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Protective Effect of Eugenol against Acetaminophen-Induced Hepatotoxicity in Human Hepatocellular Carcinoma Cells via Antioxidant, Anti-Inflammatory, and Anti-Necrotic Potency

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Abstract

BACKGROUND: Overdoses acetaminophen (APAP) could cause acute liver failure, even though it used is for analgesics. APAP could cause hepatotoxicity due to multiple mediators of inflammation and oxidative stress. Eugenol has been reported to have anti-inflammatory and antioxidant activity but its hepatoprotective effect has not been widely reported.

AIM: The purpose of this research is to know if eugenol could protect HepG2 cells from APAP.

METHODS: HepG2 that induced by APAP as hepatotoxicity cells model was treated by using eugenol at 6.25 and 25 μg/mL. The protective effects of eugenol toward hepatotoxicity were evaluated by determine tumor necrosis factor-α (TNF-α) concentration, apoptotic activity, reactive oxygen species (ROS) level, also cytochrome (CYP)2E1 and GPX gene expression.

RESULTS: Eugenol at 6.25 and 25 μg/mL concentration can reduce TNF-α concentration, the apoptotic, necrotic, dead cells, and ROS level. Besides it can increase the gene expression (GPX and CYP2E1). The best hepatoprotective effect was found when using the eugenol at 25 μg/mL.

CONCLUSION: Therefore, eugenol can be used to protect HepG2 cells against APAP.

Introduction

Acetaminophen (paracetamol) or known as APAP, is frequently used as analgesic [1]. Overdose of APAP has been reported can lead to acute liver failure [2]. In several countries such as USA, and UK, the most frequent cause of acute liver failure is APAP overdoses [3]. But, the mechanism of APAP-induced hepatotoxicity was still unclear. Based on various research, APAP can induce acute liver damage by mediators' inflammation and oxidative stress [4]. One of the mediators was tumor necrosis factor-α (TNF-α) [5].

The mechanism of APAP hepatotoxicity is dominated by intracellular events including the Hepatotoxicity by APAP induction was intracellular events, including GSH depletion, protein adduct formation, and the formation of a reactive metabolite. It initiates mitochondrial oxidant stress and peroxynitrite formation [6]. At present, natural medicines have been investigated for their hepatoprotective ability, because it has many active compounds. Therefore, new treatment protocols were needed urgently to be investigated [7], [8].

Eugenol or 4-allyl 2-methoxyphenol has many pharmacological activity such as antioxidant, antibacterial, antiviral, hypoglycemic, and anti-inflammatory function in diabetes [9], [10]. These effects have been researched, but there are few reports concerning the hepatoprotective effects. It has been reported that eugenol at 25 μg/mL has the best hepatoprotective effects by decrease of lactate dehydrogenase (LDH) level also aspartate aminotransferase (AST) and alanin aminotransferase (ALT) activities in APAP-induced hepatotoxicity model

[11]. The focus of this research is to see if eugenol could protect HepG2 cells against APAP.

Methods

HepG2 cells culture and APAP-induced HepG2

This research using human hepatocellular carcinoma (HepG2) cells line (ATCC, HB-8065TM). The cell was obtained from Aretha Medika Utama, Biomolecular and Biomedical Research Center, Bandung, Indonesia. The cells were thawed and grown in Modified Eagle Medium (MEM) (Biowest, L0416-500). Grown medium enhanced by 1% (v/v) antibiotic-antimycotic (Gibco, 15240062), 10% (v/v) fetal bovine serum (FBS) (Biowest, S1810), and 1% (v/v) nanomycopulitine (Biowest, LX16). The cells were maintained in environment 37oC 5% CO2, and change the grown medium per 3 days.

APAP-induced HepG2 was used to create the hepatotoxic model in vitro. Confluent cells were rinsed with PBS before being incubated at 37°C with trypsin EDTA to extract the cells from the flask. After that, cells were counted using a hemocytometer and planted into 6 well plates (5 x 105 cells per well). For 24 hours, cells were cultured in a 37°C incubator with 5% CO2. It was added by 40 mM APAP after the cells were connected (Sigma Aldrich, A7085-100G). Control normal cells (with medium complete); DMSO control (1 percent); APAP control 40 mM; APAP 40 mM + Eugenol 6.25 g/mL; APAP 40 mM + Eugenol 25 g/mL; APAP 40 mM + Eugenol 6.25 g/mL; APAP 40 mM + Eugenol 25 g/mL; APAP 40 mM + Eugenol 25 g/ After the chemical was induced, the cell was incubated for another 24 hours at 37°Cwith 5% CO2. The conditioned media was obtained for ELISA assays after incubation and centrifuged for 10 minutes at 1600 rpm. For Tumour Necrosis Factor-α (TNF-α) assay, the supernatant was collected and kept at -80° C[11], [12], [13], [14], [15], [16].

Total protein assay

Bovine Standard Albumin (BSA) standard (Sigma, A9576) as much as 2 mg was dilute in 1000 μL ddH2O. Then, 20 μL of standard solutions and 200 μL Quick Start Dye Reagen 1X (Biorad, 5000205) was added into well plate, then incubate at 5 min in room temperature. Absorbance of sample was measured by microplate reader (MultiskanTM GO Micro plate Spectrophotometer, Thermo Scientific, Waltham, MA, USA) at 595 nm [17]. The result from this assay was used for fibronectin data calculation [18].

TNF-α assay

For each treatment, the level of TNF-α was determined. According to the manual kit, the measurement was performed using an ELISA assay (BioLegend, ELISA kit 421701). The plates that would be used in the assay were coated with capture antibody solution and incubated overnight at 4°C before the assay began. The plate was rinsed four times with wash buffer before being incubated in an orbital shaker for one hour. Each sample and standard well received up to 50 μL of matrix C and assay buffer. The detection antibody solution was then added to each well and incubated at room temperature for 1 hour on an orbital shaker. After four washes, 100 μL of diluted Avidin-HRP solution was added to each well and incubated at room temperature for 30 minutes on an orbital shaker. The plate was then rinsed five times and 100 μL of substrate solution were added to each well. For ten minutes, the plate was incubated in the darkroom. To stop the reaction, then add the 100 μL of stop solution. The Multikan GO Microplate Reader (Thermo Fisher, 51119300) was then used to read the absorbance at 450 nm [19].

Apoptotic, necrotic, dead cells assay

The hepatotoxic HepG2 cell line was cultivated in MEM, 10% FBS, and 1% Antibiotic Antimycotic as a hepatotoxic model. For 24 hours, the cells were incubated at 37°C in a humidified environment with 5% CO2. Then, APAP was used to induced the cells. Each cell combined with administration of eugenol, and then the cells were incubated at 37°C and 5% CO2 for 24 h. The pellet cell was washed with 500 μL Annexin Binding Buffer 1X (130-092-820, Miltenyi Biotec) twice, before centrifuged at 1600 rpm for 5 minutes. The pellet cell was then stained with Annexin V-FITC (BioLegend, Part79998) and Propidium Iodide (BioLegend, Part79997) after being rinsed with 100 μL Annexin Binding Buffer (Miltenyi Biotec, 130-092-820). Apoptotic, necrotic, dead, and viable cells percentages of HepG2 cells were examined using MACSquant Analyzer 10 after the cells were kept in darkness at 4°C (Miltenyi Biotec) [20].

Reactive oxygen species (ROS) level assay

Flow cytometry was used to detect intracellular ROS levels using a DCF-DA fluorescent probe (Invitrogen) in accordance with Widowati et al method with minor modifications [14]. After being cultured for seven days, HepG2 cells were detached with trypsin-EDTA. As much as 2.5×104 cells/0.5 mL cells were incubated with 20 μM DCF-DA at 37ºC for 45 min. After incubated, eugenol was added (25 and 100 μg/mL) to the cells, then incubated again for 4 h. Miltenyi Flow Cytometer was used to measure the intracellular ROS levels (MAQS quant). The control used for ROS assay were HepG2 cells treated

with H2O2 without eugenol treatment. The fluorescence readings that were evaluated were expressed as a percentage of the control.

Table 1: Primer sequences of CYP2E1, GPX, and β-Actin gene used in RT-PCR

Gene symbols	Primer sequences (5' to 3') Upper strand: sense Lower strand: Antisense	Annealing (°C)	Cycle	References
β-Actin	5'-TCTGGCACCACACCTTCTACAATG-3' 5'-AGCACAGCCTGGATAGCAACG-3'	63	40	[23]
CYP2E1	5'-GTTCTTTGCGGGGACAGAGA-3' 5'-GAGGGTGATGAACCGCTGAA-3'	59	40	[24]
GPX	5'-CCAAGCTCATCACCTGGTCT-3' 5'-TCGATGTCAATGGTCTGGAA-3'	59	40	[25]

CYP: Cytochrome

Cytochrome (CYP)2E1 and GPX gene expression assay

The HepG2 cell line was cultured in complete medium (MEM + 10% FBS + 1% Antibiotic Antimycotic) and incubated at 37°C with 5% CO2 for 24 h. After that, APAP was used to induce the cells, with the administration of 6.26 μg/mL and 25 μg/mL eugenol for each cell. The cells were incubated for 24 h at 37°C and 5% CO2. The cells were then collected and processed for RNA isolation using AurumTM Total RNA mini Kit (Bio-Rad, 732-6820). The genes expression for CYP2E1, GPX, as well as the constitutively expressed β-actin gene, was analyzed using RT-qPCR (Clever, GTC96S) [20,21,22]. The primer sequences, purity, and concentration of RNA could be seen respectively in Table 1 and Table 2.

Table 2: Concentration and purity of isolated RNA

Sample	Concentration (ng/μL)	Purity (Absorbance 280/260)
Normal cells	92.90	2.3212
APAP-induced cells	90.10	2.0904
APAP-induced + eugenol 6.25 μg/mL	77.20	2.1842
APAP-induced + eugenol 25 μg/mL	83.00	2.3621

Statistical analysis

The experiment was carried out three times. SPSS software was used for statistical analysis (version 20.0). The data was presented in the form of a mean and standard deviation. On the basis of the normality of the data, significant differences in the groups were established using analysis of variance (One Way ANOVA) with >P 0.05. The post-hoc analytical statistics followed by Tukey's HSD, Games-Howell, or Mann-Whitney Post-Hoc Tests with a 95% confidence interval.

Results

TNF-α concentration

Figure 1 shows the TNF-α concentration in HepG2 cells that were induced by APAP. According to the results, APAP can significantly elevate TNF-α concentration ($P < 0.05$) when compared to normal cells. Treatments of 6.25 and 25 μg/mL eugenol decreased the concentration of TNF-α significantly, it is means $P < 0.05$ compare to cell treated only with APAP (positive control). This result shows that eugenol has potential in suppressing TNF-α production and it can be beneficial in liver damage treatment.

Apoptotic, Necrotic, and Dead Cells

The effect of APAP-induced and eugenol treatments can be seen in Figure 2. Based on the result, APAP induced were increased the percentage of cells in apoptotic, death, and necrotic. It means, the percentage of live cells was decreased compare to normal cells (without any induction) from 92.46 ± 0.68% to 68.41 ± 5.07%. Both eugenol treatments in HepG2 cells were decreased the apoptotic cells with the value of live cells are 79.98 ± 0.42% and 82.39 ± 0.66%, respectively.

ROS Level

Figure 3 shows the effect of eugenol on the level of ROS in HepG2 cells induced by APAP. According to the findings, APAP induced in HepG2 cells could significantly increase the ROS levels in HepG2 cells with $P < 0.05$ compared to cell without treatment or normal cells. Eugenol of 6.25 and 25 μg/mL was decreased the ROS level compare to APAP-induced significantly based on statistical analysis ($p > 0.05$).

CYP2E1 Gene Expression

The CYP2E1 gene expression in APAP-induced HepG2 cells was significantly decreased ($P < 0.05$). Eugenol at 6.25 μg/mL and 25 μg/mL could increase the CYP2E1 gene expression significantly ($P < 0.05$). The results could be seen in Figure 4.

GPX Gene Expression

In Figure 5, the induction of APAP in HepG2 cells has the same effect as CYP2E1 gene expression in GPX gene expression. The APAP-induced could significantly decrease the GPX gene expression ($P < 0.05$) compared to normal cells. The GPX gene expression increased in eugenol-treated cells (6.25 and 25 μg/mL) compared with cells induced by APAP. Based on the statistical analysis, eugenol at 25 μg/mL could significantly increase the GPX gene expression. While the addition of eugenol at 6.25 μg/mL didn't show any significant difference.

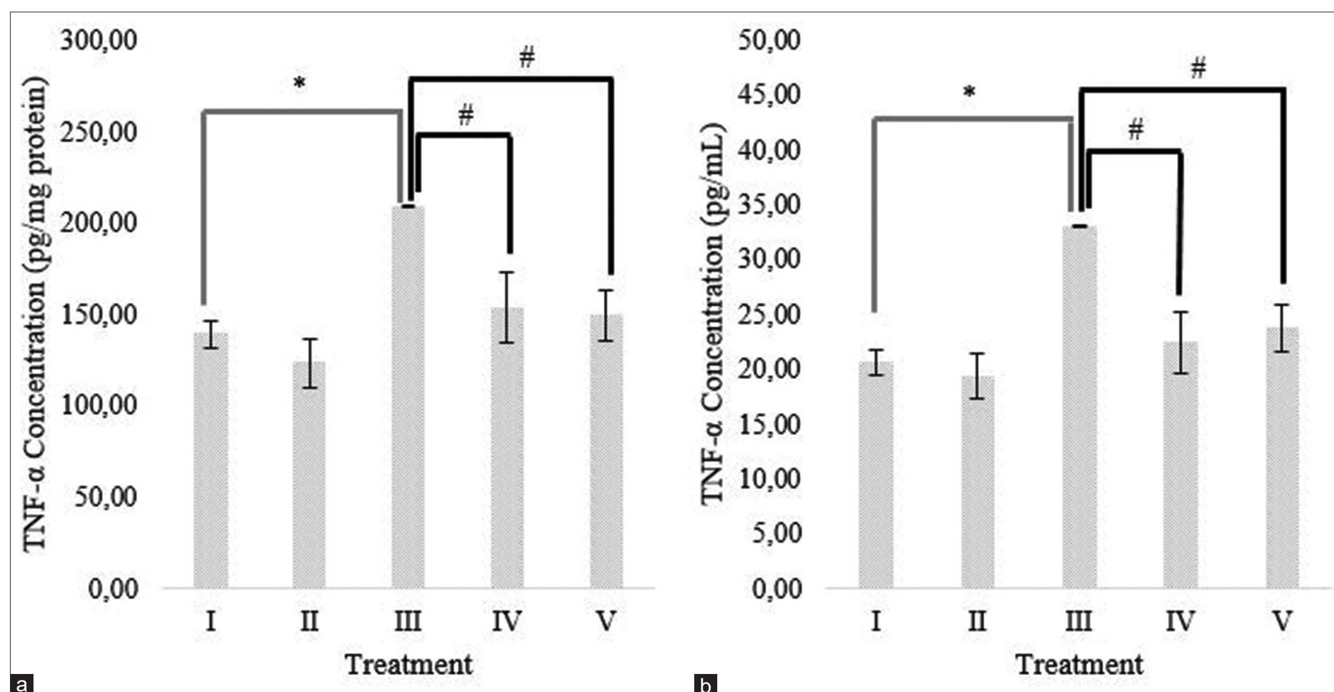

*Figure 1: Effect eugenol toward tumor necrosis factor-α (TNF-α) concentration in Acetaminophen (APAP)-induced human hepatocellular carcinoma (HepG2) cells as hepatotoxicity model. (a) TNF-α level (pg/mg protein) on APAP-induced HepG2 cells. (b) TNF-α level (pg/mL) on APAP-induced HepG2 cells. *n=3, the data is illustrated as mean + standard deviation. (I) Normal cells; (II) Vehicle control (APAP-induced HepG2 cells + DMSO 1%); (III) APAP-induced cells; (IV) APAP-induced cells + eugenol 6.25 μg/mL; (v) APAP-induced cells + eugenol 25 μg/mL. Single star sign (*) marks statistical difference between control group and APAP-induced cells group and at 0.05 significance level based on Tukey HSD post hoc test, single hashtag (#) marks statistical difference in treatment groups compared to APAP-induced cells model group at 0.05 significance level based on Tukey HSD post hoc test*

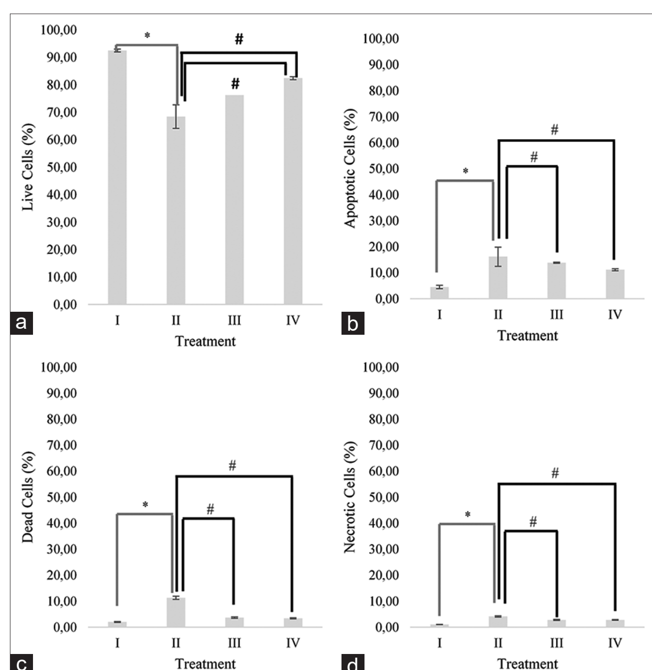

*Figure 2: Effect of eugenol towards apoptotic, necrotic, dead cells of Acetaminophen (APAP)-induced human hepatocellular carcinoma cells. (a) Live cells (%); (b) Apoptotic cells (%); (c) Dead cells (%); (d) Necrotic cells (%). *n=3, the data is illustrated as mean + standard deviation. (I) Normal cells; (II) APAP-induced cells; (III) APAP-induced cells + eugenol 6.25 μg/mL; (IV) APAP-induced cells + eugenol 25 μg/mL. Single star sign (*) marks statistical difference between control group and APAP-induced cells group and at 0.05 significance level based on Tukey HSD post hoc test, single hashtag (#) marks statistical difference in treatment groups compared to APAP-induced cells model group at 0.05 significance level based on Tukey HSD post hoc test*

Discussion

Overdoses of APAP have been reported can cause hepatotoxicity [26]. APAP can cause liver damage by inducing oxidative stress, which is triggered by the toxic metabolite NAPQI [27]. Inflammation also may potentially play a role in the pathophysiology of APAP-induced hepatotoxicity [4]. Eugenol belongs to the class of phenylpropanoids and is a phenolic compound [28]. Eugenol has pharmacological activities such as antioxidant, anti-inflammatory, anticancer, and antibacterial [10], [29], [30], [31]. Based on a study, eugenol has hepatoprotective effects in an APAP-induced hepatotoxicity model. Also, eugenol could decrease LDH levels as well as AST and ALT activity [11].

TNF-α was major key pro-inflammatory cytokines involved in oxidative stress injury [2]. APAP-induced can increase liver tissue of TNF-α [32]. Increased circulating TNF-α stimulates cell surface TNF-α receptors, which activate the stress-related protein kinases, JNK and IKKβ. This results in increased inflammatory cytokine production and decreased insulin sensitivity [33]. As a result, TNF-α inhibition was considered as a therapeutic way for fatty liver and liver injury [34], [35]. TNF has been manipulated pharmacologically and genetically to treat liver disease. Because low "basal" TNF levels are required for liver regeneration, down regulating but not completely

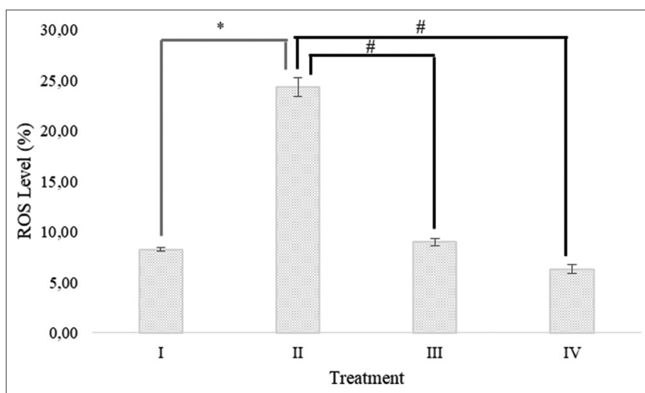

*Figure 3: Effect of eugenol toward reactive oxygen species level in Acetaminophen (APAP)-induced human hepatocellular carcinoma cells as hepatotoxicity model. *n=3, the data is illustrated as mean + standard deviation. (I) Normal cells; (II) APAP-induced cells; (III) APAP-induced cells + eugenol 6.25 μg/mL; (IV) APAP-induced cells + eugenol 25 μg/mL. Single star sign (*) marks statistical difference between control group and APAP-induced cells group and at 0.05 significance level based on Tukey HSD post hoc test, single hashtag (#) marks statistical difference in treatment groups compared to APAP-induced cells model group at 0.05 significance level based on Tukey HSD post hoc test*

inhibiting TNF activity is a desirable therapeutic option for liver disease [34], [36].

Based on the results, treatments using eugenol can decreased TNF-α concentration in APAP-induced HepG2 cells. The results were in line with Yuan *et al*. study that used ferulic acid, a phenolic compound, at doses 100 mg/kg that decreased TNF-α concentration in the mice that received APAP at doses 350 mg/kg [37].Eugenol improves liver function to near-normal levels by inhibiting lipid peroxidation and cytokine release. Thus, it is obvious that the mechanism of eugenol protection may be due to a decrease in ROS production, inflammatory cell infiltration, and cytokine production by Kupffer cells [38].

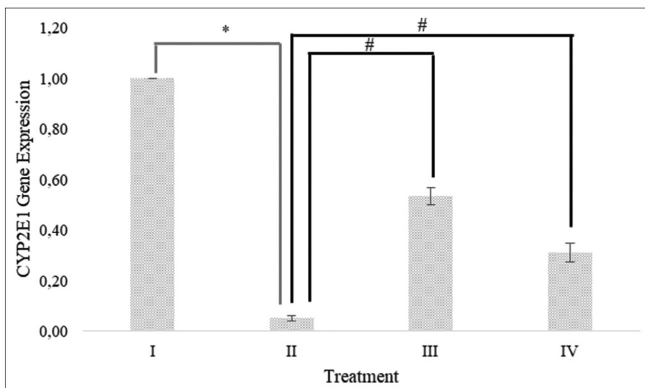

*Figure 4: Effect of eugenol toward CYP2E1 relative gene expression in Acetaminophen (APAP)-induced human hepatocellular carcinoma cells as hepatotoxicity model *n=3, the data is illustrated as mean + standard deviation. (I) Normal cells; (II) APAP-induced cells; (III) APAP-induced cells + eugenol 6.25 μg/mL; IV) APAP-induced cells + eugenol 25 μg/mL. Single star sign (*) marks statistical difference between control group and APAP-induced cells group and at 0.05 significance level based on Tukey HSD post hoc test, single hashtag (#) marks statistical difference in treatment groups compared to APAP-induced cells model group at 0.05 significance level based on Tukey HSD post hoc test*

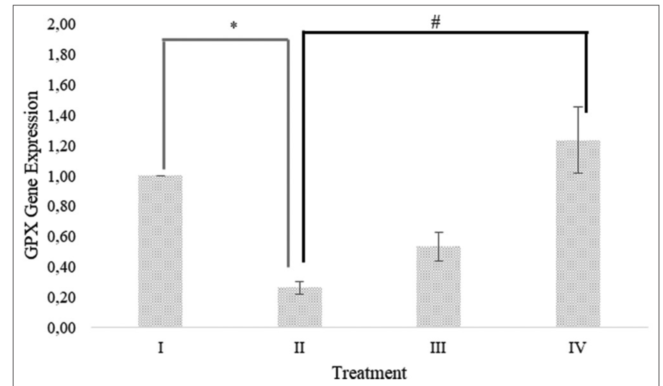

*Figure 5: Effect of eugenol toward GPX gene expression relative in Acetaminophen (APAP)-induced human hepatocellular carcinoma cells as hepatotoxicity model. *Data is presented as mean ± standard deviation. (I) Normal cells; (II) APAP-induced cells; (III) APAP-induced cells + eugenol 6.25 μg/mL; (IV) dsx-induced cells + eugenol 25 μg/mL. Single star sign (*) marks statistical difference between control group and APAP-induced cells group and at 0.05 significance level, single hashtag (#) marks statistical difference in treatment groups compared to APAP-induced cells model group at 0.05 significance level*

Apoptosis and necrosis frequently coexist in pathological conditions of the liver, and the balance of cell death may be dictated by the particular insult [1]. Based on the results, when HepG2 cells were induced by APAP the TNF-α concentration also the apoptotic activity was increased. Eugenol treatments could decrease the TNF-α concentration including the apoptotic, necrotic, dead cells. The result was also in agreement with Yuan et al. study that said APAP-induced could cause severe hepatocellular necrosis, while fewer apoptotic cells were seen in the APAP-induced hepatotoxicity model treated with ferulic acid [37]. This result was validated with previous research that eugenol 3.125–25 μg/mL increased cell viability on HepG2 cells [11].

APAP treatment can increased ROS production [39]. APAP is metabolized mainly by the CYP2E1 isoform of CYP to NAPQI, which depletes intracellular GSH and covalently binds to proteins, including many mitochondrial proteins, triggers mitochondrial damage and production of ROS [40]. It was leading to an overwhelming mitochondrial oxidant stress and mitochondrial dysfunction [41]. Based on the results, eugenol could decrease ROS level in HepG2 cells that induced by APAP. It's because eugenol is an antioxidant and a scavenger of ROS [9], [38]. The findings are in agreement with Parikh et al. who found that quercetin and catechin, phenolic compounds found in Brassica juncea hydromethanolic extract, may lower ROS levels in HepG2 cells when induced by APAP [42]..

CYP2E1 plays a crucial role in the metabolism of a wide range of endogenous and exogenous chemicals, and it has been linked to chemical toxicity and liver carcinogenesis [43]. ROS produced by

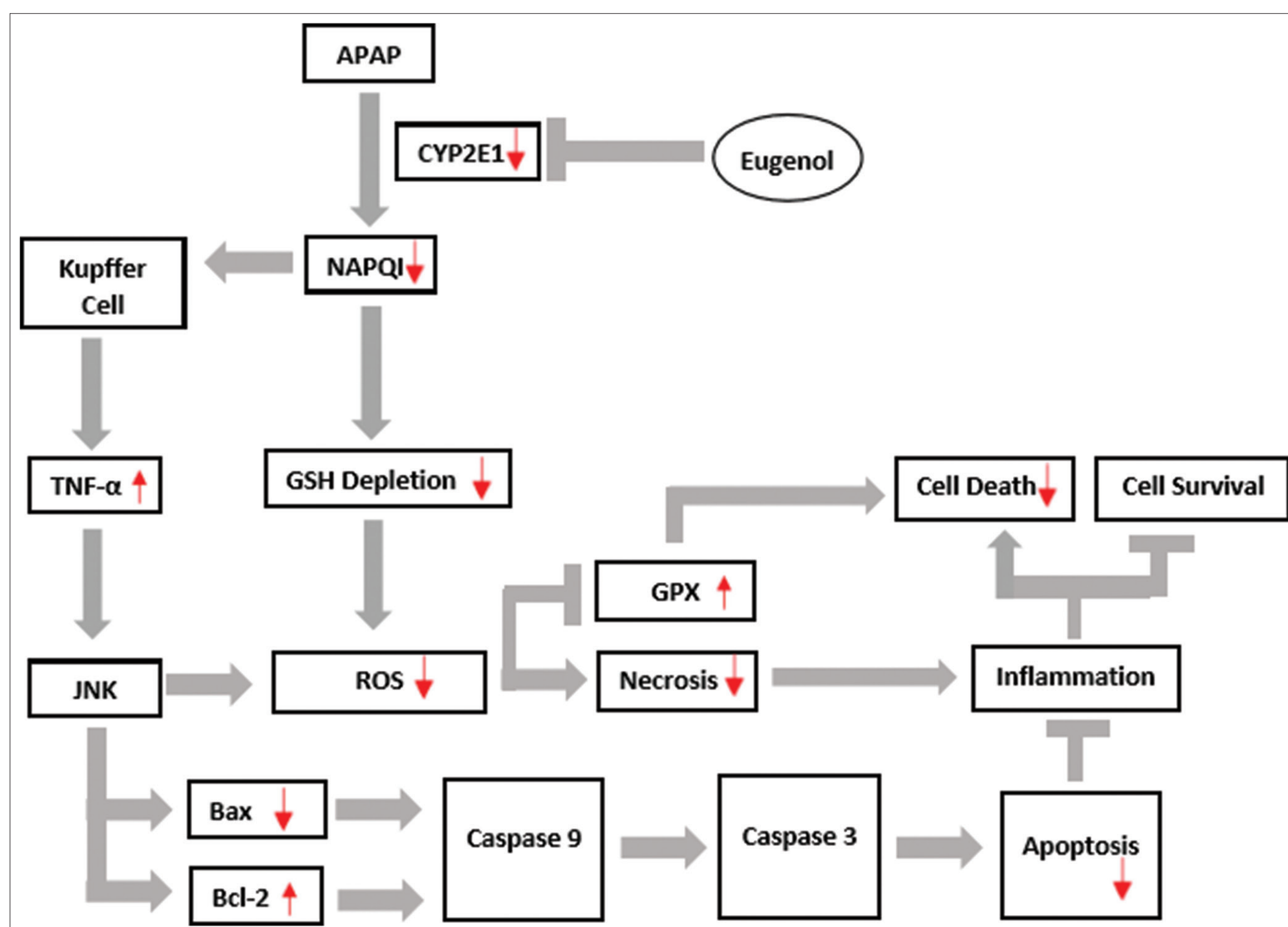

Figure 6: Proposed mechanism on how eugenol could act as a hepatoprotective agent in liver injury model. Acetaminophen is transformed into NAPQI mainly by CYP2E1. The presence of NAPQI could induce GSH depletion and leads to production of reactive oxygen species (ROS). The excessive ROS led to down regulation of GPX gene expression and increasing the cell death. The presence of NAPQI could activate the kupper cell and causing the production of tumor necrosis factor-α (TNF-α). It was induced JNK signaling pathways resulting in increased ROS and leads to unregulated cell death, necrosis; increase inflammation; and promote cell death. On the other hand, JNK-induced cause downregulated of Bcl-2 and upregulated of Bax resulting in activation of caspase 9 and caspase 3. The activiation of caspase 9 and 3 leads to apoptotic. Treatment of eugenol could inhibit the CYP2E1 enzyme and suppress the NAPQI production. It leads to decrease the ROS level, TNF-α production, and necrosis that leads to lower inflammation. The treatment also could suppress the apoptotic by increasing the Bcl-2 level and decreasing the Bax level. So, the eugenol treatment could decrease the cell death and increase the survival of hepatic cells

CYP2E1 have been shown to increase lipid peroxidation and mitochondrial membrane permeability, release pro-apoptotic proteins, and activate caspase 3 to induce apoptosis [44]. ROS produced by CYP2E1 have been shown to increase lipid peroxidation and mitochondrial membrane permeability, release pro-apoptotic proteins, and activate caspase 3 to induce apoptosis [37]. It was shown that eugenol has a protective effect in APAP hepatotoxicity that directly influences on APAP metabolism by inhibiting CYP2E1.

The oxidative stress response can be evaluated using GPX [45]. Based on the results, APAP decreased the GPX gene expression while eugenol treatment increased the gene expression after the addition of APAP. It means eugenol has hepaprotective activity involves antioxidant gene expression restoration. The result was in agreement Zhao *et al*. study that used 4-hydroxyphenylacetic, a phenolic compound, at doses 25 mg/kg resulting an increment of GPX gene expression in APAP-induced liver injury in mice [46]. Eugenol improved liver injury by decreasing LDH, AST, and ALT in APAP-induced liver injury [11].

Based on the findings of this research, eugenol has been shown to have hepatoprotective properties due to its anti-necrotic, anti-inflammatory, and antioxidant properties. We proposed a mechanism on how eugenol act as hepatoprotective agent in liver injury (Figure 6).

Conclusion

Eugenol at 6.25 and 25 μg/mL concentration can reduce TNF-α concentration, the apoptotic activity, and ROS level also increases the GPX and CYP2E1 gene expression in APAP-induced HepG2 cells. The best hepatoprotective effect was found when using the

eugenol at 25 μg/mL. Therefore, eugenol can be used against APAP-induced in HepG2 cells.

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