Journal of Advanced Zoology
ISSN: 0253-7214
Volume 45 Issue S-01 Year 2024 Page 213:229

Assessment Of *In-Vivo* Anti-Fibrotic Potential Of Fruit Seed Extract Of Indian Jujube (*Ziziphus Mauritiana*) Using 2-Nitropropane Induced Hepatic Fibrosis Model.

Praveen Kumar[1*], Dr. Dinesh Kumar Sharma[2], Dr. Nasiruddin Ahmad Farooqui[3]

[1*]*Research scholar, Sanskriti university, Mathura, U.P.*
[2]*Dean and professor, Sanskriti university, Mathura, U.P.*
[3]*Professor, Translam Institute of Pharmaceutical Education and Research, Meerut, U.P.*

***Corresponding Author:** Praveen Kumar*
[*]*Research scholar, Sanskriti university, Mathura, U.P.*

Abstract

Injury to the liver is a significant public health issue that affects people all over the world, which necessitates the creation of innovative treatments that are both effective and safe. Due to the antioxidant activity that it possesses, *Ziziphus mauritiana (Z. mauritiana)* has traditionally been considered to have therapeutic potential against many organ toxicity disorders. In the current work, the objective was to assess the antioxidant activities in vitro and the potential hepatoprotective effects of hydroethanolic extracts from *Z. mauritiana* seeds (ZMSE) against 2-nitropropane (2-NP) produced liver damage (Fibrosis) in rats. Additionally, the phytochemical contents of the extracts were to be identified. Through in vitro testing, the extract demonstrated significant antioxidant properties, as well as a high quantity of flavonoids and other phenolic compounds respectively. By activating the hepatic antioxidant defense mechanisms, modulating hepatic functions, and decreasing the production of lipid peroxidation, pro-inflammatory mediators, and collagen content, oral administration of ZMSE (especially high dose) significantly suppressed the incidence and severity of 2-NP-induced liver toxicity. However, the degree of suppression varied depending on the dose. There is a possibility that the presence of phenolic acids, flavonoids, and diterpenoids is responsible for these activities. These findings demonstrate the antioxidant and anti-inflammatory properties of ZMSE when administered in varying doses (200, 400, or 800 mg/kg body weight), demonstrate the protective and beneficial effects of the seed against 2-NP-induced hepatic toxicity in rats, and provide support for its consumption, traditional uses, and the promotion of its valorization as a nutraceutical product.

Keywords:-2-nitropropane (2-NP), Liver fibrosis, Ziziphus mauritiana, Antioxidant, Pro-inflammatory mediators, Anti-inflammatory

Introduction

On a global scale, chronic liver disease is becoming more prevalent and affects everyone. The condition known as cirrhosis is characterized by fibrosis, structural distortion, and renewing nodules. Cirrhosis is the result of several liver illnesses that are accompanied by a variety of clinical symptoms (Bataller & Brenner, 2005) It is estimated that chronic liver illnesses claim the lives of over 35 million people across the globe. In 2016, cirrhosis was responsible for the deaths of 2.2% of persons and the disability of 1.5% of people worldwide. 1.32 million people were died by CLD in 2017, with men making up two-thirds of the victims (James et al., 2018; Xu et al., 2020). The Global Burden of Disease (GBD) Project of the World Health Organization (WHO) calculates mortality and morbidity rates according to age, gender, and geographic location. There are a number of factors that contribute to determining the burden of a disease, including its incidence, prevalence, mortality, morbidity, quality of life, and direct or indirect cost expenditures. Knowledge of the disease burden is essential to the establishment of public health priorities and preventative measures (WHO., 2022).

Chronic liver inflammation is the root cause of fibrosis, cirrhosis, and hepatocellular carcinoma (HCC) (Dhar et al., 2020). Extracellular matrix (ECM) proteins, which are primarily cross-linked collagens type I and III, are responsible for the development of hepatic fibrosis. These proteins eventually replace normal tissue that has been damaged (Ortiz et al., 2021). Fibrosis is caused by damage to the liver that is hepatotoxic and cholestatic. The condition known as non-alcoholic steatohepatitis (NASH) is brought on by chronic hepatocyte injury brought on by HBV, HCV, alcohol, or metabolic syndrome. cholestatic damage is caused by an obstruction in the flow of bile in patients with PBC, PSC, and biliary atresia (Trauner & Fuchs, 2022).

Free radicals (FR) and xenobiotics are both metabolized and detoxified by the liver, which is a beneficial organ (Casas-Grajales, 2015). Antioxidants eliminate free radicals and maintain a healthy balance between liver oxidation and defense. An imbalance between the liver's oxidative and antioxidant processes leads to oxidative stress, which hurts the liver. As a result, antioxidants bring about equilibrium. Antioxidants can be replenished by the consumption of antioxidant-rich foods and plants (Sharifi-Rad et al., 2020). Anti-inflammatory, antibacterial, antiviral, anti-aging, and anticancer properties are exhibited by certain polyphenols and carotenoids (Ullah et al., 2020).

Damage caused by antioxidants can result in reactive oxygen species, which can lead to dementia, diabetes, and cancer. Antioxidants repair damage caused by reactive oxygen species (Tan et al., 2022). The formation of 8-hydroxydeoxyguanosine (8-OHdG) is the manifestation of oxidative DNA damage in the liver, which is caused by 2-nitropropane. This damage is associated with oxidative DNA damage. It is believed that this chemical plays a part in the development of problems that are associated with oxidative stress, such as liver fibrosis and hepatocarcinogenesis, respectively (Sai et al., 1998). The vast majority of Ziziphus species are rich in triterpenoids, saponins, and polyphenolics, which are antioxidants. Both the triterpenes and the triterpenic acids found in *ziziphus* have biological activities, one of which is an antioxidant capability. The fruit of the *Z. jujube* plant included high concentrations of the acids maslinic, ursolic, oleanolic, betulinic, and alphitolic. There is a possibility that *Z. jujube* is an antioxidant because significant triterpenic acids have a good association with the antioxidant activity of the genus. *Z. oxyphylla* is used in Pakistani traditional medicine as an anti-inflammatory and analgesic for the treatment of chronic liver diseases such as hepatitis. The ziziphus fruit, which is high in triterpenoid, is an antioxidant. Pentacyclic triterpenes from a wide variety of Ziziphus species require additional research on cell signaling pathways that activate antioxidant enzymes such as superoxide dismutase (SOD) or catalase (Sakna et al., 2022).

Deserts and wild temperate regions in India, Algeria, Egypt, and southern Africa are ideal environments for the growth of *Z. mauritiana* vegetation. Numerous other names for Z. mauritiana include ber, jujube, desert apple, Indian plum, Malay apple, and Chinese apple. Triterpenoids, flavonoids, alkaloids, leucoanthocyanidins, and sterols are all components of the *Z. mauritiana* plant variety. Thiamine, riboflavin, niacin, and ascorbic acid are all found in the fruits of the ziziphus plant. The utilisation of *Z. mauritiana* was both therapeutic and nourishing. *Z. mauritiana* yields fruits that are both antioxidants and anti-diabetic. The bark of *Z. mauritiana* has anti-inflammatory, anti-cancer, and anti-allergic properties (Alsayari & Wahab, 2021).

We have hypothesized that the utilization of *Z. mauritiana* species will be advantageous for the treatment of chronic liver disease situations such as liver fibrosis. This is the context in which we are discussing this information. When it comes to the fight against chemically induced liver fibrosis, the antioxidant activity of *Z. mauritiana* seed extract will be utilized in the context of experimental animals.

Chemicals and reagents

Olive oil was procured from MP biomedicals, India. Kits for serum biochemical analysis was purchased from Span Diagnostic LTD, Sachin, Gujarat, India. The proinflammatory cytokines were measured by commercially available kits (R&D systems, Minneapolis, MN) as per the manufacturer's instruction.

Plant source and preparation of extract

Fruits of *Z. mauritiana* (Lamk.), were collected from wild area of Meerut district of Uttar Pradesh, India and authenticated by Prof. (Dr.). Vijai Malik, Botany Department, CCS University, Meerut, Uttar Pradesh, India. Plant sample has been kept in Voucher specimen Bot/730/13-5-2022 at CCS University, Meerut, Uttar Pradesh, India.

Preparation of Extracts

Seed extract

The pulp was peeled off and the seeds were shade dried at room temperature and reduced to coarse powder. The dried and powdered seeds of *Z. mauritiana* (250g) were percolated six times with ethanol: water (8:2) at room temperature. The combined extracts were filtered (Whatmann paper), centrifuged (10000×g, 4°C, 10 min) and concentrated under reduced pressure in a rotatory evaporator at 40±5°C. Finally, the extract was completely dried under vacuum in the desiccator and refrigerated at 4°C until further use. The whole procedure resulted in 12-19% (w/w) yield of the extract (ZMSE) in terms of dried starting material.

Preliminary Phytochemical Screening

On preliminary phytochemical screening of Seed extract and Leaf extract using the reported method, the ethanolic extract of the seed and leaf showed positive tests for Cardiac glycosides, Coumarins, Emodins, Phenol/Polyphenols, Quinones, Saponins, Tannins and Terpenoids, while negative results for Alkaloids, Phlobatannins, Steroids, flavonoids.

Determination of the Total Phenolic Content

The total phenolic content (TPC) in the extracts was determined using Folin reagent (Molole et al., 2022). Briefly, 1.5 mL Folin–Ciocalteu reagent (diluted 1:10) and 1.2 mL of 7.5% (w/v) Na_2CO_3 were added to 0.3 mL of the extract. After incubation at room temperature for 1 h in the dark, the absorbance was measured at 765 nm against a blank. The TPCs were estimated using a standard curve of the gallic acid developed and expressed as gallic acid equivalents (GAE) in mg g^{-1} extract.

Determination of the Total Flavonoid Content

The total flavonoid content (TFC) of the extracts was estimated using the aluminium chloride colorimetric method (Mahboubi et al., 2013). Briefly, 1.5 mL of methanol, 0.1 mL of aluminium chloride (10%), 0.1 mL of 1 M potassium acetate, and 2.8 mL of distilled water were mixed with 0.5 mL of extract solution. The absorbance of the reaction mixture was determined at 415 nm after incubation at room temperature for 30 min. Quercetin was used as the reference standard and the results were expressed as quercetin equivalents (QE) in mg g^{-1} extract.

Antioxidant Activity

DPPH Radical Scavenging Capacity

Briefly, 0.1 mL of plant extract at various concentrations was added to 2.9 mL of a 0.002% methanolic solution of DPPH. The reaction mixtures were incubated for 30 min at room temperature and the absorbance at 517 nm was measured against a blank (Baliyan et al., 2022). Ascorbic acid was used as the standard. The ability to scavenge the $DPPH^{\cdot}$ radical was calculated as:

% of antioxidant activity= [(Ac−As) ÷Ac] × 100

where: Ac-Absorbance of negative control at the moment of solution preparation; As-Absorbance of sample after 30 min.

ABTS Radical Scavenging Capacity

ABTS radical scavenging was estimated by measuring the decolorization of free radical $ABTS^{+}$ (González-Palma et al., 2016) with some modifications. $ABTS^{+}$ was produced by reacting 7 mM ABTS stock solution in water with 2.45 mM potassium persulfate solution in equal amount and the mix was allowed to stand for 12–16 h at room temperature in the dark until the reaction was complete and the absorbance was stable. The $ABTS^{+}$ solution was diluted to give an initial absorbance of 0.70 ± 0.02 at 734 nm in phosphate buffered

saline (pH 7.4) solution. Then, 1 mL of $ABTS^{+}$ solution was added to the test samples. The absorbance was recorded 6 min after mixing and the percentage of inhibition was calculated as:

$\%inhibition = [(A_{control} - A_{sample})/A_{control}] \times 100$

$A_{control}$ = Absorbance of negative control at the moment of solution preparation; A_{sample} = Absorbance of sample after 6 min.

Animals

Hepatoprotective activity was carried out on albino rats of either sex (110-145 g), supplied by the central animal house facility of TIPER, Meerut. The rats were maintained in a 12 h light/dark cycle at 25±2°C. They were allowed free access to a standard pellet diet (Amrut Laboratory Rat Feed, Pune, India) and water *ad libitum*. The study was approved by the ethics committee CPCSEA with reference letter no IAEC/PH-22/TIPER/170 and ethical norms were strictly followed during all experimental procedures.

Acute toxicity studies

ZMSE was administered to the female rats under overnight fasting by using oral gavage in a volume of 10ml/kg body weight. Animals were divided into 3 groups of 3 rats in each as mentioned previously (Al-Afifi et al., 2018). The starting dose of ZMSE at 50 mg/kg was administered to group 1. All rats were observed for behavioral changes, toxicity, and mortality after treatment for the first 4h, then up to 48 h. Group 2 was administrated successively at 48 h intervals with the next higher dose 2000 mg/kg body weight of ZMSE when there were no signs of toxicity or mortality showed in group 1. Separately, group 3 treated vehicles as negative control group according to the OECD guideline. All animals observed every 30 minutes up to 4h and then up to 24h after administration and then once daily for 14 days. This observation was done to assess the onset of toxic symptoms including changes in skin and fur, eyes and mucous membranes and behavioral changes were recorded. Furthermore, animals were also observed for sign convulsions, tremors, diarrhea, salivation, lethargy, sleep, coma, and mortality. The food consumption and water intake recorded daily. The body weights of animals were recorded weekly. The percentage of body weight change calculated according to equation.

$$\%\ Body\ weight\ change = \frac{Body\ weight\ at\ the\ end\ of\ each\ week - \ Initial\ body\ weight}{Initial\ body\ weight} \times 100$$

Drugs and dosing schedule

The animals were divided into six groups: group I (Control treatment), group II (Disease control treatment), 2NP, group III (Standard treatment), group IV (Extract Treatment 1), V (Extract Treatment 2) and VI (Extract Treatment 3). Animals of group I were given distilled water in a volume of 10 mL/kg body weight. Animals of groups II, III, IV, V and VI were administered 2-NP at a dose of 100 mg/kg body weight twice weekly *i.p.* for 12 weeks dissolved in olive oil via the s.c. route. Simultaneously but at different hours of the day, animals of groups III were fed with silymarin suspension orally (50 mg/kg body weight) once daily for 12 weeks respectively. Group IV, V and VI were fed orally with ethanolic extract in doses of 200 mg/kg, 400 mg/kg, and 800 mg/kg body weight respectively.

Collection of Blood Samples

Blood samples were collected by retro-orbital venous plexus puncture method without anticoagulant under mild anesthesia. The blood sample collected at the end of study period was for the estimation of all biochemical parameters. All animals in different groups were sacrificed 24h after last dose by cervical dislocation and liver tissue was extracted and weight, cleaned with ice-cold saline, and blotted dry with the help of filter paper. The biggest lobe of liver was kept in 10% formaldehyde solution for histopathological study. The remaining part of liver tissue was stored at -80°C and further utilized to assess the tissue biochemical parameters.

Estimation of Biochemical Parameters

Liver function test

Assessment of hepatic parameters including AST, ALT, ALP, and total bilirubin by using kits (Span Diagnostic LTD, Sachin, Gujarat, India.

Preparation of Cytosolic and Microsomal Fraction

Frozen liver tissue samples (stored at -80°C) of different experimental groups of rats were thawed, blotted quickly, and weighed. The hepatic tissues were separately homogenized with ice-cold 0.1 N KCl (pH-7.4) in precooled teflon-coated glass homogenizer for few minutes to make 10% w/v tissue homogenate. Liver homogenate was centrifuged at 8000×g for 15 minutes (at 4°C) and the supernatant was subjected to ultracentrifugation (WX-Ultra 90, T-890 rotor, Thermoscientific, USA) at 78,000×g for 90 minutes. The supernatant thus obtained, was used as cytosolic fraction and a portion of it was undergone ultracentrifugation at 1,05,000×g for 1h to obtain microsomes (precipitate) to assay UDPGT activity, CYP-450 content, lipid peroxidation, and the cytosolic fraction was used to determine SOD, CAT, GPx, and reduced GSH. (Eugenia Letelier Muñoz et al., 2009)

Assay for membrane bound enzymes such as CYP-450 assay was estimated by the method described Yuan Xie et al. and UDPGT activity towards p-nitrophenol was determined according to the standard method described by Black, 1994. The activity of GST was determined according to the method of Hossain *et al* with some modifications GST by Habig et al.(Habig et al., 1974). Hossain *et al*., 2013. Na^+-K^+-ATPase activity was assayed in two reaction mixtures as reported by Pradip K. Sarkar (Sarkar, 2002). Ca^{2+}-ATPase in the plasma membranes estimated by the reported method of Lotersztajn. SOD and CAT were measured in liver tissue homogenate based the method of Marklund et al.(MARKLUND & MARKLUND, 1974) and Sinha (Sinha, 1972) respectively. GPx by Rotruck et al.(Rotruck et al., 1973), and total GSH by Moron et al.(Moron et al., 1979). In the liver tissue homogenates, LPO was calculated using TBARS based on the method of Ohkawa et al.(Ohkawa et al., 1979).

Estimation of Proinflammatory Cytokines

The proinflammatory cytokines including interleukin-1β(IL-1β), tumor necrosis factor-α (TNF-α), IL-6 and IL-10, and profibrotic cytokines (TGF-β) were measured by commercially available kits (R&D systems, Minneapolis, MN) as per the manufacturer's instruction with enzyme-linked immunosorbent assay and their amount represented as pg/mg protein.

Determination of Hydroxyproline in Liver Tissue

The liver tissue was removed, weight of 30–100 mg and homogenized in 5% trichloroacetic acid solution (x10 volume) using a cell homogenizer at 8,000×g (4°C) for 2 min in an ice bath. Cells were centrifuged (2,500 × g, 4°C) for 20 min and the supernatant washed twice with distilled water. Then, 6N HCl was added at 110°C completely at the beginning and reacted for 16 h. Following completion of the reaction, toluene (3 ml) was added, and the mixture was agitated for 20 min. Following centrifugation (3,000×g, 20°C) for 10 min, the organic layer was collected and p-dimethylaminobenzaldehyde added. Hydroxyproline in the sample was detected by spectrophotometrically at 565 nm using a colorimetric analyser (Qiu et al., 2014).

Reference Measurement of hydroxyproline in collagen with three different methods

The formula for content calculation was as follows: hydroxyproline content (μg/mg wet weight) = (measured OD value − blank OD value)/ (standard OD value-blank OD value) × standard concentration (5 μg/mL) × total hydrolysate volume (10 mL/tissue wet weight (mg)

Histopathological studies

For histological examination, liver tissue was fixed in 10% buffered formalin. Standard laboratory technique is used for liver histology. After fixing the liver tissue in formalin for 48h, the tissues were washed with tap water for about 4h. The tissues were dehydrated by soaking for 1h each in 70%, 80% and 100% alcohol followed by soaking in xylene for about 30 minutes. The tissue was finally soaked overnight in hot liquid paraffin and was proceeded to form blocks. The block was cooled at -20ºC for 1h, sectioned to 5 μm using a microtome (Leica, Bensheim, Germany), and dried in a hot air oven. The sections were deparaffinized with excess xylene and rehydrated with 100%, 95%, 80%, and deionized water. Before haematoxylin staining, the slides are dried. Slides were kept in haematoxylin solution for 3 minutes, then rinsed for 5 minutes. The slides were then soaked in acid ethanol and washed with water. Excess water was blotted from the slide. The slides were stained with eosin for 30-45 seconds, dehydrated with 95% and 100% ethanol, and then treated with xylene for 15 minutes. After staining, the sections were mounted by DPX, covered using a cover slip and observed under light microscope using Zeiss microscope (Axioplan 2 Imaging, Axiovision software). The degree of hepatic damage was assessed by examining hepatocyte structure, portal vein, hepatic and bile ducts and inflammatory cell infiltration.

7.3.5. Statistical analysis

The results were expressed as mean ±SEM; n=6 animals in each group; $^{*}P<0.05$: Statistically significant, $^{**}P<0.01$: Statistically very significant and $^{***}P<0.001$: Statistically highly significant from normal control. $^{\#}P<0.05$: Statistically significant, $^{\#\#}P<0.01$: Statistically very significant and $^{\#\#\#}P<0.001$ Statistically highly significant from disease control. Statistical analysis was carried out using Graph Pad Prism software (version 8.1). One way ANOVA or two-way ANOVA was used, followed by Bonferroni multiple comparison tests. Normal control groups and disease control group were compared with other.

Results and Discussion

Phytochemical analysis plays a crucial role in comprehending the chemical makeup of plants and exploiting their potential advantages in the fields of health, nutrition, agriculture, and environmental science. It has a pivotal function in advancing diverse scientific disciplines and has practical applications in enhancing human health and well-being.

The *Z. mauritiana* seeds were extracted using a mixture of ethanol and water (8:2) at room temperature. The extraction process yielded 12-19% (w/w) of the extract (ZMSE) based on the weight of the dried starting material. The ethanolic extracts of *Z. mauritiana* were subjected to qualitative preliminary screening for phytoconstituents, which revealed the presence of several phytoconstituents. The seed extract was subjected to preliminary phytochemical screening using the established method. The ethanolic extract of the seed exhibited positive results for Cardiac glycosides, Coumarins, Emodins, Phenol/Polyphenols, Quinones, Saponins, Tannins, and Terpenoids. However, it yielded negative results for Alkaloids, Phlobatannins, Steroids, and flavonoids (Table 1).

Metabolites	Observation	Results
Alkaloids	No Brownish-red precipitate formed	Negative
flavonoids	No deep yellow color appeared	Negative
Cardiac glycosides	Brown ring formed at interphase	Postitive
Coumarins	Yellow coloration formed	Postitive
Emodins	Yellow coloured precipitate formed	Negative
Phenol/Polyphenols	Blue green to dark blue coloration formed	Postitive
Quinones	Formation of red coloration observed	Postitive
Saponins	Formation of continues effervescence observed	Postitive
Steroids	No dark green coloration observed	Negative
Tannins	Dark blue or greenish grey coloration observed	Postitive
Terpenoids	Reddish brown colour formed at interface	Postitive

Table 1: Phytochemical analysis of ethanolic extract of *Z. mauritiana* Lam. Seed

The quantification of total phenolic and flavonoids content is a powerful analytical technique with wide-ranging applications in evaluating the antioxidant capacity, health advantages, and quality of plant-derived compounds. This method contributes to diverse scientific disciplines and enterprises (Aryal et al., 2019).

The extraction methods and solvents are accountable for dissolving the naturally occurring components of the plants. Furthermore, plant components can exhibit either polar or non-polar characteristics. Phenolic compounds exhibit enhanced solubility in polar organic solvents because of the hydroxyl group present. Consequently, hydroethanolic was chosen as the solvent for extraction. The hydroxyl groups present in plant extracts play a crucial role in helping the process of scavenging free radicals. Phenolic content in each extract was tested using the Folin–Ciocalteu reagent as a reference. The data were obtained from a calibration curve ($y = 0.037x$, $R^2 = 0.9996$) of gallic acid (0–30 µg/mL) (Figure. 1A) and reported as gallic acid equivalents (GAE) per gramme of dry extract weight (Table 2). The hydroethanolic extracts have a phenolic component concentration of 302.94± 11.8mg GAE/g.

Flavonoids are a type of secondary metabolite that possesses antioxidant properties. The effectiveness of their antioxidant activity is determined by the quantity and arrangement of unbound hydroxyl (OH) groups. The flavonoid concentrations in selected plant extracts were quantitatively measured using the colorimetric technique with aluminium chloride. The results were obtained from the calibration curve ($y = 0.0382x - 0.0221$, $R^2 = 0.9979$) of quercetin (0–30 µg/mL) (Figure. 1B) and were represented in quercetin equivalents (QE) per gramme of dry extract weight (Table 2). The hydroethanolic extracts have a flavonoid concentration of 128.16 ± 11.00 mg QE/g.

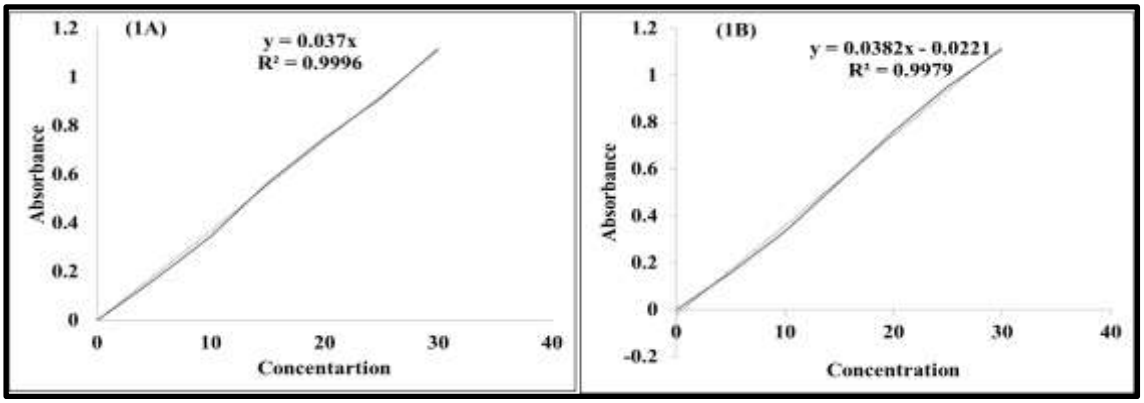

Figure 1. A. Calibration curve of gallic acid. **B.** Calibration curve of Catechin. Each point represents the mean of three experiments.

Table. 2. Total phenolic and flavonoids content in hydroethanolic extract of *Z. mauritiana* Lam. Seed.

Total phenolic mg GAE/g extract	Total flavonoids mg Catechin/g extract
302.94± 11.8	128.16 ± 11.00

In figure 2A displays the DPPH radical scavenging capabilities of hydroethanolic extract of *Z. mauritiana* Lam. Seed. The seed extracts, which were prepared using a mixture of water and ethanol, exhibited an increase in their ability to scavenge radicals as the concentration of the extracts increased. The highest DPPH radical scavenging efficacy was seen at a concentration of 1000 µg/ml. DPPH is an enduring organic radical that undergoes a loss of its absorption spectrum range at 515–528 nm upon receiving an electron or a free radical entity. The DPPH test is a straightforward, generally accepted approach that is commonly used to assess the ability of plant extracts to scavenge radicals. Antioxidants are plant components that can visibly neutralize the stable purple DPPH radical, causing it to become yellow (Aryal et al., 2019).

In figure 2A, A comparison was made between the general BTH and the relative antioxidant capacity of ABTS+ to scavenge for radicals. Through the utilisation of potassium persulfate, the ABTS radical cation was successfully generated in its stable state. The antioxidant plant extract was added to the reaction medium after the steady absorbance was obtained, and the antioxidant potency was evaluated by observing the decolorization process. At a dose of 1000 µg/ml, the ABTS radical scavenging efficacy was seen, which was comparable to the DPPH assay exhibited.

The obtained results agreed with those presented in literature. The studied the antiradical and antioxidant activity (by ABTS and DPPH) using aqueous ethanolic extract (80%) of *Zizyphus mauritiana* found that the activities could be correlated with the polyphenolic compounds in the extract (Koley et al., 2016).

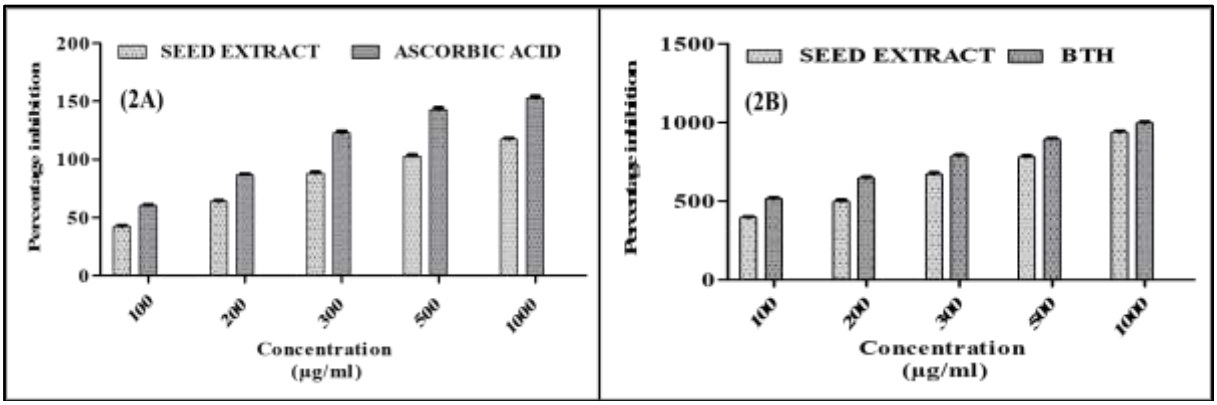

Figure. 2. A. DPPH Assay and **B.** ABTS assay of hydroethanolic extract of *Z. mauritiana* Lam. Seed.

The acute toxicity test using the OECD guideline at an oral limit dose of 50 and 2000 mg/kg of the ZMSE caused no death in the mice and rats. No lethal effects were noted throughout the short and long-term observation period. No toxicity signs were observed in the animals throughout the 14 days study period (Table 3). Therefore, the extract may be safe at these doses and the oral LD50 is considered greater than 2000 mg/kg in rats.

The extracts of ZMSE were exposed to a 14-day acute toxicity investigation, and the results showed that they did not result in any mortality or behavioural, motor-neuronal abnormalities in rats. According to Owolarafe et al., the administration of an aqueous extract of *Z. mauritiana* leaves over an extended period may result in serious unfavorable effects on the biological system. This conclusion contrasted with the one that we found here, and slo the one that Ramar et al. reported, which stated that methanol extract of *Ziziphus mauritana* Lam leaves does not cause any symptoms of toxicity or mortality in acute toxicity study (Owolarafe et al., 2022; Ramar et al., 2022) .

Table 3. General observation and behavioral analysis

Observations	**Control**		**ZMSE (50 mg/kg)**		**ZMSE (2000 mg/kg)**	
	4h	**24h**	**4h**	**24h**	**4h**	**24h**
Eyes	No Change	No Change	No Change	No Change	No Change	No Change
Skin and fur	No Change	No Change	No Change	No Change	No Change	No Change
Lethargy	Not Observed	Not Observed	Not Observed	Not Observed	Not Observed	Not Observed
Sleep	Normal	Normal	Normal	Normal	Normal	Normal
Diarrhea	Not Observed	Not Observed	Not Observed	Not Observed	Not Observed	Not Observed
Coma	Not Observed	Not Observed	Not Observed	Not Observed	Not Observed	Not Observed

During the research, the rats in the treatment group did not exhibit any changes in their body weight that could be considered statistically significant when compared to the rats in the control group Table 4. Throughout the course of the trial, the rats maintained a regular and constant pattern of consumption of both feed and water (the data for this experiment are not shown here).

While researching the toxicity and safety of a natural product, it is crucial to keep track of the experimental animals' body weight and feed/water consumption because these data provide insight into their physiological and metabolic status and prevent the researcher from drawing any "false" conclusions based on the rats' abnormal nutritional status. The weight gain that each rat in the current study displayed was similar and followed a broad pattern. Since none of the experimental groups experienced weight loss or gain, it is likely that the ZMSE extracts had no adverse impact on the rats' overall health or metabolic development, nor did they cause any appreciable changes in appetite. Additionally, it was observed that the rats' feed and water intake followed a consistent pattern from the beginning of the trial until its conclusion. The administration of hydroethanolic extracts of ZMSE did not substantially (>0.05) change the pattern of body weight and feed consumption, indicating that the plant

extracts had no negative impacts on the rats' growth and development (Pariyani et al., 2015).

Table 4. Body weight and percentage change in body weight at 0 day, 1 and 2 week in acute oral toxicity

Group	**0 Day**	**Week 1**	**Week 2**	**% Change Week 1**	**% Change Week 2**
Control	219.3 ± 3.68	226.0 ± 1.08	236.7 ± 3.180	4.90 ± 1.53	6.56 ± 0.17
ZMSE (50 mg/kg)	223.6 ± 2.48	239.4 ± 4.17	248.8 ± 4.72	5.04 ± 1.95	8.70 ± 1.83
ZMSE (2000 mg/kg)	225.6 ± 4.19	236.2 ± 3.92	249.0 ± 4.39	5.35 ± 1.72	8.26 ± 1.98

Data were expressed as mean±SEM (n=3). The P<0.05 was considered as significant (*), p<0.01 considered as very significant (**), and p<0.001 considered as highly significant (***).

Table 5 displays the impact that a single oral treatment of ZMSE (50 and 200 mg/kg) had on the hematological parameters of rats following these administrations. The parameters that were analyzed did not record any changes that were statistically significant when compared to the control groups that were being considered. Evaluation of the haematological parameters is crucial since it might immediately indicate the systemic effects that the extract that was delivered was causing. According to the findings, there was no discernible change (> 0.05) between the haematological profiles of all treated rats and the control group.

This toxicity study also includes a number of significant biochemical markers. The kidney and liver are the main organs that are vulnerable to the harmful effects of medications. Serum creatinine, urea, and total protein levels were measured to evaluate renal function, while the levels of AST and ALT were measured to evaluate liver function. The experiment's findings revealed that neither the treated female rats' kidney nor liver functions were changed. Between the treatment and control groups, there were no statistically significant changes in the levels of creatinine, urea, total protein, AST, or ALT. Therefore, these results imply that ZMSE did have any negative impact on the rats' liver and kidneys.

Table 5. Hematological and Biochemical parameters of hydroethanolic seed extract of *Z. mauritiana* Lam in acute oral toxicity**.**

Parameter/Group	Control	ZMSE (50 mg/kg)	ZMSE (2000 mg/kg)
CBC			
HGB	124.3 ± 1.12	139.2 ± 1.81	140.7 ± 1.28
WBC	7.2 ± 0.11	7.30 ± 0.12	7.33 ± 0.13
Differential count			
Neutrophil	0.52 ± 0.31	0.54 ± 0.22	0.56 ± 0.21
Lymphocyte	4.90 ± 0.12	5.10 ± 0.13	4.97 ± 0.17
Monocyte	0.13 ± 0.00	0.14 ± 0.00	0.13 ± 0.01
Eosinophil	0.07 ± 0.00	0.08 ± 0.16	0.08 ± 0.00
Basophil	0.02 ± 0.00	0.01 ± 0.00	0.02 ± 0.00
Renal function test			
Urea	2.90 ± 0.10	3.18 ± 0.26	3.23 ± 0.30
Creatinine	29.47 ± 1.2	30.32 ± 1.1	29.88 ± 0.3
Liver function test			
Albumin	32.17 ± 1.15	35.20 ± 0.73	34.5 ± 1.3
Globulin	23.92 ± 0.26	23.00 ± 0.35	23.20 ± 0.13
Total bilirubin	1.07± 0.01	1.08 ± 0.01	1.03 ± 0.01
Conjugate bilirubin	1	1	1
ALP	171.2 ± 0.6	173.9 ± 0.2	175.0 ± 0.15
ALT	26.00 ± 1.12	25.87 ± 0.20	27.84± 1.15
AST	94.00 ± 1.21	93.13 ± 1.12	92.42 ± 1.12

Data were expressed as mean±SEM (n=3). The P<0.05 was considered as significant (*), p<0.01 considered as very significant (**), p<0.001 considered as highly significant (***), and p>0.05 considered as non-significant (^{NS}).

The microscopic evaluation of the different tissue sections of *Z. mauritiana* treated rats did not show any lesions or abnormal histopathological changes as compared to their respective control groups (figure 3). The organs retained normal texture and appearance on gross examination. The relative organ weight index of the rats treated with *Z. mauritiana* extracts did not show significant changes as compared to their respective control groups (data no shown).

Histopathological studies were conducted on brain, heart, kidney, liver, and lungs of all the rats. Gross examination of the organs did not show any signs of necropsy and abnormal morphological changes. The microscopic examination of the hematoxylin eosin-stained tissue sections also recorded insignificant changes as compared with the control rats' tissues.

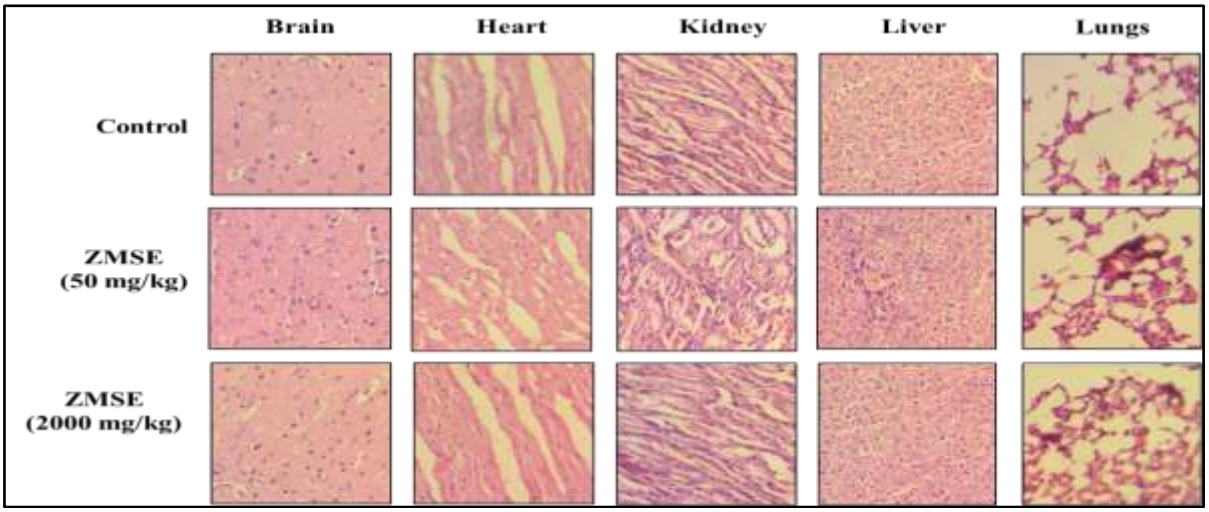

Figure 3. Histopathological examination of various organs of the rat in acute oral toxicity study; Brain, heart, kidney, liver, and lungs of the control group and treated animals; All organs show normal architecture with no sign of toxicity. (Haematoxylin & Eosin, (H&E) ×100).

Liver fibrosis is a catastrophic medical condition that leads to significant morbidity and mortality. Hepatic fibrosis is the common result of prolonged liver injury, ultimately leading to cirrhosis and its related effects, including portal hypertension, liver failure, and hepatocellular carcinoma (Rajapaksha, 2022). 2-NPhave been used induce liver toxicity as an ideal model for assessing the effectiveness of diphenyl diselenide on liver damage (Borges et al., 2006). The reason for this is the widely acknowledged fact that 2-Nitropropane, a substance toxic to the liver, may induce fibrosis in rats that may closely mimic the irreversible fibrosis observed in humans.

The administration of 2-NP resulted in an increase in serum AST and ALT activities, as well as liver TNF- α, IL-6, and MDA levels, and the production of vascular endothelial growth factor (VEGF) and caspase-3. Conversely, the levels of GSH contents and SOD activity were reduced (Shaheen et al., 2021).

Table 6 demonstrates the impact of 2-NPand hydroethanolic seed extract of *Z. mauritiana* on both enzymatic and non-enzymatic indicators of liver injury in this research. Rats exposed to 2-NPfor 12 weeks exhibited a notable increase ($p<0.05$) in the levels of AST, ALT, ALP, and TB. The administration of hydroethanolic extract of *Z. mauritiana* seed at doses of 200, 400, or 800mg/kg bw in rats resulted in a significant ($p<0.05$) dose-dependent reduction in levels of AST, ALT, ALP, and TB. Conversely, the administration of 2-NPled to a significant ($p<0.05$) increase in levels of AST, ALT, ALP, and TB, indicating its hepatotoxic potential. Silymarin 50mg/kg as standard produce very significant effect to ameliorate the effect of 2-NPon the AST, ALT, ALP, and TB level.

The repeated treatment of 2-NPresulted in significant hepatocellular damage in rats. The measurement of serum bilirubin levels and the assessment of AST, ALT, and ALP activities are the most reliable and accurate assays used to diagnose liver illnesses. The elevated concentration of ALP is a result of the compromised structural integrity of liver cells. This is since the enzyme alkaline phosphatase is situated in the cytoplasm and is discharged into the bloodstream following cellular injury.

If an injury affects organelles, such as mitochondria, the soluble enzymes, like AST, that are contained within these organelles will also be released, suggesting disruption to the cell membrane. Prior studies have demonstrated that when hepatocytes are exposed to 2-nitropropane, it disrupts the structure and functioning of the cell membrane, leading to an increase in the leakage of AST. Bilirubin, a naturally occurring chemical anion, forms a reversible bond with albumin and is then transferred to the liver. In the liver, it undergoes conjugation with glucuronic acid and is ultimately expelled in the bile. Hepatobiliary illness is diagnosed when the level of bilirubin fraction exceeds the normal range. Elevated levels of bilirubin in the bloodstream indicate abnormalities in the functioning of the liver. Hyperbilirubinemia is a highly sensitive and valuable test for assessing the liver's functional integrity and the severity of necrosis. The measurement quantifies the ability of hepatocytes to bind, conjugate, and excrete substances, which is directly linked to the rate at which erythrocytes are broken down. An elevated total bilirubin level indicates the severity of jaundice, whereas increased aminotransferases and alkaline phosphatase clearly indicate cellular leakage and loss of cellular function. Prior administration of hydroethanolic extracts from *Z. mauritiana* seeds to rats, followed by alcohol consumption, led to a notable reduction in levels of AST, ALT, ALP, and TB. This reduction occurred in a dose-dependent manner, in comparison to rats treated with 2-nitropropane. This suggests that the hydroethanolic extracts from *Z. mauritiana* seeds may have shielded both the plasma membrane and liver cells from damage caused by 2-NPtoxicity. As a result, the release of serum enzyme markers into the bloodstream was reduced. The reduced levels of the enzymes indicate a defense mechanism that protects the liver from the harmful effects of hepatotoxin. Additionally, it is plausible that administering the extract to rats along with 2-NP enhanced the rats antioxidant capacity when given with 2-nitropropane. Literature has revealed that rats co-treated with methanol extract of *Z. mauritiana* leaf before exposure to CCl4 toxicity showed reduced levels of blood AST, ALT, ALP, and total bilirubin (Rajopadhye & Upadhye, 2016).

Table 6. Effect of 2-NPand hydroethanolic seed extract of *Z. mauritiana* on both enzymatic and non-enzymatic indicators of liver injury**.**

Groups	ALT (IU/L)	AST(IU/L)	ALP(IU/L)	TB (mg/dl)
Control	145.4 ± 3.2	56.86 ± 2.5	160.0 ± 3.40	0.2860 ± 0.02
2-NP	338.5 ± 4.4	331.9 ± 6.8	444.3 ± 6.85	7.780 ± 0.18
Silymarin-50	187.6 ± 4.4	184.5 ± 4.4	254.8 ± 6.40	2.840 ± 0.19
ZMSE-200	246.6 ± 8.3	270.5 ± 2.5	379.5 ± 6.51	5.800 ± 0.21
ZMSE-400	204.8 ± 6.6	244.0 ± 3.6	303.3 ± 4.88	4.820 ± 0.19
ZMSE-800	178.2 ± 4.6	198.1 ± 4.03	280.0 ± 3.60	3.600 ± 0.38

The results of treatments on membrane bound enzymes such as cytochrome p-450 (CYP-450), UDP glucuronosyltransferase (UDPGT), Na+/K+-ATPase, and Ca+-ATPase are shown in table 6.

The level of CYP-450 in 2- nitropropane treated rats significantly decreased when compared to control rats at the end of 12 weeks. Silymarin treatment significantly ($P<0.01$) improve the level of tissue CYP-450, which has been decreased due to administration of 2-NP. Similarly, the level of UDPGT in 2- nitropropane treated rats significantly increases when compared to normal control rats at the end of 12 weeks. Silymarin treatment significantly ($P<0.01$) improve the level of tissue UDPGT, which has been decreased with the administration of 2-NP. Similar results were seen with the Na+/K+-ATPase, and Ca+-ATPase.

The administration of hydroethanolic extract of *Z. mauritiana* seed at doses of 200, 400, or 800mg/kg bw in rats resulted in a significant ($p<0.05$) dose-dependent improvement of membrane bound enzymes.

Hepatotoxicity is known to occur because of the activation or inhibition of CYP-p450 enzymes by drugs or chemicals. Cytochrome P-450 is an isoenzyme present in a supergene family of oxidases that include heme. It has a role in the initial phase of drug metabolism and is crucial for removing various foreign chemicals and natural molecules in liver cells. Studies have shown that hepatotoxicity leads to a reduction in cyt P-450 levels, which in turn affects the maintenance of homeostasis (Shin et al., 2018). In this study, it was observed that 2-NP decreased the activity of the CYP-p450 enzyme. However, when rats were treated with different doses (200, 400, or 800mg/kg bw) of the hydroethanolic extract of *Z. mauritiana* seed, there was a substantial ($p<0.05$) improvement in enzyme activity, which was depending on the dose administered.

UDP-glucuronosyltransferases (UGT) are enzymes involved in the glucuronidation process, which is a phase II metabolic response responsible for the detoxification and removal of several endogenous and exogenous compounds, including drugs and poisons. Glucuronidation is predominantly carried out in the liver, where UDP-glucuronic acid binds with molecules to augment their solubility in water and facilitate their excretion from the body. Hepatic damage can impair the functionality of UDP-glucuronosyltransferases, leading to a reduction in their enzymatic activity(Shin et al., 2018). In this study, it was revealed that the presence of 2-NPresulted in a decrease in the activity of UDP-glucuronosyltransferases. This drop can be attributed to the development of oxidative stress. Nevertheless, upon administering various doses (200, 400, or 800mg/kg bw) of the hydroethanolic extract derived from *Z. mauritiana* seed to rats, a significant ($p<0.05$) enhancement in enzyme activity was seen, with the extent of increase being dose dependent.

ATPases are membrane proteins that rely on thiol groups and phospholipids to preserve their structure and function. Peroxidation of membrane phospholipids not only changes the lipid environment and the structural and functional integrity of the cell membrane, but also impacts the functions of different enzymes located in the membrane, such as Na+K+-ATPase and Ca2+ ATPase (Singh et al., 2005). The Na+K+-ATPase pump facilitates the maintenance of osmotic balance and membrane potential in cells. The administration of paracetamol resulted in a decrease in the activity of Na+/K+-ATPase in plasma membranes, which is a characteristic feature of hepatotoxicity (Corcoran et al., 1987). In this study, it was observed that 2-NP caused a decrease in the activity of Na+/K+-ATPase and Ca2+ ATPase . However, when rats were treated with different doses (200, 400, or 800mg/kg bw) of a hydroethanolic extract from *Z. mauritiana* seed, a significant improvement in Na+/K+-ATPase and Ca2+ ATPase activity was observed. This improvement was found to be dependent on the dose administered. This demonstrates that the hydroethanolic extract of *Z. mauritiana* seed safeguards the Na+K+ and Ca2+ ATPase pump, enabling the preservation of osmotic equilibrium and membrane potential in hepatocytes. Lipid peroxidation can impact the functionality of Ca2+ and Mg2+-ATPases, as well as the activity of membrane Ca2+-translocase. The observed decline in the functioning of membrane-bound ATPases may also result from the depletion of protein-SH, because of heightened lipid peroxidative harm to cellular membranes (Yücebilgiç et al., 2003).

Table 6. Effect of 2-NP and hydroethanolic seed extract of *Z. mauritiana* on membrane bound enzymes.

Groups	CYP-450 (nmol/mg protein)	UDPGT (µmol/mg protein)	Na+/K+-ATPase (mM of Pi liberated/mg protein)	Ca+-ATPase (mM of Pi liberated/mg protein)
Control	1.48 ± 0.13	2.94 ± 0.29	2.7 ± 0.15	1.2 ± 0.20
2-NP	0.44 ± 0.10	1.12 ± 0.20	0.9 ± 0.07	0.7 ± 0.06
Silymarin-50	1.26 ± 0.15	2.82 ± 0.16	2.1 ± 0.24	1.0 ± 0.13
ZMSE-200	0.84 ± 0.12	1.72 ± 0.23	1.4 ± 0.06	0.8 ± 0.03
ZMSE-400	0.96 ± 0.08	2.32 ± 0.27	1.7 ± 0.04	0.8 ± 0.02
ZMSE-800	1.06 ± 0.09	2.54 ± 0.14	2.2 ± 0.05	0.9 ± 0.08

The rats that received 2-NP treatment displayed tissue damage, as indicated by decreased levels of tissue SOD, CAT, GPx, and diminished GSH, along with elevated levels of MDA, a biomarker for lipid peroxidation. Silymarin enhances the reduction in Superoxide Dismutase (SOD), Catalase (CAT), Glutathione Peroxidase (GPx), and diminished Glutathione (GSH), while simultaneously elevating levels of Malondialdehyde (MDA). Nevertheless, administration of varying doses (200, 400, or 800mg/kg bw) of the hydroethanolic extract from *Z. mauritiana* seeds to rats resulted in a significant ($p<0.05$) elevation in SOD, CAT, GPx, and a reduction in GSH levels, accompanied by an increase in MDA levels, which exhibited a dose-dependent pattern.

The superoxide dismutase (SOD) and catalase (CAT) enzymes act as scavengers, preserving cellular integrity by regulating cellular homeostasis and defending against oxidative stress. The Superoxide Dismutase (SOD) enzyme selectively eliminates and converts the superoxide anion (O2 −) into oxygen (O2) and hydrogen peroxide (H2O2). In addition, the ubiquitous Catalase (CAT) enzyme facilitates the generation of water and oxygen through the process of hydrogen peroxide neutralization. The GPx also plays a role in the elimination of H2O2, like CAT.

The reduced enzymatic antioxidant capabilities seen in fibrotic rats indicate a compromised ability and uncontrolled utilization in neutralizing the reactive oxygen species (ROS) produced by the chemical modification of 2-nitropropane. Furthermore, evaluating the LPO (lipid peroxidation) of unsaturated fatty acids is regarded as a definitive verification of oxidative stress resulting from the direct interaction between oxygen and lipids. Consequently, this process produces reactive intermediates and partially stable peroxides such as malondialdehyde (MDA). The elevated levels of MDA in the rats with fibrosis indicate an intensified process of lipid peroxidation, leading to liver damage and a compromised ability of the body to protect against oxidative stress. The observed increase in lipid peroxidation (LPO) levels corresponds to prior research findings, which were reported in the liver tissues of mice subjected to liver injury in experimental studies. However, administration of the hydroethanolic extract obtained from *Z. mauritiana* seeds orally to rats resulted in a significant improvement in the functioning of these enzymatic antioxidants. This repair demonstrates the extract's ability to remove reactive oxygen species (ROS) and hence prevent further damage caused by oxidative stress induced by 2-nitropropane.

The administration of 2-NP resulted in hepatic injury and a reduction in the levels of non-enzymatic antioxidants, specifically reduced glutathione (GSH), due to the generation of reactive oxygen species (ROS) and subsequent oxidative stress. The primary function of GSH is to suppress oxidative stress and counteract lipid peroxidation (LPO). Nevertheless, administration of the hydroethanolic extract obtained from *Z. mauritiana* seeds by oral route resulted in a noteworthy elevation in the levels of reduced glutathione (GSH) in rats, as compared to rats treated with 2-nitropropane. The test substances effectively elevated the level to near-normal, thereby confirming their ability to protect against and prevent oxidative stress caused by 2-nitropropane.

Table 7. Effect of 2-NP and hydroethanolic seed extract of *Z. mauritiana* on enzymatic and non-enzymatic oxidative stress markers.

Groups	SOD (U/mg protein)	CAT (U/mg protein)	GPx (nmol/min/mg protein)	Reduced GSH (nmol/mg protein)	MDA (nmol/mg protein)
Control	111.8 ± 4.0	36.7 ± 2.4	78.1 ± 2.0	11.8 ± 0.6	7.7 ± 0.2
2-NP	18.9 ± 1.5***	13.2 ± 0.5***	27.9 ± 1.8***	3.7 ± 0.2 ***	15.1 ± 0.5***
Silymarin-50	91.6 ± 4.2$^{###}$	25.8 ± 2.8$^{###}$	80.7 ± 2.8$^{###}$	10.0 ± 0.4$^{###}$	8.2 ± 0.4$^{###}$
ZMSE-200	42.7 ± 4.5	19.6 ± 0.8	38.0 ± 4.1	6.9 ± 0.2	11.7 ± 0.7
ZMSE-400	69.8 ± 2.7	22.9 ± 0.4	49.7 ± 2.8	8.7 ± 0.2	11.9 ± 0.6
ZMSE-800	82.8 ± 4.1	26.8 ± 1.6	70.9 ± 2.3	9.2 ± 0.3	8.9 ± 0.3

Oxidative stress and excessive inflammation are interrelated. The present study assessed TGF-β1, TNF-α, IL-6, IL-1β, and IL-10 as noteworthy inflammatory cytokines in plasma. The level of inflammatory cytokines in 2-NP exhibits a considerable rise when compared to the control rats. The elevation of inflammatory cytokines is a result of the enhancement of liver damage caused by the presence of 2-NP after 84 days. Compared to the control group, the presence of 2-NP resulted in a significant ($P<0.01$) rise in the levels of inflammatory cytokines such as TGF- β1, TNF-α, IL-1β, and IL-6, while simultaneously decreasing the levels of IL-10. This indicates that 2-NP induced a pronounced inflammatory response in the liver. The oral treatment of hydroethanolic extract derived from *Z. mauritiana* seeds considerably ($P<0.01$) reduced the synthesis of these proinflammatory components, which were elevated by the administration of 2-NP. The results indicate that

the hydroethanolic extract derived from *Z. mauritiana* seeds, when taken orally, effectively induced a reduction in inflammation caused by 2-NP. The latest study's findings align with the results of the previous studies.

The injection of 2-NP considerably increased the levels of TNF- β1 and TGF- α ($P<0.01$). However, silymarin efficiently lowered these raised levels. The intervention resulted in significant reductions ($P<0.01$) in IL-6 levels and a notable improvement ($P<0.01$) in IL-10 levels. The silymarin treatment groups exhibit reduced levels of inflammatory mediators compared to the 2-NP group. The results indicate that the hydroethanolic extract derived from *Z. mauritiana* seeds has the ability to potentially decrease liver fibrosis induced by 2-NP. This effect may be attributed to its anti-inflammatory capabilities.

Table 7. Effect of 2-NP and hydroethanolic seed extract of *Z. mauritiana* on inflammatory markers**.**

Groups	**TGF-β (ng-L^{-1})**	**TNF-α (ng-L^{-1})**	**IL-1β (ng-L^{-1})**	**IL-6 (ng-L^{-1})**	**IL-10 (ng-L^{-1})**
Control	32.87 ± 4.3	25.37 ± 4.0	3.465 ± 0.5	61.57 ± 1.8	134.8 ± 5.0
2-NP	209.2 ± 8.1	62.45 ± 3.3	16.16 ± 0.2	93.89 ± 2.9	54.85 ± 3.2
Silymarin-50	76.63 ± 3.1	25.40 ± 4.5	5.350 ± 0.8	70.29 ± 1.2	119.0 ± 2.6
ZMSE-200	157.0 ± 3.4	49.52 ± 2.9	9.475 ± 0.7	81.97 ± 0.8	79.15 ± 3.6
ZMSE-400	113.8 ± 6.9	34.24 ± 4.4	6.825 ± 0.6	65.98 ± 0.6	90.05 ± 3.1
ZMSE-800	89.84 ± 2.6	25.09 ± 2.2	5.150 ± 0.4	71.55 ± 0.8	100.0 ± 3.0

The liver can develop fibrous tissue due to an excessive buildup of extracellular matrix (ECM), where collagen is the main component of the ECM. The accumulation of collagen constituents is crucial in the progression of liver fibrosis. Afterwards, we evaluated collagen expression by measuring hepatic hydroxyproline levels, which acted as a marker of collagen synthesis in the rats with liver fibrosis.

The hepatic hydroxyproline concentration exhibited a significant increase in comparison to the control group, suggesting an excessive buildup of collagen in the liver. The graph in Figure 4 illustrates the concentration of hydroxyproline in the liver tissue.

Hydroxyproline is a prevalent amino acid that is abundantly present in collagen. It is formed by the process of hydroxylation of the proline component. Hydroxyproline, present in the extracellular matrix (ECM) produced by activated hepatic stellate cells (HSCs), preserves the structural and functional integrity of liver cells. The concentration of this factor in liver tissues is a crucial characteristic that precisely indicates the rates and progression of liver fibrogenesis. Quantifying hydroxyproline in different research initiatives serves as a diagnostic indicator or a method of monitoring the anti-fibrotic activity. The hydroethanolic extract derived from *Z. mauritiana* seeds exhibits the capacity to mitigate hepatic fibrosis induced by 2-NP through the reduction of hydroxyproline levels.

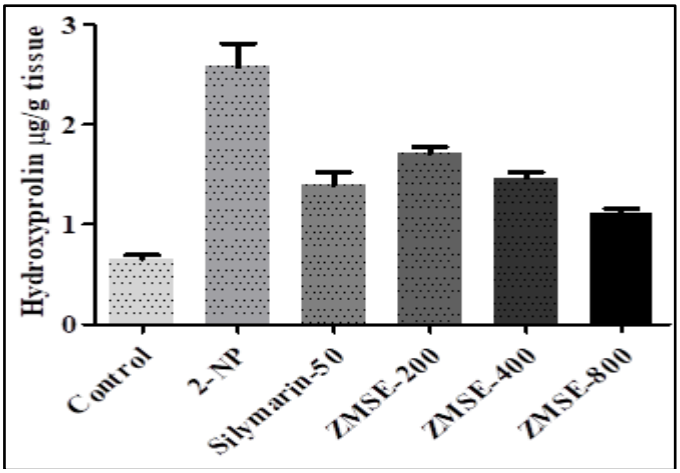

Figure 4: The estimation of hydroxyproline content At the end of the 12 week as an indicator of indirect collagen level estimation

Following the 12-week period, livers from different treatment groups were processed for histological study. Analysis of liver tissue stained with hematoxylin and eosin under a microscope revealed that the liver structure in control rats (Fig. 5A) was within the normal range. The liver's connective tissue clearly divides the hepatic parenchyma into distinct functioning hepatic lobules. The arrangement of hepatocytes in each

section of the liver lobule, especially in the middle and central areas, was not clearly defined. The plates are made up of liver cells that extend from the area around the portal to the central vein. No signs of liver necrosis, steatosis, inflammation, or fibrosis were found. The sinusoidal capillaries, which are blood vessels found between the layers of liver cells, appear to protrude from a small central vein. The region of Disse, situated between the sinusoidal lining cells and hepatocytes, comprises Küpffer cells, hepatic stellate cells, and pit cells (natural killer lymphocytes). The identification of portal areas, which include small arteries, veins, bile ducts, and lymphatic vessels, was less apparent.

Hepatic slices from rats treated with 2-NP (Fig. 5B) showed significant extensive damage to the liver structure. These abnormalities were identified by tissue damage with cell death in certain areas of the liver, presence of abnormal structures within dead liver cells, swelling and thickening of certain blood vessels, and excessive inflammation and scarring around the liver. Additionally, there were abnormal vacuoles within liver cells, clusters of enlarged immune cells and scavenger cells, enlarged liver cells responsible for storing fat, and noticeable differences in the size, shape, and appearance of liver cells.

Hepatic slices from rats treated with silymarin (Fig. 5C) appeared to be in good condition; the livers in this group exhibited less nodular alterations and a smoother surface compared to the control group. The liver changes were shown by decreased death of liver cells and swelling degeneration in central, middle, and periportal regions. Some dead liver cells contained pinkish hyaline inclusions. There was also increased blood flow and widening of the portal vein, inflammation around the portal areas, some small and large fluid-filled sacs inside the cells, enlarged Kupffer cells, and hepatic stellate cells in widened blood vessels. Indications of liver cell regrowth were also seen.

Different amounts (200, 400, or 800mg/kg bw) of the hydroethanolic extract from *Z. mauritiana* seeds significantly improved the structure of liver cells in the traditional liver lobules when compared to rats treated with 2-NP (Fig. 5D, 5E and 5F), although it was not as efficient as silymarin. The hydroethanolic extract from *Z. mauritiana* seeds (800mg/kg body weight) has a similar effect to silymarin (Figure 5F). The text states that the hydroethanolic extract from *Z. mauritiana* seeds shows action that varies depending on the dose.

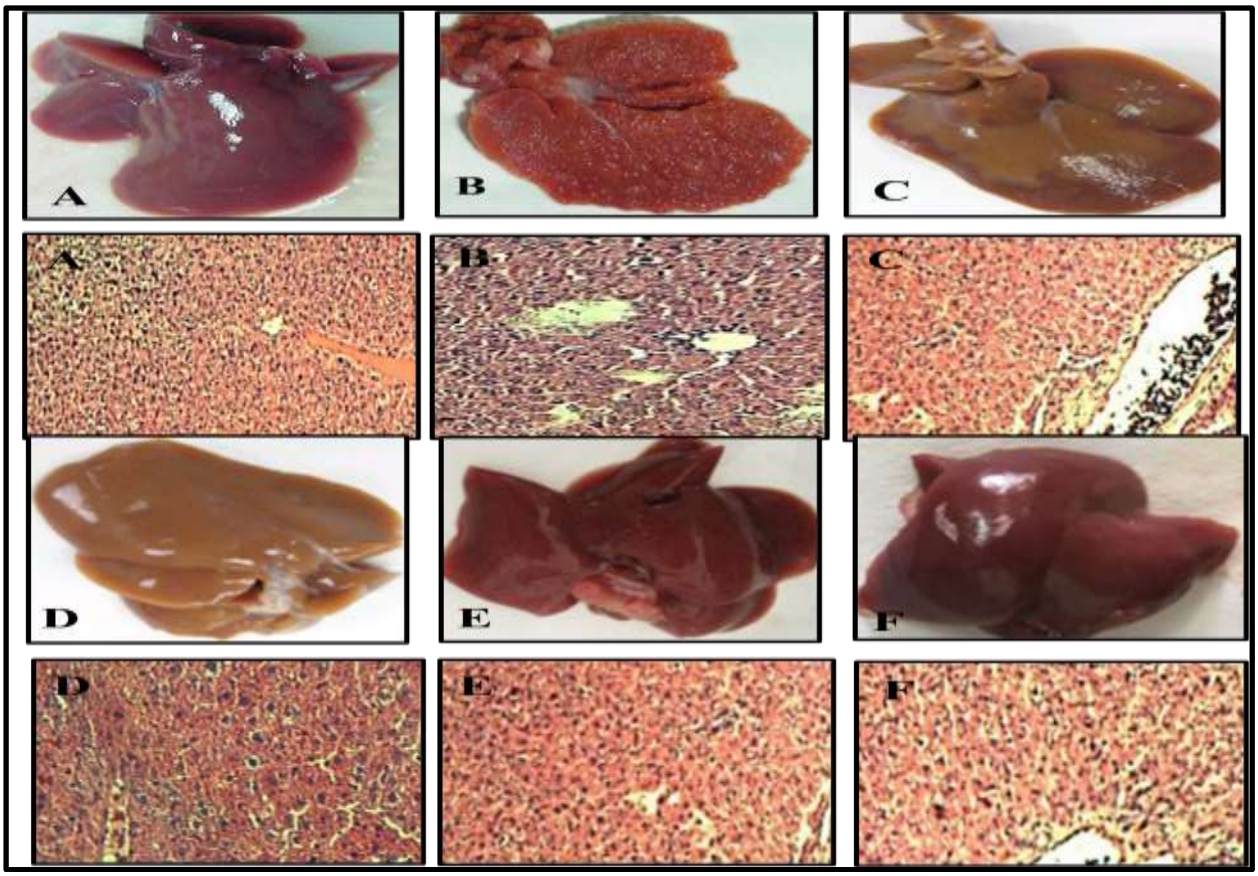

Figure 5: Hematoxylin and eosin-stained liver sections under the microscope (x100); A: Control Rats, B: 2-NP treated rats, C: Silymarin treated rats, D: ZMSE-200 treated rats, E: ZMSE-400 treated rats, F: ZMSE-800 treated rats.

Conclusion

Both silymarin and *Z. mauritiana* were effective in ameliorating 2-NP induced liver fibrosis and parenchymal changes in adult male albino rats. However, *Z. mauritiana* proved to be more effective when given to patients as Nutraceutical.

Conflict of interest: None

Funding: This work was personally supported.

Acknowledgements

The authors would like to thank Dr. Mandeep Kumar Singh, Assistant Professor from Amity institute of Pharmacy, Amity University Madhya Pradesh for his aid in statistical analysis.

Reference

1. Al-Afifi, N. A., Alabsi, A. M., Bakri, M. M., & Ramanathan, A. (2018). Acute and sub-acute oral toxicity of Dracaena cinnabari resin methanol extract in rats. *BMC Complementary and Alternative Medicine*, *18*(1), 1–14. https://doi.org/10.1186/s12906-018-2110-3
2. Alsayari, A., & Wahab, S. (2021). Genus Ziziphus for the treatment of chronic inflammatory diseases. *Saudi Journal of Biological Sciences*, *28*(12), 6897–6914. https://doi.org/10.1016/j.sjbs.2021.07.076
3. Aryal, S., Baniya, M. K., Danekhu, K., Kunwar, P., Gurung, R., & Koirala, N. (2019). Total Phenolic Content, Flavonoid Content and Antioxidant Potential of Wild Vegetables from Western Nepal. *Plants*, *8*(4). https://doi.org/10.3390/PLANTS8040096
4. Baliyan, S., Mukherjee, R., Priyadarshini, A., Vibhuti, A., Gupta, A., Pandey, R. P., & Chang, C. M. (2022). Determination of Antioxidants by DPPH Radical Scavenging Activity and Quantitative Phytochemical Analysis of Ficus religiosa. *Molecules*, *27*(4). https://doi.org/10.3390/MOLECULES27041326
5. Bataller, R., & Brenner, D. A. (2005). Liver fibrosis. *Journal of Clinical Investigation*, *115*(2), 209–218. https://doi.org/10.1172/JCI24282
6. Borges, L. P., Nogueira, C. W., Panatieri, R. B., Rocha, J. B. T., & Zeni, G. (2006). Acute liver damage induced by 2-NP in rats: effect of diphenyl diselenide on antioxidant defenses. *Chemico-Biological Interactions*, *160*(2), 99–107. https://doi.org/10.1016/J.CBI.2005.12.010
7. Casas-Grajales, S. (2015). Antioxidants in liver health. *World Journal of Gastrointestinal Pharmacology and Therapeutics*, *6*(3), 59. https://doi.org/10.4292/wjgpt.v6.i3.59
8. Corcoran, G. B., Chung, S. J., & Salazar, D. E. (1987). Early inhibition of the Na+/K+-ATPase ion pump during acetaminophen-induced hepatotoxicity in rat. *Biochemical and Biophysical Research Communications*, *149*(1), 203–207. https://doi.org/10.1016/0006-291X(87)91624-X
9. Dhar, D., Baglieri, J., Kisseleva, T., & Brenner, D. A. (2020). Mechanisms of liver fibrosis and its role in liver cancer. *Experimental Biology and Medicine*, *245*(2), 96–108. https://doi.org/10.1177/1535370219898141
10. Eugenia Letelier Muñoz, M., Jara, J., Iturra, P., & Faúndez, M. (2009). *Evaluation of the antioxidant properties and effects on the biotransformation of commercial herbal preparations using rat liver endoplasmic reticulum Antioxicant capacity of natural products View project Heteroaryl-acrylonitrile derivatives as inhibitors of NADPH Oxidases in an isoform-specific manner. View project*. http://redalyc.uaemex.mx/src/inicio/ArtPdfRed.jsp?iCve=85611769007
11. González-Palma, I., Escalona-Buendía, H. B., Ponce-Alquicira, E., Téllez-Téllez, M., Gupta, V. K., Díaz-Godínez, G., & Soriano-Santos, J. (2016). Evaluation of the antioxidant activity of aqueous and methanol extracts of Pleurotus ostreatus in different growth stages. *Frontiers in Microbiology*, *7*(JUL), 206086. https://doi.org/10.3389/FMICB.2016.01099/BIBTEX
12. Habig, W. H., Pabst, M. J., And, ~, & Jakoby, W. B. (1974). Glutathione S-Transferases THE FIRST ENZYMATIC STEP IN MERCAPTURIC ACID FORMATION*. *Journal of Biological Chemistry*, *249*(22), 7130–7139. https://doi.org/10.1016/S0021-9258(19)42083-8
13. James, S. L., Abate, D., Abate, K. H., Abay, S. M., Abbafati, C., Abbasi, N., Abbastabar, H., Abd-Allah, F., Abdela, J., Abdelalim, A., Abdollahpour, I., Abdulkader, R. S., Abebe, Z., Abera, S. F., Abil, O. Z., Abraha, H. N., Abu-Raddad, L. J., Abu-Rmeileh, N. M. E., Accrombessi, M. M. K., … Murray, C. J. L.

(2018). Global, regional, and national incidence, prevalence, and years lived with disability for 354 diseases and injuries for 195 countries and territories, 1990–2017: a systematic analysis for the Global Burden of Disease Study 2017. *The Lancet*, *392*(10159), 1789–1858. https://doi.org/10.1016/S0140-6736(18)32279-7

14. Koley, T. K., Kaur, C., Nagal, S., Walia, S., Jaggi, S., & Sarika. (2016). Antioxidant activity and phenolic content in genotypes of Indian jujube (Zizyphus mauritiana Lamk.). *Arabian Journal of Chemistry*, *9*, S1044–S1052. https://doi.org/10.1016/J.ARABJC.2011.11.005
15. Mahboubi, M., Kazempour, N., & Nazar, A. R. B. (2013). Total Phenolic, Total Flavonoids, Antioxidant and Antimicrobial Activities of Scrophularia Striata Boiss Extracts. *Jundishapur Journal of Natural Pharmaceutical Products*, *8*(1), 15. https://doi.org/10.5812/jjnpp.7621
16. MARKLUND, S., & MARKLUND, G. (1974). Involvement of the Superoxide Anion Radical in the Autoxidation of Pyrogallol and a Convenient Assay for Superoxide Dismutase. *European Journal of Biochemistry*, *47*(3), 469–474. https://doi.org/10.1111/J.1432-1033.1974.TB03714.X
17. Molole, G. J., Gure, A., & Abdissa, N. (2022). Determination of total phenolic content and antioxidant activity of Commiphora mollis (Oliv.) Engl. resin. *BMC Chemistry*, *16*(1), 1–11. https://doi.org/10.1186/S13065-022-00841-X/TABLES/2
18. Moron, M. S., Depierre, J. W., & Mannervik, B. (1979). Levels of glutathione, glutathione reductase and glutathione S-transferase activities in rat lung and liver. *Biochimica et Biophysica Acta (BBA) - General Subjects*, *582*(1), 67–78. https://doi.org/10.1016/0304-4165(79)90289-7
19. Ohkawa, H., Ohishi, N., & Yagi, K. (1979). Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Analytical Biochemistry*, *95*(2), 351–358. https://doi.org/10.1016/0003-2697(79)90738-3
20. Ortiz, C., Schierwagen, R., Schaefer, L., Klein, S., Trepat, X., & Trebicka, J. (2021). Extracellular Matrix Remodeling in Chronic Liver Disease. *Current Tissue Microenvironment Reports*, *2*(3), 41–52. https://doi.org/10.1007/s43152-021-00030-3
21. Owolarafe, T., Ihegboro, G., Salawu, K., Ononamadu, C., Fadilu, M., & Musa, B. (2022). Toxicological Investigation of Aqueous Extract of Ziziphus mauritiana Leaves on Wistar Rats. *International Journal of Traditional and Complementary Medicine Research IJTCMR*, *2*, 91–100. https://doi.org/10.53811/ijtcmr.1056770
22. Pariyani, R., Safinar Ismail, I., Azam, A. A., Abas, F., Shaari, K., & Sulaiman, M. R. (2015). Phytochemical screening and acute oral toxicity study of Java tea leaf extracts. *BioMed Research International*, *2015*. https://doi.org/10.1155/2015/742420
23. Qiu, B., Wei, F., Sun, X., Wang, X., Duan, B., Shi, C., Zhang, J., Zhang, J., Qiu, W., & Mu, W. (2014). Measurement of hydroxyproline in collagen with three different methods. *Molecular Medicine Reports*, *10*(2), 1157–1163. https://doi.org/10.3892/MMR.2014.2267/HTML
24. Rajapaksha, I. (2022). Liver Fibrosis, Liver Cancer, and Advances in Therapeutic Approaches. *Livers 2022, Vol. 2, Pages 372-386*, *2*(4), 372–386. https://doi.org/10.3390/LIVERS2040028
25. Rajopadhye, A., & Upadhye, A. S. (2016). Estimation of bioactive compound, maslinic acid by HPTLC, and evaluation of hepatoprotective activity on fruit pulp of Ziziphus jujuba Mill. Cultivars in India. *Evidence-Based Complementary and Alternative Medicine*, *2016*. https://doi.org/10.1155/2016/4758734
26. Ramar, M. K., Chidambaram, K., Chandrasekaran, B., & Kandasamy, R. (2022). Standardization, in-silico and in-vivo safety assessment of methanol extract of Ziziphus mauritiana Lam leaves. *Regulatory Toxicology and Pharmacology : RTP*, *131*. https://doi.org/10.1016/J.YRTPH.2022.105144
27. Rotruck, J. T., Pope, A. L., Ganther, H. E., Swanson, A. B., Hafeman, D. G., & Hoekstra, W. G. (1973). Selenium: Biochemical Role as a Component of Glutathione Peroxidase. *S*00073-8
28. Sakna, S. T., Maghraby, Y. R., Abdelfattah, M. S., & Farag, M. A. (2022). Phytochemical diversity and pharmacological effects of triterpenes from genus Ziziphus: a comprehensive review. *Phytochemistry Reviews*. https://doi.org/10.1007/s11101-022-09835-y
29. Sarkar, P. K. (2002). A Quick Assay for Na+-K+-ATPase Specific Activity. *Zeitschrift Fur Naturforschung - Section C Journal of Biosciences*, *57*(5–6), 562–564. https://doi.org/10.1515/znc-2002-5-628
30. Shaheen, S., Arafah, M. M., Alshanwani, A. R., Fadda, L. M., Alhusaini, A. M., Ali, H. M., Hasan, I. H., Hagar, H., Alharbi, F. M. B., & AlHarth *cience*, *179*(4073), 588–590. https://doi.org/10.1126/SCIENCE.179.4073.588
31. Sai, K., Kai, S., Umemura, T., Tanimura, A., Hasegawa, R., Inoue, T., & Kurokawa, Y. (1998). Protective Effects of Green Tea on Hepatotoxicity, Oxidative DNA Damage and Cell Proliferation in the Rat Liver Induced by Repeated Oral Administration of 2-Nitropropane. *Food and Chemical Toxicology*, *36*(12), 1043–1051. https://doi.org/10.1016/S0278-6915(98)

32. ii, A. (2021). Chitosan nanoparticles as a promising candidate for liver injury induced by 2-nitropropane: Implications of P53, iNOS, VEGF, PCNA, and CD68 pathways. *Science Progress*, *104*(2), 1–19. https://doi.org/10.1177/00368504211011839
33. Sharifi-Rad, M., Anil Kumar, N. v., Zucca, P., Varoni, E. M., Dini, L., Panzarini, E., Rajkovic, J., Tsouh Fokou, P. V., Azzini, E., Peluso, I., Prakash Mishra, A., Nigam, M., el Rayess, Y., Beyrouthy, M. el, Polito, L., Iriti, M., Martins, N., Martorell, M., Docea, A. O., … Sharifi-Rad, J. (2020). Lifestyle, Oxidative Stress, and Antioxidants: Back and Forth in the Pathophysiology of Chronic Diseases. *Frontiers in Physiology*, *11*. https://doi.org/10.3389/fphys.2020.00694
34. Shin, D. S., Seo, H., Yang, J. Y., Joo, J., Im, S. H., Kim, S. S., Kim, S. K., & Bae, M. A. (2018). Quantitative Evaluation of Cytochrome P450 3A4 Inhibition and Hepatotoxicity in HepaRG 3-D Spheroids. *International Journal of Toxicology*, *37*(5), 393–403. https://doi.org/10.1177/1091581818780149/ASSET/IMAGES/LARGE/10.1177_1091581818780149-FIG7.JPEG
35. Singh, S., Mehrotra, S., Pandey, R., & Sandhir, R. (2005). Hepatotoxic effects of tert-butyl hydroperoxide (t-BHP) and protection by antioxidants. *Indian Journal of Experimental Biology*, *43*(8), 728–731.
36. Sinha, A. K. (1972). Colorimetric assay of catalase. *Analytical Biochemistry*, *47*(2), 389–394. https://doi.org/10.1016/0003-2697(72)90132-7
37. Tan, Y., Cheong, M. S., & Cheang, W. S. (2022). Roles of Reactive Oxygen Species in Vascular Complications of Diabetes: Therapeutic Properties of Medicinal Plants and Food. *Oxygen*, *2*(3), 246–268. https://doi.org/10.3390/oxygen2030018
38. Trauner, M., & Fuchs, C. D. (2022). Novel therapeutic targets for cholestatic and fatty liver disease. *Gut*, *71*(1), 194–209. https://doi.org/10.1136/gutjnl-2021-324305
39. Ullah, A., Munir, S., Badshah, S. L., Khan, N., Ghani, L., Poulson, B. G., Emwas, A.-H., & Jaremko, M. (2020). Important Flavonoids and Their Role as a Therapeutic Agent. *Molecules*, *25*(22), 5243. https://doi.org/10.3390/molecules25225243
40. Xu, J.-H., Yu, Y.-Y., & Xu, X.-Y. (2020). Management of chronic liver diseases and cirrhosis: current status and future directions. *Chinese Medical Journal*, *133*(22), 2647–2649. https://doi.org/10.1097/CM9.0000000000001084
41. Yücebilgiç, G., Bilgin, R., Tamer, L., & Tükel, S. (2003). Effects of lead on Na+-K+ ATPase and Ca+2 ATPase activities and lipid peroxidation in blood of workers. *International Journal of Toxicology*, *22*(2), 95–97. https://doi.org/10.1080/10915810305096