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The Biochemical and Kidney Histopathological Parameters in Hyperoxaluria Rats Treated with Breadfruit Leaf Extract

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Abstract

Purpose: This study was to analyze the administration of breadfruti leaf extract to changes in biochemical parameters and renal histology in hyperoxaluria rats.

Methodology: The experimental study used 6 groups, each of 5 mice that are EG, Normal, Vitamin E, an ethanol extract of breadfruit 100, 200 and 400 mg/kg BW. Blood data for the analysis of biochemical parameters and kidney were taken at the end of the experiment for histological analysis of kidney changes.

Results: Research shows that the purified ethanol extract of breadfruit leaf can prevent increases in MDA levels, reduce creatinine and BUN levels, increase body weight, prevent hypertrophy and kidney damage

Conclusion: Purified ethanol extract of AA leaves has an antioxidant, nephroprotective activity, protect kidney inflammation, and kidney damage in nephrolithiasis rats and the effective dose is 400 mg/kg BW. The higher the extract dose, the more effective to reduce the biochemical and histopathological parameters in hyperoxaluria rats.

Applications/Originality/Value: as a basis for futher studies of molecular biology in the prevention of kidney disorder.

Keywords: *Biochemical, histopathology, hyperoxaluria, Artocarpus altilis.*

INTRODUCTION

Urolithiasis or nephrolithiasis is one of the major diseases of the urinary tract and is a major source of morbidity. Kidney stone disease is associated with systemic conditions such as diabetes mellitus, high blood pressure, chronic kidney disease and cardiovascular disease (Liu, *et al*, 2014).

Recurrence is a problem in the world, more than 10% of emergency patients require a return visit of less than 30 days, thus worsening the patient's cost and morbidity (Scale, *et al*, 2012). A total of 10-20% in 1-2 years, 35% (5 years) and 60% (10 years) (Brener, *et al*, 2011), 50% (5-10 years) and 75% (20 years) (Yadav , *et al*., 2011). Kidney stones are economically the most expensive, recurrent and lifetime urological disease, estimated to cost the health care system more than $ 10 billion per year (Litwin, and Saigal, 2012).

Pharmacological management showed that inadequate dosing of HCTZ – either too low, or given only once daily – may be responsible for many so-called failures of thiazide in stone prevention. The dose-dependent side effects of thiazide diuretics include hypokalemia, hyperglycemia, hyperlipidemia, hyperuricemia, hypomagnesemia, and hypocytraturia. (Vigen, *et*

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al, 2011). In the case of kidney stones that cannot be excreted out of the urinary tract, lithotripsy is performed with shock waves or if failure occurs, invasive surgery is performed with percutaneous nephrolithotomy or through ureteroscopy (Tiselius *et al.*, 2011). The use of extracorporeal shock wave lithotripsy (ESWL) shows the conversion of Calcium Oxalate stones increases the level of Calcium phosphate (CaP) stones (Parks, *et al*, 2009), and because of increased hardness, it becomes increasingly difficult to smooth with ESWL (Klee, *et al*, 1991). Although clinical management is largely performed surgically, the increased prevalence of kidney stone disease in Western societies is a significant economic and health burden (Ferraro, *et al*., 2015)

The chemical content of breadfruit (*Artocarpus altilis,* AA) leaf that have been isolated are: Propanon (Wang, *et al*, 2007), Chalcone, Xanthon and Flavonone (Hakim, *et al*. 2006). Adaramoye and Akanni's research proves that all biochemical and histological influences have been corrected by AA treatment. AA extract has a protective effect against cholesterol-induced hypercholestereolemia. Thus treatment with antioxidants can be proven effective in reducing crystal-induced inflammation and inflammation and possibly end-stage renal disease (ESRD) (Adaramoye and Akanni, 2014). However, antioxidant activity, anti-inflammatory, antiurolithiasis activity of AA has not been approved in full. This study assessed the potential benefits of AA leaf extract against urolithiasis, including anti-oxidative and nephroprotective activities in rat hyperoxaluria models

METHODS

Plant source and preparation of purified leaf extract of *A altilis* (EAA)

The leaf of plants, AA, were collected from Ungaran, Central Java, Indonesia. The leaves were dried under shade and pulverized. The extract was prepared through maceration by adding ethanol to the pulverized plant material at ratio 1:5 (w/v) and soaked for 8 hours, following which it was filtered. The obtained crude extract was purified with n-hexane, and the extract AA (EAA) was used for further analysis and urolithiasis treatment.

Total flavonoid content (TFC) assay

The EAA was made to the residu, by allowing solvent to evaporate. To the residu, 5 ml of 0.1 M ammonium chloride was added and allow for incubation (40 min) at room temperature. The absorbance was measured at 415 nm (Shimadzu UV mini 1240). A standard plot of quercetin was used to evaluate the TFC and expressed as mg QE/g DW of the EAA material.

Plasma Preparation

At the end of the experiment, blood samples were collected from the retro-orbital plexus under anesthetic conditions and analyzed for MDA. To confirm the incidence of urolithiasis, the animals were sacrificed and their kidneys were subjected to determination of the wet kidney weight, and histopathological studies

Malondealdehyde (MDA) analysis

A total of 100 µl samples (blood plasma) were put into centrifuge tubes plus 0.9 ml aquabidest then 0.5 ml reagent TBA was added. The tube was heated in a water bath at 95 ^{0}C for 1 hour, centrifuged at 7000 RPM for 10 minutes. The absorbed supernatant was measured using a spectrophotometer at λ_{max} 532 nm.

Creatinine levels

Determination of plasma creatinine levels is carried out on protein-free plasma (Folin Wu filtrate). Protein-free plasma is reacted with an alkaline picrate solution and the color is stable for 30 minutes. The absorbance is measured at λ_{max} 520 nm.

Blood Urea Nitrogen levels

The measurement of BUN levels was carried out using diacetyl monoxime methods and measured at λ_{max} 525 nm.

Change in body weight and wet kidney weight

On the final day of the experimental procedure, the body weights were measured to evaluate the change in the body weight from the initial body weight (Harlalka, *et al.*, 2007). After the blood collection, the kidneys were excised, weighed, and the result was expressed as wet kidney weight/100 g of body weight to assess the change in kidney weight among the experimental groups. (Feyissa *et al.*, 2013)

Histopathological study

The kidneys were fixed rapidly with 10% neutralized formalin (pH 7.4). Sections of kidney fixed in paraffin were prepared and stained with hematoxylin and eosin and observed for pathological changes. Histopathological examination was carried out blinding by the researcher and re-examination by other researchers to minimize bias. The changes in the kidney tubules are observed with parameters of degeneration, infiltration of inflammatory cells and necrosis using a software J* image in each group.

Statistical analysis

The results are expressed as mean ± SD. Statistical analysis was carried out using one-way ANOVA followed by Posthoc test. A value of $P < P_{tab}$, CI: 95%, was considered significant.

RESULTS

Ethical approval

The study protocol was approved by the Research Ethics Committee, Faculty of Medicine, Universitas Islam Sultan Agung Semarang, Indonesia. The study was conducted in accordance with The 2004 National Health Etics Guidelines, The Indonesian Ministry of Health.

Malondealdehyde (MDA) levels

TBARS analysis of the TEP standards produced a pink chromogen that had a maximal absorption at λ_{max} 531,3 nm. The linearity of the TBARs assay was demonstrated over the range of 0–30 μM, and the precision of assay response was acceptable with a mean slope of 0.0716 (% relative standard deviation (RSD) = 0.069, n = 4). Linear regression of the standard TEP curve is y = 0,0716x + 0,069.

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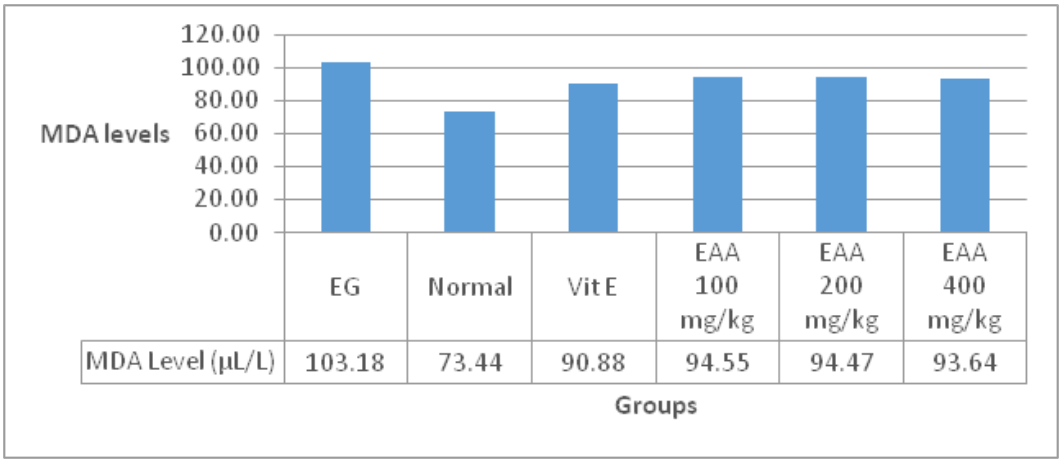

Figure 1. MDA levels

Table 1. Post Hock analysis of MDA level

No.	Groups	Mean ± SD (µL/L)
1	EG	103,182[bc*] ± 7,784
2	Normal	73,439[ac*] ± 1,033
3	Vitamin E	90,881[ab*] ± 3,060
4	EAA 100 g/kg BW	94,547[ab*] ± 0,828
5	EAA 200 g/kg BW	94,472[ab*] ± 1,686
6	EAA 400 g/kg Bw	93,645[ab*] ± 1,428

* The mean difference is significant at the 0.05 level
** The mean difference is not significant at the 0.05 level
[a]Compared EG; [b]Compared Vit E

Statistical analysis was carried out using one-way ANOVA (SPSS 20 program), MDA concentration is F_{value} : 36.671 > $F_{tab.}$: 2,51; CI: 95% , it means that the mean difference is significant. Post Hoc analysis shows that MDA level treatments were significantly different.

Creatinine levels

The decrease in creatinine levels measured on 14th and 28th days is presented in following figure 2. One way Anova analysis show that F_{cal} : 70.018 > F_{tab}: 2.51; CI: 95%, it means that the mean difference is significant at the 0.05 level, to find the differences between groups that affect creatinine levels, the Post Hoc test was conducted. The Post Hoc statistical analysis obtained significant differences between EG, and control with vitamin E, EAA 100, EAA 200 and 400 mg/kg BW groups at the 0.05 level, but the mean difference vitamin E and EAA 400 mg/kg BW groups is not significant, $P_{val.}$: 0.01200.

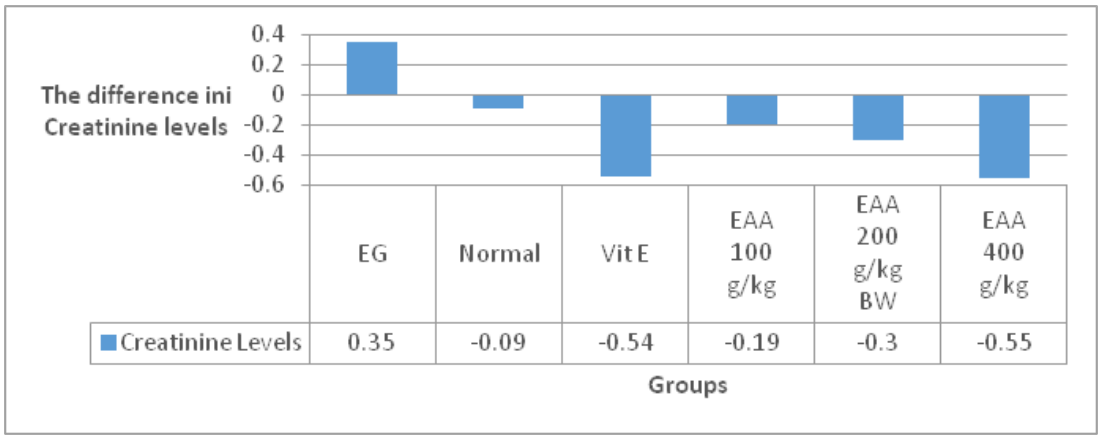

Figure 2. The difference in creatinine levels after and before treatments.

Table 2. Post Hock analysis of the difference in Creatinine level

No.	Groups	Mean ± SD (µL/L)
1	EG	0,35 ±0,011*b
2	Normal	-0,09 ±0,041*a,b
3	Vitamin E	-0,54 ±0,146*a
4	EAA 100 g/kg BW	-0,19 ±0,055*a,b
5	EAA 200 g/kg BW	-0,30 ±0,048*a,b
6	EAA 400 g/kg Bw	-0,55 ±0,087*a**b

* The mean difference is significant at the 0.05 level
** The mean difference is not significant at the 0.05 level
[a]Compared EG; [b]Compared Vit E

BUN levels

The decrease in BUN levels measured on 14th and 28th day is presented in figure 3. The difference in BUN levels after and before treatments.

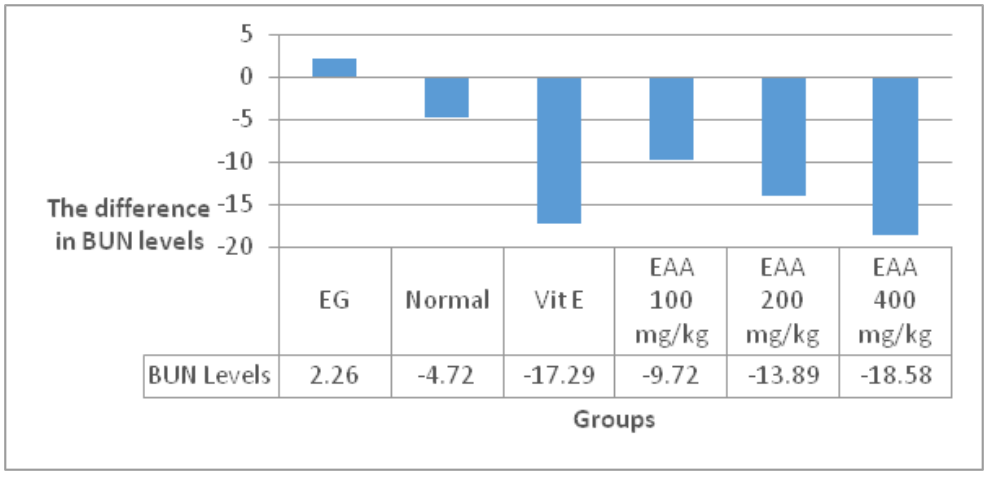

Figure 3. The difference in BUN levels after and before treatments

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One way Anova analysis show that F_{cal} : 189,048 > F_{tab}: 2.51; CI: 95%, it's means that the mean difference is significant at the 0.05 level.

Table 3. Post Hock analysis of the difference in BUN levels

No.	Groups	Mean ± SD (µL/L)
1	EG	2,26±1,953*b
2	Normal	-4,72±0,765*a,b
3	Vitamin E	-17,29±0,655*a,b
4	EAA 100 g/kg BW	-9,72±0,730*a,b
5	EAA 200 g/kg BW	-13,89±0,975*a,b
6	EAA 400 g/kg Bw	-18,58±0,168*a**b

* The mean difference is significant at the 0.05 level
** The mean difference is not significant at the 0.05 level
[a]Compared EG; [b]Compared Vit E

The Post Hoc statistical analysis obtained significant differences between EG, and control with vitamin E, EAA 100, EAA 200 and 400 mg/kg BW groups at the 0.05 level, but the mean difference of vitamin E and EAA 400 mg/kg BW groups is not significant, P_{val}. : 1.29200, CI : 95%.

Change in Body Weight Gain

Figure 4. shows the increase in weight gain in all rats, the highest increase was shown by the EAA 400 group (54,0 g), followed by EAA 200 (27,2 g), EAA 100 (24,0 g), Vitamin E (10,2 g), EG (10,0 g) and Normal (8,6 g).

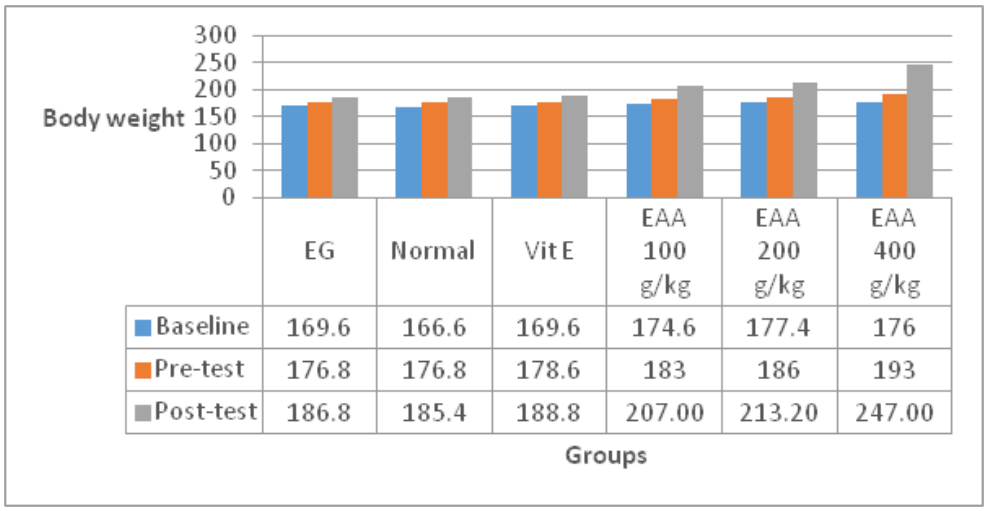

Figure 4. The rats weight were measured on days 1, 14 and 28

Statistical analysis showed that there were significant differences between treatments, F_{cal}: 7,08 > F_{tab}: 2,51, CI : 95%, giving an extract increases the body weight of experimental animals.

Table 4. The body weight on day 28

No.	Groups	Mean ± SD (g/100 g)
1	EG	0,9800 ± 0,09487
2	Normal	0,8725 ± 0,05377
3	Vitamin E	0,8250 ± 0,11121
4	EAA 100 g/kg BW	0,9225 ± 0,10905
5	EAA 200 g/kg BW	0,8100[*a] ± 0,18276
6	EAA 400 g/kg BW	0,6075[*a,b,c,d] ± 0,13672

*P < 0,05 : Significance, [a]Compared EG and Normal, [b]Compared Vitamin E, [c]Compared EAA 100 and [d]Compared EAA 200 mg/kg BW

Wet kidney weight per 100 g body weght

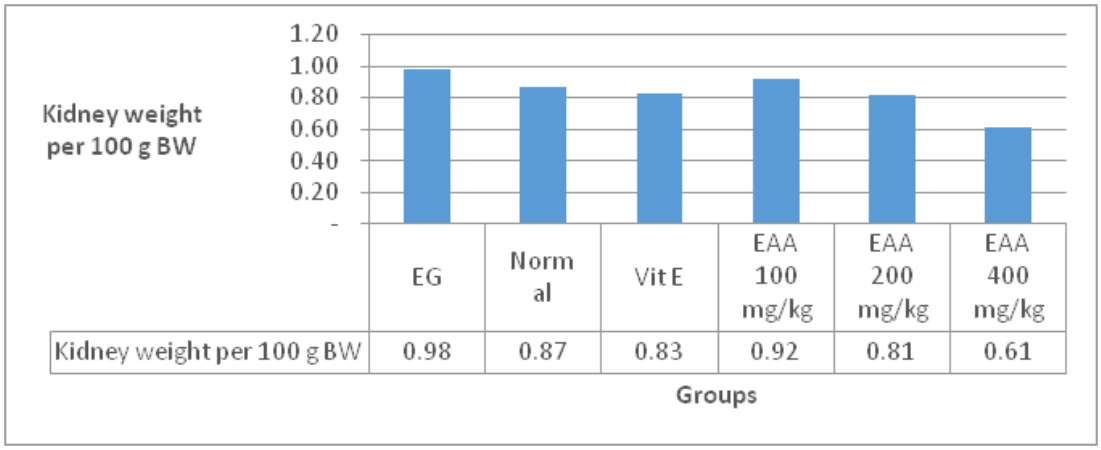

Figure 5. The wet kidney weight per 100 g body weight

The analysis of variant of wet kidney weight per 100 g BW showed significant differences between treatments, F_{cal}: 4,491 > $F_{tab.}$: 2,51; CI: 95% and LSD test showed that the mean difference between EAA 400 mg / kg BW with the other groups are significant different.

Table 5. The wet kidney weight per 100 g body weight

No.	Groups	Mean ± SD (g/100 g)
1	EG	0,9800 ± 0,09487
2	Normal	0,8725 ± 0,05377
3	Vitamin E	0,8250 ± 0,11121
4	EAA 100 g/kg BW	0,9225 ± 0,10905
5	EAA 200 g/kg BW	0,8100 ± 0,18276
6	EAA 400 g/kg BW	0,6075* ± 0,13672

*P < 0,05 : Significance,

Administering EAA 400 mg / kg BW shows the lowest, at this dose showed the lowest changes in kidney weight compared to other treatments, thus this dose can prevent hypertrophy.

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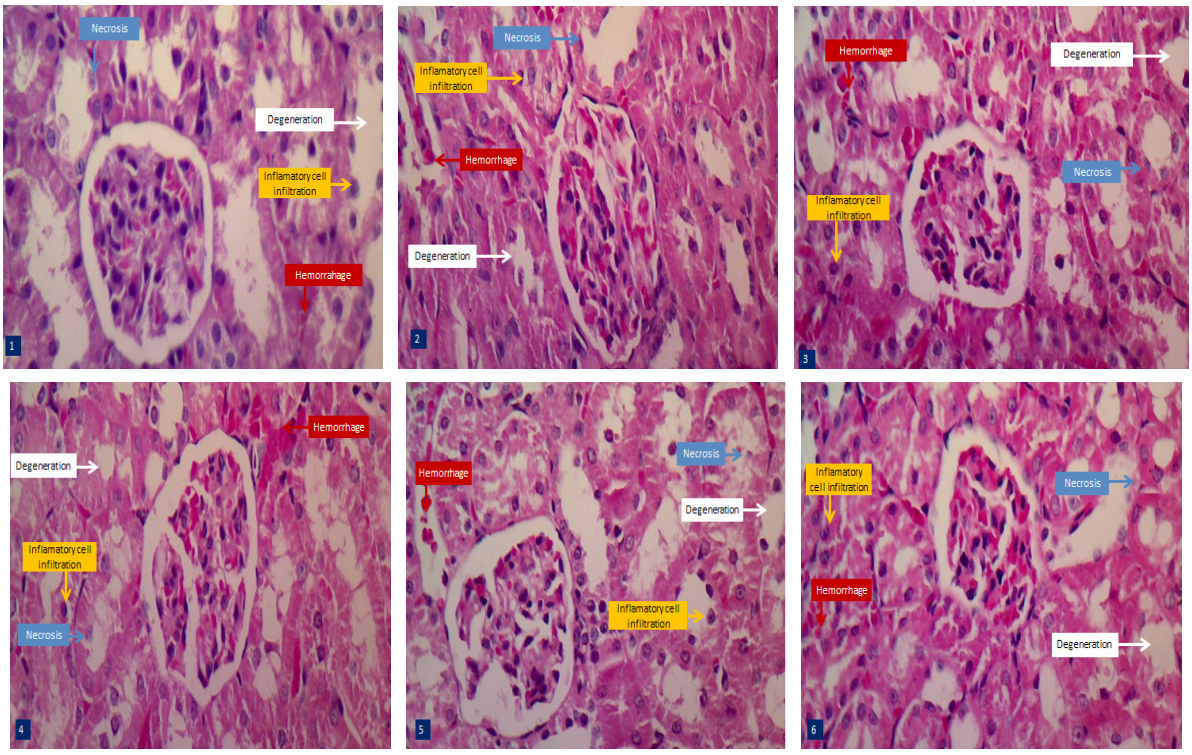
Kidney Histopathological



1 EG group; 2 : normal; 3 : Vitamin E; 4 : EAA 100; 5 : EAA 200 and EAA 400 mg/kg BW (HE 400x)

Figure 6. Histopathology of kidney

Table 6. The degree of kidney histopathological damage after treatment

Groups	Parameters	Damage levels
EG	degeneration +++, hemorrhage +++, necrosis +++, infiltration +++	Severe
Normal	normal kidney cells	Normal
Vitamin E	degeneration ++, hemorrhage +++, necrosis ++, infiltration ++	Moderate
EAA100 mg/kg	degeneration +++, hemorrhage +++, necrosis ++, infiltration ++	Severe
EAA 200 mg/kg	degeneration ++, hemorrhage ++, necrosis ++, infiltration ++	Moderate
EAA 400 mg/kg	degeneration +, hemorrhage +, necrosis +, infiltration +	Mild

DISCUSSION

MDA levels

Hyperoxaluria and crystal formation in the kidney tubules cause stress on the tubular epithelial cells (TEC) and increase ROS, activate RAS so that angiotensin II rises, an increase in ROS, and an increase in OPN synthesis. Hyperoxaluria and CaOx crystal deposition trigger morphological and pathophysiological changes in the kidney and affect urine composition (Zuo, *et al.*, 2011). These NADPH oxidase complexes play a crucial role in host defense, various signaling pathways leading to regulation of gene expression, and protein functions under normal conditions of oxidative

balance, when this oxidative balance is disturbed (Joshi, *et al*, 2013).

There are a variety of markers in the kidneys which increase during oxidative stress. Oxidative damage is characterized by increased levels of macromolecular oxidation products such as reactive substances thiobarbituric acid (TBARS), and protein carbonyls (Catala, 2006). Animals secrete significantly higher amounts of thiobarbituric acid reactive substances (TBARS) in the urine, generated as a byproduct of lipid peroxidation and an indication of oxidative stress in the kidneys (Huang, *et al*, 2003). There is greater excretion of 8-Isoprostane, PGF2α, and many type of aldehydes (hexanal, malondialdehyde (MDA) and 4-hydroxynonenal) as a result of lipid peroxidation by long-time infusion of angiotensin II in rats (Preminger, and Curhan, 2009).

The supplementation with adequate antioxidants, such as α-tocopherol, natural antioxidant plants, etc, will keep sensitive cells and organs in healthy condition and increase lifespan (Repetto *et al.*, 2012). Holoch and Tracy suggests that higher levels of antioxidants may confer protection from stone formation (Holoch and Tracy, 2011) because antioxidant activity will inhibit enzymes that play a role in the formation of oxygen species such as NADPH oxidase. Exogenous antioxidants can be obtained from phenolic compounds of flavonoid groups derived from natural ingredients (plants) (Pojsak *et al*, 2013)

Refer to the Post Hoc test results that MDA levels of EG groups differ from control, it means there is an effect of EG induction to increase MDA levels, increase lipid peroxidation.

MDA levels between treatments were significantly different, MDA levels between vitamin E, extract doses of EAA 100, 200 and 400 mg/kg are not significantly difference, thus, EAA 100, 200 and 400 mg / kg BW have the ability to inhibit lipid peroxidation based on MDA parameters.

A compound is said to have antioxidant activity when there is capture of free radicals through the hydrogen atom donor from the compound group (Palanisamy, *et al.*, 2011). Flavonoids contained in the purified leaf extract of AA can act as antioxidants.

Creatinine levels

The National Kidney Disease Education program recommends using serum creatinine to measure glomerular filtration ability and to monitor the course of kidney disease. Glomerrular Filtration Rate (GFR) cannot be measured by direct means, but it can be assessed by measuring the urinary clearence exogenous filtration markers such as insulin, iohexol or iothalamate (Levey *et al*, 1993)

Creatine is a protein derived from the end result of muscle metabolism that is released from the muscle at almost constant speed and is excreted in the urine at the same speed. Creatinine is excreted by the kidneys through a combination of filtration and secretion, the concentration is relatively constant in plasma from day to day, levels greater than the normal value indicate the existence of impaired kidney function. (Hall, 2011).

The graph 2 shows that there is an increase in creatinine levels by the administration of EG, while the provision of Vitamin E, and EAA can reduce creatinine levels in rats that are induced by EG. Research shows that EAA has ability to reduce the formation of creatinine, thus protecting against kidney dysfunction. The protective ability of EAA 400 mg / kg BW against impaired kidney function is equivalent to vitamin E.

BUN levels

BUN parameters can be used for safety testing and cytotoxic activity of a preparation. BUN levels in blood can also be used to evaluate hydration status, nitrogen balance in the body, and

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assess the severity of kidney disease (Weiner, *et al*, 2015). Increased concentrations of BUN in the blood can be influenced by factors outside the kidney, it can occur due to shock conditions, decreased kidney vascularization, dehydration, bleeding, renal cortex necrosis, ureteric obstruction and kidney failure.

Clinical conditions associated with altered urine concentrating ability or water homeostasis can result in changes in urea excretion and urea transporters. Clinical conditions associated with altered ammonia excretion can have important effects on nitrogen balance (Weiner *et al*, 2015).

The graph 3 shows that there is an increase in BUN levels by giving EG, while the provision of Vitamin E, and EAA can reduce the BUN levels of rats induced EG. In this study showed that EAA 100, 200, 400 mg / kg BW groups influence BUN levels differently from the EG induction group, the vitamin E and EAA 400 mg / kg BW groups have an activity to decrease BUN levels which is equivalent to vitamin E.

Change in Body Weight gain

Figure 4 shows a significant increase in body weight in all experimental animals, but greater changes occured in the extract groups after administration of the EAA. The outcome in this study showed that EAA improve percentage weight gain on acute dosing (14 days) as observed 2,70, 2,99 and 5,76 % weight gain when compared with 4,86% observed in the normal group, respectively. These increases in percentage weight gain may be as a result of the presence of flavonoids contained in EAA which produce nutritive effects, thereby increasing feed consumption, and no tannin content was detected in purified EAA leaves with antinutritive effects.

Wet kidney weight per 100 g BW

Figure 5 shows the difference in wet kidney weight per 100 g BW, this changes as a mechanism compensatory growth, a type of regeneration that can take place in organs after the organs are either damaged, removed, poor cease to function (Widmaier, *et al*, 2006). The growth can be a result of increased cell size (compensatory hypertrophy) or an increase in cell division (compensatory hyperplasia) or both. (*Compansatory Growth*, 2011). The factors that regulate growth, such as chemical factors e.g. EG, the highest level of hypertrophy ware proven in the EG group, whereas the extract gropus showed a significant decrease in hypertrophy, the higher the dose the smaller the incidence of hypertrophy. It means that the extract of AA can reduce compensatory growth due to EG.

Kidney Histopathological

The kidney histopathological shows that the EAA 400 mg/kg BW group had lower damage than the other treatment groups, but the level of damage is still below the normal group.

Chemicals that are too much in the kidneys can result in cell damage, such as infiltration of inflammatory cells, vacuum tubule lumen, bleeding. Nephron death occurs due to cell degeneration. The process of nephrocalcinosis in this study caused tubular obstruction that can cause ischemia of the tubules, thus triggering degeneration and necrosis. This is in accordance with research Kumar, *et al*, (2005) that the proximal tubule is the most frequently degenerated due to exposure to nephrotoxic substances (Kumar *et al.*, 2005).

Inflammation of cells, vacuoles, enlarged and closed cells is an indication of the occurrence of hydrophic degenerative. Hydrophic degenerativen is a reversible cell injury with more intracellular accumulation if albumin is followed. Hydrophic degeneration generally occurs in epithelial cells (Suhita, *et al.*, 2013).

Impaired cell function will occur if the excess fat deposits which will then cause fatty changes in cells and can cause necrosis (Suhita *et al.*, 2013). Necrosis as an advanced form of degeneration. Necrosis of tubular epithelial cells can occur due to toxins, viruses, and lack of oxygen. The presence of protein deposits in the tubular lumen is influenced by various factors, including an increase in glomerular capillary permeability so that the protein can escape. In addition, decreased absorption ability of tubules due to tubular epithelium has degenerated to necrosis is also a factor in the presence of protein deposits. Necrosis is cells undergoing changes that lead to cell death, which is caused by the presence of toxic substances that enter along with blood flow to the kidneys (Angelina *et al.*, 2000)

Damage in the form of infiltration of inflammatory cells is also found in the kidney tubules. CaOx crystal deposition causes inflammation and attracts many inflammatory cells including leukocytes, monocytes, and macrophages (Khan, *et al.*, 2006), and many nucleated giant cells are identified around the crystal. Kidney inflammation contributes to progressive kidney injury, which can cause glomerulonephritis, end-stage kidney disease, or acute or chronic kidney disease (CKD) (Ernandez, and Mayadas, 2016).

CONCLUSION

Purified ethanol extract of AA leaves has an antioxidant, nephroprotective activity, protect kidney inflammation, and kidney damage in nephrolithiasis rats and the effective dose is 400 mg/kg BW.

ACKNOWLEDGMENT

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