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Research Article

Eugenol protects human liver HepG2 cells from H_2O_2-induced oxidative hepatotoxicity by maintaining ROS homeostasis, increasing IL-10 level, and upregulating CYP1B1 gene expression

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ABSTRACT

Liver injury occurs due to continuous exposure to chemical or biological hazards. Eugenol (EUG), a phenolic compound derived from red betel (*Piper crocatum*) leaves, has multivalent effects, primarily on antioxidant and antibacterial applications. The conducted research sought to investigate the safeguarding activities of EUG on hydrogen peroxide (H_2O_2)-induced toxicity of human liver HepG2 cells by examining homeostasis, interleukin 10 (IL-10), malondialdehyde (MDA) levels, reactive oxygen species (ROS) level and cytochrome P450 family 1 subfamily B member 1 (CYP1B1) gene expression. The H_2O_2-induced hepatotoxic cells, which served as a positive control, were treated with EUG. The measurements for IL-10 and MDA levels were made using enzyme-linked immunosorbent assay (ELISA), flow cytometry was employed for ROS level, and reverse transcription-quantitative real-time PCR (RT-qPCR) was used for CYP1B1 gene expression assay. EUG 6.25; 25 µg/mL increased IL-10 levels at 55.33 and 47.52 pg/mL compared to the positive control at 21.66 pg/mL. EUG 6.25; 25 µg/mL lowered MDA levels at 679.07 and 651.60 ng/mL compared to the positive control at 845.33 µg/mL. EUG 6.25; 25 µg/mL suppressed ROS level at 15.83 and 19.40% compared to the positive control at 24.10%. EUG 6.25; 25 µg/mL also up-regulated CYP1B1 gene expression. In conclusion, EUG exhibited excellent hepatoprotective effects in hepatotoxic cells model by maintaining ROS homeostasis, increasing IL-10, decreasing MDA levels, and upregulating CYP1B1 gene expression.

Keywords:
CYP1B1, Eugenol, Hepatotoxicity, HepG2, IL-10

1. INTRODUCTION

The liver is the largest solid organ and is considered a vital organ in the human body because it can detoxify hazardous chemicals. Thus, protecting the liver from damage or injury is very crucial[1]. The liver is composed of various cells. Hepatocytes make up ±60% of liver cells, while the rest consists of epithelial cells of the biliary system[2]. Hepatocytes cells have important roles in liver, they are responsible for a variety of cellular functions including carbohydrate, lipid, and protein metabolism, detoxification and immune cell activation to maintain liver homeotasis. Hence, if hepatocytes cell has a damage, it will affect the liver[3]. Liver disease is among the most severe diseases[4]. Liver disease can be caused by viruses, toxic chemicals, and alcohol[5]. A condition where liver cells are damaged due to toxic chemicals is called hepatotoxicity. Large amounts of hepatotoxic substances injure liver cells due to oxidation activities such as lipid peroxidation[6]. Tissue damage can be initiated by fat deposition in the

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liver and pro-inflammatory conditions, in which visceral adipose tissue (VAT) is involved in the metabolism and alteration of lipids and glucose. When the body is not able to counteract the inflammation due to oxidative stress, impaired regulation of protein unfolding-mediated endoplasmic reticulum (ER) stress, lipid deposition in non-adipose tissues, and progressive cell death occur. These conditions can lead to severe outcomes, promoting liver injury, disruptive fibrosis-mediated hepatic cirrhosis, and hepatocellular carcinoma (HCC) or liver cancer progression in patients[7].

Cytokines are crucial in clearing viral and bacterial infections, suppressing inflammation, and mediating tissue regeneration. There are two distinct types of immune responses by the cluster of differentiation 4 $(CD4)^+$ T cells. Firstly, T helper type 1 (Th1)-like responses are required to activate cytotoxic T lymphocytes and natural killer (NK) for host defense against viruses (antiviral immune responses). On the other hand, T helper type 2 (Th2)-like responses secrete the pleiotropic anti-inflammatory cytokines such as IL-4 and IL-10, which support the magnification of antibody production and prevent Th1 responses. It has been proven that IL-10, mainly secreted by macrophages, Th2 cells, and B lymphocytes, can suppress inflammatory reactions in chronic liver disease[8-9]. Interleukin-10 (IL-10) is a potent cytokine that plays an important role in preventing inflammation and autoimmune pathologies[10]. IL-10 can reduce ROS production by macrophages and dendritic cells.

The elevated oxidative stress is one of the significant causes of tissue damage and inflammation driven by free radicals, resulting in lipid degradation products, such as malondialdehyde (MDA). This highly reactive compound can alter self-molecules (e.g., proteins and phospholipids) to form hapten-like substances known as MDA epitopes[11]. On the other hand, reactive oxygen species (ROS), oxygen-contained highly reactive species, and antioxidants are endogenously present in living organisms and involved in certain diseases, including carcinogenesis. However, in oxidative stress, ROS or pro-oxidant species are more dominant than the antioxidant defense capability[12]. When ROS is excessive, homeostatis will be disrupted, resulting in oxidative stress which plays an important role in liver disease and other chronic and degenerative disorders[13]. Therefore, regulating ROS homeostasis is required to prevent oxidative hepatotoxicity. ROS homeostasis in cells can be obtained through a balance between their production and release[14].

The cytochrome P450 family 1 subfamily B member 1 (CYP1B1) gene assists the production of cytochrome P450 enzymes, which are involved in different biochemical reactions, such as drug degradation, fat production, and oxygen transfer[15]. More importantly, redox homeostasis is significantly regulated by CYP1B1 enzymes. Lack of the enzymes leads to oxidative stress by increasing oxygen availability (hyperoxia) and ROS production[16]. In addition, CYP1B1 enzymes mediate the metabolism of endogenous substrates such as dietary plant flavonoids and have a significant impact on developmental and tissue homeostasis processes[17-18].

Eugenol (EUG) is a phenolic derivative substance that has been used in different medicinal applications[19], especially in antioxidant, anti-inflammatory, antibacterial, anti-metastatic, and anti-obesity applications[20]. Originating from red betel (*Piper crocatum*) leaves, EUG presents as essential oil. EUG has been classified by United State Food and Drug Administration (U.S. FDA) as a food additive in cosmetic industries and dentistry, with GRAS (Generally Recognized as Safe) status. EUG is a natural ingredient that has excellent potential to be developed. Natural ingredient can lower the liver damage, unlike synthetic drugs such as antiepileptic drugs, which can cause liver damage[21]. There is a growing focus on evaluating natural ingredients for hepatoprotective activity[22]. The protective role of EUG has been linked to enhanced pro-inflammatory cytokine production and the inhibition of dehydrogenase enzymes[23]. It is believed that the main structural characteristic of EUG responsible for its antioxidant activity is the presence of hydroxyl (OH) groups, which can donate hydrogen atoms and interrupt chain propagation during the oxidative process[24]. According to a current study, EUG could elevate the antioxidant glutathione (GSH) level, inhibiting oxidative stress during the hepatotoxicity process[20]. In this research, we examined ROS homeostasis, IL-10 secretion, and the expression of CYP1B1 gene to investigate the hepatoprotective activities of EUG on the established hepatotoxic cells model by inducing hydrogen peroxide (H_2O_2).

2. MATERIALS AND METHODS

2.1. Cell culture and H_2O_2-induced toxicity of HepG2 cells

The Biomolecular and Biomedical Research Center, Aretha Medika Utama, Bandung, Indonesia provided the HepG2 cell line (ATCC, HB-8065™). Then, the cell medium was prepared from modified eagle's medium (MEM) (Biowest, L0416-500) with 1% (v/v) antibiotic-antimycotic (Gibco, 15240062), 1% (v/v) nanomycopulitine (Biowest, LX16), and 10% (v/v) fetal bovine serum (FBS) (Biowest, S1810), added for cell thawing and cell culture purposes. HepG2 cells were incubated at 37°C with 5% CO_2 and the medium was replaced per three days to maintain cell viability. Cells with 80-90% confluency were PBS-washed and trypsin-EDTA-detached. Approximately 5×10^5 detached cells (counted by hemocytometry) were seeded per well in 96-well plates. Then, HepG2 cells were incubated at 37°C for 24 h with 5% CO_2. The hepatotoxic-model cells were generated *in vitro* by incubating HepG2 cells with 15 mM H_2O_2 (Merck, 8.22287.100). The established model cells were treated

with and without EUG (purchased commercially from Biopurify Phytochemicals Ltd., Chengdu, China, 95-99% purity), as follows: (I) normal cells, (II) normal cells+ DMSO 1%, (III) normal cells+H_2O_2 15 mM (hepatotoxic-model cells); (IV) hepatotoxic-model cells+EUG 6.25 µg/mL, and (V) hepatotoxic-model cells+EUG 25 µg/mL. After treatments, cells were co-incubated for another 24 h at 37°C and 5% CO_2 in a conditioned incubator. After co-incubation, the conditioned medium's supernatant was collected, centrifuged at 1,600 rpm for 10 min, and then kept at -80°C for the quantification of IL-10 and MDA levels[24-25].

2.2. Total protein assay

To measure the total protein of samples, the bovine serum albumin (BSA) solution was prepared by diluting BSA (Sigma, A9576, Lot. SLB2412) stock with ddH_2O and made some series of dilution. To make the BSA stock, 2 mg of BSA (Sigma, A9576, Lot SLB2412) was first dissolved in 1 mL of ddH_2O. Each well in a 96-well plate was than filled with samples, 20 µL of the standard solutions, and 200 µL Quick Start Dye Reagen 1X (Biorad, 5000205). After incubation at 27°C for 5 min, the absorbance of each sample was measured using a microplate reader (Multiskan GO, ThermoScientific) at a 595-nm wavelength[26-27].

2.3. Measurement of IL-10 level

ELISA kit (Elabscience, E-EL-H0103) was used to measure IL-10 level according to the company's instruction. Then, a microplate reader (Multiskan GO, ThermoScientific) was used to predict the absorbance of each sample at a 450-nm wavelength. In addition, the color changes of each sample were evaluated and read at the same wavelength. The IL-10 level was determined based on the protein-standard curve[25,28].

2.4. Measurement of MDA level

ELISA kit (Elabscience, E-EL-0060) was used to measure MDA level according to the company's instruction. Then, a microplate reader (Multiskan GO, ThermoScientific) was used to predict the absorbance of each sample at a 450-nm wavelength[25,29].

2.5. Measurement of ROS level

With a modest modification methodology[25], DCF-DA fluorescent probes (Abcam, ab113851) were used for flow cytometry analysis, especially to detect and quantify intracellular ROS[30]. Briefly, after seven days of cell culture, confluent HepG2 cells (80-90%) were detached using trypsin-EDTA. Then, for the next 45 min, approximately 2.5×10^5 cells/0.5 mL were treated with 20 µM DCF-DA at 37°C. After adding EUG (25 and 6.25 µg/mL), the cells were co-incubated for another 4 h. The level of intracellular ROS was analyzed using a Miltenyi flow cytometer (MACSQuant10). For the positive control, HepG2 cells without any treatments were used, while for the negative control, HepG2 cells treated with H_2O_2 and without EUG were used. Each sample fluorescence intensity was compared to the positive control and expressed in a percentage. DCF-DA fluorescent probes were used to detect intracellular ROS (excitation and emission at 485 and 535 nm, respectively), detected in the fluorescein isothiocyanate (FITC) region[25,31].

2.6. Measurement of CYP1B1 gene expression using RT-qPCR

RNA was used to measure CYP1B1 gene expression. Briefly, Aurum Total RNA mini kit (Bio-Rad, 7326820) was used to extract RNA from HepG2 cells. The purity and concentration of RNA from each sample was predicted at a 260/280 nm wavelength (Table 1). Based on

Table 1. RNA concentration and purity.

No	Samples	Average RNA concentration (ng/µL)	RNA purity (260/280 nm)
1	Normal cells (HepG2 cells)	775.70	2.38
2	HepG2 cells + H_2O_2 15 mM (hepatotoxic cells)	96.40	2.43
3	Hepatotoxic cells + EUG 6.25 µg/mL	60.50	2.27
4	Hepatotoxic cells + EUG 25 µg/mL	34.75	1.88

Table 2. RT-qPCR details of CYP1B1 gene.

Gene Symbols	Primer Sequence (5' to 3') Upper strand : sense Lower strand : antisense	Product Size (bp)	Annealing (°C)	Cycles	Ref.
β-actin	5'-TCTGGCACCACACCTTCTACAATG-3' 5'-AGCACAGCCTGGATAGCA ACG-3'	24	63	40	Palumbo et al. (2018)[31] Widowati et al. (2020)[30]
CYP1B1	5'- CCAAGCTCATCACCTGGTCT -3' 5'- TCGATGTCAATGGTCTGGAA -3'	294	60	40	Falero-Perez et al. (2018)[32]

the company's protocol, iScript cDNA synthesis kit (Bio-Rad, 1708841) was used to reverse transcript 1-15 µL RNA from each sample to cDNA[25,27,31-32], following incubation in Piko Real Time PCR (Thermofisher Scientific, TCR0096) with the following reaction conditions: (1) priming: 2537°C, 5 min, (2) reversion: 42°C, 30 min, (3) inactivation: 85, 5 min, and (4) holding: 4°C. A total of 1-2 µL cDNA samples were inserted into PCR tubes, added with 10 µL of SsoFast EvaGreen Supermix (Bio-Rad, 1725200), 2 µL of mixed forwarded primers and reversed primers, and nuclease-free water until 20 µL. The samples were then put into PikoReal Time PCR (Thermo Scientific, TCR0096) and closed with ASF-0020 seals, following incubation in qPCR under these reaction conditions: (1) pre-denaturation: 95°C, 7 min, (2) denaturation: 95°C, 30 s, 40 cycles, (3) annealing: 60°C, 40 s, 40 cycles, (4) pre-elongation: 72°C, 1 min, (5) elongation: 72°C, 5 min, (6) melting curve: 55-95°C, and (7) holding: 4°C. The primer sequences of the CYP1B1 gene can be seen in Table 2.

3. RESULTS

3.1. Effect of EUG towards IL-10 level in H_2O_2-induced HepG2 cells

The anti-inflammatory cytokine IL-10 is mainly secreted by macrophages to maintain normal tissue homeostasis. Figure 1A and 1B show that H_2O_2 could significantly decreased (p<0.01) IL-10 level in HepG2 cells. EUG 6.25; 25 µg/mL treatment significantly (p<0,01) increased IL-10 level in hepatotoxic cells model. EUG 6.25 µg/mL was the most active to increase IL-10 level, it was com-parable to normal cells. DMSO treatment (II) was non-significant (ns) compared to normal cells. The data shows that EUG could normalize IL-10 level in hepatotoxic cells model, meanwhile DMSO as a solvent treatment didn't affect the IL-10 level.

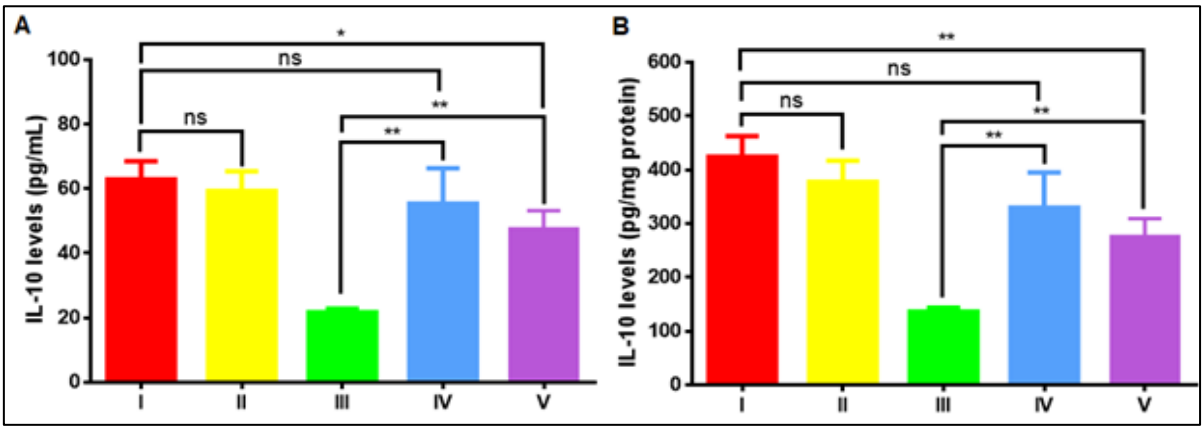

Figure 1. The effect of EUG toward IL-10 level of hepatotoxic-model cells (H2O2-treated HepG2 cells).
*(NC) Normal cells (HepG2 cells), (DC) Normal cells+DMSO 1%, (PC) Hepatotoxic cells, (EUG 6.25) Hepatotoxic cells+EUG 6.25 µg/mL, and (EUG 25) Hepatotoxic cells+EUG 25 µg/mL.
*(A) IL-10 level (pg/mL) (B) IL-10 level (pg/mg protein). Data is presented as mean±standard deviation (n=3). Unpaired Student's t-Test was conducted to analyze a significant difference between two groups (ns: non significant p>0.05, *p<0.05, **p<0.01, ***p<0.001).

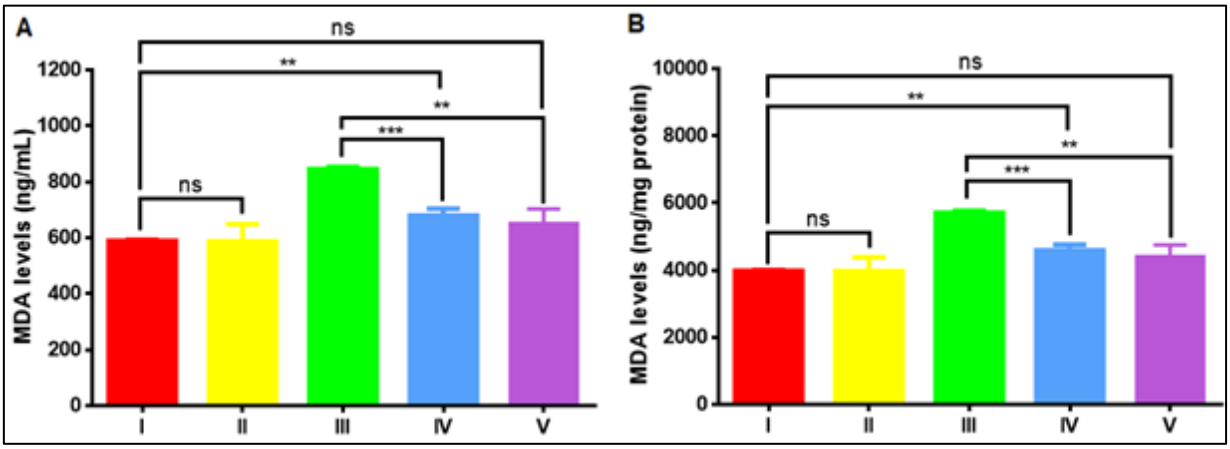

Figure 2. The effect of EUG toward MDA level in H_2O_2-induced HepG2 cells.
*(NC) Normal cells (HepG2 cells), (DC) Normal cells+DMSO 1%, (PC) Hepatotoxic cells, (EUG 6.25) Hepatotoxic cells+EUG 6.25 µg/mL, and (EUG 25) Hepatotoxic cells+EUG 25 µg/mL.
*(A) MDA level (ng/mL) in hepatotoxic cells. (B) MDA level (ng/mg protein) in hepatotoxic cells. Data is presented as mean±standard deviation (n=3). Unpaired Student's t-Test was conducted to analyze a significant difference between two groups (ns: non significant p>0.05, *p<0.05, **p<0.01, ***p<0.001).

3.2. Effect of EUG towards MDA and ROS level in H_2O_2-induced cells

Lipid peroxidation is a metabolic process involving ROS to induce oxidative damage of molecules containing lipids. As a lipid-peroxidation by-product, MDA is a highly reactive species and becomes a specific marker for oxidative stress[33]. The presence of antioxidants is vital to eliminate MDA and maintain cellular homeostasis. Figure 2A and 2B show that H_2O_2 significantly increased MDA level in HepG2 cells (p<0.001). EUG 6.25; 25 µg/mL treatment significantly decreased MDA level (p<0.01). EUG 25 µg/mL was the most active to decrease MDA level, it was comparable with normal cells. DMSO treatment (II) was non-significant (ns) compared to normal cells. Based on the data, EUG could decrease MDA level in hepatotoxic cells model, meanwhile DMSO as a solvent treatment didn't affect the MDA level.

Similar to MDA, increasing ROS impair antioxidant defense systems and lead to oxidative stress[28]. Figure 3 shows that H_2O_2 could significantly elevate ROS level in HepG2 cells (p<0.0001). EUG 6.25; 25 µg/mL treatment significantly lowered ROS level (p<0.0001). EUG 6.25 µg/mL was the most active to decrease ROS level. Based on the data, EUG could lower ROS level in hepatotoxic cells model. Figure 4 shows the dot blot representative of EUG treatment towards ROS level.

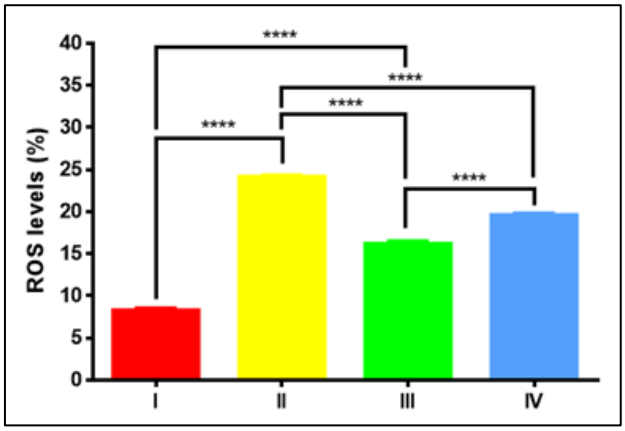

Figure 3. The effect of EUG toward ROS level in H_2O_2-induced HepG2 cells.
*(NC) Normal cells (HepG2 cells), (PC) Hepatotoxic cells, (EUG 6.25) Hepatotoxic cells+EUG 6.25 µg/mL, (EUG 25) Hepatotoxic cells+EUG 25 µg/mL. Data is presented as mean±standard deviation (n=3). Unpaired Student's t-Test was conducted to analyze a significant difference between two groups (non-significant p>0.05, ****p<0.0001).

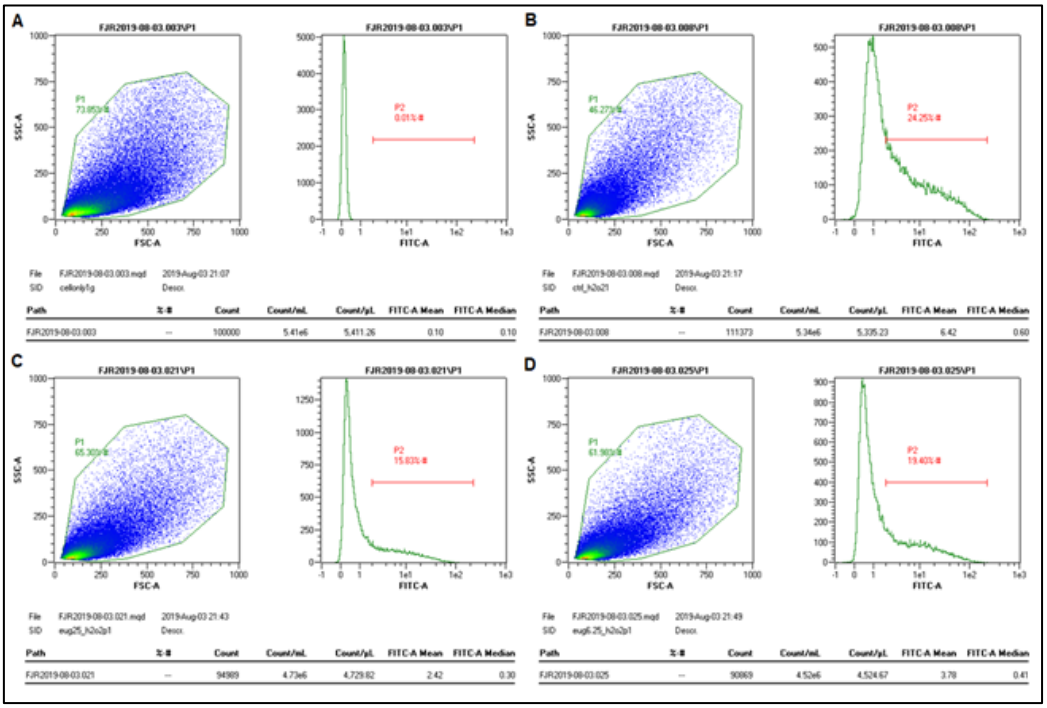

Figure 4. The representative of dot blots of EUG in H_2O_2-induced HepG2 cells toward ROS level by flow cytometry.
*(A) Normal cells (untreated HepG2 cells): 73.85%, (B) Hepatotoxic cells model: 46.27%, (C) Hepatotoxic cells model+EUG 6.25 µg/mL: 65.30%, (D) Hepatotoxic-model cells+EUG 25 µg/mL: 61.98%.

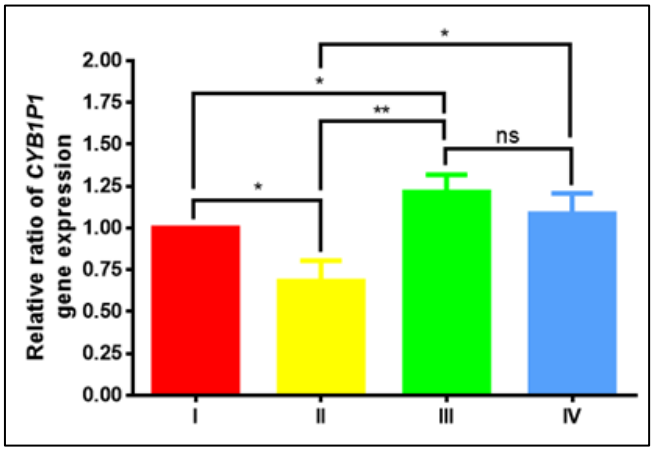

Figure 5. The effect of EUG toward CYB1P1 gene expression in H_2O_2-induced HepG2 cells.
*(NC) Normal cells (HepG2 cells), (PC) Hepatotoxic cells, (EUG 6.25) Hepatotoxic cells+EUG 6.25 µg/mL, (EUG 25) Hepatotoxic cells+EUG 25 µg/mL. Data is presented as mean±standard deviation (n=3). Unpaired Student's t-Test was conducted to analyze a significant difference between two groups (ns: non -significant $p>0.05$, *$p<0.05$, **$p<0.01$).

Table 3. Raw data of CYP1B1 gene expression in H_2O_2-induced HepG2 cells with different treatments.

Treatments (µg/mL)	Cq CYP1B1			Cq *β*-Actin			Relative ratios of CYP1B1 gene expression			
	Sample 1	Sample 2	Sample 3	Sample 1	Sample 2	Sample 3	Sample 1	Sample 2	Sample 3	Mean ± SD
NC	32.38	32.81	30.95	21.82	22.42	21.49	1.00	1.00	1.00	1.00 ± 0.00
PC	35.64	35.42	34.88	24.66	24.96	24.22	0.56	0.80	0.70	0.68 ± 0.12
EUG 6.25	34.92	34.49	35.48	25.00	24.77	25.54	1.16	1.33	1.15	1.21 ± 0.10
EUG 12.5	36.23	36.21	35.33	26.17	26.05	25.48	1.05	0.98	1.22	1.09 ± 0.12

*(NC) Normal cells (HepG2 cells), (PC) Hepatotoxic cells (H_2O_2-treated HepG2 cells), (EUG 6.25) Hepatotoxic cells+EUG 6.25 µg/mL, and (EUG 25) Hepatotoxic cells+EUG 25 µg/mL.

3.3. Effect of EUG towards CYP1B1 gene expression in H_2O_2-induced HepG2 cells

As an aryl hydrocarbon receptor (AhR) repressor, the CYP1B1 gene plays a vital role as the negative regulator of this signaling. It has been known that the AhR mediates toxic effects of dioxins, chloracne, atrophy, teratogenesis, and hepatocellular damage[34]. Based on Figure 5, q-RTPCR results show that H_2O_2 significantly decreased CYP1B1 gene expression ($p<0.05$). EUG 6.25; 25 µg/mL could significantly upregulate CYP1B1 gene expression in hepatotoxic cells model ($p<0.01$). EUG 6.25 µg/mL and EUG 25 µg/mL did not have a significant difference in increasing CYP1B1 gene expression (Table 3).

4. DISCUSSION

Liver injury occurs due to continuous exposure to chemical and biological hazards, such as alcohols, acids, viruses, and bacteria-mediated auto-immune diseases[36]. It cannot be viewed as a single whole disease, but rather as the accumulation and death of cells, where many different mechanisms cause hepatotoxicity[36]. For *in vitro* study purposes, one of the accessible ways to build hepatotoxic-model cells is by inducing human liver HepG2 cell line with various inducers. For instance, Lister et al.[37] used acetaminophen to induce cytotoxicity of HepG2 cells and evaluate the antioxidant activities of red betel extract (*P. crocatum*) leaf extract[19]. Meanwhile, Ghaffari et al.[38] investigated the safeguarding potentials of *Hyptis suaveolens* methanolic extract against cytotoxicity in H_2O_2-induced HepG2 cell culture. In the present study, H_2O_2 was chosen to induce oxidative hepatotoxicity in HepG2 cell culture because H_2O_2 can produce highly reactive hydroxyl radicals, killing different cell types rapidly. H_2O_2 from external sources can enter the cells quickly, facilitated by high membrane permeability[38]. More importantly, we aimed to evaluate EUG potential effects in reducing the toxicity of the established hepatotoxic-model cells. It is known that EUG can suppress oxidative stress as an antioxidant agent[39]. EUG also has anti-carcinogenic, anti-cytotoxic, and antitumor properties[12]. However, a high concentration of EUG might induce toxicity *in vitro*. We used EUG concentrations of 6.25 and 25 µg/mL in the present study as the safe concentration for treatments in the HepG2 cell line based on our previous cytotoxicity study[39-40]. In addition, EUG could suppress lactate dehydrogenase (LDH) secretion and inhibit the activity of alanine amino-transferase (ALT) and aspartate aminotransferase (AST) in acetaminophen-induced hepatotoxic cells[40]. In other ways, a recent study revealed that EUG administration in rats (5 mg/kg body weight for 30 days) could diminish arsenic trioxide accumulation in the liver with similar liver histology to the positive control[20].

The anti-inflammatory cytokine IL-10 can reduce inflammation throughout hepatotoxic symptoms. In this

case, IL-10 maintains the equilibrium between host protection and inflammatory tissue damage by dampening innate and adaptive immunity elements. Furthermore, IL-10 inhibits the secretion of pro-inflammatory cytokines at some stages during the contamination period. By costimulatory molecules and chemokines production and downregulating major histocompatibility complex (MHC) class II, IL-10 also changes the role of antigen-presenting dendritic cells and macrophages. This indirectly inhibits T cell responses. On top of its immunosuppressive activities, IL-10 enhances the B cells' proliferation, regulates T cells' differentiation, and activates NK cells and cytotoxic T cells[41]. Hepatotoxic cells are modeled in this research using HepG2 cells that have been exposed to H_2O_2. Figure 1 shows that H_2O_2 decreased IL-10 levels in HepG2 cells, while EUG 6.25 and 25 μg/mL increased IL-10 levels significantly in hepatotoxic-model cells. A similar result by Mateen et al.[42] disclosed that cinnamaldehyde and EUG treatments could enhance IL-10 level in arthritic rats[32]. Their further study found that EUG inhibited oxidative damage in rat myocardium by protecting the thiol group, including protein and non-protein-bound thiols, from oxidative stress and enhancing the thioredoxin activity[43].

MDA epitopes oxidation-specific epitopes (OSEs) found in oxidized lipoproteins, dying cells, and microvesicles[11]. MDA is a frequently used biomarker of lipid peroxidation that is produced when lipid peroxyl radicals, one of the signs of oxidative stress, breakdown[6,40]. The presence of antioxidants can inhibit MDA formation[44]. It is clear that EUG 6.25 and 25 μg/mL could suppress MDA formation significantly in hepatotoxic-model cells. Low MDA levels will prevent cells from oxidative stress associated with injury and inflammation[11]. Similar to MDA, ROS formation could induce oxidative stress in cells. According to Mateen et al.,[42] the ROS level of arthritic-model rats treated with cinnamaldehyde and EUG was significantly lowered. In arthritic-model rats, it was found that EUG was more dominant than cinnamaldehyde at reducing the levels of ROS and nitric oxide. A recent study by the same group showed that EUG decreased the MDA and ROS levels significantly in mononuclear cells of rheumatoid arthritis patients[25]. From flow cytometry analysis (Figure 4), it can be seen clearly that the intracellular ROS level in hepatotoxic-model cells was the highest, around 24.25%. In comparison, the figure for the positive control was negligible (only 0.01%). After treatment with EUG 6.25 and 25 μg/mL, the ROS level decreased to 15.83 and 19.40% (Figure 3), respectively. Elevated ROS production may be associated with the pathogenesis of many inflammatory diseases due to its impact on impaired antioxidant defense systems[28]. Without appropriate treatments, continuous ROS exposure can result in severe liver injury and hepatic disorders, including cirrhosis[45].

On the other hand, the CYP1B1 gene is known as the AhR repressor, which prevents the toxic effects of dioxins, chloracne, atrophy, teratogenesis, and hepatocellular damage[34]. This gene assists the production of CYP1B1 enzymes, the key regulator of redox homeostasis. It is crucial to maintain adequate CYP1B1 enzymes to prevent hyperoxia and increased ROS production, which can lead to oxidative stress[16]. In addition, CYP1B1 enzymes mediate the metabolism of endogenous substrates such as dietary plant flavonoids and significantly impact developmental and tissue homeostasis processes[17-18]. As confirmed by qRT-PCR, H_2O_2 could decrease CYP1B1 gene expression significantly in HepG2 cells. On the other hand, treatments with EUG 6.25 and 25 μg/mL could upregulate the expression of CYP1B1 gene significantly in hepatotoxic-model cells compared to the positive control.

Based on previous report, red betel leaves extract contains numerous phytochemicals that could be utilized for the treatment of liver damage. Eugenol, one of the predominant red betel leaves extract compounds, is responsible for their antioxidant properties[39]. Eugenol has the ability to scavenge free radicals, such as HO· in H_2O_2-HepG2 cells, leading to improved cellular conditions following exposure to oxidative stress[24,37]. Another research demonstrated that upturn antioxidant markers (GSH, glutathione peroxidase (GPX), and catalase (CAT)) leads to hepatoprotective properties[37].

5. CONCLUSION

In conclusion, EUG exhibited hepatoprotective effects in H_2O_2-induced hepatotoxic cells by maintaining ROS homeostasis, decreasing MDA level, increasing IL-10 level, and upregulating CYP1B1 gene expression. Therefore, considering the positive effects of EUG on suppressing hepatotoxicity *in vitro*, planned further studies in diseased animal models are needed.

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Conflict of interest
None to declare.

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Ethics approval
None to declare.

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Author contribution
The authors confirm contribution to the paper as follows: study conception and design: TS, LL, CNG, INEL; data collection: FHZ, RR; analysis and interpretation of results: EG, HSWK, WW; draft manuscript preparation: HSWK, WW, TS. All authors reviewed the results and approved the final version of the manuscript.

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