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Protective effect of Ethanolic Extract of Jati Belanda (*Guazuma ulmifolia* L.) by Inhibiting Oxidative Stress and Inflammatory Processes in Cisplatin-induced Nephrotoxicity in Rats

ABSTRACT

The accumulation of high concentrations of cisplatin in the kidneys ~~renal~~ may causes nephrotoxicity. Cisplatin-induced nephrotoxicity happens through the inflammation of the tubules, and apoptosis, necrosis, and vascular defects ~~factors~~. Jati Belanda leave contain various chemical and the ethanolic extract that can decreased cholesterol level. According to the author's knowledge, no comprehensive work was dedicated to nephroprotective effect of Jati Belanda (*Guazuma ulmifoliaL*.L.) ethanolic extract (EEJB) on cisplatin induced ~~-~~ nephrotoxic rat. Two doses of EEJB 300 mg/kg bw, EEJB 600 mg/kg bw were used on rats induced nephrotoxicity by cisplatin 8 mg/kg bw. The measurement of MDA and CRE were done using colorimetric methods while IFN-γ. IL-1β, and TNF-α expression was done by using immunochemistry (IHC) assay. The results of this study showed that EEJB 300 and 600 mg/kg bw could decrease MDA and CRE level also IFN-γ. IL-1β, and TNF-α expression when compared with positive control. Thus, ethanolic extract of Jati Belanda may give protective effect in patients being treated with chemotherapy using cisplatin but further toxicity effect and clinical trials studies are needed.

Keywords: Cisplatin, ethanolic extract *of Jati Belanda,* inflammation, nephrotoxicity, oxidative stress.

~~1.~~ INTRODUCTION

Nephrotoxicity is a condition in which renal functions are decreased due to the toxic effects of chemicals and drugs. About 20% of nephrotoxicity is induced and caused by drugs. This percentage is higher on the elderly due to an increase in lifespan and drugs consumed (Marwa et al., 2019). There are several drugs that can affect renal functions predominantly, thus the dosage needs to be adjusted to prevent a decrease in renal function (e.g. heparin). The nephrotoxic effects of most drugs are more profound in patients suffering from renal failure. The markers of nephrotoxicity and early renal dysfunction are blood~~y~~ urea and serum creatinine with low sensitivity~~)~~ (Campos et al., 2018).

Protective effect of Ethanolic Extract of Jati Belanda (*Guazuma ulmifolia* L.) by Inhibiting Oxidative Stress and Inflammatory Processes in Cisplatin-induced Nephrotoxicity in Rats

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~~1.~~ INTRODUCTION

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Cisplatin (CP) is a chemical commonly used in chemotherapy. It is usually used in neck, testicular, head, and ovarian cancers treatments. This drug is correlated with nephrotoxicity in 28–36% of patients when they receive an initial dose (50–100 mg/m^2) of cisplatin (Badary et al., 2007; Rabik et al., 2007). The accumulation of high concentrations of cisplatin in the renal tissues can cause nephrotoxicity by inducing inflammation of the tubules, and other apoptotic ~~sis~~ factors , necrosis, and vascular factors (Li et al., 2017; Zhu et al., 2017; Luke et al., 1992; Kumar et al., 2017). Clinical uses of the chemical are limited because of these serious complications (Lebwohl and Canetta, 1998). Cisplatin can also cause~~s~~ the development of renal tubule lesion by inducing oxidative stress (Chtourou et al., 2015; Lin et al., 2015; Oh et al., 2017; Saral et al., 2016). The reactive nitrogen species (RNS) and reactive oxygen species (ROS) productions in oxidative stress change the function and structure of cellular membranes Pedraza-Chayerri et al., 2004; Diyya et al., 2016). Moreover, their accumulations in the renal and lysosomes cause CP-induced acute nephropathy (Romeo et al., 2009). Therefore, antioxidants and scavengers of free radicals may be capable to prevent the nephrotoxicity that cisplatin induced (Alhoshani et al., 2017).

Natural products that are obtained from plants and animals offer vast resources of new potential medicinal agents for clinical use. A high number of modern drugs have been made and isolated from natural sources. Those drugs have the potentials to be used in the treatments for various diseases in the world (Patil and Biradar, 2013). Indonesia represents one of the tropical countries that are rich with various species of tropical plants. One of the tropical plants that have long been used to promote health is *Guazuma ulmifolia* L. which is called "Jati Belanda" in Indonesia (Rozqie et al., 2012; Prahastuti et al., 2019).

~~Many~~The ~~-~~chemicals in Jati Belanda leaves are tannins, alkaloids, mucilage, saponins, beta-sitosterol, and flavonoids. The results of previous studies that evaluated extract of Jati Belanda leaves on blood lipid levels in male rats showed that ethanolic extract of Jati Belanda can decrease cholesterol levels in test animals (Sukandar et al., 2009). Those compounds ~~can~~ may be used to decrease the amount of cholesterol in the intestine, resulting in a decreased cholesterol level in the blood (Rozqie et al., 2014). This study is aimed to measure levels of malonaldehyde (MDA), creatinine (CRN), Fibronectin (FN), blood urea nitrogen (BUN) by ELISA, and kidney weight, protein expression of IL1-β, IFN-γ, PCNA using Immuno Histochemistry (IHC) in cisplatin-induced rats that were administered with extracts of Jati Belanda (EEJB).

MATERIAL AND METHODS

~~2.~~ Ethical approval of the study ??????????

~~2.1~~ Extract Preparation

Jati Belanda was collected from Bumi Herbal Dago plantation, Bandung, West Java, Indonesia and the plant was identified by one of the staffs of herbarium of the Department of Biology from the School of Life Science and Technology at Bandung Institute of Technology, Bandung, West Java, Indonesia (0020218-A002). The leaves were collected, chopped, and kept in drier tunnel. The extractions to collect EEJB was done based on the maceration method. The solvent was ethanol (70%) (Widowati et al., 2018; Prahastuti et al., 2020)

~~2.2~~ Nephrotoxicity Rat Model

White male Sprague Dawley rats aged 1.5 months with an average body weight of 140 – 170 g were gotten from the National Agency of Drugs and Food Control (Jakarta, Indonesia) and acclimatized for 1 week. The research was then approved by the Research Ethic Committee of Immanuel Hospital, Bandung, Indonesia and the Faculty of Medicine of Maranatha Christian University (No: 138/KEP/IV/2019). The rats were divided into 4 groups according to the number of treatments that were used (I : Normal Control : Aquadest; II : Positive Control; Cisplatin 8 mg/kg bw + Aquadest; III : Cisplatin + EEJB 300 mg/kg bw; IV : Cisplatin + EEJB 600 mg/kg bw). Each treatment was repeated 5 times (5 rats). In all groups beside normal control group, the rats were induced with 8 mg/kg bw of cisplatin (Kalbe Farma) by intraperitoneal injection on the third day of pre-treatment. Then, the rats were given the dissolved 500 µL of extract by gavage for 18 days. On days 0, 9 and 18 after treatment, the blood was collected and processed as plasma for further assays. On the 18th day, the renals were extracted surgically after the rats were euthanized using the CO_2 chamber. The weights of the extracted organs were measured using Analytical Balance (AXIS, Kartuska) (Ajith et al., 2007; Soliman et al., 2016).

~~2.3~~ Immunohistochemistry (IHC)

For IHC, antigens retrieval (Abcam, ab208572) was done in citrate buffer with a pH of 6.0 at 121^{o}C for 10 minutes. Endogenous peroxidase was blocked in 3% H_2O_2 (Merck, 107209) and methanol (Merck, 106009) for 15 minutes in room temperature. The primary antibodies such as IFN-γ (Elabscience, E-AB-40075), rabbit-anti rat TNF-α (ElabScience, E-AB-40015), and rabbit-anti rat IL-1β (ElabScience, E-AB-66749) were incubated overnight in room temperature. Then, the target proteins were visualized using Rabbit-Specific HRP/DAB (ABC)

Detection IHC Kit (Abcam, ab64261). Haematoxylin was used for counterstaining agent. The stained tissues were observed in primostar (Zeiss) microscope and lumenera infinity 1-3c was used for photography (Pham et al., 2007; Ponti et al., 2005).

~~2.4~~ Malondialdehyde (MDA) Content

The measurement of MDA was done using Malondialdehyde (MDA) Assay Kit (Elabscience, E-BC-K142). Blood plasma was used as a sample for MDA measurement. The blood sampling was taken on three different days (0, 9 and 18 days) after treatment. MDA content from each samples were measured according to the manufacturer protocol and read at 532 nm and 600 nm using the microplate reader (MultiskanTM GO Microplate Spectrophotometer, Thermo Scientific, Waltham, MA, USA (Hanny et al., 2019).

~~2.5~~ Creatinine (CRE) Content

Blood samples were collected on three different days (0, 9 and 18 days) after treatment. Blood plasma was separated by centrifugation and then used for Creatinine (CRE) content measurement. CRE content was measured using Creatinine (CRE) Assay Kit (Elabscience, E-BC-K186). Assays were read at 546 nm using the microplate reader according to the manufacturer protocol (Al-Kuraishy et al., 2019).

~~2.6~~ Statistical Analysis

The data were analyzed using R software version 1.0.143 (R Studio). One-way ANOVA with Tukey post hoc test, Games-Howell post-hoc test, or Pairwise Wilcoxon non-parametric test were used to show the significance values between treatments.

3. RESULT

3.1 EEJB Effect on MDA Content

Cisplatin treatment (8 mg/kg bw) showed increased MDA content compared to normal control (Figure 1), indicating increased rate of lipid peroxidation. Treatment with EEJB 300 mg/kg.bw did not show a significant increase in MDA content compared to positive control on day 9 and 18 ($p<0.05$). Meanwhile, treatment with EEJB 600 mg/kg.bw showed a significant decrease in MDA content compared to positive control on day 9 and 18 ($p<0.05$).

3.2 EEJB Effect on CRE Content

The CRE contents in EEJB treatments can be seen in Figure 2. In this study, treatment with cisplatin (8 mg/kg.bw) showed an increased CRE content compared to control. Treatments using EEJB 300 and 600 mg/kg.bw showed significant decreases in CRE contents compared to positive control on day 9 and 18.

3.3 EEJB Effect on Kidneys ~~Renal~~ Weight

In Figure 3, cisplatin induction showed significant increase in renal weights of the rats which marked hypertrophy. Treatment with EEJB seemed to protect the nephrotic renal from hypertrophy. This can be seen by the significant decrease in renal weight in EEJB groups compared to positive control ($p<0.05$).

3.4 EEJB Effect on IFN-γ Expression

The expression of IFN-γ can be seen in Figure 4 and Figure 5(A). Scattered, and weak cytoplasmic staining of IFN-γ in renal tubules were found in negative control with a score of 10.556. In positive control, the IFN-γ expressions were diffuse and showed strong cytoplasmic staining with highest scores (43.861) than negative control and treatment group with $p<0.05$. The EEJB300, EEJB600 were lower compared to positive control with score 39.383, 36.107 respectively. Based on the Pairwise Wilcoxon test ($p<0.05$) showed EEJB 600 was significantly difference compared to positive control, the EEJB600 was effective in reducing renal IFN-γ expression.

3.5 EEJB Effect on IL-1β Expression

The expressions of IL-1β by the treatment groups can be seen in Figure 5(B) and Figure 5(C). Expression seemed to be scattered and showed weak cytoplasmic staining of IL-1β on renal tubules epithelium with a score of 9.541. The positive control showed diffuse, strong cyst~~i~~toplasmic staining of IL-1β in renal tubules epithelium with highest scores (36.178) than negative control and treatment group with $p<0.05$. The EEJB300, EEJB600 were lower compared to positive control with score 32.133, 28.657 respectively (Figure 5(C)). Based on the Pairwise Wilcoxon test ($p<0.05$) showed EEJB 600 was significantly difference compared to positive control, the EEJB600 was effective in reducing renal IL-1β expression.

3.6 EEJB Effect on TNF-α Expression

TNF-α expression can be seen in Figure 6 and Figure 7. Scattered, and weak staining of TNF-α in renal tubules epithelium can be found in negative control with a score of 9.307. The scores of EEJB300 and EEJB600 groups were higher in Figure 6 (11.182 and 11.810 respectively). Compared to the other groups, positive control showed strong cytoplasmic staining of TNF-α with a score of 31.447. The treatment groups were shown to be effective in reducing the renal TNF-α expression. Based on the statistic test, there was a significant difference between positive control and normal control and the treatment groups (EEJB 300 and EEJB 600) based

on Pairwise Wilcoxon test ($p<0.05$) (Figure 7), both EEJB300 and EEJB600 were effective to reduce renal TNF-α expression.

4. DISCUSSION

Cisplatin is an anticancer agent. This compound is potent when used in treatments against tumors of the bladder, testes, ovaries, breasts, and lungs. Nevertheless, in practice, using cisplatin as a cancer treatment is limited by the nephrotoxicity it may causes (Antunes et al., 2000; Chirino et al., 2004). Cisplatin can bind to lipid membrane and causes lipid peroxidation that produces MDA and destroys the cells of renal tubules (Divya et al., 2016; Rehman et al., 2014). Cisplatin can induce the production of free radicals such as hydrogen peroxide, superoxide anions, and hydroxyl radicals. They can cause reactions that result in peroxidation of the lipid membranes on tubule cells. It has been proposed that the MDA that is the byproduct of lipid peroxidation is a main characteristic of oxidative stress. Oxidative stress has been shown to contribute to cisplatin-induced nephrotoxicity by increasing MDA level in a previous study (Rehman et al., 2014; El-Besbishy et al., 2011).

Alternative treatments for oxidative stress can be found in medicinal plants. EEJB that was used in this study is a medicinal plant. Medicinal plants contain several phytochemicals such as phenolic compounds that have antioxidant properties. They are able to maintain redox homeostasis and protect cells against damage caused by excess ROS (Prahastuti et al., 2019; Do et al., 2014). Decreasing the side effects of cisplatin using natural antioxidants are interesting to research on. In this study, we found that EEJB 600 mg/kg bw could decrease MDA levels. Several compounds were identified in EEJB. They are phenolic acids, flavan-3-ol-derived flavonoids (monomers and dimers), and condensed tannins, including epicatechin, epigallocatechin, procyanidins, catechin, prodelphinidin–procyanidin, and procyanidin-profisetinidin might have played a role in decreasing MDA levels in our study (Hör et al., 1996; Lopes et al., 2009). EEJB contain high phenol, flavonoid and showed many antioxidant activities including 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenger, 2,2'-azinobis-(3ethylbenzothiazoline-6-sulfonate (ABTS) scavenger, hydroperoxide (H_2O_2) scavenger activities and ferric reducing antioxidant power (FRAP) (Prahastuti et al., 2020), antioxidant potential in EEJB played role in reducing MDA level in renal nephrotoxicity rat.

In the serum biomarkers, nephrotoxicity is characterized by a reduction in glomerular filtration, and an increase of serum CRE (Mashhadi et al., 2013). In the previous study, cisplatin could increase serum levels of creatinine significantly as biomarkers for cisplatin-induced

nephrotoxicity (Oh et al., 2014). CRE is distributed throughout the total body water and is normally removed from the blood by the renal (Alibakhshi et al., 2018). This study showed EEJB 300 and 600 mg/kg bw could decrease plasma CRE level in renal nephrotoxicity rat. This result is in agreement with a previous study in which phenolic compounds in EEJB might have prevented the increase of CRE level because of their antioxidant properties and by inhibiting arginase activity (Akomolafe et al., 2014).

In this result, treatments with EEJB were effective in decreasing renal weight and preventing renal hypertrophy. Renal injuries can occur because of high oxidative stress and high rate of free radicals production in the renal tissue. The antioxidants in EEJB might have contributed to the decreased occurrence of renal hypertrophy that is usually induced in cisplatin administration by defending against oxidative stress (Himmelfarb et al., 2004; Dennis et al., 2017).

Th1 cells are capable of producing cytokines such as IFN-γ. Cytokines that are produced by these cells are categorized as Th1-type cytokines. These cytokines are important in the body's defense mechanism against pathogens by activating immune cells like macrophages and NK cells. Gene transcription and translation of IFN-γ are regulated by NF-κB signaling. This signaling pathway is regulated by oxidative stress that are affected negatively by the antioxidant properties of phenolic compounds. A previous study showed that the phenolic compound, kaempferol, could decrease IFN-γ expression (Miles et al., 2005). This study showed that treatments with EEJB were effective in decreasing IFN-γ expression which is in line with another study. Phenol compounds in EEJB as anti-inflammatory potential (Gonzalez et al., 2011; Bouriche et al., 2016). Flavonoids in EEJB were effective to have anti-inflammatory activity (Bouriche et al., 2016; Rathee et al., 2009).

Antioxidants such as the phenolic compounds in EEJB have anti-inflammatory activities that regulate cytokine-induced inflammation. IL-1β is a cytokine with pro-inflammatory properties that is produced during the activation of neutrophils and can cause inflammation. Phenolic compounds can reduce neutrophil activities or activations at sites of inflammation. They can also reduce oxidative stress which contributes to the initiation of inflammatory process with their antioxidant properties. Thus, other studies have shown the role of phenolic compounds in decreasing IL-1β expression (Da Rosa et al., 2019; Gauliard et al., 2008). However, the result of this study is not in line with the other studies. In this study, treatments with EEJB were shown not to be effective in reducing IL-1β expression in the rats. Although many studies showed different result in the effect of phenolic compounds on IL-1β expression, one study is in line with our result. The study showed an increased expression of IL-1β in treatments using

some phenolic compounds such as coumaric and cinnamic acids (Bachiega et al., 2012). EEJB is able to reduce ROS level in chronic kidney disease cells model through their antioxidant potential, phenol and flavonoid content (Prahastuti et al., 2019).

TNF-α is a ctyokine which produced by macrophages, neutrophil, T cells, NK cells. This cytokine regulates the production of nitrogen oxide (NO) that is linked to the increase of oxidative stress in the body that plays an important role in nephrotoxicity by cisplatin. In this study, TNF-α expression was reduced by treatment with EEJB. The phenolic compounds in EEJB might have interfered with the transcriptional regulation and post-transcriptional modification of TNF-α, thus decreasing its expression which in turn decreases the induction of nephrotoxicity by cisplatin (Wang et al., 2002). Extracts are rich phenolic, flavonoids compound have antioxidant activity and reducing stress oxidative which play rolling in inflammation (Bouriche et al., 2016). Oolong tea contained high polyphenol has anti-inflammatory activities by reducing NO, cyclooxygenase-2 (COX-2), IL-6, IL-1β, and TNF-a level in inflammatory cells model (Novila et al., 2017). Hence, we proposed the mechanism of EEJB in improving nephrotoxicity in cisplatin-induced rats in Figure 8 based on our results and study literature.

This study showed that oxidative stress plays an important role in nephrotoxicity induced by cisplatin. Moreover, the antioxidant effect from ethanolic extract of Jati Belanda (EEJB) is responsible for reducing MDA and CRE content, also IFN-γ. IL-1β, and TNF-α expression resulting in improvement of renal function in rats that were treated with cisplatin. Therefore, pre-treatment combining ethanolic extract of Jati Belanda (EEJB) may be useful for patients undergoing chemotherapy using cisplatin but further *in vivo* studies and clinical trials are required.

(Discussion is too long ????????)

Authors contribution:

All Authors designed the project. SJ and EA constructed and made sample preparation. SJ, EA, HSWK, STH, RW executed the experiment and analyzed the tissue samples. SJ, MH, RW, HSWH, RR, WW, and MS analyzed the data. All authors critically revised the manuscript and approved the final version.

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Figure 1. Effects of EEJB on serum MDA content in nephrotoxic rats

*Control: normal rat; Positive control: nephrotoxicity rats; EEJB 300: Positive control + EEJB 300 mg/kg bw; EEJB 600: Positive control + EEJB 600 mg/kg bw.

*Data was presented as mean±standard deviation, the experiment had three replications. Hashtag symbol (#) shows significant difference between control and positive control. Asterisk (*) shows significant differences between treatment and positive control based on Tukey HSD post hoct test ($p<0.05$) on day 9 and 18.

Figure 2. Effects of EEJB on serum CRE content of nephrotoxic rats

*Control: sample without any inducer; Positive control: nephrotoxicity rats; EEJB 300: Positive control + EEJB 300 mg/kg bw; EEJB 600: Positive control + EEJB 600 mg/kg bw.

*Data was presented as mean±standard deviation, the experiment had three replications. Hashtag symbol (#) shows significant difference between control and positive control. Asterisk (*) shows significant differences between treatment and positive control based on Tukey HSD post hoct test ($p<0.05$) on day 9 and 18.

Figure 3. Effects of EEJB on renal weight in nephrotoxic rats

*Negative Control: normal rat; Positive control: rat acute renal disease; EEJB300: Positive control + EEJB 300 mg/kg.bw; EEJB600: Positive control + EEJB 600 mg/kg bw

*Data was presented as mean ± standard deviation, the data was three replication. Hashtag symbol (#) shows significant difference between negative control and positive control. While asterisk symbol (*) marks significant difference between positive control and treatment groups. All the significant differences were based on One Way Anova followed by Tukey post hoct test ($p<0.05$).

Figure 4. Effects of EEJB toward renal IFN-γ expression in nephrotoxicity rats by IHC assay

*A-D are IFN-γ expression at magnification 400x while E-H are IFN-γ expression at magnification 1000x.

*A and E are negative control (normal rat); B and F are positive control (acute renal disease model); C and G are EEJB300 (Positive control + EEJB 300 mg/kg bw) and; D and H are EEJB600 (Positive control + EEJB 600 mg/kg bw).

Figure 5. Effect of EEJB toward in nephrotoxic rats using quantitative IHC assay

(A) IFN-γ expression; (B) IL-1β expression

*Data was presented as mean ± standard deviation, the data was ten observations. Hashtag symbol (#) shows significant difference between negative control and positive control. While

asterisk symbol (*) marks significant difference between positive control and EEJB600. The significant differences were based on Pairwise Wilcoxon test ($p<0.05$)

(C) TNF-α expression

*Data was presented as mean ± standard deviation, the data was ten observations. Hashtag symbol (#) shows significant difference between negative control and positive control. While asterisk symbol (*) marks significant difference between positive control and EEJB300, positive control and EEJB600. The significant differences were based on Pairwise Wilcoxon test ($p<0.05$)

Figure 6. Effect of EEJB toward renal IL-1β expression in nephrotoxicity rats by IHC assay

*A-D are IL-1β expression at magnification 400x while E-H are IL-1β expression at magnification 1000x.

*A and E are negative control (normal rat); B and F are positive control (acute renal disease model); C and G are EEJB300 (Positive control + EEJB 300 mg/kg bw) and; D and H are EEJB600 (Positive control + EEJB 600 mg/kg bw).

Figure 7. Effect of EEJB toward TNF-α expression in nephrotoxicity rats by IHC assay

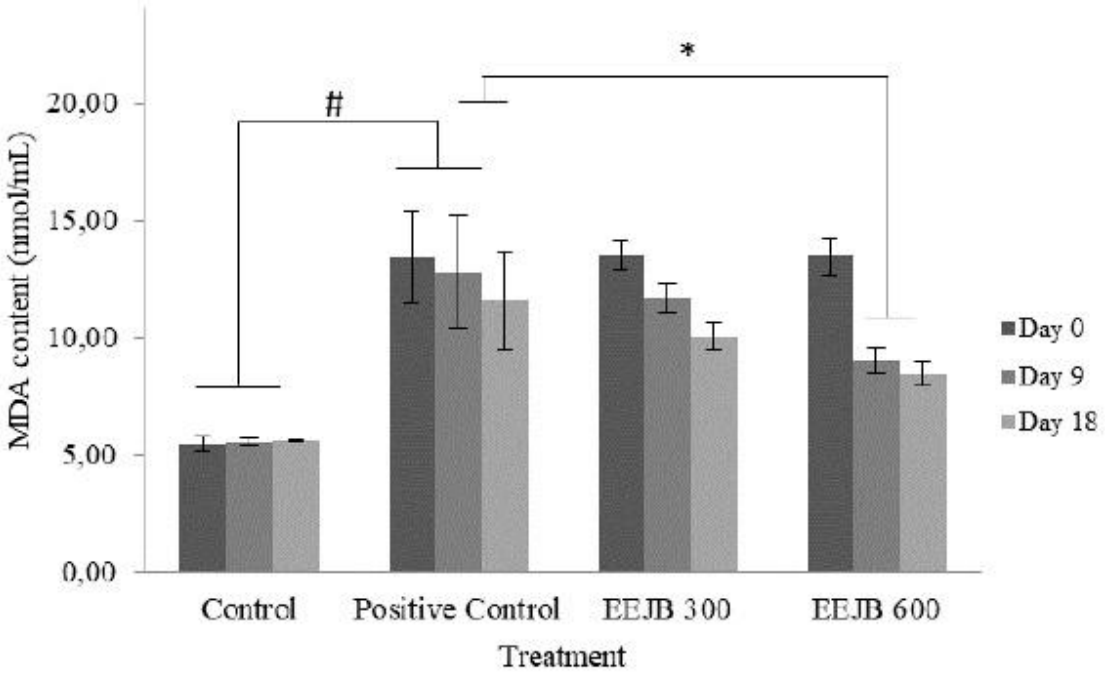
*A-D are TNF-α expression at magnification 400x while E-H are TNF-α expression at magnification 1000x.

*A and E are negative control (normal rat); B and F are positive control (acute renal disease model); C and G are EEJB300 (Positive control + EEJB 300 mg/kg bw) and; D and H are EEJB600 (Positive control + EEJB 600 mg/kg bw).

Figure 8. Proposed mechanism of EEJB in improving nephrotoxic condition

Cisplatin could induce the ROS production. The exposure of ROS could damage cell membrane indicated by the increment of MDA that leads to lipid peroxidation. On the other hand, ROS also could increase the apoptosis and cell death in renal. The treatment of Jati Belanda ethanolic extract which containing phenols, flavonoids as antioxidant, anti-inflammatory reduced ROS,. It can reduce the production of MDA also neutralize the pro-inflammatory cytokines (TNF-α, IL-1β, IFN-γ) thus protecting the renal from injury.

461 **Figure 1**



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464 **Figure 2**

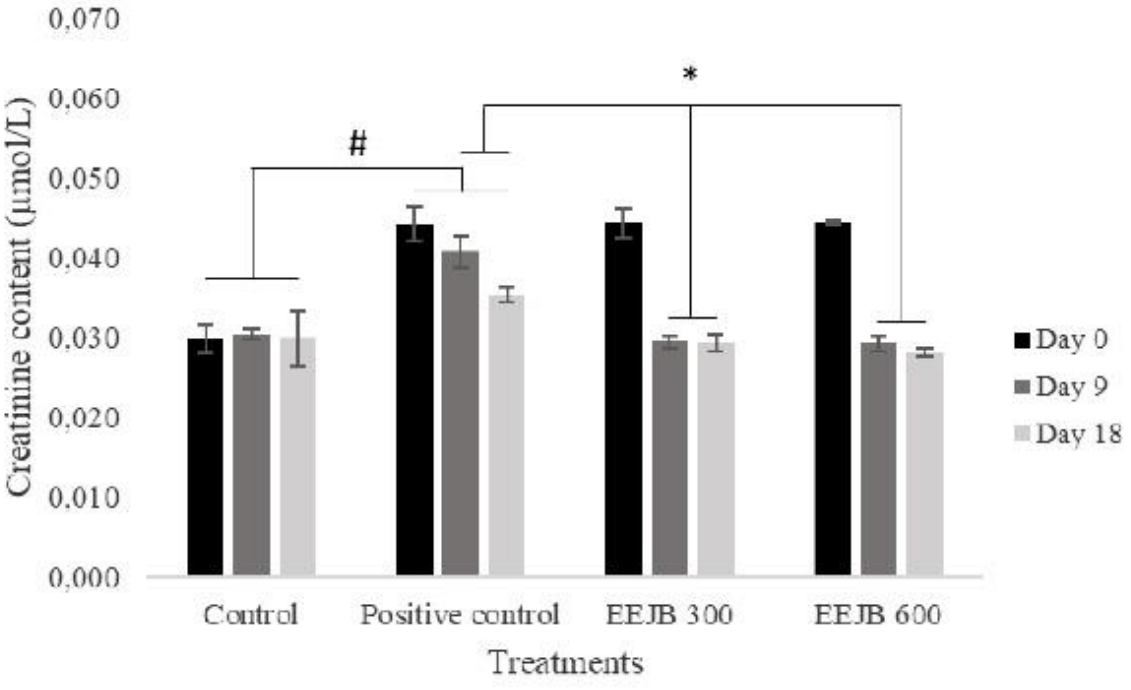

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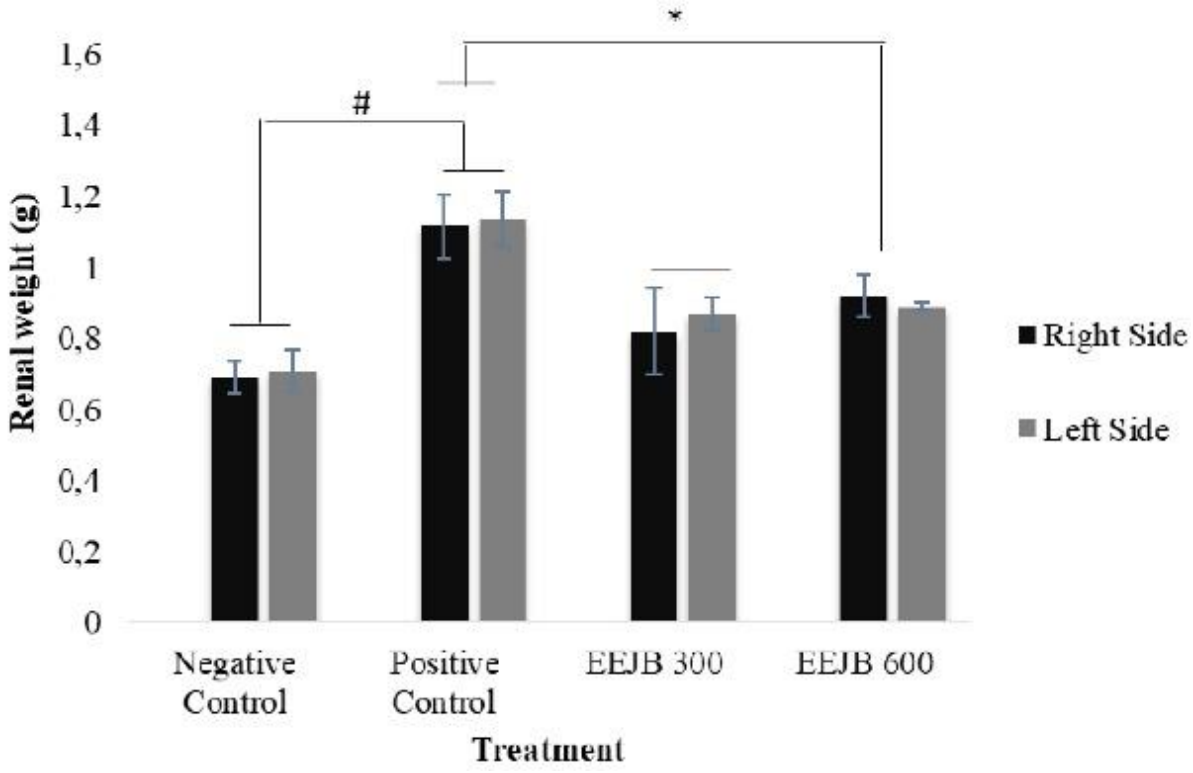
Figure 3



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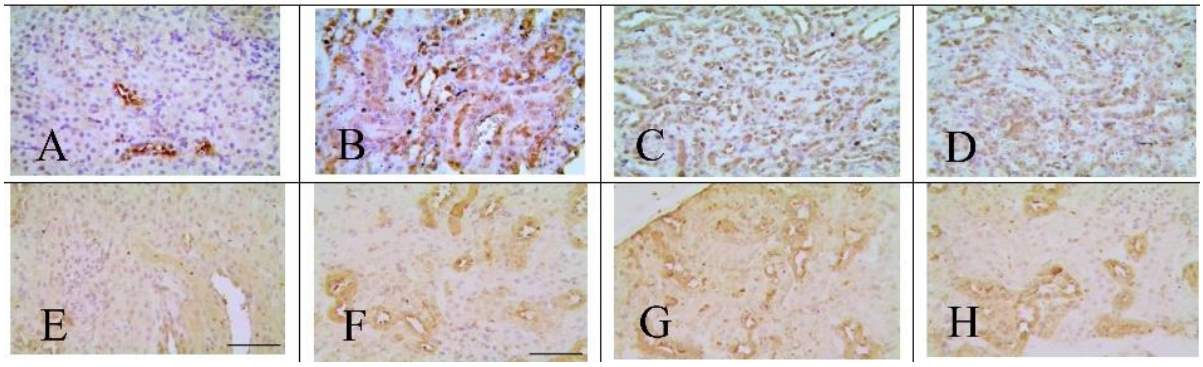
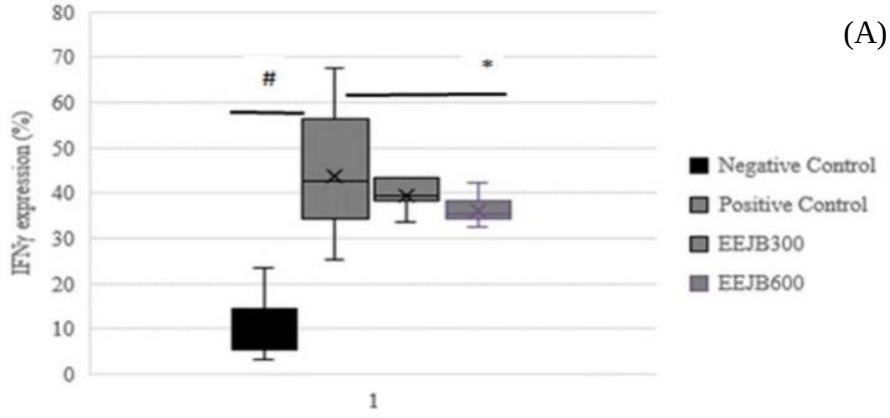


471 **Figure 5**



Effect EEBS toward quantitative IFNγ expression

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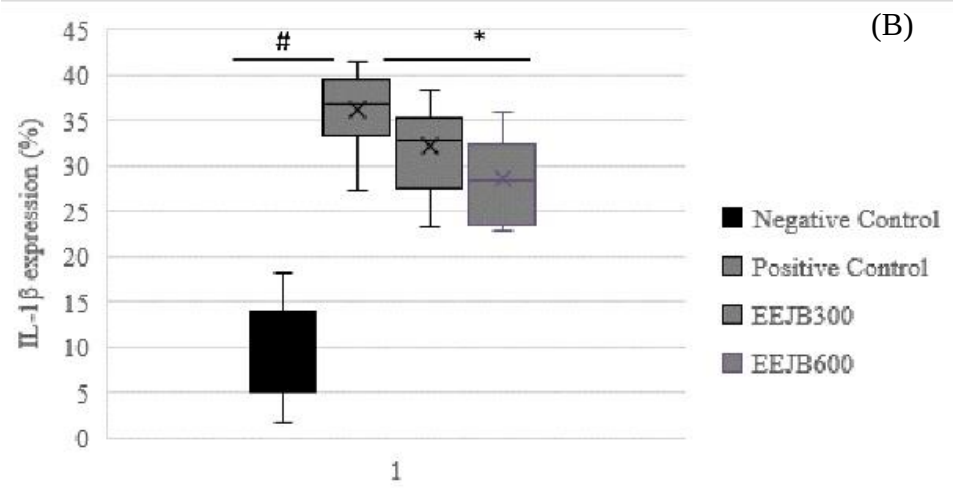

Effects of EEBS toward quantitative IL-1β expression

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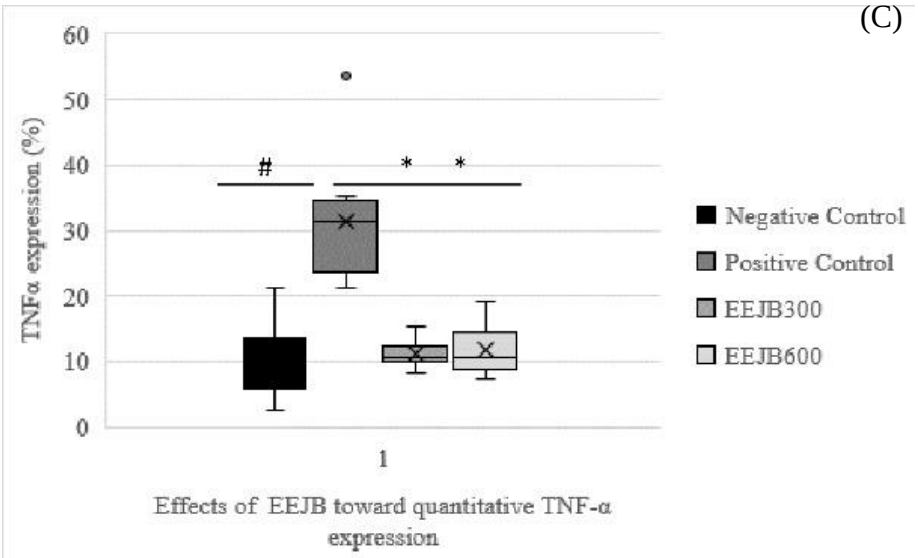

Effects of EEJB toward quantitative TNF-α expression

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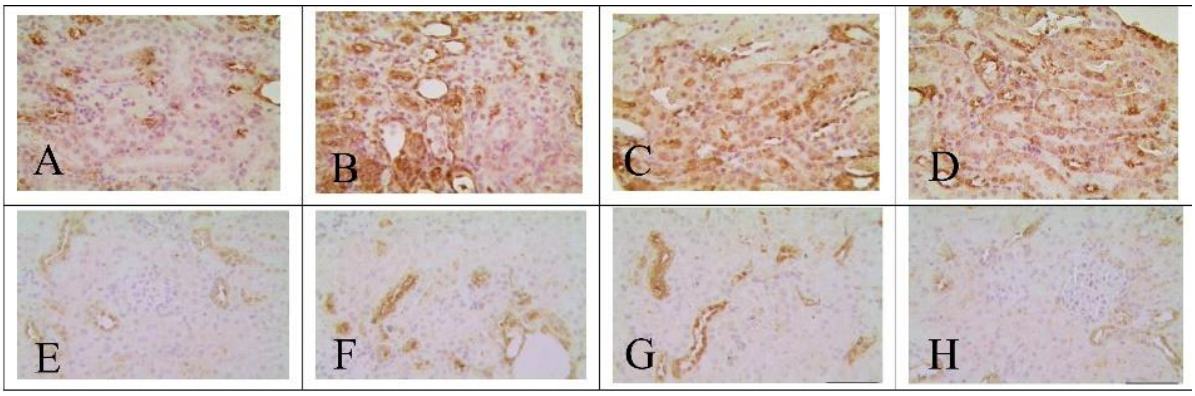
475 **Figure 6**



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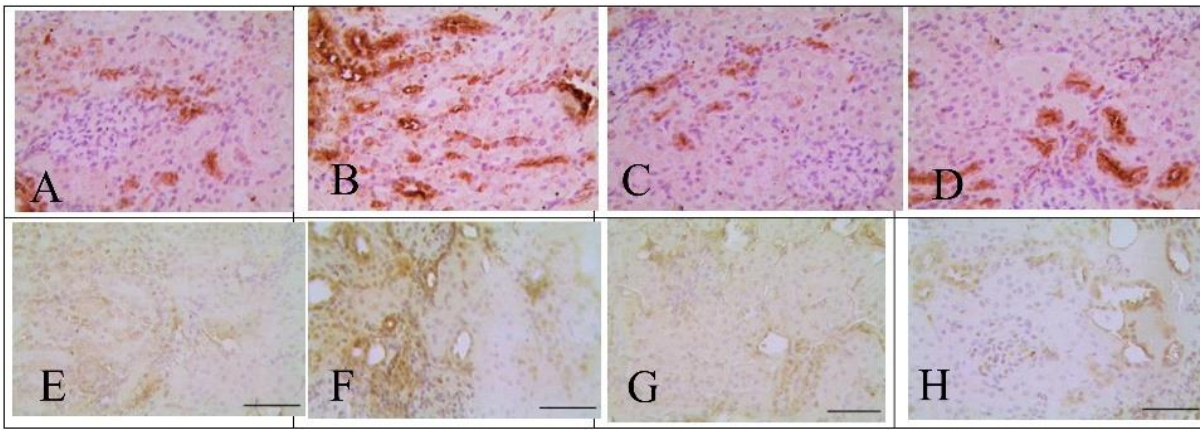
478 **Figure 7**



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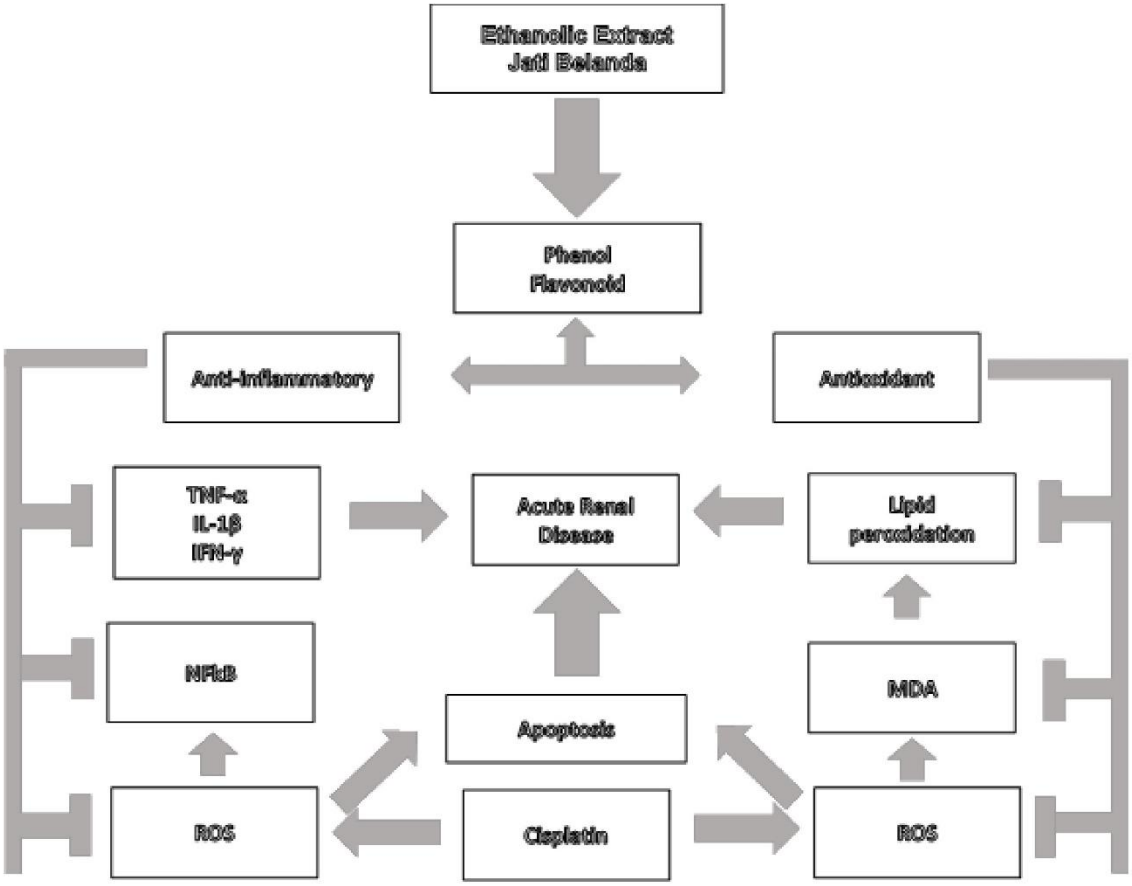
482 **Figure 8**



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PAKISTAN VETERINARY JOURNAL
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MS # PVJ-21-311		**Protective effect of Ethanolic Extract of Jati Belanda (*Guazuma ulmifolia* L.) by Inhibiting Oxidative Stress and Inflammatory Processes in Cisplatin-induced Nephrotoxicity Rats**					
Pages	19	Tables	Nil	Figures	8	Colored Figures	Nil
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The manuscript is intresting however its of regional importance because this specific plant may not be available globally its of regional importance.
Some refrences are missing in the test however present in the list.
Discussion is too long and it may be reduced.
Detail comments are present in the manuscript with track changes.
Ethical statement is missing.

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RESEARCH ARTICLE

Protective effect of Ethanolic Extract of Jati Belanda (*Guazuma ulmifolia* L.) by Inhibiting Oxidative Stress and Inflammatory Processes in Cisplatin-induced Nephrotoxicity in Rats

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ABSTRACT

The accumulation of high concentrations of cisplatin in the kidneys may causes nephrotoxicity. Cisplatin-induced nephrotoxicity happens through the inflammation of the tubules, and apoptosis, necrosis, and vascular defects. Jati Belanda (*Guazuma ulmifolia* L.) leave contain various natural compounds and the ethanolic extract of Jati Belanda decreased Reactive Oxygen Species (ROS) level in diabetic glomerulosclerosis by in vitro study. According to the author's knowledge, no comprehensive work was dedicated to nephroprotective effect of Jati Belanda ethanolic extract (EEJB) in cisplatin-induced nephrotoxic rat. Two doses of EEJB 300 mg/kg bw, EEJB 600 mg/kg bw were administered in nephrotoxicity rats by inducing cisplatin 8 mg/kg bw for three days before EEJB treatment. The measurement of MDA and CRE were done using colorimetric methods while IFN-γ. IL-1β, and TNF-α expression was done by using immunochemistry (IHC) assay. The results of this study showed that EEJB 300 and 600 mg/kg bw could decrease MDA and CRE level also IFN-γ. IL-1β, and TNF-α expression compared with positive control. Thus, ethanolic extract of Jati Belanda may give protective effect in patients being treated with chemotherapy using cisplatin but further toxicity effect and clinical trials studies are needed.

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INTRODUCTION

Nephrotoxicity is a condition in which renal functions are decreased due to the toxic effects of chemicals or drugs. About 20% of nephrotoxicity is induced and caused by drugs. This percentage is higher on the elderly due to an increase in lifespan and drugs consumed (Marwa *et al*., 2019). There are several drugs that can affect renal functions predominantly, thus the dosage needs to be adjusted to prevent a decrease in renal function (e.g. heparin). The nephrotoxic effects of most drugs are more profound in patients suffering from renal failure. The markers of nephrotoxicity and early renal dysfunction are blood urea nitrogen (BUN) and serum creatinine (CRE) with low sensitivity (Campos *et al*., 2018; Hameed *et al*., 2017).

Cisplatin (CP) is a chemical commonly used in chemotherapy against tumors of the bladder, testes, ovaries, breasts, and lungs (Chirino *et al*., 2004). This drug is correlated with nephrotoxicity in 28–36% of patients when they received an initial dose (50–100 mg/m^2) of CP (Badary *et al*., 2005; Rabik and Dolan 2007). The accumulation of high concentrations of CP in the renal tissues can cause nephrotoxicity by inducing inflammation of the tubules, and other apoptotic factors, necrosis and vascular factors (Li *et al*., 2017; Zhu *et al*., 2017; Kumar *et al*., 2017). Clinical uses of CP are limited because of these serious complications (Dasari and Tchounwou 2014). CP can also causes the development of renal tubule lesion by inducing oxidative stress (Chtourou *et al*., 2015; Lin *et al*., 2015; Saral *et al*., 2016; Oh *et al*., 2017). The reactive nitrogen species (RNS) and reactive oxygen species (ROS) productions in oxidative stress change the function and

structure of cellular membranes (Pedraza-Chaverrí *et al*., 2004; Divya *et al*., 2016). Moreover, their accumulations in the renal and lysosomes cause CP-induced acute nephropathy (Romero *et al*., 2009). Therefore, antioxidants and scavengers of free radicals may be capable to prevent the nephrotoxicity that cisplatin induced (Alhoshani *et al*., 2017). CP increase ROS level and activate Tumor Necrosis Factor Alpha (TNF-α), caspase-3 (Casp-3) and leads to apoptosis. TNF-α also activates other inflammatory related cytokines including Interferon-γ (IFN-γ) and Interleukin-1β (IL-1β) (Widowati *et al*., 2022).

Natural products that are obtained from plants and animals offer vast resources of new potential medicinal agents for clinical use. A high number of modern drugs have been made and isolated from natural sources. Those medicinal plants have the potentials to be used in the treatments for various diseases in the world (Patil and Biradar, 2013). Indonesia represents one of the tropical countries that are rich with various species of tropical plants. One of the tropical plants that have long been used to promote health is *Guazuma ulmifolia* L. which is called "Jati Belanda" in Indonesia (Rozqie *et al*., 2014; Prahastuti *et al*., 2019). The natural compounds in Jati Belanda leaves are tannins, alkaloids, mucilage, saponins, β-sitosterol, and flavonoids. Ethanolic extract of Jati Belanda (EEJB) can decrease cholesterol levels in hypercholesterol animals model (Rozqie *et al*., 2014), lower ROS level in diabetic glomerulosclerosis by in vitro study (Prahastuti *et al*., 2019). This study aimed to measure the levels of malonaldehyde (MDA), CRE, BUN, and kidney weight, protein expression of IFN-γ, IL-1β, TNF-α in cisplatin-induced rats as nephrotoxic rat model that were administered with EEJB.

MATERIALS AND METHODS

Ethical approval: The research studies had received approval from a Research Ethics Committee from Faculty of Medicine, Maranatha Christian University Bandung, West Java, Indonesia (No: 138/KEP/IV/2019).

Extract preparation: Jati Belanda was collected from Bumi Herbal Dago plantation, Bandung, West Java, Indonesia and the plant was identified by one of the staffs of herbarium of the Department of Biology from the School of Life Science and Technology at Bandung Institute of Technology, Bandung, West Java, Indonesia (0020218-A002). The leaves were collected, chopped, and kept in drier tunnel. The extractions to collect EEJB was done based on the maceration method. The solvent was ethanol (70%) (Widowati *et al*., 2018; Prahastuti *et al*. 2019; Prahastuti *et al*., 2020).

Nephrotoxicity rat model: White male Sprague Dawley rats aged 1.5 months with an average body weight of 160 – 180 g were gotten from the National Agency of Drugs and Food Control (Jakarta, Indonesia) and acclimatized for 1 week. The rats were divided into 4 groups according to the number of treatments that were used (I: Negative Control: Aquadest; II: Positive Control: Cisplatin 8 mg/kg bw + Aquadest; III: Cisplatin + EEJB 300 mg/kg bw; IV: Cisplatin + EEJB 600 mg/kg bw). Each treatment was repeated 5 times (5 rats). In all groups beside negative control group, the rats were induced with 8 mg/kg bw of cisplatin (Kalbe Farma) by intraperitoneal injection for three days and stopped on fourth days Then, the rats were given the dissolved 500 µL of EEJB by gavage for 18 days. On days 0, 9 and 18 after treatment, the blood was collected and processed as plasma for further assays. On the 18th day, the renals were extracted surgically after the rats were euthanized using the CO_2 chamber. The weights of the extracted organs were measured using Analytical Balance (AXIS, Kartuska) (Ajith *et al*., 2007; Soliman *et al*., 2016).

Immunohistochemistry (IHC): For IHC, antigens retrieval (Abcam, ab208572) was done in citrate buffer with a pH of 6.0 at 121ºC for 10 minutes. Endogenous peroxidase was blocked in 3% H_2O_2 (Merck, 107209) and methanol (Merck, 106009) for 15 minutes in room temperature. The primary antibodies of IFN-γ polyclonal antibody (Elabscience, E-AB-40075), TNF-α polyclonal antibody (ElabScience, E-AB-40015) IL-1β polyclonal antibody (ElabScience, E-AB-66749) were incubated overnight in room temperature. Then, the target proteins were visualized using Rabbit-Specific HRP/DAB (ABC) Detection IHC Kit (Abcam, ab64261). Haematoxylin was used for counterstaining agent. The stained tissues were observed in primostar microscope (Zeiss) and lumenera infinity 1-3c was used for photography (Pham *et al*., 2007; Ponti *et al*., 2005; Widowati *et al*., 2022).

MDA content: The measurement of MDA was done using Malondialdehyde (MDA) Assay Kit (Elabscience, E-BC-K025-S). Blood serum was used as a sample for MDA measurement. The blood sampling was taken on three different days (0, 9 and 18 days) after treatment. MDA content from each samples were measured according to the manufacturer protocol and read at 532 nm and 600 nm using the microplate reader (MultiskanTM GO Microplate Spectrophotometer, Thermo Scientific) (Hanny *et al*., 2019; Ginting *et al*., 2021).

CRE content: Blood samples were collected on various collection (0, 9 and 18 days) after treatment. Blood plasma was separated by centrifugation and then used for CRE content measurement. CRE content was measured using Creatinine (CRE) Assay Kit (Elabscience, E- BC-K186). Assays were read at 546 nm using the microplate reader according to the manufacturer protocol (Al-Kuraishy *et al*., 2019).

Statistical analysis: The data were analyzed using R software version 1.0.143 (R Studio). One-way ANOVA with Tukey post hoc test, Games-Howell post-hoc test, or Pairwise Wilcoxon non-parametric test were used to show the significance values between treatments.

RESULTS

EEJB effect on MDA content: CP treatment (8 mg/kg bw) increased MDA content compared to negative control (Fig. 1), indicating of lipid peroxidation. Treatment with EEJB 300 mg/kg bw show didn't show significant decrease in MDA content compared to positive control on day 9 and 18 ($P>0.05$). Meanwhile, treatment with EEJB 600 mg/kg bw showed a significant decrease in MDA content compared to positive control on day 9 and 18 ($P<0.05$).

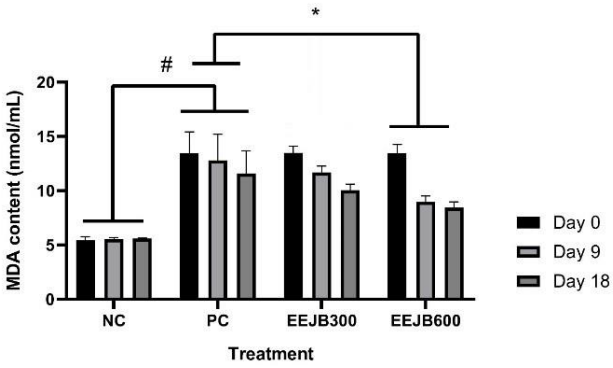

Fig. 1: Effects of EEJB on plasma MDA content in nephrotoxic rats. *NC: negative control/normal rat; PC: nephrotoxic rats; EEJB 300: Positive control + EEJB 300 mg/kg bw; EEJB 600: Positive control + EEJB 600 mg/kg bw. *Data was presented as mean±standard deviation, the experiment had three replications. Hashtag symbol (#) shows significant difference between control and positive control. Asterisk (*) shows significant differences between treatment and positive control based on Tukey HSD post hoc test (P<0.05) on day 9 and 18.

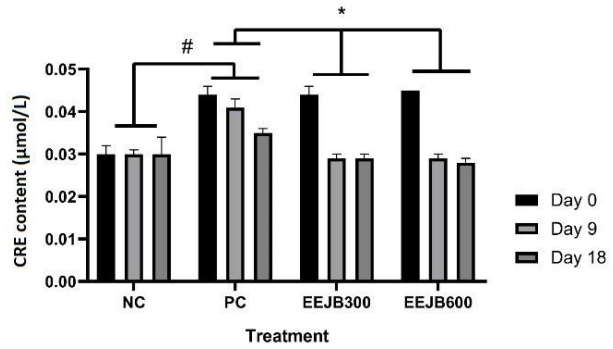

Fig. 2: Effects of EEJB on plasma CRE content of nephrotoxic rats. *NC: negative control/normal rat; PC: nephrotoxic rats; EEJB 300: Positive control + EEJB 300 mg/kg bw; EEJB 600: Positive control + EEJB 600 mg/kg bw. *Data was presented as mean±standard deviation, the experiment had three replications. Hashtag symbol (#) shows significant difference between negative control and positive control. Asterisk (*) shows significant differences between treatment and positive control based on Tukey HSD post hoc test (P<0.05) on day 9 and 18.

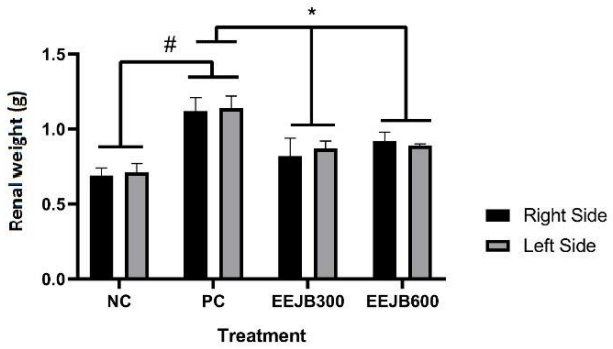

Fig. 3: Effects of EEJB on renal weight in nephrotoxic rats *NC: negative control/normal rat; PC: nephrotoxicity rats; EEJB 300: Positive control + EEJB 300 mg/kg bw; EEJB 600: Positive control + EEJB 600 mg/kg bw. *Data was presented as mean ± standard deviation, the data was three replications. Hashtag symbol (#) shows significant difference between negative control and positive control. While asterisk symbol (*) marks significant difference between positive control and treatment groups. All the significant differences were based on One-way ANOVA followed by Tukey post hoc test (P<0.05).

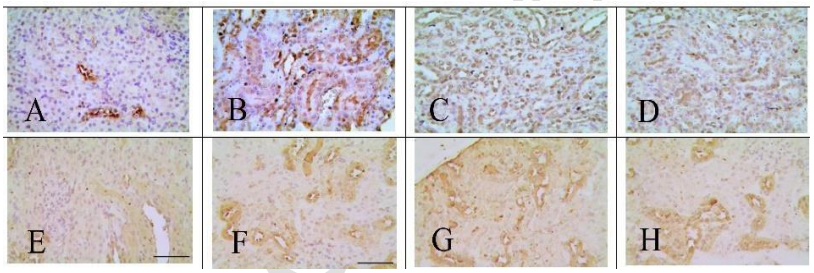

Fig. 4: Effects of EEJB toward renal IFN-γ expression in nephrotoxic rats by IHC assay *A-D are IFN-γ expression at magnification 400x while E-H are IFN-γ expression at magnification 1000x. *A and E are negative control (normal rat); B and F are positive control (acute renal disease model); C and G are EEJB300 (Positive control + EEJB 300 mg/kg bw) and; D and H are EEJB600 (Positive control + EEJB 600 mg/kg bw).

EEJB effect on CRE content: The CRE contents in EEJB treatments can be seen in Fig. 2. In this study, treatment with CP (8 mg/kg bw) showed an increasing CRE content compared to negative control. Treatments using EEJB 300 and 600 mg/kg.bw showed significant decreases in CRE contents compared to positive control on day 9 and 18.

EEJB effect on kidneys weight: In Fig. 3, CP induction showed significant increase in kidneys weight of the rats which marked hypertrophy. Treatment with EEJB seemed to protect the nephrotic renal from hypertrophy. This can be seen by the significant decrease in renal weight in EEJB groups compared to positive control (P<0.05).

EEJB effect on IFN-γ expression: The expression of IFN-γ can be seen in Fig. 4 and Fig. 5(A). Scattered, and weak IFN-γ stained cytoplasm in renal tubules were found negative control with a score of 10.556. In positive control, the IFN-γ expressions were diffuse and showed strong IFN-γ stained cytoplasm with highest scores (43.861) than negative control and treatment group with P<0.05. The EEJB300, EEJB600 were lower compared to positive control with score 39.383, 36.107 respectively. Based on the Pairwise Wilcoxon test (P<0.05) showed EEJB300 and EEJB600 were significantly difference compared to positive control, the EEJB600 was effective in reducing renal IFN-γ expression.

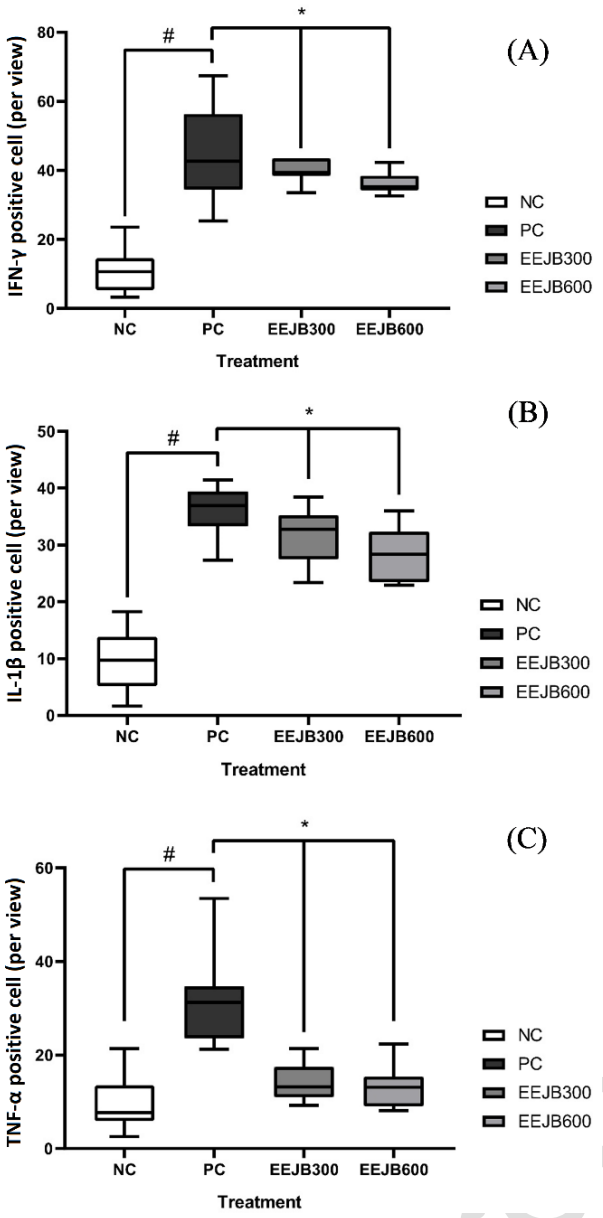

Fig. 5: Effect of EEJB toward in nephrotoxic rats using quantitative IHC assay (A) IFN-γ expression; (B) IL-1β expression *Data was presented as mean ± standard deviation, the data was ten observations. Hashtag symbol (#) shows significant difference between negative control and positive control. While asterisk symbol (*) marks significant difference between positive control and EEJB600. The significant differences were based on Pairwise Wilcoxon test (P<0.05): (C) TNF-α expression: *Data was presented as mean ± standard deviation, the data was ten observations. Hashtag symbol (#) shows significant difference between negative control and positive control. While asterisk symbol (*) marks significant difference between positive control and EEJB300, positive control and EEJB600. The significant differences were based on Pairwise Wilcoxon tests (P<0.05).

EEJB effect on IL-1β expression: The expressions of IL-1β by the treatment groups can be seen in Fig. 5(B) and Fig. 6. Expression seemed to be scattered and weak IL-1β stained cytoplasm in renal tubules epithelium with a score of 9.541. The positive control showed diffuse, strong IL-1β stained cytoplasm in renal tubules epithelium with highest scores (36.178) than negative control and treatment group with P<0.05. The EEJB300, EEJB600 were lower compared to positive control with score 32.133, 28.657 respectively (Fig. 5(C)). Based on the Pairwise Wilcoxon test (P<0.05) showed EEJB600 was significantly difference compared to positive control, the EEJB600 was effective in reducing renal IL-1β expression.

EEJB effect on TNF-α expression: TNF-α expression can be seen in Fig. 5C and Fig. 7. Scattered, and weak TNF- α stained cytoplasm in renal tubules epithelium can be found in negative control with a score of 9.307. The scores of EEJB300 and EEJB600 groups were higher in Fig. 5C (11.182 and 11.810 respectively). Compared to the other groups, positive control showed strong TNF-α stained cytoplasm with a score of 31.447. The treatment groups were shown to be effective in reducing the renal TNF-α expression. Based on the statistic test, there was a significant difference between positive control and normal control and the treatment groups (EEJB300 and EEJB600) were effective to reduce renal TNF-α expression based on Pairwise Wilcoxon test (P<0.05) (Fig. 5C).

DISCUSSION

CP can bind to lipid membrane and causes lipid peroxidation that produces MDA and destroys the cells of renal tubules (Divya *et al*., 2016; Rehman *et al*., 2014). CP can induce the production of free radicals such as hydrogen peroxide (H_2O_2) superoxide anions (O_2^{*-}) and hydroxyl radicals ($^{*}OH$) which triggering oxidative stress. They can cause reactions that result in peroxidation of the lipid membranes on tubule cells (Divya *et al*., 2016; Rehman *et al*., 2014).

Oxidative stress has been shown to contribute to CP-induced nephrotoxicity by increasing MDA level in a previous study (Rehman *et al*., 2014; El-Beshbishy *et al*., 2011). EEJB and EEJB600 decreased MDA level significantly (P<0.05) (Fig. 1), this result was validated by previous research that flavonoid of extract of *Sambucus nigra* L. reduced MDA in nephrotoxic rat (Ungur *et al*., 2021). EEJB contained phenols, flavonoids and had antioxidant activity (Prahastuti *et al*., 2019; Prahastuti *et al*., 2020).

In the serum biomarkers, nephrotoxicity is characterized by a reduction in glomerular filtration, and an increase of serum CRE (Mashhadi *et al*., 2013). In the previous study, CP could increase serum levels of CRE significantly as biomarkers for CP-induced nephrotoxicity (Oh *et al*., 2014). CRE is distributed throughout the total body water and is normally removed from the blood by the renal (Alibakhshi *et al*., 2018). This study showed EEJB 300 and 600 mg/kg bw could decrease plasma CRE content in renal nephrotoxic rat. This result is validated by previous study that phenolic compounds might prevents the increase of CRE level because of their antioxidant properties and by inhibiting arginase activity (Akomolafe *et al*., 2014).

In this result, treatments with EEJB were effective in decreasing renal weight and preventing renal hypertrophy. Renal injuries can occur because of high oxidative stress and high rate of phenolic compounds such as coumaric and cinnamic acids free radicals scavenger in the renal tissue. The antioxidants in EEJB might have contributed to decrease the occurrence of cisplatin induced-renal hypertrophy in administration by defending against oxidative stress (Himmelfarb *et al*., 2004; Dennis and Witting 2017).

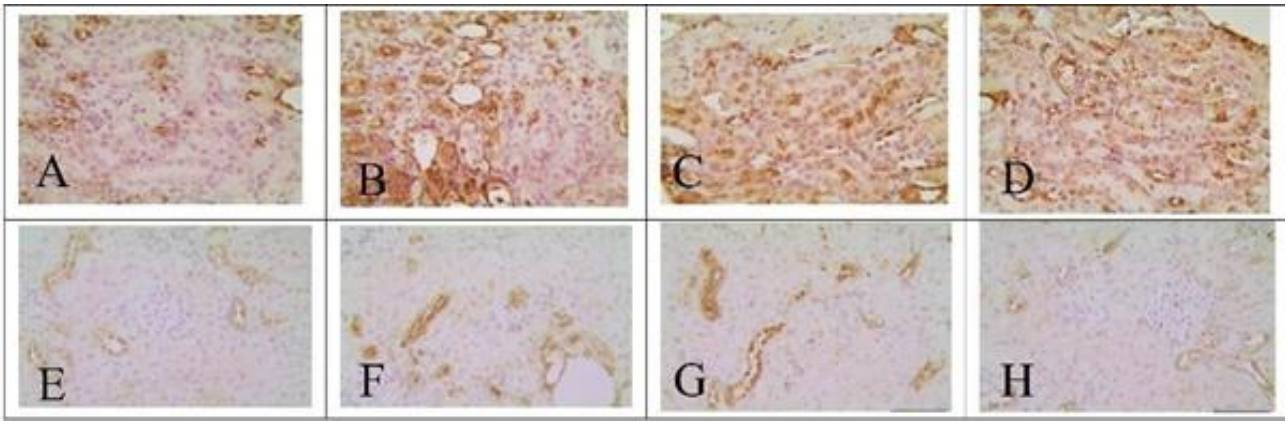

Fig. 6: Effect of EEJB toward renal IL-1β expression in nephrotoxic rats by IHC assay *A-D are IL-1β expression at magnification 400x while E-H are IL-1β expression at magnification 1000x. *A and E are negative control (normal rat); B and F are positive control (acute renal disease model); C and G are EEJB300 (Positive control + EEJB 300 mg/kg bw) and; D and H are EEJB600 (Positive control + EEJB 600 mg/kg bw).

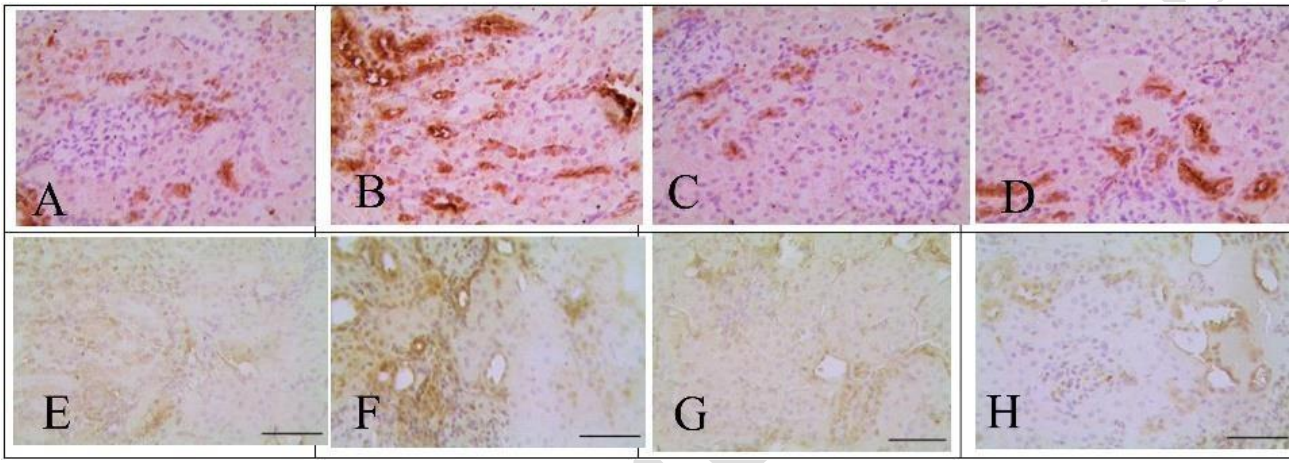

Fig. 7: Effect of EEJB toward TNF-α expression in nephrotoxic rats by IHC assay *A-D are TNF-α expression at magnification 400x while E-H are TNF-α expression at magnification 1000x. *A and E are negative control (normal rat); B and F are positive control (acute renal disease model); C and G are EEJB300 (Positive control + EEJB 300 mg/kg bw) and; D and H are EEJB600 (Positive control + EEJB 600 mg/kg bw).

Based on the result showed that EEJB could reduce IFN-γ expression of significantly ($p<0.05$) of kidneys (Fig. 4, 5A). A previous study showed that the phenolic compound, kaempferol, could decrease IFN-γ expression (Miles *et al*., 2005). Phenol compounds in EEJB as anti-inflammatory potential (Gonzalez *et al*., 2011; Bouriche *et al*., 2016). Flavonoids in EEJB were effective to have anti- inflammatory activity (Bouriche *et al*., 2016; Rathee *et al*., 2009). EEJB lowered IL-1β expression of renal tissue significantly (Fig. 5B, 6), previous studies have shown the phenolic compounds in decreasing IL-1β expression (Da Rosa *et al*., 2019; Gauliard *et al*., 2008). The phenolic compounds such as coumaric and cinnamic acids decreased expression of IL-1β (Rafiee *et al*., 2020). EEJB is able to reduce ROS level in chronic kidney disease cells model through their phenol and flavonoid content (Prahastuti *et al*., 2019). TNF-α is expressed mainly by macrophages renal tubular cells, mesangial cells, TNF-α regulates damage, promoting inflammation and cell death signaling (Black *et al*., 2019). TNF-α regulates the production of nitrogen oxide (NO) that is linked to the increase of oxidative stress in the body that plays an important role in nephrotoxicity by cisplatin. In this study, TNF-α expression of kidneys was reduced by EEJB treatment (Fig. 5C, 7), this result was validated by previous research that extracts are rich phenolics, flavonoids, compound have antioxidant activity and reducing stress oxidative which play rolling in inflammation (Bouriche *et al*., 2016). Oolong tea contained high polyphenol has anti-inflammatory activities by reducing NO, cyclooxygenase-2 (COX-2), IL-6, IL-1β, and TNF-α level in inflammatory cells model (Novilla *et al*., 2017).

Hence, we proposed the mechanism of EEJB in improving nephrotoxicity in Fig. 8 based on our results and study literature. This study showed that oxidative stress plays an important role in CP-induced nephrotoxicity. Moreover, the antioxidant effect of EEJB is responsible for reducing MDA and CRE content, IFN-γ. IL-1β, and TNF-α expression resulting in improvement of renal function in nephrotoxic rat. Therefore, pre-treatment combining EEJB may be useful for patients undergoing chemotherapy using CP but further *in vivo* studies and clinical trials are required.

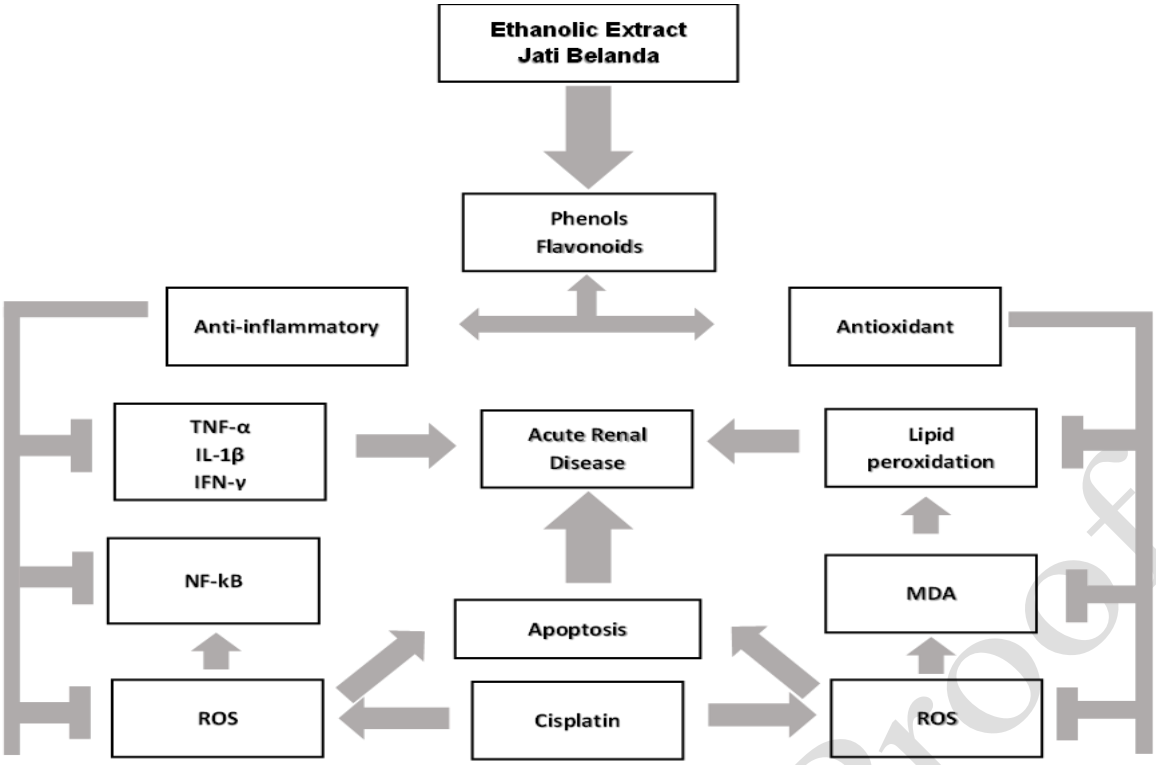

Fig. 8: Proposed mechanism of EEJB in improving nephrotoxic condition. CP could induce the ROS production. The exposure of ROS could damage cell membrane indicated by the increment of MDA that leads lipid peroxidation. On the other hand, ROS also could increase the apoptosis and cell death in renal. The treatment of Jati Belanda ethanolic extract which containing phenols, flavonoids as antioxidant, anti- inflammatory reduced ROS. It can reduce the production of MDA also neutralize the pro- inflammatory cytokines (TNF-α, IL-1β, IFN-γ) thus protecting the renal from injury.

Authors contribution: All Authors designed the project. SP and EA constructed and made sample preparation. SP, EA, HSWK, STH, RW executed the experiment and analyzed the tissue samples. SP, MH, RW, HSWK, RR, WW, and MS analyzed the data. All authors critically revised the manuscript and approved the final version.

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