Hepatoprotective effect of Eugenol on Acetaminophen-Induced Hepatotoxicity in HepG2 cells

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Abstract. Imbalance in liver metabolism lead to oxidative stress mainly caused by free radicals or termed as reactive oxidative oxygen (ROS). Prolonged ROS exposure without proper treatment induce severe liver damage and serious hepatic diseases including cirrhosis. Eugenol (4-allyl 2-methoxyphenol) is phenolic derivative compound that showed antioxidant, anti-inflammatory, analgesic, antibacterial, antifungal, and antitumor activities. This study aims to evaluate the hepatoprotective effect of eugenol through biochemical markers analysis. Cytotoxic assay was performed in various concentrations of eugenol (3.125; 6.25; 12.5; 25; 50; 100 µg/mL) using (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) to determine the safe concentrations for next assays. Aspartate aminotransferase (AST), alanin aminotransferase (ALT), and lactate dehydrogenase (LDH) assay were performed using colorimetric method to evaluate the levels and activity of liver-related enzymes which are elevated in damaged liver as they were used as hepatotoxicity markers. The viability of HepG2 cells increased in eugenol concentration 3.125 µg/mL and then decreased along with the rise of eugenol concentrations. From this cytotoxic assay, two concentrations of eugenol were choosen (6.25 and 25 ug/ml) to be evaluated in the next assays. The level of LDH, ALT, and AST decreased after eugenol treatment compared to negative control. The most effective concentration of eugenol to seemed different in certain hepatotoxicity markers. This study suggests that eugenol was safe to use for cells culture environment in large ranges of concentrations and shows hepatoprotective effect in APAP-induced hepatotoxicity model by the decrease of LDH level and AST and ALT activities.

Keywords: Hepatoprotective, Hepatotoxicity, Eugenol, HepG2, LDH, ALT, AST

1. Introduction
Liver is one of crucial organ in human body. It plays important role in maintaining metabolism, especially in toxic substances elimination and biotransformation. Imbalance in liver metabolism lead to oxidative stress which mainly caused by free radicals or termed as reactive oxidative oxygen (ROS). ROS generation within hepatocytes induces oxidative damage, cell metabolism, cell necrosis, moreover cell death [1]. Prolonged ROS exposure without proper treatment can cause severe liver damage and
serious hepatic diseases including cirrhosis [2]. According to World Health Organization (WHO), around 800 thousand of 2.4 million deaths yearly that related to liver disease are caused by cirrhosis [3]. The incidence of hepatic diseases has not decreased although there were many modern medications available nowadays [4].

Acetaminophen (APAP), or widely known as paracetamol, is one of most common drugs that is prescribed for mild pain treatment. It has analgesic and antipyretic properties which is safe and effective at specific doses. APAP overdose produces the toxic effects through mitochondrial damage and increased oxidative stress [5][6]. Several studies have revealed that the mechanism of APAP hepatotoxicity is initiated by the formation of reactive and toxic metabolite called Nacetyl-P-benzoquinoneimine (NAPQI) that is mainly occurred in hepatic cytochrome P-450. Normal amount of NAPQI could be detoxified by conjugating with glutathione (GSH) – natural antioxidant – to form mercapturic acid and excreted in bile [7][8]. The excess generation of NAPQI is occurred when the availability of GSH is insufficient. They increased the reaction of NAPQI with sulphydryl groups of proteins to form protein adducts. Next, protein adducts formation is the critical process in hepatotoxicity because it induces mitochondrial oxidative especially in superoxide production [8][9]. Other studies revealed that the initial event of APAP-induced liver injury is the changes of macrophages microenvironment and secondary innate immune response activation by upregulating proinflammatory cytokines production [10][11].

Eugenol (4-allyl 2-methoxyphenol) is phenolic derivative compound that showed antioxidant, anti-inflammatory, analgesic, antibacterial, antifungal, and antitumor activities [12]. It is derived from Eugenia caryophyllata, especially from the leaves and buds, and is formed clove essential oil. Eugenol is considered safe to use in the food industry, cosmetics industry and in dentistry by the US Food and Drug Administration (FDA). Eugenol is known to have an inhibitory effect of proinflammatory cytokines production and potential to decrease the activity of dehydrogenase enzymes at dose dependent manner [13]. Other studies showed that eugenol could elevate the level of GSH as the important protein to maintain during the occurrence of stress oxidative in hepatotoxicity process [12]. Therefore, this study aims to evaluate the hepatoprotective effect of eugenol through analysis of specific enzymes production which elevated during hepatotoxicity induced by APAP.

2. Materials and Methods

2.1. Cytotoxic Assay

Human hepatocellular carcinoma (HepG2) cells line (ATCC, HB-8065™) was obtained from Biomolecular and Biomedical Research Center, Aretha Medika Utama, Bandung, Indonesia. The cells were thawed and grown in Dulbecco’s Modified Eagle Medium (DMEM) (Biowest, L0102-500) supplemented with 10% (v/v) fetal bovine serum (FBS) (Biowest, S1810), addition of 1% (v/v) antibiotic-antimycotic (Gibco, 15240062) and 1% (v/v) nanomycopulitine (Biowest, LX16). The cells were incubated in 37°C and 5% CO₂. The excess generation of NAPQI is occurred when the availability of GSH is insufficient. They increased the reaction of NAPQI with sulphydryl groups of proteins to form protein adducts. Next, protein adducts formation is the critical process in hepatotoxicity because it induces mitochondrial oxidative especially in superoxide production [8][9]. Other studies revealed that the initial event of APAP-induced liver injury is the changes of macrophages microenvironment and secondary innate immune response activation by upregulating proinflammatory cytokines production [10][11].

Eugenol (4-allyl 2-methoxyphenol) is phenolic derivative compound that showed antioxidant, anti-inflammatory, analgesic, antibacterial, antifungal, and antitumor activities [12]. It is derived from Eugenia caryophyllata, especially from the leaves and buds, and is formed clove essential oil. Eugenol is considered safe to use in the food industry, cosmetics industry and in dentistry by the US Food and Drug Administration (FDA). Eugenol is known to have an inhibitory effect of proinflammatory cytokines production and potential to decrease the activity of dehydrogenase enzymes at dose dependent manner [13]. Other studies showed that eugenol could elevate the level of GSH as the important protein to maintain during the occurrence of stress oxidative in hepatotoxicity process [12]. Therefore, this study aims to evaluate the hepatoprotective effect of eugenol through analysis of specific enzymes production which elevated during hepatotoxicity induced by APAP.

2.2. LDH, ALT, and AST Activity

Lactate dehydrogenase (LDH), Aspartate Aminotransferase (AST), and Alanin Aminotransferase (ALT) assay were performed using colorimetric method to evaluate the levels and activity of metabolic enzymes which are elevated in damaged tissues especially in liver tissue. HepG2 cells were plated in 6-wells plate at cells density of 1 x 10⁶ cells/well and incubated in 37°C and 5% CO₂ to attain confluency.
After 24 hours, cells were treated with 1.8 mL 2% FBS supplemented DMEM containing 40 mM acetaminophen (Sigma Aldrich, A7085), 1% (v/v) DMSO (Merck, 1029521000), and medium only for 24 hours in 37°C and 5% CO\textsubscript{2}. The cells were added with eugenol solution at concentrations 6.25 µg/mL and 25 µg/mL for 24 h. LDH levels, ALT and AST activity were determined using LDH assay kit (Elabscience, E-BC-K045), ALT assay kit (Elabscience, E-BC-K235), and AST assay kit (Elabscience, E-BC-K236). All assays were conducted as described in manufacturer protocol [2][7].

3. Results and Discussion
3.1. Cytotoxicity of Eugenol on HepG2 Cells
In this study, APAP-induced HepG2 cells line is used as hepatotoxic model. It is widely known that APAP can induce liver injury through oxidative stress mechanism which is initiated by toxic metabolite, NAPQI [8]. APAP induced hepatotoxicity in overdose condition and there was a variability result of induction. Therefore, this study used acetaminophen at concentration 40 mM because it resulted in best inducer for cells injury as reported by Gonzalez et al. (2017) [7].

The cytotoxic assay was measured based on the MTS assay and colorimetric principle for assessing cell metabolic activity. In the presence of the viable cells, yellow-colored MTS will be reduced to purple-colored formazan product by NAD(P)H-dependent dehydrogenase enzymes that is produced by metabolically active cells [15][17]. From the Figure 1, the viability of HepG2 cells increased significantly around 120% in eugenol at concentration 3.125 µg/mL compared to the normal cells (without eugenol treatment). But, it decreased along with the rise of eugenol concentrations, and result in lowest cell viability after treating with eugenol at concentration 100 µg/mL among other groups of concentrations.

![Figure 1](image)

**Figure 1.** Cell viability of HepG2 after treatment by various concentrations of eugenol *The data was presented as a histogram of mean±standard deviation. Different letters (a, ab, bcd, cd,de e) symbolized a significant different among various treatments of eugenol concentrations, based on One-Way Anova analysis and it was followed by Tukey HSD post hoc test (p<0.05).

Based on the result of viability assay (Figure 1), eugenol is considered safe in broad range of concentrations for HepG2 cells line. FDA also approved the use of eugenol in several types of industry [13]. It has been reported that eugenol has toxic effect at dose dependent level and less cytotoxic effect compared to its derivative, isoeugenol [18]. from this assay, two concentrations of eugenol were chosen (6.25 and 25 µg/mL) to be evaluated in the next assays. Those two concentrations were chosen because they had similar percentage of viability with the normal one.
3.2. LDH, AST, and ALT activity

The toxic effect of APAP are shown in Figure 2 based on the level of LDH, as it is known as marker for the damage tissues. The induction of APAP significantly increased LDH level compared to normal cells and vehicle control. The addition of both concentrations of eugenol reduced significant level of LDH. It is evident from the result that eugenol at concentration 6.25 µg/mL reduced LDH level. Furthermore, at concentration 25 µg/mL had better to approaching the normal level of LDH in control groups.

Figure 2. The effect of eugenol concentrations on APAP-induced HepG2 cells towards LDH production. The data was presented as a histogram of mean±standard deviation. Different group of letters (a, b) symbolized a significant different among various group treatments, based on One-Way Anova analysis and it was followed by Tukey HSD post hoc test (P<0.05). Normal cells: cells without any treatments; Vehicle control: cells with DMSO 1% treatment; APAP control: cells with APAP treatment; APAP+Eug6.25: cells with APAP + Eugenol 6.25 µg/mL treatment; APAP+Eug25: cells with APAP + Eugenol 25 µg/mL treatment.

LDH is a non-organ specific enzyme distributed throughout the body and elevated in different types of damage tissues including skeletal, cardiac muscle, kidney, and liver. LDH levels indicates the increased level of cells necrosis and was regarded as biomarker of cell cytotoxicity [19]. In this study, the levels of LDH significantly decreased in the addition of 6.25 µg/mL eugenol. The antioxidant properties of eugenol such as elevating GSH level may cause the reduction of enzymes leakage production by the damage cells [2].

ALT is a specific metabolic enzyme that predominantly found in liver organ specifically located in the cytoplasm. As it is found mainly in liver, ALT was considered as valuable biomarker of liver damage. AST is another enzyme that is used to monitor various liver diseases including alcoholic hepatitis and cirrhosis in combination with ALT. However, AST is not only found in liver tissue which is similar to LDH, but also in several damage tissues such as heart, skeletal muscles, kidney, pancreas, lungs and brain tissues [19]. The ratio of AST and ALT can be an indication of liver-related disease, higher level of ALT than AST indicates acute hepatitis caused by virus. On the other hand, alcoholic hepatitis or cirrhosis caused by stress damage result in higher AST:ALT ratio. The result of this study revealed that AST produced higher range of activities in U/mg protein than that of ALT [20][19].
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Figure 3. The effect eugenol concentrations on APAP-induced HepG2 cells toward ALT activity. *The data was presented as a histogram of mean±standard deviation. Different group of letters (a, b) symbolized a significant different among various group treatments, based on One-Way Anova analysis and it was followed by Tukey HSD post hoc test (p<0.05). Normal cells: cells without any treatments; Vehicle control: cells with DMSO 1% treatment; APAP control: cells with APAP treatment; APAP+Eug6.25: cells with APAP + Eugenol 6.25 µg/mL treatment; APAP+Eug25: cells with APAP + Eugenol 25 µg/mL treatment.

The ALT and AST activities can be seen at Figure 3 and 4 after APAP induction for 24 hours and it was followed by the treatment with two different concentrations of eugenol. Eugenol at concentration 25 µg/mL could reduce the activity of ALT compared to the negative control (APAP control), and it was significant different with the ALT activity that slightly reduce in a group treatment of eugenol at concentration 6,25 µg/mL (Figure 3).

It suggested that eugenol at concentration 25 µg/mL had better capacity of reducing ALT activity. The AST activity show the same result, eugenol at concentration 25 µg/mL showed drop of AST activity among the other group treatments and APAP control (Figure 4). This finding clearly revealed that eugenol at concentration 25 µg/mL had better capacity to lower the AST activity.

Figure 4. The effect eugenol concentrations on APAP-induced HepG2 cells toward AST activity. The data was presented as a histogram of mean±standard deviation. Different group of letters (a, ab, b, c) symbolized a significant different among various group treatments, based on One-Way Anova analysis and it was followed by Tukey HSD post hoc test (P<0.05). Normal cells: cells without any treatments; Vehicle control: cells with DMSO 1% treatment; APAP control: cells with APAP treatment; APAP+Eug6.25: cells with APAP + Eugenol 6.25 µg/mL treatment; APAP+Eug25: cells with APAP + Eugenol 25 µg/mL treatment.
The addition of eugenol in APAP-induced HepG2 showed reductions in ALT and AST activities (Figure 3 and 4). This finding has similar result with the study demonstrated by Binu et al. (2018) that eugenol exhibited an hepatoprotective effects through the rise of specific antioxidant markers including GSH, catalase (CAT), and glutathione peroxidase (GPX) as well as reduction in AST and ALT activities, decreased NO and Thiobarbituric acid reactive substances (TBARS) in arsenic trioxide-induced hepatotoxicity [12]. The elevation of AST and ALT activities may cause by the leakage of plasma membrane as the consequence of defect transport function of hepatocytes [21]. The decrease of these enzyme levels in APAP-induced hepatotoxicity model after eugenol treatment has clearly proved its hepatoprotective effects at dose dependent. This study suggested that eugenol at concentration 25 µg/mL is way more effective in reducing the activity of ALT or AST. However, further studies of eugenol antioxidant and anti-inflammatory capacity will be necessary to support this finding.

4. Conclusion

This study suggests that eugenol was safe to use for cells culture environment in broad ranges of concentrations. The best hepatoprotective effects exhibited by eugenol at concentration 25 µg/mL by the decrease of LDH level and AST and ALT activities in APAP-induced hepatotoxicity model.

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