Detam 1 black soybean against cisplatin-induced acute renal failure on rat model via antioxidant, anti-inflammatory and antiapoptosis potential

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ABSTRACT

Background and aim: Cis-Diaminedichloroplatinum (II) (Cisplatin) is one of the most synthetic anticancer drug but have several adverse effects and one of them is acute renal failure. Cisplatin can induce nephrotoxicity occur via the toxic generation of reactive oxygen species (ROS). Black soybean (Glycine max L. Merr.) has been reported contain high levels of phenolics and anthocyanins that has antioxidant activity. This study aims to determine the effect of ethanol extract of black soybean (EEBS) against cisplatin-induced nephrotoxicity in rats.

Experimental procedure: Cisplatin-induced nephrotoxicity rats treated with EEBS and the blood samples taken on days 0, 9, and 18. The effects of EEBS was evaluated by determining Interferon-gamma (IFN-γ), Caspase-3 (Casp-3), and Interleukin-1beta (IL-1β) expression using immunohistochemistry (IHC), blood urea nitrogen (BUN), Uric Acid (UA) content and catalase (CAT) content in the blood plasma with colorimetric assay kit.

Results and conclusion: Based on the results, EEBS treatment had successfully reduced pro-inflammatory cytokines IL-1β and IFN-γ, and improved physiological condition by lowering BUN and UA content while increasing CAT activity. No significant effect was found in Casp-3 expression. EEBS has potential to improve acute renal failure condition through inflammatory suppression and renal function improvement.

1. Introduction

Many anticancer drugs have teratogenic and other serious effects on biological systems which prompting restricted usage. Cis-Diaminedichloroplatinum (II), typically known as cisplatin, is one of the most effective anti-neoplastic drugs currently available because of its high-therapeutic efficacy. However, several adverse effects of cisplatin have been reported, mainly neurotoxicity and nephrotoxicity. Long-term cisplatin nephrotoxic effects causes Acute Kidney Injury (AKI) which occurs in 20–30% of patients. As reported in previous studies, nephrotoxicity was most likely caused by generation of Reactive Oxygen Species (ROS) which causes molecular damage to the cells. Therefore, compounds with antioxidant activity will offer therapeutic effect to inhibit cisplatin side-effect.
AKI is an abrupt decrease in kidney function caused by damage in renal parenchyma or reduction in renal perfusion. Cisplatin has been known to cause kidney damage, mainly through ROS generation. Several antioxidant enzymes in the kidney play a role in protecting against nephrotoxicity. Among the antioxidant enzymes, hydrogen peroxide (H₂O₂) is considered the most effective target molecule to reduce ROS toxicity. However, cisplatin has been known also to block the innate antioxidant enzymes activity, including catalase (CAT). Cisplatin nephrotoxicity is also highly related to kidney proximal tubule uptake of cisplatin at higher concentrations compared to other tissues. Cisplatin-induced ROS causes activation innate Tumor Necrosis Factor-α (TNF-α) which activates caspase-3 (Casp-3) that leads to apoptosis. TNF-α also activates other inflammatory related cytokines including Interleukin-1β (IL-1β) and Interferon-γ (IFN-γ). AKI causes decrease in renal perfusion which leads to reduction of glomerular filtration rate (GFR). Blood uric acid and urea nitrogen often used as indicator of AKI that these two substances are regularly excreted through reduction of serum alanine aminotransferase (ALT), which has antioxidant activity and also has hepatoprotective effect. Therefore, black soybean is a potential candidate to reduce cisplatin side-effect. Black soybean (Glycine max L. Merr.) has long been consumed in Indonesia as one of Indonesia’s traditional food, the soybean sauce. Black soybean contains plenty of protein, isoflavone, and vitamins. Black soybean has also been reported to contain high levels of anthocyanins in the seed coat and also has polysaccharides which has ability for preventing such oxidation-related diseases, antioxidant activity, and antiinflammatory potential. Therefore, black soybean is a potential candidate to reduce cisplatin side-effect. Besides that, black soybean has arabinogalactan compound that has antioxidant activity and also has hepatoprotective effect through reduction of serum alanine aminotransferase (ALT), alkaline phosphatase (ALP), aspartate aminotransferase (AST), increase activity superoxide dismutase (SOD) and CAT. The novelty of this research rest in the usage of ethanol extract of black soybeans (EEBS) to improve nephrotic kidney condition and acute renal failure condition through inflammatory suppression by lowering proinflammatory cytokines, blood urea nitrogen (BUN), and uric acid (UA) level, while increasing the activity of CAT and improving physiological condition. This study aims to determine the effect of ethanol extract of black soybeans (EEBS) against cisplatin-induced nephrotoxicity in rats by measuring BUN, UA content and CAT activity in the blood plasma and immunohistochemistry (IHC) of kidney for IFN-γ, Casp-3, IL-1β expression.

2. Materials and methods

2.1. Extract preparation

Detam variety of black soybeans (Glycine max L. Merr.) was collected from Unit Pengelolaan Benih Sumber (UPBS) Balai Penebian Tanaman Aneka Kacang dan Umbi, Malang, East Java, Indonesia. The plants were identified by herbarium staff, Department of Biology, School of Life Science and Technology, Bandung Institute of Technology, Bandung, West Java, Indonesia. Black soybean seed was kept in drier tunnel service and chopped. The extraction of detam black soybeans was performed based on maceration method using distilled ethanol 70% as the solvent. The filtrate was evaporated using a rotary evaporator (Zhengzhou Well-known, RE-201D) until a paste extract. The ethanolic extract of black soybeans (EEBS) stored at −20 °C. The extract were then analyzed by liquid chromatography and liquid chromatography mass spectrometry (LC-MS/MS) profiling for identifying the qualitative bioactive compound composition using isoflavone as standard and methanol as solvent. Analysis using Hypersil Gold column (150 mm × 2.1 mm × 1.9 mm). The sample injected 1 μl to the column. The mobile phase was 0.1% formic acid with the flow rate 350 μl/min. MS/MS Triple Q (quadrupole) mass spectrophotometer TSQ Quantum Access with Electrospray Ionization (ESI) be used and controlled by software TSQ Tune operated with positive polarity with condition such as voltage 3 kV; evaporation temperature 275 °C; capillary temperature 300 °C; nitrogen 40 psi; and Aux 10 psi with argon gas.

2.2. Acute renal disease rat model

Male Sprague-Dawley (SD) rats (6 weeks old, 160–180 g BW) were obtained from the National Agency of Drugs and Food Control (Jakarta, Indonesia). The research has been approved by the Research Ethic Committee from Faculty of Medicine, Maranatha Christian University and Immanuel Hospital, Bandung, Indonesia (No.138/KEP/IV/2019). The rats were housed in plastic-bottom wire-upper cages and acclimated for 1 week under laboratory conditions (25–27 °C; humidity 60%; 12-h light/dark cycle). The acute renal disease rat model were randomly divided into four different groups containing five rats each group. Group 1 (negative control) rats treated with 0.5 mL distilled water once every day.
Group II (positive control) containing cisplatin-induced rats, while group III and IV were positive control given EEBS at the dose of 300 and 600 mg/kg BW. Rats were kept in single system cages, each cage contain 1 rat. Rats were fed with basal diet (water content 12%, crude protein 15%, crude fat 3–7%, crude fiber 6%, Ca 0.9–1.1%, P 0.6–0.9%, energy 4400 kcal). Rats were induced with cisplatin 8 mg/kg BW intraperitoneally for three days before EEBS treatment except the negative control group. On the fourth day, cisplatin induction was stopped and EEBS treatment was started for 18 days by giving orally 300 and 600 mg/kg BW, except for negative and positive control group for 18 days. Bloods were collected from the orbital veins at day 0, 9, and 18 during treatment for BUN, UA, and CAT level analyses. At day 18, rats were anesthetized using ketamine HCl (Ikapharmindo Putramas) 100 mg/kg BW, xyla (Interchemie, 361453) 15 mg/kg BW intraperitoneal and rats were terminated, the kidneys were collected. Kidneys were weighted on Analytical Balance (AXIS, ACN/G) and prepared for IHC assay.13

2.3. Immunohistochemistry assay of kidney IFN-γ, Casp-3, IL-1β expression

Kidneys were embedded in paraffin and fixed with formalin. Deparaffinization was done by placing the slides in 56 °C oven for 15 min and baking in xylene. Later serial dehydration was done by placing the slides in absolute ethanol, 90% ethanol, 70% ethanol and final washing with water. Slides were place in PBS bath for further dehydration for 30 min. Antigen retrieval were done in citrate buffer pH 6.0 (Abcam, ab208572) in 121 °C for 10 min. Endogenous peroxidase were blocked with 3% H2O2 (Merck, 107209) in methanol (Merck, 106009) for 15 min at room temperature. Samples were then pre-incubated with 5% Bovine Serum Albumin for 10 min prior to primary antibody reaction. The primary antibody reaction using rabbit-anti rat IFN-γ (Elabsci, E-AB-R0009), rabbit-anti rat Casp-3 (ElabScience, E-AB-63602), and rabbit-anti rat IL-1β (Elabsci, E-AB-70048) were done overnight in room temperature. The protein target were visualized with rabbit specific HRP/DAB (ABC) detection IHC kit (Abcam, ab64261). Haematoxyline was used for counterstain. The stained tissue were observed in primostar (Zeiss) microscope and lumenera in primostar. The IHC result ware captured using digital camera NIKON. The IHC result were analyzed with ImageJ software to assess indices of positive on immunohistochemical slides.14

2.4. Blood urea nitrogen assay

The BUN level was measured using Blood Urea Nitrogen (BUN) Assay Kit (Elabscience, E-BK-K183). The reaction absorbance was read at 580 nm according to the manufacturer’s protocol using the micro-plate reader (Multiskan™ GO Micro plate Spectrophotometer, Thermo Scientific).15

2.5. Uric acid assay

UA level was measured using Uric Acid (UA) Assay Kit (Elabscience, E-BK-K016). The absorbance was then read at 690 nm using the microplate reader according to the manufacturer’s protocol.16

2.6. Catalase assay

CAT activity was measured using Catalase (CAT) Assay Kit (Elabscience, E-BK-K031). The absorbance was read at 405 nm using the microplate reader according to the manufacturer’s protocol.17

2.7. Statistical analysis

The data was analyzed using IBM SPSS Statistics 20.0 version. One-way ANOVA with Tukey HSD post hoc test were used to show significance among treatments. P-value <0.05 was considered as significant.

3. Results and discussion

3.1. Secondary metabolite composition of extract

The results of the LC-MS/MS analysis can describe difference in the compound of the soybean extract. The content of these differences is illustrated by the peak chromatogram of compounds with different molecular weights. Based on the result of LC-MS/MS analysis in our study and compared to the standard (isolavone), as shown in Figs. 1 and 2, daidzein has retention time at 2.58 min, daidzin at 1.21 min, genistin at 1.43 min, biochanin A at 1.22 min, while glycitin has retention time at 2.72 min. This result was indicated that soybean extract contained daidzein, daidzin, genistin, biochanin A, and glycitin. These results show the most well-known isolavone in soybeans as shown in the previous study except biochanin A. Biochanin A is also a part of isolavones but have small concentration in soybean so in the previous study biochanin A was not determined. The difference between our results and previous study can caused by the variation of external condition which is can reduce the isolavone before detected.24 Soybeans contain high amount of secondary metabolite especially their isoflavones, as shown in Fig. 1 so that isolavone used as the standard in this study. The soybeans isolavone have been shown to have many pharmacological effect such as antioxidant, anti-inflammatory, anticarcinogenic, and antiviral activity.25 All the isolavone determined in our study has an potential activity to reducing oxidative stress or inflammatory disease.12,14

3.2. Effect of EEBS toward IFN-γ expression in acute renal disease rat model

Cisplatin is one of the most effective drugs used to treat a variety of cancer but has side effect that can cause nephrotoxicity. Based on our result, cisplatin induction caused inflammation as marked by significant elevation of IL-1β and IFN-γ and cell apoptosis as marked by Casp-3 elevation. Moreover, cisplatin induction has resulted in slight renal hypertrophy. Cisplatin induction caused nephrotoxicity because of cisplatin high affinity copper transporter (CTR-1) and organic cation transporter (OCT-2) located at basolateral membrane of proximal tubule cells. There are several proposed mechanism how cisplatin caused nephrotoxicity. The well-known mechanism is through generation of ROS and binding to various cytoplasmic anti-oxidant including glutathione (GSH), superoxide dismutase (SOD) and CAT which shift the redox status within cells leading to ROS toxic level. Nephrotoxicity effects caused by cisplatin can occur via ROS generation. For protecting from this effect, compound with antioxidant activity have been studied and in our research was tested by EEBS. The seed coat of black soybeans has been known to contain high concentration of flavonoids especially proanthocyanidin and anthocyanin which possess high level of antioxidant activity.26 Anthocyanins is a phenolic compound from flavonoid class which is soluble in polar solvents and highly deposited in flowers, fruits, and seed coats that gives red to black pigmentation. Black soybean seed and its compound daidzein, genistein have been studied to possess anti-inflammatory activity which reducing IL-1β, TNF-α, prostaglandin 2 (PGE-2) by in vitro study toward inflammatory macrophage cells.14 The best studied mechanism is through its antiradical
activity.\textsuperscript{26} Flavonoid or more specifically isoflavones including genistein, daidzein, and glycitein dose-dependently suppress NO might, attenuate excessive NO generation at inflammatory sites in LPS-induced macrophage cells.\textsuperscript{27}

The inflammation caused by cisplatin induction has been reported in several studies as marked by increased in several cytokine such as IL-1\textbeta, IL-1\alpha, IL-6, TNF-\alpha.\textsuperscript{28,29} In our study, we also found that cisplatin induction trigger releasing pro-inflammatory cytokine IFN-\gamma as marked by significant elevation of the expression which is supported that higher expression of pro-inflammatory cytokine correlated with renal injury.\textsuperscript{30} As shown in Fig. 3A and E, presents that the negative control (healthy rats) showed scattered and weak cytoplasmic staining of IFN-\gamma in renal tubules epithelium as shown by brown coloration inside the cytoplasm compared to the positive control group (cisplatin-induced rat) as shown in Fig. 3B and F, that has high expression of IFN-\gamma that showed by diffuse and strong cytoplasmic staining (see Fig. 3). The IFN-\gamma expression of positive control significant difference compare to negative control (\(p < 0.05\)) as present in Fig. 4. The increasing IFN-\gamma expression has been demonstrated that it has an associated with the NACHT, LRR, and PYD domains-containing protein-3 (NRLP-3) downstream secretion of IL-18.\textsuperscript{31} To reduce the severity happened by inflammation, the expression of cytokine should be reduced with one of them is flavonoid specifically isoflavone compound found in black soybeans as mentioned in section 3.1. Several isoflavone has already shown a protective effect such as anti-inflammatory for acute renal injury and improved kidney function on in vivo model studies especially daidzein as mentioned in that ameliorated kidney injury.\textsuperscript{32} Genistein and daidzein have anti-inflammatory by inhibiting inflammatory marker TNF-\alpha, IL-1\beta in LPS-induced macrophage cells as inflammation cells model.\textsuperscript{33} Based on our results, the treatment of EEBS caused a significant reduction in cytoplasmic IFN-\gamma expression dose dependently (Fig. 3C and G, D and H). This study showed that EEBS 600 mg/kg

Fig. 1. Chromatogram of Isoflavones as a standard using LC-MS/MS method. (A. Daidzein (RT 2.58), B. Daidzin (RT 1.23), C. Genistein (RT 3.77), D. Genistin (1.43), E. Biochanin A (RT 1.22) and Glycitein (RT 2.72)).
Fig. 2. Chromatogram of EEBS using LC-MS/MS method. EEBS contained A. Daidzein (RT 2.58), B. Daidzin (RT 1.21), C. Genistin (RT 1.43), D. Biochanin A (RT 1.22) and Glycitein (RT 2.72).
BW had significantly (p < 0.05) reduced inflammation as marked by reduced pro-inflammatory cytokines IFN-γ expression (Fig. 4).

3.3. Effect of EEBS toward Casp-3 expression in acute renal disease rat model

ROS generation within cells trigger Mitogen-Activated Protein Kinase (MAPK) activation which in turn trigger the intercellular production of TNF-α. TNF-α production activates extrinsic pathway of apoptosis, where it triggers the activation of Casp-8 and Casp-3. In on our result, cisplatin induction caused cell apoptosis as marked by the significant elevation of Casp-3 compared to the negative control (p < 0.05) (Fig. 5B and 5F). The negative control (healthy rats) showed scattered, weak cytoplasmic staining of Casp-3 in renal tubules epithelium as shown by brown coloration inside the cells cytoplasm) (Fig. 5A and 5E), while the positive control group (cisplatin-induced rats) showed diffuse, strong cytoplasmic staining as an indication of high expression of Casp-3 (Fig. 5B and 5F). EEBS treatment (EEBS 300, EEBS 600) reduced Casp-3 expression compared to positive control (p < 0.05) Fig. 6. This results was similar with the previous study that Casp-3 as an apoptotic marker will be down regulated their expression after flavonoid specifically daidzein as part of isoflavone treatments. Genistein decreased cisplatin-induced apoptosis by regulating P53 induction in kidney injury.

3.4. Effect of EEBS toward IL-1β expression in acute renal disease rat model

Increased levels of inflammatory cytokines such as IL-1β, TNF-α, and monocyte chemoattractant protein-1 (MCP-1) have become a focus of study in kidney injury. In Fig. 7 showed the immunohistochemical protein expression of IL-1β in renal cortex. The brown color has indicated the positive staining area of IL-1β. Furthermore, IHC study revealed that cytoplasmic immunoreactivity of IL-1β in the renal cortex was significantly increased compared to negative control group (Fig. 7). The treatment of EEBS in the highest dose (600 mg/kg BW) can decrease the IL-1β production, this result indicate the lowest of IL-1β protein expression (Fig. 7D and 7H, Fig. 8). Based on He (2019) research showed that soybean product significantly improved the histopathological damage in chronic kidney disease mice model. The soybean treatments decreased the serum levels of kidney toxicity and inflammatory biomarkers such as IL-6, IL-1β, Transforming Growth Factor-β1(TGF-β1), Toll Like Receptor-4 (TLR-4), F4/80 and TNF-α in kidney samples.

3.5. Effect of EEBS toward blood urea nitrogen, uric acid in acute renal disease rat model

Edema in the kidney caused by inflammation will caused increasing in intratubular pressure, hypoxia, and decrease the GFR. The decrease in GFR causes elevation of BUN which is excreted through urine. Moreover, the renal proximal tubules cells have high energy demand (ATP) as they reabsorb nearly 80 meq Na/g kidney/
Hypoxic condition causes drop in renal ATP concentrations and increase in ADP and AMP formation. The activity of 5'-nucleotidase with AMP forms adenosine, inosine and hypoxanthine. Hypoxanthine is converted by xanthine oxidase to UA. Our result shows significant increase in BUN and UA level in nephrotic rat models (p < 0.05). Increasing level of BUN and UA has an associated with the renal injury or any disease in kidney. Therefore, reducing both level will be ameliorate the kidney’s function. In our study, EEBS treatment had improved physiological condition as marked by lower BUN and UA level. The BUN and UA with EEBS 600 mg/kg BW were comparable to negative control (Figs. 9 and 10). This treatment showed a potential effect from EEBS to reduce the progression of kidney disease in cisplatin-induced rat models. This results was similar with previous study that the urea content was decreased significantly in cisplatin-induced rats by using Hibiscus sabdariffa treatment that contain high level of anthocyanins.

A significant correlation has been found between renal proinflammatory cytokines and level of BUN and UA. Isoflavonoid in EEBS improves reverse glomerular functions in cisplatin-administered rats. Moreover, the considerable reduction in BUN and UA in cisplatin-administered rats due to EEBS treatment improved renal tubular function.

3.6. Effect of EEBS toward catalase level in acute renal disease rat model

Increasing ROS in our models indicate of kidney injury (see Fig. 8). To regulate the level of ROS especially in kidney injury, there are many antioxidants system that has been described in many studies. One of the mostly used is SOD which will form oxygen (O₂) and hydrogen peroxide (H₂O₂) from dismutation of superoxide (O₂⁻*), and H₂O₂ converted by CAT into molecular O₂ and H₂O. CAT is free radical scavengers or electron donors that can react with free radicals to form harmless product which is it would converting H₂O₂ into molecular O₂ and H₂O. Therefore, cisplatin-induced rat inhibits the renal activities of CAT which indicated by decrement of CAT activity. Our result shows that cisplatin-induced rats shows significantly decrease plasma CAT level (p < 0.05) Fig. 11. Cisplatin also directly bind to cytochrome P450 in mitochondria which leads to mitochondrial ROS generation. In our study, EEBS treatment had improved physiological condition as marked by higher CAT content. After the EEBS treatment, CAT level elevated significantly compared to positive control (p < 0.05). EEBS was able to increase CAT level in nephrotoxic rat (Fig. 11). This results was validated with previous study that CAT activity increased in cisplatin-induced nephrotoxic mice when using antioxidant vitamins C and E treatments 500 mg/kg. The flavonoid content of soybean extract has been known exhibited nephroprotective activity due to preventing oxidative damage in renal tubular mediate by iron (Fe) and also blocking of P38 MAPK activation mechanisms. These findings reveal that Adenosine Monophosphate cyclic (AMPc) protects renal tubular cells from cisplatin-induced oxidative stress by obliterating ROS and reducing the generation of proinflammatory cytokines through
suppressing P38 MAPK activation.42,49 The P38 MAPK may be an important mediator of proinflammatory cytokines production in a variety of forms of renal injury in proximal tubule cells in response to cisplatin.51 Genistein reduced ROS production in cisplatin-induced human kidney HK-2 cells, genistein one of EEBS compound have ability to prevent cisplatin-induced renal injury.33 Genistein possesses protective effects on renal ischemia-reperfusion injury through apoptosis inhibition and regeneration.52

3.7. Effect EEBS toward renal weight in acute renal disease rat model

Inflammation in the kidney tubular cells can lead to injury of the endothelium. This may lead to renal vasoconstriction which causes water retention and edema (swelling) of the kidney.37 Our model shows cisplatin-induced rats experienced significant renal weight gain that probably is caused by edema (Fig. 12) which marked renal hypertrophy. The increased of renal weight can be caused by the

Fig. 7. Effect of EEBS towards IL-1β expression in acute renal disease rat model The slides is seen at 400× (A–D) and 1000× (E–H) magnification. *A and E are Negative Control (normal rat); B and F are Positive Control (acute renal disease); C and G are EEBS 300 (Positive Control + EEBS 300 mg/kg BW); D and H are EEBS600 (Positive Control + EEBS 600 mg/kg BW).

Fig. 8. Effect of EEBS towards IL-1β expression in acute renal disease rat model Data are presented as mean ± SD. NC: negative control (normal rat + untreated EEBS); PC: positive control (Cisplatin-induced rat); EEBS 300: positive control + EEBS 300 mg/kg BW; EEBS 600: positive control + EEBS 600 mg/kg BW. Different letters (a, b, c) show significant difference of IL-1β expression among treatments based on Tukey HSD post hoc test (p < 0.05).

Fig. 9. Effect EEBS toward BUN level in acute renal disease rat model Data are presented as mean ± SD. NC: negative control (normal rat + untreated EEBS); PC: positive control (Cisplatin-induced rat); EEBS 300: positive control + EEBS 300 mg/kg BW; EEBS 600: positive control + EEBS 600 mg/kg BW. Different letters (a,b, c) show significant difference of BUN content among treatments in day 0, different letters (a,b,c,d) show significant difference of BUN content among treatments in day 9, and different letters (a,b,c) show significant difference of BUN content among treatments in day 18. All statistical different was based on Tukey HSD post hoc test (p < 0.05).

Fig. 10. Effect EEBS toward UA level in acute renal disease rat model Data are presented as mean ± SD. NC: negative control (normal rat + untreated EEBS); PC: positive control (Cisplatin-induced rat); EEBS 300: positive control + EEBS 300 mg/kg BW; EEBS 600: positive control + EEBS 600 mg/kg BW. Different letters (a,b) show significant difference of UA content among treatments in day 0, different letters (a,b,ab) show significant difference of UA content among treatments in day 9, and different letters (a,b,c) show significant difference of UA content among treatments in day 18. All statistical different was based on Tukey HSD post hoc test (p < 0.05).
Data are presented as mean ± SD. NC: negative control (normal rat + untreated EEBS); PC: positive control (Cisplatin-induced rat); EEBS 300: positive control + EEBS 300 mg/kg BW; EEBS 600: positive control + EEBS 600 mg/kg BW. Different letters (a,b) show significant difference of CAT content among treatments in day 0, different letters (a,b,c) show significant difference of CAT content among treatments in day 9, and different letters (a,b,c,d) show significant difference of CAT content among treatments in day 18. All statistical different was based on Tukey HSD post hoc test (p < 0.05).

**Fig. 11.** Effect EEBS toward CAT activity in acute renal disease rat model*.

**Fig. 12.** Effect EEBS toward renal weight in acute renal disease rat model*.

There is no conflict of interest.

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