



## Original Article

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Protective effects of turmeric extract, curcumin, demethoxycurcumin, *bis*-demethoxycurcumin, and ar-turmerone from *Curcuma longa* L. rhizomes on acetaminophen-induced hepatotoxicity in HepG2 cells

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## ABSTRACT

**Objective:** To assess the effects of turmeric extract and its compounds on oxidative stress, inflammation, and apoptosis in acetaminophen-induced liver injury.

**Methods:** HepG2 cells were administered with acetaminophen (40 mM) to induce hepatotoxicity, followed by treatment with turmeric extract and its isolated compounds including curcumin, demethoxycurcumin, *bis*-demethoxycurcumin and ar-turmerone at 5, 25, and 125 µg/mL. IL-1β, IL-6, and IL-10 levels were quantified with ELISA kits. Further, qRT-PCR was used to analyze the mRNA expression of *JNK*, *Casp-9*, and *Casp-3*. Meanwhile, the levels of nitric oxide and lactate dehydrogenase were analyzed using colorimetric assay.

**Results:** Acetaminophen administration caused an increase in the levels of lactate dehydrogenase, nitric oxide, IL-1β, IL-6, and the mRNA expression of *JNK*, *Casp-9*, and *Casp-3* in HepG2 cells while reducing IL-10 levels. Treatment with turmeric extract, curcumin, demethoxycurcumin, *bis*-demethoxycurcumin, and ar-turmerone lowered IL-1β, IL-6, nitric oxide, and lactate dehydrogenase levels, downregulated the mRNA expression of *JNK*, *Casp-9*, and *Casp-3*, and increased IL-10 levels.

**Conclusions:** Turmeric extract and its compounds have significant hepatoprotective activity and could be further explored for the treatment of liver damage.

**KEYWORDS:** Acetaminophen; Apoptosis; *Curcuma longa*; Curcumin; Hepatotoxicity; Inflammation; Oxidative stress

## Summary

**Question:** Do turmeric extract and its bioactive compounds exhibit hepatoprotective effects on acetaminophen-induced liver injury?

**Findings:** This *in vitro* study using HepG2 cells demonstrated that turmeric extract, curcumin, demethoxycurcumin, *bis*-demethoxycurcumin, and ar-turmerone significantly reduced acetaminophen-induced oxidative stress, inflammation, and apoptosis by decreasing the levels of IL-1β, IL-6, LDH, NO, and the expression of *JNK*, *Casp-3*, and *Casp-9* genes, while increasing IL-10 levels.

**Meaning:** Turmeric extract and its bioactive compounds show potential as hepatoprotective agents, offering a promising therapeutic strategy for mitigating drug-induced liver injury.

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## 1. Introduction

The liver plays an essential function in detoxifying and metabolizing drugs, foreign substances, and harmful compounds. It serves as a critical organ responsible for neutralizing toxins and facilitating their elimination from the body, thereby safeguarding overall health and well-being. Misuse or excessive use of drugs can result in liver dysfunction and other associated problems[1]. Drug-induced liver injury (DILI) is a common consequence of drug-related liver dysfunction. The rate of DILI was determined to be 13.9% cases per 100 000 people. Out of these, 12% needed hospital care, and 6% resulted in fatalities[2,3]. A 2019 study in China reported a DILI incidence of 23.8 cases/100 000 people annually[4]. Acetaminophen, known as paracetamol or *N*-acetyl-*p*-aminophenol (APAP), has been extensively studied for its potential to cause liver damage in cases of overdose in animal and human experiments[5,6]. Whether taken intentionally or unintentionally, the consumption of excessive amounts of APAP can damage the liver and in severe cases, it can progress to acute liver failure. By creating a liver injury mouse model by APAP, researchers have gained a significant understanding of its toxicity mechanisms. These insights include the creation of a reactive byproduct that relies on the activity of cytochrome P450[7]. Moreover, APAP induces cell death *via* centrilobular necrosis[8].

Drugs and their reactive metabolites can cause initial cell damage by forming reactive substances that harm mitochondria. This damage triggers oxidative stress and activates stress-detecting signaling pathways, ultimately leading to impaired mitochondrial function and stress within the endoplasmic reticulum. The elevated oxidative stress will increase free radicals such as lactate dehydrogenase (LDH) and nitric oxide (NO). Excessive reactive oxygen species (ROS) contribute to inflammatory responses, increasing the production of cytokines such as interleukin-6 (IL-6) and IL-10[9]. It also induces the production of pro-inflammatory cytokines including IL-1 $\beta$ . The elevation of IL-1 $\beta$  levels suggests the existence of inflammation[10]. The damaged mitochondria and the release of pro-inflammatory cytokines result in cell death mediated by activating apoptosome to recruit apoptotic genes such as caspase-3 (Casp-3) and Casp-9 family. The chemical exposure resulting from drug consumption can induce stress in the cells, activating c-Jun N-terminal kinase (JNK), a member of the mitogen-activated protein kinase (MAPK) family known for its pro-apoptotic effect[11]. In living cells, Casp-3 typically exists in an inactive pro-caspase form. However, during the process of apoptosis, it becomes activated, ultimately orchestrating and executing the programmed cell death pathway.

In recent years, researchers have focused extensively on investigating herbal extracts as potential protective agents for

the liver. The aim is to reduce the adverse effects caused by APAP-induced toxicity[12]. The abundant presence of bioactive phytochemicals in medicinal plants, which have shown various physiological and pharmacological activities, has sparked a surge in research efforts in this field. Scientists are increasingly exploring the potential of these compounds to provide therapeutic benefits, particularly in mitigating the harmful effects of APAP-induced toxicity on the liver[13].

Turmeric (*Curcuma longa* L.) is a perennial herb with distinctive rhizomes. This plant is classified within the Zingiberaceae family, renowned for its rich history of culinary and medicinal use[14]. Turmeric is used traditionally to treat loss of appetite, cough, sinusitis, asthma, parasitic worm infections, gonorrhea, and ailments related to the kidneys and liver, showcasing its versatility and significance in traditional and herbal medicine[15,16]. Turmeric is composed of numerous chemical compounds, each with distinct biological and pharmacological properties. Curcuminoids, which are prominent phenolic compounds, are derived from the turmeric plant[17]. These substances are widely recognized for their various biological and therapeutic properties, making them a topic of thorough investigation and interest in the scientific community. Notably, it exhibits antioxidant and anti-inflammatory activities, attracting significant attention in natural medicine field[18].

This investigation aimed to evaluate the impact of turmeric extract (TE) and phytoconstituents on oxidative stress, inflammatory cytokine production, and the apoptosis-related gene expression including *Casp-3*, *Casp-9*, and *JNK*.

## 2. Materials and methods

### 2.1. Extraction preparation

TE was acquired from PT Fathonah Amanah Siddiq Tabligh (Depok, West Java, Indonesia) with CoA No. Batch 001102010070. TE was processed by extracting the rhizome of turmeric with 70% ethanol as the solvent. The resulting crude ethanolic TE was then mixed with lactose as an additional component and subsequently dried to yield a powdered form of the extract[19].

### 2.2. Isolation of compounds

Isolation of pure compounds was carried out by subjecting the ethanol extract to preparative liquid chromatography and mass spectrometry using methanol and water at 1:1 (*v/v*) at 10 mL/min flow rate, while the structures were elucidated by NMR spectroscopy (<sup>1</sup>H NMR, 500 MHz and <sup>13</sup>C NMR, 125 MHz)[19].

### 2.3. Cell culture and hepatotoxicity induction

HepG2 cells, a human hepatocellular carcinoma cell line (ATCC<sup>®</sup> HB-8065<sup>™</sup>) were obtained from Aretha Medika Utama, Bandung, West Java, Indonesia. After the cells reached 90% confluence,  $5 \times 10^3$  cells were planted into a 6-well plate (Costar, 3516) and co-cultured in a complete medium. To induce hepatotoxicity, APAP (40 mM) (Sigma Aldrich, A7085) was administered and underwent a 24-hour incubation in a humid environment (37 °C with 5% CO<sub>2</sub>). TE was then administered to the cells, along with its constituent before an additional 24 h incubation. The experimental groups were divided into: I ) APAP group (HepG2 cells induced with APAP), II ) normal control (non-treated HepG2 cells); III) vehicle control (HepG2 cells induced with APAP + DMSO 1%), IV) APAP + curcumin, V ) APAP + demethoxycurcumin, VI) APAP + *bis*-demethoxycurcumin, VII) APAP + ar-turmerone, VIII) APAP + TE, with various concentrations such as 5, 25, and 125 µg/mL. The concentrations were determined based on a preliminary study, which showed that the extract at 125 µg/mL exhibited little cytotoxicity against HepG2 cells with cell viability above 90%. However, concentrations above 125 µg/mL resulted in decreased cell viability. Therefore, concentrations of 5, 25, and 125 µg/mL were selected for this study. Subsequently, the collected cells were spinned down for 10 min at 1 600 rpm. For total protein, ELISA, and colorimetric assays, the supernatant was collected, and the pellet was reserved for relative mRNA expression quantification[20].

### 2.4. Protein assay

Standard solution of bovine serum albumin (BSA) from Sigma Aldrich (A9576) was dissolved in double distilled water with a ratio of 1:50. The supernatant of treated HepG2 cells was used as a sample, and plated into a 96 well-plate along with 200 µL Quick Start Dye Reagent 1× from Biorad (5000205). Following a five-minute incubation, the absorbance was recorded using a spectrophotometer at a 595 nm wavelength[20].

### 2.5. Quantification of IL-10, IL-6, and IL-1β levels

The measurement of IL-10, IL-6, and IL-1β levels was conducted using the Elabscience Human IL-6 ELISA kit (E-EL-H6156), Human IL-1β ELISA kit (E-EL-H0149) and Human IL-10 ELISA kit (E-EL-H6154). Samples (conditioned medium from the treatment) and standard working solutions were prepared at approximately 100 µL per well in the ELISA kit. The plates were then incubated at 37 °C for 90 min. After incubation, the liquid in each well was discarded, and 100 µL of biotinylated detection antibody working solution was added. The solution was thoroughly mixed and incubated for 60 min at 37 °C. Following incubation, the solution was discarded,

and 350 µL of wash buffer was added and soaked for 1-2 min. This washing step was repeated three times. Next, 100 µL of horseradish peroxidase (HRP) conjugate working solution was added and incubated for 30 min at 37 °C. The wells were then washed five times with 350 µL of wash buffer. Subsequently, substrate reagent (90 µL) was added, the plate was covered with a new sealer, and incubation continued for 15 min at 37 °C in the dark until a blue color developed. Finally, 50 µL of stop solution was added, turning the solution yellow. The absorbance was measured at a wavelength of 450 nm. The IL-10, IL-6, and IL-1β concentrations were calculated based on their percentage values compared to the standard curve[21].

### 2.6. Quantification of NO levels

Nitrite levels, indicative of NO production, were assessed using the NO Colorimetric Assay from ElabScience (E-BC-K035-M). A standard curve was generated using sodium nitrite to quantify the nitrite levels[22].

### 2.7. Quantification of LDH level

LDH assay was conducted by colorimetric LDH assay kit (Elabscience, E-BC-K046-S) based on the manufacturer's instruction. The LDH that is released in the medium was collected and quantified using a spectrophotometer[23].

### 2.8. Quantification of JNK, Casp-9, and Casp-3 gene expression

The gene expressions of *JNK*, *Casp-9*, and *Casp-3* were measured using a qRT-PCR instrument. Direct-zol RNA Miniprep Plus (Zymo, R2073) and SensiFAST cDNA Synthesis Kit (Meridian Bioscience, BIO-65053 and BIO-65054) were used to extract mRNA and converted into cDNA respectively. The quantity of total RNA obtained was determined by a microplate reader at 260/280 nm. To quantify the gene expressions, AriaMC PCR (Agilent) was used with a pre-incubation cycle at 95 °C, annealing 57 °C and elongated at 72 °C. The procedure was carried out for 30 s, 40 cycles, and 1 min, respectively[24]. The primer sequence used was obtained from www.ncbi.nlm.nih.gov, as written in Supplementary Table 1.

### 2.9. Statistical analysis

Data analysis was performed using SPSS software (version 25.0). A one-way analysis of variance (ANOVA) was conducted to compare treatment outcomes. Subsequently, significant differences among the treatments were further confirmed using Tukey's *post hoc* tests, with the significance level set at  $P < 0.05$ [25].

### 3. Results

#### 3.1. TE quality

The parameters checked met the requirements based on the Attachment to the Regulation of the Head of the Indonesian Food and Drug Authority Number 32 of 2019, which addresses Quality Requirements for Traditional Medicines as evident from the data presented in Supplementary Table 2.

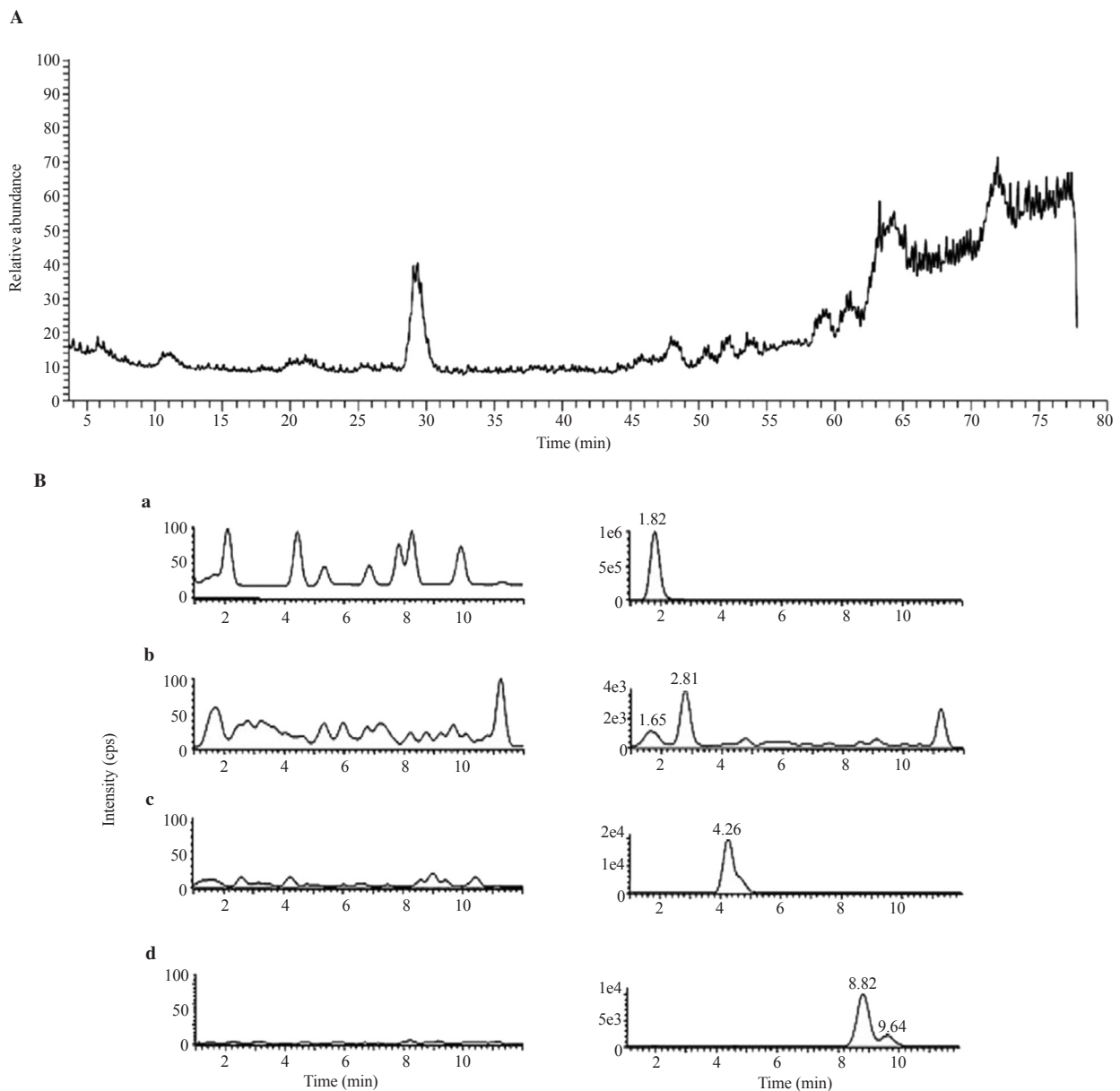
#### 3.2. Bioactive compounds in TE

The analysis using liquid chromatography showed that TE

contained numerous bioactive compounds such as curcumin, demethoxycurcumin, *bis*-demethoxycurcumin, and ar-turmerone. The chromatogram of this result is shown in Figure 1, with the mass spectra of each compound elucidated in Figure 2 and Supplementary Table 3. Meanwhile, the structure of targeted compounds was explained by NMR spectroscopy, as illustrated in Supplementary Figure and Supplementary Table 4.

#### 3.3. Effects of TE on total protein level in HepG2 cells

The effects of TE and the isolated compounds on the level of total protein in activated HepG2 cells is presented in Figure 3A. APAP induction reduced protein levels compared to the normal control group. TE treatment increased the protein level significantly,

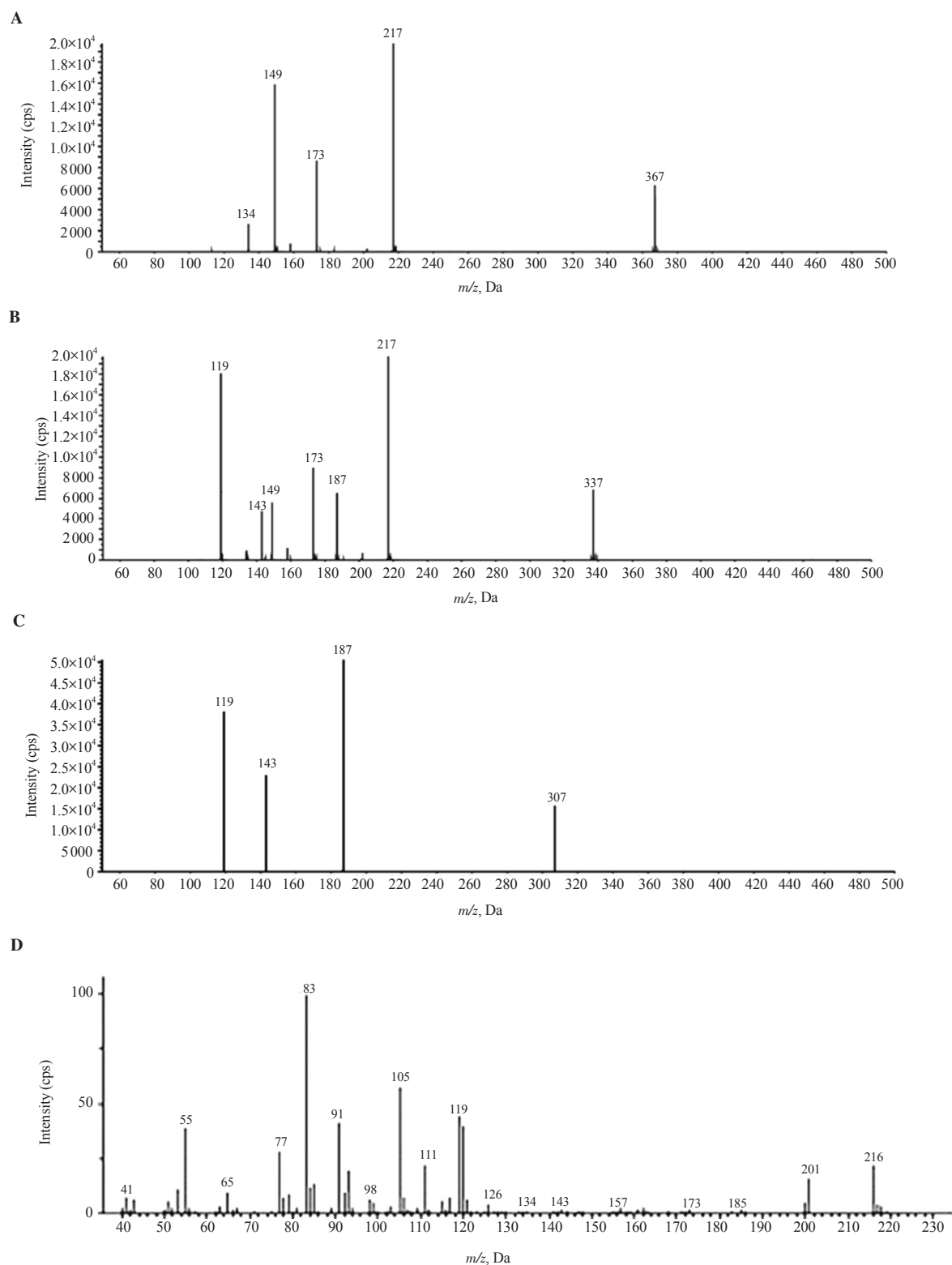


**Figure 1.** LC-MS profile of (A) turmeric extract and (B) multiple reaction monitoring transitions setting for the isolated compounds from turmeric extract (a: curcumin; b: demethoxycurcumin; c: *bis*-demethoxycurcumin; d: ar-turmerone).

with a significant effect observed at 5  $\mu\text{g/mL}$ . Among all isolated compounds in TE, ar-turmerone showed no significant result compared to TE, followed by curcumin, demethoxycurcumin, and bis-demethoxycurcumin.

### 3.4. Effects of TE and isolated compounds on IL-1 $\beta$ , IL-6, and IL-10 levels

The impact of TE on the level of IL-1 $\beta$  and IL-6 levels is presented in Figure 3B and 3C. TE at 25  $\mu\text{g/mL}$  showed the lowest level of IL-



**Figure 2.** Mass spectra of (A) curcumin, (B) demethoxycurcumin, (C) bis-demethoxycurcumin and (D) ar-turmerone.

1 $\beta$  (28.04 pg/mL) and IL-6 (40.97 pg/mL) compared with the APAP-administered group. The results showed that APAP significantly heightened IL-1 $\beta$  and IL-6 levels. Concerning the isolated compounds, *bis*-demethoxycurcumin exhibited the most pronounced inhibitory effect on IL-1 $\beta$  expression (30.63 pg/mL), whereas demethoxycurcumin demonstrated superior activity in suppressing IL-6 production (38.50 pg/mL) at 25  $\mu$ g/mL.

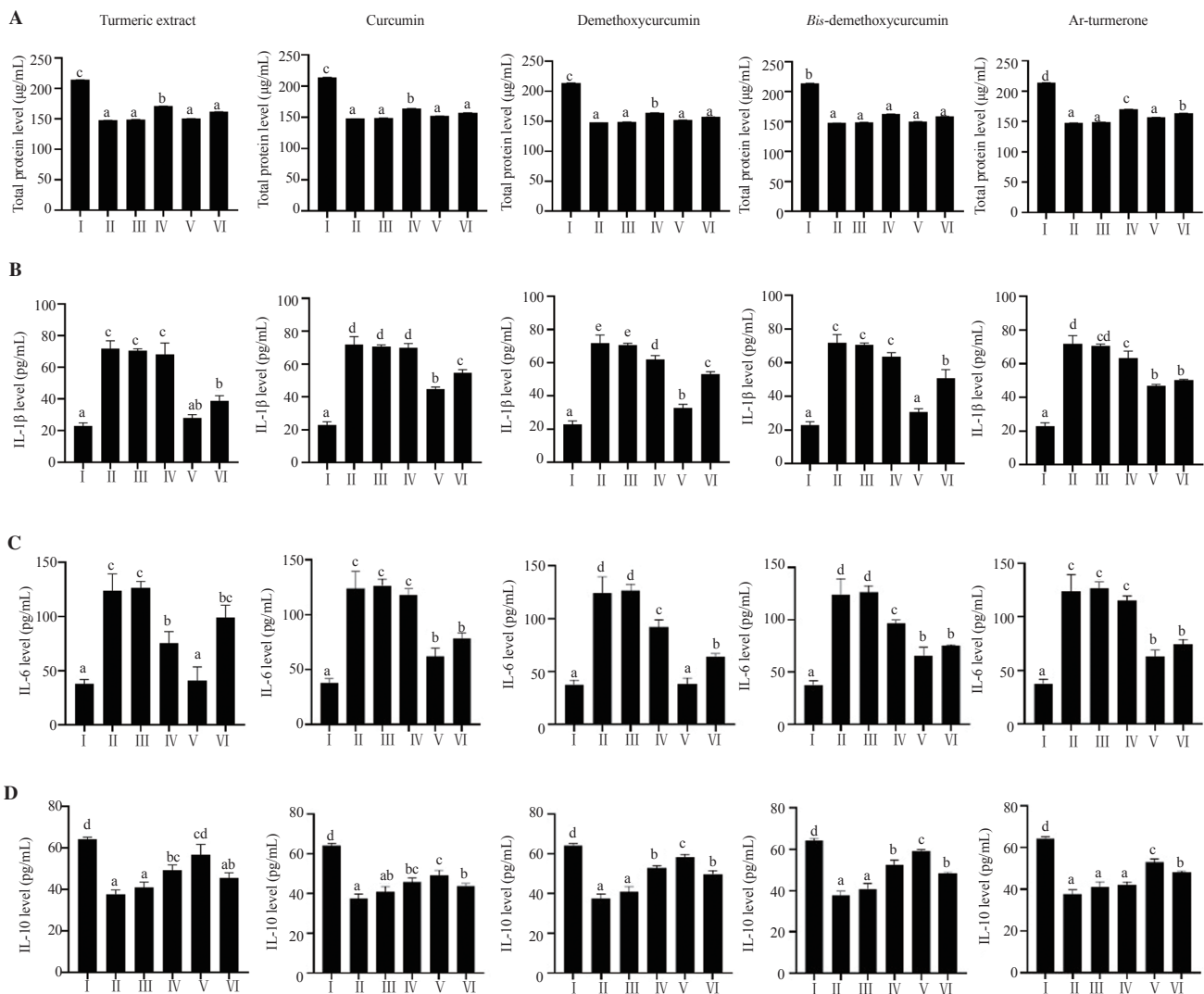
The effect of TE on IL-10 level is presented in Figure 3D, Notably, TE at 5, 25, and 125  $\mu$ g/mL increased IL-10 levels. Furthermore, TE at 25  $\mu$ g/mL was found to increase the IL-10 level (56.69 pg/mL) most significantly. Among the isolated compounds, *bis*-demethoxycurcumin at 25  $\mu$ g/mL exhibited a significant increase in the IL-10 level of 59.22 pg/mL followed by demethoxycurcumin (58.23 pg/mL), ar-turmerone (52.97 pg/mL), and curcumin (49.35 pg/mL).

### 3.5. Effects of TE and isolated compounds on NO levels

TE at 5, 25, and 125  $\mu$ g/mL decreased the NO concentration in HepG2 cells compared to the APAP-administered group (Figure 4A). TE at 25  $\mu$ g/mL was the most effective in reducing NO level (96.17  $\mu$ mol/L). In addition, all compounds exhibited a considerable decrease in the NO levels with *bis*-demethoxycurcumin as the most active (66.94  $\mu$ mol/L), followed by demethoxycurcumin (69.11  $\mu$ mol/L), ar-turmerone (72.22  $\mu$ mol/L), and curcumin (84.28  $\mu$ mol/L).

### 3.6. Effects of TE and isolated compounds on LDH levels

TE at all concentrations markedly lowered the LDH level compared

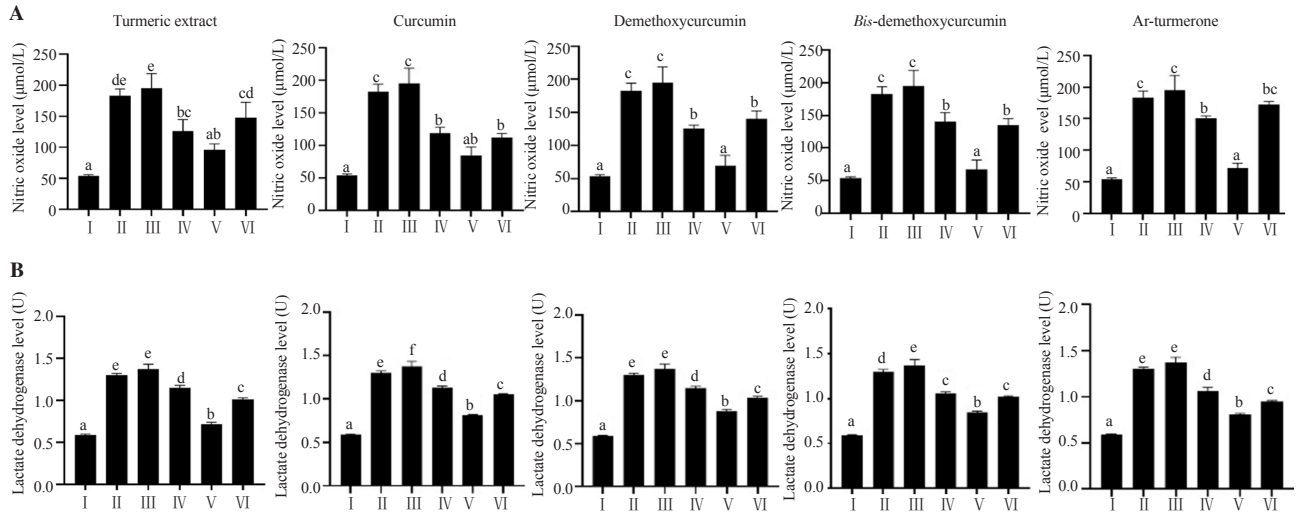


**Figure 3.** Effects of turmeric extract and its phytoconstituents on total protein (A), IL-1 $\beta$  (B), IL-6 (C), and IL-10 (D) levels in HepG2 cells with acetaminophen-induced hepatotoxicity. (I) normal control; (II) acetaminophen only; (III) vehicle control (acetaminophen + DMSO 1%); acetaminophen + turmeric extract, curcumin, demethoxycurcumin, *bis*-demethoxycurcumin, or ar-turmerone at doses of (IV) 5, (V) 25 and (VI) 125  $\mu$ g/mL. Results are given as mean  $\pm$  SD of three independent experiments and analyzed by ANOVA analysis followed by Tukey *post hoc* test. Distinct letters represent statistically significant differences between different groups at  $P < 0.05$ .

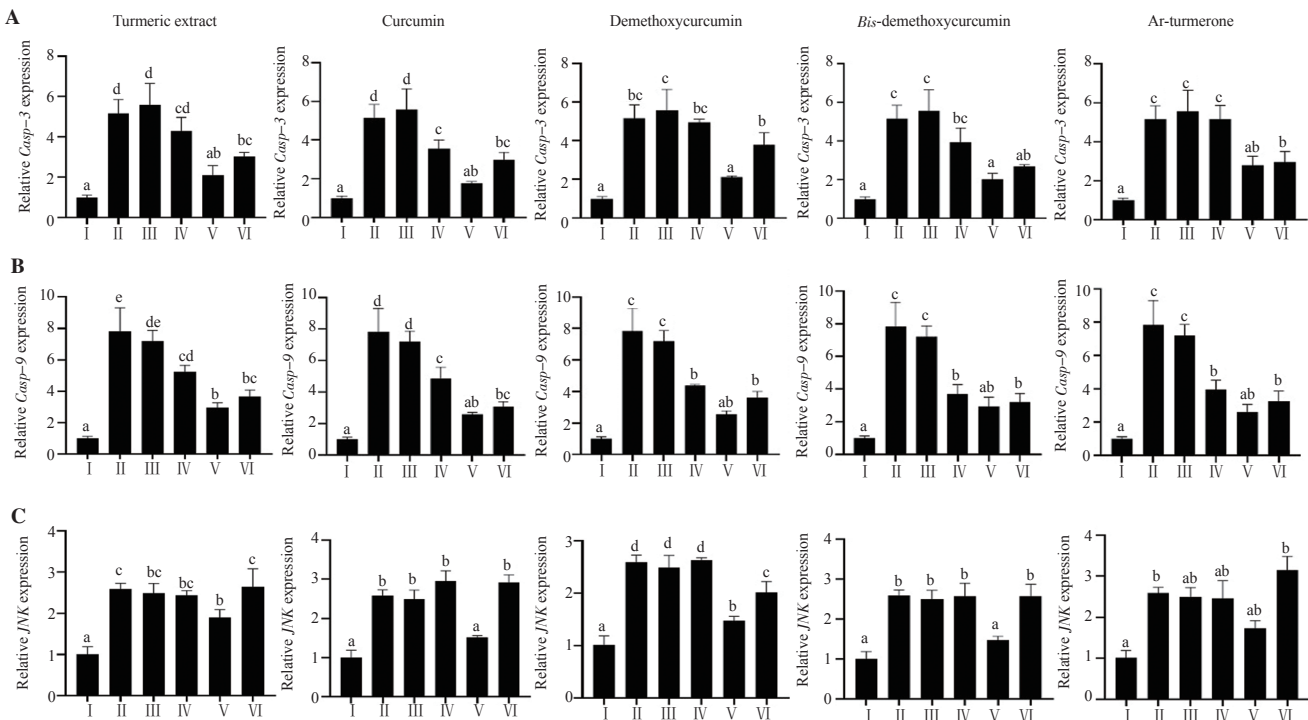
with the APAP-administered group (Figure 4B). The most significant effect was found at 25  $\mu\text{g}/\text{mL}$  with a LDH level of 0.72 U. Moreover, among isolated compounds from TE, a considerable decrease in the LDH levels was noticed in the 25  $\mu\text{g}/\text{mL}$  curcumin-treated group with a LDH level of 0.81 U.

### 3.7. Effects of TE and isolated compounds on *Casp-3*, *Casp-9*, and *JNK* gene expressions

The gene expressions of *Casp-3* and *Casp-9* were substantially downregulated by treatment with all concentrations of TE, especially at 25  $\mu\text{g}/\text{mL}$  (Figure 5A-5B). Furthermore, 25  $\mu\text{g}/\text{mL}$  of TE



**Figure 4.** Effects of turmeric extract and its phytoconstituents on nitric oxide (A) and lactate dehydrogenase (B) levels in HepG2 cells with acetaminophen-induced hepatotoxicity. ( I ) normal control; (II) acetaminophen only; (III) vehicle control (acetaminophen + DMSO 1%); acetaminophen + turmeric extract, curcumin, demethoxycurcumin, *bis*-demethoxycurcumin, or ar-turmerone at doses of (IV) 5, (V) 25 and (VI) 125  $\mu\text{g}/\text{mL}$ . Results are given as mean  $\pm$  SD of three independent experiments and analyzed by ANOVA analysis followed by Tukey *post hoc* test. Distinct letters represent statistically significant differences between different groups at  $P < 0.05$ .



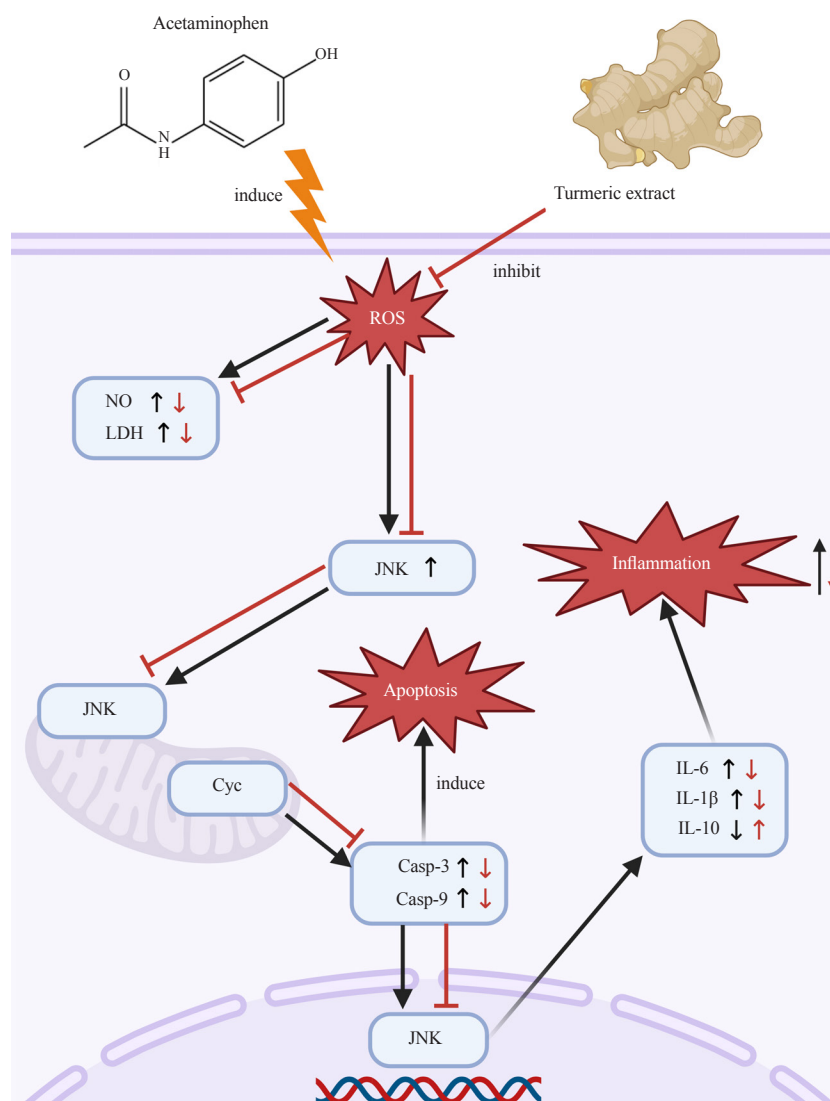
**Figure 5.** Effects of turmeric extract and its phytoconstituents on *Casp-3* (A), *Casp-9* (B), and *JNK* (C) gene expression in HepG2 cells with acetaminophen-induced hepatotoxicity. ( I ) normal control; (II) acetaminophen only; (III) vehicle control (acetaminophen + DMSO 1%); acetaminophen + turmeric extract, curcumin, demethoxycurcumin, *bis*-demethoxycurcumin, or ar-turmerone at doses of (IV) 5, (V) 25 and (VI) 125  $\mu\text{g}/\text{mL}$ . Results are given as mean  $\pm$  SD of three independent experiments and analyzed by ANOVA analysis followed by Tukey *post hoc* test. Distinct letters represent statistically significant differences between different groups at  $P < 0.05$ .

noticeably downregulated the gene expression of *JNK* (Figure 5C) while other concentrations showed no decreasing effects on its expression. Among four isolated compounds, curcumin presented the most remarkable effect on downregulating these gene expressions.

#### 4. Discussion

Hepatotoxicity, a potentially fatal illness brought on by an overabundance of APAP, is characterized by severe liver damage. Understanding the mechanisms and consequences of APAP overdose is essential for improving patient safety and medical intervention strategies[26]. The present study revealed a substantial increase in IL-1 $\beta$ , IL-6, LDH, and NO levels following APAP administration along

with a decrease in IL-10 level. APAP-caused hepatotoxicity is also distinguished by severe oxidative stress[27]. Oxidative stress leads to an increase in NO levels[28]. NO is a highly volatile and transient free radical that was once primarily recognized as an air pollutant[29]. This research demonstrates that TE, curcumin, demethoxycurcumin, *bis*-demethoxycurcumin, and *ar*-turmerone significantly reduce the levels of NO. This is consistent with previous research that examined the impact of nano-curcumin supplementation on synovial collagenase- II and NO levels and found that it effectively decreased synovial NO levels. These results highlight the potential of both TE and nano-curcumin in modulating NO levels, highlighting their roles in mitigating NO-related physiological effects[30]. The potential of rhizome extract can be linked to the existence of the major substances isolated but the synergistic effects of other compounds



**Figure 6.** Proposed mechanism of turmeric extract in acetaminophen-induced HepG2 cells. Continuous consumption of acetaminophen can lead to liver damage by increasing free radicals, inflammatory markers, and apoptosis markers. Turmeric extract can counteract NO, and reduce IL-6, IL-1 $\beta$ , JNK, LDH, Casp-3, and Casp-9, while increasing IL-10. Black arrows depict acetaminophen-induced activation of liver injury pathways, while red arrows show the inhibitory actions of turmeric extract on oxidative stress, apoptosis, and inflammation. NO: nitric oxide; LDH: lactate dehydrogenase.

cannot be neglected.

Besides that, elevated LDH levels in the bloodstream may serve as an indicative marker for various medical conditions, including liver disease[31]. LDH is a crucial enzyme in anaerobic metabolic processes and takes a part in conversion of lactate into pyruvate[32]. The findings indicate a significant decrease in LDH levels following the TE administration and isolated compounds. Aqueous extract from turmeric could decrease LDH levels in H<sub>2</sub>O<sub>2</sub>-induced fish hepatotoxicity[32]. These results show that APAP administration triggers Kupffer cell activation, resulting in anti-inflammatory and pro-inflammatory cytokines elevation. Cytokines play pivotal roles in immune responses, inflammation, cell growth, differentiation, and regulation of cell death, underscoring their significance in understanding the broader implications of APAP-induced effects[33]. IL-1 $\beta$  serves as a key cytokine renowned for potent inflammatory properties. This cytokine plays a critical part in the body's natural defense mechanisms against injury and infection, contributing significantly to the orchestration of immune responses and tissue repair[34]. Our research has uncovered a noteworthy reduction in IL-1 $\beta$  levels achieved through the use of TE and isolated compounds. This aligns with findings from previous studies, which reported that curcumin treatment effectively prevented NF- $\kappa$ B activation and led to a substantial reduction in IL-1 $\beta$  expression[35]. Curcumin also reduced the protein and gene expressions of IL-1 $\beta$  in the myocardium. This implies that curcumin exhibits anti-inflammatory effects and has potential to modulate IL-1 $\beta$ -associated pathways[35].

IL-6 is a cytokine recognized for its inflammatory attributes and plays a multifaceted part in various biological processes, including immune dysregulation and cancer development. Its diverse functions underscore its significance in both health and disease[36,37]. Nonetheless, excessive IL-6 production is linked to the development of multiple diseases, highlighting its dual role in immune regulation and disease development[38]. The present study observed that TE and isolated compounds effectively reduced the IL-6 levels and is consistent with the previous finding which showed the combination of capsaicin and curcumin was capable of decreasing IL-6 expression in human peripheral blood mononuclear cells[38]. In another study, curcumin demonstrated complete inhibition of IL-6-induced proliferation of U266 cells[39]. These results highlight the broad potential of curcumin as an effective agent against IL-6-mediated cell proliferation. IL-10 is widely acknowledged for its robust anti-inflammatory attributes and its capability to modulate the immune system. Consequently, IL-10 possesses the potential to alleviate tissue damage stemming from inflammation, making it a pivotal regulator of immune responses and tissue homeostasis[40]. This study revealed that IL-10 levels were increased significantly by treatment with TE and its compounds, suggesting that these compounds might be implicated in a proper dosage form to regulate

the immune responses and tissue homeostasis.

Furthermore, APAP upregulated *Casp-3*, *Casp-9*, and *JNK* gene expressions. *Casp-3*, a member of the cysteine protease group, holds a pivotal role in initiating cellular apoptosis. *Casp-3* serves as an important marker in apoptosis, with increased *Casp-3* expression signifying a heightened level of apoptotic activity[10]. *Casp-9* is commonly recognized as a pivotal protein involved in initiating apoptosis in various types of cancer cells. This process requires a cascade of phases leading to programmed cell death. *Casp-9* is also important for regulating and executing this critical pathway[41]. In a previous study, the supplementation of curcumin nano-micelles showed a noteworthy reduction in *Casp-9* expression levels[41], suggesting the potential of the nano curcumin micelles in modulating *Casp-9* gene expression and highlighting their key roles in influencing apoptotic pathways[42]. Consistent with the previous study, a significant decrease in *Casp-3* and *Casp-9* gene expression was observed in the groups treated with TE and its compounds.

One of the genes that are involved in the extrinsic apoptotic pathway is JNKs[43]. JNKs directly modulate distinct phosphorylation events that regulate the activities of mitochondrial proteins that either promote or inhibit apoptosis[43]. A previous study reported that curcumin significantly reduced the phosphorylation of JNK[43]. This is in accordance with this study, in which the *JNK* gene expression was significantly downregulated by TE and its compounds. Another study also revealed that curcumin markedly diminished IL-1 $\beta$ -mediated phosphorylation of JNK and p38[44] and the findings of the present study align with those of previously published reports.

The hepatoprotective activity of curcumin, a compound found in TE, is primarily attributed to its capacity to effectively neutralize free radicals and its anti-inflammatory attributes. Curcumin's ability to counter oxidative stress and reduce liver inflammation has made it a subject of extensive research and a possible treatment agent for liver health[45]. Curcumin has a significant part in promoting the healing of liver cells by mitigating inflammation. Anti-inflammatory properties have been shown to contribute to the restoration and regeneration of damaged liver tissues, establishing it as a promising option for liver health interventions. When the ethanolic extract of turmeric is administered orally, it exhibits a hepatoprotective effect that varies with dosage[17]. Moreover, turmeric is enriched with volatile oils, which further contribute to its anti-inflammatory attributes. The current study demonstrates that administration of TE and compounds in APAP-induced HepG2 cells effectively mitigated the levels of all the parameters investigated. The use of APAP-induced HepG2 cells is expected to better demonstrate the hepatoprotective potential of TE. If TE can exert hepatoprotective effects in a hepatotoxicity model, it is likely that normal liver cells would also benefit from its protective effects. This study suggests TE and compounds as a potential protective agent against APAP-

induced effects on these genes, with its possible mechanism shown in Figure 6.

However, the study was conducted using an *in vitro* model with HepG2 cells, which may not fully replicate the complex physiological responses of the liver *in vivo*. The absence of liver-specific metabolic processes may limit the generalizability of the results. Further validation using *in vivo* pharmacokinetic and pharmacodynamic studies is necessary to confirm their efficacy and bioavailability. Additionally, this study primarily focused on selected oxidative stress, inflammation, and apoptosis markers; other pathways involved in hepatoprotection, such as autophagy and mitochondrial function, were not explored. Future research should involve *in vivo* studies, clinical trials, and broader mechanistic evaluations to further establish the therapeutic relevance of TE in drug-induced liver injury.

Based on our findings, prolonged APAP exposure contributes to liver damage by increasing free radical production, inflammatory markers, and apoptosis-related gene expression. TE and its isolated compounds demonstrated antioxidant activity, reducing NO, IL-1 $\beta$ , IL-6, and LDH levels while downregulating *Casp-3*, *Casp-9*, and *JNK* gene expression. Additionally, TE increased the anti-inflammatory cytokine IL-10 levels. These results suggest that TE and its bioactive compounds may have hepatoprotective properties. However, further studies are required to elucidate their precise mechanisms of action and evaluate their potential therapeutic applications in liver-related conditions.

### Conflict of interest statement

The authors declare that no competing interests exist.

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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

WW conceptualized, supervised the research project, contributed to manuscript drafting and revision, and secured funding for the project. DRL, DKP, and DR were responsible for the design and execution of the cell-based experiments and data analysis. SRK and JAD performed compound isolation and characterization. DP contributed to the methodology and interpretation of the gene expression data. NSMD, AFS, and RA conducted ELISA and colorimetric assays and performed statistical analyses. DSH was involved in data curation, graphical illustrations, and manuscript editing. ME provided critical revisions and ensured the scientific integrity of the manuscript. All authors have read and approved the final version of the manuscript.

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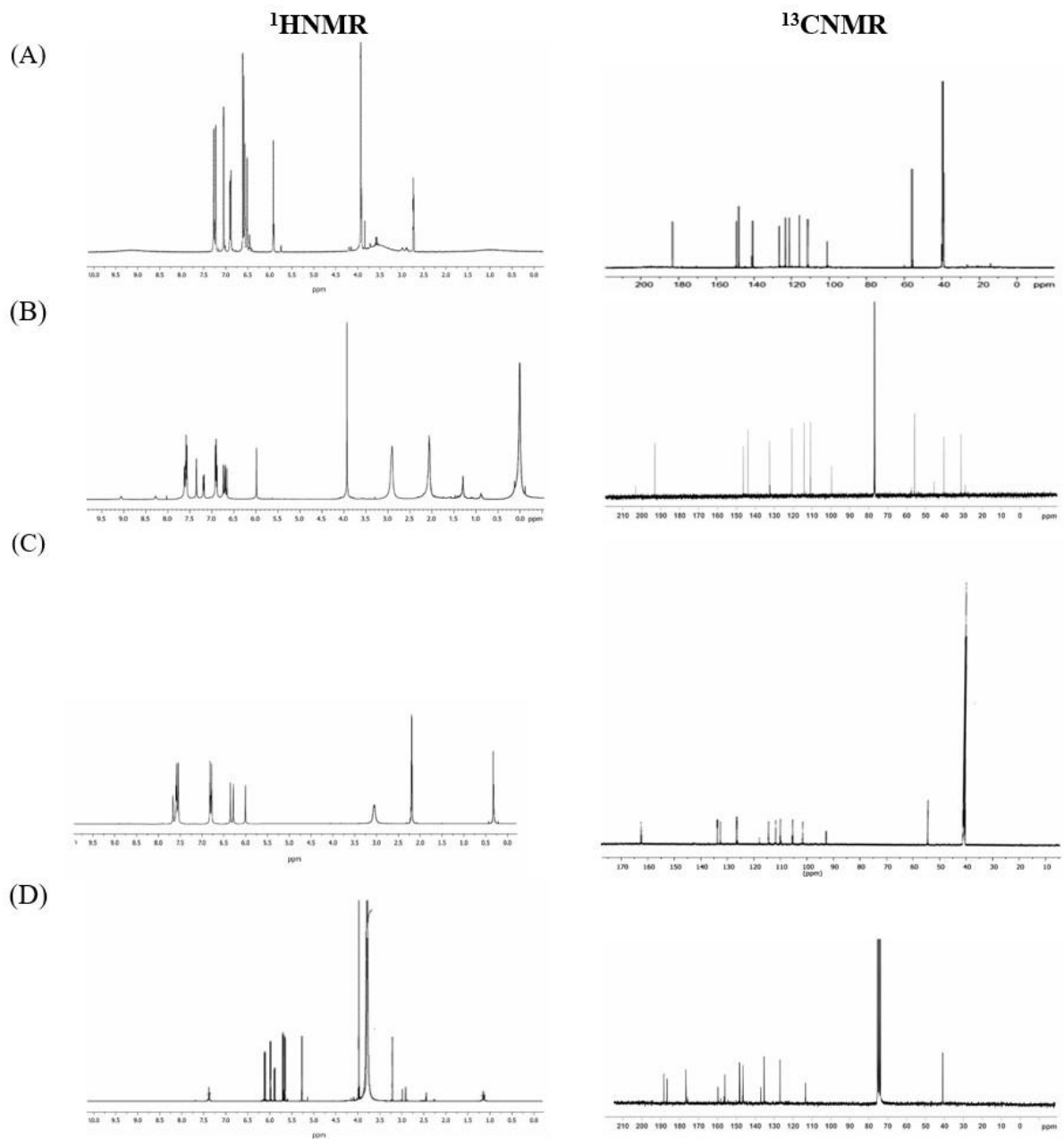
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**Supplementary Figure.**  $^1\text{H}$ NMR and  $^{13}\text{C}$ NMR spectrum of (A) curcumin, (B) demethoxycurcumin, (C) *bis*-demethoxycurcumin, and (D) ar-turmerone.

**Supplementary Table 1.** Primer sequence.

<b>Gene</b>		<b>Primer sequence</b>	<b>Product size (bp)</b>	<b>Annealing (°C)</b>	<b>References</b>
Human	F	5'-AGACCTGTACGCCAACACAG-3'	213	54	M28424.1
<i>β-Actin</i>	R	5'-TTCTGCATCCTGTCGGCAAT-3'			
Human	F	5'-CATGCTCAGGATGTAAGCCA-3'	241	60	NM_011577.2
<i>Casp-9</i>	R	5'-AGGTTCTCAAGACCGAAACA-3'			
Human	F	5'-AGAACTGGACTGTGGCATTGAG -3'	258	58	NM_011339.2
<i>Casp-3</i>	R	5'-CAGTGTTCTCCATGGATACCTTTATT-3'			
Human	F	5'-GTCTTGCATCTGTTTCTCCA-3'	163	58	NM_010548.2
<i>JNK</i>	R	5'-CACCAAGAAGCCTTGACAG-3'			

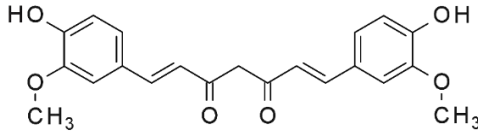
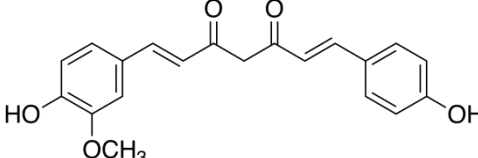
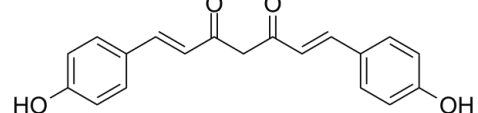
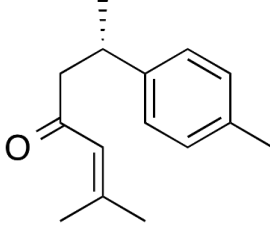
**Supplementary Table 2.** The TE quality test.

Item	Specification	Method	Result
<b>Organoleptic</b>			
Form	Fine powder	Sensory analysis	Qualified
Color	Yellow	Sensory analysis	Qualified
Odor	Typical aromatic odor	Sensory analysis	Qualified
Flavor	Bitter	Sensory analysis	Qualified
<b>Physical characteristic</b>			
Extract ratio	1:1	-	Qualified
80 mesh testing	≥ 90%	Testing sieve	Qualified
Solubility	Low solubility in water	-	Qualified
Water content	< 10%	432/01/2019/QC	Qualified
<b>Microbiological contamination /1 g</b>			
Total plate count (TPC)	≤10 <sup>5</sup> colony	415/03/2019/QC	3.2 x 10 <sup>2</sup>
Yeast cell count	≤10 <sup>3</sup> colony	415/03/2019/QC	1.6 x 10 <sup>1</sup>
<i>E. Coli</i>	≤10 colony	415/03/2019/QC	< 1.0 x 10 <sup>1</sup>
<i>Enterobacteriaceae</i>	≤10 <sup>3</sup> colony	415/03/2019/QC	< 1.0 x 10 <sup>1</sup>
<i>Clostridia</i>	Negative	415/03/2019/QC	Negative
<i>Salmonella</i>	Negative	415/03/2019/QC	Negative
<i>Shigella</i>	Negative	415/03/2019/QC	Negative

**Supplementary Table 3.** Targeted compounds identification.

TE targeted compounds	MW (g/mol)	MS [M+H] <sup>+</sup>	[M+NH <sub>4</sub> ] <sup>+</sup>	[M+Na] <sup>+</sup>	[M+K] <sup>+</sup>	[M-H] <sup>-</sup>	[M+Na-2H] <sup>-</sup>	[M+Cl] <sup>-</sup>	[M+K-2H]
Curcumin	364.40	369.40	386.43	391.39	407.36	367.39	389.37	403.37	405.35
Demethoxycurcumin	338.40	339.41	356.43	361.39	377.36	337.39	359.37	373.37	375.35
<i>bis</i> -demethoxycurcumin	308.30	309.31	326.33	331.29	347.26	307.29	329.27	343.27	345.25
ar-turmerone	216.32	217.33	234.35	239.31	255.28	215.31	237.29	251.29	253.27

Supplementary Table 4. Chemical structure of TE phytoconstituent by NMR.

Chemical structure	<sup>1</sup> H NMR (DMSO, 500MHz, δ ppm)	<sup>13</sup> C NMR (CDCl <sub>3</sub> , 125MHz, δ ppm)
 <p>Curcumin</p>	7.58 (2H, d, <i>J</i> =15.9 Hz), 7.27 (2H, d, <i>J</i> =1.9 Hz), 7.15 (2H, ddd, <i>J</i> =0.3, 1.9, 8.2 Hz), 6.89 (2H, d, <i>J</i> =8.2Hz), 6.70 (2H, d, <i>J</i> =15.9 Hz), 5.94 (1H, s), 3.93 (6H, s)	56.6, 102.1, 111.8, 116.2, 122.4, 124.2, 128.2, 141.4, 148.8, 149.8, 184.2
 <p>Demethoxycurcumin</p>	7.59 (1H, d, <i>J</i> =16 Hz), 7.58 (2H, d, <i>J</i> =16 Hz), 7.56 (2H, d, <i>J</i> =8 Hz), 7.32 (1H, d, <i>J</i> =1 Hz), 7.17 (1H, dd, <i>J</i> =1Hz, 8Hz), 6.88 (2H, d, <i>J</i> =8Hz), 6.87 (1H, d, <i>J</i> =8Hz), 6.68 (1H, d, <i>J</i> =16 Hz), 6.64 (1H, d, <i>J</i> =16 Hz), 5.97 (1H, s), 3.91 (3H, s)	56.6, 102.1, 111.8, 116.2, 116.9, 122.1, 122.4, 124.1, 127.7, 128.2, 131.2, 141.2, 141.3, 148.8, 149.8, 160.3, 184.1, 184.4
 <p>bis-demethoxycurcumin</p>	7.58 (2H, d, <i>J</i> =15.9 Hz), 7.56 (4H, d, <i>J</i> =8 Hz), 6.89 (4H, d, <i>J</i> =8.1Hz), 6.65 (2H, d, <i>J</i> =15.9 Hz), 5.94 (1H, s)	116.9, 122.1, 127.5, 131.2, 141.2, 160.3, 184.3
 <p>ar-turmerone</p>	7.10 (4H, d), 6.02 (1H, m), 3.29 (1H, m), 2.71 (1H, dd, <i>J</i> = 15.5, 6.0 Hz), 2.61 (1H, dd, <i>J</i> = 15.5, 8.3 Hz), 2.31 (3H, s), 2.10 (3H, s), 1.85 (3H, d, <i>J</i> = 1.2 Hz), 1.24 (3H, d, <i>J</i> =6.9 Hz)	20.7, 21.2, 22.1, 27.7, 35.2, 52.7, 124.3, 126.8, 129.1, 135.6, 143.5, 155.1, 199.8