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Research Article

Dietary Supplementation of Lotus (*Nelumbo nucifera* Gaertn.) Extract Alleviates Liver Injury Induced by 2-Methoxyethanol in Mice Model

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Abstract
2-Methoxyethanol (2-ME), a phthalic acid ester widely used in the plastics industry, can cause liver injury. Natural plant substances with antioxidant properties are promising candidates for alleviating such injury. While hepatoprotective effects of *Nelumbo nucifera* seeds and leaves have been documented, the potential of *N. nucifera* flower extract against 2-ME-induced hepatotoxicity remains unexplored. This study investigated the hepatoprotective effects of *N. nucifera* flower extract on male Balb/C mice exposed to 2-ME and identified its secondary compounds. The study design included five groups: normal control (CN) group, positive control (CP) group receiving 2-ME only for 7 days, and treatment groups receiving 2-ME for 7 days followed by *N. nucifera* flower extract at 50 (low), 150 (moderate), and 450 (high) mg/kg body weight (bw) for 28 days. Liver histology and biochemistry parameters results revealed that the *N. nucifera* flower extract significantly ($p < 0.05$) mitigated hepatocyte damage caused by 2-ME by decreasing the levels of liver enzyme (AST, ALT, and ALP), oxidative stress (MDA), and cytokine pro inflammatory (IL-6 and TNF-α). The middle and high dose provided good protection for liver injury against 2-ME. Spearman correlation analysis showed strong positive correlations between MDA, IL-6, TNF-α levels and liver histopathological scores ($r > 0.8$; $p < 0.05$), suggesting that the reduction in oxidative and inflammatory markers is closely associated with decreased hepatocellular damage. These protective effects are attributed to the antioxidant activity (IC_{50} = 63.42 ppm) and active compounds in *N. nucifera* flower extract, which include cycloartenol acetate, hexadecanoic acid, 6beta-bicyclo(4.3.0)nonane, delta-guaiene, 24-norursa-3,12-diene, campesterol, lupeol, stearic acid, gamma-sitostenone, and 3-methylphenol. *In silico* analysis showed that 24-norursa-3,12-diene has a high binding afinity (-8.2 kcal/mol) with prekursor of cytokine proinflammatory (NF-κB). Further studies are recommended to confirm the optimal dosage and elucidate the underlying mechanisms.

Keywords: dietary supplementation, *Nelumbo nucifera*, antioxidant, liver injury, 2-methoxyethanol

1. INTRODUCTION

2-Methoxyethanol (2-ME) is a phthalic acid ester used in the plastic industry. The 2-ME contains phthalic acid esters, which are widely used in the industry due to their colorless and transparent properties, making them excellent solvents, and as plasticizers, they improve the flexibility of other plastic materials [1]. Items that contain 2-ME can increase the risk of absorption or inhalation by the human body. The body oxidizes 2-ME using the enzyme alcohol dehydrogenase to produce 2-methoxyacetaldehyde (2-MALD), which is further metabolized into a toxic and teratogenic compound called methoxyacetic acid (MAA). Reports suggest that MAA can damage liver tissues, spermatotoxicity, and cause fetal defects [2][3].

The liver plays a central role in detoxification and metabolism, as well as the production and storage of proteins, lipids, carbohydrates, and vitamins [4][5]. Furthermore, 2-MALD induces toxicity that has the potential to activate lipid peroxidation in hepatocytes, leading to the overproduction of reactive oxygen species (ROS). This increase in ROS levels may harm the liver by affecting oxidative metabolism and lowering membrane fluidity [6]. The increase in ROS can be marked by an increase in malondialdehyde (MDA) levels, which is a byproduct of ROS production. Moreover, excessive ROS levels in the cells may trigger apoptosis and necrotic, partly due to the overexpression of proinflammatory cytokines like interleukin-6 (IL-6), interleukin-1 (IL-1), and tumor necrotic factor-alpha (TNF-α), which serve as early markers [7].

Biochemical markers such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) are frequently used to assess liver function [8]. The

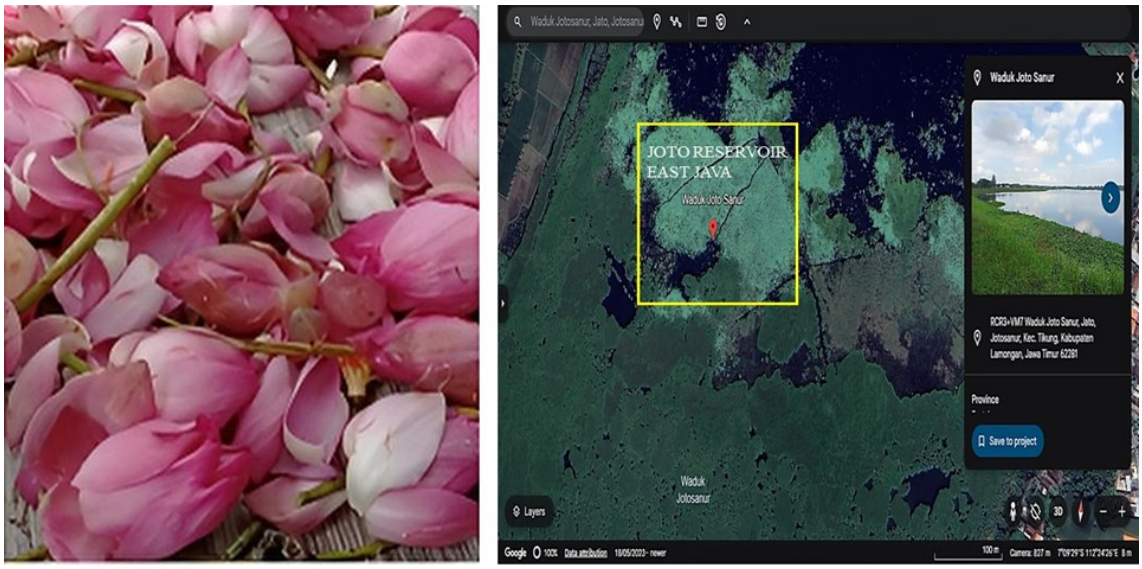


Figure 1. The (a) petals of *Nelumbo nucifera* and the (b) location of the plant in Joto Reservoir, Lamongan District, East Java, Indonesia based on Google Earth.

ALT and AST are enzymes within liver cells, and the rise of these enzymes in the circulation indicates liver injury, as they are released from damaged cells. The ALP is involved in the function of the bile ducts and metabolism of bones, and higher concentrations are observed in liver diseases such as hepatitis, bile duct injury, or cirrhosis [9]. Regularly monitoring these markers reveals important information regarding liver health and the extent of damage inflicted by various toxins, including 2-ME. Thus, resolving liver damage due to 2-ME toxicity requires natural substances from plants with antioxidant properties.

Lotus (*Nelumbo nucifera*) is an aquatic plant commonly found throughout Asia, including India, China, Vietnam, Japan, Thailand, and Indonesia. This plant is extensively used in traditional medicine for its flowers as spermatoprotective [10], leaves as anti-inflammatory [11], seeds as antidiabetic [12], and roots as antiobesity [13]. Besides that, its prized for its antibacterial, anti-inflammatory, antioxidant, and pain-relief properties [14]. Because of these properties, the use of *Nelumbo nucifera* as a dietary supplement has expanded. The *N. nucifera* extracts, particularly from flowers, contain secondary metabolite compound such as tannins, alkaloids, flavonoids, terpenoids, steroids, glycosides, coumarins, and quinones, which are believed to have hepatoprotective effects [15]. In addition, *N. nucifera* flower contains secondary metabolites such as quercetin, kaempferol, lupeol, and nuciferine, which are known for their activity in reducing free radicals [16].

However, there has been no comprehensive study exploring the hepatoprotective potential of phytocompounds derived from *N. nucifera* flowers using a combined approach involving *in vitro*, *in vivo*, and *in silico* methods against 2-ME. Among these, *in silico* approach has gained prominence in drug discovery due to its cost-effectiveness and time efficiency. Specifically, molecular docking plays a key role by predicting the binding orientation and position of candidate compounds within the active site of target proteins, offering valuable insights into their potential as therapeutic agents [17]. In this study, NF-κB was selected as the target protein due to its critical role as a transcription factor that regulates the expression of pro-inflammatory cytokines. Thus, its inhibition may offer valuable insights into the anti-inflammatory potential of *N. nucifera* flower extract, particularly regarding its hepatoprotective properties. This study aims to investigate the therapeutic potential of *N. nucifera* flower extract by performing phytochemical screening and GC-MS-based profiling of its secondary metabolites. Additionally, we aimed to evaluate its activity in

repairing hepatocyte damage and restoring liver function caused by 2-ME exposure, as well as to determine the optimal dosage through both in vivo and in silico approaches. The identification of bioactive secondary metabolites and the confirmation of their hepatoprotective activity through both experimental and computational methods provide a comprehensive foundation for future drug development based on *N. nucifera.*

2. MATERIALS AND METHODS

2.1. Materials and Chemicals

The plant material (*N. nucifera*) was taxonomically identified by Heri Santoso, S. Si, a certified taxonomist affiliated with the Generasi Biologi Indonesia Foundation, Indonesia (certificate number: BT.08.2482). Ethanol (96%, p.a., Merck Millipore, Darmstadt, Germany), Whatman No. 1 filter paper (GE Healthcare Life Sciences, UK), rotary evaporator (Büchi Rotavapor R-300, Switzerland), and 2-ME (Sigma-Aldrich, St. Louis, MO, USA) were used in this work. Neutral buffered formalin (NBF, 10%), hematoxylin-eosin, aquadest, ethanol, methanol, and xylene, along with paraffin pastilles were purchased from (E. Merck, Germany), while reagent kits for ALT, AST, and ALP assays were purchased from Glory Diagnostics (Linear Chemicals, SLU., Montgat, Barcelona, Spain). Enzyme-linked immunosorbent assay (ELISA) kit were used for cytokine pro inflammatory and oxidative stress analysis: IL-6: Mouse IL-6 ELISA Kit (E0049Mo, BT-Lab, China), TNF-α: Mouse TNF-α ELISA Kit (E0117Mo, BT-Lab, China), MDA: Rat MDA ELISA Kit (E0156Ra, BT-Lab, China). Microplate reader (Thermo Scientific Waltham, Massachusetts, AS), UV-Vis spectrophotometer (DLAB UV-VIS model SP-UV1000, Qingdao, China), and 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich St. Louis, MO, USA) were also purchased. Reagents for qualitative phytochemical screening tests (Mayer, aluminum chloride ($AlCl_3$), ferric chloride ($FeCl_3$), Salkowski, Liebermann-Burchard, and sodium hydroxide (NaOH), all other chemicals and solvents employed in this study were of analytical grade and used without further purification.

2.2. Methods

2.2.1. Study Area

The flowers of *N. nucifera*, specifically the petals (Figure 1(a)), were collected from the Joto Reservoir in Lamongan, East Java, Indonesia (geographical coordinates: 7°09'27"S 112°24'19"E (Figure 1(b)).

2.2.2. Animal and Ethical Approval

This study employed an *in vivo* experimental laboratory design using male BALB/c mice (*Mus musculus*), aged 6–8 weeks and weighing approximately 25 g, were obtained from the Pusat Veteriner Farma, Surabaya, Indonesia. Prior to

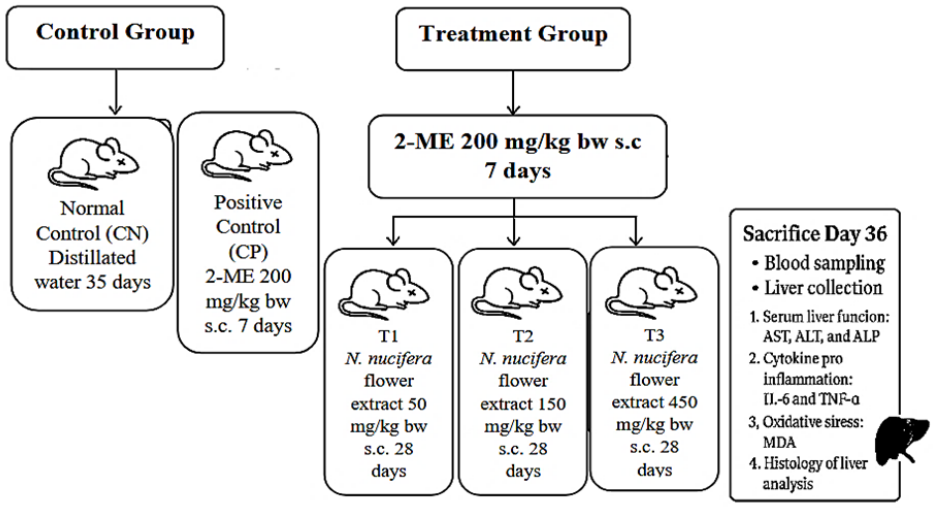


Figure 2. Experimental design of the research employing control (CN, CP) and treatment (T1, T2, T3) groups.

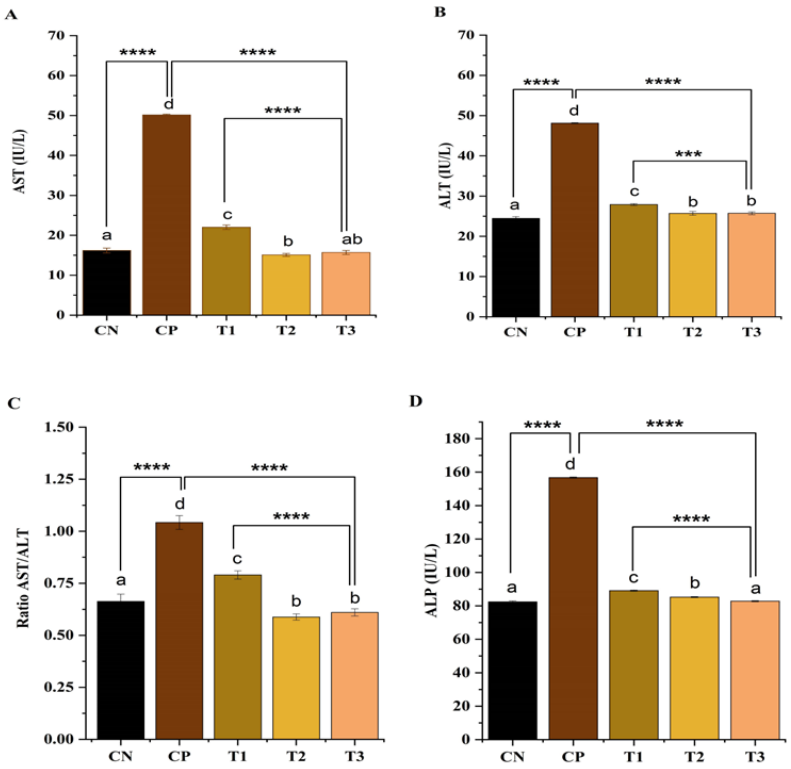


Figure 3. Effect of *Nelumbo nucifera* flower extract following 2-ME induction on biochemical of liver function, including (a) AST, (b) ALT, (c) ALP enzyme, and (d) ratio AST/ALT ratio levels. Data are expressed as mean ± standard deviation. Statistically significant differences between treatment groups are indicated by different letters (one-way ANOVA followed by Tukey's post hoc test) **** $p < 0.0001$; *** $p < 0.001$.

treatment, all mices underwent a 7-day acclimatization period under standard laboratory conditions, with access to commercial feed and water provided ad libitum. All procedures were approved by the Ethical Clearance Commission of Brawijaya University, East Java, Indonesia with an approval number of 120-KEP-UB-2024.

2.2.3. In Vivo Experimental Design

The experimental design followed a post-test only control group model. A total of 25 mices were randomly divided into five groups (n = 5 per group). In this study, both of 2-ME and *N. nucifera* flower extract were administered via subcutaneous injection. This route was selected to ensure consistent absorption and to minimize first-pass metabolism in the gastrointestinal tract [18], especially for the short exposure duration of 7 days for 2-ME. The subcutaneous injection was also selected to minimize variability and dosing errors associated with oral administration in mice. Oral gavage can result in inconsistent dosing due to regurgitation or incomplete swallowing, which may affect the reliability of the hepatoprotective assessment. Although subcutaneous (SC) administration is not the most common route for hepatotoxicity studies, it provided a technically manageable and reproducible method under our experimental conditions.

The variation dosages of *N. nucifera* flower extract used in this study (50, 150, and 450 mg/kg bw) are based on previous research that compares the biological effects of this extract with similar dosages in the reproductive system [10]. Although there has been no specific report regarding hepatoprotective activity at these doses, a previous toxicology study [19] showed that lotus flower extract has an lethal dose (LD_{50}) of more than 5000 mg/kg bw in Sprague-Dawley rats and a 90-day subchronic toxicity study at a daily dose of 200 mg/kg bw found no significant differences in body weight, hematological parameters nor organ histopathology between the treatment and control

groups. Therefore, the dose used in this study is within the range that is considered safe. However, further toxicity evaluation will be conducted in future studies to ensure the safety of long-term use.

The positive control group (CP) received a subcutaneous injection of 0.05 mL of 2-ME at a dose of 200 mg/kg body weight (bw) for 7 days, while the normal control group (CN) received only distilled water for 35 days. Following a seven-day subcutaneous injection of 0.05 mL of 2-ME at a dose of 200 mg/kg bw, the three treatment groups received 0.2 mL of *N. nucifera* flower extract subcutaneously for 28 days at three different doses low (50 mg/kg bw; T1), moderate (150 mg/kg bw; T2); and high (450 mg/kg bw; T3). On the 36 day, all mice were sacrificed using kloroform by inhalation technique, the blood were collected from cardiac vein, then the blood was centrifuge to collect the serum for liver function analysis. The liver organ was collected and weighed, for histological and biochemical anaylsis (Figure 2).

2.2.4. Extraction of N. nucifera Flower

The ethanol extract of *N. nucifera* flower was prepared by cutting the petals, drying them at 40 °C for 1 h, and grinding them into a fine powder. The powder was soaked in 96% ethanol at a 1:10 (w/v) ratio for 3 days at room temperature, with occasional stirring. After maceration, the mixture was filtered using Whatman No. 1 filter paper. The residue was re-macerated twice under the same conditions. All filtrates were pooled and evaporated under reduced pressure at 40 °C using a rotary evaporator. The resulting extract was then stored in a sealed container at 4 °C for subsequent analysis.

2.2.5. Biochemical Parameters

2.2.5.1. Measurement of Biochemical Tests for Liver Functions

Serum levels of AST, ALT, and ALP were analyzed using commercial diagnostic reagent kits (Glory Diagnostics®), according to the manufacturer's instructions. With amount of 100 µL serum sample were mix with 1000 µL reagent of AST, ALT, and ALP. Then incubate one minutes in room temperature. Enzymatic activities were measured spectrophotometrically using a spectrophotometer UV-VIS at the recommended wavelengths (ALT and AST at 340 nm; ALP at 405 nm). All assays were conducted in duplicate to ensure reproducibility. The activities of ALT, AST, and ALP were calculated using the following kinetic Equation 1;

$$\text{Enzyme activity (IU/L)} = \Delta A/\text{min} \times \text{Factor} \quad (1)$$

where, ΔA/min is the average change in absorbance per 1 min, calculated from readings at 1 -minute intervals over a 3-min period. Factor is a kit-specific constant derived from the Beer-Lambert law and provided by the manufacturer. It is based

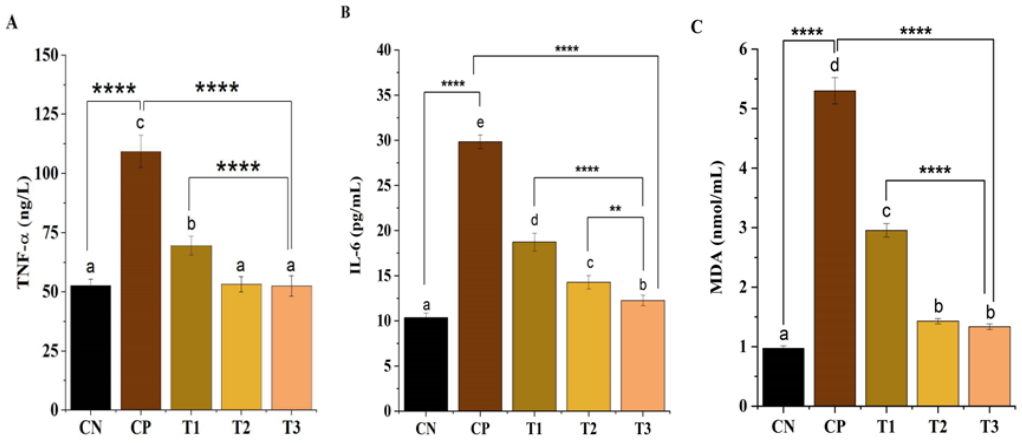


Figure 4. Effect of *Nelumbo nucifera* flower extract following 2-ME induction on biochemical of cytokine inflammatory and stress oxidative, i.e., (a) IL-6, (b) TNF-α, and (c) MDA. Data are expressed as mean ± standard deviation. Statistically significant differences between treatment groups are indicated by different letters (one-way ANOVA followed by Tukey's post hoc test) ****$p < 0.0001$; **$p < 0.01$.

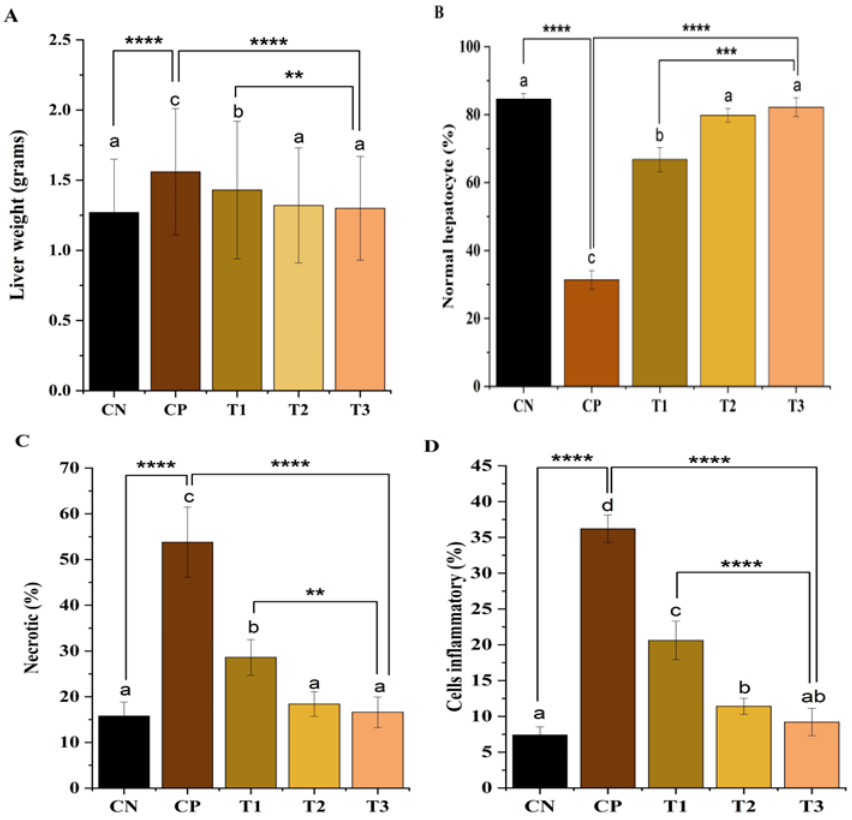


Figure 5. Effect of *Nelumbo nucifera* flower extract following 2-ME induction on (a) liver weight, (b) normal hepatocyte, (c) necrotic, and (d) cell inflammatory. Data are expressed as mean ± standard deviation. Statistically significant differences between treatment groups are indicated by different letters (one-way ANOVA followed by Tukey's post hoc test) ****p < 0.0001; ***p < 0.001; **p < 0.01.

on the reaction conditions, including cuvette path length (1 cm), reaction volume, and the molar absorptivity (ε) of the chromogenic molecule. For ALT and AST, the factor is 1746, based on the oxidation of NADH at 340 nm (ε = 6.22 mM^{-1} cm^{-1}). For ALP, the factor is 2754, based on the formation of *p*-nitrophenol at 405 nm (ε = 18.75 mM^{-1} cm^{-1}). These constants allow the conversion of the observed absorbance change into international enzyme activity units (IU/L), where one unit is defined as the amount of enzyme that catalyzes the conversion of 1 µmol of substrate per minute under the assay conditions [20].

2.2.5.2. Measurement of Biochemical Tests for Cytokine Pro-inflammatory and Oxidative Stress

The concentrations of cytokine pro-inflammatory (IL-6, TNF-α), and oxidative stress (MDA) in liver tissue were determined using the ELISA procedure. Liver homogenates were prepared by grinding tissue in 50 mM phosphate-buffered saline (PBS, pH 7.4) at a 1:4 ratio under cold conditions. The homogenate was centrifuged at 3,000 rpm for 15 min at 4 °C, and the supernatant was collected for further analysis. Quantification was conducted using commercially available ELISA kits, following the protocols provided by the manufacturer. In brief, 50–100 µL of standards, controls, and sample supernatants were pipetted into 48-well ELISA plates pre-coated with specific capture antibodies. Plates were incubated at 37 °C for 60–90 min, followed by multiple washes with the provided buffer. Biotin-conjugated detection antibodies were then added, followed by another incubation and washing step. Subsequently, horseradish peroxidase (HRP)-labeled streptavidin was introduced and incubated. A TMB substrate solution was added and allowed to develop in the dark for 10–20 min. The enzymatic reaction was stopped using a stop solution, and absorbance was measured at 450 nm with a microplate reader. Standard curves were generated from the known standards, and sample concentrations were calculated automatically based on absorbance values.

2.2.6. Histological Parameters

2.2.6.1. Measurement the Percentage of Normal, Necrosis, and Cell Inflammatory

Histological preparations of hepatic tissue were obtained by first fixing the hepatic organs in 10% NBF solution for 24–48 h, followed by routine paraffin embedding. Tissue sections of 4–5 µm thickness were prepared using a rotary microtome and mounted onto glass slides. Staining was performed using hematoxylin and eosin (H&E) to evaluate liver architecture and cellular integrity. Histological analysis focused on examining hydropic degeneration and hepatic necrotic using semi-quantitative methods [20]. The number of hepatocytes in hepatic histology preparations was counted using a digital counter in 100 hepatocytes randomly, under a light binocular miscroscope (Sinher XSZ-107, China) at 40× magnification, and the percentage of normal hepatocytes, necrotic hepatocytes, and inflammatory cells were calculated. Normal hepatocytes are cells with clearly visible nuclei, without vacuolization, clear cytoplasm, and normochromatic nuclei. Necrotic hepatocytes with marked changes in the cell nucleus and protrusion of the plasma membrane. Inflammatory cells was counted by the number of polimorfonuclear (PMN). The Equation 2 to count the percentage of normal, necrotic, and inflammatory cell.

$$\text{Percentage of Normal, Necrotic, and Inflamantory Cell (\%)} = \frac{\text{number of normal hepatocyte, necrotic, inflammatory cells}}{\text{100 hepatocytes}} \times 100\% \quad (2)$$

Then, the results of the biochemical parameters, including AST, ALT, ALP, IL-6, TNF-α, MDA and histological parameters, including percentage of normal hepatocytes, necrotic, and cell inflammatory were analysis for each correlation using spearman test.

2.2.7. Antioxidant Activity and Phytochemical Analysis of Nelumbo Nucifera Flower

2.2.7.1. Antioxidant Activity

The method for evaluated antioxidant activity in *N. nucifera* flower ethanolic extract using DPPH test as outlined by Astutiningsih et al. [21]. Ethanolic extracts of the flower were prepared at concentrations of 20, 40, 60, 80, and 100 ppm. Absorbance measurements were taken at 517 nm with a spectrophotometer, and the percentage of radical scavenging activity was determined using

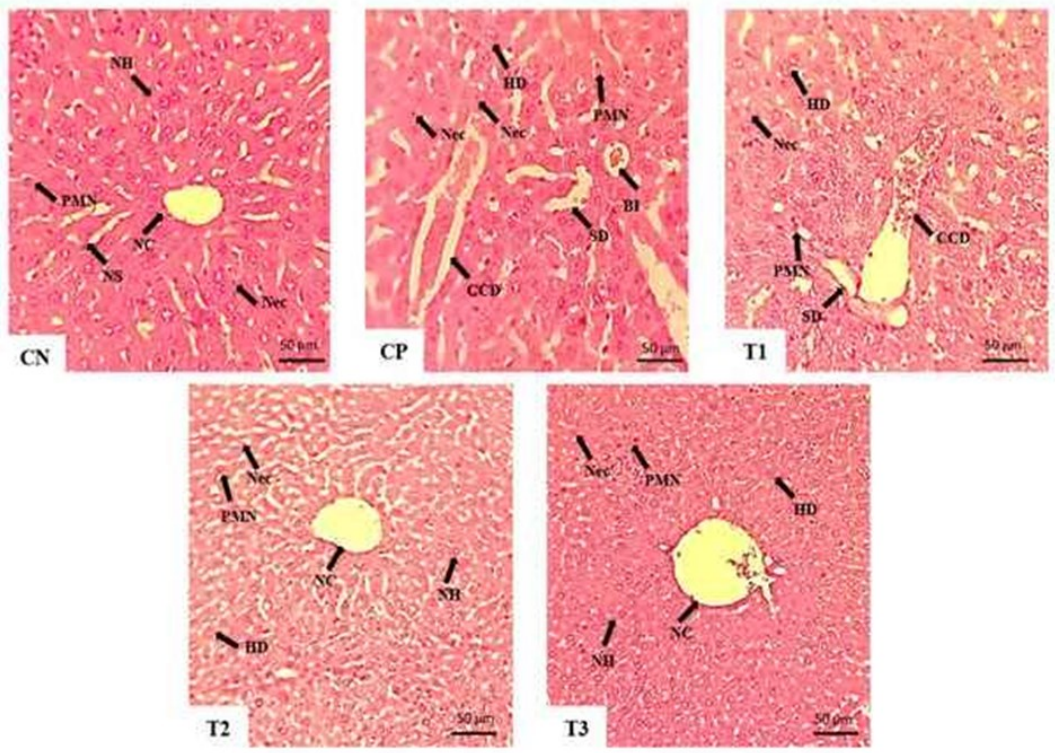


Figure 6. Histopathology results of the liver with HE staining at 40× magnification. Scale bar: 50 µm.

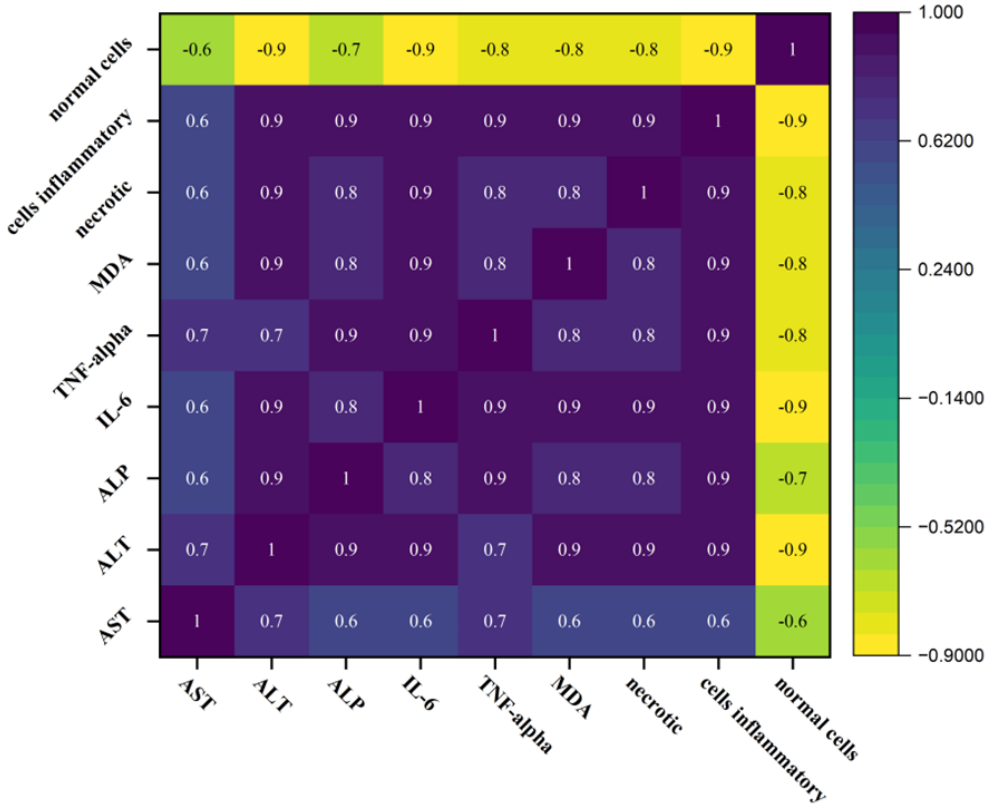


Figure 7. The relationship between biochemical parameters (AST, ALT, ALP, IL-6, TNF-alpha, and MDA) and histological parameters (necrotic, cells inflammatory, and normal cells) using the Spearman test. A negative correlation (value range -1.0 to -0.6) (indicated by the green color spectrum). Meanwhile, a positive correlation (value range 1.0–0.6) (indicated by the blue color spectrum). (2 tailed test of significance is used ($p < 0,05$). Note: p-values corresponding to each Spearman correlation coefficient are presented in Table 1 for validation of statistical significance.

the Equation 3.

$$\text{Inhibition (\%)} = \frac{(\text{Absorbance of control} - \text{Absorbance of sample})}{\text{Absorbance of control}} \times 100\% \quad (3)$$

The IC_{50} value was then determined by plotting a linear regression between extract concentrations (x-axis, ppm) and their corresponding inhibition percentages (y-axis), based on the equation $y = ax + b$. Ascorbic acid was used as the reference standard for comparison.

2.2.7.2. Phytochemical Analysis

2.2.7.2.1. Qualitative Phytochemical Screening of Secondary Metabolite

The ethanolic extract of *N. nucifera* flower underwent preliminary qualitative phytochemical analysis to identify key groups of secondary metabolite compounds, including alkaloids, phenols, flavonoids, steroids, terpenoids, and tannins [22]. The screening procedures followed the methods outlined by Khedr et al. [23], with slight modifications. For each test, 2 mL of the extract was reacted with specific reagents under controlled laboratory conditions using the following protocols test.

Alkaloids were identified using Mayer's and Wagner's reagents, where the formation of a cream-colored precipitate (Mayer's) or reddish-brown precipitate (Wagner's) signified a positive result. Phenolic compounds were detected by adding 1 mL of 5% $FeCl_3$ solution, which produced a deep blue or green color. Flavonoids were assessed via the alkaline reagent (NaOH) test, where an intense yellow color that faded upon adding dilute HCl indicated their presence. Steroids were tested using the Liebermann–Burchard reaction, with a green or blue color confirming their presence. Terpenoids were detected using the Salkowski test, where a reddish-brown layer at the interface indicated a

positive result. Tannins were evaluated by adding a few drops of 1% ferric chloride solution, producing a blue-black or greenish-black color if tannins were present. All tests were conducted in triplicate. The presence or absence of each compound class was determined based on characteristic visual indicators (such as precipitate formation or color change), though no quantitative or intensity measurements were recorded. Results were reported qualitatively as either positive (+) or negative (–).

2.2.7.2.2. GC-MS based Profiling of Secondary Metabolite

The chemical composition of secondary metabolite in *N. nucifera* flowers crude extract was analyzed using gas chromatography–mass spectrometry (GC-MS) on a Perkin-Elmer Clarus 500 system equipped with an AOC-20i auto-sampler. The analysis was performed using a capillary column HP-5MS (30 m length × 0.25 mm internal diameter × 0.25 µm film thickness). Helium was used as the carrier gas at a constant flow rate of 1.0 mL/min. The injection volume was 1 µL with a split ratio of 10:1. The GC oven temperature was programmed as follows: initial temperature 60°C (held for 2 min), ramped to 280 °C at 10 °C/min, and held at 280 °C for 10 min. GC-MS analysis was conducted using electron ionization at 70 eV, scanning m/z values from 40 to 600. Detector and injector were maintained at 280 and 250 °C, respectively. Then, the compound identification was performed by comparing retention indices and mass spectra with entries in the National Institute of Standards and Technology (NIST) library database, as described by Tran et al. [24].

2.2.8. Molecular Docking Analysis

2.2.8.1. Molecular Docking Materials Preparation

Testing with an *in silico* approach was carried out with hardware specifications of Dell Vostro 14 3000 and Intel® AMD Ryzen 5 3500U models. The operating system used was Windows 10 Ultimate 64-bit with Radeon Vega MobilrGfx 2.10 GHz. The test compounds used were cycloartenol acetate (CID: 13023741), delta-guaiene (CID: 94275), lupeol (CID: 259846), campesterol (CID: 173183), and 24-norursa-3,12-diene (CID: 91735342). The test compounds were obtained from PubChem (https://pubchem.ncbi.nlm.nih.gov/). Meanwhile, the type of receptor used was NF-κB (Uniprot ID: AF-Q63369) from *Rattus norvegicus.* The test receptor was retrieved from UniProt webserver (https://www.uniprot.org/) and further testing was performed.

2.2.8.2. Compound Screening

Compound screening was conducted using the SCFBio webserver (http://www.scfbio-iitd.res.in/software/drugdesign/LIP1.jsp). Screening of drug compounds is based on the Lipinski Rule of Five to determine the potential similarity of compounds as raw materials for drugs (drug-likeness). Lipinski Rule of Five regulates the minimum value of similarity of compounds as drug ingredients based on five parameters: mass (g/mol), hydrogen bond acceptors, hydrogen bond donors, log P (high lipophilicity), and molar refractivity. A compound is said to be able to act as a medicinal substance if it fulfils at least two of the five Lipinski Rule of Five [25].

2.2.8.3. Molecular Docking Experiment and Visualization of Complexes

Molecular docking experiments were conducted using PyRx 0.8 software (SourceForge Headquarters, San Diego, California, USA). The molecular docking experiment technique was carried out to computationally combine ligands in the form of test compounds and test receptors. The test ligand-receptor complex will give rise to a binding affinity value obtained from the strength of molecular binding. The more negative the binding affinity value, the greater the binding ability between the ligand and the receptor [26]. Furthermore, the chemical interaction visualization of molecular docking results was carried out using PyMol v1.74 software (Schrodinger, LLC, USA) and BIOVIA Discovery Studio 2019 software (Dassault Systemes BIOVIA, San Diego, California, USA).

2.2.8.4. Validation of Molecular Docking Experiment (Molecular Dynamics Simulation)

Validation of molecular docking test was conducted using CABS-flex webserver (https://biocomp.chem.uw.edu.pl/CABSflex2/index). Parameter used in this simulation were protein

Table 1. Spearman and p value result.

		AST	ALT	ALP	IL-6	TNF-α	MDA	necrotic	cell inflam.	normal cell
AST	**Spear**	1	0.7	0.6	0.6	0.7	0.6	0.6	0.6	-0.6
	p value	0.001*	0.18	0.28	0.28	0.18	0.28	0.28	0.28	0.28
ALT	**Spear**	0.7	1	0.9	0.9	0.7	0.9	0.9	0.9	-0.9
	p value	0.18	0.001*	0.03*	0.03*	0.18	0.03*	0.03*	0.03*	0.03*
ALP	**Spear**	0.6	0.9	1	0.8	0.9	0.8	0.8	0.9	-0.7
	p value	0.28	0.03*	0.001*	0.04*	0.03*	0.04*	0.04*	0.03*	0.18
IL-6	**Spear**	0.6	0.9	0.8	1	0.9	0.9	0.9	0.9	-0.9
	p value	0.28	0.03*	0.04*	0.001*	0.03*	0.03*	0.03*	0.03*	0.03*
TNF-α	**Spear**	0.7	0.7	0.9	0.9	1	0.8	0.8	0.9	-0.8
	p value	0.18	0.18	0.03*	0.03*	0.001*	0.04*	0.04*	0.03*	0.04*
MDA	**Spear**	0.6	0.9	0.8	0.9	0.8	1	0.8	0.9	-0.8
	p value	0.28	0.03*	0.03*	0.03*	0.04*	0.001*	0.04*	0.03*	0.04*
necrotic	**Spear**	0.6	0.9	0.8	0.9	0.8	0.8	1	0.9	-0.8
	p value	0.28	0.03*	0.04*	0.03*	0.04*	0.04*	0.001*	0.03*	0.04*
cells inflam.	**Spear**	0.6	0.9	0.9	0.9	0.9	0.9	0.9	1	-0.9
	p value	0.28	0.03*	0.03*	0.03*	0.03*	0.03*	0.03*	0.001*	0.03*
normal cells	**Spear**	-0.6	-0.9	-0.7	-0.9	-0.8	-0.8	-0.8	-0.9	1
	p value	0.28	0.03*	0.188	0.03*	0.04*	0.04*	0.04*	0.03*	0.001*

*) showed significant differences $p < 0.05$

rigidity, restraints, C-alpha restraints weight, side-chain restraint weight, trajectory, temperature range, number of cycles, and RNG seed [27].

2.2.9. Data Analysis

The effects of *N. nucifera* flower extract on biochemical parameters (AST, ALT, ALP, IL-6, TNF-α, and MDA) and the histopathological parameters including the numbers of normal, cell inflammatory, and necrotic cells in the control and treatment groups were investigated using one way ANOVA test (Tukey post hoc test). The correlation test between biochemical parameters, normal hepatocytes, and liver weight results were analyzed using spearman test. The data were analyzed using GraphPad Prism 10.4.1 (Inc.California, USA) software at probably 95% confidence level (α = 0.05) to assess substantial variations among the groups. The phytochemical analysis was qualitative, focusing on determining the compound's bioactivity.

3. RESULTS AND DISCUSSIONS

3.1. Biochemical Results

3.1.1. Biochemical of Liver Function

Based on liver enzyme measurements (Figure 3), CN showed AST, ALT, AST/ALT ratio, and ALP values within the normal range, which were 16.192±0.604 IU/L, 24.457±0.438 IU/L, 0.663±0.035, and 82.434±0.552 IU/L, respectively. In contrast, CP, which was exposed to a toxic agent, exhibited a highly significant increase in all parameters: AST at 50.136±0.198 IU/L, ALT at 48.130±0.163 IU/L, AST/ALT ratio at 1.042±0.033, and ALP at 156.720±0.433 IU/L. This elevation indicated severe hepatocellular damage due to toxic exposure.

In the T1, which received the lowest dose of *N. nucifera* flower extract, AST and ALT levels decreased to 22.021±0.559 and 27.893±0.239 IU/L, respectively, with an AST/ALT ratio of 0.790±0.020 and ALP level of 89.128±0.397 IU/L. These values indicated improvement compared to

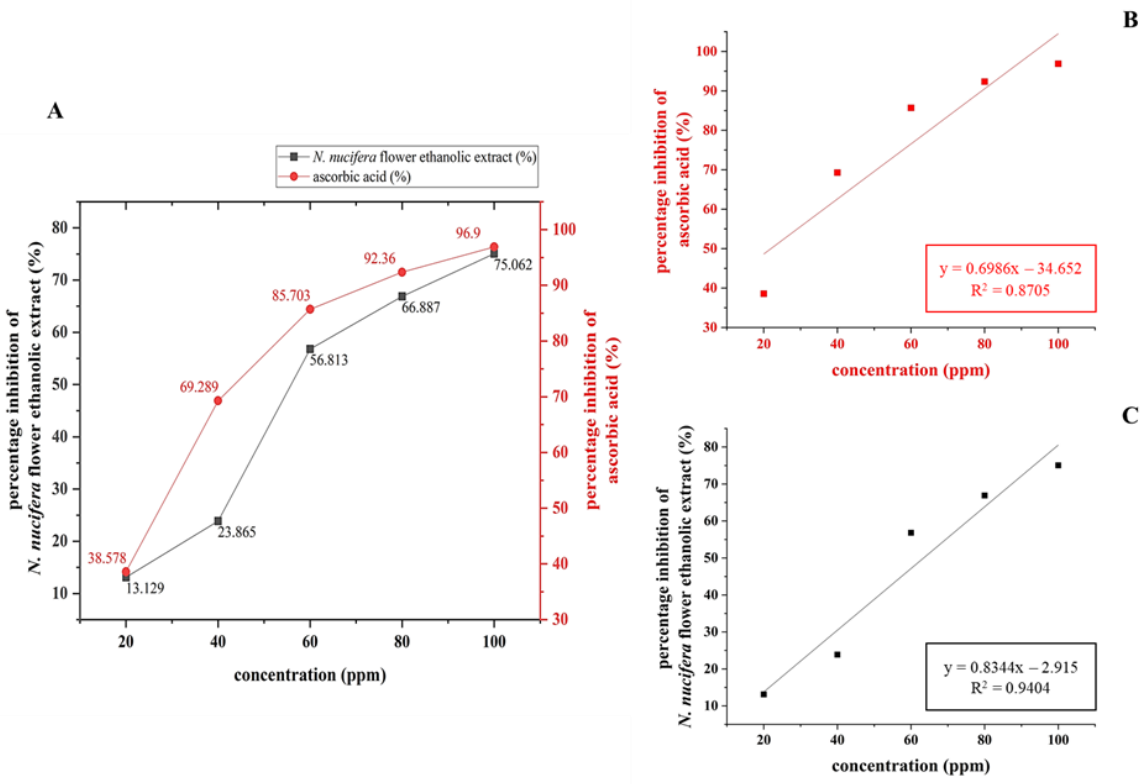


Figure 8. The relationship between concentration of *Nelumbo nucifera* flower ethanolic extract and percentage of inhibition also compare with (a) ascorbic acid as th standard. The (b) linier curve of ascorbic acid and (c) *N. nucifera* flower ethanolic extract.

Table 2. Antioxidant activity of *Nelumbo nucifera* flower ethanolic extract at various concentrations, compared with ascorbic acid.

Concentration (ppm)	PI. *N. nucifera* flower (%) ± SD	IC_{50} (ppm)	PI. Ascorbic acid (%) ± SD	IC_{50} (ppm)
20	13.13 ± 0.14[a]		38.58 ± 0.18[a]	
40	23.87 ± 0.20[b]		69.29 ± 0.17[b]	
60	56.81 ± 0.12[c]	63.42	85.70 ± 0.02[c]	21.97
80	66.89 ± 0.26[d]		92.36 ± 0.14[d]	
100	75.06 ± 0.12[e]		96.90 ± 0.13[e]	

Note: Difference in letter shown sigificantly difference ($p < 0.05$). PI: percentage inhibition.

the cp group, although the parameters had not yet fully returned to normal. Further improvements were observed in the T2 and T3 groups. In T2, AST decreased to 15.098±0.368 IU/L and ALT to 25.699±0.488 IU/L, with an AST/ALT ratio of 0.588±0.015 and ALP level of 85.250±0.326 IU/L. The T3 group showed similar results, with AST at 15.696±0.499 IU/L, alt at 25.733±0.359 IU/L, AST/ ALT ratio at 0.610±0.017, and ALP at 82.844±0.298 IU/L. These results indicated that the T2 and T3 groups had liver enzyme profiles close to those of the CN group, suggesting that administration of *N. nucifera* flower extract at moderate to high doses was effective in improving liver function impaired by toxic exposure.

Liver function is typically evaluated by quantifying AST, ALT, and ALP levels. ALT serves as a better marker for liver damage than AST because it is mainly localized in the cytoplasm of hepatocytes, whereas AST is localized in the cytoplasm and mitochondria. Elevated levels of both ALT and AST indicate cellular injury or necrosis as a result of hepatocyte damage [9]. However, increased ALP activity is related to bile duct injury and cholestasis [28] and this is correlated with the histopatological results of our findings, in the group that treat only with 2-ME show bile duct injury. The treatment with *N. nucifera* flower extract (T2 and T3) led to significantly ($p < 0.0001$) lower AST, ALT, and ALP levels, as opposed to the CP. These findings are in accordance with previous research; for instance, Nuchniyom et al. [29] reported that lotus extract mitigates the damage done to AML-12 hepatocytes by *acetaminophen* (APAP). Our findings demonstrate that *N. nucifera* flower extract significantly reduced liver enzyme levels (AST, ALT, ALP) and oxidative stress markers (MDA) in 2-ME-induced hepatotoxicity in male ddy mice. These results are in line with Tseng et al. [30], who reported hepatoprotective effects of lotus seedpod extract against chemically induced liver injury. However, some differences are apparent. The previous study also observed a more pronounced reduction in ALT levels at lower doses, which may be due to the use of seedpod extract rather than flower extract in our study. The phytochemical profiles between flower and seedpod extracts differ substantially, potentially leading to variations in bioactive compounds and their efficacy. Additionally, the toxin model used is different, in the previous study involved carbon tetrachloride (CCl_4) induced hepatotoxicity, whereas our study used 2-ME, which may induce liver injury through distinct mechanisms. These differences in plant part, dosing regimen, and hepatotoxin model likely contribute to the variation in hepatoprotective outcomes observed. Therefore, while both studies support the hepatoprotective potential of *N. nucifera*, our study uniquely highlights the efficacy of flower extract against 2-ME toxicityextract of *N. nucifera* has been observed to act as an antioxidant in nature by mitigating the oxidant effect of 2-ME. The extract may activates Krüppel-like factor 2 (KLF2), which suppresses the activation of hepatic stellate cells (HSC), a factor that contributes to liver injury [31]. This is also consistent with previous research, in which the lotus extract markedly diminished serum AST and ALT levels in hepatocytes treated with paracetamol [32]. The AST/ALT ratio values were also obtained, showing that all treatment groups (T1, T2, and T3) experienced a decrease in values compared to the CN. This indicates that the administration of *N.*

nucifera flower extract has a positive effect on the improvement of liver function. The normal AST/ALT ratio value is no more than 1, if it exceeds this value, it indicates chronic liver damage due to oxidative stress [33]. Although no adverse effects were observed during the 28-day administration, the long-term safety of *N. nucifera* extract at high doses requires further chronic toxicity studies. Previous research on lotus extract has shown a favorable safety profile, but this should be confirmed in extended studies with repeated dosing.

Although oral administration is typically used in herbal hepatoprotection studies, the SC route was chosen in this study to enhance dosing precision and ensure consistent systemic exposure. SC injection bypasses first-pass hepatic metabolism and gastrointestinal degradation, which are known to reduce the oral bioavailability of many phytoconstituents, including flavonoids and triterpenoids commonly found in *N. nucifera*. These compounds have poor solubility and are subject to enzymatic breakdown in the digestive tract, potentially limiting their therapeutic efficacy when given orally [18]. Thus, SC administration allowed for improved reproducibility and systemic absorption during the short-term treatment period. However, further studies simulating dietary intake via oral routes are still needed to fully understand the translational potential of this extract in clinical or nutritional settings.

3.1.2. Biochemical of Cytokine Pro-inflammatory and Oxidative Stress

As shown in Figure 4, the administration of the tested treatment significantly influenced the levels of pro-inflammatory cytokines IL-6 and TNF-α, as well as the oxidative stress marker MDA, across all experimental groups ($p < 0.0001$). As shown in Figure 3, the normal control group (CN) exhibited baseline concentrations of IL-6 (10.359±0.484 pg/mL), TNF-α (52.687±2.674 ng/L), and MDA (0.972±0.041 nmol/mL), representing normal physiological conditions.

In contrast, the positive control group (CP), which was exposed to the toxicant without any treatment, showed a marked increase in all three biomarkers: IL-6 (29.832±0.756 pg/mL), TNF-α (109.313±6.895 ng/L), and MDA (5.300±0.220 nmol/mL), indicating a pronounced inflammatory and oxidative stress response. Treatment with the test substance resulted in a dose-dependent amelioration of these biomarkers. Group T1 demonstrated a partial reduction in IL-6 (18.736±0.980 pg/mL), TNF-α (69.563±3.918 ng/L), and MDA (2.955±0.113 nmol/mL), suggesting a moderate anti-inflammatory and antioxidant effect. A more pronounced improvement was observed in the T2 group, with IL-6 levels reduced to 14.274±0.747 pg/mL, TNF-α to 53.188±3.325 ng/L, and MDA to 1.428±0.045 nmol/mL. Notably, the T3 group achieved near normal levels of IL-6 (12.260±0.584 pg/mL), TNF-α (52.563±4.300 ng/L), and MDA (1.335±0.048 nmol/mL), which were statistically comparable to those of the CN group.

The role of the oxidative stress of 2-ME may activated the nuclear factor kappa β (NF-κB) signaling pathway, leading to the transcriptional upregulation of pro-inflammatory cytokines such as IL-6 and TNF-α [34]. The overproduction of these pro-inflammatory cytokines contributes to liver inflammation, hepatocyte apoptosis, and overall hepatotoxicity. In parallel, oxidative stress increases lipid peroxidation, as indicated by elevated MDA levels, which serve as a sensitive and direct biomarker of oxidative injury to cellular membranes [35]. Given the central role of oxidative stress and

Table 3. Results of qualitative phytochemical screening of *Nelumbo nucifera* flower extract.

Secondary Metabolites	Methods	Qualitative Results
Alkaloid	Mayer	Positive
Phenol	$FeCl_3$	Positive
Flavonoid	NaOH	Positive
Terpenoid	Salkowski	Positive
Steroid	Liebermann-Burchard	Positive
Tannin	$FeCl_3$	Positive

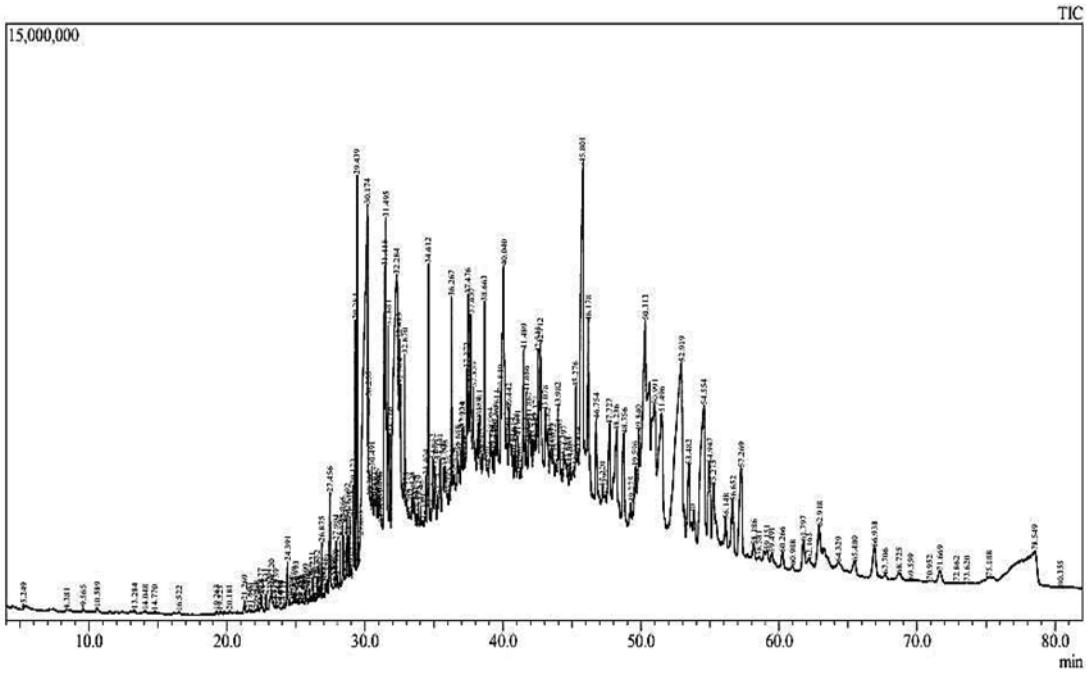


Figure 9. GC-MS chromatogram of *Nelumbo nucifera* flower extract.

inflammation in mediating hepatic injury, therapeutic agents with dual antioxidant and anti-inflammatory properties are of particular interest. In this context, the secondary metabolite compound on *N. nucifera* such us flavonoids, polyphenols, and alkaloid act as potent antioxidants that scavenge ROS through decreasing MDA level and inhibit the activation of the NF-κβ pathway. Similar results from the extract of *Rosmarinus officinalis* provided hepatoprotective effects through its antioxidant activity by diminishing ROS and preventing necrosis of hepatocytes [36]. Research by Sranujit *et al*. [37] revealed that the ethanol extract of *N. nucifera* flowers contains phenol levels of 351.08 mg/gallic acid equivalent/g dry extract. This compound is a strong antioxidant that can exhibit immunomodulatory properties by suppressing TNF-α secretion in inflammatory-induced human macrophages. Another study [29] indicated the effects of *N. nucifera* flower extract tea on hepatotoxicity and oxidative stress caused by mancozeb. The extract was safely used and supresses mancozeb toxicity on histological changes, liver and body weight, oxidative stress, and gluthathione contens. The previous study in line with this study, *N. nucifera* flower extract expresses the suppression of MDA coincides with reduced inflammatory cytokine expression, indicating a dual protective mechanism: inhibition of oxidative damage and attenuation of inflammatory signaling. To further validate these biochemical findings, histological analysis was performed to assess the extent of hepatic damage and tissue recovery.

3.2. Liver Weight, Histological of Liver, and the Correlation on Biochemical Parameters

As shown in Figure 5(a), the liver weight was highest in the CP group (1.56±0.45 g) and showed a statistically significant difference ($p < 0.0001$) compared to the CN group (1.27±0.38 g) as well as all treatment groups: T1 (1.43±0.49 g), T2 (1.32±0.41 g), and T3 (1.30±0.37 g). These findings indicate that liver weights in all treatment groups approached values observed in the CN group. As shown in Figure 5(b), the percentage of normal hepatocytes was lowest in the CP group (31.4±2.70%) and differed significantly ($p < 0.0001$) from the CN group (84.6±1.67%) and all treatment groups (T1: 66.8±3.56%; T2: 79.8±1.92%; T3: 82.2±2.78%). Notably, the percentage of normal hepatocytes in the T2 and T3 groups did not differ significantly ($p < 0.0001$) from that of the CN group, indicating that administration of *N. nucifera* at moderate and high doses effectively restored hepatocyte morphology to near-normal conditions. Furthermore, the percentage of necrotic hepatocytes, as shown in Figure 5(c), was highest in the CP group (53.8±7.69%) and was

significantly higher ($p < 0.0001$) than in the CN (15.8±7.69%), T1 (28.6±3.91%), T2 (18.4±2.70%), and T3 (16.6±3.36%) groups. While the percentage of necrotic cells did not differ significantly between T1 and T2, the T1 group showed a significantly higher percentage of necrosis compared to both CN and T3 ($p < 0,001$). These results suggest that higher doses of *N. nucifera* are more effective in preventing hepatocellular necrosis.

Figure 5(c) showed the percentage of inflammatory cells observed in liver tissue serves as a key histological marker of hepatic inflammation. In this study, the extent of inflammatory cell was quantified to assess the degree of inflammatory response. The CN exhibited minimal infiltration, with an inflammatory cell percentage of 7.40±1.14%, indicating normal physiological conditions. In contrast, the CP showed a significantly ($p < 0.0001$) elevated inflammatory cell percentage (36.2±1.92%), consistent with severe hepatic inflammation following toxicant exposure. Treatment with *N. nucifera* flower extract resulted in a dose-dependent reduction in the percentage of inflammatory cells. T1 showed moderate inflammation (20.6±2.70%), while T2 exhibited a marked reduction to 11.4 ± 1.14%, classified as mild infiltration. Notably, the T3 showed only 9.20±1.92%, closely approximating the CN, indicating near complete resolution of inflammation. These findings suggest that *N. nucifera* flower extract exerts a protective anti-inflammatory effect on liver tissue, particularly at higher doses.

Control normal (CN) demonstrates normal liver histoarchitecture with polygonal hepatocytes exhibiting central round nuclei (NH), organized hepatic cords radiating from the central vein (NC), and clear sinusoidal spaces (NS) without evidence of cellular degeneration or inflammation. Control positive (CP) with 2-ME exposure displays severe hepatic injury characterized by extensive coagulative necrosis (Nec), pronounced hydropic degeneration (HD), sinusoidal dilatation (SD), infiltration of polymorphonuclear inflammatory cells (PMN), and bile duct injury (BI). Central vein congestion and cytoplasmic vacuolation are also evident. T1 group (low-dose *N. nucifera* flower extract) shows moderate lesions with residual hepatocyte necrosis (Nec) and inflammatory infiltration (PMN). Hydropic swelling (HD) and sinusoidal dilatation (SD) persist, but with reduced intensity compared to CP. T2 group (medium-dose *N. nucifera* flower extract reveals noticeable improvement in liver structure. Necrotic regions (Nec) are fewer and smaller, with partial restoration of hepatocyte integrity (NH) and reduced inflammatory cell presence. Central veins (NC) and sinusoids (NS) appear closer to normal. T3 group (high-dose *N. nucifera* flower extract) exhibits nearly normal hepatic architecture with minimal evidence of necrosis or degeneration. Hepatocytes (NH) are largely intact, and inflammatory cell infiltration (PMN) is negligible, indicating substantial hepatoprotective activity at this dosage.

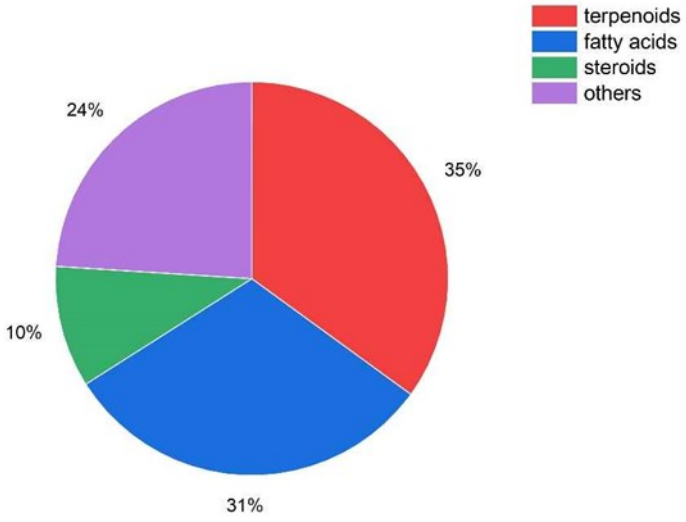


Figure 10. Chemical group composition of selected compound in *Nelumbo nucifera* flower extract.

Abbreviations: NH – Normal hepatocyte; NC – Central vein; NS – Sinusoid; Nec – Necrosis; HD – Hydropic degeneration; PMN – Polymorphonuclear cell infiltration; BI – Bile duct injury; SD – Sinusoidal dilatation.

The results of the Spearman correlation analysis (Figure 7 and Table 1) demonstrate significant relationships between liver biochemical parameters, inflammation, oxidative stress, and liver tissue histology. For instance, ALT and IL-6 ($r = 0.90$, $p = 0.03$), and ALT and TNF-α ($r = 0.70$, $p = 0.18$) show positive correlations, indicating that inflammatory cytokines are key mediators of hepatocellular injury. Similarly, MDA levels, representing lipid peroxidation, also correlate positively with ALT and inflammatory markers, reinforcing the intertwined role of oxidative stress and inflammation in 2-ME-induced hepatotoxicity.

Histological examination of liver tissue revealed the degree of damage and recovery. Mice treated with 2-ME showed marked histological damage, including hydropic degeneration, cytoplasmic vacuolization, and necrosis (Figure 5). Such pathological alterations are thought to be a consequence of elevated levels of ROS, which cause inflammation and, ultimately, hepatocyte death [38]. In moderate and high dose of *N. nucifera* flower extract were found to aid the regeneration of hepatocytes, demonstrating its reparative capacity. This was supported by other studies on *N. nucifera* extracts from other plant parts, such as seedpods and leaves, which also showed hepatoprotective effects [39][40]. Allameh et al. [41] stated that the presence of the bioactive compound in *N. nucifera* flower like quercetin may explain the observed decrease in inflammatory cell infiltration within the liver tissue and blocking adhesion molecule expression in sinusoidal endhotelial cells. This analysis further explains the effects of distinct doses of *N. nucifera* flower extract. Although moderate and high dose improved liver histology and significantly ($p < 0.05$) reduced biochemical parameters, low dose was less effective. This indicates a threshold effect where a minimum concentration of bioactive compounds appears to be necessary for hepatic protection. A similar dose-dependent relationship in other studies has been aligned with Andrian et al. [42], who reported that greater amounts of *Andrographis paniculata* extract seemed to lower liver enzymes and histological damage to a greater extent in rats with alcohol-induced liver injury. The dose dependent efficacy with 150 mg/kg bw as the minimal effective dose implies a pharmacokinetic threshold for bioactive compounds to achieve hepatic accumulation sufficient to inhibit inflammation and lipid peroxidation. This aligns with studies on flavonoid bioavailability, where doses below 100 mg/kg fail to reach therapeutic concentrations in hepatocytes [12].

These study indicate a close systemic relationship between hepatocellular damage, inflammation, and oxidative stress following 2-ME exposure. A strong positive correlation between liver enzymes (AST, ALT, ALP) and IL-6 and TNF -α reinforces the central role of pro-inflammatory cytokines in worsening hepatic function.This is consistent with the report by Somade et al. [43], which showed that exposure to the hepatotoxic compound 2-ME increased the expression of cytokine pro-inflammatory along with elevated ALT and AST levels in rats, as indicators of liver tissue damage. Moreover, the strong correlation between MDA with IL-6 and TNF-α supports the findings by Aurellia et al. [44], which indicate that in liver fibrosis, oxidative stress plays a role in the activation of inflammatory pathways through increased lipid peroxidation and cytokine production.

The relationship between inflammatory cells, pro -inflammatory cytokine parameters and oxidative stress is also in line with the previous study conducted by Kurdy and Kazaal [45] which reported that the accumulation of macrophages and neutrophils in liver tissue contributes to the increase of TNF-α and ROS, exacerbating tissue damage.The decrease in the number of normal cells that negatively correlates strongly with TNF-α, IL-6, and MDA indicates the destructive effects of chronic inflammatory processes. The observed liver weight gain, indicates inflammation and oxidative stress play central roles in hepatic enlargement following 2-ME. The pro-inflammatory cytokine promote immune cell infiltration and fluid retention in hepatic tissues, leading to edema and swelling [46]. Simultaneously, increased MDA levels reflect lipid peroxidation-induced hepatocyte damage, which triggers hepatocellular hypertrophy,

Table 4. GC–MS-identified secondary metabolites in *Nelumbo nucifera* flower extract.

No.	Compound Name	Group of Compound	Molecular weight (g/mol)	Molecular Formula	Retention Time (RT)	Area (%)
1.	Cycloartenol acetate	Terpenoid	468.80	$C_{32}H_{52}O_2$	52.919	4.17
2.	Hexadecanoic acid	Fatty acid	256.42	$C_{16}H_{32}O_2$	30.174	3.10
3.	10*E*,12*Z*-Octadecadienoic acid	Fatty acid	280.40	$C_{18}H_{32}O_2$	32.284	2.95
4.	delta-Guaiene	Terpenoid	204.35	$C_{15}H_{24}$	50.991	2.91
5.	10-Nonadecanol	Alifatic alcohol	284.52	$C_{19}H_{40}O$	45.801	2.86
6.	24-Norursa-3,12-diene	Terpenoid	394.67	$C_{29}H_{46}$	52.919	1.99
7.	6beta-bicyclo(4.3.0)nonane	Terpenoid	332.10	$C_{13}H_{24}O$	54.554	1.89
8.	Campesterol	Steroid	400.68	$C_{28}H_{48}O$	48.236	1.75
9.	9*Z*-Octadecenoic acid	Fatty acid	594.90	$C_{37}H_{70}O_5$	27.253	1.66
10.	Lupeol	Terpenoid	426.71	$C_{30}H_{50}O$	48.756	1.09
11.	Hexadecyloxirane	Aldehid	268.47	$C_{18}H_{36}O$	50.313	1.09
12.	Solanesol	Polyphenol	631.10	$C_{45}H_{74}O$	47.727	1.07
13.	Thunbergol	Terpenoid	290.50	$C_{20}H_{34}O$	55.275	1.07
14.	2-Hydroxy-hexadecanoic acid	Fatty acid	330.50	$C_{19}H_{38}O$	37.657	0.85
15.	Fern-7-en-3beta-ol	Terpenoid	426.70	$C_{30}H_{50}O$	56.652	0.83
16.	Stearic acid	Fatty acid	284.50	$C_{18}H_{36}O_2$	32.564	0.77
17.	Triacontane	Hydrocarbon	422.81	$C_{30}H_{62}$	36.688	0.76
18.	Dotriacontane	Alkanes	450.87	$C_{32}H_{66}$	39.064	0.76
19.	gamma-Sitostenone	Steroid	412.70	$C_{29}H_{48}O$	53.482	0.75
20.	Methyl nonahexacontanoate	Fatty acid	999.80	$C_{69}H_{138}O_2$	43.078	0.75
21.	3,7,11-Trimethyl-, (Z)-2,10-dodecadien-1-ol	Terpenoid	224.38	$C_{15}H_{28}O$	56.148	0.72
22.	Methyl tetracosanoate	Fatty acid	382.67	$C_{25}H_{50}O_2$	40.442	0.72
23.	Octadecyl eicosanoate	Fatty acid	565.00	$C_{38}H_{76}O_2$	53.720	0.71
24.	Farnesol	Terpenoid	222.36	$C_{15}H_{26}O$	44.085	0.61
25.	Celidoniol	Alkaloid	424.80	$C_{29}H_{60}O$	35.706	0.56
26.	cis-Methyl 11-eicosenoate	Fatty acid	324.50	$C_{21}H40O$	34.932	0.50

Table 4. *Cont.*

No.	Compound Name	Group of Compound	Molecular weight (g/mol)	Molecular Formula	Retention Time (RT)	Area (%)
27.	5alpha-Stigmastane-3,6-dione	Steroid	428.69	$C_{29}H_{48}O_2$	58.186	0.48
28.	Epilupeol	Terpenoid	426.70	$C_{30}H_{50}O$	71.669	0.32
29.	Neophytadiene	Terpenoid	278.52	$C_{20}H_{38}$	28.366	0.13
30.	(Z,Z)-Octadecadienoic acid	Fatty acid	294.50	$C_{19}H_{34}O_2$	30.745	0.12
31.	3-methylphenol	Phenol	108.14	C_7H_8O	8.381	0.11

ballooning degeneration, and in some cases, regenerative responses [47].

The hepatoprotective effect of *N. nucifera* flower is also attributed to the presence of bioactive compounds such as campesterol, lupeol, and cycloartenol acetate, as revealed by phytochemical studies (Table 4). Bioactive compound such as campesterol have an ability as anti-inflammatory by inhibiting NF-κB activation, thereby reducing the transcription of pro-inflammatory cytokines like IL-6 and TNF-α [48]. This mechanism likely underlies the observed reductions in cytokine levels and inflammatory cell infiltration in treated groups. Additionally, 24-norursa-3,12-diene, lupeol and cycloartenol acetate, belonging to the triterpenoid class, they may activate the Nrf2 pathway, which plays a crucial role in enhancing the transcription of cellular antioxidant enzymes, including HO-1 and NQO1 [49], ultimately reducing oxidative damage as reflected by decreased MDA levels and improved hepatocyte architecture in treated mice. Future studies should validate these pathways through molecular assays like western blot or gene expression for NF-κB, Nrf2, HO-1, IκBα to confirm the proposed mechanism.

Overall, these findings reinforce the evidence that the hepatotoxicity observed in this model involves interactive mechanisms between oxidative stress, inflammation, and hepatocyte death. Therefore, therapeutic approaches targeting pro-inflammatory pathways and oxidative stress hold promising potential for reducing tissue damage and supporting hepatocyte regeneration. Additionally, the results of this study also confirm that the administration of *N. nucifera* flowers ethanolic extract has been proven to have protective potential against liver damage caused by 2-ME. In other words, *N. nucifera* flowers not only reduce the inflammatory process but also help repair damage that has already occurred in liver cells, making them a promising candidate in the treatment of liver damage caused by toxic compound like 2-ME.

3.3. Antioxidant Activity and Phytochemical Analysis

3.3.1. Antioxidant Activity

The antioxidant capacity of *N. nucifera* flower ethanolic extract was evaluated using a DPPH

radical scavenging assay and compared with ascorbic acid as a standard antioxidant. As shown in Table 2 and Figure 8, both samples exhibited a dose -dependent increase in percentage inhibition of DPPH radicals, indicating effective free radical scavenging potential. At the lowest concentration tested (20 ppm), *N. nucifera* flower extract demonstrated 13.13% inhibition, whereas ascorbic acid showed a significantly higher inhibition of 38.58%. As the concentration increased, the extract's antioxidant activity improved substantially, reaching 75.06% inhibition at 100 ppm. For comparison, ascorbic acid achieved 96.90% inhibition at the same concentration. The linear regression equation for the *N. nucifera* extract was $y = 0.8344x - 2.915$ with a R^2 value of 0.9404, suggesting a strong linear relationship between concentration and antioxidant activity. Meanwhile, ascorbic acid followed a regression trend $y = 0.6986x + 34.652$, showing a slightly stronger correlation with a R^2 value of 0.8705.

These results indicate that *N. nucifera* flower extract possesses significant antioxidant potential, although not as potent as ascorbic acid (IC_{50} = 21.97 ppm). However, the IC_{50} value of the extract was calculated to be approximately 63.42 ppm, which classifies it as a strong antioxidant (< 100 ppm), according to commonly accepted thresholds [21]. This strong antioxidant property may be attributed to the presence of bioactive compounds such as flavonoids, polyphenols, and alkaloids, which act as potent radical scavengers and contribute to hepatoprotective mechanisms observed in this study.

3.3.2. Phytochemical Analysis

3.3.2.1. Qualitative Phytochemical Screening of Secondary Metabolite

The phytochemical screening in the *N. nucifera* flower extract were analyzed using standard methods for the qualitative measurement of secondary metabolite compounds. According to the results, indicated that the *N. nucifera* flower extract contains various secondary metabolites such as alkaloids, phenols, flavonoids, steroids, terpenoids, and tannins. These compounds have been extensively studied for their antioxidant and hepatoprotective activities in medicinal plants [50]

Table 5. Drug-likeness test of compounds based on Lipinski Rule of Five.

Compound	Compound ID	Drug-likeness Parameters				
		Mass (g/mol)	Hydrogen Bond Acceptors	Hydrogen Bond Donor	Log P (High Lipophilicity)	Molar Refractivity
Cycloartenol acetate	13023741	468	0	2	8.739	140.267
Delta-guaiene	94275	204	0	0	4.725	66.742
Lupeol	259846	426	1	1	8.025	130.649
Campesterol	173183	400	1	1	7.635	123.599
24-norursa-3,12-diene	91735342	394	0	0	7.444	143.596

Table 6. Molecular docking experiment results.

Receptor	Compounds	Complex Binding Affinity (kcal/mol)	Type of Interaction	Amino Acids Involved in Interaction
NF-κB	Cycloartenol acetate	-7.1	vdW	Gln357, Glu421, Arg424, Ile435, Gln436
			HI	Ile416, Lys417, Val420, Phe439,
	Delta-guaiene	-6.5	vdW	Thr279, Gln330, Asp333, Pro339, Glu341
			HI	Trp329, Ile334, Lys338, Tyr340, Val343
	Lupeol	-7.6	vdw	Asp354, Gln357, Gln436
			HI	Ile416, Lys417, Val420, Ile435
	Campesterol	-6.6	vdw	Ile416, Arg424, Gln436,Thr441
			HI	Lys417, Val420, Ile435
			PHI	Phe439
	24-norursa-3,12-diene	-8.2	vdW	Leu284, Asp348, Gln352, Val411
			HI	Ile245, Arg280, Ala283, Tyr340, Pro342, Phe344, Leu350
	Silymarin (control)	-7.4	vdW	Ile372, Pro373, Asn378, Trp379, Thr381
			HI	Ala380, Pro401
			PHI	Pro375, Gln384, Arg396

Note: PHI: polar hydrogen interaction; HI: hydrophobic interaction; vdW: Van der Walls interaction.

(Table 3).

3.3.2.2. GC-MS based Profiling of Secondary Metabolite

To complement the qualitative secondary metabolites findings, GC-MS analysis was performed to identify individual secondary metabolites present in the extract, providing a detailed chemical profile of the bioactive compounds. The results from GC-MS analysis, revealed the presence of 235 compounds in the ethanol extract of *N. nucifera* flowers, with the highest compound at a retention time of 52.92 min, namely cycloartenol acetate (Figure 9). A total of 31 out of 235 identified compounds, selected based on the highest percentage area values, were predominantly composed of terpenoids (35%), fatty acids (31%), and steroids (10%). The remaining 24% consisted of several groups of compounds, including phenols, alkanes, and hydrocarbons (Figure 10 and Table 4).

The biochemical and histological changes in this study are in line with the antioxidant activity and profile of secondary metabolites of the *N. nucifera* flower extract, as determined by GC-MS. The antioxidant activity (IC_{50}) in *N. nucifera* flower is 63.42 ppm, its indicate that ethanolic extract of *N.* nucifera flower has strong antioxidant (< 100 ppm). This result suggests that the extract has a significant capacity to scavenge free radicals, thereby potentially reducing oxidative stress in hepatic tissue. Then, according to the GC-MS results, ethanolic extract of *N. nucifera* flower has many bioactive compounds which included cycloartenol acetate (4.17%) and hexadecanoic acid (3.10%) as the most abundant compounds with hepatoprotective activity. In particular, it induces Nrf-2 protein, which is crucial for the protection of the liver from damage through the inhibition of pro-inflammatory cytokines [51][52]. Besides these compounds, other bioactive molecules are found in the extract, including 24-norursa-3,12-diene (terpenoid), 6beta-bicyclo(4.3.0)nonane (terpenoid), lupeol (terpenoid), campesterol (steroid), stearic acid (terpenoid), γ-sitostenone (steroid), eicosanoic acid, octadecyl ester (fatty acid), neophytadiene (terpenoid), (Z,Z)-octadecadienoic acid (fatty acid), and 3-methylphenol (phenol).

Phenolic compounds such as 3-methylphenol, and compounds of the terpenoid category, such as 24-norursa-3,12-diene, lupeol, thunbergol, farnesol, and epilupeol, have the ability to increase the biosynthesis of endogenous antioxidants like catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) [53]. Both these phenolic and terpenoid compounds have activity as hepatoprotective by its hydroxyl groups within their molecular structure. It serves as a free radical capture agent and the ability of hydroxyl group to attract MAA via a nucleophilic attack on the nucleophilic target, thus making MAA less toxic and increasing its water solubility so that it can be better excreted [33][54]. As a compound group in *N. nucifera* flowers extract, the specific compounds of the fatty acid found here have hepatoprotectant effects due to their phospholipid main component. These compounds act to control inflammation-related liver fibrosis, but not all liver fibrosis, the former is accompanied by a rising serum ALT level [5]. Stearic acid has activity in protecting hepatocyte mitochondria by preventing the release of *cytochrome* C, which can trigger hepatocyte apoptosis [55], while hexadecanoic acid has activity in preventing lipid peroxidation in liver cells [56]. Agents like *Pistia stratiotes* leaves and *Curcuma longa* have corroborated these findings, which have also shown comparable effects on lowering liver enzyme levels and enhancing liver function [57] [58].

In addition to its hepatoprotective effects, *N. nucifera* flower extract contains campesterol, oxirane, and farnesol are some of the most significant anti-inflammatory compounds [59][60]. Other important antioxidants include delta-guaiene and thunbergol. In support of Laoung-on et al. [61], who documented *N. nucifera* flower extract in bioactive compounds, the results showed that these extracts had antioxidant properties. Moreover, GC-MS analysis has detected solanesol, a compound that appears in a number of Solanaceae plants, such as tomato, potato, and tobacco. Solanesol has received attention due to its varied biological activities, including anti-ulcer, antibacterial, and anticancer [62]. The potential hepatoprotective action of *N. nucifera* flower extract is supported by phytochemical screening and the profiling of secondary metabolites. The remarkable decrease in liver biochemical markers, along with the notable

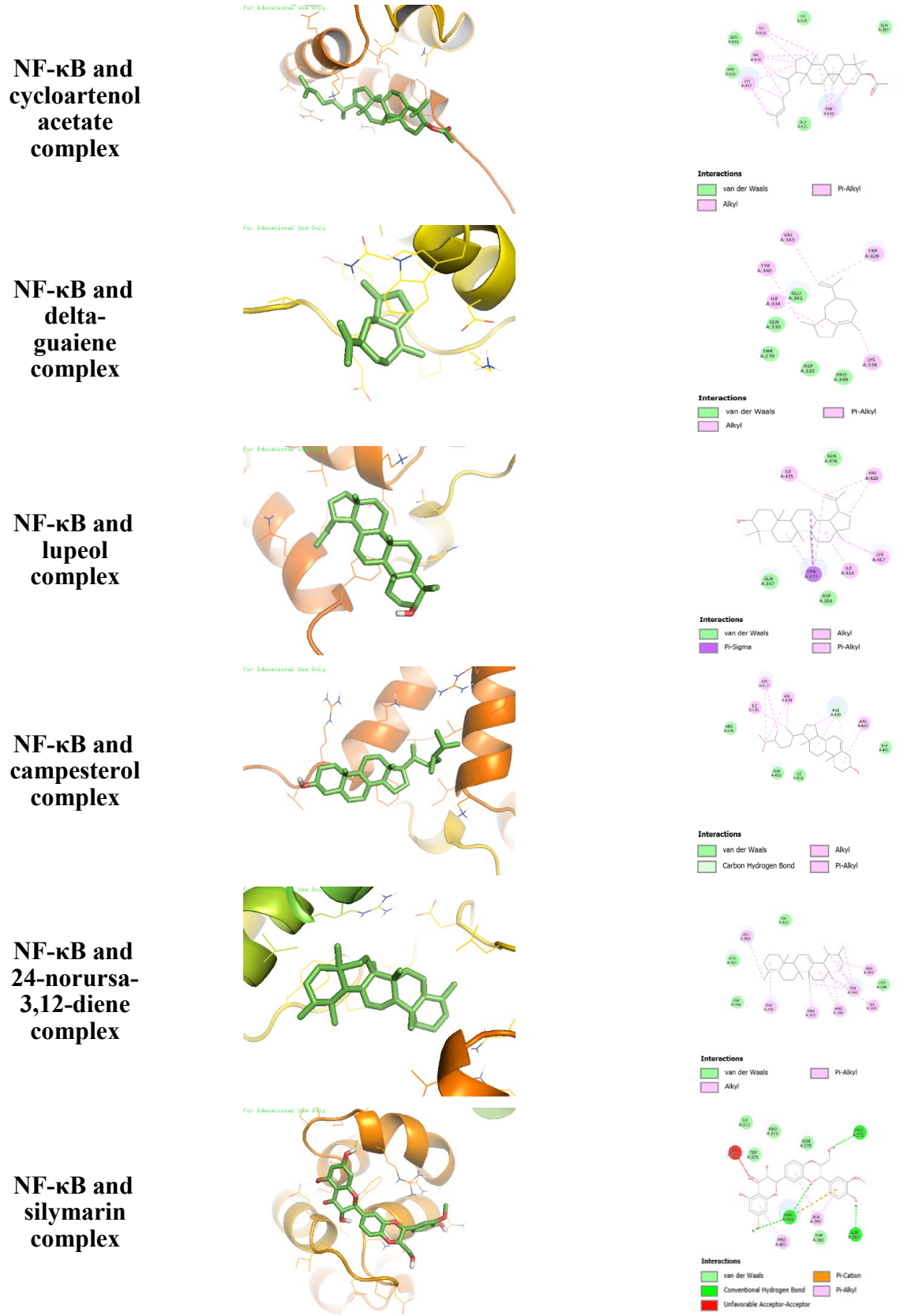


Figure 11. Visualization of each complexes binding (left: three-dimensional molecular interaction binding; right: two-dimensional molecular interaction binding with amino acids involved).

improvement in liver histology, demonstrates efficacy. Increased oxidative stress following 2-ME induction is associated with liver injury and its biochemical and histological parameters, so this improvement suggests reduced injury.

However, this study had some limitations. First, although promising hepatoprotective effects were observed at moderate and high doses, further research is necessary to assess the long-term safety of high-dose administration and to compare its efficacy with established antioxidant compounds such as quercetin, curcumin, or silymarin. Second, it is important to note that the GC-MS profiling conducted in this study was performed without pure reference standards. As a result, the identification and relative abundance of phytoconstituents were based solely on retention times and peak area percentages matched to the NIST library. While this approach allows for the identification of major and minor components, it does not permit absolute quantification (e.g., mg of compound per gram of extract). Therefore, caution should be exercised when linking specific compounds to observed bioactivities. Further quantitative phytochemical analysis using validated analytical techniques, such as HPLC, LC-MS/MS, or standard-calibrated GC-MS is recommended to accurately determine the concentrations of key bioactive constituents such as lupeol, cycloartenol acetate, and 24-norursa-3,12-diene. Despite these limitations, the semi-quantitative GC-MS analysis remains a widely accepted method for initial natural product screening and provides valuable foundational insight into the extract's chemical composition [24].

3.4. In-silico Analysis

The screening results of the test compounds based on the Lipinski Rule of Five using the SCFBio webserver showed that all compounds met the drug-likeness parameters. The delta-guaiene compound has the highest similarity characteristics with drug compounds compared to other compounds. This can be seen in the molecular mass value < 500 g/mol (204 g/mol), hydrogen bond acceptors and donors < 5, log P < 5, and molar refractivity in the interval 40–130 (Table 5).

According to molecular docking, showed that the compound 24-norursa-3,12-diene was able to bind most strongly to the NF-κB receptor, as evidenced by the binding affinity value of -8.2 kcal/mol. The types of bonds involved in the 24-norursa-3,12-diene and NF-κB ligand complexes are Van der Walls bonds and hydrophobic bonds. In van der Walls bond, the complex binds to amino acids Leu284, Asp348, Val411, and Gln352. Meanwhile, in hydrophobic bonding through alkyl groups, the complex binds to amino acids Ile245, Arg280, Ala283, Tyr340, Pro342, Phe344, and Leu350. Meanwhile, the weakest binding strength was shown by the delta-guaiene compound on the NF-κB receptor with a binding affinity value of -6.5 kcal/mol. The entire complex appeared to bind to the A chain of the NF-κB receptor. This indicates that the A chain of the NF-κB receptor tends to be active and can trigger biological responses when interacting with ligand groups (Table 6 and Figure 11). The 24-norursa-3,12-diene is a triterpenoid compound that known to have useful biological activities and anti-inflammatory agent [63]. In this study, molecular docking results showed that the compound had a strong binding affinity for the NF-kb receptor (-8.2 kcal/mol). Translocation of NF-κB to the nucleus can potentionally be inhibited by this interaction of inflammatory cytokines [8].

The validation of molecular docking experiment using CABS-Flex molecular dynamics simulation showed that all complexes have RMSF values < 3 Å. This means that the compound and receptor complexes are relatively stable to work in the body and have the potential to elicit physiological responses (Table 7). This value is in line with previous research [64] which states that the stability of fluctuations formed from ligand and protein interactions can be said to be stable if it has an RMSF value of < 3 Å. The molecular docking analysis presented in this study was conducted to support the in vivo findings by providing preliminary mechanistic insight into the interaction of major phytocompounds in *N. nucifera* flower extract with NF-κB, a key regulator of inflammation. In response to reviewer feedback, this in silico analysis focused on selected dominant terpenoid compounds identified via GC-MS. According to the docking experiments, the binding affinity values observed for 24-norursa-3,12-diene (−8.2 kcal/mol), lupeol (−7.6 kcal/mol), cycloartenol acetate (−7.1 kcal/mol), silymarine (as a positive control, -7.4 kcal/mol) indicate a

Table 7. Molecular dynamics results.

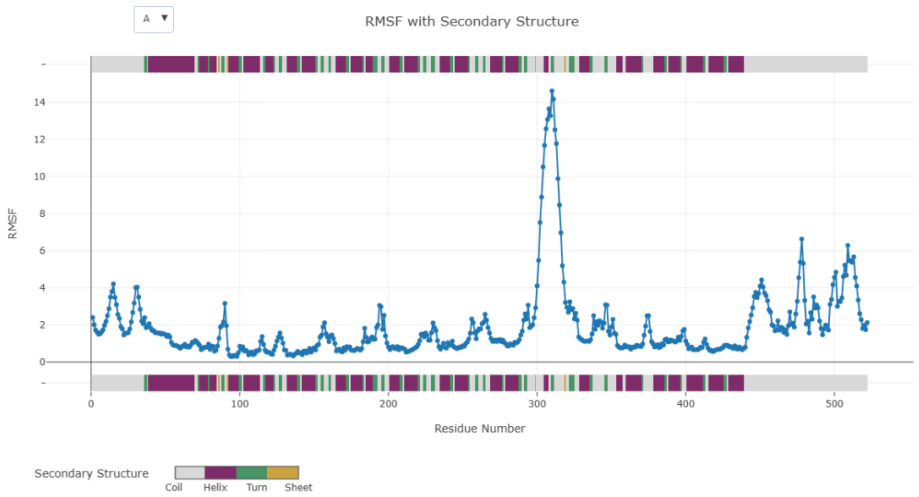
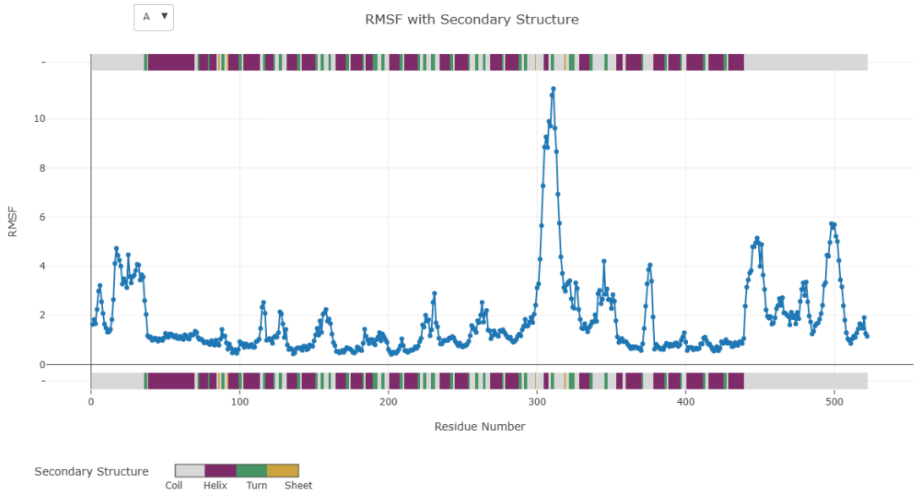
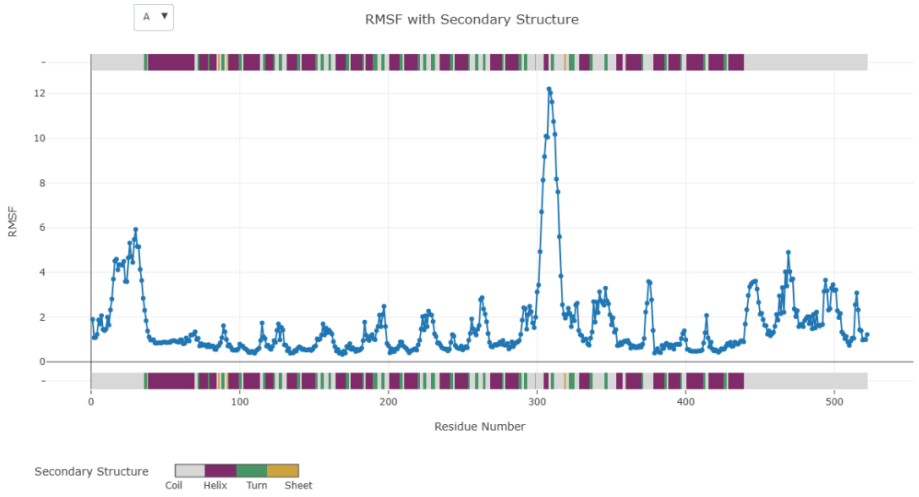
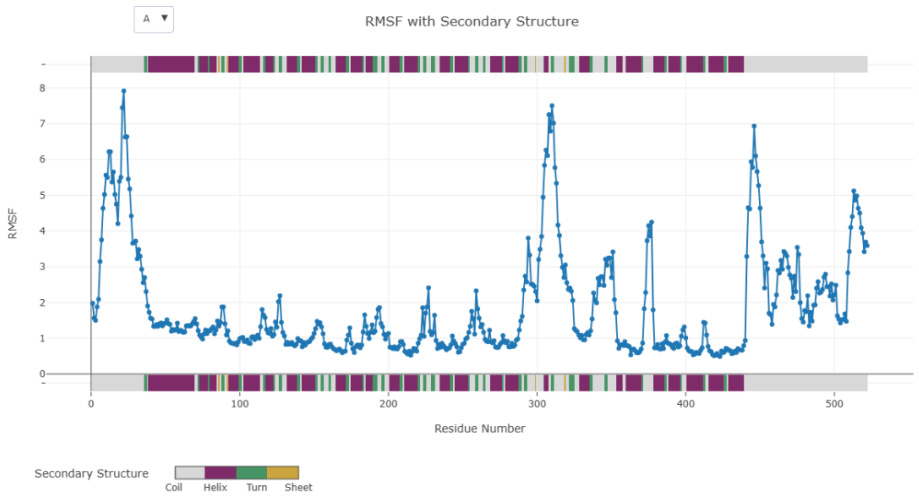
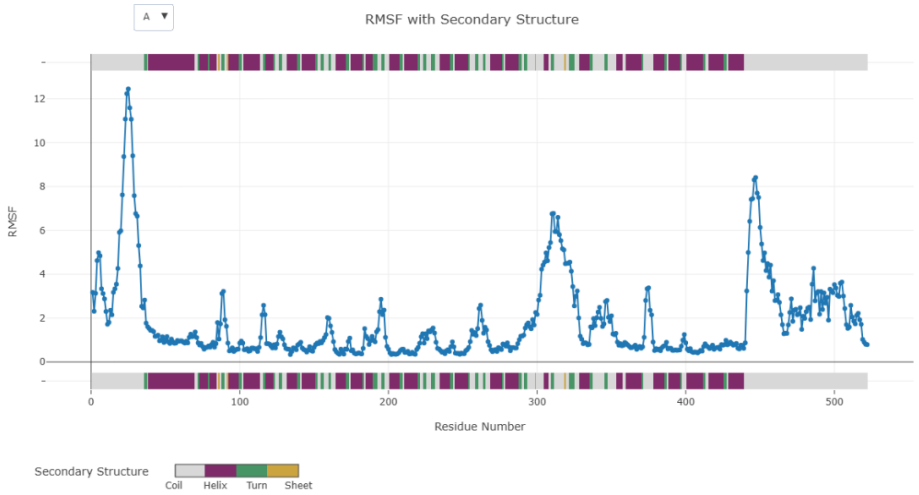
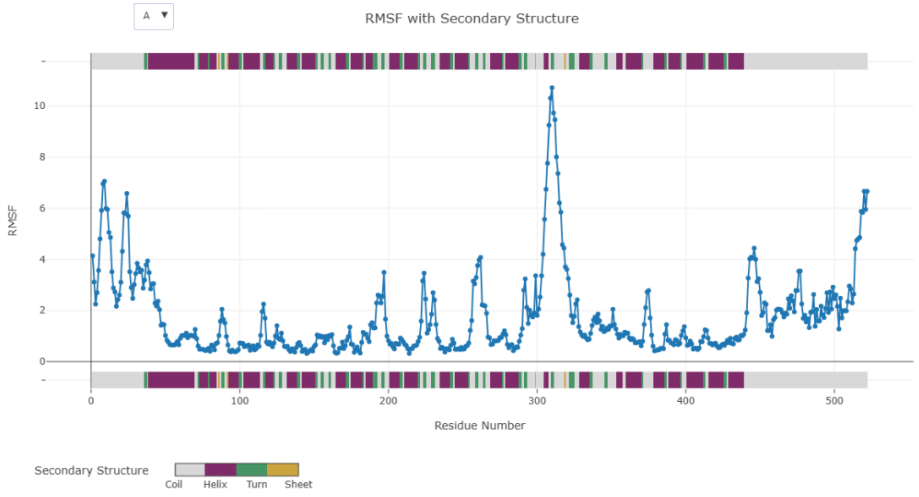
Fluctutation Plot	RMSF Value (Å)
NF-κB and cycloartenol acetate complex	
	2.605
NF-κB and delta-guaiene complex	
	2.377
NF-κB and lupeol complex	
	2.540

Table 7. *Cont.*

Fluctutation Plot	RMSF Value (Å)
NF-κB and campesterol complex	
	2.455
NF-κB and 24-norursa-3,12-diene complex	
	2.969
NF-κB and silymarin complex	
	2.639

reasonable potential for interaction with the target site. These values fall within the range generally considered biologically relevant for small-molecule binding in virtual screening studies [65]. Nonetheless, the lack of a known inhibitor as a docking comparator limits the ability to quantitatively assess the relative binding strength of the tested compounds.

The NF-κB pathway is a key regulator for the expression of pro-inflammatory cytokines such as TNF-α and IL-6, which play an important role in mediating oxidative stress induced hepaotocytes damage [66]. According to the in vivo data, showing a decrease in ALT, AST, as well as ALP levels, and a reduced percentage of hepatocyte necrosis. This effect may indicate the potential of 24-norursa-3,12-diene compound to be hepatoprotective through the mechanism of inhibition of the inflammatory pathway. Not only this compound, but also another compound like cycloartenal acetate and lupeol that also have activity binding with NF-κB protein. Moreover, these results were comparable to the effects observed with silymarin, a well-established hepatoprotective agent used as a reference compound in this study. Silymarin is known to exert its protective effects by stabilizing hepatocyte membranes, scavenging free radicals, and downregulating inflammatory mediators via NF-κB inhibition [67]. The similar trend observed between silymarin and the tested plant derived compounds suggests that the extract may act through converging molecular pathways. Therefore, the hepatoprotective effect observed in this study may be attributed, at least in part, to the modulation of the NF-κB axis and suppression of downstream pro-inflammatory cytokines.

To the best of our knowledge, this is the comprehensive study to investigate the hepatoprotective effect of *N. nucifera* flower extract against 2-ME induced liver injury *in vivo* by integrating both biochemical, histopathological, immunological, and molecular docking parameters. The identification of key bioactive compounds through GC-MS analysis and their activity through *in silico* analysis, alongside the demonstration of a dose-dependent effect on cytokine suppression and antioxidant defense, provides a novel insight into the mechanistic pathways underlying its therapeutic potential. Unlike previous studies on *N. nucifera* seeds or leaves, this work provides the first evidence that its flower extract exerts hepatoprotection against 2-ME toxicity through a unique dual mechanism, the first by ROS scavenging via cycloartenol acetate, lupeol, and 24-norursa-3,12-diene complexes (identified here as the dominant phytochemical) and the second by suppression of NF-κB mediated inflammation, as demonstrated by correlated reductions in TNF-α, IL -6, and hepatic necrosis.

4. CONCLUSIONS

In summary, the findings from this study emphasize the interplay between oxidative stress, inflammatory responses, and hepatic injury following 2-ME exposure, and how *N. nucifera* flower extract exerts a hepatoprotective effect through the variation of dose. The optimal dose of *N. nucifera* flower extract as a treatment against oxidative stress induced by 2-ME was 150–450 mg/ kg bw. This dose is optimal because it could reduction in liver enzyme levels, improvement in histological architecture, and suppression of pro-inflammatory cytokines collectively suggest that the extract not only mitigates liver damage but also supports hepatocyte regeneration. These protective effects are attributed to the antioxidant activity (IC_{50} = 63.42 ppm) and active compounds in *N. nucifera* flower extract, which include cycloartenol acetate, hexadecanoic acid, 6beta-bicyclo(4.3.0) nonane, delta-guaiene, 24-norursa-3,12-diene, campesterol, lupeol, stearic acid, gamma-sitostenone, and 3-methylphenol. The compound 24 -norursa-3,12-diene, lupeol, and cycloartenol acetate had strong binding afinity to NF-kb. These findings underscore the potential of *N. nucifera* flower as a natural hepatoprotective agent, and warrant further investigation into its clinical relevance and long-term safety.

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Conflicts of Interest

The authors declare no conflict of interest.

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