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Journal: Heliyon

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Kind regards,

Anna Lewinska
Associate Editor - Pharma & Toxicology
Heliyon

Editor and Reviewer comments:

Reviewer #1: Methods: OK
Results: OK
Interpretation: OK
Other comments: no comments

Reviewer #3: After a first revision, the manuscript on "Prevention of Acetaminophen-Induced Hepatotoxicity in HepG2 Cells by Red Betel (Piper crocatum Ruiz and Pav) from Indonesia via Antioxidant, Anti-inflammatory, and Anti-necrotic Potency" has been improved in its content, but carelessly revised in the writing. Therefore, in order of accepting the manuscript for publication, authors must clean it, particularly the writing because there are still multiple English issues along the document:
Revise particularly abstract and conclusions, which are showing notorious clerical mistakes. A pdf file is attached with some highlighted comprehensive amendments.

Reviewer #4: Methods: Acceptable
Results: Acceptable
Interpretation: Acceptable
Other comments: Acceptable

Prevention of Acetaminophen-Induced Hepatotoxicity in HepG2 Cells by Red Betel (*Piper crocatum* Ruiz and Pav) extract from Indonesia via Antioxidant, Anti-inflammatory, and Anti-necrotic Potency

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Abstract

Acetaminophen (APAP) is a widely used analgesic. Long term usage and overdose may cause liver injury (*hepatotoxicity)* via *N*-acetyl-*p*-benzoquinone imine (NAPQI)-induced oxidative stress. Multiple inflammatory mediators were also found to contribute for this effect. Red betel (*Piper crocatum* Ruiz and Pav), one of Indonesia medicinal plants, was known for its antioxidant and anti-inflammatory activities. This study was done to determine the red betel leaves extract (RBLE) protective effect against APAP-induced HepG2 cells. APAP-induced HepG2 as hepatotoxicity cell model was treated with RBLE at 25 and 100 µg/mL. Protective effects of RBLE toward hepatotoxicity were evaluated by several parameters: tumor necrosis factor-α (TNF-α) concentration, reactive oxygen

species (ROS) level, live cells percentage, apoptotic cells percentage, necrotic cells percentage, death cells percentage, CYP2E1 and GPX gene expression. The RBLE treatments (both 25 and 100 µg/mL) increased live cells percentage, CYP2E1 and GPX gene expression, while decreased the TNF-α, ROS level, necrotic and death cells percentage. Red Betel leaves ethanol extract has hepatoprotective effect towards liver injury model via antioxidant, anti-inflammatory, and anti-necrotic potency.
Keywords: Red Betel Leaves Extract, Acetaminophen, HepG2 Cells, Hepatoprotective

1. Introduction

Liver is the major site of drug metabolism. Drug-induced liver injury continues to be a problem for many commonly used drugs, and represents a major challenge in designing potential therapies (Noh et al., 2015). Acetaminophen (paracetamol, APAP) is considered as first line analgesics. However, excessive use of APAP leads to liver injury and even liver failure in animals and human (Ganey et al., 2007; Ni et al., 2012). APAP is majorly metabolized by UDP-glucuronosyltransferase (UGT) and sulfotransferase (SULT) to phenolic glucuronide and sulfate inactive conjugations and then they are excreted into the urine and bile. Small percentage of APAP is oxidized by cytochrome P450 2E1 (CYP2E1) enzymes to N-acetyl-p-benzoquinone imine (NAPQI), a highly reactive intermediate, which is detoxified by covalent binding with glutathione (GSH). APAP poisoning generates excess NAPQI which evokes the depletion of GSH and then binds to macromolecules triggering mitochondrial dysfunction, oxidative stress and ultimately resulting in hepatocellular death (Salminen et al., 2012; Uzi et al., 2013; Yuan et al., 2016). Until now, although the exact mechanisms underlying APAP-induced hepatotoxicity still remain unclear, growing evidence indicates that multiple mediators of inflammation and oxidative stress contribute to the pathology process of APAP-induced acute liver damage (Uzkeser, 2012). One of the mediators was tumor necrosis factor-α (TNF-α) (Dragomir, et al., 2012).

Red betel (*Piper crocatum* Ruiz and Pav) is one type of betel often used as ornamental plant in the 1990s, but now it has shifted to medicinal function since its introduction by Sudewo (2010), a medicinal plants producer in Blunyahrejo (Rinanda et al., 2012). It can be used to treat diabetes, gout, hepatitis, hypertension, and eye inflammation (Werdhany et al., 2014; Anugrahwati et al., 2016). In previous study, red betel leaves were found to have some secondary metabolite content like flavonoids, alkaloids, tannins, saponins, triterpenoids steroids, polyphenolics, quinones, and essential oil groups (Arambewela et al., 2005; Wulandari et al., 2018). In addition, red betel contains phenolic compounds in the form of hydrochavicol, cavibetol acetate and eugenol (Swapna et al., 2012; Dervis et al., 2017). Previous studies had shown that red betel leaves extract (RBLE) had antioxidant activity (Lister et al., 2019a); anti-inflammatory and antifungal properties (Misra et al., 2009); and also have antimigration activity towards metastatic breast cancer (Zulharini et al., 2018) as well as anticancer property towards cervical cancer (Widowati et al., 2013).

This study aims to determine the potential of RBLE to suppress liver injury in APAP-induced HepG2 cells. The observed parameters of this study include TNF-α level; apoptotic, necrotic cells, and death cells percentage; ROS level; CYP2E1 and GPX gene expression.

2. Materials and Methods

2.1 Red Betel Leaves Extract Preparation

Red betel (*Piper crocatum* Ruiz and Pav) leaves were obtained from Pabuaran Cilendek Timur, Bogor, West Java, Indonesia. The plant was identified by Herbarium Bogoriense, Botanical Field Research Center for Biology-Indonesian Institute of Science, Bogor, Indonesia. The extraction used maceration

method, the RBLE was obtained from our previous research and stored at -20ºC (Lister et al., 2019a; 2019b).

2.2 HepG2 Cells Culture and APAP-Induced HepG2

Human hepatocellular carcinoma (HepG2) cell line (ATCC, HB-8065™) was obtained from Aretha Medika Utama Biomolecular and Biomedical Research Center, Bandung, Indonesia. The cells were thawed and grown in complete medium (Modified Eagle Medium (MEM) (Biowest, L0416-500) supplemented with 10% (v/v) fetal bovine serum (FBS) (Biowest, S1810), with 1% (v/v) antibiotic-antimycotic (Gibco, 15240062) and 1% (v/v) nanomycopulitine (Biowest, LX16) addition). Induction of hepatotoxicity was done using 40 mM Acetaminophen (Sigma Aldrich, A7085). Confluent cells were rinsed with PBS and detached with trypsin EDTA with incubation at 37°C. Then cells were counted with hemocytometer and seeded in 6 well plates (5 x 10^5 cells / well). Cells were incubated in a 37°C incubator with 5% CO_2 for 24 h. Cells were divided into following groups: I) Normal Cells; II) DMSO1%; III) APAP 40 mM; IV) APAP 40mM+ RBLE 25 µg/mL; V) APAP 40 Mm + RBLE 100 µg/mL. After the compound was induced, the cell was incubated again for 24 h. After incubation, the conditioned medium was taken for ELISA assays and centrifuged at 1600 rpm for 10 min. The supernatant was collected and stored at -80° C for the quantification of TNF-α (Luo, et al., 2016; Aouache et al., 2018; Lister et al., 2019b).

2.3 Total Protein Assay

Bovine Serum Albumin (BSA) standard was obtained by dissolving 2 mg of BSA (Sigma Aldrich, A9576) in 1000 µL ddH_2O. Briefly 20 µL of standard solutions or samples and 200 µL Quick Start Dye Reagen 1X (Biorad, 5000205) were added into each well plate. After 5 min of incubation in room temperature, the absorbance was measured by microplate reader (Multiskan™ GO Micro plate Spectrophotometer, Thermo Scientific, 51119300) at 595 nm. The result from this assay was used for normalization of TNF-α data calculation (Pluemsamran et al., 2012; Widowati et al., 2019).

2.4 TNF-α Assay

TNF-α levels were measured using ELISA assay according to the manufacturer's kit manual (BioLegend, ELISA kit 421701). The absorbance was read at 450 nm using Multiskan GO Microplate Reader (Widowati et al., 2019).

2.5 Apoptotic Activity Assay

Treated and control HepG2 cells were washed using PBS 1x and harvested with trypsin-EDTA for apoptotic assay. The pellet was washed using Annexin Binding Buffer 1X (Miltenyi Biotec, 130-092-820) 500µL and stained with Annexin V-FITC (BioLegend, 79998) and Propidium Iodide (BioLegend, 79997). Cells were incubated at 37ºC for 30 minutes in the dark. Cells were later suspended in Annexin Binding Buffer 1x. The apoptotic percentage of HepG2 cells were analyzed using MACSquant Analyzer 10 (Miltenyi Biotec) (Widowati et al., 2018a).

2.6 Reactive Oxygen Species (ROS) Level Assay

After 7 days of culture, HepG2 cells were digested with trypsin-EDTA (Gibco, 25200072) and 2.5x10^4 cells/0.5 mL cells were incubated with 20 µM DCF-DA at 37ºC for 45 min, after that incubated with RBLE (25 and 100 µg/mL) for 4 h. Intracellular ROS was measured using 2',7'–dichlorofluorescin diacetate (DCFDA)–Cellular Reactive Oxygen Species Detection Assay Kit (Abcam, ab113851) with modifications (Widowati et al., 2014, Prahastuti et al., 2019).

2.7 CYP2E1 and GPX Gene Expression Assay

The HepG2 cell line was culture in complete medium (MEM + 10% FBS + 1% Antibiotic Antimycotic) and incubated at 37°C with 5% CO_2 for 24 h. Cells were induced using APAP, each combined with administration of RBLE (25 and 100 µg/mL) then incubated for 24 h. Then, the cells were harvested and processed for RNA isolation for further assay. RNA isolation was conducted using Aurum™ Total RNA mini Kit (Bio-Rad, 732-6820). The CYP2E1 and GPX gene expression along with the constitutively expressed β-actin gene was analyzed using RT-qPCR (Clever, GTC96S) (Widowati et al., 2018a; Widowati et al., 2018b). The primer sequences can be seen at Table 1 also the concentration and purity of RNA can be seen at Table 2.

2.8 Statistical Analysis

All measurements were done in triplicate. Statistical analysis was conducted using SPSS software (version 20.0). Data were presented as mean ± standard deviation. Data with normal distribution were analyzed using ANOVA and *Post Hoc* Test using Tukey HSD with $p< 0.05$. Data which are not normally distributed were analyzed with Kruskal Wallis and *Post Hoc* Test Mann Whitney.

3. Result

3.1 Effect of RBLE towards TNF-α Concentration in APAP-induced HepG2 Cells

APAP increased TNF-α concentration in HepG2 cells. RBLE 25 µg/mL and 100 µg/mL treatments were found to decrease TNF-α concentration (**Figure 1**). This results indicated that RBLE has potential in supressing TNF-α production in HepG2 cells.

3.2 Effect of RBLE towards Apoptotic, Necrotic, and Cell Death in APAP-induced HepG2 Cells

APAP decreased live cell percentage compare to normal HepG2 cells (**Figure 2A**). RBLE treatment decreased the percentage of apoptotic and necrotic significantly in APAP-induced HepG2 cells (**Figure 2B, 2C, 2D**). RBLE treatment increased live cells percentage and reduced dead cells percentage in the liver injury model.

3.3 Effect of RBLE towards ROS level in APAP-induced HepG2 Cells

ROS level increased significantly after APAP induction and reduced significantly when injured HepG2 cells were treated with RBLE **(Figure 3**). RBLE had potential to decrease ROS level in liver injury model.

3.4 Effect of RBLE on CYP2E1 and GPX Gene Expression in HepG2 cells induced by APAP

CYP2E1 gene expression decreased significantly in APAP-induced HepG2 cells. RBLE treatments increased the CYP2E1 gene expression significantly compare to the APAP-induced HepG2 cells group (**Figure 4**). GPX gene expression decreased in APAP-induced HepG2 cells. RBLE treatments could increase the GPX gene expression significantly (**Figure 5**). RBLE treatments had ability to increase the CYP2E1 and GPX gene expression.

4. Discussions

Betel leaves had been known to contain many active compounds, mainly hydrochavicol, cavibetol acetate and eugenol (Begam et al., 2018). Based on previous study, it had been demonstrated that red betel leaves extract, along with its active constituents: eugenol and hydroxychavicol, had

antioxidant activity as indicated by their ability in scavenging DPPH and H_2O_2; as well as reducing ABTS and FRAP radicals (Lister et al., 2019a). Eugenol had been reported could decrease the AST and ALT activities and LDH level in APAP-induced hepatotoxicity model (Lister, et al., 2019b).

TNF-α is an inflammatory cytokine involved in oxidative stress injury and its formation induced after APAP overdose (Barman et al., 2016; Jaeschke et al., 2011). The presence of APAP toxic metabolite NAPQI caused activation of Kupffer cells that leads to TNF-α release (Legert et al., 2015). TNF-α mediated death receptor pathway apoptosis by activating caspase 3 that act as a central effector to cleave various cellular substrates and trigger cell apoptosis eventually (Nagase et al., 2002; Truong et al., 2016). While apoptosis and necrosis frequently coexist in pathological conditions of the liver, and the balance of cell death may be dictated by the particular insult (Antoine, *et al*., 2010).

In this study, RBLE treatment decreased TNF-α level in liver injury model (APAP-induced HepG2 cells). One active compound in RBLE, eugenol, had been studied to have ability to suppress TNF-α level in liver injury model. It also had effect on reduction of inflammatory cells infiltration and generation of cytokines from Kupffer cells (Yogalaskhmi et al., 2010). Phenolic compound were hypothesized to give the anti-inflammatory effect as another study from Yuan et al. (2016) also stated that a phenolic compound ferulic acid could decrease the TNF-α level in mice induced with APAP.

Figure 2 shows that the APAP induction increased the apoptotic, necrotic, and death cells precentage, while RBLE treatments had successfully reduce death cells and maintain live cells at higher level. This data was in line with previous research that less apoptotic cells were seen in ferulic acid treatment in injury liver model (Yuan et al., 2016).

In APAP-induced hepatotoxicity model, oxidative stress played an important role and it was characterized by ROS accumulation (Nagi et al. 2010; Du et al. 2016). NAPQI, a reactive metabolite formed from APAP, could react rapidly with GSH and induced hepatocellular damage by aggravating oxidative stress in conjuction with mitochondrial dysfunction (Smith et al., 2016; Kang et al., 2017). ROS normally was detoxified by the enzymatic antioxidant defense system. Previous study exhibited that RBLE had antioxidant potential (Lister et al., 2019a). Based on the result, RBLE proved to suppress the ROS level in liver injury model, this result was in line with Parikh et al. (2015) that found phenolic compounds in *Brassica juncea* hydromethanolic extract such as quercetin and cathecin could reduce the ROS level in APAP-induced HepG2 cells. Thus, the RBLE hepatoprotective mechanism might result from diminishing generation of ROS.

In the metabolism of a wide variety of endogenous and exogenous compounds, CYP2E1 has important functions and is relevant to chemical toxicity and carcinogenesis in liver (Gonzalez, 2007). CYP2E1-generated ROS could increase lipid peroxidation and mitochondrial membrane permeability, which caused the release of pro-apoptotic factors and activate caspase 3 to induce apoptosis (Lee and Wei, 2007). Based on the result, RBLE treatments decreased CYP2E1 gene expression in APAP-induced HepG2 cells, probably by its high phenolic compounds. This result was in line with previous research where ferulic acid could inhibit the upregulation of CYP2E1 expression in APAP-induced hepatotoxicity (Yuan *et al*., 2016).

Glutathione Peroxidase, GPX, constitute the primary part of enzymatic antioxidant defense system against oxidative stress via directly eliminating ROS (Truong et al., 2016). When free radicals formed rapidly, GPX functions will become inefficient and leads to hepatocytes damage (Roh et al., 2018). GPX level can be used as indicator of the oxidative stress response (Wang et al., 2016). Based on the result, it was shown that APAP could decrease the GPX expression, however RBLE treatments could counter this effect. These results indicated that RBLE protects cells/livers from APAP-inducer through the enhancement of an antioxidant defense system. Truong et al. (2016) also stated that a

phenolic compound, mainly quercitrin could restore GPX expression and attenuates APAP-induced liver damage.

Based on this study, RBLE demonstrated hepatoprotective effect through antioxidant, anti-necrotic, and anti-inflammatory activities. Based on our study and literature review, we proposed a mechanism on how RBLE could act as hepatoprotective agent in liver injury (**Figure 6**).

5. Conclusion

Red betel leaves extract treatments could reduce TNF-α level, reduce cell's apoptosis and increase live cells percentage, reduce intracellular ROS, reduce CYP2E1 and increase glutathione peroxidase level in HepG2 cells. This marked the hepatoprotective potential of RBLE through antioxidant, antinecrotic, and anti-inflammatory activities. Further research on *in vivo* model is needed to confirm current result.

Declarations

Author contribution statement

Participated in research design: I Nyoman Ehrich Lister, Ermi Girsang, Chrismis Novalinda Ginting, Wahyu Widowati

Conducted experiments: Dewani Tediana Yusepany

Performed data analysis: Dewani Tediana Yusepany, Alya Mardhotillah Azizah

Contributed to the writing of the manuscript: Alya Mardhotillah Azizah, Hanna Sari Widya Kusuma

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There is no funding was received.

Competing interest statement

The authors declare that they have no conflict of interests.

Additional information

No additional information is available for this paper.

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Figure Legends

Figure 1. Effect of RBLE toward TNF-α concentration in APAP-induced HepG2 cells as hepatotoxicity model
(A) TNF-α concentration (pg/mL) on APAP-induced HepG2 cells
(B) TNF-α concentration (pg/mg protein) on APAP-induced HepG2 cells
*Data is presented as mean ± standard deviation. I) Normal cells (Negative control); II) Vehicle control (Normal cells + DMSO 1%); III) APAP-induced cells (Positive control); IV) Positive control + RBLE 25 μg/mL; V) Positive control + RBLE 100 μg/mL. Different letters (a,b) are significant among treatments toward TNF-α concentration based on Tukey HSD post hoc test ($P<0.05$).

Figure 2. Effect of RBLE toward apoptotic, necrotic, dead cells in APAP-induced HepG2 cells as hepatotoxicity model
(A) Live cells on APAP-induced HepG2 cells
(B) Early apoptotic on APAP-induced HepG2 cells
(C) Late apoptotic on APAP-induced HepG2 cells
(D) Necrotic on APAP-induced HepG2 cells
*Data is presented as mean ± standard deviation. I) Normal cells (Negative control); II) Vehicle control (Normal cells + DMSO 1%); III) APAP-induced cells (Positive control); IV) Positive control + RBLE 25 μg/mL; V) Positive control + RBLE 100 μg/mL. There are significant different between all groups based on normality test ($P < 0.05$). Single star (*) marks statistical difference between positive control and negative control and hashtag (#) mark statistical difference between treatment and positive control. It was based on normality test ($P < 0.05$) followed by Kruskal-Wallis Test ($P < 0.05$) and Mann-Whitney Test ($P < 0.05$).

Figure 3. Effect of RBLE toward ROS level in APAP-induced HepG2 cells as hepatotoxicity model
*Data is presented as mean ± standard deviation. I) Normal cells (Negative control); II) Vehicle control (Normal cells + DMSO 1%); III) APAP-induced cells (Positive control); IV) Positive control + RBLE 25 μg/mL; V) Positive control + RBLE 100 μg/mL. Single star (*) marks statistical difference between positive control and negative control and hashtag (#) mark statistical difference between treatment and positive control. It was based on normality test ($P < 0.05$) followed by Kruskal-Wallis Test ($P < 0.05$).

Figure 4. Effect of RBLE toward CYP2E1 gene expression in APAP-induced HepG2 cells as hepatotoxicity model
*Data is presented as mean ± standard deviation. I) Normal cells (Negative control); II) Vehicle control (Normal cells + DMSO 1%); III) APAP-induced cells (Positive control); IV) Positive control + RBLE 25 μg/mL; V) Positive control + RBLE 100 μg/mL. Single star (*) marks statistical difference between positive control and negative control and hashtag (#) mark statistical difference between treatment and positive control. It was based on normality test ($P > 0.05$) followed by ANOVA ($P < 0.05$) and Games-Howell ($P < 0.05$).

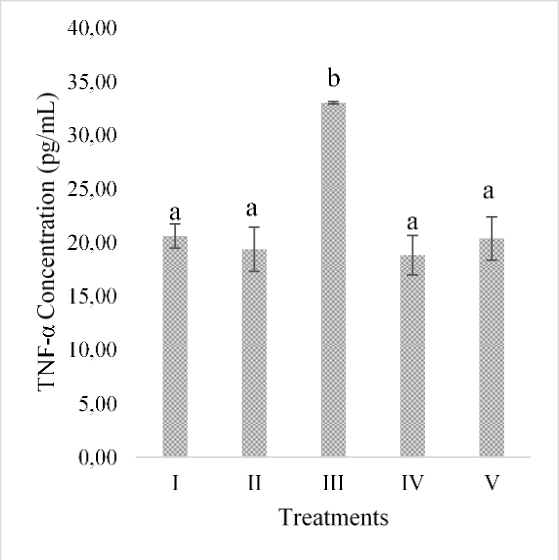
Figure 5. Effect of RBLE toward GPX gene expression in APAP-induced HepG2 cells as hepatotoxicity model
*Data is presented as mean ± standard deviation. I) Normal cells (Negative control); II) Vehicle control (Normal cells + DMSO 1%); III) APAP-induced cells (Positive control); IV) Positive control + RBLE 25 μg/mL; V) Positive control + RBLE 100 μg/mL. Single star (*) marks statistical difference between positive control and negative control and hashtag (#) mark statistical difference between treatment and positive control. It was based on normality test ($P < 0.05$) followed by Kruskal-Wallis Test ($P < 0.05$) and Mann-Whitney Test ($P < 0.05$).

Figure 6. Proposed RLBE hepatoprotective mechanism in liver injury model
*CYP2E1 act to transform APAP to NAPQI. It induce GSH depletion then induce production of ROS. The excessive of ROS decrease the GPX gene expression leads to increase cell death. On the other hand, NAPQI activate the kupper cell leads TNF-α production, it induce the JNK signaling pathways that also increase ROS

level and leads to upregulate cell death, necrosis; increase inflammation; and induce cell death. While JNK induced the down regulation of Bcl-2 and up regulation of Bax, resulting in caspase 9 and caspase 3 activation, it leads apoptosis cells. The RLBE treatments could inhibit the excessesive ROS and TNF-α. It also could lowering the necrosis and apoptosis that leads to lowering inflammation. RBLE treatments decrease the cell death and increase the survival hepatic cells.

Figure 1:

A.


40,00
35,00
30,00
25,00
20,00
15,00
10,00
5,00
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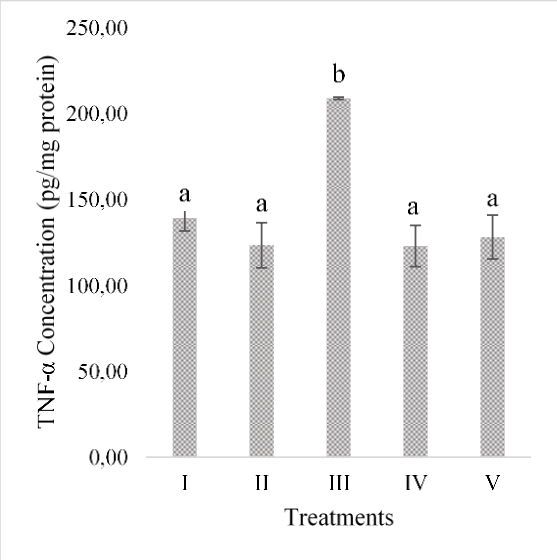
TNF-α Concentration (pg/mL)
a
a
b
a
a
I
II
III
IV
V
Treatments

B.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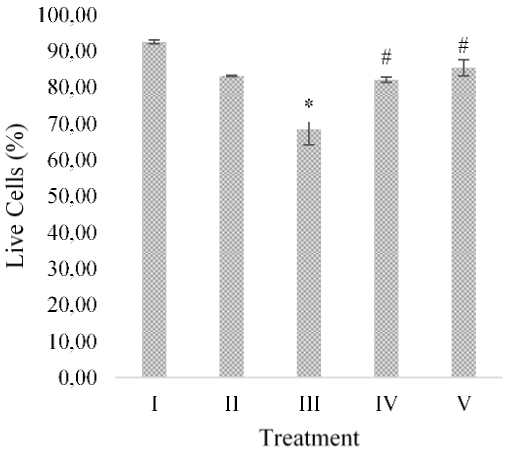
150,00
100,00
50,00
0,00
TNF-α Concentration (pg/mg protein)
a
a
b
a
a
I
II
III
IV
V
Treatments

Figure 2:

A.



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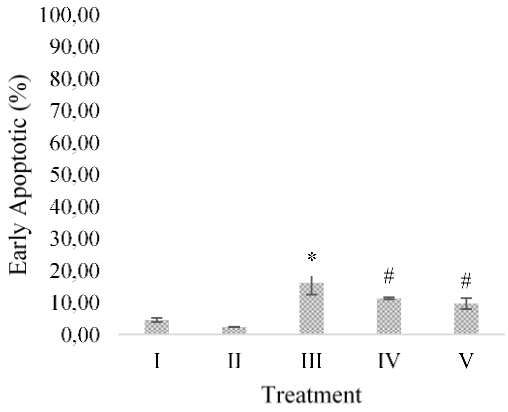

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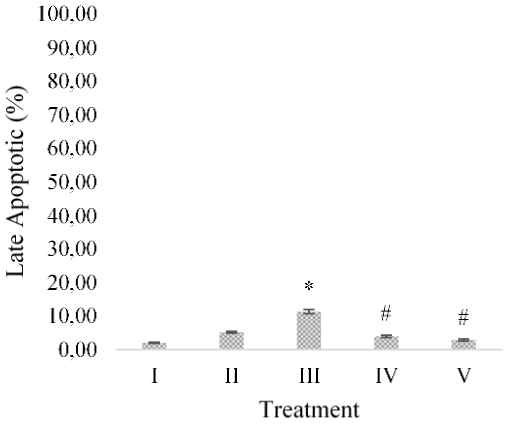


D.

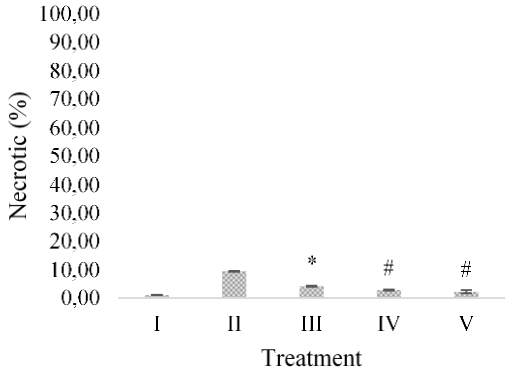
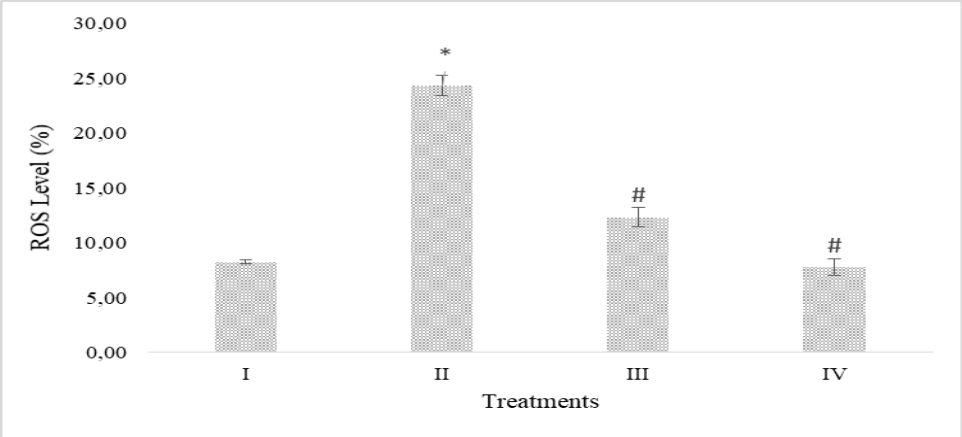


Figure 3:



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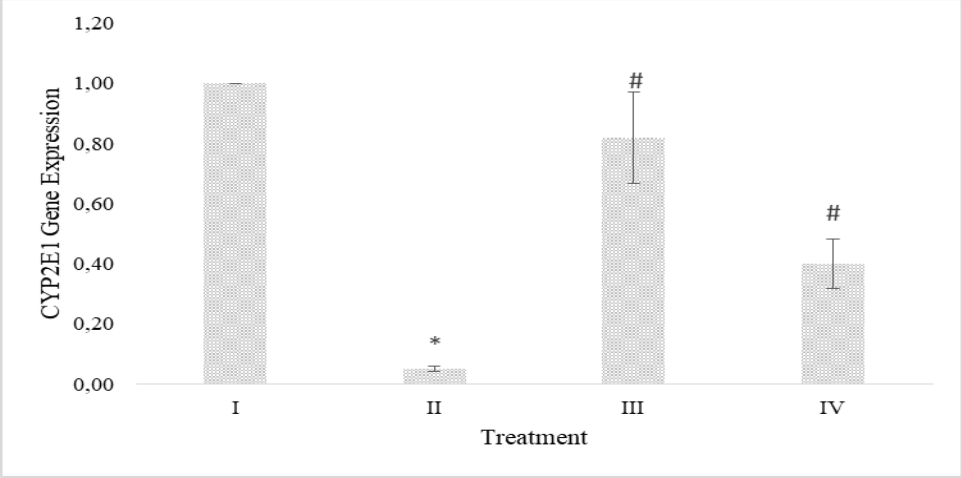

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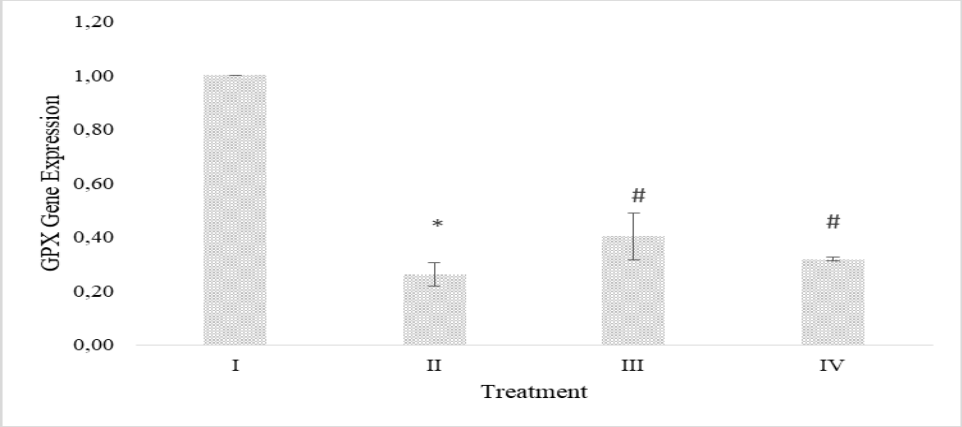

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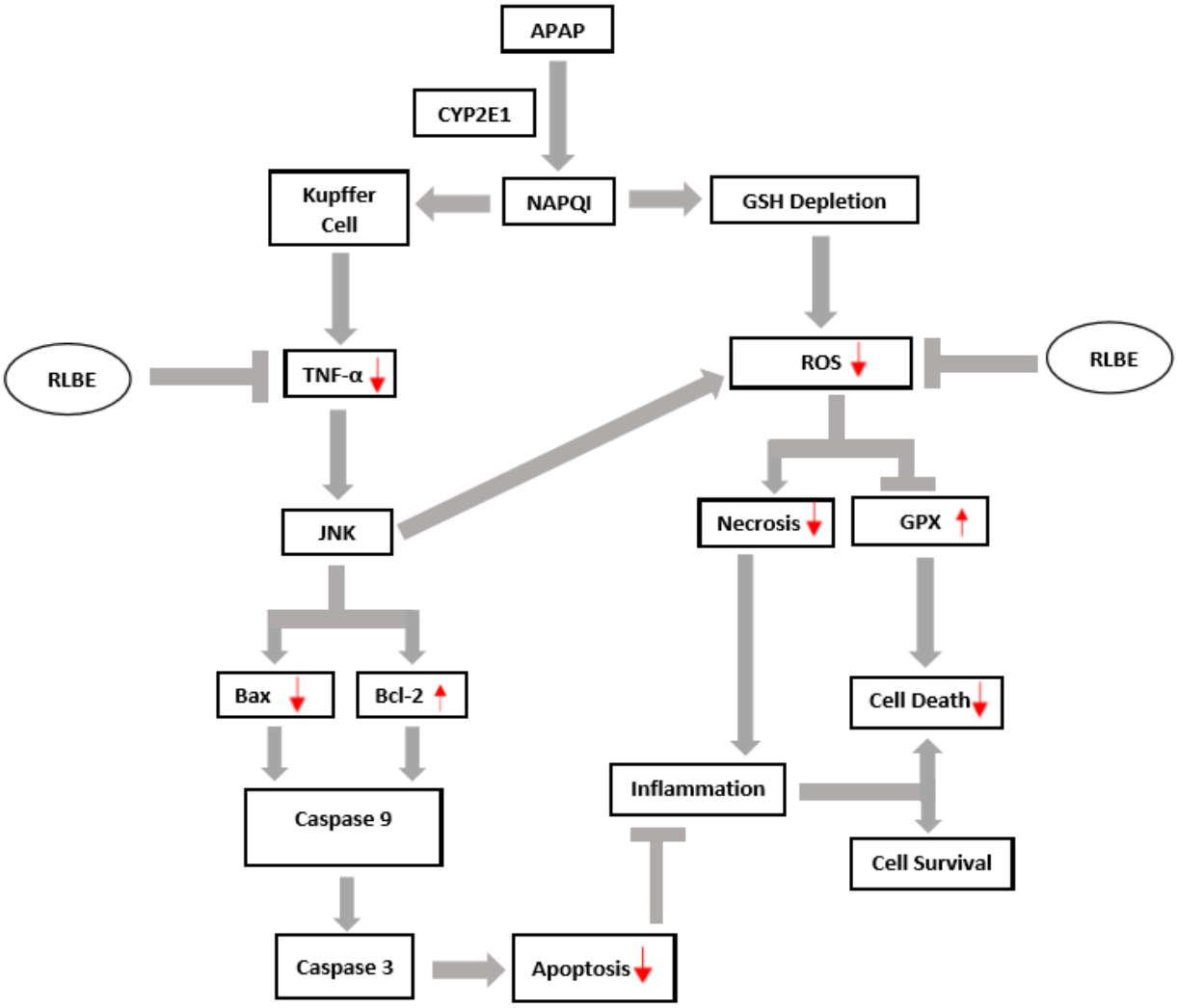
APAP
CYP2E1
NAPQI
Kupffer Cell
GSH Depletion
RLBE
TNF-α
ROS
RLBE
JNK
Necrosis
GPX
Bax
Bcl-2
Cell Death
Caspase 9
Inflammation
Cell Survival
Caspase 3
Apoptosis

Table 1. RT-PCR details of β-Actin, CYP2E1, and GPX gene.

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	Widowati et al., 2018a; Widowati et al., 2018b
CYP2E1	5'-GTTCTTTGCGGGGACAGAGA-3' 5'-GAGGGTGATGAACCGCTGAA-3'	59	40	Kim et al., 2018
GPX	5'-CCAAGCTCATCACCTGGTCT-3' 5'-TCGATGTCAATGGTCTGGAA-3'	59	40	Ugusman et al., 2011

Table 2. Concentration and purity of RNA.

No.	Sample	Concentration (ng/μL)	Purity (Absorbance 280/260)
1.	Control cells	92.90	2.3212
2.	Positive control	90.10	2.0904
3.	Positive control + RBLE 25 μg/mL	36.20	1.9676
4.	Positive control + RBLE 100 μg/mL	40.00	2.0366