149), β-actin (#4970), were from Santa Cruz Biotechnology, Inc, (Dallas, TX, USA). Caspase 3 (ARG54938) was from Arigo Biolaboratories (East District, Taiwan); cleaved caspase 3 (#9661), cleaved caspase-9 (#9509), and BAX (#2772) were from Cell Signaling Technology (Danvers, MA, USA). We bought horseradish peroxidase-conjugated anti-rabbit (#7074) and anti-mouse (#7076) IgG secondary antibodies and cell lysis buffer (9803S) from Cell Signaling Technology (Danvers, MA, USA). Alexa Fluor 647-conjugated goat anti-rabbit (A21245), AlexaFluor 488-conjugated anti-rabbit (A11008), and Hoechst 33342 (H3570) were from Invitrogen, Thermo Fisher Scientific (Waltham, MA, USA). All other routine chemicals were purchased from Sisco Research Laboratory Pvt. Ltd (Mumbai, MH, India) and SD Fine Chemicals (Mumbai, MH, India).

Mice

All animal experiments were approved by the Institutional Animal Ethical Committee (Approval No: UOM/IAEC/08/2021), Department of Studies in Zoology, University of Mysore, Mysuru and were conducted in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals.

All mice (Male and Female, both sexes were utilized) were kept in 12 h light/dark cycles with adequate ventilation, access to food and water, and constant observation throughout the experiments. All experiments involving mice were performed in three independent replicates, with six animals used in each experimental group.

Effect of Liv52 pre-treatment on APAP-induced hepatotoxicity

To determine the effects of Liv52 on APAP-induced mouse death, we divided Wistar albino mice into 6 groups of 6 mice per group: vehicle (saline), APAP (150 mg/kg) alone, APAP (250 mg/kg) alone, Liv52 (2.5 ml/kg) + APAP (150 mg/kg), Liv52 (2.5 ml/kg) + APAP (250 mg/kg), and Liv52 (2.5 ml/kg) alone. Liv52 aliquots (2.5 ml/kg) were administered orally to the mice daily for 7 days (saline was used to prepare Liv52 aliquots in a total volume of 0.5 ml). After 7 days, mice were deprived of food for 16 h and were then orally administered APAP (non-lethal dose, 150 mg/kg; lethal dose, 250 mg/kg). We dissolved 20 mg of APAP in 1 ml of warm saline to make an APAP stock solution. Control mice were given the vehicle (saline) used for making the APAP and Liv52 preparations. We monitored survival time for up to 24 h. In a separate experiment, we divided Wistar albino mice into 6 groups of 6 mice per group as shown above. The mice were also treated as described above, with a 7-day regimen of Liv52, followed by APAP (non-lethal dose: 150 mg/kg; toxic dose: 250 mg/kg). Then, all mice were anesthetized using xylazine (5-10 mg/kg), and blood and liver samples were collected within 4 hours after APAP treatment (ie, before death induced by a toxic dose [250mg/kg]). Note: In the research conducted by Ahmed and Urooj (Ahmed, & Urooj, 2010) Liv52 liquid was given at a dosage of 2.5 ml/kg as a +ve standard, produced significant results, we therefore adopted the same dosage for our experiments. Our earlier research (Indumathi et al., 2024) indicated that a non-lethal dose of acetaminophen (APAP) at 150 mg/kg could cause liver toxicity, while a lethal dose of 250 mg/kg resulted in a 50% mortality rate. This study was designed to assess the protective effects of Liv52 against both moderate (150 mg/kg) and high (250 mg/kg) doses of APAP.

Assays for liver-specific biomarkers

To analyze specific liver injury biomarkers, such as SGOT, SGPT, LDH, and ALP, we collected blood from the mouse carotid artery (using a 1ml syringe) and separated out the serum by centrifugation. Agape kits were used to determine the levels of SGOT, SGPT, LDH, and ALP, and serum enzyme activity was calculated according to the instructions provided by the manufacturer. The liver homogenate's (~100 µg protein) SGOT and SGPT levels were determined, and the results were expressed as micromoles of NADH oxidized per milligram of protein. To prepare liver homogenate, we weighed and homogenized the liver samples in cold phosphate buffer (pH 7.4) with 1 mM phenylmethylsulphonyl fluoride (10% homogenate, 0.1 g in 1 mL buffer). The homogenate was centrifuged at 12,000 x g for 15 minutes at 4 °C. The supernatant was used to measure liver SGOT and SGPT.

Protein estimation

The protein content was determined using Lowry's method, with BSA as the standard (Lowry et al., 1951).

Histological analysis

Hematoxylin and eosin staining

Freshly excised liver samples were sliced, fixed for 24 hours in 10% formalin solution, dehydrated with ethanol (by gradually increasing concentration), and embedded in paraffin. The samples were then cut into thick sections (5-µm) with a microtome (R. Jung AG in Germany), and the deparaffinized sections were stained with haematoxylin and eosin. Tissue sections were viewed under a bright field microscope, and images were captured (Feldman & Wolfe, 2014).

Picrosirius red staining

Freshly excised liver samples were handled as above. The 5-µm deparaffinized sections were stained with collagen-specific picrosirius red to determine fibrosis. Tissue sections were viewed under bright field microscopy, and images were captured (Jain et al., 2019).

Transmission electron microscopy analysis

For liver ultrastructural studies, excised liver samples from mice were sliced into small pieces and fixed using 3% glutaraldehyde (0.1M sodium cacodylate/phosphate buffer was used in preparation) at 4°C for 24 h. Then, fixed tissues were washed using phosphate buffer and post-fixed with 1% osmium tetroxide for 1-2 h at 4°C. The tissues were dehydrated with gradually increased concentrations of ethanol (70% ethanol for 1 h and 80% ethanol for 1 h). During the

dehydration process to improve fine structural preservation, we embedded the tissues with 2% uranyl acetate (95% ethanol used for preparation) at 40°C for 1-2 h, then at 100% ethanol, followed by clearing in propylene oxide. The tissues were impregnated with propylene oxide and araldite resin overnight at a ratio 1:1, which was increased to a 1:3 ratio for 1-2 h, followed by pure araldite resin for 2-3 h. To facilitate polymerization, we implanted the tissues in a flat embedding mold and stored them at 60°C for 48 h. Tissue blocks were sliced under an EM UC6 ultramicrotome (Leica, Austria). To locate the appropriate area of interest and to examine light microscopic characteristics, we first stained 1-µm thick sections collected on plain glass slides with 1% toluidine blue. The ultrathin (50–60 nm) were then placed on copper grids and stained with uranyl acetate (saturated with 50% methanol) and 0.1% lead citrate, in accordance with the protocol provided by (Frasca & Parks, 1965). A transmission electron microscope (Tecnai G2 Spirit Bio-twin, FEI, Netherlands) was used for examining the ultrathin slices, and an inbuilt Megaview III CCD camera was used to take images of representative areas.

Immunohistochemistry

Liver tissues were fixed in 10% neutral buffered formalin for 24 h, washed three times with phosphate buffer saline, dehydrated with gradually increasing alcohol concentrations, and embedded in solidifying paraffin wax. The samples were then sliced into 10-µm thick slices with a microtome. The sections were deparaffinized by incubating the slides overnight at 55 °C and then cleared with xylene for 5 minutes. The sections were rehydrated with varying levels of alcohol (100-50%). The antigens were retrieved by steaming the slides in Tris-EDTA solution (pH 9.0) for 45 min. The tissues were permeabilized with 1% Triton X-100 and incubated overnight at 4 °C with primary antibodies (cleaved caspase 3 [1:500]/cleaved caspase-9 [1:500]) followed by incubation with an AlexaFluor 488-conjugated anti-rabbit (1:500)/ AlexaFluor 647-conjugated goat anti-rabbit (1:500) secondary antibody for 2 h in the dark at room temperature. Hoechst 33342 (1:10,000) was used to stain DNA. Images were photographed using confocal microscope (63X-Carl Zeiss LSM) (Katkar et al., 2016).

Oxidative stress marker analysis

Preparation of liver samples for liver specific and oxidative stress marker analysis

Liver samples were weighed and homogenized in cold phosphate buffer (pH 7.4) with 1 mM phenylmethylsulphonyl fluoride (10% homogenate - 0.1 g in 1 ml buffer). The homogenate was centrifuged for 15 minutes at 4 °C at 12,000 X g. The supernatant was used for the protein analysis in the tissue homogenate, which was measured by Lowry's method using BSA as the standard (Lowry et al., 1951).

Protein carbonyls

The total concentration of PCs in the liver homogenate was measured using the method of (Levine et al., 1990) which allowed for the determination that protein oxidation results from oxidative stress. In brief, 500 µl of 10 mM 2, 4-dinitrophenyl hydrazine was mixed with liver homogenate (~200 µg protein) at 28 °C for 20 min in the dark. Following the addition of 250 µl of 50% trichloroacetic acid, the reaction mixture was centrifuged for 10 min at 28 °C at 23640 X g. The resulting pellet was centrifuged at 23640 X g for 5 min at 28 °C after being washed three times with ethanol: ethyl acetate (1:1). After that, the pellet was suspended in 1 ml of 6 M guanidine hydrochloride, and the absorbance at 370 nm was measured. The final results are expressed as micromoles of PCs formed per milligram of protein.

Lipid peroxidation (LPO)

The extent of lipid oxidation in the liver was measured using the method of (Ohkawa et al., 1979). Liver homogenate (~200 µg protein) was mixed with 5 ml of the reaction mixture containing 20% acetic acid, 8.1% sodium dodecyl sulphate, and 0.8% thiobarbituric acid and incubated for 1h at 95 °C. After incubation, the tubes were cooled, and 5 ml of n-butanol was added. The mixture was centrifuged for 10 minutes at 28°C at 1503 X g. After separating the top organic phase, we measured the absorbance at 532 nm. The final results are expressed as micromoles of MDA formed per milligram of protein.

Catalase

The (Aebi, 1984) was used to evaluate the antioxidant enzyme catalase's activity. Liver homogenate (~25 µg protein) was added to 1 ml of phosphate buffer (100 mM, pH 7.6) containing 15 mM hydrogen peroxide to initiate the process. The decreased absorbance was measured (for up to 3 min) at 240 nm. The final results are presented as micromoles of H_2O_2 decomposed per milligram of protein.

Superoxide dismutase (SOD)

The method of (Kostyuk & Potapovich, 1989) was used to measure the level of SOD, an antioxidant enzyme. Liver homogenate (~25 µg protein) was added to 1 ml of phosphate buffer (16 mM, pH 7.8) containing 0.08 mM ethylenediaminetetraacetic acid, 0.15% w/v quercetin, and 8 mM tetramethylethylenediamine; the decline in absorbance was measured at 406 nm for 1 min. The amount of protein that inhibited quercetin autoxidation by 50% was considered as one unit of activity.

Myeloperoxidase (MPO)

The inflammation index, MPO activity, was determined using the process described by (Bradley et al., 1982). Liver homogenate (10%-0.1 g in 1 ml buffer) was prepared with 0.5% hexadecyl trimethyl ammonium bromide in sodium phosphate buffer (50 mM, pH 6). The homogenized samples were centrifuged for 20 min at 4 °C at 12,000 X g. the absorbance change at 460 nm was recorded for 3 min. The final MPO activity was expressed as micromoles of O-dianisidine oxidized per milligram of protein.

Glutathione-S-transferase (GST)

The activity of the most important detoxifying enzyme, GST, was determined using the approach of (Mozer et al., 1983). To initiate the reaction, we mixed 1 ml of phosphate buffer solution (10 mM, pH 6.5) containing 1 mM 1-chloro-2, 4-dinitrobenzene (DNB) and 1 mM reduced GSH with liver homogenate (~25 µg protein). GS-DNB conjugation was measured to increase at 340 nm for 3 min. The results are expressed as nanomoles of GS-DNB conjugate formed per milligram of protein.

Glutathione reductase (GR)

The enzyme, GR, is responsible for maintaining reduced glutathione concentration in the liver. The Mavis et al. methodology (Mavis & Stellwagen, 1968) was used to determine GR activity. We mixed 1 ml of phosphate buffer solution (100 mM, pH 7.6) containing 100 µM NADPH, 900 µM GSSG, and 3.4 mM ethylenediamine tetra acetic acid with liver homogenate (~25 µg of protein) and measured the decreased absorbance for 3 min at 340 nm. The results are expressed as nanomoles of NADPH oxidized per milligram of protein.

Glutathione peroxidase (GPx)

The activity of the antioxidant enzyme GPx was assessed using methodology (Mannervik, 1985). We mixed 1 ml of Tris HCl (50 mM, pH 8.0) buffer solution containing 0.5 mM 0.5 mM NADPH, ethylenediamine tetra acetic acid, 4.2 mM reduced GSH, and 1U GR with the liver homogenate (~25 µg protein). The reaction was initiated by adding 0.15 mM hydrogen peroxide to the reaction mixture. After three minutes, we monitored the absorbance drop at 340 nm. The results are expressed as nanomoles of NADPH oxidized per milligram of protein.

Immunoblotting

Liver samples were homogenized using phosphate buffer (10 mM, pH 7.4) containing a cocktail of protease and phosphatase inhibitors. Homogenates were centrifuged for 15 minutes at 4 °C at 13000 X g, and the protein concentration was determined using Lowry's method. Cytosolic and nuclear proteins were isolated using the Boster extraction kit, as per the manufacturer's instructions, and protein concentration was determined using Lowry's method (Lowry et al., 1951). About 40 µg of proteins were boiled with the sample buffer for 5 min, separated on 10% or 12% SDS-PAGE, and electro transferred onto nitrocellulose membrane. The membrane was then blocked for 1 h with 5% BSA (made in Tris-buffered saline with polysorbate 20 [TBST]). Then, we treated the membrane with primary antibodies (1:1000 dilution in TBST) against pro-caspase-3, cleaved caspase-3, BAX and Bcl2, β-actin, phosphorylated-ERK, total-ERK, phosphorylated-SAPK/JNK, phosphorylated-P-p38, total-p38, Nrf2, Keap-1, HO-1 for 12 h in separate experiments. After that, the membranes were incubated for two hours with corresponding horseradish peroxidase-conjugated anti-rabbit/anti-mouse IgG secondary antibodies (1:5000 dilution in TBST). Chemiluminescence was used to visualize the immunoreactive bands.

Statistical analyses

All in vitro and in vivo experiments are indicative of at least three separate experiments (n=6 mice). For histology and Western blot analyses, one representative image from each experimental group is shown in the Figures. The data were evaluated using GraphPad Prism Version 8.0. To determine the statistical significance of group comparisons, we used One-way ANOVA (one-way analysis of variance followed by Tukey's multiple comparison test). The significance of the survival study was determined by Kaplan-Meier survival analysis. P ≤0.05 was considered statistically significant for all tests.

Results

Liv52 protects mice against APAP-induced hepatotoxicity

To examine the protective effect of Liv52 against APAP, we treated mice orally with Liv52 (2.5 ml/kg) for 7 days before administering either a non-lethal (150 mg/kg) or lethal (250 mg/kg) dose of APAP. Survival time was monitored for up to 24 h.

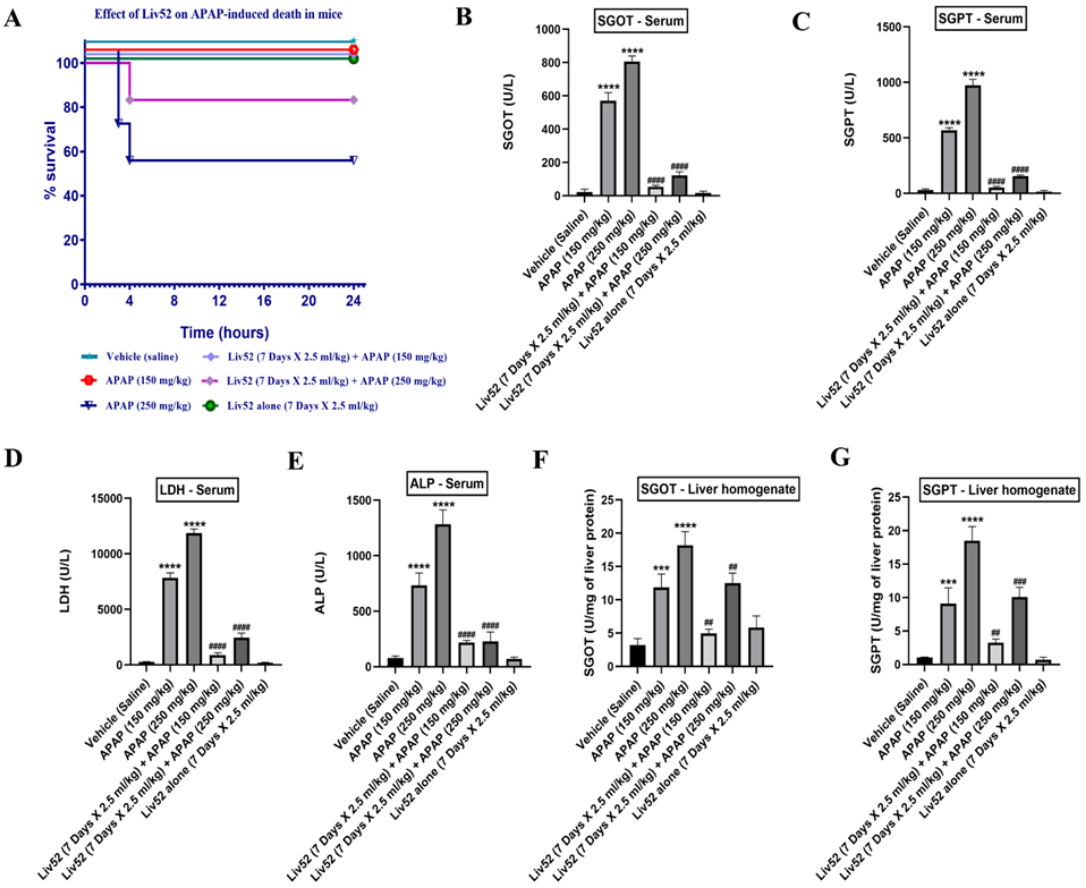

Figure 1. Liv52 pretreatment protects mice from APAP-induced hepatotoxicity.

Mice (n=6) were treated with Liv52 (2.5 mL/kg) orally for 7 days before being given APAP (150 mg/kg or 250 mg/kg). After APAP treatment, survival time was monitored for 24 h. (A) Survival was determined by Kaplan-Meier survival analysis, in a separate experiment, we divided wister albino mice (n=6) and the (B) liver-specific biomarkers of serum glutamic-oxaloacetic transaminase (SGOT), (C), serum glutamate pyruvate transaminase (SGPT), (D) serum lactate dehydrogenase (LDH), (E) serum alkaline phosphatase (ALP), (F) liver SGOT, and (G) liver SGPT were measured from the respective groups. The results are representative of three individual experiments. P values were calculated using one-way analysis of variance (One-way analysis of variance followed by Tukey's multiple comparison test.). ***P<0.001, ****P<0.0001 compared with mice treated with vehicle. ##P<0.01, ###P<0.001, ####P<0.0001, as compared with APAP treatment.

We previously found that 50% of mice died within 4 h of receiving a 250 mg/kg dose of APAP. At 150 mg/kg, APAP did not cause death but did result in increased liver hypertrophy, deep coloration, and distorted lobular architecture and a

significant rise in liver biomarkers within 4 h as compared with control (Indumathi et al., 2024). Therefore, in the current study, we have used 150 mg/kg as the non-lethal standard dose and 250 mg as the toxic (lethal) dose needed to cause hepatotoxicity, indicating both moderate and severe liver toxicity. Here, we observed that pre-treatment with Liv52 protected mice against APAP-induced mortality at 250 mg/kg (Figure 1A). Considering these findings, we conducted an additional experiment in which mice received oral Liv52 (2.5 ml) for 7 days followed by APAP treatment (either 150 or 250 mg/kg) on the 8th day, but mice were anesthetized within 4 h of APAP treatment to collect blood and liver samples for analyzing hepatic injury markers. As expected, we found elevated levels of biomarkers such as SGOT (Figure 1B), SGPT (Figure 1C), LDH (Figure 1D), and ALP (Figure 1E) in both serum (8 to 12-fold) and liver (2 to 8-fold) when compared to levels in mice treated with vehicle alone. Pretreatment with Liv52 (2.5 ml/kg) significantly decreased the elevated levels of liver injury biomarkers in both serum (4 to 10-fold) (Figure 1B-1E) and liver homogenate (2 to 3-fold) (Figure 1F and Figure 1G) as compared with APAP treatment.

Effect of Liv52 pretreatment on liver morphology and histology in APAP-induced injury

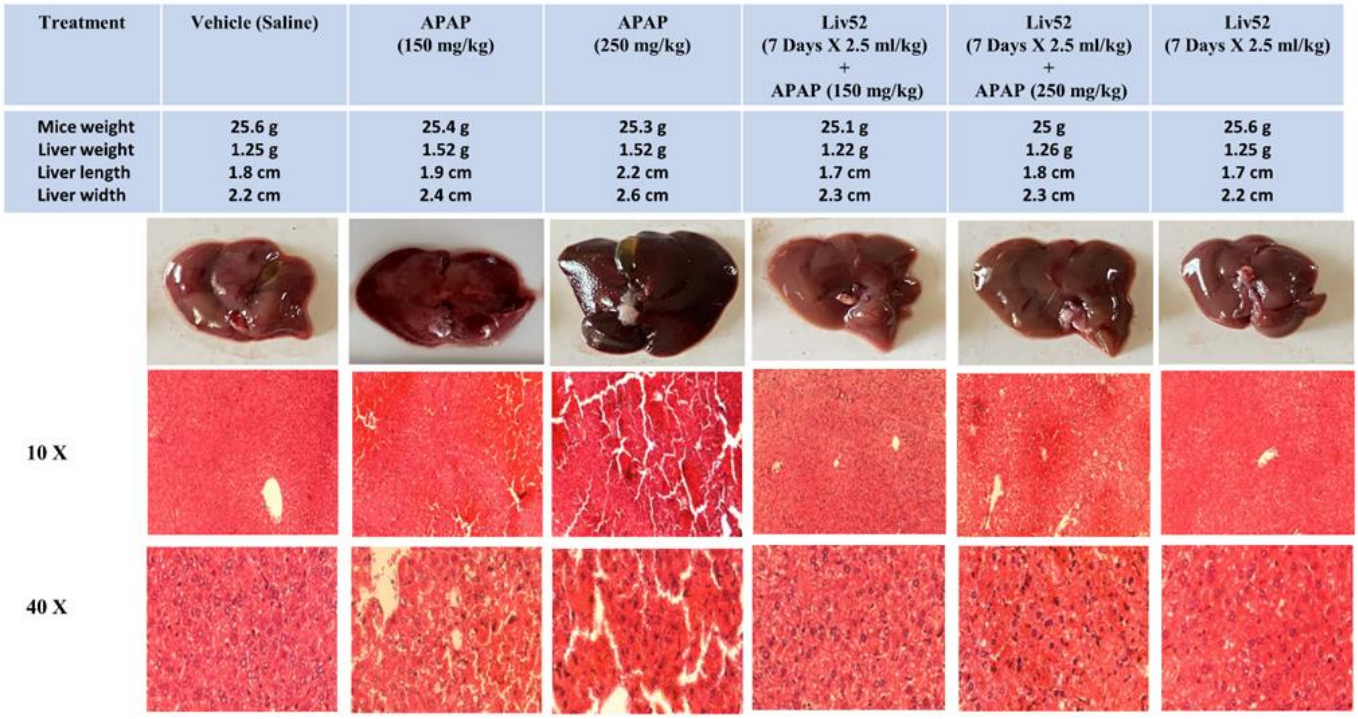

Figure 2. Liv52 restored the morphological and histological integrity of the liver.

Representative mouse weight and liver dimensions are provided, as well as images of excised liver and liver sections stained with hematoxylin and eosin (magnification, 10X; scale, 50 µm and magnification, 40X; scale, 20 µm) are shown. Control liver had normal morphology and histology. The liver in mice treated with APAP exhibited pathological alterations such as hypertrophy, necrosis, cell infiltration, and increased blood flow, particularly in mice treated with 250 mg/kg APAP. All of these alterations were mitigated by Liv52 pretreatment.

The liver of APAP-treated mice showed abnormalities such as liver hypertrophy and deep coloration when compared to control mice (Figure 2). Moreover, hematoxylin and eosin staining showed both APAP groups (150 mg/kg and 250 mg/kg) had significant liver injury, as evidenced by structural damage, intrahepatic hemorrhage, necrosis, and inflammatory infiltration when compared with control mice, which had normal liver tissue, cell nuclei, cellular arrangement, and hepatic central vein (Figure 2). We evaluated liver fibrosis histologically by the staining of collagen fibers with Sirius red. Collagen fibers were highly accumulated around central veins and portal tracts in APAP-treated groups and extended from the central vein to the portal tract, which was not seen in the control group (Figure 3). Transmission electron microscopy showed normal ultrastructural appearance of hepatocytes in the vehicle-treated group, with centrally localized nuclei, clear cytoplasmic matrix, and abundant mitochondria. In contrast, extensive ultrastructural changes were seen in the APAP group, including condensed mitochondrial matrix and high lipid deposition, blood flow, and necrosis. The histological and ultrastructural changes induced by APAP were significantly ameliorated by Liv52 pre-treatment (Figure 4). Immunohistochemistry with immunofluorescence of liver tissue demonstrated that caspase-3 and caspase-9 were lower or had not accumulated in the vehicle-treated group. APAP-treated groups demonstrated enhanced apoptotic caspase-3 and caspase-9 accumulation in a dose-dependent manner that was dramatically reduced after Liv52 administration (Figure 5).

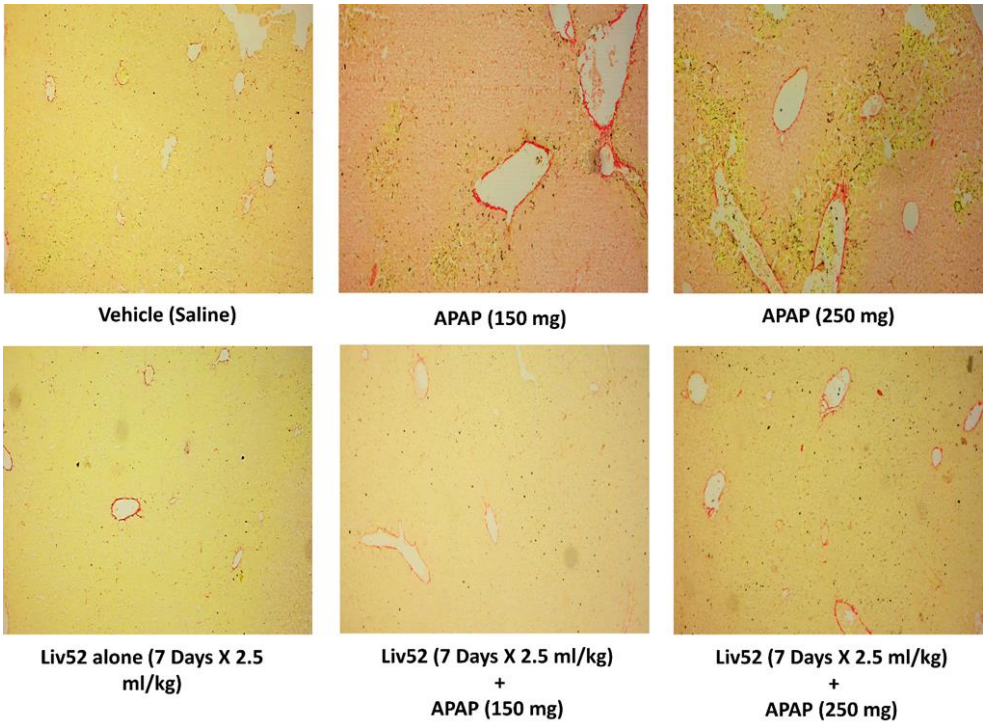

Figure 3. Histopathological examination of mouse livers by sirius red staining.

Representative photographs of sirius red stained liver sections (magnification, 10X; scale, 50 µm) are shown. The control group showed less collagen fiber deposition. In APAP-treated groups, collagen fibers were heavily deposited around the portal tract and central veins, which was significantly decreased with Liv52 pre-treatment.

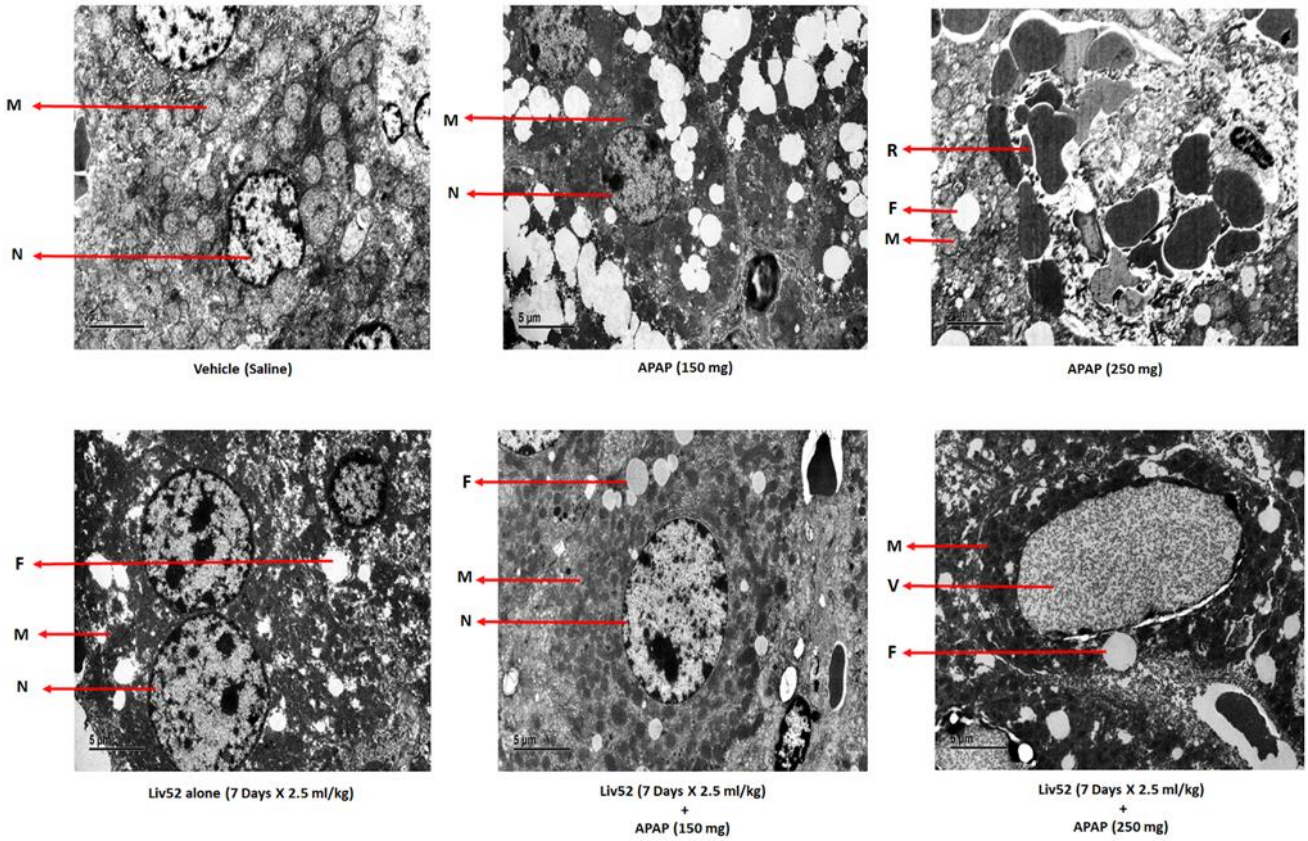

Figure 4. Electron micrograph of excised liver sections.

The ultrathin sections were examined with a transmission electron microscope, and representative areas were photographed with an inbuilt Mega view III CCD camera (magnification, 500-800X-low power; scale, 5 µm). The vehicle group showed a normal hepatocellular arrangement, with centrally localized nuclei, clear matrix, and abundant

mitochondria. APAP-treated groups showed extensive ultrastructural changes including condensation of mitochondria, lipid deposition, necrosis, and high blood flow. Liv52 pretreatment significantly ameliorated these detrimental changes. M, mitochondria; N, nucleus; R, blood cells; V, vacuole; and F, fat droplet.

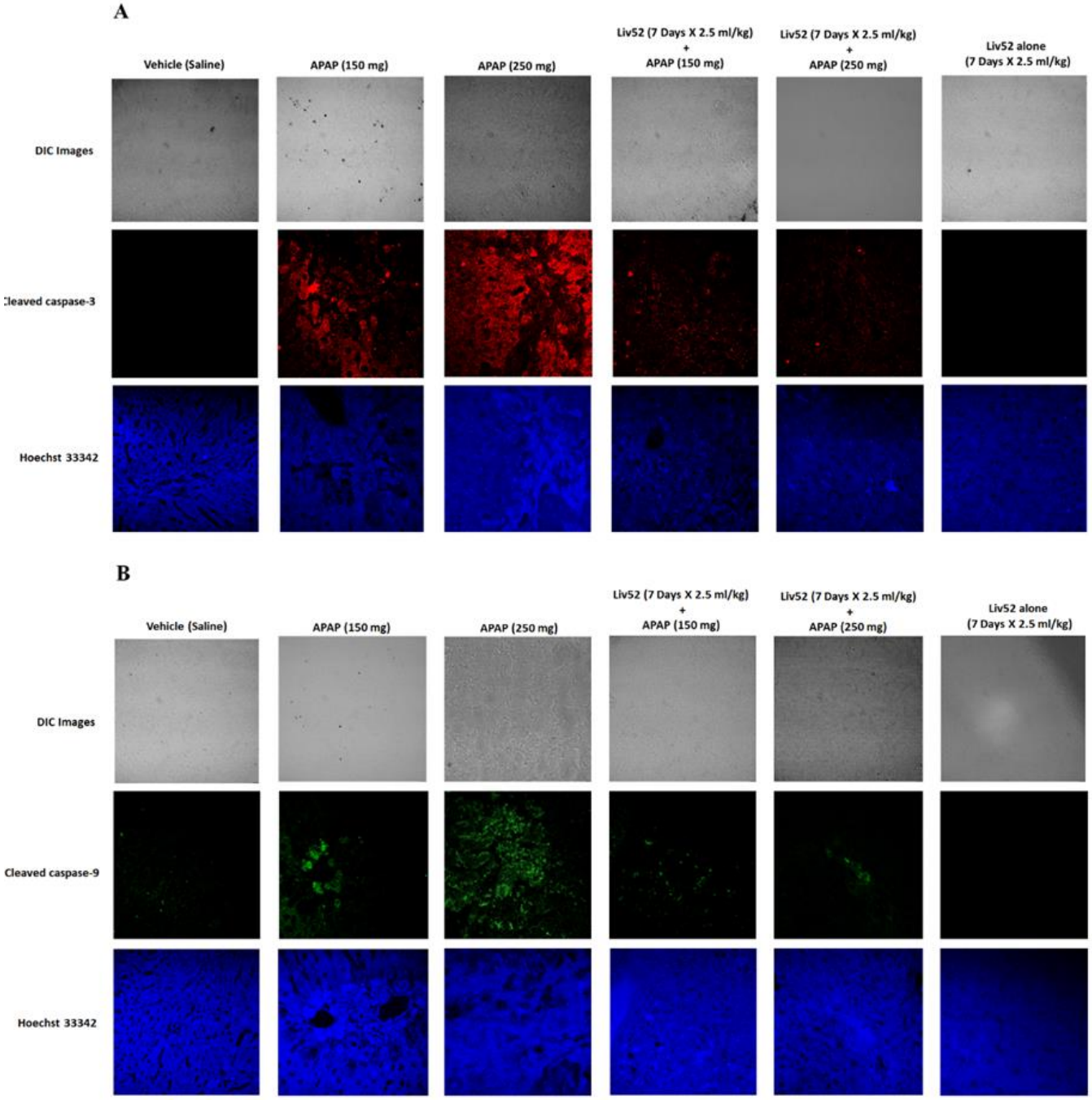

Figure 5. Pre-treatment with Liv52 protects mice against APAP-induced apoptosis (Immunohistology study). The fluorescent images (magnification-63X, scale-20 µm) were acquired using a confocal microscope (Carl Zeiss LSM). (A) Differential interference contrast image, cleaved caspase-3 (red fluorescence) and DAPI (blue fluorescence) staining images of liver tissues from each group. (B) Differential interference contrast image, cleaved caspase-9 (green fluorescence) and DAPI (blue fluorescence) staining images of liver tissues from each group. APAP-treated groups showed significant accumulation of caspase-3 and -9, whereas the vehicle group accumulated neither. These harmful alterations were considerably lessened by Liv52 pretreatment, suggesting that Liv52 protects against APAP-induced apoptosis.

Liv52 protects mice against APAP-induced oxidative stress

Because Liv52 protects mice from APAP-induced liver damage, we investigated its effect on APAP-induced oxidative stress. We measured the levels of PCs, MPO, LPO, catalase, SOD, GR, GST, and GPx in liver samples obtained from

mice treated with Liv52 for 7 days before APAP treatment. In mice treated only with APAP, levels of PCs, MPO, and LPO (Figure 6 A-C) in the liver were increased, and antioxidant enzymes (SOD, catalase, GPx, GR, and GST) were decreased (Figure 6 D-H). Liv52 protected mice from APAP-induced oxidative stress by significantly reducing liver levels of PCs, MPO, and LPO (Figure 6 A-C) and increasing antioxidants such as SOD, catalase, GST, GR, and GPx (Figure 6 D-H). These findings suggest that Liv52 protects against both oxidative stress and APAP-induced liver injury.

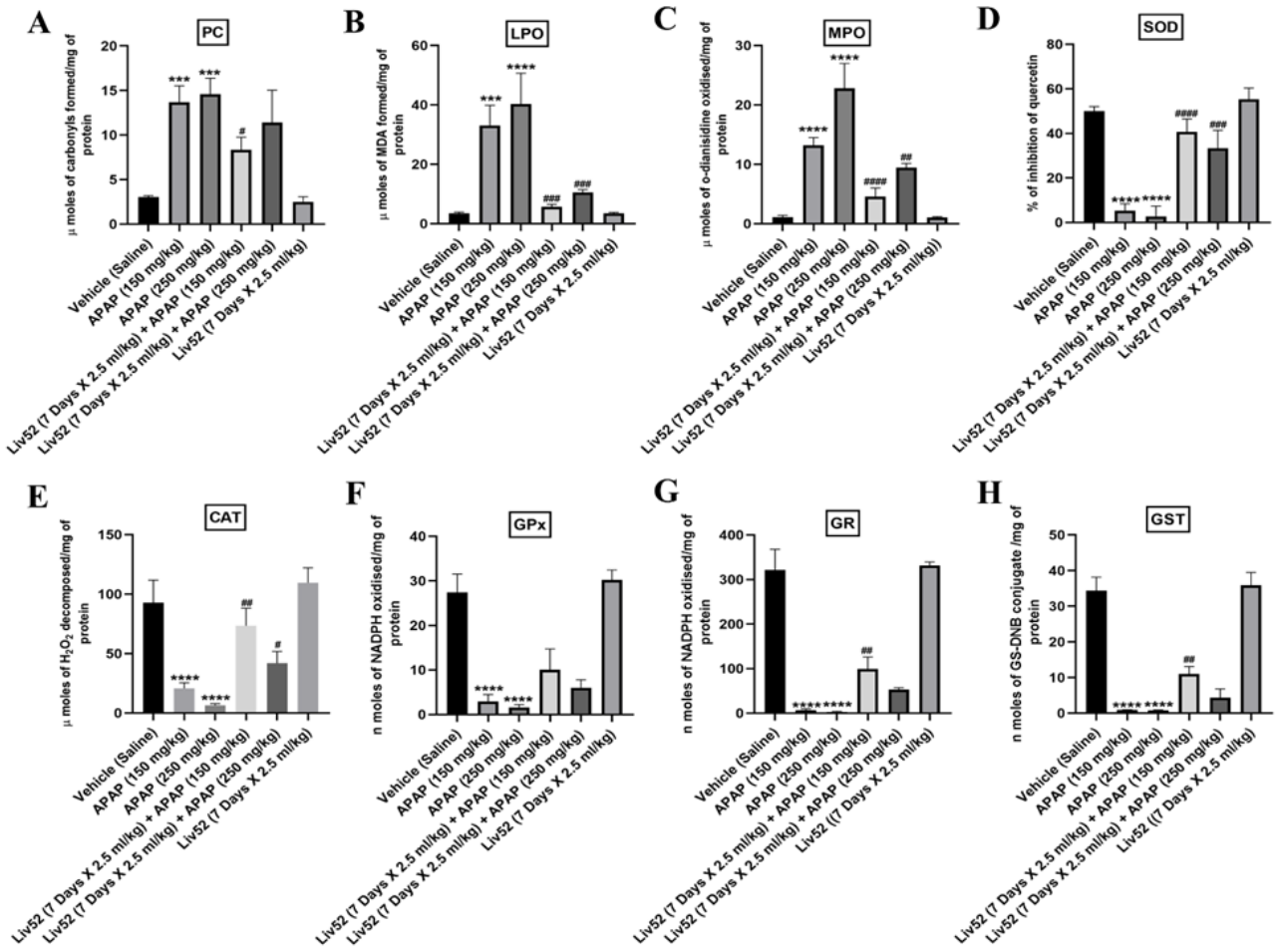

Figure 6. Pretreatment with Liv52 protects mice against APAP-induced oxidative stress.
Oxidative stress indicators were measured in liver homogenates (A-H). P values have been calculated using one-way ANOVA (One-way analysis of variance followed by Tukey's multiple comparison test.), and the results are indicative of three individual experiments with six mice used per experimental group. ***P<0.001, ****P<0.0001, as compared with vehicle. #P≤0.05, ##P<0.01, ###P<0.001, ####P<0.0001, as compared with APAP treatment.

Protective role of Liv52 in APAP-induced MAPK, caspase-3, and BAX activation and Nrf2, HO, and Bcl2 reduction

Previous investigations have shown an increase in caspase-3 enzyme activity and Bax protein after hepatic damage induced in APAP-treated mice, implying that the damage may be caused by mitochondrial malfunction and apoptosis (Abu-Ajamieh et al., 2020). In a previous study, we found that oxidative stress caused by APAP decreases Nrf2 signaling and increases MAPK signaling (Indumathi et al., 2024). However, in acetaminophen-induced stress, investigators have reported an increase in Keap-1 expression, which acts as a negative regulator of the antioxidant response pathway, implying that acetaminophen-stressed cells create more Keap-1 and suppress the beneficial antioxidant response (Shen et al., 2023). As a result, we investigated the effect of Liv52 on APAP-induced MAPK, keap-1, caspase-3, and BAX activation, as well as Nrf2, HO, and Bcl2 reduction. Liv52 pretreatment significantly decreased the phosphorylation of all three MAPKs (Figure 7 A-D), reduced Keap1 (Figure7 I) expression, and marginally reduced BAX (Figure 7 E) and caspase-3 (Figure.7 G) production. In response to APAP, Liv52 also substantially elevated total Nrf2 expression and the Nrf2-mediated antioxidant defense system by raising both cytosol and nucleus levels (particularly at the 250 mg/kg dose) (Figure7 K and L), while partially increasing HO1 (Figure7 H) and Bcl2 (Figure 7 F) expression.

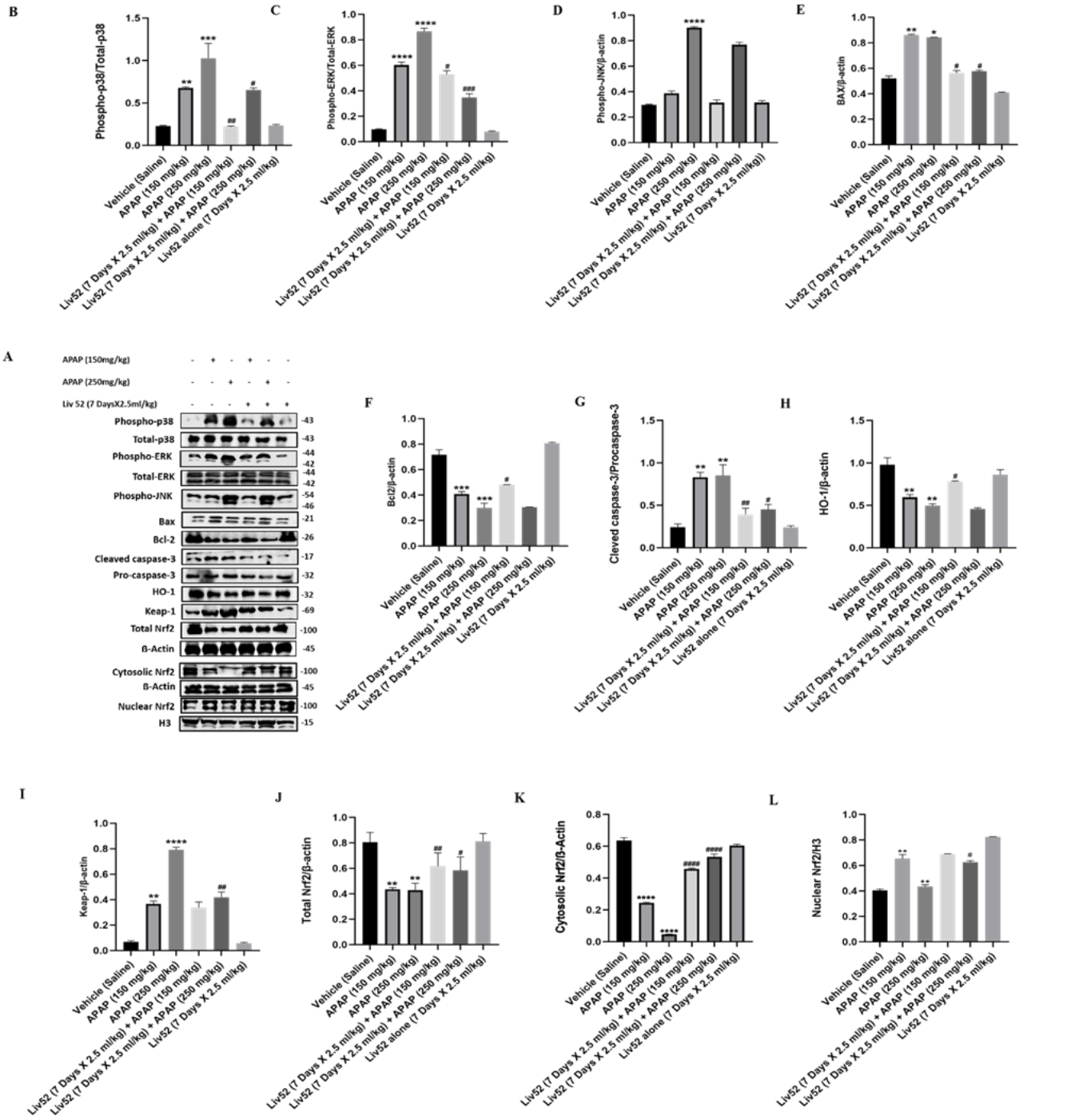

Figure 7. Effect of pretreatment with Liv52 on APAP-induced MAPK, BAX, and caspase 3 activation and Nrf2, HO, and Bcl2 reduction in mouse liver.

Liver homogenate protein (40 µg) was separated on 10% SDS-PAGE and electro-transferred onto a nitrocellulose membrane. The samples were incubated with the appropriate primary and secondary antibodies and visualized using chemiluminescence. The bar graph depicts the fold change for P-p38, P-ERK, P-JNK, HO-1, Nrf2, keap1, BAX, Bcl2, and caspase 3. P-values were calculated using one-way ANOVA (One-way analysis of variance followed by Tukey's multiple comparison test.) *P≤0.05, **P<0.01, ***P<0.001, ****P<0.0001, as compared with vehicle (saline). #P≤0.05, ##P<0.01, ###P<0.001, as compared with APAP treatment.

Discussion

The liver plays a critical role in detoxifying xenobiotics but is susceptible to the adverse consequences of detoxification (Devarbhavi, 2012). More than a thousand prescription drugs and other over-the-counter medications have the potential

to harm the liver, but APAP, an extremely common over-the-counter medication, leads the list (Alempijevic et al., 2017; Bjornsson, 2015; Chun et al., 2009; Devarbhavi, 2012; Grant, 1991; Maddrey, 2005; Zhao et al., 2021). In contrast, hepatoprotective agents for preventing drug-induced hepatotoxicity are few (Delgado-Montemayor et al., 2022; Sun et al., 2022). Herbal products and traditional indigenous medicines are increasingly being recognized as therapeutic alternatives because of their effectiveness and safety profile (Bhowmik et al., 2009; Candan et al., 2003). Few bioactive compounds extracted from plants have been investigated for hepatoprotective and antioxidant effects against hepatotoxicity (Desai et al., 2021; Harikrishnan & Balasundaram, 2020). In this study, we assessed the ability of Liv52 to protect against APAP-induced toxicity and found that Liv52 can prevent the adverse effects (mitochondrial dysfunction, hepatocyte apoptosis, and oxidative stress) associated with APAP via alterations in MAPK, Nrf2/Keap-1, and caspases. Increased levels of biomarkers of injury are early indicators of disintegrating hepatocytes. Hepatocellular injury causes liver biomarkers such as SGOT, SGPT, LDH, and ALP to enter the bloodstream. Increased morphological and histological abnormalities such as hypertrophy, deep coloration, intrahepatic hemorrhage, necrosis, and inflammatory infiltration lead to a dysfunctional liver, which impairs detoxification (Indumathi et al., 2024; Chun et al., 2009; Placke et al., 1987). Here, we have confirmed previous findings that showed significant increases in hepatic injury biomarkers in response to APAP and accompanying abnormalities in liver architecture and morphology (Figure 1 and Figure 2). Liv52 has been effective in treating various liver diseases (Kantharia et al., 2023; Siregar et al., 2021). Our findings support those of a previous study in which pretreatment with Liv52 significantly decreases APAP-induced hepatotoxicity by reducing SGOT, SGPT, LDH, and ALP levels and the associated histological and morphological alterations (Figure 1 and 2). Most severe liver illnesses result in the excessive buildup of extracellular matrix proteins, especially collagen, thus leading to liver fibrosis. Liver failure and cirrhosis result from advanced liver fibrosis (Bataller & Brenner, 2005) because a collagen-rich tissue replaces the degenerated hepatic parenchyma (Bataller & Brenner, 2005). In this study, our histologic examination showed that APAP caused fibrosis in a dose-dependent fashion. Pretreatment with Liv52 decreased the development of fibrosis (Figure 3), suggesting that Liv52 may be able to lessen the detrimental long-term effects of hepatocyte damage such as necrosis and liver failure. Mitochondria are critical cell organelles for generating energy and for playing important roles in different cell signaling pathways in APAP-induced hepatotoxicity (Chandel, 2014; McGill et al., 2012). Because of their significant function in energy metabolism and their high concentration in hepatocytes, mitochondria are prime targets for hepatotoxicity caused by APAP (Jaeschke et al., 2018). In our electron microscopy study of the liver, we found severe ultrastructural abnormalities including lipid deposition (appearance of lipid droplets), vacuolar degeneration, and a decrease in mitochondria number in response to APAP treatment when compared to control liver. These changes were ameliorated, especially the mitochondrial damage, in mice treated with Liv52 before APAP exposure (Figure 4). Multiple components present in polyherbal preparation may likely have exerted a protective effect against APAP-induced toxicity in a complex manner. We have previously reported that a single micronutrient selenium exhibited ameliorative effects for APAP-induced toxicity (Indumathi et al., 2024). We hypothesized that selenium was present in Liv52 preparations in appreciable amounts. Professor Sandeep K. Prabhu and his team from the Pennsylvania State University, Pennsylvania, USA, quantified the amount of selenium in Liv52 formulation using an Agilent 5800 inductivity coupled plasma-optical emission spectrometer (ICP-OES). The results indicated that the formulation contains 0.52233 mg/L of selenium, which is much lower that we used in selenium study (Indumathi et al., 2024) suggested that selenium is present in trace amounts in Liv52 and not likely to exert the beneficial effects. It is possible that the multiple active ingredients of Liv52 may have exerted beneficial effects. Nevertheless, a traditional drug used in Indian Ayurvedic pharmacopoeia exhibited hepatoprotective effects. Oxidative stress is important in the progression of hepatotoxicity.(Jaeschke et al., 2018) Protein carbonylation and lipid peroxidation (LPO) are processes frequently involved in liver injury and cell death induced by reactive oxygen species (Halder & Bhattacharyya, 2014). Increased MPO is a sign of inflammation and oxidative stress as well as neutrophil infiltration (Aratani, 2018). Antioxidants are the primary initial defense against oxidative stress. Two crucial enzymes that help maintain equilibrium in redox homeostasis in the liver are SOD and catalase (Kyle et al., Farber, 1987). Enzymes such as Gpx, GR, and GST maintain constant amounts of GSH in the body (Forman et al., 2009). Endogenous GSH is necessary for the conversion of xenobiotics into GSH conjugates, which is one of the crucial stages of the detoxification process (Kidd, 1997). Our investigation revealed that Liv52 pre-treatment ameliorated APAP-induced oxidative stress by reducing the levels of protein carbonyls, LPO, and MPO and enhancing the antioxidant defense system by moderately increasing GST, GR, and GPx levels in the liver (Figure 6). Multiple mechanisms are involved in hepatotoxicity caused by APAP in response to oxidative stress (Jaeschke et al., 2018; Ramachandran & Jaeschke, 2017; . Wang et al., 2010). Overexposure to APAP may cause mitochondrial dysfunction, which can activate MAPK signaling, cause oxidative stress, and result in a substantial energy deficit (Jaeschke et al., 2018; Wang et al., 2010). We previously showed that APAP induces oxidative stress through the MAPK and Nrf2 pathways (Indumathi et al., 2024). MAPK signaling and oxidative stress are closely connected (Abhilasha et al., 2019; Gaitanaki et al., 2003; Kacimi et al., 2011; Luo et al., 2005; Mannam et al., 2013). Oxidative stress increases NF-kB transcription (Luo et al., 2005) and activates the phosphorylation of major MAPKs such as p38, ERK, and JNK (Indumathi et al., 2024; Nguyen & Stamper, 2017); this leads to increased production of reactive oxygen species, which further damages hepatocytes (Cha et al., 2018; Saito et al., 2010) and results in mitochondrial death and hepatocyte apoptosis (Nowak, 2002; Yu & Kim, 2015). The Nrf2/Keap1/ARE pathway controls phase II detoxifying and antioxidant enzymes, which are a key cell defense

mechanism against oxidative stress. Inflammatory reactions are triggered by the activation of MAPK pathways causing loss of Nrf2 (Indumathi et al., 2024). Evidence of elevated Keap-1 expression, a negative regulator of the antioxidant response pathway, has also been shown in cases of severe acetaminophen-induced stress (Shen et al., 2023). Previous studies also have demonstrated that APAP administration downregulates Nrf2/ARE expression. Nrf2 upregulation helps to prevent APAP toxicity through Akt/GSK3β signaling (Aratani, 2018; Indumathi et al., 2024). Liv52 has both hepatoprotective and antioxidant effects. The protective effects of Liv52 are thought to be due in part to its high content of multiple phenolic compounds (Vidyashankar & Patki, 2010). However, this has not been validated with hepatotoxicity benefits. Here, we explored Liv52-mediated hepatoprotection against APAP liver toxicity by studying the MAPK and Nrf2-mediated pathway. Liv52 pretreatment protected mice from APAP-induced hepatotoxicity by suppressing MAPK signaling and promoting total Nrf2 expression (Figure 7). Our findings also indicate that exposure to APAP decreases Nrf2 levels in the cytoplasm in a dose-dependent manner, which correlates with a moderate increase in Nrf2 translocation to the nucleus compared to controls. In contrast, APAP exposure increased the expression of the Keap1 protein, consistent with previous studies (Shen et al., 2023). However, pre-treatment with Liv52 significantly reduces Keap1 expression and enhances the Nrf2-mediated antioxidant defense by increasing Nrf2 levels in both the cytoplasm and nucleus compared to APAP alone. The elevated Keap1 expression after APAP exposure may account for the decreased Nrf2 levels in APAP alone groups, as noted when compared to the Liv52 pre-treatment. Additionally, the Liv52-alone group exhibited a slight decrease in Keap1 levels, normal cytoplasmic Nrf2 levels, and efficiently stimulated nuclear Nrf2 levels compared to controls, indicating that Liv52 effectively promotes Nrf2 translocation to the nucleus and inhibits Keap1 expression, thereby mitigating oxidative stress induced by APAP. Apoptosis plays an important role in the etiology of several liver diseases. Caspases are the essential terminal mediators of apoptosis; the most common among them, caspase-9 and caspase-3, catalyze the precise cleavage of numerous important cellular proteins (Jaeschke et al., 2018). Caspase-9 is a key initiator in the intrinsic apoptosis pathway. It cleaves and activates caspase-3, an executioner apoptosis molecule induced by APAP (Yiang et al., 2015). The apoptotic response mediated by several drugs is significantly affected by the Bcl-2/Bax ratio and the amount of caspase-3 (Kumari & Kakkar, 2012). Anti-apoptotic proteins, such as Bcl-2, are primarily located in the outer membranes of the mitochondria and alter the membrane permeability. Pro-apoptotic proteins, such as Bax, translocate into the mitochondria from the cytosol and help permeabilize the mitochondrial membrane, which causes the release of cytochrome c and, ultimately, apoptosis (Kumari & Kakkar, 2012). We found Liv52 upregulates the antiapoptotic protein, Bcl-2 and downregulates the pro-apoptotic Bax protein along with caspase-3 and -9 (Figure 5 and Figure 7). These findings suggest that Liv52 protects against APAP-induced apoptosis. Overall, after administration, APAP is first metabolized by cytochrome P4502E1 (CYP2E1) and generates NAPQI, which in turn depletes hepatic GSH and causes overproduction of ROS. ROS can cause the phosphorylation of MAPK, leading to further increases in ROS generation. In addition, ROS can increase the production of the caspases and BAX, which accelerate mitochondrial dysfunction and hepatocyte apoptosis, culminating in severe liver damage. Liv52 pretreatment not only alleviated the changes in ROS/MAPKs-mediated apoptotic signaling cascade during APAP challenge but also promoted Nrf2 expression and enhanced the Nrf2-mediated antioxidant defense system, ultimately leading to reduced oxidative stress and cell death. Furthermore, Liv52 has been shown to have hepatoprotective, antioxidant, antiviral, and anti-inflammatory properties with no adverse effects. It is licensed for the symptomatic improvement and supportive management of mild-to-moderate liver disease, such as viral hepatitis and non-alcoholic fatty liver disease. Studies have shown that it is powerful, safe, and effective for up to 14 days of continuous use (Shivnitwar et al., 2024). Almost all of the findings in the Liv52-treated group in our study, including liver and stress marker investigations, upstream and downstream mechanisms, and histological/ultrastructural assessments, were identical or comparable to those seen in the vehicle control group. This scenario gives additional evidence of the safety of Liv52 use.

Conclusion

Our results demonstrate that oral APAP at doses of 150 mg/kg and 250 mg/kg (moderate and severe liver toxicity, respectively) causes oxidative damage, hepatocyte apoptosis, mitochondrial dysfunction, and eventually hepatotoxicity. Pretreatment with Liv52 for 7 days before APAP administration prevented the severe damaging effects of APAP via the Nrf2, MAPK, and caspase (both caspase-3 and -9) pathways. Thus, Liv52 may potentially be a useful herbal formulation for preventing or treating APAP-induced hepatotoxicity. Because of the increasing number of hepatotoxicity cases around the globe, we believe our findings are important and relevant. Further studies are being undertaken in our laboratory to identify the mechanisms of action for Liv52.

Acknowledgments

The authors thank the Department of Studies in Biochemistry, University of Mysore for providing all the resources required for the study, and also the Institute of Excellence at the University of Mysore for instrumentation facilities; the Central Animal Facility, Department of Studies in Zoology, University of Mysore for the animal facility; the Department

of Neuropathology, National Institute of Mental Health and Neurosciences for Transmission Electron Microscope facilities; the Department of Veterinary and Biomedical Sciences, the Centre for Molecular Toxicology and Carcinogenesis, the Centre for Molecular Immunology and Infectious Disease at The Pennsylvania State University for optical emission spectrometer facility; and Scientific Publications at The Texas Heart Institute for editorial assistance. Authors also thank the State Ayurveda Research Centre, Government Ayurvedic Medical College, Mysuru and Shri Dharmasthala Manjunatheshwara (SDM) education trust (R), Ujjire, Dakshina Kannada for moral support. Rebecca Bartow, PhD, of the Department of Scientific Publications at The Texas Heart Institute, contributed to the editing of this manuscript.

Author contributions

Gopal Kedihithlu Marathe: Supervision, review & editing, Conceptualization, Methodology, Validation. Mylanayakanahosahalli Chandrashekar Indumathi: Original draft, Investigation, Methodology, Visualization, Software, Data curation, Formal analysis. Kamatam Swetha: Methodology. Bhadravathi Kenchappa Chandrasekhar Sagar: Methodology, Visualization. Santhosh Kumar Rashmi: Methodology, Visualization. K. Sandeep Prabhu: Methodology. Lakshminarayana Shenoy: Methodology. Chu-Huang Chen: review & editing.

Funding

The authors received no financial support for the research, authorship, and publication of this article.

Conflict of interest

The authors declare that they have no competing financial or any other conflict of interests.

Ethics approval

All the animal experiments were approved by the Institutional Animal Ethical Committee (Approval No: UOM/IAEC/08/2021), Department of Studies in Zoology, University of Mysore, Mysuru and were conducted in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals.

AI tool declaration

Artificial intelligence/AI programs have not contributed to the compilation of our manuscript.

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