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Research Article
Expression of TNF-α on Wistar Rat (*Rattus norvegicus* L.) with Extract of Pletekan Leaves (*Ruellia tuberosa* L.)

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Abstract

Background and Objective: Alcoholic drink produced traditionally by Maluku-Indonesia is called sopi, if consumed in excessive doses, it causes kidney damage. The ability of pletekan leaf extract (*Ruellia tuberosa* L.) is used as an alternative pharmacy because it has strong antioxidant activity. This study aimed to determine the expression of TNF-α in the kidney of Wistar rats (*Rattus norvegicus* L.) exposed to sopi alcoholic beverages after being treated with a pletekan herbal extract (*Ruellia tuberosa* L.). **Materials and Methods:** Thirty Wistar rats with an average weight of 200 g, divided by group I control (-) were given demineralized water, group II control (+) only given sopi, group III-V were given sopi 2.5 mL/head/body weight and pletekan leaf extract with a concentration of 5.04 mg/100 mL water, 10.08 mg/100 mL water and 12.12 mg/100 mL water for each group. This treatment is done for 24 days by giving sopi 3 times a week, then taking the kidney organ to make kidney histology. Observation of TNF-α expression by immunohistochemistry methods. **Results:** Wistar rats (*R. norvegicus* L.) exposed to sopi had decreased creatinine levels then were treated with ethanol extract of pletekan leaf (*R. tuberosa* L.) at a dose of 6% (12.12 mg/100 mL water) which was more effective in reducing creatinine levels and decreased the expression of TNF-α kidney Wistar rats. **Conclusion:** Pletekan leaf (*Ruellia tuberosa* L.) ethanol extract has the potential to prevent/repair kidney damage which is characterized by a decrease in the expression of TNF-α kidney Wistar rats (*Rattus norvegicus* L.) in line with an increase in the extract dosage in all treatments of sopi.

Key words: Pletekan leaf extract, antioxidants, immunohistochemistry, TNF-α expression, sopi, methanol, multi-focal inflammatory cells

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Two motives for alcohol consumption have been emphasized in the etiological and the reasons-for-drinking literature: (a) People drink alcohol to cope with stress and (b) People drink alcohol because of social influences[1]. Beverage ethanol production via fermentation is an age-long tradition in many parts of the world. In the tropical world and elsewhere, indigenous people are involved in the entire value chain of traditional alcohol fermentation[2]. Alcohol misuse by older adults is an important public health concern with significant consequences. It is associated with poor mental health functioning, increased odds of suicide and increased risk of accidental and recurrent falls[3]. In human communities where alcohol was traditionally consumed, production of alcoholic beverages commonly occurred on a small scale as a household or artisanal activity, particularly where or when agricultural surpluses were available. In many cultures, drinking alcohol was an occasional activity shared by people within the communities, often associated with festivals or other special occasions. In many places around the world, traces of these traditional customs originating from tribal and village societies persist, including in Maluku[4].

The habit of drinking alcohol is not only done by people in big cities but also in rural areas. This is proven by the presence of traditional alcoholic drinks that are typical in each region such as *sagoer* (Manado), *tuak* (Java), *arak* (Bali), *lapen* (Yogyakarta), *ciu* (Banyumas), *milo* (Papua), *congyang* (Semarang), *sopi* (Maluku) and others. Traditional alcoholic drinks generally have varying levels of alcohol because they are traditionally produced and do not yet have licensing standards for alcoholic drinks.

Illegally produced alcoholic beverages regularly contain a higher percentage of alcohol (above 45% by volume), but at a much lower cost than legal drinks, potentially leading to more problematic drinking patterns with death rates from cirrhosis of the liver[5]. Cheap alcohol drinks usually add additives that are harmful to the body. This phenomenon makes cheap alcoholic drinks can cause death. Data from the Masohi General Hospital in Central Maluku Regency in the Masohi District in 2011, showed the incidence of illness due to alcohol consumption in a year is increasing every month. Beginning 52 people with kidney disease, 32 people with chronic heart disease and 36 people with cirrhosis (damage to liver function). This is due to a lack of public awareness about the dangers of consuming alcohol, so the incidence of this disease has increased from year to year.

The toxic effect produced by alcohol is an imperfect absorption by the body. The absorption occurs after alcohol enters the stomach and is absorbed in the small intestine. Only 5-15% is excreted directly through the lungs, sweat and urine. Alcohol undergoes metabolism in the kidneys, lungs and muscles[1]. Considering the greater levels of alcohol metabolizing enzymes in the liver compared to the stomach, it seems likely that the liver plays a major role in alcohol metabolism[6]. Alcohol affects various neurotransmitter systems in the brain including the adrenergic, cholinergic, dopaminergic, GABAergic, glutamatergic, peptidergic and serotonergic systems. Due to the myriad of neurotransmitter and neuromodulator systems affected by alcohol[7]. Ethanol has a direct and indirect toxic effect on the body[1]. Various effects of alcohol have been reported including increasing and decreasing blood flow. Indirect poisoning due to alcohol also affects organs that process blood such as the liver and kidneys. Consuming alcoholic drinks like sopi is very dangerous because the chemical compounds contained in sopi drinks can undergo chemical reactions in the cell, disrupting cell function and cell death (necrosis). Necrosis can occur in kidney cells and tissues, thus interfering with the kidney's function as a filtration organ.

Robin *et al.*[8] even moderate alcohol consumption increases liver disease in obese patients. Both ethanol abuse and obesity can cause similar liver lesions, including steatosis, steatohepatitis, fibrosis and cirrhosis. Both ethanol intoxication can increase the formation of reactive oxygen species (ROS) and trigger lipid peroxidation, mitochondrial dysfunction and Tumour Necrosis Factor α (TNF-α) formation. Moreover, whereas normal hepatocytes express Fas but not Fas ligand, alcohol increases Fas expression and triggers Fas ligand expression. The TNF-α and Fas ligand, in turn, act on their cognate hepatic receptors to activate caspase-8. Although death receptor-mediated caspase-8 activation is weak in hepatocytes, it can be amplified by several turns of a mitochondrial loop involving in particular the release of mitochondrial cytochrome c into the cytosol.

Kidney disease is classified as a chronic disease that is difficult to cure. Humans often use traditional medicine to minimize the use of synthetic drugs. One of the plants used as a traditional medicine which is efficacious as an acute kidney failure drug is the pletekan herb *Ruellia tuberosa* L. *Ruellia tuberosa* L. belongs to the Magnoliophyta division, Magnoliopsida class and the Acanthaceae tribe. *Ruellia tuberosa* L. contains at least 5 classes of secondary metabolites, namely flavonoids, glycosides, phenols, saponins and minerals[9]. In addition, *R. tuberosa* L. seeds contain unsaturated fatty acids and apigenin and luteolin[10]. The content of secondary metabolites in *R. tuberosa* L. is often used by people to treat inflammation (anti-inflammatory)[11].

A study of raw water extracts from the roots of *R. tuberosa* L. in alcohol-induced gastric lesion models of rats showed strong and dose-dependent gastroprotective activity. The plant extract also showed mild erythropoietic and moderate analgesic activity[12].

Roosdiana *et al.*[13] stated that *Ruellia tuberosa* L. is one of the medicinal plants that is widely distributed in tropical countries, i.e., Indonesia. In herbal medicine, this plant has been widely used as an antidiuretic, antidiabetic, analgesic and antihypertensive. The study showed the effect of hydroethanolic root extract of *R. tuberosa* L., on histopathological profile, MDA concentration and renal TNF-α expression. In addition to the conversion of the acetaldehyde molecule, ALDH is also involved in other cellular functions[14]. Pletekan extract (*Ruellia tuberosa* L.) has been studied to have a strong antioxidant activity and cause a decrease of alcohol in the body[13,15].

The ability of *Ruellia tuberosa* L. extract has been investigated to have strong antioxidant activity so that pletekan leaf extract can be used as an alternative pharmacy for patients with kidney damage. This can prevent apoptosis of kidney cells through decreased TNF-α expression. Therefore, it is necessary to research the effects of herbal extracts of pletekan (*Ruellia tuberosa* L.) on TNF-α expression in Wistar rats (*Rattus norvegicus* L.) kidneys exposed to alcoholic drinks sopi, as well as to overcome kidney problems due to consumption of alcoholic drinks, especially excessive sopi drinking.

Thus, the study aimed to determine the ethanol extract of pletekan leaf (*Ruellia tuberosa* L.) could increase the expression of TNF-α kidney Wistar rats (*Rattus norvegicus* L.) which were exposed to sopi type alcoholic beverages, in addition, to determining creatinine levels in Wistar rats (*Rattus norvegicus* L.) exposed to sopi alcohol after being treated with ethanol extract (*Ruellia tuberosa* L.) and know the potential of pletekan leaf extract (*Ruellia tuberosa* L.) in preventing kidney damage to rats (*Rattus norvegicus* L.).

MATERIALS AND METHODS

Study area: The study was conducted from 8 May to 21 June, 2018. This is an experimental study, to see the expression of TNF-α in the kidney of Wistar rats (*Rattus norvegicus* L.) exposed to alcoholic drink sopi after being treated with pletekan leaf (*Ruellia tuberosa* L.). The study was conducted at the Zoology Laboratory of the Faculty of Mathematics and Natural Sciences, Pattimura University Ambon. The extract process was carried out at the Basic Chemistry Laboratory, Department of Mathematics and Natural Sciences, Pattimura University, Ambon. Measurement of creatinine levels was carried out at the Maluku Provincial Health Laboratory. While testing the expression of TNF-α based on histopathological tests using hematoxylin-eosin and immunohistochemistry techniques was carried out at Brawijaya University Malang. The population in this study was the Wistar rat (*Rattus norvegicus* L.). The sample in this study was the kidney of Wistar rats (*Rattus norvegicus* L.) totalling 30 rats.

Research procedure: The research was carried out with several stages of analysis, namely: (1) Preparation of raw materials and animals testing, (2) Determination of sopi dosage, (3) Determination of raw material dosage (pletekan leaf), (4) Extraction of active ingredients from pletekan leaf, (5) Determination of the antioxidant activity of the extract, (6) Phytochemical test of pletekan leaf extract, (7) Preparation and application of sopi and extract to test the animals and (8) Analysis of TNF-α levels and histopathological observations of the rat kidneys.

Preparation of pletekan (*Ruellia tuberosa* L.) and animal testing: Preparation of raw materials in the form of wet sorting aims to separate dirt or other foreign materials from the plants which will be studied. The process continued with washing to remove soil and other impurities that are still attached to the material that has been sorted. Plant parts are sorted and separated (cut). The plants were weighed and calculated their yields to the total weight.

Determination of sopi dosage: The initial dose used is the dose of chronic use in the community which is about 100 mL (with a body weight of 50 kg). If used for humans weighing 70 kg, then $70/50\times100$ mL = 140 mL. Anroop[16], states the conversion factor for humans (70 kg) to mice (200 g) = 0.018. So that the dose of sopi in Wistar rats is 0.018×140 mL = 2.5 mL. So based on these results the administration of the dose of sopi used in Wistar rats (*R. overgicus* L.) in this study was 2.5 mL/head/body weight.

Determination of pletekan (*Ruellia tuberosa* L.) leaf dosage: States that if a human weighing 50 kg (the average weight of the Indonesian human body) consumes 250 mL of pletekan leaf (*Ruellia tuberosa* L.) with a concentration of pletekan water extract as much as 2% then the usual dose for rats Wistar with conversion calculation based on the

concentration value of pletekan leaf extract is: 70/50 kg $\times$0.018$\times$2% = 0.0504%. So based on the results above, the ethanol extract of pletekan leaf used in rats is 2, 4 and 6% (5.04, 10.08 and 12.12 mg/100 mL water) each from 3 different types of sopi, nira sopi, coconut sopi and koli sopi[17].

Pletekan (*Ruellia tuberosa* L.) extraction: The leaf of the pletekan plant was extracted with an ethanol solvent to get a crude extract. Parts of the plant were chopped using a knife into smaller pieces. Diminution was intended to simplify the extraction process. The extraction method used in this study was maceration in 70% ethanol solvent. The use of 70% ethanol as a solvent is based on its polarity but can dissolve non-polar compounds so it is expected to absorb non-polar and polar compounds. The pletekan leaf that has been chopped were then weighed 500 g each and then put in a maceration vessel. Each of the ethanol solvents was slowly poured into maceration vessels containing Simplicia powder until it soaked. Simplicia extraction process lasts for 3$\times$24 hrs, then filtered to get a liquid extract and evaporated the results of the search (with a rotary evaporator) on 40 until a thick extract is obtained. The extracted sample which has been concentrated with a rotary evaporator is then added with several mL of ethanol until all the ethanol has evaporated. The sample extract was weighed and the yield was calculated using the following formula[18]:

$$\text{Extract rendemen (\%)} = \frac{a}{(1-x)b} \times 100$$

a = Extract weights (g)
b = Sample weights (g)
x = Water content

Determination of alcohol content of various types of sopi: The sopi alcoholic drinks used are sopi from aren (fermented *Arenga pinnata* L. sap), coconut sopi (fermented *Cocos nucifera* L. sap) and koli sopi (the result of the fermentation of *Borassus flabellifer* L.) produced by the local community in Ambon, Maluku. About 5 mL samples (sopi) was then put in a test tube. Samples were diluted with distilled water in a volumetric flask to 100 mL. Diluted samples of 1 mL were added with 5 mL of K_2CrO_7 reagent (Sigma-Aldrich USA with catalogue number: B1760-1G) in a test tube, then heated to a temperature of 80°C for 15 min then cool. After cooling down, the sample was put into an Erlenmeyer flask and 3 drops of ferrozine were added as an indicator. Samples were added with aluminium ferrous sulfate Fe $(NH_4)^2$ by titration method until the sample solution changed colour.

Pletekan extract application: Preparation of experimental animals was carried out for 4 weeks in the Biology Laboratory, Department of Biology, Faculty Mathematics and Natural Science, Pattimura University, Ambon-Maluku. Rats used were male Wistar rats (*Rattus novegicus*) aged 2-4 weeks with a weight of 200 g, totalling 30 rats. Experimental animals were divided into two groups, namely, (1) Control group that is not getting any treatment for as many as five rats and (2) Groups of rats induced by each alcohol from coconut sopi, sopi nira and sopi koli for 1 month (interval of 3 times in 1 week) as many as five animals. After that, the rats were treated with pletekan plant extracts with different concentrations of 2, 4 and 6% of the body weight of mice with a volume of 2.5 mL/head/body weight for each rat orally.

Histopathological observation of Wistar rat kidneys

Harvesting of kidney organs: Kidney organ harvesting in Wistar rats was carried out on day 22 with treatment according to the group. Before taking the kidney organ, the rats were euthanized by dislocating their neck and then surgery was performed. Surgery was performed on the abdomen, where the rat is placed with the abdomen position on the surgical board. Then the kidney is taken and cut using surgical scissors. The kidneys were first rinsed with cold 0.9% NaCl-fis. Then the left kidney is stored in a PBS-azide solution pH 7.4 and stored in a refrigerator as an isolation agent for the protease enzyme. Whereas the right kidney is included in a 10% PFA solution for the histology sample preparations.

Kidney embedding: The first step of embedding the kidney organ was soaking it in 10% formaldehyde solution and then immersing in 70% ethanol for 24 hrs, transferring it in 80% ethanol for 2 hrs, 90% ethanol for 20 min, 95% ethanol for 20 min and absolute ethanol for 20 min, where this step is carried out three times then the kidney organs are transferred to the xylol solution for 20 min 2 times, put back into the xylol solution and carried out at a temperature of 60-63 for 30 min. Then, the kidney is dipped in liquid paraffin which has been poured into a container. After a while, the paraffin will solidify and the kidney organs are in the paraffin block[19].

Making kidney histology sample: Making a kidney histology sample is done by first inserting the kidneys in paraffin blocks from the previous process on the mitochrome brace and arranging them parallel to the mitochrome blades. Before cutting, the thickness was set by incision above 10 µm to accelerate the achievement of tissue cutting areas. Then, the kidneys were cut to the size of 5 µm, slices were taken with a brush and put in water at room temperature. Next, the slices were transferred with a brush into 38-40°C warm water and

chosen based on the flatness of a glass object. The selected slices are dried and placed on a 38-40°C hot plate to dry and after that, the histological sample was stored in an incubator at 38-40°C for 24 hrs[19].

Hematoxylin and Eosin (H&E) staining: The first Hematoxylin and Eosin (H&E) staining was carried out with the stages of deparaffinization, wherein the sample preparation was put in 1-3 levels of xylol each for 5 min. Next, a rehydration stage was carried out where the samples were put into multilevel ethanol starting from absolute ethanol 1-3, ethanol 95, 90, 80 and 70% for 5 min, respectively. Then, samples were soaked in distilled water for 5 min. After that, hematoxylin and eosin staining was done[20].

Measurement of creatinine serum levels in Wistar rat blood: Creatinine serum was taken during the adaptation period of rats every 4 days (up to the 12th day) to determine the average creatinine serum level produced by rats (assuming normal activity). Creatinine serum was also taken after induction of sopi alcohol and after being given leaf pletekan extract. The serum was incubated for 5 min at 37°C. Then 50 μL of serum was taken and placed into a cuvette and then added with 1000 μL of Jaffe reagent (Sigma-Aldrich, Jerman). Jaffe monoreagen was made by mixing sodium hydroxide (NaOH) with concentrated picric acid, a ratio of 4:1 (v/v). The Jaffe reagent was reacted with creatinine in the sample to produce a colour change in the mixture. This colour change was read using a UV-Vis spectrophotometer (Supelco™ (former Sigma-Aldrich-USA, Cat. No. 173018 Spectroquant® UV/VIS)) with a wavelength of 492 nm. Average creatinine serum level from 11 groups was obtained and its data were analyzed[8].

Immunohistochemistry staining and observation of TNF-α expression: Renal histopathological observations were performed on the glomerular and tubular sections by Hematoxylin Eosin (HE) staining with immunohistochemistry staining to determine TNF-α expression. Glomerular and tubular images were observed qualitatively using an Olympus BX51 microscope with a magnification of 400× and observation of TNF-α expression with immunohistochemistry was carried out by observing five fields of view then an average percentage of the area was assessed using the Axio Vision Program[21].

Research design and data analysis: Data were analyzed to find out information on the relationships between treatments and interactions between treatments. Each data analysis has a different purpose and interpretation of the data. Data were analyzed descriptively (for nominal and ordinal data) in the form of determining the average value, value variance and data presentation. Experimental quantitative data were also analyzed by inference. Inference analysis can only be done if a group of data on each of the observed parameters meets the assumptions. Testing assumptions used include normality, homogeneity, linearity and collinearity. Hypothesis testing is carried out after the data meets all assumption tests (parametric tests). Data that did not meet the assumption test were analyzed in a non-parametric way[22]. Hypothesis testing used to compare (comparative) the effect between the treatments of responses from the sample is the variants analysis and the t-test. If there is a significant influence, then further tests are carried out for the degree of difference. Hypothesis testing used to classify (associative) the effect between the treatment of responses from the sample is a regression test and correlation test[23].

Descriptive analysis: Interpretation or analysis of quantitative data (intervals or ratios) in the form of tables, graphs and diagrams. The purpose of this analysis is to make it easier for readers to have a direct understanding. Data captions or conditions in this analysis include the mean (μ) and standard deviation (Δx).

F-Test (Analysis of Variance Analysis/ANOVA): Data on creatinine levels in each experimental group were analyzed using Analysis of Variance (ANOVA) and will be continued with the Difference Test if there is a significant effect at a significant level of 0.05. TNF-α expression was analyzed descriptively. The expression of TNF-α is marked in brown on the photomicrograph of the kidney of the Wistar rat (*Rattus norvegicus* L.).

RESULTS

Measurement of creatinine levels of Wistar rats (*Rattus norvegicus* L.): The results of creatinine levels measurements in *R. norvegicus* L., showed an increase in creatinine levels after administration of sopi (coconut, nira and koli) alcohol. Increased creatinine levels are an indicator of kidney damage. However, a decrease in creatinine levels was seen after the ethanol extract of pletekan (*R. tuberosa* L.) leaves was administrated. The results of the analysis of creatinine levels before and after giving ethanol extract *R. tuberosa* L., can be seen in Table 1.

The results of creatinine levels measurements from coconut sopi type in Table 1 show that the average creatinine levels in negative controls were 0.43 mg dL^{-1} and after

Table 1: Average creatinine levels of Wistar rats (*R. norvegicus* L.) with coconut sopi

	Average coconut sopi±SD			
Treatment groups	Pre coconut sopi	After giving sopi	After administration of the extract	Averaging coconut sopi
Control (-)	0.43±0.05[a]	0.53±0.25[a]	0.53±0.25[a]	0.06±0.05
Control (+)	0.63±0.15[b]	1.46±0.30[b]	1.46±0.30[b]	0.07±0.05
Dose 5.04 mg/100 mL water (1)	0.66±0.05[c]	1.43±0.11[b]	1.43±0.11[b]	0.17±0.10
Dose 10.08 mg/100 mL water (2)	0.76±0.05[b]	1.53±0.15[b]	1.13±0.11[c]	0.00±0.06
Dose 12.12 mg/100 mL water (3)	0.80±0.17[a]	1.76±0.20[c]	0.80±0.10[b]	0.06±0.07

Superscript with the same letter shows no significant difference and (p<0.05)

Table 2: Average creatinine of Wistar rats (*R. norvegicus* L.) with sopi nira

	Average nira sopi drinks±SD			
Treatment groups	Pre sopi nira	After giving sopi nira	After administration of the extract	Averaging sopi nira
Control (-)	0.50±0.10[a]	0.53±0.23[a]	0.47±0.15[a]	0.03±0.05
Control (+)	1.23±0.57[c]	1.36±0.25[b]	1.16±0.20[c]	0.07±0.37
Dose 5.04 mg/100 mL water (1)	1.20±0.30[c]	1.36±0.10[b]	1.16±0.15[c]	0.03±0.15
Dose 10.08 mg/100 mL water (2)	1.13±0.30[b]	1.46±0.15[b]	1.13±0.15[b]	0.00±0.15
Dose 12.12 mg/100 mL water (3)	1.06±0.15[b]	1.73±0.15[c]	0.90±0.10[b]	0.17±0.05

Superscript with the same letter shows no significant difference and (p<0.05)

induction of coconut sopi increased to 0.53 mg dL^{-1}. In the positive control group, the initial average creatinine was 0.63 mg dL^{-1} and after induction of coconut sopi, it doubled to 1.46 mg dL^{-1}. Creatinine levels before treatment in the rat group with a dosage of 5.04 mg/100 mL of water are 0.66 mg dL^{-1} and increased to 1.43 mg dL^{-1} after the administration of coconut sopi, whereas in the rat group with the dosage of 10.08 mg/100 mL water, the initial creatinine level is 0.76 mg dL^{-1} and after administration of coconut sopi its increased to 1.53 mg dL^{-1}. The treatment dose of 12.12 mg/100 mL of water, showed an initial creatinine level of 0.80 mg dL^{-1} and after administration of coconut, sopi increased to 1.76 mg dL^{-1}.

After an increase due to alcohol induction of coconut sopi type, the samples were then given ethanol extract of pletekan leaf for 14 days, the results showed that in the negative control group of rats, the average creatinine level increased from 0.53-0.06 mg dL^{-1}, whereas in the positive control of rat group mean creatinine levels increased from 1.46-0.07 mg dL^{-1}. In the rat group with a dosage of 5.04 mg/100 mL of water, a decrease in creatinine level was seen, from 1.43-0.17 mg dL^{-1}, as well as at a dose of 10.08 mg/100 mL of water, creatinine level was decreasing from 1.53-1.13 mg dL^{-1} and a dose of 12.12 mg/100 mL of water there was a decrease in creatinine level of 1.76-0.80 mg dL^{-1}.

Based on the analysis of variance test results showed that the administration of ethanol extract of pletekan leaf (*R. tuberosa* L.) significantly affected the decrease in creatinine levels in Wistar rats (*R. norvegicus* L.) (p<0.05). The results of further tests (LSD) showed that the group of 5.04 mg/100 mL of water, 10.08 mg/100 mL of water and 12.12 mg/100 mL of water were significantly different from negative controls and positive controls, but the dose of 5.04 mg/100 mL of water and 10.08 mg/100 mL of water were not significantly different from the dose of 12.12 mg/100 mL of water (Table 2).

The results of measurements of creatinine levels in the type sopi nira in Table 2 show that the average creatinine level from negative control was 0.50 mg dL^{-1} and after the induction of sopi nira, it is increased to 0.53 mg dL^{-1}, in the positive control group the initial average creatinine was 1.23 mg dL^{-1} and after the induction of sopi nira it is increased to 1.36 mg dL^{-1}, in the rat group dosage of 5.04 mg/100 mL of water the average initial creatinine was 1.20 mg dL^{-1} and after the administration of sopi nira it is increased to 1.36 mg dL^{-1}, whereas in the rat group dosage of 10.08 mg/100 mL water, the initial creatinine level was 1.13 mg dL^{-1} after administration of the sopi nira it is increased to 1.46 mg dL^{-1} and a dose of 12.12 mg/100 mL water, initial creatinine level was 1.06 mg dL^{-1} and after administration of sopi nira, it is increased to 1.73 mg dL^{-1}.

After an increase due to alcohol induction of sopi nira type, the rats were then given an ethanol extract of pletekan leaf for 14 days, the results showed that in the group of negative control rats the average creatinine level increased by 0.03 mg dL^{-1} so that creatinine levels became 0.47 mg dL^{-1}. Whereas in the positive control group of rats the average creatinine level increased by 1.16 mg dL^{-1}. In the rat group with the dose of 5.04 mg/100 mL of water, creatinine levels

Table 3: Average Wistar rats creatinine (*R. norvegicus* L.) with coli sopi

	Average koli sopi drinks±SD			
Treatment groups	Pre sopi koli	After giving sopi koli	After administration of the extract	Averaging sopi koli
Control (-)	0.43±0.15^{a}	0.53±0.15^{a}	0.40±0.10^{a}	0.03±0.05
Control (+)	1.30±0.26^{b}	1.53±0.25^{b}	1.23±0.23^{c}	0.07±0.03
Dose 5.04 mg/100 mL water (1)	1.33±0.15^{c}	1.36±0.15^{b}	1.23±0.23^{c}	0.10±0.08
Dose 10.08 mg/100 mL water (2)	1.16±0.15^{b}	1.43±0.15^{b}	1.16±0.05^{c}	0.00±0.10
Dose 12.12 mg/100 mL water (3)	1.03±0.25^{b}	1.83±0.25^{c}	0.73±0.15^{b}	0.30±0.10

Superscript with the same letter shows no significant difference and (p<0.05)

decreased from 1.36-1.16 mg dL^{-1}. With the dose of 10.08 mg/100 mL of water, creatinine levels decreased to 1.13 mg dL^{-1} and the creatinine levels in the rat group with the dosage of 12.12 mg/100 mL of water decreased by 0.17-0.90 mg dL^{-1}.

Based on the analysis of variance test results showed that the administration of ethanol extract of pletekan leaf (*Ruellia tuberosa* L.) significantly affected the decrease in creatinine levels of white rats (*Rattus norvegicus* L.) (p<0.05). The results of further tests (LSD) showed that the dose groups 5.04, 10.08 and 12.12 mg/100 mL of water were significantly different from negative controls and positive controls, but the doses of 5.04 and 10.08 mg/100 mL of water were not significantly different from the dose of 12.12 mg/100 mL of water (Table 2).

Furthermore, the average results of measurements of creatinine levels after administration of sopi coli can be seen in Table 3.

The results of creatinine levels sopi koli measurements in Table 3 show that the mean creatinine level from the negative control was 0.43 mg dL^{-1} and after induction of koli sopi increased to 0.53 mg dL^{-1}, in the positive control group the initial creatinine level average was 1.30 mg dL^{-1} and after induction sopi koli it is increased to 1.53 mg dL^{-1}, in the rat group of 5.04 mg/100 mL of water dosage, mean creatinine initial was 1.33 mg dL^{-1} and after administration of sopi koli it is increased to 1.36 mg dL^{-1}, whereas in the rat group with the dose of 10.08 mg/100 mL of water, the initial creatinine level was 1.16 mg dL^{-1} given sopi koli it is increased to 1.43 mg dL^{-1} and the dose of 12.12 mg/100 mL of water, the initial creatinine level was 1.03 mg dL^{-1} after giving sopi koli increased to 1.83 mg dL^{-1}.

After an increase due to alcohol induction of sopi coli, the rats were then given ethanol extract of pletekan leaf for 14 days, the results showed that in the negative control group of rats the average creatinine level increased from 0.53-0.03 mg dL^{-1} so, that creatinine levels became 0.40 mg dL^{-1}. Whereas in the positive control group of rats there is an increased creatinine level of 0.07 mg dL^{-1} so the average creatinine level is from 1.53-1.23 mg dL^{-1}. In the rat group with a dosage of 5.04 mg/100 mL of water, the creatinine level decreased by 1.36 mg dL^{-1} so the creatinine level from 0.10-1.23 mg dL^{-1}. The dosage of 10.08 mg/100 mL of water, became 1.16 mg dL^{-1} and at the dosage of 12.12 mg/100 mL of water, the creatinine level decreased by 0.30 mg dL^{-1} so it becomes 1.83 mg dL^{-1}.

Based on the result from variance test analysis showed that the administration of pletekan leaf (*Ruellia tuberosa* L.) ethanol extract significantly affected the decrease in creatinine levels in Wistar rats (*Rattus norvegicus* L.) (p<0.05). The results of further tests (LSD) showed that the dose group 5.04, 10.08 and 12.12 mg/100 mL of water were significantly different from negative controls and positive controls, but the doses of 5.04 and 10.08 mg/100 mL of water were not significantly different from the dose of 12.12 mg/100 mL of water.

Photomicrograph results of Wistar rat (*Rattus norvegicus* L.): Histology results of rat's renal cells before treatment, after induction of sopi alcohol (coconut, nira and koli) and ethanol extract of pletekan leaf in Fig. 1(a-e).

The dosage group of 5.04 mg/b.wt./day, shows that the cells have high damage, which is characterized by tubular epithelial cells undergoing necrosis and mild apoptosis is characterized by karyorrhexis and pyknosis and some cells have glomerular fibrosis (Fig. 1a-e). The dosage group of 10.08 mg/g/b.wt./day shows little cell damage only in cells undergoing karyorrhexis and most cells undergo cell regeneration (Fig. 1d). In normal cell regeneration treatment, with cell parts visible, that is the cell nucleus, cytoplasm appears homogeneous red with an enlarged basophilic nucleus and wider tubular lumen.

Whereas the dose group of 12.12 mg/g/b.wt./day showed normal-looking kidney conditions marked by the absence of necrosis or cell apoptosis (Fig. 1e). So that it can be stated that the ethanol extract of pletekan leaf at a dose of 12.12 mg/g/b.wt./day can repair and regenerate kidney cells that have been negatively affected by the administration of sopi (coconut, nira and koli) alcohol.

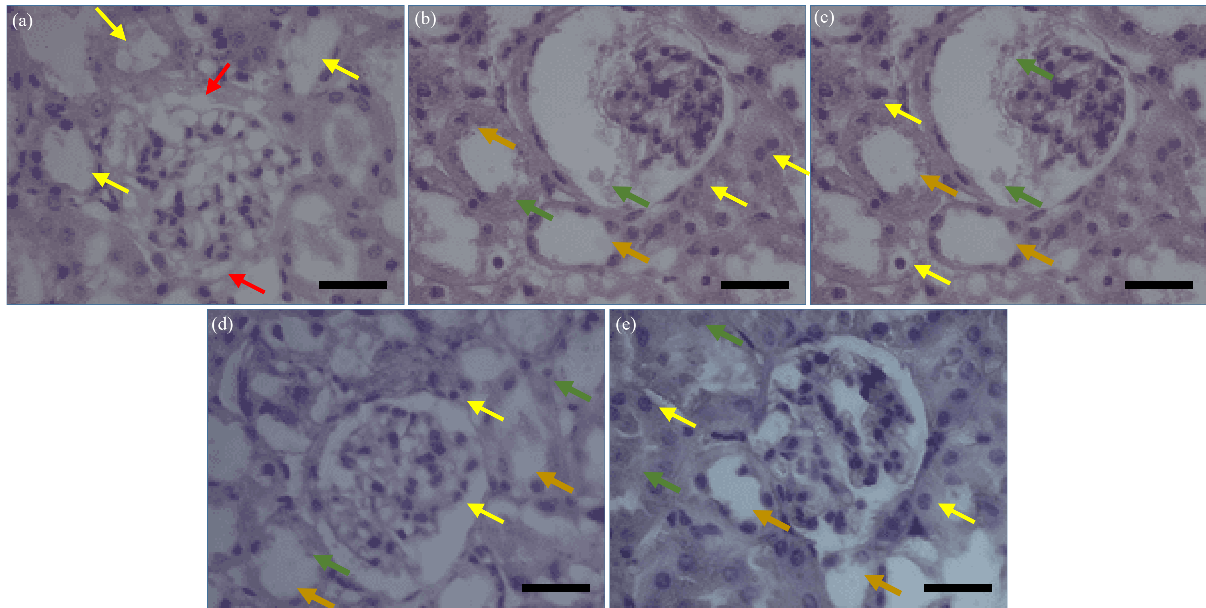

Fig. 1(a-e): Histology of Wistar rat's kidney cells before treatment, (a) Post induction of coconut sopi, (b) Nira, (c, d) Koli and (e) Ethanol extract of pletekan leaf

Before treatment normal glamorous (red arrow) and tubule (yellow arrow) were seen, after induction (b, c, d and e), the tubular epithelial cells were swollen (yellow arrows), vacuole cytoplasm (brown arrows) and missing nuclei (karyolysis) (green arrows) and in the picture, you can see a desquamated tubule (H&E, 400×)

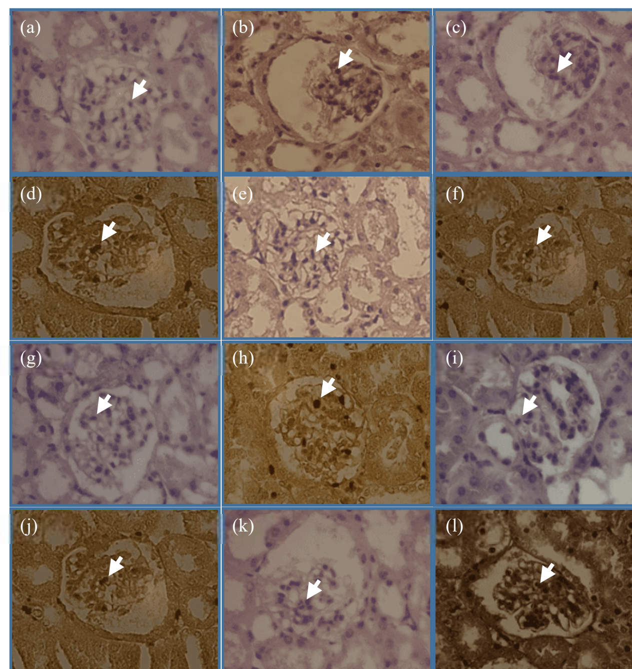

Fig. 2(a-l): Photomicrograph results (TNF-a expression) in renal cells of Wistar rats (*Rattus norvegicus* L.), (a) Provision of alcohol for the coconut sopi, (b) Dose 1 (5.04 mg/100 mL water), (c) Dose 2 (10.08 mg/100 mL water), (d) Dose 3 (12.12 mg/100 mL water), (e) Provision of sopi koli type, (f) Dose 1 (5.04 mg/100 mL water), (g) Dose 2 (10.08 mg/100 mL water), (h) Dose 3 (12.12 mg/100 mL water), (i) Provision of sopi nira type, (j) Dose 1 (5.04 mg/100 mL water), (k) Dose 2 (10.08 mg/100 mL water) and (l) Dose 3 (12.12 mg/100 mL water)

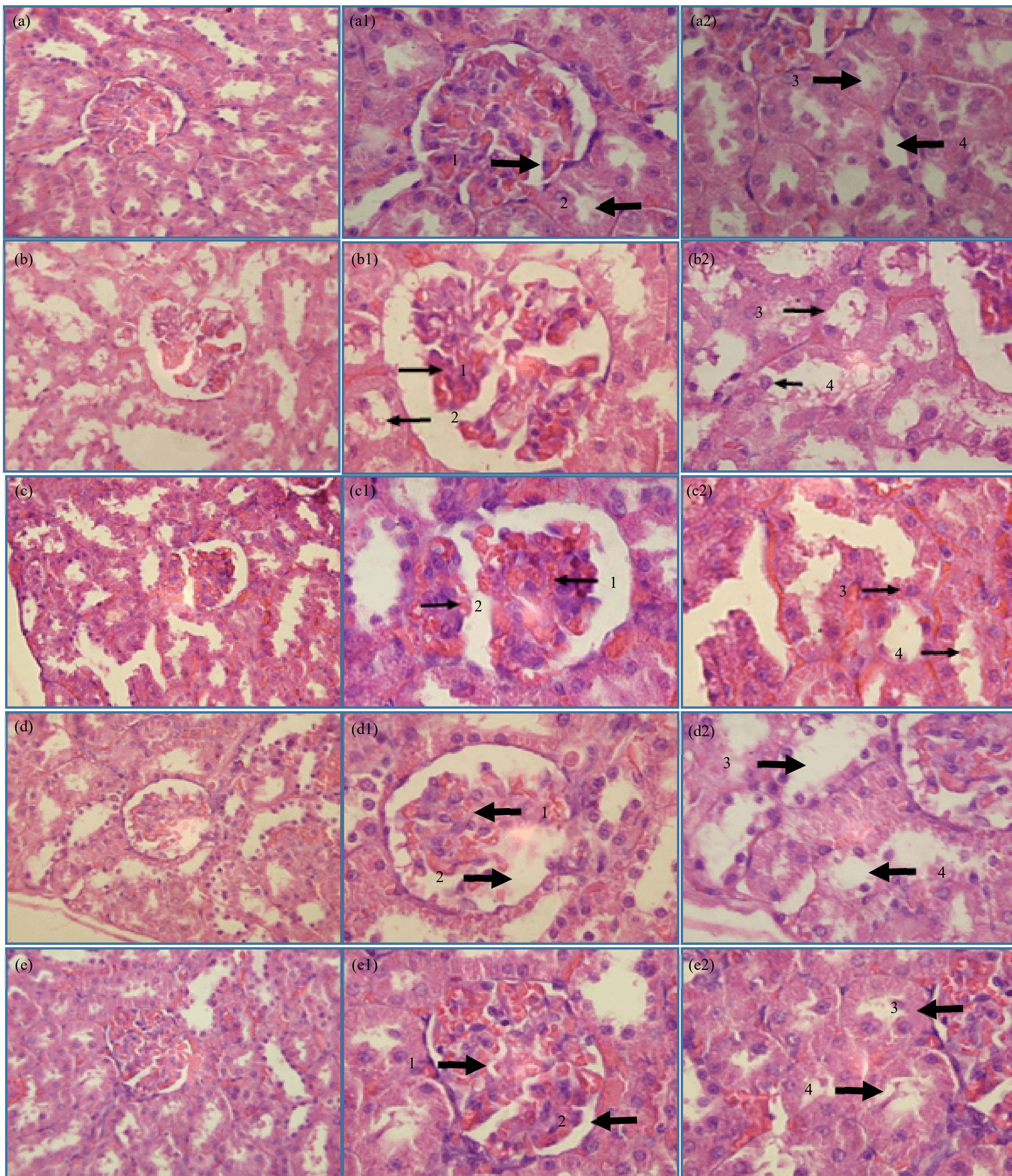

Fig. 3(a-e2): Histology of renal Wistar rats in control and treatment group, (a) Histology of renal Wistar rats in the control group, (b) Kidney histology of Wistar rats with a dose of 2.5 mL/200 g b.wt., (c) Kidney histology of Wistar rats with 2.5 mL/200 g b.wt., dose group and ethanol extract of pletekan leaf (*Ruellia tuberosa* L.) 5.04 mg/100 mL water, (d) Histology of Wistar rat Kidney with doses of 2.5 mL/200 g b.wt. and ethanol extract of pletekan leaf (*R. tuberosa* L) 10.08 mg/100 mL of water, (e) Histology of kidney Wistar rats with 2.5 mL/200 g b.wt., dose group and ethanol extract of pletekan leaf (*R. tuberosa* L) 12.12 mg/100 mL water, (a1, b1, c1, d1, e1) Histology of kidney glomerulus of Wistar rats in the control group, magnification 1000× and (a2, b2, c2, d2, e2) Histology of kidney tubules of control group Wistar rats, magnification 1000×

1: Glomerular atrophy, 2: Distal tubular protein deposits, 3: Tubular necrosis and 4: Proximal tubular edema

TNF-α expression in renal cells of Wistar rats (*Rattus norvegicus* L.) through immunohistochemistry test: The TNF-α expression was directly observed through immunohistochemistry tests by observing renal cell damage which triggered an increase in TNF-α production. Based on the results of photomicrographs in renal cells differences were found in TNF-α expression, which can be observed in Fig. 2.

The expression of TNF-α in the kidney organ is indicated by the presence of brown colour in the renal immunohistochemistry (IHC) picture indicated by an arrow (↑). Results of analysis of kidney damage using Hematoxylin and Eosin (H&E) staining in the histology of the kidney organs of Wistar rats that were given sopi alcoholic drinks for 60 days (2 months) showed that the kidneys were damaged in both the glomerulus and tubules, but after the Wistar rat was given ethanol extract pletekan leaf (*R. tuberosa* L.) with a dose of 5.04, 10.08 and 12.12 mg/100 mL of water for 60 days showed improvement in the kidney organs.

Based on the kidney cell photomicrograph above, the negative control group (Fig. 2a), shows a normal kidney condition by having a compact tissue structure, bowman capsules around the glomerulus also clearly did not experience any dilation or bowman atrophy. Another case with positive control shows damage to kidney cells with cell necrosis and also bowman dilatation (enlargement of the bowman space). Inflammation occurs in the glomerular region marked by erythrocyte inflammation and there is necrosis of epithelial cells which are characterized by cells undergoing karyolysis, karyorrhexis and apoptosis which are characterized by cells undergoing pyknosis (Fig. 2b-l).

Histological picture of the kidneys from rats that were given distilled water, a group of Wistar rats who were given sopi alcoholic drinks for 60 days (2 months) and given pletekan leaf ethanol extract (*R. tuberosa* L.) for 30 days (1 month) can be seen in Fig. 3a-e2.

The kidney histology of the Wistar rats control group showed that there was no damage to the kidneys. The glomerulus, proximal tubules and distal tubules are normal. Kidney histology of Wistar rats grouped with sopi alcoholic drinks showed damage to rat kidneys. This can be seen by the presence of glomerular atrophy, distal tubular protein deposits, proximal tubular necrosis and proximal tubular oedema. Kidney histology of Wistar rats grouped with 2.5 mL/200 gr BB sopi alcoholic drinks and given ethanol extract of pletekan leaf (*Ruellia tuberosa* L.) 5.04 mg/100 mL of water showed that there was still damage to the rat kidneys. This can be seen by the occurrence of glomerular atrophy, proximal tubular protein deposits and proximal tubular oedema, but no tubular necrosis has occurred.

Kidney histology of Wistar rats grouped with 2.5 mL/200 gr BB sopi alcoholic drinks and given ethanol extract of pletekan leaf (*Ruellia tuberosa* L.) 10.08 mg/100 mL of water showed that damage to the kidneys began to decrease. This can be seen by the absence of proximal tubular protein deposits, necrosis of the tubules and oedema of the proximal tubules. But still occurs atrophy of the glomerulus. Kidney histology of Wistar rats grouped with 2.5 mL/200 gr BB sopi alcoholic drink and given leaves ethanol extract (*Ruellia tuberosa* L.) 12.12 mg/100 mL of water showed that the rat kidney had returned to normal condition. The glomerulus, proximal tubules and distal tubules are normal.

DISCUSSION

The results of analysis of kidney damage in this study using Hematoxylin Eosin (HE) staining in the histology of the kidney organs of Wistar rats that were given sopi alcoholic drinks for 60 days (2 months) showed that the kidney has been damaged (Fig. 1b). This can be seen by the occurrence of glomerular atrophy, distal tubular protein deposits, necrosis of the proximal tubules and proximal tubular oedema. Proximal tubule damage occurred in Wistar rats who were given 2.5 mL/200 g of alcoholic drink for 60 days (2 months). The results of this study are supported by a theory that states that the chemical excretion process that takes place in the kidneys can harm kidney disease[24].

Alcohol consumption can increase blood pressure which is a risk factor for kidney damage[25]. In addition, alcohol consumption can cause direct damage to the kidneys due to the formation of strong nephrotoxins which can result in disruption of cell function and death (necrosis) in proximal tubular cells. Under certain circumstances, alcohol abuse or dependence is associated with certain renal pathologies, including renal papilla necrosis, infection-related glomerulonephritis and acute renal failure due to non-traumatic rabdomiolysis[26].

The results of this study indicate the presence of protein deposits in the lumen of the tubules. This is influenced by various factors including an increase in glomerular capillary permeability so that proteins cannot escape. In addition, decreased absorption of tubules due to tubular epithelium has degenerated to necrosis which is also a factor in protein deposition[27]. Sopi excretion process can cause tubular damage in the form of acute tubular necrosis (NTA) which is reversible and if not handled properly can continue to become an irreversible NTA. Reversible NTAs occur because proximal tubular epithelial cells have good regenerative ability[26,28]. Morphologically, NTA is characterized by the destruction of proximal tubular epithelial cells but the basement membrane of the tubules is still good.

Microscopic features of proximal tubular epithelial cells that swell with the granular cytoplasm due to a shift in extracellular water into the cell[24]. This fluid shift occurs because of changes in the electrical charge surface of the tubular epithelial cells, active transport of ions and organic acids and the ability to concentrate from the kidneys which ultimately results in damaged tubules disrupted urinary flow, increased intra-tubular pressure, decreased glomerular filtration speed all of which are caused by toxic which exposes the kidneys[28]. This picture of cell swelling is called degeneration of albuminous or degeneration of parenchymmosis or cloudy swelling, which is a form of the mildest and reversible degeneration[29]. This is what might have caused the proximal tubular lumen to be narrowed to close in this study. If the tubular system is left and continues, it will be followed by preglomerular arteriole vasoconstriction, which can cause ischemia. Ischemic can cause various changes in the structure and function of epithelial cells, from reversible damage as mentioned above to irreversible damage that is characterized by the occurrence of necrosis and apoptosis[30].

Pletekan leaf extract (*Ruellia tuberosa* L.) with a dose of 5.04, 10.08 and 12.12 mg/100 mL of water showed an improvement in the kidney of Wistar rats. This can be seen in the group of Wistar rats who were given 2.5 mL sopi alcoholic drinks and given a leaf extract with a dose of 5.04 mg/100 mL of water (Fig. 1c) showed that there was no necrosis in the proximal tubules but glomerular atrophy still occurred, protein deposition distal tubules and proximal tubular oedema. The group of Wistar rats who were given 2.5 mL of sopi alcoholic drinks and given leaf extract with a dose of 10.08 mg/100 mL of water (Fig. 1d) showed the rate of improvement in the kidneys had improved. This can be seen with no distal tubule protein deposition, proximal tubular necrosis and proximal tubular oedema but glomerular atrophy still occurs. The group of Wistar rats that were given 2.5 mL of sopi alcoholic drinks and given leaf extract with a dose of 5.04 mg/100 mL of water (Fig. 1e) showed that the renal histology of experimental animals (Wistar rats) was in normal condition. The glomerulus, proximal tubules and distal tubules are normal. There was an improvement in the histopathology of renal Wistar rats fed with 2.5 mL/200 gr sopi alcoholic drinks and ethanol extract of pletekan leaf (*R. tuberosa* L.) with a dose of 5.04, 10.08 and 12.12 mg/100 mL of water caused by the ethanol extract of *R. tuberosa* L. contains natural antioxidants that can neutralize free radicals due to administration of sopi to Wistar rats. Antioxidants work by donating one electron to the oxidant compound, in this case, free radicals, so that the activity of the oxidant compound can be inhibited.

This research has been carried out to see the effect of giving alcohol drink types of coconut sopi, coli and nira to Wistar rat subjects for 60 days. The results obtained are elevated blood creatinine levels in Wistar rats. Alcohol is one of the factors that can affect kidney function directly, through acute or chronic consumption, or indirectly, as a consequence of liver disease[31]. Basille *et al.*[24] reported that Wistar rats exposed to alcohol for 12 weeks had significantly increased urea and creatinine concentrations compared to Wistar rats exposed to alcohol for 4 weeks and rats as a control group. Chung and Martin[32] added that alcohol consumption can influence the regulation of vasoactive substances so that it affects renal hemodynamics, glomerular filtration rate to damage to the glomerulus, which can affect creatinine concentration in the blood. This is in line with research conducted by Islam *et al.*[33] showed that the administration of 10% alcohol in rats could increase blood creatinine levels in Wistar rats.

Types of sopi used in the treatment are coconut sopi, koli and nira. Nira can be consumed directly as a fresh drink or allowed to ferment by microbes naturally containing alcohol and become a traditional beverage of the community called sopi or tuak. Coconut sopi is sopi which is made from shoots from coconut trees which are then taken into the juice and processed to become coconut sopi, while sopi koli is made from the essence of a coli tree trunk which is then processed to become sopi koli[4]. In this study, three doses of pletekan leaf extract were used to test the dosage that was able to reduce blood creatinine levels in Wistar rats due to exposure to coconut sopi, koli sopi and sopi nira.

The ANOVA results on the measurement of creatinine levels in Wistar rats before and after exposure to alcohol sopi coconut, coli and nira type showed a significant effect ($p<0.05$). A decrease in creatinine levels in the blood of Wistar rats after administering doses of pletekan leaf extract is influenced by flavonoid and antioxidant content. Pletekan plant leaves *(Ruellia tuberosa* L.) contain flavonoids, phenols, saponins, antioxidants, anti-microbial anti-inflammatory and anti-cancer[34-36]. The same research results were also conveyed by Seo *et al.*[37] Guava leaves have phenolic compounds and flavonoids with high antioxidant activity. The main active substances in guava leaves are gallic acid, tannins, carotenoids and triterpenoids. Based on these studies it can be concluded that the content of flavonoids derived from ethanol extracts of leaves of pletekan plants can improve the function of renal glomerulus as filtration to reduce creatinine excretion in the blood serum of Wistar rats as experimental animals. Vargas *et al.*[38] added that flavonoids reduce cisplatin-induced

kidney injury in mice and reduce the increase in creatinine and nitrogen waste in the form of urea in blood serum, while flavonoids inhibit apoptosis in cisplatin-induced kidney cells, thereby benefiting kidney cell survival. Flavonoids are also reported to be able to prevent cell damage and improve the morphology and kidney function of rats given alcohol[39,40].

Tumor Necrosis Factor (TNF-α) is secreted by macrophages and is synthesized mainly by monocytes and macrophages which are sensitive indicators of inflammation[41]. TNF-α plays a role in cell defence when an infection occurs. Body cells, especially kidney cells will be damaged when exposed to alcohol which will increase TNF-α production. The TNF-α can induce cell survival, proliferation, differentiation, apoptosis and necrotic cell death under certain conditions[42]. Lee *et al.*[43] study showed that circulating TNF-α levels are significantly higher in chronic kidney disease patients compared to controls and the levels of TNF-α are independently associated with the severity of chronic kidney disease. Research by Liakopoulos *et al.*[44] researched on 23 dialysis patients and 16 patients with chronic kidney failure without dialysis and 28 healthy patients in the United Kingdom showed that TNF-α concentrations increased in patients with chronic kidney failure compared with controls.

This is in line with the results of research that has been done, namely in the histology of renal Wistar rats with Hematoxylin and Eosin (H&E) staining seen damage after being given a type of sopi alcoholic drink for 60 days (2 months), where the damage occurred both glomerular and tubular. Damage that occurs in the kidney organ triggers the formation of TNF-α which is seen in Fig. 3. Histological changes in kidney Wistar rats are seen after the Wistar rat is given ethanol extract of pletekan leaf (*R. tuberosa* L.) at a dose of 5.04, 10.08 and 12.12 mg/100 mL of water for 30 days showed an improvement in the kidney organs. At a dose of 5.04 mg/100 mL of water, it shows that there is still damage to the rat kidneys in glomerular atrophy, proximal tubular protein deposits and proximal oedema tubules, but no tubular necrosis has occurred. This shows that there is a significant influence on the ethanol extract of pletekan leaf on cell repair, which directly influences the decrease in TNF-α production.

The TNF-α expression was seen with the presence of brown spots formed in the histological preparations of renal Wistar rats. The emergence of the brown colour is caused by the Immunohistochemical (IHC) staining antigen in the kidneys bound to the primary antibody (rat anti TNF-α) then labelled by secondary antibodies (goat anti rat biotin labelled), after all, binding the addition of the Diaminobenzidine (DAB) substrate which is then aimed at being labelled by secondary antibodies (goat anti rat biotin labelled) to produce a brown colour in cytokines (TNF-α)[45]. Koivunen and Krogsrud[46] state that the addition of Diaminobenzidine (DAB) substrate will give a brown colour to antigens that bind to primary antibodies labelled with secondary antibodies[21].

Extract leaf pletekan (*Ruellia tuberosa* L.) has the potential to be developed as a medicinal plant in repairing kidney damage because it has strong antioxidant activity so that it can be used as an alternative pharmaceutical for people with kidney damage. Further research is needed for another in-vivo test regarding the effect of giving leaf extract pletekan (*Ruellia tuberosa* L.) to measure other indicators in the process of kidney damage and physiological work mechanisms in the kidney repair process caused by excessive use of liqueur in the body.

CONCLUSION

Based on the results of the study, it can be concluded that: 1) Ethanol extract of pletekan leaf (*Ruellia tuberosa* L.) has an effect in determining the TNF-α expression of kidney Wistar rats (*Rattus norvegicus* L.) exposed to sopi alcoholic drinks, 2) Wistar rats (*Rattus norvegicus* L.) exposure to sopi type alcohol drinks have decreased creatinine levels after being treated with ethanol extract of pletekan leaf (*Ruellia tuberosa* L.) on all treatments of sopi type for a dose of 6% (12.12 mg/100 mL water) which was more effective in reducing creatinine levels and TNF-α and 3) Ethanol extract of pletekan leaf (*Ruellia tuberosa* L.) has the potential to prevent/repair kidney damage characterized by decreased expression of TNF-α kidney Wistar rats (*Rattus norvegicus* L.) along with an increase in extract dosage in all treatments of all types of sopi.

SIGNIFICANCE STATEMENT

Thus, the results of the study revealed that the flavonoid content in the leaves of pletekan leaf was able to improve the function of renal glomerulus as filtration thereby reducing creatinine excretion in the blood serum of Wistar rats as experimental animals. Thus, this plant can be used as an alternative treatment for patients with kidney damage after being forwarded to a clinical trial.

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