Piper crocatum Ameliorates APAP-Induced Hepatotoxicity through Antioxidant and Anti-Inflammatory Mechanisms

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Abstract. Cirrhosis is a serious hepatic disease that occurs worldwide and is caused by progressive fibrosis in the liver. Free radicals are the major cause of reactive oxygen species (ROS) generation, which affects the balance of the liver metabolism. *Piper crocatum*, commonly called red betel, is widely used in ethnomedicine because it has biological capabilities, including antioxidant and anti-inflammatory properties. The present study was undertaken to examine the possibility of hepaprotective activity of red betel extract on acetaminophen (APAP)-induced HepG2 cells as the cirrhosis *in vitro* model. Assessment of red betel extract (RBE) was performed using the colorimetric method for the quantification of LDH, AST, and ALT. As preliminary study, a cytotoxicity assay was performed at various RBE concentrations (100.00; 50.00; 25.00; 12.50; 6.25; 3.13 μg/ml) using an MTS assay to decide the safe concentration based on cell viability. The RBE treatment did not affect cell viability, even at a high concentration (100 μg/ml, $p > 0.05$). RBE at concentrations 25 and 100 μg/ml successfully reduced LDH, AST, and ALT activities in the hepatotoxic model in a dose-dependent manner. In the APAP-induced hepatotoxicity model, RBE positively indicated a hepatoprotective effect and cell amelioration by a decrease in hepatotoxic markers.

Keywords: *aminotransferase; alanine aminotransferase; cirrhosis; lactate dehydrogenase; red betel.*

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1 Introduction

Reactive oxygen species (ROS) are free radicals and reactive molecules that have oxygen content and can react easily with other molecules. ROS can cause damage to DNA, RNA, and proteins, and even cause cell death. Thus, they can affect the functioning of the liver, especially in biotransformation and eliminating toxic substances. Free radicals can trigger a liver metabolism imbalance. The generation of ROS within the hepatocytes causes cell metabolism disorder, oxidative damage, cell necrosis, and cell death [1]. Continuous exposure to ROS can cause serious damage to the liver, including cirrhosis [2]. The World Health Organization (WHO) states that cirrhosis causes about 800,000 of the 2.4 million liver-related deaths each year [3]. The incidence of liver disease does not decrease in spite of the many modern drugs available today [4].

Acetaminophen (APAP), also identified as paracetamol, is one of the most commonly prescribed medicines for pain relief (analgesics) and fever control (antipyretics), and is safe and effective at certain dosages. The use of excessive doses of APAP can lead to toxic effects due to enhanced oxidative stress and mitochondrial harm [5][6]. A small amount of acetaminophen is metabolized into N-acetyl-P-benzoquinone imine (NAPQI), a reactive and toxic metabolite, through the P-450 hepatic cytochrome pathway. NAPQI can be detoxified by glutathione (GSH), a hepatic natural antioxidant, and is then excreted in bile [7][8]. If the GSH concentration is inadequate, an excess of NAPQI will be generated. The increase in NAPQI will form protein adducts on mitochondrial proteins. Later, it will induce mitochondrial oxidative stress by superoxide production [8][9]. Other studies have revealed that APAP induced liver damage causes a change in the macrophage microenvironment and activates secondary immune responses by increasing pro-inflammatory cytokine production [10][11].

Active compounds of medicinal plants are a promising source for therapeutic drugs. The *Piper* genus is very diverse in geography, with about 700 species found in tropical America and almost 340 in tropical Asia, including Indonesian rain forests [12]. Red betel is a medicinal plant with red leaves of the genus *Piper*. Many indigenous people plant this herb in the yard as an herbal remedy. It is known that red betel contains phytochemicals, such as phenolics, essential oils, flavonoids, and terpenes, that have positive bioactivity effects. Therefore, this bioactive compound has potential as antimicrobial agent, antioxidant, antiseptic, and antihyperglycemic [13][14]. Red betel leaf extract and its compounds, including eugenol and hydroxychavicol, are potent antioxidants [14]. A recent study has shown the anti-inflammatory potential of red betel through inflammatory mediator inhibition [15]. Piperine compound from black pepper (*Piper nigrum)* improves liver functioning and can reduce APAP-induced hepatotoxicity mediated via its antioxidant, anti-inflammatory, and anti-apoptotic

properties, as well as Transforming Growth Factor Beta Receptor Associated Protein 1 (TGFBRAP1) modulation [16]. The novelty of this research is the investigation of red betel extract (RBE) for its hepatoprotective effect. Hence, the objective of this research was to elucidate the hepatoprotective potential of RBE by analyzing the development of some enzymes that are elevated during APAP-induced hepatotoxicity.

2 Materials and Methods

2.1 Cytotoxicity Assay (MTS Assay)

A cytotoxicity assay was conducted to determine the safe concentration of RBE toward liver cells. Human hepato-cellular carcinoma (HepG2) (ATCC, HB-8065^{TM}) cell line was provided by Biomolecular and Biomedical Research Center, Aretha Medika Utama, Bandung, Indonesia. Cells were grown in Dulbecco's Modified Eagle Medium (DMEM) (Biowest, L0102-500), fetal bovine serum 10% (v/v) (FBS) (Biowest, S1810), antibiotic antimycotic 1% (v/v) (ABAM) (Gibco, 15240062), nanomycopulitine 1% (v/v) (Biowest, LX16) and incubated in 37 °C, 5% CO_2 incubator. For maintenance, the culture medium was replaced after three days. The cells with density $5x10^3$ cells in 96 well plate, cytotoxic analysis was performed at various levels of RBE (100; 50; 25; 12.5; 6.25; 3.125 µg/ml incubated in 37 °C and 5% CO_2 for 24 hours. Subsequently, 20 µl of 3-4,5-dimethylthiazol-2-(yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) solution was applied to each sample. Then, the cell culture was re-incubated for 3 hours. The number of live cells was measured by spectrophotometric analysis at 490 nm wave length (MultiSkan GO Microplate Reader, Thermo Scientific) [17][18][19].

2.2 Hepatotoxic Model and Treatments

HepG2 cells were grown in a six-well plate with density 1×10^6 cells/well in DMEM complete medium and incubated in an incubator at 5% CO_2, 37 °C to achieve confluence. Five experimental groups were designed as follows: 1) control group (HepG2 cells in medium only); 2) vehicle control – to ensure that DMSO as extract solvent does not affect cell growth that may result in outcome bias (control + 1% DMSO (v/v) (Merck, 1029521000)); 3) hepatotoxic model (control + 40 mM APAP (Sigma Aldrich, A7085)); 4) treated groups (hepatotoxic model + RBE 25 µg/ml based on cytotoxicity assay); 5) treated groups (hepatotoxic model + RBE 100 µg/ml based on cytotoxicity assay). All experimental groups were incubated for 24 hours in an incubator at 37 °C, 5% CO_2 [17].

2.3 Bradford Assay

Total protein contained in the treated and control HepG2 cells was measured with Bradford assay. Protein standard curves were formed using Bovine Standard Albumin (BSA, Sigma Aldrich, A9576), 2 mg BSA in 1000 µl ddH_2O in serial dilution. Briefly 20 µl of standard protein and samples were plated in a 96-well plate, and were added with 200 µl Quick Start Dye Reagent 1X (Bio Rad, 5000205) for 5 minutes at room temperature. The absorbance was measured by a microplate reader at 595 nm wave length [22].

2.4 LDH, ALT, AST Assay

Lactate dehydrogenase (LDH), alanine aminotransferase (ALT), aspartate aminotransferase (AST) were evaluated as parameters of detoxification in hepatic tissue. LDH, ALT and AST were measured using E-BC-K236, E-BC-K045, E-BC-K235 kits from Elabscience according to the manufacturers' protocol [2][7][16].

2.5 Result and Discussion

2.6 Cytotoxicity of Red Betel Extract on HepG2 Cells

The HepG2 cells line induced by APAP were used as the hepatotoxic model in this research. APAP is commonly known to cause liver damage through oxidative stress triggered by NAPQI [8]. An overdose of APAP causes hepatotoxicity; the outcome of induction is variable. This research used APAP at a concentration of 40 mM, referring to Gonzalez *et al.* [7].

The viability of RBE-treated HepG2 cells increased significantly compared to normal cells by about 130 percent, with a concentration of 3,125 µg/ml (without APAP treatment) (Fig. 1). This suggests that RBE may have cytoproliferative effects at low concentration. Cell viability decreased in a dose-dependent manner. Nevertheless, at the highest dose (100 µg/ml), there was no significant cell reduction compared to normal cells. It can be concluded that RBE does not have a cytotoxic effect on hepatic cells.

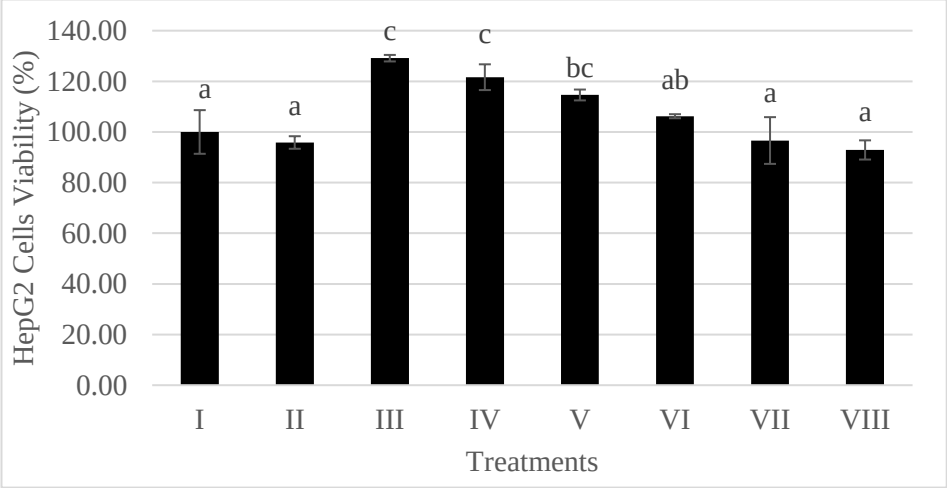

Figure 1 The effect of RBE concentration on HepG2 cell viability. The data is represented as a mean±standard deviation histogram. Different superscript letters (a, ab, bc, c) designate significant differences among various concentration of RBE at $p < 0.05$ based on a one-way ANOVA, followed by a Tukey HSD post-hoc test. I: HepG2 normal cells, II: HepG2 cells added 1% DMSO, III: HepG2 cells treated 3.125 µg/ml. IV: HepG2 cells treated 6.25 µg/l, V: HepG2 cells treated 12.50 µg/ml, VI: HepG2 cells treated 25.00 µg/ml, VII: HepG2 cells treated 50.00 µg/ml, VIII: HepG2 cells treated 100.00 µg/ml.

Then, two concentrations of RBE (25 and 100 µg/ml) were chosen from this assay to be tested in later assays. These concentrations were chosen because their viability did not differ significantly compared to control.

2.7 Total Content of Protein

The Bradford assay is useful for estimating protein concentrations in a variety of biochemistry and biology applications [22]. The total protein contents in all tested groups are listed in Table 1.

Table 1 The total content of protein

Treatments	Average (mg/ml)
Normal cells	0.148±0.003
DMSO solvent control	0.157±0.010
APAP-induced HepG2	0.158±0.106
APAP-induced HepG2+ RBE 25 µg/ml	0.153±0.008
APAP-induced HepG2+ RBE 100 µg/ml	0.159±0.008

The total contents of protein were used for calculating LDH, AST, ALT assay.

2.8 LDH, ALT, AST Levels

As can be seen in Figure 2, the LDH level was significantly increased in the hepatotoxic model. RBE treatment successfully reduced the LDH level in the hepatotoxic model. The best treatment was 25 µg/ml of RBE, which nearly reached the LDH level of normal cells. However, at a higher concentration, there was only a slight increase in LDH level, which means that a high dose of RBE has a hepatotoxic effect.

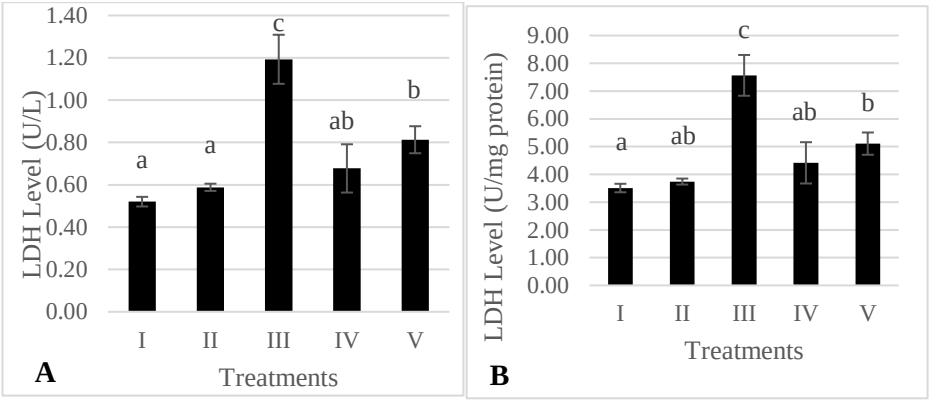

Figure 2 Effect of RBE on hepatotoxic cells towards LDH level. Figure 2A: LDH level in U/L (units/Liter). Figure 2B: LDH level in U/mg protein. The data is represented as a mean±standard deviation histogram. The different superscript letters (a, b, ab, c) designate significant differences among the treatments at $p < 0.05$ based on a one-way ANOVA, followed by a Tukey HSD post-hoc test. I: un-induced HepG2 cells, II: normal cells treated with 1% DMSO, III: APAP-induced HepG2 cells, IV: APAP-induced HepG2 cells treated with RBE 25 µg/ml, V: APAP-induced HepG2 cells treated with RBE 100 µg/ml.

LDH is an enzyme distributed throughout the body that plays an important role in the conversion of reversible lactate to pyruvate. LDH is elevated in damaged tissue, including skeletal, cardiovascular, kidney and liver tissues, and can thereby be considered a biomarker of cytotoxicity [23]. LDH was significantly decreased by the addition of RBE. RBE possesses antioxidant properties that may compensate for GSH depletion in the cirrhosis model, thereby substituting its role in NAPQI detoxification [2].

ALT is a type of metabolic enzyme that is specifically located in the cytoplasm of hepatocytes. Due to its specific location, ALT is considered a more valuable and accurate liver damage biomarker compared to others [24].

AST is another enzyme used in conjunction with ALT to monitor various liver diseases. AST is present not only in LDH-like liver tissue but also in other damaged tissues, such as tissues of heart, kidney, pancreas, brain, skeletal muscles and lungs [23]. ALT to AST comparison can be a useful indicator for the cause of cirrhosis. A higher ALT to AST ratio suggests virus-induced acute hepatitis. In contrast, a higher AST to ALT ratio indicates stress damage caused by cirrhosis such as alcoholic hepatitis. As shown in Figures 3 and 4, the AST level is higher than the ALT level, meaning that APAP causes stress to the hepatocytes [23][25].

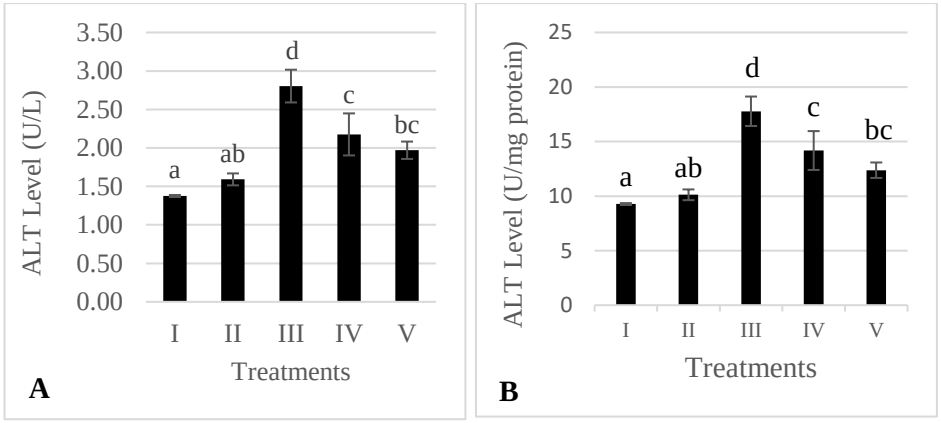

Figure 3 The effect of RBE on hepatotoxic cells towards ALT level. Figure 3A: ALT level in U/L (units/Liter). Figure 3B: ALT level in U/mg protein. The data is represented as a mean±standard deviation histogram. The different superscript letters (a, b, ab, bc, c, d) designate significant differences among the treatments at $p < 0.05$, based on a one-way ANOVA, followed by a Tukey HSD post-hoc test. I: un-induced HepG2 cells, II: normal cells treated with 1% DMSO, III: APAP-induced HepG2 cells, IV: APAP-induced HepG2 cells treated with RBE 25 µg/ml, V: APAP-induced HepG2 cells treated with RBE 100 µg/ml.

The activities of ALT and AST are shown in Figures 3 and 4 after 24-hour induction with APAP, followed by two different RBE treatments. RBE treatment could reduce the ALT level significantly in a dose-dependent manner. RBE also significantly caused a decrease in AST activity in the hepatotoxic model. A low dose already gave a normal level of AST, as can be seen in Figure 4.

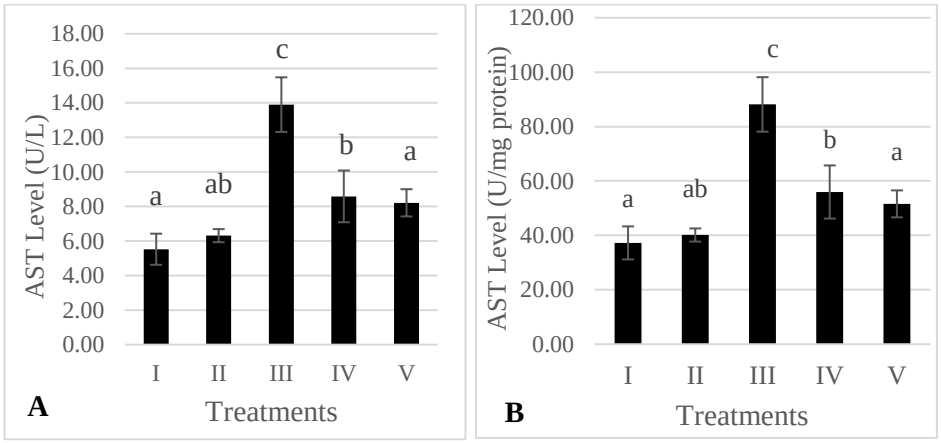

Figure 4 The effect of RBE concentration on hepatotoxic cells towards AST level. Figure 4A: AST level in U/L (units/Liter). Figure 4B: AST level in U/mg protein. The data is represented as a mean±standard deviation histogram. The different superscript letters (a, ab, b, c) designate significant differences among the treatments at $p < 0.05$, based on a one-way ANOVA, followed by a Tukey HSD post-hoc test. I: un-induced HepG2 cells, II: normal cells treated with 1% DMSO, III: APAP-induced HepG2 cells, IV: APAP-induced HepG2 cells treated with RBE 25 µg/ml, V: APAP-induced HepG2 cells treated with RBE 100 µg/ml.

RBE treatment successfully reduced the ALT and AST level in the cirrhosis model. Our findings are consistent with Novilia *et al.* [24], who found that red betel leaf ethanol extract (*Piper crocatum* Ruiz and Pav.) 600 mg/kg bw daily for 15 days exhibited curative effects via reducing AST, ALP and ALT levels in CCl_4-induced rats [24]. The higher AST and ALT activities can be caused by plasma membrane leakage as a result of a hepatocyte transport function defect [26]. The present study suggests that RBE treatment at 25 and 100 µg/ml reduced the AST level.

RBE contains phytochemical compounds such as alkaloids, flavonoids and also tannin as antioxidants [29]. In HepG2 cells induced by APAP and H_2O_2, RBE treatment at 25 and 100 µg/ml reduced the TNF-α level, cell apoptosis, intracellular ROS, cytochrome P450 2E1 (CYP2E1) gene expression, and increased the proportion of living cells, and glutathione peroxidase (GPX) levels [26][27]. Based on Morsy *et al.* (2020), piperine in *Piper nigrum* has anti-inflammatory and anti-apoptotic actions in the liver through lowering nuclear factor kappa B (NF-kB) p65 expression and modulating hepatic caspase3 and Bcl2, and also enhanced hepatic TGFBRAP1 expression [16]. Red betel containing eugenol [14][30][31], which has antioxidant activities [14], helps maintain the GSH level during oxidative stress in the hepatotoxicity process [32].

Eugenol showed hepatoprotective potential on the APAP-induced HepG2 cells model by decreasing the LDH, AST and ALT activities [17]. However, RBE had a hepatoprotective effect towards the liver injury model through its anti-inflammatory and antioxidant properties [27].

3 Conclusion

According to the findings of this research, red betel extract is safe to use in a wide range of concentrations in an HepG2 cell culture environment. At the highest dose (100 µg/ml), no significant cell reduction was seen. RBE has hepatoprotective effects by lowering LDH, ALT, and AST levels in hepatotoxic cells.

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Conflict of Interest

There are no conflicts of interest to declare by any of the contributing authors.

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