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Verbascum thapsus L. ethanol fraction mitigates CCl_4-induced hepatic damage *via* inhibition of TNFα-NF-κB signaling and oxidative stress in rats

Amina Ishfaq[1], Hafiz Muhammad Zubair[2]✉, Farah Abid[3], Naeem Ur Rehman[4], Umair Ikram Dar[5], Shahid Muhammad Iqbal[6], Ali Sharif[7], Muhammad Saad Majeed[8], Muhammad Akhlaq[9], Samiullah Khan[10,11]

[1]*Faculty of Pharmacy, The University of Lahore, Lahore, Pakistan*

[2]*Post Graduate Medical College, Faculty of Medicine and Allied Health Sciences, The Islamia University of Bahawalpur, Bahawalpur, Pakistan*

[3]*Department of Pharmacy, University of South Asia, Lahore, Pakistan*

[4]*Department of Pharmacology, Faculty of Pharmacy, Gomal University, DIKhan, Pakistan*

[5]*Department of Pharmaceutical Chemistry, Lahore College of Pharmaceutical Sciences, Lahore, Pakistan*

[6]*Department of Pharmacology, Faculty of Pharmacy, The Islamia University of Bahawalpur, Bahawalpur, Pakistan*

[7]*Faculty of Pharmaceutical and Allied Health Sciences, Lahore College for Women University, Lahore, Pakistan*

[8]*Department of Human Nutrition and Dietetics, Faculty of Medicine and Allied Health Sciences, The Islamia University of Bahawalpur, Bahawalpur, Pakistan*

[9]*Department of Pharmacy, Hazara University, Mansehra, Pakistan*

[10]*Faculty of Health and Life Sciences, INTI International University, Persiaran Perdana BBN Putra Nilai, 71800 Nilai, Negeri Sembilan, Malaysia*

[11]*Faculty of Pharmaceutical Sciences, University of Central Punjab, Lahore, Pakistan*

ABSTRACT

Objective: To evaluate the hepatoprotective effects of the ethanol fraction of *Verbascum thapsus* L. (EFVT) against CCl_4-induced liver injury and elucidate its underlying mechanisms.

Methods: The assessment of antioxidant properties and cell viability was conducted using the 2,2-diphenyl-1-picrylhydrazyl assay and HepG2 cells, respectively. The *in vivo* hepatoprotective efficacy of EFVT was evaluated in a rat model of carbon tetrachloride (CCl_4)-induced liver injury by determining biochemical parameters, and oxidative stress- and inflammation-related markers. Gas chromatography-mass spectrometry was also employed for the qualitative analysis of its phytochemical composition.

Results: GC-MS analysis of EFVT revealed the presence of several bioactive compounds such as 3 methyl mannoside and 9,12-octadecadienoic acid. Oral administration of EFVT significantly mitigated CCl_4-induced liver injury, as evidenced by reduced levels of total bilirubin, alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase, and malondialdehyde, boosted activities of catalase and superoxide dismutase, as well as enhanced glutathione levels. Histopathological examinations indicated EFVT restored abnormal liver architecture and reduced inflammation. Additionally, EFVT substantially downregulated the mRNA levels of *IL-6*, *IL-1β*, *TNF-α*, and *NF-κB*, and upregulated *IL-10* expression.

Conclusions: These findings underscore the therapeutic potential of EFVT in ameliorating liver damage associated with oxidative

Summary

Question: Does the ethanol fraction of *Verbascum thapsus* L. mitigate carbon tetrachloride (CCl_4)-induced hepatic injury in rats?

Findings: The ethanol fraction of *Verbascum thapsus* L. significantly attenuated CCl_4-induced liver injury by inhibiting the TNFα-NF-κB signaling and reducing oxidative stress.

Meaning: The ethanol fraction of *Verbascum thapsus* L. shows great potential as a hepatoprotective agent through anti-inflammatory and antioxidant mechanisms and may be used for the treatment of liver injury, which needs further investigation.

✉To whom correspondence may be addressed. E-mail: dr.zubair83@gmail.com

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stress, providing scientific validation for its traditional utilization in ethnomedicine.

KEYWORDS: Liver injury; *Verbascum thapsus*; CCl_4; Xenobiotics; Oxidative stress; HepG2; NF-κB

1. Introduction

The liver is a vital organ essential for maintaining body homeostasis. It performs diverse functions, including energy generation, regulation of glucose, protein, lipid metabolism, bile production, and vitamin storage[1]. Liver diseases encompass a wide range of conditions, such as alcoholic and non-alcoholic liver diseases, viral hepatitis, metabolic liver disorders, cholelithiasis, and drug- or chemical-induced liver injuries, all of which significantly impact health[2,3]. Oxidative stress is one of the key factors in the initiation and progression of liver diseases. The overproduction of reactive oxygen species (ROS) in the liver leads to increased levels of oxidative stress, damaging lipids, proteins, and nucleic acids, ultimately causing cellular dysfunction and death[4]. Chemical-induced oxidative stress further aggravates hepatotoxicity by weakening cellular defense mechanisms. Carbon tetrachloride (CCl_4) is a halogenated hydrocarbon historically utilized as a solvent, in fire suppression systems, and in the synthesis of refrigerants and propellants. Upon administration, CCl_4 undergoes bioactivation primarily in the liver, resulting in the formation of reactive hepatotoxic metabolites. The excessive generation of free radicals, primarily through the cytochrome P450 system, leads to oxidative stress, which is a key mechanism driving CCl_4-induced hepatotoxicity[5]. Antioxidants, which can be endogenous or obtained through diet and supplements, are crucial in counteracting oxidative stress[6]. Historically, medicinal plants have significantly contributed to human health and are widely used to treat various diseases. Approximately 80% of the global population relies on natural medicines reported by the World Health Organization. Globally, 50 000-80 000 flowering plant species are consumed for medicinal purposes, with 600-700 species traditionally used in Pakistan. Thus, plants remain essential for nutrition, healthcare, and shelter[7].

Plant-derived antioxidants, including polyphenols and flavonoids, are suggested to offer neuroprotective benefits owing to their capacity for free radical scavenging, potent antioxidant activity, and metal chelation[8]. Natural secondary metabolites present in plants have demonstrated protective capabilities in addressing a range of medical conditions, encompassing neurodegenerative and liver diseases. Plant-based phytochemicals also hold the potential to protect the structure and function of hepatocytes, safeguarding against liver ailments[9].

Verbascum thapsus L. (*V. thapsus*), commonly referred to as common mullein, is a highly regarded medicinal plant renowned for its myriad health benefits. This robust herb thrives in various regions across the world, particularly in temperate climates such as Pakistan. Throughout history, it has been used in traditional medicine to treat a wide range of health issues, including respiratory infections, gastrointestinal ailments, skin disorders, and as a natural sedative[10,11]. Various plant components have been harnessed to create infused oils, teas, and tinctures, all serving therapeutic purposes. *V. thapsus* holds a wealth of valuable compounds, including alkaloids, saponins, tannins, and coumarins, which are believed to impart antibacterial, antiviral, antifungal, and anti-inflammatory properties[12]. Its anti-inflammatory attributes make it an excellent option for alleviating pain, inflammation, and swelling associated with injuries or chronic conditions. Additionally, its antispasmodic properties have been used in the treatment of bronchial asthma, diarrhea, and various digestive disorders[13]. Beyond its extensive use in traditional Spanish folk medicine for addressing issues related to liver or gut inflammation and diarrhea[14]. *V. thapsus* has also found recognition in Canadian folk medicine. Furthermore, it has been subjected to *in vitro* evaluation to investigate its potential anti-hepatoma activity. Additionally, a biostimulating herbal mixture has been devised, aiming to treat hematopoietic, liver, and respiratory disorders in humans, with *V. thapsus* extract as a prominent component[15]. *V. thapsus* is a powerful medicinal plant and can be used both internally and externally to promote healing and health[12]. The rising side effects and costs of pharmaceuticals have increased interest in alternative treatments. Plants, valued for their natural antioxidants, are among the most promising options[16].

Hepatic disorders represent a major global health burden, underscoring the need for novel therapeutic strategies. A wide range of studies have shown that plant-derived extracts possess strong antioxidant activity, attenuating hepatotoxicity through inhibition of lipid peroxidation and enhancement of antioxidant enzymes[17]. Therefore, this study aimed to evaluate the hepatoprotective effects of *V. thapsus* and to explore its underlying molecular mechanisms.

2. Materials and methods

2.1. Chemicals and reagents

The chemicals were used in the experiments, including silymarin, CCl_4, olive oil, ethanol, methanol, formaldehyde, chloroform, *n*-hexane, carboxymethyl cellulose, dimethyl sulfoxide (DMSO), and ascorbic acid. These chemicals were procured from Sigma-Aldrich (USA) and employed as standard reagents.

2.2. Collection and preparation of plant extract

In September 2020, plant specimens were collected from the vicinity of the Chenab River in Sialkot, Pakistan. The taxonomic

identification was performed by Dr. Zaheer-uddin Khan from Department of Botany, Government College University, Lahore, Pakistan. A voucher specimen of the plant (Gc.Herb.Bot.3709) was deposited in the herbarium for future reference.

Fresh leaves of *V. thapsus* were thoroughly washed with running tap water, shade-dried, and subsequently ground into a coarse powder. The powdered material was subjected to maceration in 1 L of 70% aqueous ethanol for 72 h at ambient temperature with intermittent stirring. Following maceration, the extract was initially filtered through muslin cloth and subsequently through Whatman No.1 filter paper. The process of extraction was repeated three times under identical conditions. The combined filtrates were concentrated under reduced pressure using a rotary evaporator at below 40 ℃. The resultant aqueous ethanolic extract of *V. thapsus* (EEVT) was air-dried, yielding approximately 13.2% *w/w* of crude extract. The semi-solid dried extract was stored in airtight glass containers at 4 ℃ until further use[18].

2.3. Fractions of plant extract

The process of fractionation involves partitioning a crude extract into multiple fractions, each enriched with specific phytoconstituents. This was typically achieved through sequential solvent extraction using solvents of increasing polarity, such as *n*-hexane, chloroform, and ethanol. EEVT was subjected to solvent-solvent partitioning employing solvents of increasing polarity, as previously described. Briefly, 40 g of EEVT was dissolved in *n*-hexane at a 1:10 (*w/v*) ratio and stirred using a magnetic stirrer to ensure thorough mixing. The *n*-hexane phase was subsequently concentrated under reduced pressure through a rotary evaporator, and the resulting residue was collected for further analysis. Chloroform was subsequently added to the *n*-hexane residue, and the same procedure was followed. Finally, ethanol was introduced to the chloroform residue, and the process was repeated[19]. The resulting three distinct fractions; *n*-hexane fraction (NFVT), chloroform fraction (CFVT), and ethanol fraction (EFVT), along with the crude extract (EEVT), were stored separately in airtight jars at 4 ℃.

2.4. Phytochemical screening

Phytochemical screening was performed to detect the presence of flavonoids, saponins, tannins, glycosides, carbohydrates, phenols, triterpenoids, alkaloids, and steroids in the crude extract (EEVT) and its fractions (NFVT, CFVT, and EFVT), following established standard protocols[17].

2.5. Free radical scavenging activity

The free radical scavenging activity of the extract was evaluated by the DPPH method with minor modifications[20,21]. A 0.16 mM solution of DPPH in ethanol was prepared, and the extract solution was added to it. The mixture was then incubated for 1 h in the dark, and the absorbance was measured at 517 nm by using a spectrophotometer (IRMECO U2020-Germany). Ascorbic acid was used as the standard, and the experiment was repeated three times to obtain an average value.

2.6. Cell culture and treatments

HepG2 cells were used to evaluate the hepatoprotective potential of the fractions[22]. Briefly, HepG2 cells were seeded at a density of approximately 1×10^4 cells per well in 96-well plates containing Dulbecco's Modified Eagle Medium (Gibco), supplemented with 10% fetal bovine serum. Following 24 h of adherence, cells were pre-treated with either DMSO (control), plant-derived fractions, or silymarin (positive control). Subsequently, 1% (*v/v*) carbon tetrachloride (CCl_4) was added to induce hepatotoxicity. After 24 h of incubation, cells were washed with phosphate-buffered saline, and MTT reagent prepared in phosphate-buffered saline was added to each well. The cells were incubated further at 37 ℃, and the resulting formazan crystals were solubilized before measuring absorbance at 540 nm through a microplate reader. Cell viability was expressed as a percentage relative to untreated control cells[17,22].

2.7. In vivo hepatoprotective activity

Adult albino rats [(180 ± 10) g] of both sexes were obtained from the animal facility of University of Lahore (UOL, Lahore) exclusively for experimental purposes. All housing, handling, and feeding protocols were conducted in accordance with the guidelines established by the Organization for Economic Co-operation and Development (OECD). Animals were housed in stainless steel cages under controlled environmental conditions, including a relative humidity of (50 ± 5)% and a 12-h light/dark cycle. They were provided *ad libitum* access to fresh drinking water and a standardized high-nutrition pellet diet. Prior to the initiation of experimental procedures, the animals were acclimatized to laboratory conditions[23,24]. The ethical approval of the study was obtained from the Institutional Research Ethics Committee (IREC) of The University of Lahore, under approval number IREC-2021-17. To induce acute hepatic injury, equal volume of carbon tetrachloride (CCl_4) and olive oil was administered intraperitoneally (*i.p.*) at a dose of 2 mL/kg body weight (BW) for a period of 21 d[17]. The animals were randomly allocated into six groups, each comprising six rats. Group Ⅰ (control) received distilled water (1 mL/kg BW, orally) daily and olive oil (2 mL/kg BW, *i.p.*) thrice weekly. Group Ⅱ (negative control) was administered distilled water (1 mL/kg BW, orally) along with the CCl_4-olive oil mixture (2 mL/kg BW, *i.p.*) thrice weekly. Group Ⅲ (Standard) received silymarin (100 mg/kg BW, orally) daily in conjunction with the CCl_4-olive oil mixture (2 mL/kg BW, *i.p.*) thrice weekly. Groups Ⅳ, Ⅴ, and Ⅵ (test groups) were

treated with EFVT at doses of 125, 250, and 500 mg/kg BW orally, along with the CCl_4-olive oil mixture (2 mL/kg BW, *i.p.*) thrice weekly. After 24 h of the final treatment, all animals were anesthetized and sacrificed for further analyses.

2.8. Determination of body weight, liver weight, and liver index

The body weights of the animals were monitored from day 0 to day 21 throughout the experimental period. Following euthanasia, the livers were excised, rinsed thrice with cold normal saline, and their wet weights were recorded. Subsequently, the liver index was calculated using the following formula:

Liver index = liver weight/body weight

2.9. Determination of serum biochemical markers

The serum biochemical parameters, including alanine aminotransferase (ALT), alkaline phosphatase (ALP), aspartate aminotransferase (AST), and total bilirubin, were quantified using the Cobas c111 automated chemistry analyzer (Roche Diagnostics, Germany)[17].

2.10. Measurement of oxidative stress markers

A 10% (*w/v*) liver homogenate was prepared in ice-cold physiological saline and subsequently centrifuged to obtain a clear supernatant. The concentrations of antioxidant biomarkers, including glutathione (GSH), superoxide dismutase (SOD), malondialdehyde (MDA), and catalase (CAT), were quantitatively assessed using standard spectrophotometric assays[17,25].

2.11. Histopathological examination

A portion of the liver was preserved in 10% formalin buffer, embedded in paraffin wax according to standard protocol[26,27], and cut into 5 μm thick tissue sections. These sections were transferred onto glass slides for deparaffinization and stained with both hematoxylin and eosin (H&E) for histopathological examination under a light microscope (BIO CELL 1 500×).

2.12. Quantitative real–time polymerase chain reaction (qRT–PCR)

RNA was extracted from liver tissues using TRIzol reagent (Sigma-Aldrich), and RNA concentration and purity were assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific). High-quality RNA was reverse-transcribed into complementary DNA (cDNA) using a commercial cDNA synthesis kit (SuperMix). Gene-specific primers were designed with Primer 5.0 software (Premier Biosoft International, Palo Alto, CA, USA). qPCR was carried out using the QuantStudio 3 system (Thermo Fisher Scientific). *β–actin* was used as the internal reference gene[17,28]. The sequences of primers used in this study are listed in Supplementary Table 1.

2.13. Gas chromatography–mass spectrometry (GC–MS) analysis

Chromatographic analysis was conducted using a Shimadzu QP2010 gas chromatograph equipped with a mass spectrometric detector (Shimadzu, Japan). Separation was achieved on a DP-5 capillary column (30 m × 0.25 mm *i.d.*, 0.25 μm film thickness; 5%-phenyl, 95%-dimethyl polysiloxane). Helium served as the carrier gas at a constant flow rate of 1.22 mL/min. The injector and detector temperatures were maintained at 220 ℃. The oven temperature was programmed to increase from 40 ℃ to 280 ℃ at a rate of 5 ℃/min. Compound identification was performed by calculating the retention indices of each analyte and comparing them with those of a hydrocarbon standard analyzed under identical conditions. Additionally, mass spectra of the detected compounds were compared with reference spectra from the National Institute of Standards and Technology library for structural confirmation[17].

2.14. Statistical analysis

The experimental data were expressed as mean ± standard deviation (SD). Statistical analysis was conducted using one-way analysis of variance (ANOVA), followed by Tukey's *post hoc* test to evaluate differences between groups. Graphs were generated *via* GraphPad Prism version 8 (GraphPad Software, Inc., USA). A *P*-value of less than 0.05 was considered statistically significant.

3. Results

3.1. Preliminary phytochemical analysis

Phytochemical screening results showed the presence of carbohydrates, phenolics, alkaloids, flavonoids, saponins, glycosides, and tannins in EEVT, NFVT, CFVT, and EFVT as shown in Supplementary Table 2.

3.2. DPPH assay results

The percentage of scavenging power exhibited an upward trend, as the concentrations of NFVT, CFVT, and EFVT increased. However, it was noteworthy that the results obtained from EFVT did not significantly differ from those of vitamin C (Figure 1A).

3.3. In vitro hepatoprotective activity assessment

An *in vitro* hepatoprotective study was conducted using HepG2 cells to preliminarily screen and identify the most potent and efficacious fractions. Cell viability assays were performed to determine the percentage of viable cells after treatment. All test fractions were evaluated at a concentration of 20 μg/mL. The results showed that CCl_4 treatment significantly reduced cell viability, whereas EFVT exhibited the most pronounced protective effect, as evidenced by markedly increased percentage of viable cells (Figure

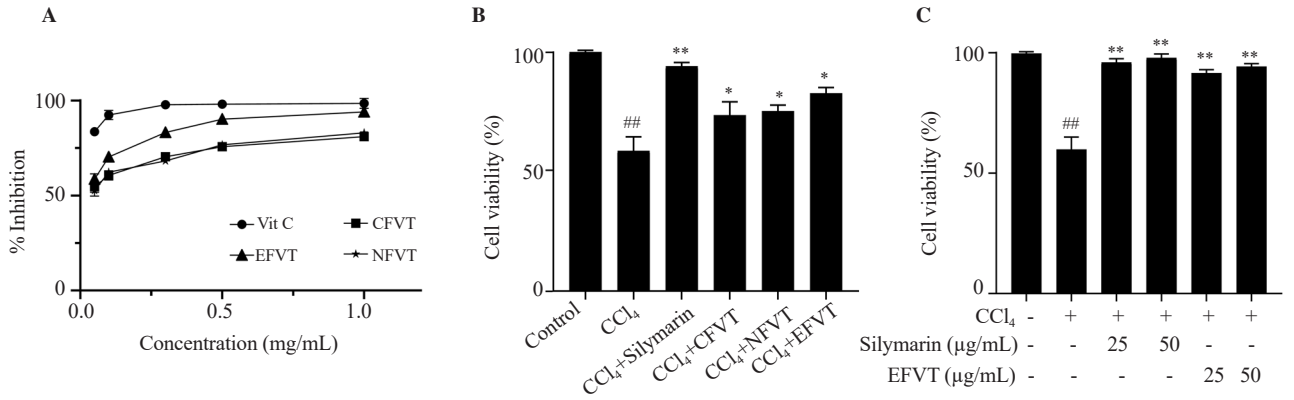

Figure 1. *In vitro* antioxidant and hepatoprotective activities of *Verbascum thapsus* leaf extract. (A) DPPH radical scavenging assay. (B) Percent cell viability of CFVT, NFVT, and EFVT in HepG2 cells. (C) Dose-response effects of EFVT. Statistically significant differences are indicated with $^{\#\#}P < 0.001$ *vs.* the control group; $^{*}P < 0.05$, $^{**}P < 0.001$ *vs.* the CCl_4 group. NFVT: *n*-hexane fraction of *Verbascum thapsus*, CFVT: chloroform fraction of *Verbascum thapsus*, EFVT: ethanol fraction of *Verbascum thapsus*, Vit C: Vitamin C.

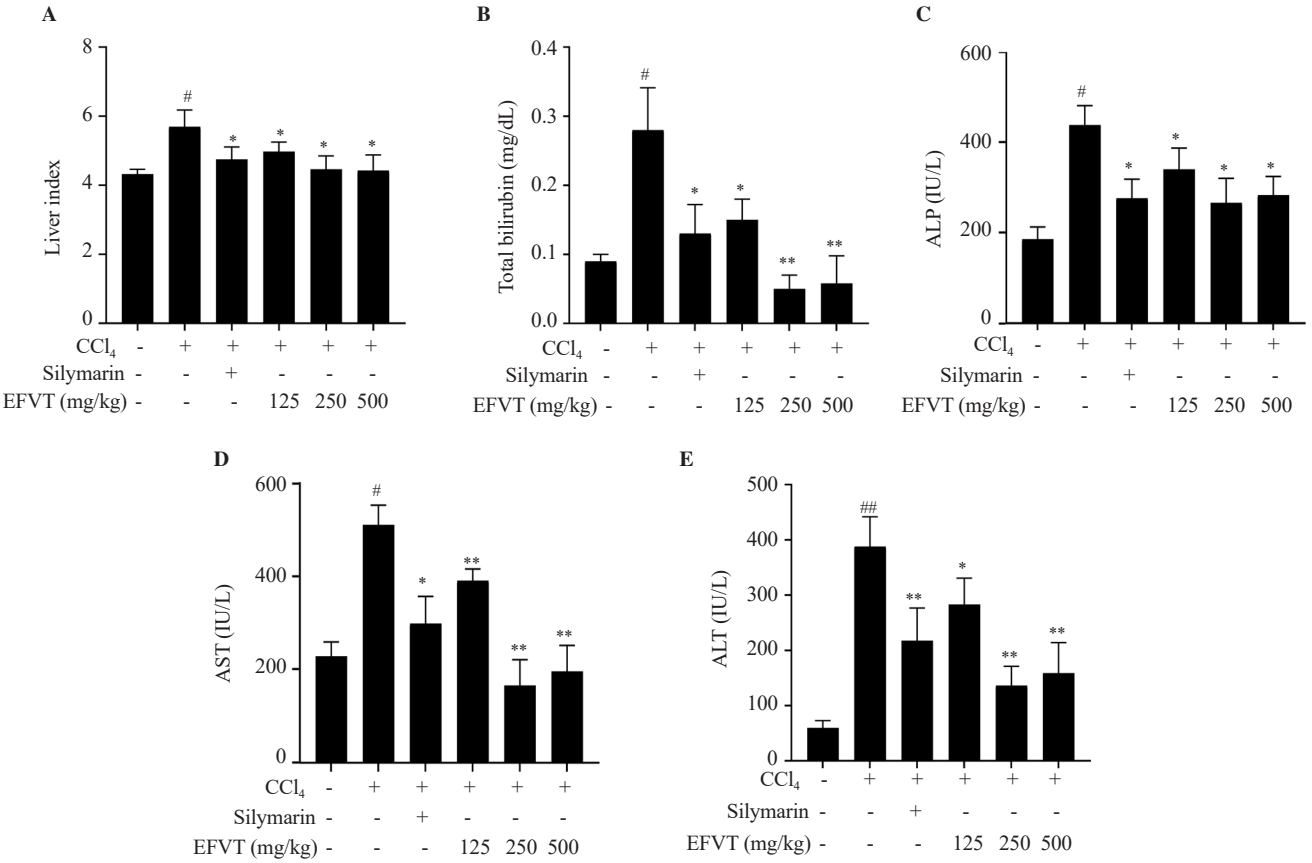

Figure 2. Effect of EFVT on liver index and hepatic markers in CCl_4-exposed rats. (A) Liver index, (B) total bilirubin, (C) ALP, (D) AST, (E) ALT. Results are expressed as mean ± SD ($n = 6$). Statistically significant differences are indicated with $^{\#}P < 0.05$, $^{\#\#}P < 0.001$ *vs.* the control group; $^{*}P < 0.05$, $^{**}P < 0.001$ *vs.* the CCl_4 group. ALP: alkaline phosphatase, AST: aspartate aminotransferase, ALT: alanine aminotransferase.

1B). Based on these findings, EFVT was selected for further *in vivo* hepatoprotective studies to evaluate its efficacy against CCl_4-induced liver injury in rats.

Furthermore, the hepatoprotective effects of EFVT were examined at concentrations of 25 and 50 µg/mL, alongside equivalent doses of silymarin, to assess a potential dose-response relationship. Both EFVT and silymarin significantly protected the cells from CCl_4-induced cytotoxicity (Figure 1C). These results suggest that EFVT possesses strong antioxidant and protective activities against CCl_4-induced hepatotoxicity in HepG2 cells.

3.4. Effect of EFVT on body weight, liver weight, and liver index

The results demonstrate that there was no obvious variation in the average body weight of each rat group from the initial to the final measurements. Additionally, there were no prominent changes in the weight of the animals' livers. However, mild hepatomegaly was observed in the CCl_4-treated group, causing a marked increase in liver index when compared to the control group (Figure 2A). Treatment with all doses of EFVT improved liver index. However, no statistically significant differences in liver index were observed among the EFVT groups at different doses.

3.5. Effect of EFVT on biochemical parameters

CCl_4 administration resulted in increased levels of ALT, AST, ALP, and bilirubin, whereas treatment with EFVT markedly decreased the levels of these biochemical parameters. Notably, EFVT at doses of 250 and 500 mg/kg showed significantly improved liver function

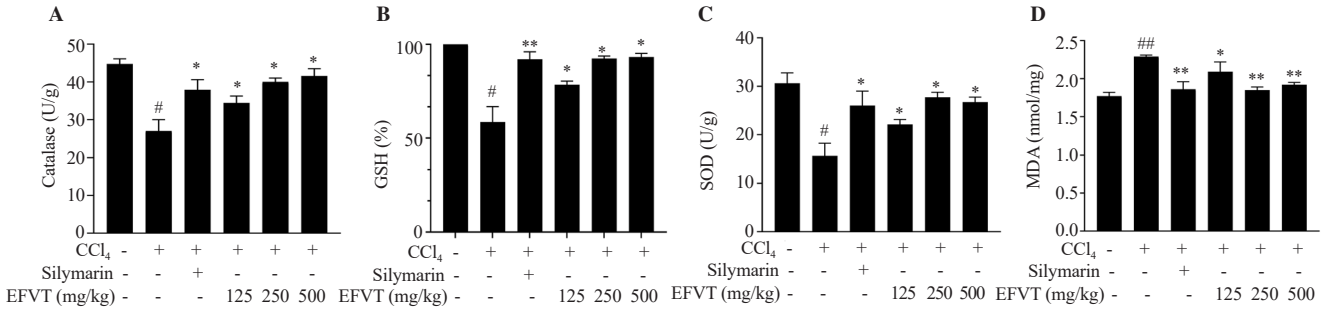

Figure 3. Effect of EFVT on antioxidant markers and lipid peroxidation in CCl_4-exposed rats. (A) Catalase, (B) GSH, (C) SOD, (D) MDA. Results are expressed as mean ± SD ($n = 6$). Statistically significant differences are indicated with $^{\#}P < 0.05$, $^{\#\#}P < 0.001$ *vs.* the control group; $^{*}P < 0.05$, $^{**}P < 0.001$ *vs.* the CCl_4 group. MDA: malondialdehyde, SOD: superoxide dismutase, GSH: glutathione.

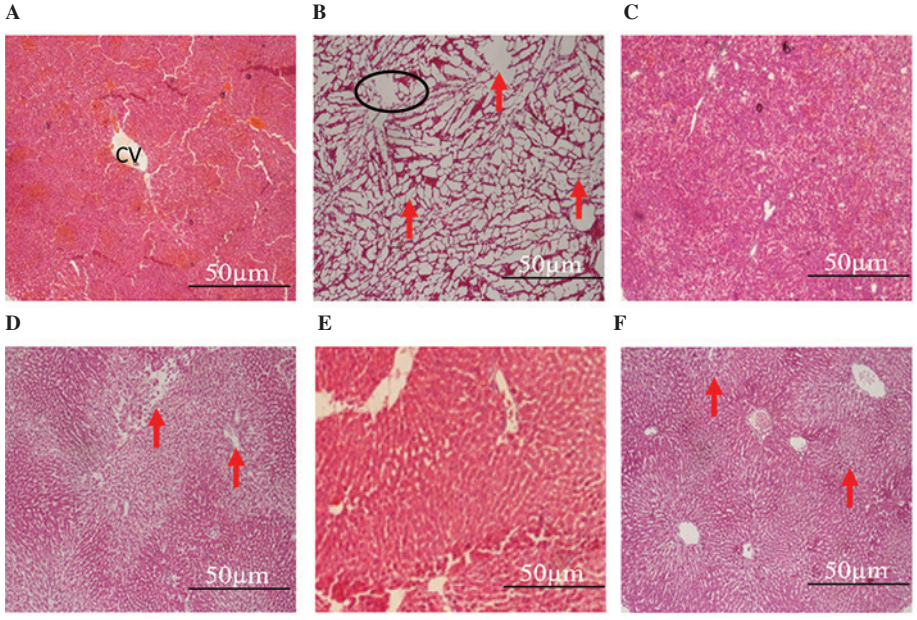

Figure 4. Effect of EFVT on CCl_4-induced histopathological alterations in liver tissue. Red arrows indicate lipid accumulation; the black circle denotes hepatocellular shrinkage. (A) Control; (B) CCl_4; (C) CCl_4 + Silymarin; (D) CCl_4 + EFVT (125 mg/kg); (E) CCl_4 + EFVT (250 mg/kg); (F) CCl_4 + EFVT (500 mg/kg). CV: central vein.

and reduced hepatocyte damage (Figure 2B-E).

3.6. Effect of EFVT on oxidative stress markers

CCl_4 triggered oxidative stress in rats, as evidenced by heightened MDA levels and diminished activities of SOD, CAT, and GSH. However, EFVT treatment could reduce oxidative stress by restoring these levels significantly (Figure 3A-D).

3.7. Histopathological results

In this study, liver damage caused by CCl_4 was examined in rats using H&E staining. The findings showed that CCl_4 disrupted the normal liver tissue structure, with noticeable fat accumulation, cell shrinkage, tissue damage, and congestion around the central vein. Treatment with EFVT significantly mitigated inflammation and restored normal liver architecture, with no visible fat accumulation or ballooning. Specifically, the treatment group receiving 250 mg/kg

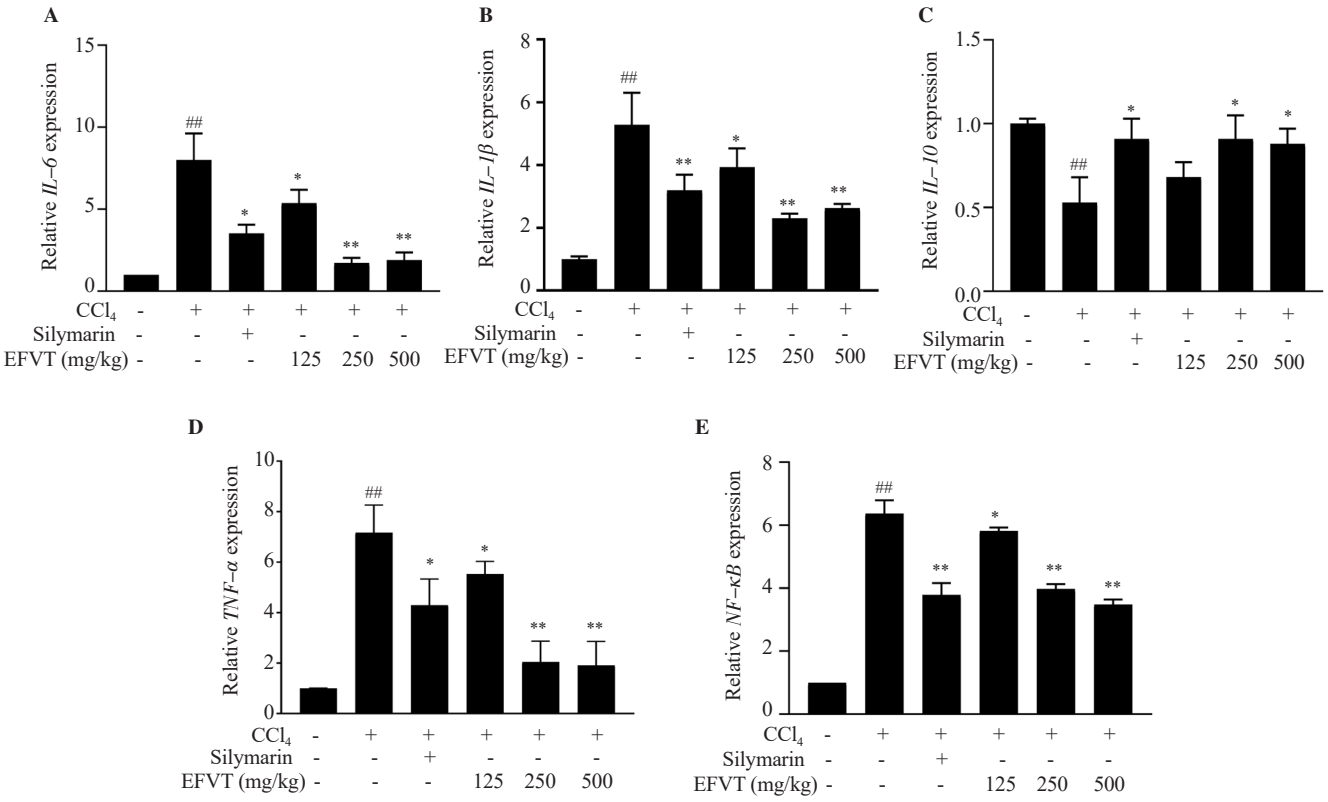

Figure 5. Effects of EFVT on the mRNA levels of (A) *IL-6*, (B) *IL-1β*, (C) *IL-10*, (D) *TNF-α*, and (E) *NF-κB* in CCl_4-exposed rats. Results are expressed as mean ± SD (n = 6). Statistically significant differences are indicated with $^{\#\#}P < 0.001$ *vs.* the control group; $^{*}P < 0.05$, $^{**}P < 0.001$ *vs.* the CCl_4 group.

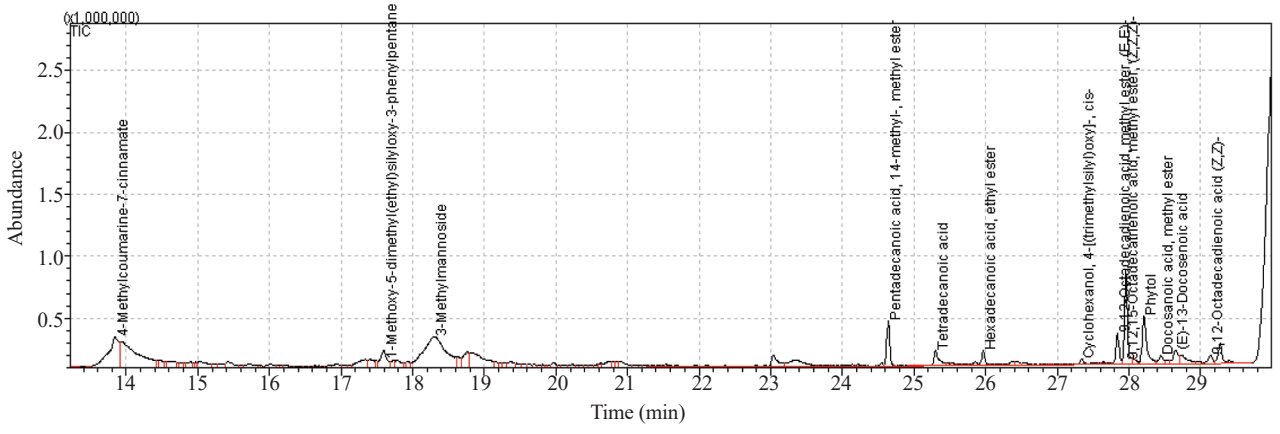

Figure 6. The GC-MS chromatogram of EFVT.

Table 1. Compounds identified by GC-MS in EFVT.

Name	Retention time (min)	Area	Concentration (%)
4-Methylcoumarine-7-cinnamate	13.840	115429	8.95
Sucrose	14.108	676689	5.95
1-Methoxy-5-dimethyl (ethyl) silyloxy-3-phenyl pentane	17.597	29689	2.30
3-Methyl mannoside	18.279	27838482	21.59
Pentadecanoic acid, 14-methyl-, methyl ester	24.639	194437	15.07
Tetradecanoic acid	25.301	52360	4.06
Hexdecanoic acid, ethyl ester	25.964	43014	3.33
Cyclohexanol, 4-[(trimethylsilyl)oxy]-, cis-	27.336	14594	1.13
9,12-Octadecadienoic acid, methyl ester, (E,E)-	27.843	64910	5.03
9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	27.961	157979	12.25
Phytol	28.211	1471774	13.32
Docosanoic acid, methyl ester	28.447	21136	1.64
(E)-13-Docosenoic acid	28.651	30990	2.40
9,12-Octadecadienoic acid (Z,Z)-	29.145	12313	0.95
7-Tetradecenal, (Z)-	29.275	38473	2.98

of EFVT exhibited no ballooning, fatty accumulation, or abnormal architecture in the liver, indicating a protective effect (Figure 4).

3.8. Effect of EFVT on the mRNA expression level of anti–inflammatory and pro–inflammatory cytokines

A further investigation was conducted to investigate the mRNA expression level of both anti-inflammatory and pro-inflammatory cytokines using qRT-PCR. CCl_4 administration upregulated the mRNA expression level of *IL–6*, *IL–1β*, *TNF–α*, and *NF–κB*, while reducing *IL–10* expression. However, EFVT treatment markedly restored *IL–10* expression and decreased the expressions of *IL–6*, *IL–1β*, *TNF–α*, and *NF–κB* (Figure 5A-E).

3.9. GC–MS analysis results

GC-MS analysis of EFVT revealed the presence of several bioactive compounds (Figure 6). The constituents were identified as 3-methyl mannoside (21.59%), pentadecanoic acid 14-methyl-methyl ester (15.07%), 9,12,15-octadecatrienoic acid methyl ester (Z,Z,Z) (12.25%), phytol (13.32%), 9,12-octadecadienoic acid, methyl ester (5.03%), and 7-tetradecenal, (Z) (2.98%), as shown in Table 1.

4. Discussion

Liver disease is one of the major health issues as the liver plays a vital role in metabolism and regulating many bodily functions. It can be damaged by various substances, including drugs, xenobiotics, liver metabolites, and microbial agents, leading to liver injury[29]. CCl_4 is a well-known hepatotoxin used to induce experimental liver injury in animal models by generating free radicals that lead to cell membrane damage, lipid peroxidation, and activation of pro-inflammatory pathways. During the metabolism, the liver generates ROS, which plays a pivotal role in hepatic damage[30]. The consumption of natural and dietary antioxidants has gained recognition as a potential therapeutic approach, demonstrating efficacy in mitigating the oxidation of susceptible cellular components, thereby inhibiting the initiation and progression of hepatic pathologies[31]. Common mullein (*V. thapsus*) of the Scrophulariaceae family, encompassing 360 *Verbascum* species, is recognized in traditional medicine for its expectorant and anti-inflammatory properties[32]. Predominantly found in Pakistan's temperate regions, its traditional use motivates this study to explore its hepatoprotective potential, extending prior research.

Our *in vitro* study's outcomes revealed the presence of various secondary metabolites in *V. thapsus*. GC-MS analysis showed that EFVT contains phytol, 3-methyl mannoside, 9,12-octadecadienoic acid, and 7-tetradecenal, with notable antioxidant activity. Additionally, phytol and 9,12-octadecadienoic acid demonstrated hepatoprotective potential[33]. Previous studies suggest that certain derivatives of methyl mannoside, pentadecanoic acid, octadecatrienoic acid, and phytol exhibit pronounced antioxidant and anti-inflammatory activities[33,34]. Moreover, 9,12-octadecadienoic acid has been reported to possess antihistaminic, anti-inflammatory, antiarthritic, and hepatoprotective activities[35,36]. Thus, our study results suggest that hepatoprotective effects of EFVT can be attributed to the presence of these bioactive compounds.

In addition, our *in vivo* study revealed no marked differences in the initial and final body weights of all groups. In the CCl_4 group, hepatomegaly was observed, as evidenced by an increased liver index. EFVT could significantly diminish liver index, and thus mitigate hepatomegaly. Liver enzymes are crucial proteins that are synthesized within liver cells and play a vital role in numerous metabolic processes. The liver produces a diverse array of enzymes, including ALT, AST, and ALP[37]. These enzymes are responsible for breaking down and metabolizing nutrients, synthesizing essential

molecules, and aiding in the detoxification of harmful substances. When the liver is damaged or afflicted by diseases, the levels of liver enzymes in the bloodstream may become elevated[38,39]. Studies have shown a correlation between elevated enzyme levels and centrilobular necrosis or hepatocytic ballooning degeneration[40]. Experimental models using substances like CCl_4 have demonstrated that hepatotoxicity leads to the disruption of cell membrane integrity, resulting in the leakage of enzymes such as ALT, AST, and ALP into the bloodstream[40,41]. Furthermore, the current research confirms the previous findings of substantial increases in AST, ALT, ALP, and total bilirubin levels in the serum following CCl_4 treatment. After EFVT treatment, noticeable changes were observed in serum enzymatic levels, which varied depending on the dosage administered. The effectiveness of EFVT as a hepatoprotective agent was found to be greatest at doses of 250 and 500 mg/kg.

Antioxidant enzymes play a crucial role in maintaining the balance of ROS within the body, protecting cells from oxidative damage, such as SOD, which is responsible for converting superoxide radicals into hydrogen peroxide and oxygen. This process helps prevent the formation of highly reactive and damaging free radicals[41]. Another enzyme is CAT, which catalyzes the breakdown of hydrogen peroxide into water and oxygen. GSH acts as both an antioxidant and a detoxifying agent. It scavenges free radicals directly and also participates in the regeneration of other antioxidants. On the other hand, MDA is a marker of lipid peroxidation, reflecting the extent of oxidative damage in cells and tissues. Monitoring MDA levels provides insights into the overall oxidative stress in the body. The increase in antioxidant enzyme levels in the treatment group suggests that EFVT enhances the liver's defense system against oxidative injury. Its ability to scavenge free radicals and boost antioxidant enzyme activity could be attributed to its rich phytochemical content, particularly polyphenols and flavonoids, which are known to exhibit strong antioxidant properties.

Histopathology is a valuable tool for evaluating liver tissue architecture and identifying histological changes associated with hepatotoxicity. In cases of liver injury, H&E staining enables the assessment of hepatocyte integrity, hepatocellular ballooning, inflammation, fibrosis, and necrosis[42]. Such histopathological changes are crucial for evaluating CCl_4-induced liver damage and serve as reliable indicators of hepatic injury. CCl_4 exposure typically results in prominent alterations, including hepatocyte necrosis, fatty degeneration, and inflammatory infiltration, which can be observed and identified in rats using H&E staining. These changes reflect the hepatotoxic effects of CCl_4, primarily mediated by oxidative stress and lipid peroxidation, leading to cellular damage and fibrosis[43]. In our study, histopathological analysis revealed significant necrosis of hepatocytes in both the periportal area and the majority of hepatic lobules, indicating severe liver damage. Additionally, hepatocytes displayed severe fat accumulation, along with multifocal areas of fatty change. However, EFVT effectively mitigated inflammation and restored the liver architecture, with no visible fat accumulation or ballooning observed. Histopathological findings suggest that EFVT effectively inhibits oxidative stress-induced hepatotoxicity.

The pathogenesis of oxidative stress-induced liver toxicity and inflammation involves intricate interactions between various molecules and signaling pathways. Among these, pro-inflammatory cytokines (IL-6, IL-1β, and TNF-α) are released in response to oxidative stress and play key roles in promoting liver inflammation[44]. The activation of NF-κB, a transcription factor that regulates immune responses and inflammation, is triggered by these cytokines[26]. Subsequently, NF-κB activation leads to the production of additional pro-inflammatory cytokines, establishing a positive feedback loop[45]. In our study, we observed elevated expression levels of *IL-6*, *IL-1β*, and *TNF-α* in the CCl_4-exposed group, indicating an overactive signaling system. However, treatment with EFVT effectively reduced the hepatic expression levels of these cytokines by inhibiting oxidative stress and NF-κB activation. Additionally, IL-10 plays a crucial role in suppressing the production of pro-inflammatory cytokines and inhibiting NF-κB activation. Acting as a negative regulator, IL-10 limits the extent of inflammation, safeguards against excessive tissue damage, and promotes tissue repair[46]. In this study, EFVT significantly upregulated the expression of *IL-10*.

EFVT treatment markedly attenuated CCl_4-induced hepatic injury, as evidenced by improvements in biochemical parameters, reduction of oxidative stress markers, mitigation of tissue necrosis, and suppression of pro-inflammatory mediators. These findings suggest that EFVT harbors bioactive constituents with notable hepatoprotective potential. Nonetheless, the study is limited by the lack of long-term toxicity evaluation and the absence of *in vivo* molecular validation of TNF-α/NF-κB pathway modulation using genetic or pharmacological approaches. Future investigations should focus on isolating the active compounds, elucidating the precise molecular mechanisms, and validating the therapeutic efficacy in clinical settings.

Conflict of interest statement

Hafiz Muhammad Zubair is an editorial board member of this journal but has no role in the decision to publish this article. No potential conflict of interest relevant to this article was reported.

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Data availability statement

The data supporting the findings of this study are available from the corresponding author upon request.

Authors' contributions

AA carried out the animal study, performed biochemical experiments, and drafted the original manuscript. HMZ supervised the project, managed research administration, contributed to conceptualization, and performed qPCR. FA conducted MTT assays and phytochemical screening tests. NUR and SMI prepared fractions and assisted in statistical analyses. SK carried out validation. AS performed histological analysis and review. UID, MA, and MSM contributed to manuscript review and editing.

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