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Red guava juice (*Psidium guajava linn.*) suppress TGF- β protein expression in secondary hyperuricemia mice (*Mus musculus*) model



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ABSTRACT

Background: Secondary hyperuricemia can increase the risk of renal dysfunction in the form of fibrosis and glomerulosclerosis mediated by TGF- β signalling. Non-pharmacological therapy through high dietary antioxidant intakes can help in controlling and preventing renal dysfunction by suppressing TGF- β expression. This study aimed to analyse the effect of red guava juice (*Psidium Guava Linn.*) on TGF- β protein expression in mice (*Mus Musculus*) kidneys with secondary hyperuricemia model.

Method: This was an experimental study with a post-test only design. Forty-eight mice (*Mus Musculus*) were chosen as samples, which were distributed into six different treatment groups. The mice were induced with potassium oxonate for 14 days and given red guava juice for 21 days. TGF- β protein expression in mice (*Mus Musculus*) kidneys was identified through immunohistochemistry

using TGF- β antibodies. The preparation was observed in the Anatomical Pathology Laboratory, Faculty of Medicine, Universitas Sebelas Maret using the Olympus XC10 light microscope. Data were statistically analysed using the Kruskal Wallis test, followed by the Post-Hoc Mann Whitney test.

Results: The results of this study showed that the administration of red guava juice had a significant effect on TGF- β expression ($p = 0.001$). There was a significant difference between the negative control treatment and treatment of red guava juice at a dosage of 5 ml/kg/day ($p = 0.043$).

Conclusion: It can be concluded that the administration of red guava juice in mice (*Mus Musculus*) with secondary hyperuricemia model can suppress TGF- β expression, and therefore, can be used as non-pharmacological therapy.

Keywords: immunohistochemistry, red guava juice, secondary hyperuricemia, TGF- β .

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INTRODUCTION

Secondary hyperuricemia is a condition caused by disorders that affect the production and excretion of uric acid in the body. Elevated uric acid levels can negatively affect many organ systems, including kidney. Hyperuricemia contributes to kidney disorders through various mechanism that leads to kidney injury.¹ Hyperuricemia is associated with increased proteinuria, decreased renal function, glomerulosclerosis, renal interstitial fibrosis, and pre-glomerular vasculopathy. Increased renin expression appears to be the other side effects of hyperuricemia on renal function.^{2,3} Abnormal increase in TGF- β expression was thought to be an essential factor in the development of renal dysfunction, which may cause fibrosis and glomerulosclerosis through the accumulation of extracellular matrix (ECM).^{4,5}

TGF- β is a mediator in renal fibrosis. TGF- β 1 suppresses the immune system by stimulating ECM components. Excessive TGF- β expression will lead to the accumulation of ECM, scar tissues and fibrosis, which could damage the tissues.⁶ This

protein affects the main pathway of Smad signalling. During fibrogenesis, Smad3 becomes very active, as opposed to the decline in the inhibition regulation of Smad7 through a degradation mechanism. The shift in the balance between Smad3 and Smad7 results in the accumulation and activation of myofibroblasts, excessive production of ECM, and decreased ECM degradation in impaired kidneys.⁷

In hyperuricemia, increased xanthine oxidation expression and activity can increase the formation of Reactive Oxygen Species (ROS), where ROS activate proinflammatory cytokines and TGF- β 1. Enhancement of antioxidant and anti-inflammatory approaches can prevent ROS formation by targeting xanthine oxidase and suppressing TGF- β through molecular pathways that can directly suppress oxidative stress and renal dysfunction.^{8,9} In several studies, vitamin C supplementation, showed antihyperuricemic, antioxidant, and nephroprotective activities in mice models induced with oxonic acid (hyperuricemia) and kidney injury. The results of the study on vitamin C supplementation can provide clinical

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therapeutic value in the treatment of hyperuricemia with renal dysfunction.³ Another study showed that the administration of *Dioscorea alata* extract containing polyphenol antioxidants was able to control fibrosis by suppressing TGF- β signalling. Quercetin, a flavonoid component, can improve the degree of renal fibrosis in mice with chronic kidney disease, as well as reducing MDA and glutathione peroxidase levels as markers of ROS.^{10,11} Other flavonoid components, such as rutin, can also potentially be used as antioxidants and inhibit TGF- β /Smad signalling when given at dosages of 15 and 45 mg/kg for 20 weeks in male Wistar mice.¹²

Red guava fruit contains antioxidants such as vitamin C, flavonoid, polyphenol, saponin, and quercetin.^{3,13} Guava fruit can protect the kidney against the development of diabetes through its anti-oxidative, anti-inflammatory, and antiglycative effects. Several studies have shown that antioxidants contained in red guava fruit have anti-hyperuricemia and anti-inflammatory properties.¹⁴ Antioxidants contained in red guava juice (vitamin C, polyphenol, and flavonoid) can significantly reduce the level of uric acid and creatinine in mice.¹⁵ Based on the previous discussion, it is possible that the administration of red guava juice (*Psidium Guava Linn.*) affects TGF- β protein expression in mice (*Mus Musculus*) and suppresses the development kidney injury associated with

hyperuricemia. Therefore, this study conducted to prove that hypothesis.

METHOD

This study was an experimental post-test only design. This study used 48 white mice (*Mus Musculus*) as the animal model samples. The criteria were male mice aged two months with a weight of 20-35 grams. The mice were given seven days to adapt to their environment before they were distributed into six different groups with different treatments in each group (Table 1). A total of eight mice were assigned to each treatment group. The different treatments conducted to the mice groups were: normal (N), groups that were given standard feed and aquadest; negative control (K-) that were given standard feed, aquadest, and potassium oxonate; positive control (K+) that were given standard feed, aquadest, potassium oxonate, and allopurinol therapy; red guava juice. Intervention group with dose 1 (O1) that were given standard feed, aquadest, potassium oxonate, and red guava juice of 5 ml/mice body weight/day; red guava juice intervention dose 2 (O2) that were given standard feed, aquadest, potassium oxonate, and red guava juice of 10 ml/mice body weight/day; and red guava juice intervention dose 3 (O3) that were given standard feed, aquadest, potassium oxonate, and red guava juice of 20 ml/mice body weight/day (Table 1).

The administration of red guava juice was done for 21 days after the mice were induced with potassium oxonate at a dosage of 250 mg/mice body weight for 14 days. The maintenance and treatment of laboratory animals were done in the Center for Food and Nutrition Studies Laboratory, Gadjah Mada University, Yogyakarta, according to the procedure for animal experiments certificate number: PSPG-UGM/02/SK/IV/2017. The feed used in this study was Comfeed that was given ad libitum. Ethical clearance was obtained from the Faculty of Medicine, Universitas Sebelas Maret, with the number: 69/UN27.6/KEPK/2018. TGF- β protein expression in mice kidneys was identified through immunohistochemistry. The making and observation of preparation were done in the Anatomical Pathology laboratory in the Faculty of Medicine, Universitas Sebelas Maret using the Olympus XC10 light microscope.

The independent variable in this study was the administration dose of red guava juice, while the dependent variable was TGF- β protein expression. Red guava juice in the study was obtained from fresh red guava, which was then juiced using a juicer without additional water. The administration of guava juice in mice was

Table 1. Mice (*Mus Musculus*) treatment groups

Treatment group	Potassium Oxonate Induction	Feed + Aquadest	Allopurinol	Red Guava Juice Intervention (ml/mice body weight/day)		
				5	10	20
N	-	+	-	-	-	-
K-	+	+	-	-	-	-
K+	+	+	+	-	-	-
O1	+	+	-	+	-	-
O2	+	+	-	-	+	-
O3	+	+	-	-	-	+

- N : Normal (standard feed, aquadest)
 K- : Negative control (standard feed, aquadest, potassium oxonate)
 K+ : Positive control (standard feed, aquadest, potassium oxonate, allopurinol)
 O1 : Red guava juice intervention dose 1 (standard feed, aquadest, potassium oxonate, red guava juice of 5 ml/mice body weight/day)
 O2 : Red guava juice intervention dose 2 (standard feed, aquadest, potassium oxonate, red guava juice of 10 ml/mice body weight/day)
 O3 : Red guava juice intervention dose 3 (standard feed, aquadest, potassium oxonate, red guava juice of 20 ml/mice body weight/day).
 + : Given
 - : Not given

given once per day. TGF- β protein expression, using which measured by immunohistochemical methods was the parameters for kidney dysfunction due to secondary hyperuricemia. The results of immunohistochemical preparations obtained in the form of images and grading of TGF- β protein expression.

The procedure for specimen processing was started with kidney fixation with 10% Neutral Buffer Formalin (BNF) solution. Then it was cut (trimmed) with a thickness of ± 1 mm and put in a tissue cassette. After that, the organ in the tissue cassette is inserted into the tissue processor to be dehydrated using alcohol with a graded composition (70%, 80%, 90%, 95%). The specimens then cleared (clearing) by a series of xylol. The next process is immersion and printing. Dehydrated preparations after planted in moulds that have been filled with paraffin, cooled using cold plates and stored in the refrigerator until ready to be trimmed. After the preparation was frozen in a paraffin block, then it was cut using a microtome with a thickness of 5 μ m to form a ribbon-like shaped and placed on the surface of warm air to prevent folds on the tape. The processed specimen then placed on an object glass and dried at room temperature.

The measurement of TGF- β Protein Expression signalling were conducted with Immunohistochemistry technique. The 4-5 microns

thick paraffin blocks were put on poly-L-lysine slide then incubated at 37°C for a night. Deparaffinization was carried out by putting preparations into several series of xylol and alcohol, washed with distilled water and given a few drops of 0,3% H₂O₂ in methanol to suppress endogenous peroxidase activity. The specimen then washed with aquadest and then PBS 2 times. Antigens retrieval was carried out in a microwave oven with Tris EDTA (pH 9) at high temperatures at close to boiling point then continued at low temperatures. After the specimens cooled into room temperature, it washed with PBS 2 times. Then few drops blocking serum were given continued with a drop of antibodies that have been prepared. The specimen then incubated at 4°C, then washed with PBS, biotin, and addition of peroxidase enzyme substrate. Few drops of hematoxylin and rinsed off with running water and then mounted. Microscopic examination was performed using the Olympus XC 10 light microscope.

The study data were analysed using the SPSS program version 20. The Normality test was performed using the Shapiro Wilk method. The difference between groups was statistically analysed using the one-way analysis of variance (ANOVA), followed with the Post Hoc test with Tukey High Significant Difference (HSD) if data were normally distributed. Data that were not normally distributed were analysed using the Kruskal Wallis test, followed by the Mann Whitney test. The difference between groups was considered significant if $p < 0.05$.

Table 2. Effect of red guava juice on TGF- β expression in mice (*Mus Musculus*) kidneys with secondary hyperuricemia model.

Group 1	Group 2	p
Normal	Negative control	0.001*
	Positive control	0.001*
	O1 (5 ml/mice body weight)	0.001*
	O2 (10 ml/ mice body weight)	0.001*
	O3 (20 ml/ mice body weight)	0.001*
Negative Control	Positive control	0.089
	O1 (5 ml/mice body weight)	0.043*
	O2 (10 ml/mice body weight)	0.164
	O3 (20 ml/mice body weight)	1.000
Positive Control	O1 (5 ml/mice body weight)	0.467
	O2 (10 ml/mice body weight)	0.811
	O3 (20 ml/mice body weight)	0.031*
O1 (5 ml/mice body weight)	O2 (10 ml/mice body weight)	0.373
	O3 (20 ml/mice body weight)	0.006*
O2 (10 ml/mice body weight)	O3 (20 ml/mice body weight)	0.051

*significant difference ($p < 0.05$) Post Hoc Mann Whitney test

RESULTS

The normality test revealed that data were not normally distributed, thus, the difference between groups was analysed using the Kruskal Wallis test. The analysis using the Kruskal Wallis test found that there was a significant difference among groups related to the TGF- β expression ($p = 0.001$). Accordingly, the Post Hoc Mann Whitney test was then performed. Table 2 presents the results of the Post Hoc Mann Whitney test, where $p < 0.05$ was considered as statistically significant.

DISCUSSION

Transforming growth factor-beta (TGF- β 1) is a multifunctioning regulator that modulates cell proliferation, differentiation, apoptosis, adhesion, the migration of various types of cells, and the induction of ECM protein production. TGF- β 1 has been recognised as the most dominant cytokine that plays a central role in the development of glomerulosclerosis or interstitial fibrosis. TGF- β 1 stimulates fibroblast cell membrane receptors to express type-I collagen as a result of interstitial

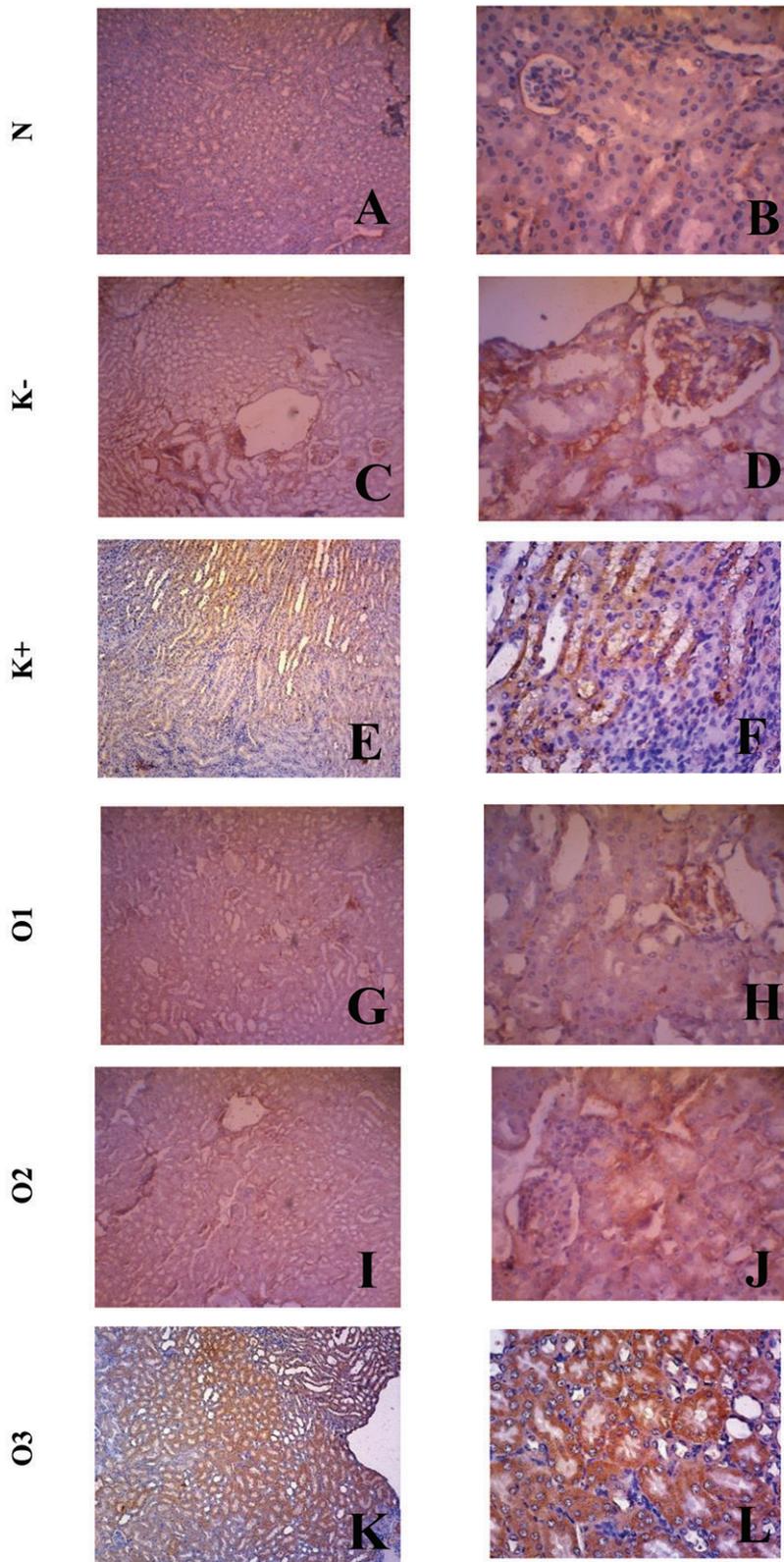


Figure 1. The immunohistochemical result of TGF- β in Kidney Mice (*Mus Musculus*). N: Normal group; K-: Pottasium oxonate without therapy; K +: Pottasium oxonate + Allopurinol therapy; O1: Pottasium oxonate + red guava juice therapy 5 ml/Kg/day; O2: Pottasium oxonate + red guava juice therapy 10 ml/Kg/day; and O3: Pottasium oxonate + red guava juice therapy 20 ml/kg/day. A, C, E, G, I, K (at 100x magnification). B, D, F, H, J, L (at 400x magnification).

fibrosis. Moreover, TGF- β 1 will also stimulate mesangial cell membrane receptors to express type-IV collagen, which could lead to glomerulosclerosis. TGF- β 1 has been known to play a critical role in renal fibrogenesis through pathways that involve SMAD signalling.^{7,16-18}

As presented in Table 2, red guava juice can significantly affect TGF- β expression in mice. A significant difference was found between the negative control treatment (no intervention) and treatment of red guava juice at a dosage of 5 ml/mice body weight/day (O1) ($p = 0.043$) and the positive control (allopurinol therapy) with the treatment of red guava juice at a dosage of 20 ml/mice body weight/day (O3) ($p = 0.031$). The administration of allopurinol can indirectly slow the progression of renal disorder by decreasing the level of uric acid in the blood through the inhibition of xanthine oxidase.² Hyperuricemia plays a role in the pathogenesis of kidney disease as it induces high blood pressure, renal afferent arteriopathy, increased glomerular hydrostatic pressure, and renal scarring. Lowering the systolic blood pressure and reducing the level of uric acid may slow the progression of renal dysfunction.⁸

Red guava juice contains vitamin C, which acts as an antioxidant. This antioxidant prevents radical-induced tissue damage by preventing the formation of radicals and suppresses the effect of lipid peroxidation, thereby slowing the progression of kidney disease. Vitamin C also affects the proliferation of damaged cells and the formation of new tissues to restore renal function.^{3,15} Polyphenol is another antioxidant that can control renal fibrosis by suppressing TGF- β signalling.¹⁹

This study showed that the administration of red guava juice affects TGF- β expression, which is a marker of renal dysfunction. Red guava fruit contains antioxidants such as vitamin C, flavonoid, polyphenol, saponin, and quercetin.^{3,13} This is in line with several studies stating that non-pharmacological treatment using antioxidants can inhibit or suppress TGF- β expression. The combination of Asiatic acid (AA), a compound from purified *Centella asiatica*, and naringenin (NG), a flavonoid from grapefruits and citrus fruits, significantly suppressed renal fibrosis by enhancing the inhibition of TGF- β /Smad signalling both *in vivo* and *in vitro* in the unilateral ureteral obstruction (UUO) model. The combination of these two compounds restores the balance between TGF- β /Smad signalling. It has been identified that AA functioned as a Smad7 agonist, whereas NG was a Smad3 inhibitor. The combination of AA and NG may represent a novel therapy for chronic kidney disease with renal fibrosis.¹⁶ Astilbin, a flavonoid

compound derived from the isolation of rhizome of *Smilax glabra* Roxb, was found to affect uric acid levels and renal function in potassium oxonate-induced hyperuricemic mice. It was thought works as modulator of renal transporters through regulating inflammatory response and oxidative stress in mice.²⁰

This study found a significant difference ($p < 0.05$) in TGF- β expression between the negative control treatment (K-) and the treatment of red guava juice with a dose of 5 ml/kg/day (O1). Additionally, it was found that increasing the dose of red guava juice did not result in a significant difference in TGF- β expression. Red guava juice intervention at a high dose also contains a high fructose diet, which does not provide the best protective function towards the kidneys and pancreas. It also could be due to a lower percentage of fibres and a higher sugar load in the guava juice than the fruit itself. A higher sugar load may induce intracellular ROS through AGE (Advanced Glycation End-products) and various cytokine so that red guava juice cannot work effectively to suppress TGF- β of mice with secondary hyperuricemia.²¹

Phenolic compounds contained in polyphenol and flavonoid antioxidants may also explain why the administration of red guava juice at dosages of 10 ml/mice body weight/day and 20 ml/mice body weight/day did not significantly affect TGF- β expression compared to a lower dose of 5 ml/mice body weight/day. The use of certain doses and the presence of metal ions can cause phenolic compounds to act as pro-oxidants. The presence of oxygen and metal ions will catalyse the redox cycle of phenolic compounds to form phenoxyl radicals, which could damage the DNA and induce lipid peroxidation that may seriously damage the cells.^{22,23}

CONCLUSION

It can be concluded that the administration of red guava juice (*Psidium Guava* Linn.) in secondary hyperuricemic mice (*Mus Musculus*) can suppress TGF- β protein expression and can therefore potentially be used as an alternative non-pharmacological therapy.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR'S CONTRIBUTIONS

RNSA was the owner of the study concept, designed the study, prepared the draft manuscript, collected and synthesised the data, and wrote the manuscript. BW and PD were in charge of data collection, helped analyse and interpret the data, and reviewed the manuscript.

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