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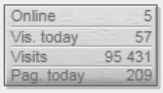

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Research Article

Protective Effect Of Ethanolic Extract *Sonchus arvensis* L. in Gentamicin-Induced Acute Tubular Necrosis on Wistar Rats

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ABSTRACT

Acute tubular necrosis (ATN) is the most common histopathologic findings of acute kidney injury (AKI). AKI is marked by the decrement of glomerular filtration rate, causing waste metabolism retention (creatinine). Gentamicin is used as it is the most common nephrotoxic agent in inducing ATN. Tempuyung (*Sonchus arvensis* L.) has been used in folklore medicine to ameliorate kidney problems as it contains antioxidants, two of which are flavonoid and phenolic acid. Yet, these active substances' benefit on gentamicin induced ATN has not been investigated in Indonesia. This research aims to analyze protective effect of ethanolic extract tempuyung leaf (EET) on gentamicin-induced ATN based on histopathological study and creatinine serum level. True experimental laboratoric study was done with simple random design on male wistar rats, randomly divided into 4 groups (n=4). Control group (CMC 0.5% aquadest); Induction group (Gentamicin 80 mg/kgBW); 1st treatment group (EET 100 mg/kgBW + Gentamicin 80 mg/kgBW) and 2nd treatment group (EET 200 mg/kgBW + Gentamicin 80 mg/kgBW) for ten days. On the 11th day, blood was taken for creatinine measurement and kidneys collection for blind observation with scoring methode on alteration degree for histopathological study. Histopathological examination on gentamicin-treated rats revealed degenerative changes in kidney tubules. Aside from that, gentamicin-treated rats also showed increment in creatinine serum level. Conversely, simultaneous administration of EET with Gentamicin ameliorated the nephrotoxic effects of gentamicin as confirmed from the significant improvement on histopathological changes and normalization of creatinine serum level. Co-administration of EET and gentamicin provides protection on gentamicin-induced ATN, based on histopathological feature and creatinine serum level.

Key words: ATN, creatinine, gentamicin, tempuyung, *S arv*ensis L.

INTRODUCTION

Acute tubular necrosis (ATN) is the most common form of Acute Kidney Injury (AKI) (80%). ATN is characterized by tubular epithelial cells damage and cast, which fill tubular lumen, which can cause renal function decline. AKI is marked by the decrement of glomerular filtration rate (GFR), which causing retention of nitrogen metabolism, which will increase ureum and creatinine level, dysregulate fluid, electrolyte and acid-base homeostasis. This study focus on intrinsic AKI in which gentamicin act as nephrotoxin agent (exogenous). (Kumar, 2018; Sharfuddin *et al.,* 2016; Steddon, *et al.,* 2014)

Gentamicin which classified under aminoglycoside, is one of the most frequently used antibiotics in clinical practice worldwide. Its highly effective in treating life-threatening gram-negative bacterial infections (meningitis and sepsis in infants). Prophylaxis gentamicin is administered systemically in intensive care units to pre-term infants and topically for major burn case. Its primary site of action located in proximal tubules epithelial cells of the nephron. (Karasawa and Steyger, 2011)

Gentamicin-induced ATN initially enter via cation channel permeation, as it binds to phosphoinositides on cell membrane. It is important messengers in intracellular signal transduction. Its complex induce the release of arachidonic acid, which act as electron donor in Fe^{2+}-gentamicin complex and generate Reactive Oxygen Species (ROS) formation. Other site of entry can also via megalin reseptor, where gentamicin is endocytosed and retrograde trafficked to ER and lysosome. Inside lysosome, gentamicin inhibits activity of phospholipase, causing lipid deposition and forming myeloid bodies (phospholipidosis). Once lysosome is saturated, it bursts into cytosol compartment, causing metabolic changes and cell death. Cytosolic gentamicin binds to ferric iron (Fe^{3+}) and generates Fe^{2+}-gentamicin complex. Gentamicin complex act as catalyzator on ROS formation from oxygen molecule. Excessive ROS production in turn will induce various cell death mechanisms, either apoptosis and necrosis. During lysosomal burst, aside from gentamicin, protease (cathepsin) also translocated to cytosol. Cathepsin will trigger massive proteolysis, resulting necrosis. Gentamicin concentration is essential in activating certain cell death mechanism. The higher gentamicin dosage, the higher chance of lysosomal burst, the higher chance of necrosis. (Karasawa and Steyger, 2011; Servais *et al.,* 2008; Tulkens and Mingeot-Leclerq, 1999, Quiros, *et al.,* 2011)

Tempuyung (*Sonchus arvensis* L.) is a herbal plant grows in Java Island. Tempuyung plant has been used empirically in folklore medicine, commonly used to minimize kidney discomfort due to its benefit in exuviating kidney stones and diuretic effect. Tempuyung has been known to contain several active substances with antioxidant property. (Hussain, *et al.,* 2010; Khan, 2012)

Active substances in tempuyung, such as flavonoid (cathecin, myricetin, rutin, orientin, quercetin kaempferol and hyperuside); phenolic acid (cinnamic acid); terpenoid (carotenoid and saponin); alkaloid; Vitamin B and Vitamin C. Flavonoid act as antioxidant by competitively inhibit ROS production as it has the potency in chelating iron. In addition, flavonoid also break the chain reaction of ROS production as its mode of action in scavenging free radical. (Hussain et al., 2010; Khan, 2012)

Previous studies investigated antioxidant potency, such as quercetin study in gentamicin-induced nephrotoxicity resulting kidney improvement; Yin study analysed antioxidant and citotoxicity activities of *Sonchus oleraceus*, which showed its antioxidant potencial benefit; Khan study on renoprotective effect of *Sonchus asper* on oxidative injury caused by carbon tetrachloride.(Khan, *et al.,* 2011; Talat, *et al.,* 2009; Yin, *et al.,* 2007)

Although the highly availability of tempuyung in Indonesia, and its versatility to be cultivated. Yet, not many research study has been done specifically, focusing on antioxidant potential of tempuyung (*Sonchus arvensis* L.) against kidney injury, induced by gentamicin.

MATERIAL AND METHODS

Plant Collection

Tempuyung leaves (*Sonchus arvensis* L.) were obtained from Manoko village, Lembang district, Bandung, West Java, Indonesia. Its specimen was identified and determinated at herbarium of School of Life Sciences and Technology Institut Teknologi Bandung, West Java, Indonesia. Qualitative phytochemical analysis was tested at Chemistry department, Faculty of Mathematics and Natural Sciences, Universitas Padjadjaran, Bandung, West Java, Indonesia.

Preparation of plant extract

The collection of crude fresh tempuyung leaves were washed, dried and cut into small pieces using clean steril knife. Further drying done in dryer machine at 40°C until it was totally dried. Following afterward, tempuyung simplicia was macerated using ethanol 96% solution for 3x 24h. The ethanolic solution was filtered and it was repeated for another maceration until the third day. The extract was then evaporized using rotary evaporator until the extract became viscous in consistency. At this stage the extract was already done and further would be reffered as ethanolic extract tempuyung leaf (EET). It was kept in a clean, closed container in the refrigerator at 4°C until it was utilized for the study.

DPPH Antioxidant Assay

A stock solution of 1,1-diphenyl 1-2-picrylhydrazyl (DPPH) was made by dissolving 1.95mg DPPH in 10mL of methanol and diluted after achieving an absorbance of (0.98±0.02) at 517 nm. 50μL of DPPH solution was mixed with 50μL of various concentration of extract. Followed by incubation for 30min in dark room at room temperature. Absorbance was recorded at 517 nm and DPPH scavenging activiy was calculated using the equation Percentage Inhibition (%) = [(Refference absorbance- sample absorbance)/ Refference absorbance x100. IC_{50} values obtained as to determine 50% inhibition of DPPH radicals. Ascorbic acid was used as standard.

Animals

Sixteen Wistar male rats aged between 8 to 10 weeks and weighed between 200-250g, were acquired for this study. Rats were provided by Animal Laboratory, ITB, Bandung. Acclimatization was done in one week periode. All rats were put in ordinary cages at a room temperature and set with 12h dark / light cycle. Rats were fed to the standard chow and had free access for drinking water. All conducted protocol procedure had followed the standard of management care for laboratory animals and approved by the Health Research Ethics Committee Faculty of Medicine Universitas Padjadjaran, Bandung, Indonesia.

Experimental design

After acclimatization periode, rats were randomly selected for each treatment group. Each group (n=4) received different treatment, accordingly as following protocols: Control group received CMC 0.5% and aquadest orally; Induction group received intraperitoneal injection of gentamicin with dosage of 80mg/kgBW; First treatment group received both EET with dosage of 100mg/kgBW orally and gentamicin. The second treatment group received both EET with dosage of 200mg/kgBW orally and gentamicin. All of these treatment were administered once daily for ten days periode. Twenty four hours afterward, all animals were anesthesized per inhalation using isoflurane and blood was taken for evaluation on creatininine measurement. Continued with collection of both kidneys from each rat to be preserved, that were taken for histopathological study.

Creatinine serum analysis

Level of creatinine in serum taken from rats was centrifuged at 7500rpm for 10min, at 4°C to obtain its serum. Creatinine was determined using spectrophometry standard cobas c311 analyzer.

Histopathological studies of kidney

Once the kidneys been excised, they were immediately fixated in neutral buffered formaldehyde 10% solution for approximately 48h. Followed with dehidration using alcohol in serial concentration, continued with clearing, infiltration and embedding proccesses with paraffin. Once they were done, continued with sectioning using rotary microtome at approximately 4-5μm. These ribbon were put on clean glass slides in water bath. After it dried, followed by staining using Haematoxylin and Eosin. Next, mounted and covered each slide and ready for viewing under a light microscope. The observation was done in blind setting using scoring method. Score (-), Histopathologic alteration is ≤5% total field examined. (Rare tubular dilatation, rare tubular necrosis and rare peritubular inflammation); Score (+), Histopathologic alteration 6-40% total field examined. (Multifocal tubular dilatation, multifocal tubular necrosis and multifocal peritubular inflammation); Score (++), Histopathologic alteration 41-80% total field examined. (Coalescing tubular dilatation, coalescing tubular necrosis and coalescing peritubular inflammation); Score (+++), Histopathologic alteration ≥81% total field examined. (Diffuse tubular dilatation, diffuse tubular necrosis and diffuse peritubular inflamation).

RESULT AND DISCUSSION

DPPH antioxidant assay

DPPH is commonly used for investigating potential antioxidant of medicinal plants. DPPH is a stable free radical that decolourized from purple once there was antioxidants. Its purple color due to its odd electron. As antioxidant act as electron donor

Table I. DPPH Antioxidant Assay result

Tube	Concentration mg/50 mL	Absorbansi				% Inhibition
		1	2	3	mean	Reff Abs – Sample Abs x 100% / Refference Abs
Refference		1.423	1.417	1.424	1.421	
1	37.500	0.457	0.455	0.457	0.456	67.909
2	25.000	0.678	0.733	0.717	0.709	50.105
3	12.500	1.054	1.059	1.047	1.053	25.897
4	6.250	1.206	1.207	1.216	1.210	14.848
5	3.125	1.246	1.242	1.244	1.244	12.456

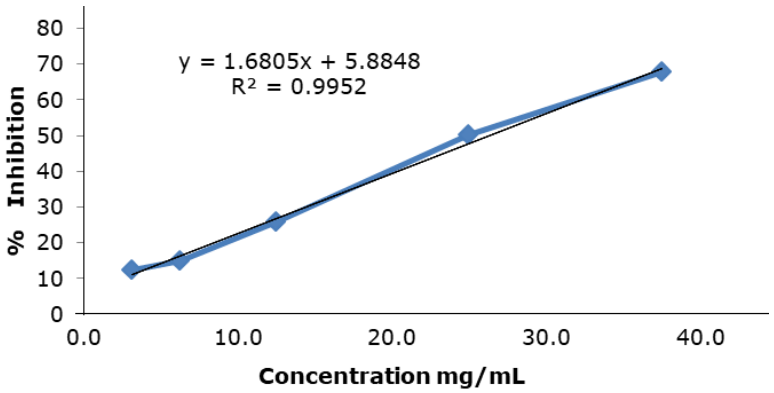

Figure 1. Correlation concentration of EET concentration to IC_{50}

and couple with previous electron, and markedly change in color. Quantification was achieved by observing its absorbance at 517nm. Various concentrations of EET resulting IC_{50} values at 26.259mg/mL (Table I, Figure 1).

This quantitave measure indicates the amount of ethanolic extract of tempuyung is needed to inhibit the process of gentamicin-induced acute tubular necrosis by half. IC_{50} value at 26.259mg/mL represent the concentration of ethanolic extract of tempuyung is required for 50% inhibition of necrosis process. As this study, using 2 different dosage of EET, in which low dose EET (100mg/kgBW) is refering to the minimum dosage to get the inhibition effect and high dose EET (200 mg/kgBW) is refering to double dosage from minimal dosage to require significant improvement against gentamicin-induced acute tubular necrosis, as its antioxidant content and activity is significantly higher than the minimum dose to require protective effect.

Creatinine serum level

After ten days of injection of gentamicin with dosage 80mg/kgBW to rats, induction group showed the highest in average of creatinine serum level was 0.558±0.025 mg/dL. In the first treatment group, co-administration EET 100mg/kgBW with gentamicin, its creatinine serum level was 0.487±0.029 mg/dL, lowered compared to induction group. In second treatment group, co-administration EET 200/mg/kgBW with gentamicin, its creatinine serum level was 0.393±0.025 mg/dL, It showed better result, which much lowered compared to first treatment group (Figure 2). Creatinine serum level in control group 0.300±0.031mg/dL was done as the baseline. This colorimetric assay is based on the Jaffe methode. In alkaline solution, creatinine forms yellow orange complex with picrate, measured at 505 nm. The rate of dye formation is proportional to the creatinine concentration in the specimen.

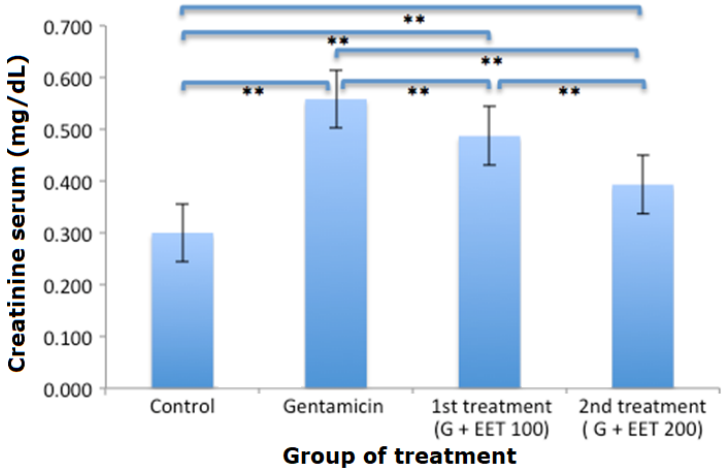

Figure 2. Effects of Gentamicin, Co-adminstration (G + EET 200mg/kgBW) and (G+100mg/kgBW) on Creatining serum compared to Control Group. Data expressed as means ± SD (n=4/group). The significance difference between two groups was determined by ANOVA followed by LSD post hoc. ** $p<0.01$, statistically significant difference between two groups

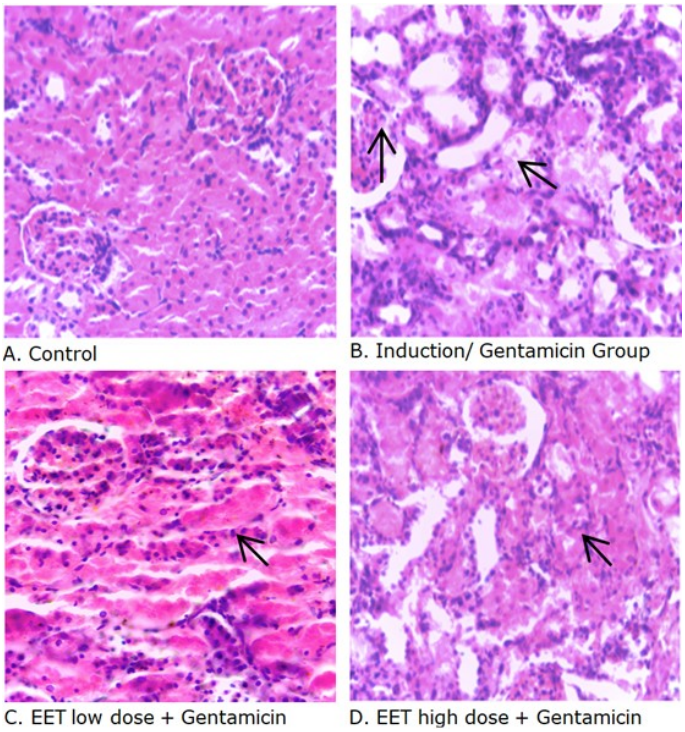

Figure 3. Control group: showed normal kidney histologic architecture. Induction group showed massive coalescing and multiple tubular degeneration with inflammation. Low dose EET with gentamicin showed multifocal peritubular inflammation (perivascular congestion), High dose EET with gentamicin showed rare histopathologic alteration (minimal inflammation)

In the examination of the level of serum creatinine serum level, injection of gentamicin to rats for ten days significantly ($p<0.01$) increased the serum level of creatinine. This is in accordance with the principle in which high dose gentamicin will trigger the release of cathepsin, causing massive proteolysis and resulting with increased waste nitrogen metabolism. These altered level of creatinine serum was reversed, with administration of EET and gentamicin simultaneously. And based on the result, we can concluded that there was dose dependent corelated to the treatment response, as it had been showed, in which,its creatinine serum level on EET high dose (200mg/kgBW) showed significant lower creatinine serum level compared to EET low dose (100 mg/kgBW). Creatinine serum level parameter from all groups of treatment showed the potencial renoprotective capability in accordance with previous research studies result. Its highly due to its active substance flavonoid and phenolic acid, which acts as iron/metal chelator, competitively inhibited ROS production by binding to iron in order to reduce power of Fe^{3+} to Fe^{2+} and its capability to stop chain reaction of ROS production with its free radical scavenging property. Based on the mechanism above, it explained in which higher dose of EET treatment group (200mg/kgBW) had lower creatinine serum level compared to lower dose of EET treatment group (100mg/kgBW).

Histopathological studies of kidney

Histologically, nephron is functional unit of kidney, consists of renal corpuscle and renal tubule. Renal corpuscle itself consist of glomerulus and bowman's capsule. Glomerulus is a network of anastomosing capillaries which invaginates bowman's capsule, a single layer squamous cells resting on a basement membrane, derived from distended blind end renal tubules, which will continued with proximal tubules, ansa henle, distal tubules, collecting ducts and ended to ducts of Bellini. Observation on kidney of animal in the control group showed normal histological findings of kidney architecture, where glomerulus and bowman's capsule dispersed in cortex area, surrounded with abundant of proximal convoluted tubules and distal convoluted tubules (Figure 3A). In renal sections from induction group with gentamicin, histologic findings were markedly disrupted as evidenced by necrosis epithelial cells lining in the proximal tubules with tubular dilatation and tubular degeneration, tubular congestion, tubular dilatation and glomerular injuries. Focal mononuclear infiltration between tubules and perivascular area with dilated blood vessels associated with edema (Figure 3B). Tempuyung restored most of histopathological alterations, which induced by gentamicin as shown from sections of both treatment groups, either using EET low dose or EET high dose. Tempuyung minimized mononuclear infiltration and alleviated congestion of perivascular area (Figure 3C). As it is dose dependently, it showed better outcome in EET high dose group, in which the degree of histopathological alteration much lesser (Figure 3D), yet both treatment groups showed significant difference of histologic architecture, where impairment is much more subtle compared to induction groups. In treatment groups, though the renal architecture is not the same as in control group, yet the tubules degeneration were not as severe as in induction groups. Epithelial cells were squamous in shape but its cytoplasm rather intact compared to the ones in induction groups.

In this study, there was significant relationship between both treatment groups response related to the dosage of ethanolic extract tempuyung. In higher dose EET (200mg/kgBW), histopathologic alterations showed significant improvement compared to induction group with minimal histopathological alterations, reffering to rare with minimal multifocal histopathologic alterations on tubular dilatation, tubular necrosis and peritubular inflamation compared to lower dose EET (100mg/kgBW) which showed dominant multifocal histopathologic alterations. Yet, both treatment groups showed histopathologic improvement compared to the induction group, in the degree of tubular dilatation, tubular necrosis and peritubular inflammation.

Table II. Phytochemical analysis of *Sonchus arvensis L.*

No	Metabolic Seconder	Method Test	Result
1	Phenolic	$FeCl_3$ reactor 5%	+
2	Flavonoid	Concentrated HCl reactor + Mg	-
		H_2SO_4 reactor 2N	+
		NaOH reactor 10%	+
3	Steroid	Lieberman-Burchard reactor	-
4	Triterpenoid		+
5	Saponin	HCl reactor + H_2O	+
6	Tannin	$FeCl_3$ reactor 1%	+

These findings are in line with theory in which administration of high dose gentamicin in certain periode of time can cause tubular injury, resulting in the destruction of the epithelial cells and affect predominantly in proximal tubules compared to distal tubules as proximal tubules has megalin, a protein receptor which corelates with the tendency of gentamicin accumulation inside epithelial cells of it and gentamicin kill these single layer cuboid cells and resulting in degeneration of tubules and sometime also with detachment of its epithelial cells into the lumen and in further condition can cause obstruction.

CONCLUSION

The present study showed how gentamicin damage the kidney with its nephrotoxic effect. In this study, it also revealed co-administration of (EET) ethanolic extract tempuyung leaf and gentamicin provide protection against Acute Tubular Necrosis, based on histopathological alterations improvement and this promising result was consistent with the normalization ofl creatinine serum level. These protection effect of ethanolic extract of tempuyung leaf against gentamicin induced ATN most likely due to its flavonoid and phenolic acid content in tempuyung, which has antioxidant properties by scavenging reactive oxygen species and metal (iron) chelating mechanism (Table II).

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