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Potential of Karamunting (*Rhodomyrtus tomentosa*) Fraction Against Kidney Damage in Diabetic Rats

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ABSTRACT

Introduction: Karamunting (*Rhodymyrtus tomentosa*) is Indonesian native plants that contain high antioxidant compounds with the potential to suppress oxidative stress activity in cells. This study is the first research to specifically explore the role of the Karamunting fraction in protecting kidney damage due to the formation of AGEs in the renal glomerulus in diabetes mellitus.

Objective: This study aimed to assess the role of Karamunting on blood glucose profile, HbA1c, insulin profile and kidney condition (AGEs expression, TNF alpha and kidney histology) on diabetic rats.

Methods. The Karamunting extraction process was carried out by maceration with ethanol. Induction of diabetes mellitus was carried out using streptozotocin. Thirty male rats (*Rattus norvegicus*) Wistar strain weighing between 200 - 250 grams were the subject in this study. HbA1c, insulin, TNF- α , AGEs levels were evaluated in the kidney tissue. Histological assessment of the kidney was carried out.

Results. Karamunting can improve blood glucose levels in diabetic rats. This improvement in blood glucose levels causes an improvement in the level of glycosylated haemoglobin (HbA1c). Administration of Karamunting fraction can prevent AGEs deposition in the renal tissue, which in turn will inhibit inflammation and prevent further damage to the kidneys.

Conclusion. Karamunting fraction can improve blood glucose regulation so that it can prevent further damage and complications of the kidney organs in diabetic rats.

Keywords: glycated haemoglobin A, insulin, protective agents, oxidative stress, *Rhodymyrtus tomentosa*.

INTRODUCTION

Diabetes mellitus is a clinical condition characterized by systemic impairment of glucose regulation which leads to accumulation of glucose products in the blood and tissues.[1] Glucose that accumulates in the blood and tissues will react with proteins/nucleic acids/lipids to form

various Amadori products which will lead to the formation of advanced glycation end products (AGEs).[1,2] AGEs are protein modification products and lipids that are responsible for the progression and complications of diabetes mellitus. The kidneys are vital organs that play a role in the filtration of blood, electrolytes and plasma. As a filtration organ, the kidneys are the organs most at risk of becoming a buildup of AGEs products.[2,3] Accumulation of AGEs products on the renal filtration tissue, the glomerulus, causes activation of the inflammatory cascade. Activation of the inflammatory cascade is responsible for the activation of death receptors from cells which will lead to cell death.[3] Cell death in the glomerulus and tubule causes the blood filtration function not to take place optimally. This condition causes chronic kidney failure. Kidney failure due to diabetes mellitus is one of the most common complications experienced by people with diabetes mellitus.[3,4] Kidney failure causes dependence on hemodialysis. Limited hemodialysis tools and hemodialysis facilities cause severe morbidity and mortality, in addition to the increasing health costs that must be borne by the patient and the state. Therefore, it is essential and very urgent to explore new therapeutic modalities to prevent severe complications of diabetes mellitus in the renal organs due to accumulation of AGEs and chronic inflammation.

Indonesia is a country with the second-largest vegetation wealth in the world. This wealth of vegetation is an opportunity for Indonesia to be developed into potential new therapeutic modalities. Karamunting (*Rhodymyrtus tomentosa*) is one of Indonesia's native plants from the Bangka Belitung archipelago. This plant is a type of shrub with high antioxidant content. Antioxidants are compounds with the potential to suppress oxidative stress activity in cells. In contrast, the accumulation of AGEs in cells and tissues plays a role in the initiation of oxidative stress in cells and tissues.[5] Oxidative stress inhibition causes inhibition of the inflammatory cascade and inhibition of cell and tissue death and damage.

This study is the first research that seeks to specifically explore the role of the Karamunting fraction in protecting kidney damage due to the formation of AGEs in the renal glomerulus in diabetes mellitus. This study will assess at the role of Karamunting on blood glucose profile, glycated haemoglobin, insulin profile and kidney condition with a focus on the expression of AGEs, TNF alpha and kidney histology.

MATERIAL AND METHODS

Animal Model

A total of thirty male rats (*Rattus norvegicus*) Wistar strain obtained from the Eureka Research Laboratory (Palembang, Indonesia) weighing between 200 - 250 grams. All experimental animals were kept in cages under controlled conditions of 12 hours of the light-dark cycle, temperature $22 \pm 1^{\circ}\text{C}$ and humidity 40-60% and given ad libitum food. The research treatments and procedures have received approval from the medical research ethics committee of the Faculty of Medicine, Universitas Sriwijaya (No. 254/kptfkunsri-rsmh/2020).

Karamunting fractionation preparation

Karamunting simplicia was obtained from the Tawangmangu Herbal Research Center, Karanganyar, Indonesia. The Karamunting extraction process was carried out by maceration in which 500 grams of simplicia were macerated with 96% ethanol for 72 hours. Furthermore, carried out a proportion between the dregs and macerate. Maserate continued with the fractionation process with n-hexane, ethyl acetate and water solvents, to obtain the n-hexane fraction (FH), ethyl acetate fraction (FE) and water fraction (FA) from Karamunting. Karamunting water fraction is a fraction that has been tested and explored more intensively.

Induction of diabetes mellitus

Induction of diabetes mellitus was carried out using streptozotocin (STZ) compounds (Sigma Aldrich, Singapore). A total of 55 mg/kg BW solution of STZ in citrate buffer (0.1 M, pH 4.5) was injected into intraperitoneal which had been fasted overnight [6]. Non-diabetic controls were injected with citrate buffer (0.1 M, pH 4.5). After seven days of STZ induction, fasting blood glucose levels were measured with a spectrophotometer (Biorad, Singapore). Rats with a blood glucose concentration of more than 200 g/dL are categorized as diabetic rats.

Chronic treatment with Karamunting fraction

The rats were grouped into six groups, namely group 1: a group of non-diabetic white rats, group 2: a group of streptozocin-induced diabetes mellitus without treatment, group 3-6: a group of rats induced diabetes mellitus given glibenclamide 5 mg/kg BW (Dexamedica, Palembang, Indonesia) [7]; Karamunting fraction dose of 10 mg/kg BW; 20 mg/kg BW and 40 mg/kg BW. Glibenclamide and fractions were given for 30 days.

Blood Sample Collection

A blood sample was taken from the orbital vein on the first day after overnight fasting. After 30 days of being given standard drugs or Karamunting fraction, the rats that had been fasted overnight were sacrificed, and blood samples were taken for biochemical examination. HbA1c was measured from blood samples that had been collected in the EDTA tube with the rat HbA1c assay kit. Insulin was measured from blood samples using an ELISA Insulin rating (Cloud Clone, Hangzhou, China). Blood glucose measurement was carried out with a spectrophotometer (Biorad, Singapore). Before being stored at -20°C, the blood samples in the EDTA tube were centrifuged

(Biorad, Singapore) at a speed of 5000 rpm at 25°C, 10 minutes. The supernatant is collected and stored to be used as a sample for biochemical testing.

Evacuation of experimental animal's kidney

Before organ evacuation, the Wistar rat was anaesthetized by injecting 10% chloral hydrate (3.5 ml/kg BW). Furthermore, the operation was performed on the flank area with an incision of about 5 centimetres to obtain the kidney. Next, the kidneys are evacuated and divided, where some are put into ice packed containers, and some are put into boxes filled with 10% NBF liquid (normal buffer formalin). The kidneys that were put into the ice-packed container were homogenized and centrifuged at a speed of 5000 rpm, for 30 minutes at 4°C. The supernatant was then taken and stored in a centrifuge tube at -20°C, to check levels of TNF alpha and AGEs. Part of the kidneys that had been placed in 10% NBF solution was prepared for histopathological examination.

ELISA (Enzyme-Linked Immunosorbent Assay): HbA1c, Insulin, TNF- α , AGEs

Rat ELISA HbA1c examined HbA1c, insulin, TNF- α , AGEs levels in the kidney tissue; Insulin; TNF- α ; AGEs (Cloud Clone, Hangzhou, China), based on the protocol contained in the manufacturer's protocols (sensitivity of ELISA kit 92%). Briefly, 50 μ l of standard diluent or serum samples were added to the well and incubated at 37°C for 30 minutes. After the plates were washed, 100 μ l of the biotinylated antibody solution was added and set for 30 minutes at 37°C. After washing three times, 50 μ l avidin-peroxidase complex solution was added and incubated for 15 minutes at 37°C. After washing, 50 μ l of tetramethylbenzidine colour solution was added and set in the dark for 15 minutes at 37°C. Finally, 50 μ l stop solution was added to stop the reaction, and the optical density (OD) was measured using an ELISA reader (Biorad, Singapore), the wavelength

of 450 nm. By analyzing the standard curve of the standard of each examination protein, the levels of HbA1c, insulin, TNF- α and AGEs were obtained.

PAS (Periodic acid-Schiff) histological examination staining

Kidney tissue that was fixed with 10% NBF was dehydrated using graded alcohol and xylene, then paraffinized and cut to a thickness of 5 μ m using a rotary microtome (Leica, Illinois, USA). The results of the cut were then placed on a glass object, added to the Schiff's reagent, let stand for 5-7 minutes and then stained with Mayer's Hemalaum solution for 3 minutes. Subsequently, washed, dehydrates, cleared in xylene and mounted with mounting media were carried out. Histopathological changes in synovial tissue were analyzed under a microscope (Olympus, Tokyo, Japan).

Phytochemical test

Test for phenols

The test was performed by using the method of Sofowora [8]. 2 ml extract was taken in a beaker glass. Then, 2 ml of ferric chloride solution was added. A deep bluish-green solution indicated the presence of phenols.

Test for terpenoids

Salkowski test was performed by using the method of Edeoga et al. [9]. 5 ml of aqueous extract was mixed in 2 ml of chloroform. Then 3 ml of concentrated sulfuric acid was poured to form a layer. A reddish-brown colouration of interface indicated the presence of terpenoids.

Test for saponins

The test was performed using the method of Edeoga et al. [9]. 2 g of the powdered sample boiled in 20 ml of distilled water in a water bath and filtered the solution. Then 10 ml of the filtrate was mixed with 5 ml of distilled water and shake vigorously for a stable, persistent foam. The foam was mixed with three drops of olive oil and shake vigorously, which leads to the formation of the emulsion; indicated the presence of saponins.

Test for flavonoids

The test was performed by using the method of Harborne [10]. 1 g powdered sample was heated with 10 ml ethyl acetate over a steam bath (40–50°C) for 5 minutes. The filtrate was treated with 1 ml dilute ammonia. A yellow colouration demonstrated positive test for flavonoids.

Test for alkaloids

The test was performed by using the method of Harborne [10]. 1 g powdered sample was extracted with 5 ml methanol and 5 ml of 2N hydrochloric acid. Then the filtrate was tested with Meyer's and Wagner's reagents. The samples were scored positive, based on turbidity.

Statistical analysis

All data were presented as mean \pm standard deviation, and all statistical analyzes were performed with the SPSS 25 (IBM, Jakarta, Indonesia) program. One way ANOVA followed by post hoc analysis was carried out to assess the difference in mean expression levels of each protein. $P < 0.05$ was determined as an indication that there was a significant difference in mean levels.

RESULTS

Table 1 shows the potential for Karamunting fraction on glucose regulation in Wistar rats. Karamunting extract was able to improve blood glucose levels in rats. This improvement in blood

glucose levels causes an improvement in the level of glycosylated haemoglobin (HbA1c). Karamunting fraction has been shown to increase insulin secretion from the pancreas, which is believed to improve blood glucose regulation.

Table 2 provides an overview of the potential for Karamunting fraction in inhibiting complications of kidney problems in people with diabetes mellitus. The Karamunting fraction can improve the directional glucose regulation so that there is inhibition of AGEs deposition in the renal tissue so that it can inhibit inflammation and the inflammatory cascade played by TNF-alpha expression.

Figure 1 is a histopathological picture, as well as cross-check of the results of biochemical and molecular markers on the cellular image. Figure 1 shows that administration of Karamunting fraction can prevent AGEs deposition in the renal tissue, which in turn will inhibit inflammation and prevent further damage to the kidneys.

Table 3 shows that each Karamunting water fraction is rich in flavonoids with very high antioxidant potential. Flavonoids are the primary, secondary metabolites believed to play a role in inhibiting inflammation and oxidants due to the accumulation of AGEs.

DISCUSSION

Karamunting water fraction is part of further exploration carried out to isolate further the presence of polar compounds in the Karamunting extract. The Karamunting water fraction shows that the flavonoid content is relatively high and is believed to be the primary, secondary metabolite that plays a role in improving glucose regulation and inhibition of AGEs deposition in kidney tissue. Karamunting is rich in flavonoids with potential as antioxidants.

Diabetes mellitus is a clinical disorder, where there is severe oxidative stress due to chronic stress conditions on cells [1,5]. Excess glucose intake will lead to overproduction of insulin in pancreatic beta cells. Pancreatic beta cells will be stimulated to produce large amounts of insulin and quickly. This condition causes a lot of misfolded insulin protein which will lead to the accumulation of misfolded insulin protein in the endoplasmic reticulum. This condition causes cell stress which leads to a cascade of oxidative stress.[7,11] Oxidative stress on pancreatic beta cells will cause the activation of the inflammatory cascade, which will lead to the activation of death receptors and cause the death of pancreatic beta cells.[12-13] This condition will lead to insulin deficiency which will worsen glucose regulation.[14-16] The absence of insulin will cause the accumulation of glucose outside the cells in blood vessels and tissues.[17-19] This condition causes a systemic glycosylation process in all organs, especially in the kidneys as the blood filtration organ. This glycosylation process causes the accumulation of AGEs products, which are the reaction products between glucose and proteins, lipids and nucleic acids.[18-20]

The accumulation of AGEs in the kidney, especially in the glomerulus, causes the activation of an inflammatory transcription factor that will trigger the activation of the proinflammatory cytokine, TNF- α [21-24]. Activation of inflammation in the kidneys leads to kidney damage [25-27]. This damage causes the kidneys to decrease their function and role as a filtration organ [28]. Karamunting acts as an antioxidant that will be able to improve oxidative stress conditions in diabetes mellitus [29,30]. This study proves the ability of Karamunting to enhance the regulation of blood glucose, insulin and HbA1c. Good glucose regulation ability will reduce the production of AGEs which will cause inhibition of AGEs accumulation in the tissue [18,19]. The decrease in AGEs will cause a reduction in the production of proinflammatory cytokines which will lead to repair of damage and deterioration of cell function.

CONCLUSION

Karamunting fraction can improve blood glucose regulation so that it can prevent further damage and complications of the kidney organs in the condition of diabetes mellitus in rat.

CONFLICT OF INTEREST

Authors declared no conflict of interest.

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Table 1. Effect Karamunting Extract in Glucose Regulation

Groups	Fasting blood glucose (mg/dL)		HbA1c ($\mu\text{g/mL}$)		Insulin (IU/mL)	
	Day 1	Day 30	Day 1	Day 30	Day 1	Day 30
1	89.9 \pm 6.87	87.8 \pm 5.37	3.9 \pm 0.17	3.8 \pm 0.27	188.2 \pm 15.32	188.1 \pm 16.65
2	389.9 \pm 26.71	399.9 \pm 28.27	12.8 \pm 0.82	12.9 \pm 0.65	32.8 \pm 0.11	32.9 \pm 0.13
3	382.2 \pm 21.13	181.3 \pm 11.27*	12.5 \pm 0.76	7.8 \pm 0.23*	32.4 \pm 0.21	123.1 \pm 10.12*
4	384.3 \pm 23.21	299.2 \pm 24.57*	12.3 \pm 0.43	10.1 \pm 0.95*	32.3 \pm 0.12	87.8 \pm 4.65*
5	383.2 \pm 27.97	197.3 \pm 16.47*	12.9 \pm 0.68	7.9 \pm 0.44*	32.1 \pm 0.22	124.3 \pm 10.12*
6	386.6 \pm 26.57	169.9 \pm 10.22*	12.6 \pm 0.77	6.9 \pm 0.33*	32.3 \pm 0.23	132.9 \pm 11.22*

*ANOVA, pos hoc Bonferroni, $p < 0,05$ VS Group 2

Group 1: non-diabetic rat; group 2: streptozocin-induced diabetes without treatment; group 3: streptozocin-induced diabetes with glibenclamide treatment (5 mg/kg BW); group 4: streptozocin-induced diabetes rats with Karamunting fraction (10 mg/kg BW); group 5: streptozocin-induced diabetes rats with Karamunting fraction (20 mg/kg BW); group 6: streptozocin-induced diabetes rats with Karamunting fraction (40 mg/kg BW).

Table 2. Effect of Karamunting Extract in AGEs and TNF- α

Groups	AGEs ($\mu\text{g/mL}$)	TNF- α ($\mu\text{g/mL}$)
1	17.7 \pm 1.37	89.9 \pm 7.76
2	989.9 \pm 58.74	1221.4 \pm 87.65
3	198.3 \pm 16.23*	453.6 \pm 32.23*
4	249.6 \pm 21.27*	567.8 \pm 43.15*
5	193.3 \pm 15.57*	447.2 \pm 40.41*
6	169.9 \pm 13.92*	398.8 \pm 34.93*

*ANOVA, pos hoc Bonferroni, $p < 0,05$ versus group 2

Group 1: non-diabetic rat; group 2: streptozocin-induced diabetes without treatment; group 3: streptozocin-induced diabetes with glibenclamide treatment (5 mg/kg BW); group 4: streptozocin-induced diabetes rats with Karamunting fraction (10 mg/kg BW); group 5: streptozocin-induced diabetes rats with Karamunting fraction (20 mg/kg BW); group 6: streptozocin-induced diabetes rats with Karamunting fraction (40 mg/kg BW).

Table 3. Phytochemical Test of Karamunting Fraction

Test Material	Saponin	Alkaloid	Triterpenoid	Steroid	Flavonoid
Karamunting fraction	+	+	+	+	+++

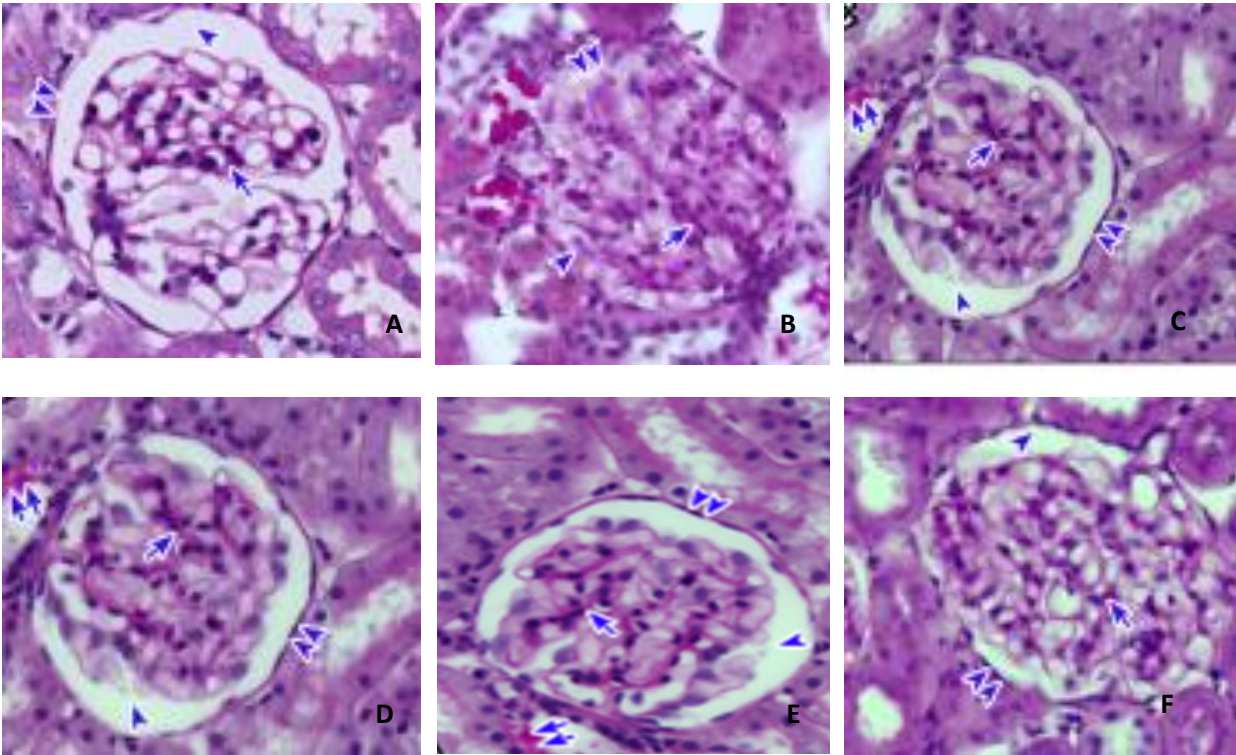


Figure 1. Histological overview of kidney tissue. PAS Staining. A: group 1, B: group 2, C: group 3, D: group 4, E: group 5, F: group 6. Glycogen deposition (double arrows), Bowman's capsule (double arrowheads), Bowman's space (arrowhead), mesangial area (arrow). 400x magnification.

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ABSTRACT

Introduction: Karamunting (*Rhodomyrtus tomentosa*) is Indonesian native plants that contain high antioxidant compounds with the potential to suppress oxidative stress activity in cells. This study is the first research to specifically explore the role of the Karamunting fraction in protecting kidney damage due to the formation of AGEs in the renal glomerulus in diabetes mellitus.

Objective: This study aimed to assess the role of Karamunting on blood glucose profile, HbA1c, insulin profile and kidney condition (AGEs expression, TNF alpha and kidney histology) on diabetic rats.

Methods. The Karamunting extraction process was carried out by maceration with ethanol. Induction of diabetes mellitus was carried out using streptozotocin. Thirty male rats (*Rattus norvegicus*) Wistar strain weighing between 200 - 250 grams were the subject in this study. HbA1c, insulin, TNF- α , AGEs levels were evaluated in the kidney tissue. Histological assessment of the kidney was carried out.

Results. Karamunting can improve blood glucose levels in diabetic rats. This improvement in blood glucose levels causes an improvement in the level of glycosylated haemoglobin (HbA1c). Administration of Karamunting fraction can prevent AGEs deposition in the renal tissue, which in turn will inhibit inflammation and prevent further damage to the kidneys.

Conclusion. Karamunting fraction can improve blood glucose regulation so that it can prevent further damage and complications of the kidney organs in diabetic rats.

Keywords: glycated haemoglobin A, insulin, protective agents, oxidative stress, *Rhodomyrtus tomentosa*.

INTRODUCTION

Diabetes mellitus is a clinical condition characterized by systemic impairment of glucose regulation which leads to accumulation of glucose products in the blood and tissues.[1] Glucose that accumulates in the blood and tissues will react with proteins/nucleic acids/lipids to form

various Amadori products which will lead to the formation of advanced glycation end products (AGEs).[1,2] AGEs are protein modification products and lipids that are responsible for the progression and complications of diabetes mellitus. The kidneys are vital organs that play a role in the filtration of blood, electrolytes and plasma. As a filtration organ, the kidneys are the organs most at risk of becoming a buildup of AGEs products.[2,3] Accumulation of AGEs products on the renal filtration tissue, the glomerulus, causes activation of the inflammatory cascade. Activation of the inflammatory cascade is responsible for the activation of death receptors from cells which will lead to cell death.[3] Cell death in the glomerulus and tubule causes the blood filtration function not to take place optimally. This condition causes chronic kidney failure. Kidney failure due to diabetes mellitus is one of the most common complications experienced by people with diabetes mellitus.[3,4] Kidney failure causes dependence on hemodialysis. Limited hemodialysis tools and hemodialysis facilities cause severe morbidity and mortality, in addition to the increasing health costs that must be borne by the patient and the state. Therefore, it is essential and very urgent to explore new therapeutic modalities to prevent severe complications of diabetes mellitus in the renal organs due to accumulation of AGEs and chronic inflammation.

Indonesia is a country with the second-largest vegetation wealth in the world. This wealth of vegetation is an opportunity for Indonesia to be developed into potential new therapeutic modalities. Karamunting (*Rhodymyrtus tomentosa*) is one of Indonesia's native plants from the Bangka Belitung archipelago. This plant is a type of shrub with high antioxidant content. Antioxidants are compounds with the potential to suppress oxidative stress activity in cells. In contrast, the accumulation of AGEs in cells and tissues plays a role in the initiation of oxidative stress in cells and tissues.[5] Oxidative stress inhibition causes inhibition of the inflammatory cascade and inhibition of cell and tissue death and damage.

This study is the first research that seeks to specifically explore the role of the Karamunting fraction in protecting kidney damage due to the formation of AGEs in the renal glomerulus in diabetes mellitus. This study will assess at the role of Karamunting on blood glucose profile, glycated haemoglobin, insulin profile and kidney condition with a focus on the expression of AGEs, TNF alpha and kidney histology.

MATERIAL AND METHODS

Animal Model

A total of thirty male rats (*Rattus norvegicus*) Wistar strain obtained from the Eureka Research Laboratory (Palembang, Indonesia) weighing between 200 - 250 grams. All experimental animals were kept in cages under controlled conditions of 12 hours of the light-dark cycle, temperature $22 \pm 1^{\circ}\text{C}$ and humidity 40-60% and given ad libitum food. The research treatments and procedures have received approval from the medical research ethics committee of the Faculty of Medicine, Universitas Sriwijaya (No. 254/kptfkunsri-rsmh/2020).

Karamunting fractionation preparation

Karamunting simplicia was obtained from the Tawangmangu Herbal Research Center, Karanganyar, Indonesia. The Karamunting extraction process was carried out by maceration in which 500 grams of simplicia were macerated with 96% ethanol for 72 hours. Furthermore, carried out a proportion between the dregs and macerate. Maserate continued with the fractionation process with n-hexane, ethyl acetate and water solvents, to obtain the n-hexane fraction (FH), ethyl acetate fraction (FE) and water fraction (FA) from Karamunting. Karamunting water fraction is a fraction that has been tested and explored more intensively.

Induction of diabetes mellitus

Induction of diabetes mellitus was carried out using streptozotocin (STZ) compounds (Sigma Aldrich, Singapore). A total of 55 mg/kg BW solution of STZ in citrate buffer (0.1 M, pH 4.5) was injected into intraperitoneal which had been fasted overnight [6]. Non-diabetic controls were injected with citrate buffer (0.1 M, pH 4.5). After seven days of STZ induction, fasting blood glucose levels were measured with a spectrophotometer (Biorad, Singapore). Rats with a blood glucose concentration of more than 200 g/dL are categorized as diabetic rats.

Chronic treatment with Karamunting fraction

The rats were grouped into six groups, namely group 1: a group of non-diabetic white rats, group 2: a group of streptozocin-induced diabetes mellitus without treatment, group 3-6: a group of rats induced diabetes mellitus given glibenclamide 5 mg/kg BW (Dexamedica, Palembang, Indonesia) [7]; Karamunting fraction dose of 10 mg/kg BW; 20 mg/kg BW and 40 mg/kg BW. Glibenclamide and fractions were given for 30 days.

Blood Sample Collection

A blood sample was taken from the orbital vein on the first day after overnight fasting. After 30 days of being given standard drugs or Karamunting fraction, the rats that had been fasted overnight were sacrificed, and blood samples were taken for biochemical examination. HbA1c was measured from blood samples that had been collected in the EDTA tube with the rat HbA1c assay kit. Insulin was measured from blood samples using an ELISA Insulin rating (Cloud Clone, Hangzhou, China). Blood glucose measurement was carried out with a spectrophotometer (Biorad, Singapore). Before being stored at -20°C, the blood samples in the EDTA tube were centrifuged

(Biorad, Singapore) at a speed of 5000 rpm at 25°C, 10 minutes. The supernatant is collected and stored to be used as a sample for biochemical testing.

Evacuation of experimental animal's kidney

Before organ evacuation, the Wistar rat was anaesthetized by injecting 10% chloral hydrate (3.5 ml/kg BW). Furthermore, the operation was performed on the flank area with an incision of about 5 centimetres to obtain the kidney. Next, the kidneys are evacuated and divided, where some are put into ice packed containers, and some are put into boxes filled with 10% NBF liquid (normal buffer formalin). The kidneys that were put into the ice-packed container were homogenized and centrifuged at a speed of 5000 rpm, for 30 minutes at 4°C. The supernatant was then taken and stored in a centrifuge tube at -20°C, to check levels of TNF alpha and AGEs. Part of the kidneys that had been placed in 10% NBF solution was prepared for histopathological examination.

ELISA (Enzyme-Linked Immunosorbent Assay): HbA1c, Insulin, TNF- α , AGEs

Rat ELISA HbA1c examined HbA1c, insulin, TNF- α , AGEs levels in the kidney tissue; Insulin; TNF- α ; AGEs (Cloud Clone, Hangzhou, China), based on the protocol contained in the manufacturer's protocols (sensitivity of ELISA kit 92%). Briefly, 50 μ l of standard diluent or serum samples were added to the well and incubated at 37°C for 30 minutes. After the plates were washed, 100 μ l of the biotinylated antibody solution was added and set for 30 minutes at 37°C. After washing three times, 50 μ l avidin-peroxidase complex solution was added and incubated for 15 minutes at 37°C. After washing, 50 μ l of tetramethylbenzidine colour solution was added and set in the dark for 15 minutes at 37°C. Finally, 50 μ l stop solution was added to stop the reaction, and the optical density (OD) was measured using an ELISA reader (Biorad, Singapore), the wavelength

of 450 nm. By analyzing the standard curve of the standard of each examination protein, the levels of HbA1c, insulin, TNF- α and AGEs were obtained.

PAS (Periodic acid-Schiff) histological examination staining

Kidney tissue that was fixed with 10% NBF was dehydrated using graded alcohol and xylene, then paraffinized and cut to a thickness of 5 μ m using a rotary microtome (Leica, Illinois, USA). The results of the cut were then placed on a glass object, added to the Schiff's reagent, let stand for 5-7 minutes and then stained with Mayer's Hemalaum solution for 3 minutes. Subsequently, washed, dehydrates, cleared in xylene and mounted with mounting media were carried out. Histopathological changes in synovial tissue were analyzed under a microscope (Olympus, Tokyo, Japan).

Phytochemical test

Test for phenols

The test was performed by using the method of Sofowora [8]. 2 ml extract was taken in a beaker glass. Then, 2 ml of ferric chloride solution was added. A deep bluish-green solution indicated the presence of phenols.

Test for terpenoids

Salkowski test was performed by using the method of Edeoga et al. [9]. 5 ml of aqueous extract was mixed in 2 ml of chloroform. Then 3 ml of concentrated sulfuric acid was poured to form a layer. A reddish-brown colouration of interface indicated the presence of terpenoids.

Test for saponins

The test was performed using the method of Edeoga et al. [9]. 2 g of the powdered sample boiled in 20 ml of distilled water in a water bath and filtered the solution. Then 10 ml of the filtrate was mixed with 5 ml of distilled water and shake vigorously for a stable, persistent foam. The foam was mixed with three drops of olive oil and shake vigorously, which leads to the formation of the emulsion; indicated the presence of saponins.

Test for flavonoids

The test was performed by using the method of Harborne [10]. 1 g powdered sample was heated with 10 ml ethyl acetate over a steam bath (40–50°C) for 5 minutes. The filtrate was treated with 1 ml dilute ammonia. A yellow colouration demonstrated positive test for flavonoids.

Test for alkaloids

The test was performed by using the method of Harborne [10]. 1 g powdered sample was extracted with 5 ml methanol and 5 ml of 2N hydrochloric acid. Then the filtrate was tested with Meyer's and Wagner's reagents. The samples were scored positive, based on turbidity.

Statistical analysis

All data were presented as mean \pm standard deviation, and all statistical analyzes were performed with the SPSS 25 (IBM, Jakarta, Indonesia) program. One way ANOVA followed by post hoc analysis was carried out to assess the difference in mean expression levels of each protein. $P < 0.05$ was determined as an indication that there was a significant difference in mean levels.

RESULTS

Table 1 shows the potential for Karamunting fraction on glucose regulation in Wistar rats. Karamunting extract was able to improve blood glucose levels in rats. This improvement in blood

glucose levels causes an improvement in the level of glycosylated haemoglobin (HbA1c). Karamunting fraction has been shown to increase insulin secretion from the pancreas, which is believed to improve blood glucose regulation.

Table 2 provides an overview of the potential for Karamunting fraction in inhibiting complications of kidney problems in people with diabetes mellitus. The Karamunting fraction can improve the directional glucose regulation so that there is inhibition of AGEs deposition in the renal tissue so that it can inhibit inflammation and the inflammatory cascade played by TNF-alpha expression.

Figure 1 is a histopathological picture, as well as cross-check of the results of biochemical and molecular markers on the cellular image. Figure 1 shows that administration of Karamunting fraction can prevent AGEs deposition in the renal tissue, which in turn will inhibit inflammation and prevent further damage to the kidneys.

Table 3 shows that each Karamunting water fraction is rich in flavonoids with very high antioxidant potential. Flavonoids are the primary, secondary metabolites believed to play a role in inhibiting inflammation and oxidants due to the accumulation of AGEs.

DISCUSSION

Karamunting water fraction is part of further exploration carried out to isolate further the presence of polar compounds in the Karamunting extract. The Karamunting water fraction shows that the flavonoid content is relatively high and is believed to be the primary, secondary metabolite that plays a role in improving glucose regulation and inhibition of AGEs deposition in kidney tissue. Karamunting is rich in flavonoids with potential as antioxidants.

Diabetes mellitus is a clinical disorder, where there is severe oxidative stress due to chronic stress conditions on cells [1,5]. Excess glucose intake will lead to overproduction of insulin in pancreatic beta cells. Pancreatic beta cells will be stimulated to produce large amounts of insulin and quickly. This condition causes a lot of misfolded insulin protein which will lead to the accumulation of misfolded insulin protein in the endoplasmic reticulum. This condition causes cell stress which leads to a cascade of oxidative stress.[7,11] Oxidative stress on pancreatic beta cells will cause the activation of the inflammatory cascade, which will lead to the activation of death receptors and cause the death of pancreatic beta cells.[12-13] This condition will lead to insulin deficiency which will worsen glucose regulation.[14-16] The absence of insulin will cause the accumulation of glucose outside the cells in blood vessels and tissues.[17-19] This condition causes a systemic glycosylation process in all organs, especially in the kidneys as the blood filtration organ. This glycosylation process causes the accumulation of AGEs products, which are the reaction products between glucose and proteins, lipids and nucleic acids.[18-20]

The accumulation of AGEs in the kidney, especially in the glomerulus, causes the activation of an inflammatory transcription factor that will trigger the activation of the proinflammatory cytokine, TNF- α [21-24]. Activation of inflammation in the kidneys leads to kidney damage [25-27]. This damage causes the kidneys to decrease their function and role as a filtration organ [28]. Karamunting acts as an antioxidant that will be able to improve oxidative stress conditions in diabetes mellitus [29,30]. This study proves the ability of Karamunting to enhance the regulation of blood glucose, insulin and HbA1c. Good glucose regulation ability will reduce the production of AGEs which will cause inhibition of AGEs accumulation in the tissue [18,19]. The decrease in AGEs will cause a reduction in the production of proinflammatory cytokines which will lead to repair of damage and deterioration of cell function.

CONCLUSION

Karamunting fraction can improve blood glucose regulation so that it can prevent further damage and complications of the kidney organs in the condition of diabetes mellitus in rat.

CONFLICT OF INTEREST

Authors declared no conflict of interest.

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Table 1. Effect Karamunting Extract in Glucose Regulation

Groups	Fasting blood glucose (mg/dL)		HbA1c (μ g/mL)		Insulin (IU/mL)	
	Day 1	Day 30	Day 1	Day 30	Day 1	Day 30
1	89.9 \pm 6.87	87.8 \pm 5.37	3.9 \pm 0.17	3.8 \pm 0.27	188.2 \pm 15.32	188.1 \pm 16.65
2	389.9 \pm 26.71	399.9 \pm 28.27	12.8 \pm 0.82	12.9 \pm 0.65	32.8 \pm 0.11	32.9 \pm 0.13
3	382.2 \pm 21.13	181.3 \pm 11.27*	12.5 \pm 0.76	7.8 \pm 0.23*	32.4 \pm 0.21	123.1 \pm 10.12*
4	384.3 \pm 23.21	299.2 \pm 24.57*	12.3 \pm 0.43	10.1 \pm 0.95*	32.3 \pm 0.12	87.8 \pm 4.65*
5	383.2 \pm 27.97	197.3 \pm 16.47*	12.9 \pm 0.68	7.9 \pm 0.44*	32.1 \pm 0.22	124.3 \pm 10.12*
6	386.6 \pm 26.57	169.9 \pm 10.22*	12.6 \pm 0.77	6.9 \pm 0.33*	32.3 \pm 0.23	132.9 \pm 11.22*

*ANOVA, pos hoc Bonferroni, $p < 0,05$ VS Group 2

Group 1: non-diabetic rat; group 2: streptozocin-induced diabetes without treatment; group 3: streptozocin-induced diabetes with glibenclamide treatment (5 mg/kg BW); group 4: streptozocin-induced diabetes rats with Karamunting fraction (10 mg/kg BW); group 5: streptozocin-induced diabetes rats with Karamunting fraction (20 mg/kg BW); group 6: streptozocin-induced diabetes rats with Karamunting fraction (40 mg/kg BW).

Table 2. Effect of Karamunting Extract in AGEs and TNF- α

Groups	AGEs ($\mu\text{g/mL}$)	TNF- α ($\mu\text{g/mL}$)
1	17.7 \pm 1.37	89.9 \pm 7.76
2	989.9 \pm 58.74	1221.4 \pm 87.65
3	198.3 \pm 16.23*	453.6 \pm 32.23*
4	249.6 \pm 21.27*	567.8 \pm 43.15*
5	193.3 \pm 15.57*	447.2 \pm 40.41*
6	169.9 \pm 13.92*	398.8 \pm 34.93*

*ANOVA, pos hoc Bonferroni, $p < 0,05$ versus group 2

Group 1: non-diabetic rat; group 2: streptozocin-induced diabetes without treatment; group 3: streptozocin-induced diabetes with glibenclamide treatment (5 mg/kg BW); group 4: streptozocin-induced diabetes rats with Karamunting fraction (10 mg/kg BW); group 5: streptozocin-induced diabetes rats with Karamunting fraction (20 mg/kg BW); group 6: streptozocin-induced diabetes rats with Karamunting fraction (40 mg/kg BW).

Table 3. Phytochemical Test of Karamunting Fraction

Test Material	Saponin	Alkaloid	Triterpenoid	Steroid	Flavonoid
Karamunting fraction	+	+	+	+	+++

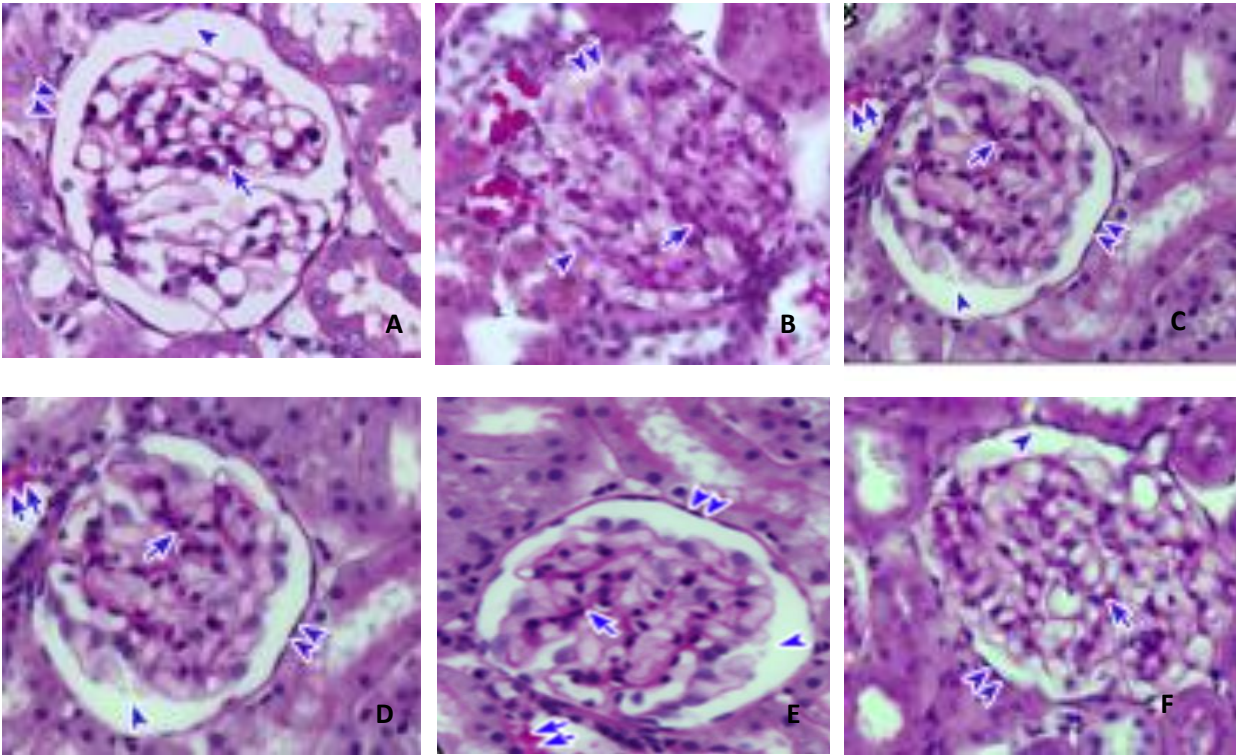


Figure 1. Histological overview of kidney tissue. PAS Staining. A: group 1, B: group 2, C: group 3, D: group 4, E: group 5, F: group 6. Glycogen deposition (double arrows), Bowman's capsule (double arrowheads), Bowman's space (arrowhead), mesangial area (arrow). 400x magnification.

Submission Acknowledgement (November 21, 2020)

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Dear authors,

Hereby I acknowledge the manuscript. We will send the MS to reviewer.

Regards,

Anna Patan

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REVIEWER EVALUATION FORM

Title: Potential of Karamunting (*Rhodomyrtus tomentosa*) Fraction Against Kidney Damage in Diabetic Rats

I have read the above manuscript and give hereafter my comments:

The manuscript by Mgs Irsan Saleh, Rachmat Hidayat, Geri Febriyanto, Nita Parisa is focusing on the potential of Karamunting fraction against kidney damage in diabetic rats.

1°/ consistency with the journal profile

non-consistency with the journal profile

2°/ a manuscript is based on extensive study with proper controls

a manuscript is not based on extensive study with proper controls

3°/ the information concerning the identification and documentation of plants is included

the information concerning the identification and documentation of plants is not included

4°/ the information concerning medicinal use of the plant is clarified

the information concerning medicinal use of the plant is not clarified

5°/ the approach to the subject is innovative

the approach to the subject is not innovative

6°/ in the article based on animal or clinical studies written approvals of Ethics/Bioethics Committee is cited

in the article based on animal or clinical studies written approvals of Ethics/Bioethics Committee is not cited

7°/ the article is written carefully

the article is written carelessly

8°/ Language of the paper:

excellent

good **X**

need corrections

9°/ the article is written according to the 'Instructions to Authors' **X**

the article is not written according to the 'Instructions to Authors'

These following concerns need to be addressed:

I. Major errors:

1. Tables should be reedited, because not all the columns with results are visible. I guess, that there is a lack of a column in Table 1.
2. The names or explanations of groups should be placed under the tables to facilitate the comparison of the results.

II. Minor errors:

1. In the Results section tin the last paragraph should be "Table 3" instead of "Table 2"
2. It is necessary to add the source (literature citation) of the dose of STZ or Glibenclamide.
3. It is necessary to add the sensitivity of ELISA kit.
4. It is necessary to emphasize how long the samples of blood were centrifuged.

Concluding,

- I recommend this paper for publication in *Herba Polonica* after strong revision and inserting the above-mentioned changes in the manuscript **X**
- I agree to evaluate the revised body text of the paper **X**
- I do not recommend this paper for publication in *Herba Polonica*
- I recommend this paper for publication in *Herba Polonica* after minor revisions
- I recommend this paper for publication in *Herba Polonica* without any changes

Editor decision

herba polonica Kotak Masuk x



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Running title right: Potential of karamunting (*Rhodomirtus tomentosa*) fraction against kidney damage in diabetic rats

Potential of karamunting (*Rhodomirtus tomentosa*) fraction against kidney damage in diabetic rats

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Summary

Introduction: Karamunting (*Rhodomyrtus tomentosa*) is Indonesian native plant that contains high antioxidant compounds with the potential to suppress oxidative stress activity in cells. This study is the first research to specifically explore the role of the karamunting fraction in protecting kidney damage due to the formation of AGEs in the renal glomerulus in diabetes mellitus.

Objective: This study aims to assess the impact of karamunting on blood glucose profile, HbA1c, insulin profile and kidney condition (AGEs expression, TNF- α and kidney histology) in diabetic rats.

Methods: The karamunting extraction process was carried out by maceration with ethanol. Induction of diabetes mellitus was carried out using streptozotocin. Thirty male rats (*Rattus norvegicus*), Wistar strain weighing between 200–250 grams were the subject in this study.

HbA1c, insulin, TNF- α , AGEs levels were evaluated in the kidney tissue. Histological assessment of the kidney was carried out. **Results:** Karamunting can improve blood glucose levels in diabetic rats. This improvement in blood glucose levels causes an improvement in the level of HbA1c. Administration of karamunting fraction can prevent AGEs deposition in renal tissue, which in turn will inhibit the inflammation and prevents further damage of kidneys.

Conclusion: Karamunting fraction can improve blood glucose regulation so that it can prevent further damage and complications of kidney organs in diabetic rats.

Key words: glycated haemoglobin A, insulin, protective agents, oxidative stress, *Rhodomyrtus tomentosa*

Słowa kluczowe: hemoglobina glikowana A, insulina, środki ochronne, stres oksydacyjny, *Rhodomyrtus tomentosa*

INTRODUCTION

Diabetes mellitus is a clinical condition characterised by systemic impairment of glucose regulation which leads to accumulation of glucose products in blood and tissues [1]. Glucose that accumulates in blood and tissues will react with proteins, nucleic acids or lipids to form various Amadori products which will lead to the formation of advanced glycation end products (AGEs) [1, 2]. AGEs are protein modification products and lipids that are responsible for the progression and complications of diabetes mellitus. The kidneys are vital organs that play a role in the filtration of blood, electrolytes and plasma. As a filtration organ, kidneys are the organs most at risk of becoming a buildup of AGEs products [2, 3]. Accumulation of AGEs products on the renal filtration tissues, the glomerulus, causes activation of the inflammatory cascade. Activation of the inflammatory cascade is responsible for the activation of death receptors from cells which will lead to cell death [3]. A consequence of cell death in the glomerulus and tubule is the decrease of blood filtration function. This condition causes chronic kidney failure. Kidney failure is one of the most common complications experienced by people with diabetes mellitus [3, 4], causing the dependence on hemodialysis. Limited hemodialysis facilities cause severe morbidity and mortality, in addition to increasing healthcare cost for patient and state. Therefore, it is essential and urgent to explore new therapeutic modes to prevent severe complications of diabetes mellitus in renal organs due to accumulation of AGEs and chronic inflammation.

Indonesia is a country with the second-largest vegetation wealth in the world. This is an opportunity to develop potential new therapeutic modalities. Karamunting (*Rhodomyrtus tomentosa*) is one of Indonesia's native plants from the Bangka Belitung archipelago. This plant is a type of shrub with high antioxidant content. Antioxidants are compounds with potential to suppress oxidative stress activity in cells. In contrast, the accumulation of AGEs in cells and tissues plays a role in the initiation of oxidative stress in cells and tissues [5]. Oxidative stress inhibition causes the inhibition of the inflammatory cascade and inhibition of cell and tissue death and damage.

This study is the first research that seeks to explore the role of the karamunting fraction in protecting against kidney damage due to the formation of AGEs in the renal glomerulus in diabetes mellitus. This study will assess the role of karamunting on blood glucose profile,

glycated haemoglobin (HbA1c), insulin profile and kidney condition with a focus on the expression of AGEs, TNF- α and kidney histology.

MATERIAL AND METHODS

Animal model

A total of thirty male rats (*Rattus norvegicus*), Wistar strain were obtained from the Eureka Research Laboratory (Palembang, Indonesia) weighing between 200–250 grams. Animals were kept in cages under controlled conditions of 12 hours of the light-dark cycle, at a temperature of $22\pm 1^\circ\text{C}$ and humidity 40–60% and given food *ad libitum*. The research treatments and procedures have received approval from the medical research ethics committee of the Faculty of Medicine, Universitas Sriwijaya (No. 254/kptfkunsri-rsmh/2020).

Karamunting fractionation preparation

Karamunting simplicia was obtained from the Tawangmangu Herbal Research Center, Karanganyar, Indonesia. The karamunting extraction process was carried out by maceration in which 500 grams of simplicia were macerated with 96% ethanol for 72 hours. Furthermore, a proportion between the dregs and macerate was carried out. Maceration continued with the fractionation process with n-hexane, ethyl acetate and water solvents, to obtain the n-hexane fraction (FH), ethyl acetate fraction (FE) and water fraction (FA) from karamunting.

Karamunting water fraction is a fraction that has been tested and explored more intensively.

Induction of diabetes mellitus

Induction of diabetes mellitus was carried out using streptozotocin (STZ) compounds (Sigma Aldrich, Singapore). A total of 55 mg/kg BW solution of STZ in citrate buffer (0.1 M, pH 4.5) was injected intraperitoneally in overnight fasted rats [6]. Non-diabetic controls were injected with citrate buffer (0.1 M, pH 4.5). After seven days of STZ induction, fasting blood glucose

levels were measured with a spectrophotometer (Biorad, Singapore). Rats with a blood glucose concentration higher than 200 g/dl were categorised as diabetic rats.

Chronic treatment with karamunting fraction

The rats were divided into six groups, namely: group 1: non-diabetic white rats, group 2: streptozocin-induced diabetes mellitus rats without treatment, group 3-6 rats with induced diabetes mellitus given glibenclamide 5 mg/kg BW (Dexamedica, Palembang, Indonesia) [7]. Karamunting fraction was administered at a dose of 10 mg/kg BW; 20 mg/kg BW and 40 mg/kg BW. Glibenclamide and fractions were given for 30 days.

Blood sample collection

A blood sample was taken from the orbital vein on the first day after overnight fasting. After 30 days of administration with standard drugs or karamunting fraction, the rats that fasted overnight were sacrificed, and blood samples were taken for biochemical examination. HbA1c was measured in blood samples that had been collected in the EDTA tube with the rat HbA1c assay kit. Insulin was measured from blood samples using an ELISA Insulin rating (Cloud Clone, Hangzhou, China). Blood glucose measurement was carried out with a spectrophotometer (Biorad, Singapore). Before being stored at -20°C , the blood samples in the EDTA tube were centrifuged (Biorad, Singapore) at a speed of 5000 rpm at 25°C , for 10 minutes. The supernatant was collected and stored to be used as a sample for biochemical testing.

Evacuation of experimental animal's kidney

Before organ evacuation, the Wistar rat was anaesthetized by injecting 10% chloral hydrate (3.5 ml/kg BW). Furthermore, the operation was performed on the flank area with an incision of about 5 centimetres to obtain the kidney. Next, the kidneys are evacuated and divided, where some are put into ice-packed containers, and some into boxes filled with 10% NBF liquid (normal buffer formalin). The kidneys that were put into the ice-packed containers were homogenized and centrifuged at a speed of 5000 rpm, for 30 minutes at 4°C . The supernatant was

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then taken and stored in a centrifuge tube at -20°C , to check the levels of TNF- α and AGEs. Parts of kidneys that had been placed in 10% NBF solution were prepared for histopathological examination.

ELISA (Enzyme-Linked Immunosorbent Assay): HbA1c, insulin, TNF- α , AGEs

Rat ELISA HbA1c was used to assess HbA1c, insulin, TNF- α , AGEs levels in the kidney tissue. Insulin, TNF- α , AGEs (Cloud Clone, Hangzhou, China) were based on the protocol contained in the manufacturer's protocols (sensitivity of ELISA kit 92%). Briefly, 50 μl of standard diluent or serum samples were added to the well and incubated at 37°C for 30 minutes. After the plates were washed, 100 μl of the biotinylated antibody solution was added and set for 30 minutes at 37°C . After washing three times, 50 μl avidin-peroxidase complex solution was added and incubated for 15 minutes at 37°C . After washing, 50 μl of tetramethylbenzidine colour solution was added and set in the dark for 15 minutes at 37°C . Finally, 50 μl stop solution was added to stop the reaction, and the optical density (OD) was measured using an ELISA reader (Biorad, Singapore) at the wavelength of 450 nm. By analysing the standard curve of the standard of each examination protein, the levels of HbA1c, insulin, TNF- α and AGEs were obtained.

PAS (Periodic acid-Schiff) histological examination staining

Kidney tissue that was fixed with 10% NBF was dehydrated using graded alcohol and xylene, then paraffinised and cut to a thickness of 5 μm , using a rotary microtome (Leica, Illinois, USA). The results of the cut were then placed on a glass object, added to the Schiff's reagent, left for 5–7 minutes and then stained with Mayer's Hemalaum solution for 3 minutes. Subsequently, wash, dehydration, clearing in xylene and mount with mounting media were carried out.

Histopathological changes in synovial tissue were analysed under a microscope (Olympus, Tokyo, Japan).

Phytochemical test

Test for phenols

The test was performed by the method of Sofowora [8]. 2 ml extract was taken in a beaker glass. Then, 2 ml of ferric chloride solution was added. A deep bluish-green solution indicated the presence of phenols.

Test for terpenoids

Salkowski test was performed by using the method of Edeoga et al. [9]. 5 ml of aqueous extract was mixed with 2 ml of chloroform. Then, 3 ml of concentrated sulfuric acid was poured to form a layer. A reddish-brown colouration of interface indicated the presence of terpenoids.

Test for saponins

The test was performed using the method of Edeoga et al. [9]. 2 g of the powdered sample was boiled in 20 ml of distilled water in a water bath and filtered the solution. Then, 10 ml of filtrate was mixed with 5 ml of distilled water and shaken vigorously for a stable, persistent foam. The foam was mixed with three drops of olive oil and shaken vigorously, which led to the formation of the emulsion, which indicated the presence of saponins.

Test for flavonoids

The test was performed with the method of Harborne [10]. 1 g of powdered sample was heated with 10 ml ethyl acetate over a steam bath (40–50°C) for 5 minutes. The filtrate was treated with 1 ml of diluted ammonia. A yellow colouration demonstrated positive test for flavonoids.

Test for alkaloids

The test was performed by using the method of Harborne [10]. 1 g powdered sample was extracted with 5 ml of methanol and 5 ml of 2N hydrochloric acid. Then the filtrate was tested with Meyer's and Wagner's reagents. The samples were scored positive, based on turbidity.

Statistical analysis

All data was presented as mean \pm standard deviation, and all statistical analysis was performed with the SPSS 25 (IBM, Jakarta, Indonesia) software. One way ANOVA followed by post hoc analysis, was carried out, to assess the difference in mean expression levels of each protein. $p < 0.05$ indicated that there was a significant difference in mean levels.

RESULTS

Table 1 shows the potential for Karamunting fraction on glucose regulation in Wistar rats. Karamunting extract was able to improve blood glucose levels in rats. This improvement in blood glucose levels causes an improvement in the level of glycosylated haemoglobin (HbA1c). Karamunting fraction has shown the increase of insulin secretion from the pancreas, which is believed to improve blood glucose regulation.

Table 2 provides an overview of the potential for karamunting fraction in inhibiting complications of kidney problems in people with diabetes mellitus. The karamunting fraction can improve the directional glucose regulation so that there is an inhibition of AGEs deposition in the renal tissue. It can inhibit inflammation and the inflammatory cascade played by TNF- α expression.

Figure 1 is a histopathological picture, as well as cross-check of the results of biochemical and molecular markers on the cellular image. Figure 1 shows that administration of karamunting fraction can prevent AGEs deposition in renal tissue, which in turn will inhibit inflammation and prevent further damage to the kidneys.

Table 3 shows that each karamunting water fraction is rich in flavonoids with very high antioxidant potential. Flavonoids are the primary and secondary metabolites believed to play a role in inhibiting inflammation and oxidants due to the accumulation of AGEs.

DISCUSSION

Karamunting water fraction is a part of further exploration carried out to isolate the presence of polar compounds in the karamunting extract. The karamunting water fraction shows that the flavonoid content is relatively high and is believed to be the primary and secondary metabolite that plays a role in improving glucose regulation and inhibition of AGEs deposition in kidney tissue. Karamunting is rich in flavonoids with antioxidant potential.

Diabetes mellitus is a clinical disorder with severe oxidative stress due to chronic stress conditions on cells [1, 5]. Excess glucose intake will lead to overproduction of insulin in pancreatic β cells. Pancreatic β cells will be stimulated to quick production of large amounts of

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insulin. This condition causes a lot of misfolded insulin protein which will lead to the accumulation of misfolded insulin protein in the endoplasmic reticulum. This condition causes cell stress which leads to a cascade of oxidative stress [7, 11]. Oxidative stress on pancreatic β cells will cause the activation of the inflammatory cascade, which will lead to the activation of death receptors and cause the death of pancreatic β cells [12-13]. This condition will lead to insulin deficiency which will worsen glucose regulation [14-16]. The absence of insulin will cause the accumulation of glucose outside the cells in blood vessels and tissues [17-19]. This condition causes a systemic glycosylation process in all organs, especially in kidneys as a blood filtering organ. This glycosylation process causes the accumulation of AGEs products, which are the reaction products between glucose and proteins, lipids and nucleic acids [18-20].

The accumulation of AGEs in the kidney, especially in the glomerulus, causes the activation of an inflammatory transcription factor that will trigger the activation of the proinflammatory cytokine, TNF- α [21-24]. Activation of inflammation in kidneys leads to kidney damage [25-27]. This damage causes decrease the function of kidneys and a role as a filtration organ [28]. Karamunting acts as an antioxidant that will be able to improve oxidative stress conditions in diabetes mellitus [29, 30]. This study proves the ability of karamunting to enhance the regulation of blood glucose, insulin and HbA1c. Good glucose regulation ability will reduce the production of AGEs which will cause the inhibition of AGEs accumulation in tissue [18, 19]. The decrease in AGEs will cause a reduction in the production of proinflammatory cytokines which will lead to repair of damage and deterioration of cell function.

CONCLUSION

Karamunting fraction can improve blood glucose regulation so that it can prevent further damage and complications of kidney organs in the condition of diabetes mellitus in rat.

CONFLICT OF INTEREST

Authors declared no conflict of interest.

FUNDING

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Table 1.

Effect of karamunting extract on glucose regulation

Groups	Fasting blood glucose [mg/dl]		HbA1c [μ g/ml]		Insulin [IU/ml]	
	Day 1	Day 30	Day 1	Day 30	Day 1	Day 30
1	89.9 \pm 6.87	87.8 \pm 5.37	3.9 \pm 0.17	3.8 \pm 0.27	188.2 \pm 15.3	188.1 \pm 16.65
2	389.9 \pm 26.7	399.9 \pm 28.27	12.8 \pm 0.82	12.9 \pm 0.65	2	32.9 \pm 0.13
3	1	181.3 \pm 11.27*	12.5 \pm 0.76	7.8 \pm 0.23*	32.8 \pm 0.11	123.1 \pm 10.12*
4	382.2 \pm 21.1	299.2 \pm 24.57*	12.3 \pm 0.43	10.1 \pm 0.95*	32.4 \pm 0.21	87.8 \pm 4.65*
5	3	197.3 \pm 16.47*	12.9 \pm 0.68	7.9 \pm 0.44*	32.3 \pm 0.12	124.3 \pm 10.12*
6	384.3 \pm 23.2	169.9 \pm 10.22*	12.6 \pm 0.77	6.9 \pm 0.33*	32.1 \pm 0.22	132.9 \pm 11.22*
	1				32.3 \pm 0.23	
	383.2 \pm 27.9					
	7					
	386.6 \pm 26.5					
	7					

*ANOVA, post hoc Bonferroni, $p < 0,05$ vs group 2

group 1: non-diabetic rat

group 2: streptozocin-induced diabetes without treatment

group 3: streptozocin-induced diabetes with glibenclamide treatment (5 mg/kg BW)

group 4: streptozocin-induced diabetes rats with karamunting fraction (10 mg/kg BW)

group 5: streptozocin-induced diabetes rats with karamunting fraction (20 mg/kg BW)

group 6: streptozocin-induced diabetes rats with karamunting fraction (40 mg/kg BW)

Table 2.

Effect of karamunting extract on AGEs and TNF- α

Groups	AGEs [$\mu\text{g/ml}$]	TNF- α [$\mu\text{g/ml}$]
1	17.7 \pm 1.37	89.9 \pm 7.76
2	989.9 \pm 58.74	1221.4 \pm 87.65
3	198.3 \pm 16.23*	453.6 \pm 32.23*
4	249.6 \pm 21.27*	567.8 \pm 43.15*
5	193.3 \pm 15.57*	447.2 \pm 40.41*
6	169.9 \pm 13.92*	398.8 \pm 34.93*

*ANOVA, post hoc Bonferroni, $p < 0,05$ vs group 2

group 1: non-diabetic rat

group 2: streptozocin-induced diabetes without treatment

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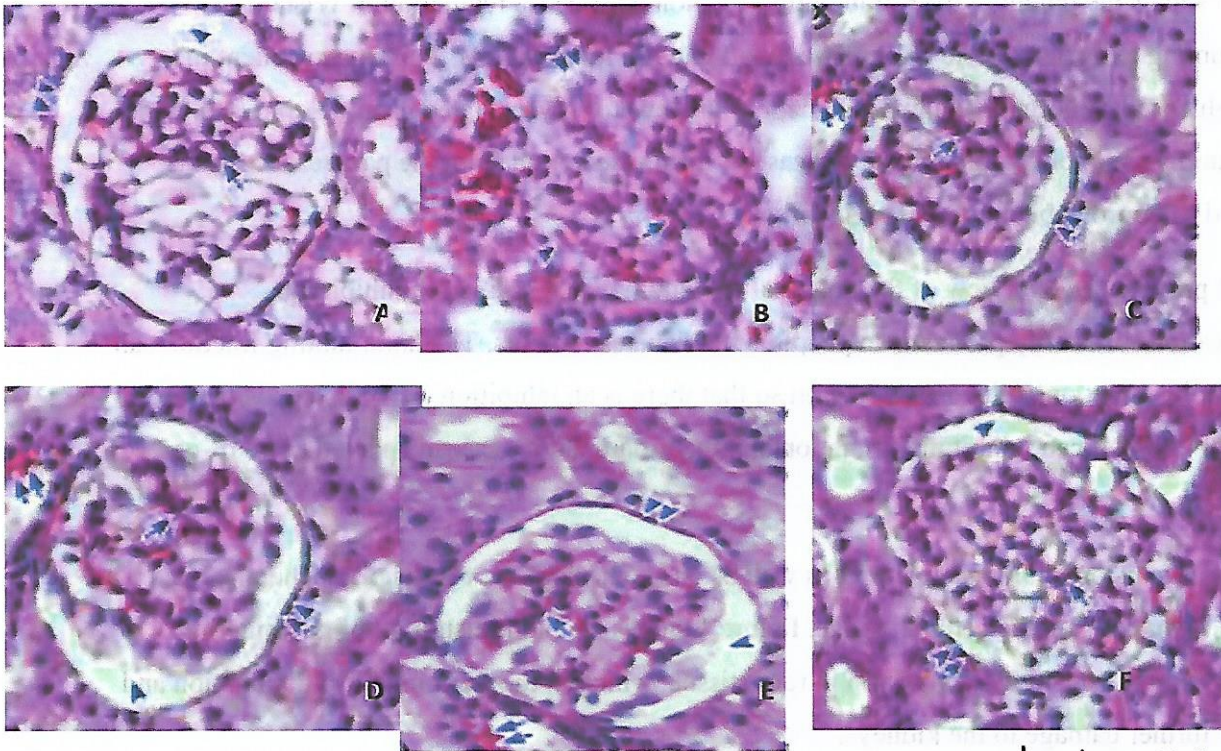
group 6: streptozocin-induced diabetes rats with karamunting fraction (40 mg/kg BW)


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Table 3.

Phytochemical test of karamunting fraction

Test material	saponin	alkaloid	triterpenoid	steroid	flavonoid
Karamunting fraction	+	+	+	+	+++



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Figure 1.

Histological overview of kidney tissue. PAS Staining. A: group 1, B: group 2, C: group 3, D: group 4, E: group 5, F: group 6. Glycogen deposition (double arrows), Bowman's capsule (double arrowheads), Bowman's space (arrowhead), mesangial area (arrow). 400 x magnification

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Potential of karamunting (*Rhodomyrtus tomentosa*) fraction against kidney damage in diabetic rats (*ahead of print*)

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Summary

Introduction: Karamunting (*Rhodomyrtus tomentosa*) is Indonesian native plant that contains high anti-oxidant compounds with the potential to suppress oxidative stress activity in cells. This study is the first research to specifically explore the role of the karamunting fraction in protecting kidney damage due to the formation of AGEs in the renal glomerulus in diabetes mellitus.

Objective: This study aims to assess the impact of karamunting on blood glucose profile, HbA1c, insulin profile and kidney condition (AGEs expression, TNF- α and kidney histology) in diabetic rats.

Methods: The karamunting extraction process was carried out by maceration with ethanol. Induction of diabetes mellitus was carried out using streptozotocin. Thirty male rats (*Rattus norvegicus*), Wistar strain weighing between 200–250 grams were the subject in this study. HbA1c, insulin, TNF- α , AGEs levels were evaluated in the kidney tissue. Histological assessment of the kidney was carried out.

Results: Karamunting can improve blood glucose levels in diabetic rats. This improvement in blood glucose levels causes an improvement in the level of HbA1c. Administration of karamunting fraction can prevent

AGEs deposition in renal tissue, which in turn will inhibit the inflammation and prevent further damage of kidneys.

Conclusion: Karamunting fraction can improve blood glucose regulation so that it can prevent further damage and complications of kidney organs in diabetic rats.

Key words: *glycated haemoglobin A, insulin, protective agents, oxidative stress, Rhodomyrtus tomentosa*

Słowa kluczowe: *hemoglobina glikowana A, insulina, środki ochronne, stres oksydacyjny, Rhodomyrtus tomentosa*

INTRODUCTION

Diabetes mellitus is a clinical condition characterised by systemic impairment of glucose regulation which leads to accumulation of glucose products in blood and tissues [1]. Glucose that accumulates in blood and tissues will react with proteins, nucleic acids or lipids to form various Amadori products which will lead to the formation of advanced glycation end products (AGEs) [1, 2]. AGEs are protein modification products and lipids that are responsible for the progression and complications of diabetes mellitus. The kidneys are vital organs that play a role in the filtration of blood, electrolytes and plasma. As a filtration organ, kidneys are the organs most at risk of becoming a buildup of AGEs products [2, 3]. Accumulation of AGEs products on the renal filtration tissues, the glomerulus, causes activation of the inflammatory cascade. Activation of the inflammatory cascade is responsible for the activation of death receptors from cells which will lead to cell death [3]. A consequence of cell death in the glomerulus and tubule is the decrease of blood filtration function. This condition causes chronic kidney failure. Kidney failure is one of the most common complications experienced by people with diabetes mellitus [3, 4], causing the dependence on hemodialysis. Limited hemodialysis facilities cause severe morbidity and mortality, in addition to increasing healthcare cost for patient and state. Therefore, it is essential and urgent to explore new therapeutic modes to prevent severe complications of diabetes mellitus in renal organs due to accumulation of AGEs and chronic inflammation.

Indonesia is a country with the second-largest vegetation wealth in the world. This is an opportunity to develop potential new therapeutic modalities. Karamunting (*Rhodomyrtus tomentosa*) is one of Indonesia's native plants from the Bangka Belitung archipelago. This plant is a type of shrub with high antioxidant content. Antioxidants are compounds with potential to suppress oxidative stress activity in cells.

In contrast, the accumulation of AGEs in cells and tissues plays a role in the initiation of oxidative stress in cells and tissues [5]. Oxidative stress inhibition causes the inhibition of the inflammatory cascade and inhibition of cell and tissue death and damage.

This study is the first research that seeks to explore the role of the karamunting fraction in protecting against kidney damage due to the formation of AGEs in the renal glomerulus in diabetes mellitus. This study will assess the role of karamunting on blood glucose profile, glycated haemoglobin (HbA1c), insulin profile and kidney condition with a focus on the expression of AGEs, TNF- α and kidney histology.

MATERIAL AND METHODS

Animal model

A total of thirty male rats (*Rattus norvegicus*), Wistar strain were obtained from the Eureka Research Laboratory (Palembang, Indonesia) weighing between 200–250 grams. Animals were kept in cages under controlled conditions of 12 hours of the light-dark cycle, at a temperature of 22±1°C and humidity 40–60% and given food *ad libitum*. The research treatments and procedures have received approval from the medical research ethics committee of the Faculty of Medicine, Universitas Sriwijaya (No. 254/kptfkunsri-rsmh/2020).

Karamunting fractionation preparation

Karamunting simplicia was obtained from the Ta-wangmangu Herbal Research Center, Karanganyar, Indonesia. The karamunting extraction process was carried out by maceration in which 500 grams of simplicia were macerated with 96% ethanol for 72 hours. Furthermore, a proportion between the

dregs and macerate was carried out. Maceration continued with the fractionation process with n-hexane, ethyl acetate and water solvents, to obtain the n-hexane fraction (FH), ethyl acetate fraction (FE) and water fraction (FA) from karamunting. Karamunting water fraction is a fraction that has been tested and explored more intensively.

Induction of diabetes mellitus

Induction of diabetes mellitus was carried out using streptozotocin (STZ) compounds (Sigma Aldrich, Singapore). A total of 55 mg/kg BW solution of STZ in citrate buffer (0.1 M, pH 4.5) was injected intraperitoneally in overnight fasted rats [6]. Non-diabetic controls were injected with citrate buffer (0.1 M, pH 4.5). After seven days of STZ induction, fasting blood glucose levels were measured with a spectrophotometer (Biorad, Singapore). Rats with a blood glucose concentration higher than 200 g/dl were categorised as diabetic rats.

Chronic treatment with karamunting fraction

The rats were divided into six groups, namely: group 1: non-diabetic white rats, group 2: streptozocin-induced diabetes mellitus rats without treatment, group 3-6 rats with induced diabetes mellitus given glibenclamide 5 mg/kg BW (Dexamedica, Palembang, Indonesia) [7]. Karamunting fraction was administered at a dose of 10 mg/kg BW; 20 mg/kg BW and 40 mg/kg BW. Glibenclamide and fractions were given for 30 days.

Blood sample collection

A blood sample was taken from the orbital vein on the first day after overnight fasting. After 30 days of administration with standard drugs or karamunting fraction, the rats that fasted overnight were sacrificed, and blood samples were taken for biochemical examination. HbA1c was measured in blood samples that had been collected in the EDTA tube with the rat HbA1c assay kit. Insulin was measured from blood samples using an ELISA Insulin rating (Cloud Clone, Hangzhou, China). Blood glucose measurement was carried out with a spectrophotometer (Biorad, Singapore). Before being stored at -20°C , the blood samples in the EDTA tube were centrifuged (Biorad, Singapore) at a speed of 5000 rpm at 25°C , for

10 minutes. The supernatant was collected and stored to be used as a sample for biochemical testing.

Evacuation of experimental animal's kidney

Before organ evacuation, the Wistar rat was anaesthetized by injecting 10% chloral hydrate (3.5 ml/kg BW). Furthermore, the operation was performed on the flank area with an incision of about 5 centimetres to obtain the kidney. Next, the kidneys are evacuated and divided, where some are put into ice-packed containers, and some into boxes filled with 10% NBF liquid (normal buffer formalin). The kidneys that were put into the ice-packed containers were homogenized and centrifuged at a speed of 5000 rpm, for 30 minutes at 4°C . The supernatant was then taken and stored in a centrifuge tube at -20°C , to check the levels of TNF- α and AGEs. Parts of kidneys that had been placed in 10% NBF solution were prepared for histopathological examination.

ELISA (Enzyme-Linked Immunosorbent Assay): HbA1c, insulin, TNF- α , AGEs

Rat ELISA HbA1c was used to assess HbA1c, insulin, TNF- α , AGEs levels in the kidney tissue. Insulin, TNF- α , AGEs (Cloud Clone, Hangzhou, China) were based on the protocol contained in the manufacturer's protocols (sensitivity of ELISA kit 92%). Briefly, 50 μl of standard diluent or serum samples were added to the well and incubated at 37°C for 30 minutes. After the plates were washed, 100 μl of the biotinylated antibody solution was added and set for 30 minutes at 37°C . After washing three times, 50 μl avidin-peroxidase complex solution was added and incubated for 15 minutes at 37°C . After washing, 50 μl of tetramethylbenzidine colour solution was added and set in the dark for 15 minutes at 37°C . Finally, 50 μl stop solution was added to stop the reaction, and the optical density (OD) was measured using an ELISA reader (Biorad, Singapore) at the wavelength of 450 nm. By analysing the standard curve of the standard of each examination protein, the levels of HbA1c, insulin, TNF- α and AGEs were obtained.

PAS (Periodic acid-Schiff) histological examination staining

Kidney tissue that was fixed with 10% NBF was dehydrated using graded alcohol and xylene, then

paraffinised and cut to a thickness of 5 μm , using a rotary microtome (Leica, Illinois, USA). The results of the cut were then placed on a glass object, added to the Schiff's reagent, left for 5–7 minutes and then stained with Mayer's Hemalaum solution for 3 minutes. Subsequently, wash, dehydration, clearing in xylene and mount with mounting media were carried out. Histopathological changes in synovial tissue were analysed under a microscope (Olympus, Tokyo, Japan).

Phytochemical test

Test for phenols

The test was performed by the method of Sofowora [8]. 2 ml extract was taken in a beaker glass. Then, 2 ml of ferric chloride solution was added. A deep bluish-green solution indicated the presence of phenols.

Test for terpenoids

Salkowski test was performed by using the method of Edeoga et al. [9]. 5 ml of aqueous extract was mixed with 2 ml of chloroform. Then, 3 ml of concentrated sulfuric acid was poured to form a layer. A reddish-brown colouration of interface indicated the presence of terpenoids.

Test for saponins

The test was performed using the method of Edeoga et al. [9]. 2 g of the powdered sample was boiled in 20 ml of distilled water in a water bath and filtered the solution. Then, 10 ml of filtrate was mixed with 5 ml of distilled water and shaken vigorously for a stable, persistent foam. The foam was mixed with three drops of olive oil and shaken vigorously, which led to the formation of the emulsion, which indicated the presence of saponins.

Test for flavonoids

The test was performed with the method of Harborne [10]. 1 g of powdered sample was heated with 10 ml ethyl acetate over a steam bath (40–50°C) for 5 minutes. The filtrate was treated with 1 ml of diluted ammonia. A yellow colouration demonstrated positive test for flavonoids.

Test for alkaloids

The test was performed by using the method of Harborne [10]. 1 g powdered sample was extracted with 5 ml of methanol and 5 ml of 2N hydrochloric acid. Then the filtrate was tested with Meyer's and Wagner's reagents. The samples were scored positive, based on turbidity.

Statistical analysis

All data was presented as mean \pm standard deviation, and all statistical analysis was performed with the SPSS 25 (IBM, Jakarta, Indonesia) software. One way ANOVA followed by post hoc analysis, was carried out, to assess the difference in mean expression levels of each protein. $p < 0.05$ indicated that there was a significant difference in mean levels.

Ethical approval: The conducted research is not related to either human or animal use.

RESULTS

Table 1 shows the potential for Karamunting fraction on glucose regulation in Wistar rats. Karamunting extract was able to improve blood glucose levels in rats. This improvement in blood glucose levels causes an improvement in the level of glycosylated haemoglobin (HbA1c). Karamunting fraction has shown the increase of insulin secretion from the pancreas, which is believed to improve blood glucose regulation.

Table 2 provides an overview of the potential for karamunting fraction in inhibiting complications of kidney problems in people with diabetes mellitus. The karamunting fraction can improve the directional glucose regulation so that there is an inhibition of AGEs deposition in the renal tissue. It can inhibit inflammation and the inflammatory cascade played by TNF- α expression.

Figure 1 is a histopathological picture, as well as cross-check of the results of biochemical and molecular markers on the cellular image. Figure 1 shows that administration of karamunting fraction can prevent AGEs deposition in renal tissue, which in turn will inhibit inflammation and prevent further damage to the kidneys.

Table 3 shows that each karamunting water fraction is rich in flavonoids with very high antioxidant potential. Flavonoids are the primary and secondary metabolites believed to play a role in inhibiting inflammation and oxidants due to the accumulation of AGEs.

Table 1.
Effect of karamunting extract on glucose regulation

Groups	Fasting blood glucose [mg/dl]		HbA1c [µg/ml]		Insulin [IU/ml]	
	Day 1	Day 30	Day 1	Day 30	Day 1	Day 30
1	89.9±6.87	87.8±5.37	3.9±0.17	3.8±0.27	188.2±15.32	188.1±16.65
2	389.9±26.71	399.9±28.27	12.8±0.82	12.9±0.65	32.8±0.11	32.9±0.13
3	382.2±21.13	181.3±11.27*	12.5±0.76	7.8±0.23*	32.4±0.21	123.1±10.12*
4	384.3±23.21	299.2±24.57*	12.3±0.43	10.1±0.95*	32.3±0.12	87.8±4.65*
5	383.2±27.97	197.3±16.47*	12.9±0.68	7.9±0.44*	32.1±0.22	124.3±10.12*
6	386.6±26.57	169.9±10.22*	12.6±0.77	6.9±0.33*	32.3±0.23	132.9±11.22

*ANOVA, post hoc Bonferroni, $p < 0,05$ vs group 2; group 1: non-diabetic rat; group 2: streptozocin-induced diabetes without treatment; group 3: streptozocin-induced diabetes with glibenclamide treatment (5 mg/kg BW); group 4: streptozocin-induced diabetes rats with karamunting fraction (10 mg/kg BW); group 5: streptozocin-induced diabetes rats with karamunting fraction (20 mg/kg BW); group 6: streptozocin-induced diabetes rats with karamunting fraction (40 mg/kg BW);

Table 2.
Effect of karamunting extract on AGEs and TNF-α

Groups	AGEs [µg/ml]	TNF-α [pg/ml]
1	17.7±1.37	89.9±7.76
2	989.9±58.74	1221.4±87.65
3	198.3±16.23*	453.6±32.23*
4	249.6±21.27*	567.8±43.15*
5	193.3±15.57*	447.2±40.41*
6	169.9±13.92*	398.8±34.93*

*ANOVA, post hoc Bonferroni, $p < 0,05$ vs group 2; group 1: non-diabetic rat; group 2: streptozocin-induced diabetes without treatment; group 3: streptozocin-induced diabetes with glibenclamide treatment (5 mg/kg BW); group 4: streptozocin-induced diabetes rats with karamunting fraction (10 mg/kg BW); group 5: streptozocin-induced diabetes rats with karamunting fraction (20 mg/kg BW); group 6: streptozocin-induced diabetes rats with karamunting fraction (40 mg/kg BW)

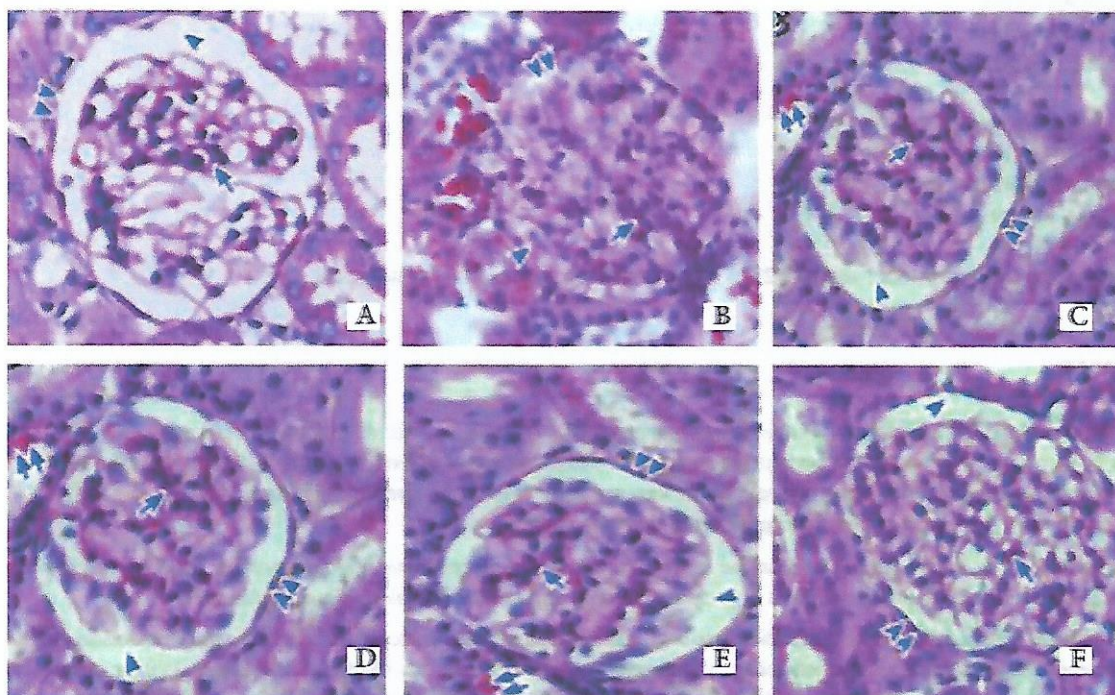


Figure 1.

Histological overview of kidney tissue. PAS Staining. A: group 1, B: group 2, C: group 3, D: group 4, E: group 5, F: group 6. Glycogen deposition (double arrows), Bowman's capsule (double arrowheads), Bowman's space (arrowhead), mesangial area (arrow). 400 x magnification

corrected

Table 3.

Phytochemical test of karamunting fraction

Test material	saponin	alkaloid	triterpenoid	steroid	flavonoid
Karamunting fraction	+	+	+	+	+++

Notes: +++= High; ++= Moderately; += Trace ✓

DISCUSSION

Karamunting water fraction is a part of further exploration carried out to isolate the presence of polar compounds in the karamunting extract. The karamunting water fraction shows that the flavonoid content is relatively high and is believed to be the primary and secondary metabolite that plays a role in improving glucose regulation and inhibition of AGEs deposition in kidney tissue. Karamunting is rich in flavonoids with antioxidant potential.

Diabetes mellitus is a clinical disorder with severe oxidative stress due to chronic stress conditions on cells [1, 5]. Excess glucose intake will lead to overproduction of insulin in pancreatic β cells. Pancreatic β cells will be stimulated to quick production of large amounts of insulin. This condition causes a lot of misfolded insulin protein which will lead to the accumulation of misfolded insulin protein in the endoplasmic reticulum. This condition causes cell stress which leads to a cascade of oxidative stress [7, 11]. Oxidative stress on pancreatic β cells will cause the activation of the inflammatory cascade, which will lead to the activation of death receptors and cause the death of pancreatic β cells [12-13]. This condition will lead to insulin deficiency which will worsen glucose regulation [14-16]. The absence of insulin will cause the accumulation of glucose outside the cells in blood vessels and tissues [17-19]. This condition causes a systemic glycosylation process in all organs, especially in kidneys as a blood filtering organ. This glycosylation process causes the accumulation of AGEs products, which are the reaction products between glucose and proteins, lipids and nucleic acids [18-20].

The accumulation of AGEs in the kidney, especially in the glomerulus, causes the activation of an inflammatory transcription factor that will trigger the activation of the proinflammatory cytokine, TNF- α [21-24]. Activation of inflammation in kidneys leads to kidney damage [25-27]. This damage causes decrease the function of kidneys and a role as a filtration organ [28]. Karamunting acts as an antioxidant that will be able to improve oxidative stress conditions in diabetes mellitus [29, 30]. This study proves the ability of karamunting

to enhance the regulation of blood glucose, insulin and HbA1c. Good glucose regulation ability will reduce the production of AGEs which will cause the inhibition of AGEs accumulation in tissue [18, 19]. The decrease in AGEs will cause a reduction in the production of proinflammatory cytokines which will lead to repair of damage and deterioration of cell function.

CONCLUSION

Karamunting fraction can improve blood glucose regulation so that it can prevent further damage and complications of kidney organs in the condition of diabetes mellitus in rat.

Conflict of interest: Authors declare no conflict of interest.

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EXPERIMENTAL PAPER

Potential of karamunting (*Rhodomyrtus tomentosa*) fraction against kidney damage in diabetic rats (*ahead of print*)

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Summary

Introduction: Karamunting (*Rhodomyrtus tomentosa*) is Indonesian native plant that contains high anti-oxidant compounds with the potential to suppress oxidative stress activity in cells. This study is the first research to specifically explore the role of the karamunting fraction in protecting kidney damage due to the formation of AGEs in the renal glomerulus in diabetes mellitus.

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Słowa kluczowe: *hemoglobina glikowana A, insulina, środki ochronne, stres oksydacyjny, Rhodomyrtus tomentosa*

INTRODUCTION

Diabetes mellitus is a clinical condition characterised by systemic impairment of glucose regulation which leads to accumulation of glucose products in blood and tissues [1]. Glucose that accumulates in blood and tissues will react with proteins, nucleic acids or lipids to form various Amadori products which will lead to the formation of advanced glycation end products (AGEs) [1, 2]. AGEs are protein modification products and lipids that are responsible for the progression and complications of diabetes mellitus. The kidneys are vital organs that play a role in the filtration of blood, electrolytes and plasma. As a filtration organ, kidneys are the organs most at risk of becoming a buildup of AGEs products [2, 3]. Accumulation of AGEs products on the renal filtration tissues, the glomerulus, causes activation of the inflammatory cascade. Activation of the inflammatory cascade is responsible for the activation of death receptors from cells which will lead to cell death [3]. A consequence of cell death in the glomerulus and tubule is the decrease of blood filtration function. This condition causes chronic kidney failure. Kidney failure is one of the most common complications experienced by people with diabetes mellitus [3, 4], causing the dependence on hemodialysis. Limited hemodialysis facilities cause severe morbidity and mortality, in addition to increasing healthcare cost for patient and state. Therefore, it is essential and urgent to explore new therapeutic modes to prevent severe complications of diabetes mellitus in renal organs due to accumulation of AGEs and chronic inflammation.

Indonesia is a country with the second-largest vegetation wealth in the world. This is an opportunity to develop potential new therapeutic modalities. Karamunting (*Rhodomyrtus tomentosa*) is one of Indonesia's native plants from the Bangka Belitung archipelago. This plant is a type of shrub with high antioxidant content. Antioxidants are compounds with potential to suppress oxidative stress activity in cells.

In contrast, the accumulation of AGEs in cells and tissues plays a role in the initiation of oxidative stress in cells and tissues [5]. Oxidative stress inhibition causes the inhibition of the inflammatory cascade and inhibition of cell and tissue death and damage.

This study is the first research that seeks to explore the role of the karamunting fraction in protecting against kidney damage due to the formation of AGEs in the renal glomerulus in diabetes mellitus. This study will assess the role of karamunting on blood glucose profile, glycated haemoglobin (HbA1c), insulin profile and kidney condition with a focus on the expression of AGEs, TNF- α and kidney histology.

MATERIAL AND METHODS

Animal model

A total of thirty male rats (*Rattus norvegicus*), Wistar strain were obtained from the Eureka Research Laboratory (Palembang, Indonesia) weighing between 200–250 grams. Animals were kept in cages under controlled conditions of 12 hours of the light-dark cycle, at a temperature of $22\pm 1^\circ\text{C}$ and humidity 40–60% and given food *ad libitum*. The research treatments and procedures have received approval from the medical research ethics committee of the Faculty of Medicine, Universitas Sriwijaya (No. 254/kptfkunsri-rsmh/2020).

Karamunting fractionation preparation

Karamunting simplicia was obtained from the Tangwangmangu Herbal Research Center, Karanganyar, Indonesia. The karamunting extraction process was carried out by maceration in which 500 grams of simplicia were macerated with 96% ethanol for 72 hours. Furthermore, a proportion between the

dregs and macerate was carried out. Maceration continued with the fractionation process with n-hexane, ethyl acetate and water solvents, to obtain the n-hexane fraction (FH), ethyl acetate fraction (FE) and water fraction (FA) from karamunting. Karamunting water fraction is a fraction that has been tested and explored more intensively.

Induction of diabetes mellitus

Induction of diabetes mellitus was carried out using streptozotocin (STZ) compounds (Sigma Aldrich, Singapore). A total of 55 mg/kg BW solution of STZ in citrate buffer (0.1 M, pH 4.5) was injected intraperitoneally in overnight fasted rats [6]. Non-diabetic controls were injected with citrate buffer (0.1 M, pH 4.5). After seven days of STZ induction, fasting blood glucose levels were measured with a spectrophotometer (Biorad, Singapore). Rats with a blood glucose concentration higher than 200 g/dl were categorised as diabetic rats.

Chronic treatment with karamunting fraction

The rats were divided into six groups, namely: group 1: non-diabetic white rats, group 2: streptozocin-induced diabetes mellitus rats without treatment, group 3-6 rats with induced diabetes mellitus given glibenclamide 5 mg/kg BW (Dexamedica, Palembang, Indonesia) [7]. Karamunting fraction was administered at a dose of 10 mg/kg BW; 20 mg/kg BW and 40 mg/kg BW. Glibenclamide and fractions were given for 30 days.

Blood sample collection

A blood sample was taken from the orbital vein on the first day after overnight fasting. After 30 days of administration with standard drugs or karamunting fraction, the rats that fasted overnight were sacrificed, and blood samples were taken for biochemical examination. HbA1c was measured in blood samples that had been collected in the EDTA tube with the rat HbA1c assay kit. Insulin was measured from blood samples using an ELISA Insulin rating (Cloud Clone, Hangzhou, China). Blood glucose measurement was carried out with a spectrophotometer (Biorad, Singapore). Before being stored at -20°C , the blood samples in the EDTA tube were centrifuged (Biorad, Singapore) at a speed of 5000 rpm at 25°C , for

10 minutes. The supernatant was collected and stored to be used as a sample for biochemical testing.

Evacuation of experimental animal's kidney

Before organ evacuation, the Wistar rat was anaesthetized. Furthermore, the operation was performed on the flank area with an incision of about 5 centimetres to obtain the kidney. Next, the kidneys are evacuated and divided, where some are put into ice-packed containers, and some into boxes filled with 10% NBF liquid (normal buffer formalin). The kidneys that were put into the ice-packed containers were homogenized and centrifuged at a speed of 5000 rpm, for 30 minutes at 4°C . The supernatant was then taken and stored in a centrifuge tube at -20°C , to check the levels of TNF- α and AGEs. Parts of kidneys that had been placed in 10% NBF solution were prepared for histopathological examination.

ELISA (Enzyme-Linked Immunosorbent Assay): HbA1c, insulin, TNF- α , AGEs

Rat ELISA HbA1c was used to assess HbA1c, insulin, TNF- α , AGEs levels in the kidney tissue. Insulin, TNF- α , AGEs (Cloud Clone, Hangzhou, China) were based on the protocol contained in the manufacturer's protocols (sensitivity of ELISA kit 92%). Briefly, 50 μl of standard diluent or serum samples were added to the well and incubated at 37°C for 30 minutes. After the plates were washed, 100 μl of the biotinylated antibody solution was added and set for 30 minutes at 37°C . After washing three times, 50 μl avidin-peroxidase complex solution was added and incubated for 15 minutes at 37°C . After washing, 50 μl of tetramethylbenzidine colour solution was added and set in the dark for 15 minutes at 37°C . Finally, 50 μl stop solution was added to stop the reaction, and the optical density (OD) was measured using an ELISA reader (Biorad, Singapore) at the wavelength of 450 nm. By analysing the standard curve of the standard of each examination protein, the levels of HbA1c, insulin, TNF- α and AGEs were obtained.

PAS (Periodic acid-Schiff) histological examination staining

Kidney tissue that was fixed with 10% NBF was dehydrated using graded alcohol and xylene, then paraffinised and cut to a thickness of 5 μm , using

a rotary microtome (Leica, Illinois, USA). The results of the cut were then placed on a glass object, added to the Schiff's reagent, left for 5–7 minutes and then stained with Mayer's Hemalaum solution for 3 minutes. Subsequently, wash, dehydration, clearing in xylene and mount with mounting media were carried out. Histopathological changes in synovial tissue were analysed under a microscope (Olympus, Tokyo, Japan).

Phytochemical test

Test for phenols

The test was performed by the method of Sofowora [8]. 2 ml extract was taken in a beaker glass. Then, 2 ml of ferric chloride solution was added. A deep bluish-green solution indicated the presence of phenols.

Test for terpenoids

Salkowski test was performed by using the method of Edeoga *et al.* [9]. 5 ml of aqueous extract was mixed with 2 ml of chloroform. Then, 3 ml of concentrated sulfuric acid was poured to form a layer. A reddish-brown colouration of interface indicated the presence of terpenoids.

Test for saponins

The test was performed using the method of Edeoga *et al.* [9]. 2 g of the powdered sample was boiled in 20 ml of distilled water in a water bath and filtered the solution. Then, 10 ml of filtrate was mixed with 5 ml of distilled water and shaken vigorously for a stable, persistent foam. The foam was mixed with three drops of olive oil and shaken vigorously, which led to the formation of the emulsion, which indicated the presence of saponins.

Test for flavonoids

The test was performed with the method of Harborne [10]. 1 g of powdered sample was heated with 10 ml ethyl acetate over a steam bath (40–50°C) for 5 minutes. The filtrate was treated with 1 ml of diluted ammonia. A yellow colouration demonstrated positive test for flavonoids.

Test for alkaloids

The test was performed by using the method of Harborne [10]. 1 g powdered sample was extracted with 5 ml of methanol and 5 ml of 2N hydrochloric acid. Then the filtrate was tested with Meyer's and Wagner's reagents. The samples were scored positive, based on turbidity.

Statistical analysis

All data was presented as mean \pm standard deviation, and all statistical analysis was performed with the SPSS 25 (IBM, Jakarta, Indonesia) software. One way ANOVA followed by post hoc analysis, was carried out, to assess the difference in mean expression levels of each protein. $p < 0.05$ indicated that there was a significant difference in mean levels.

RESULTS

Table 1 shows the potential for karamunting fraction on glucose regulation in Wistar rats. Karamunting extract was able to improve blood glucose levels in rats. This improvement in blood glucose levels causes an improvement in the level of glycosylated haemoglobin (HbA1c). Karamunting fraction has shown the increase of insulin secretion from the pancreas, which is believed to improve blood glucose regulation.

Table 2 provides an overview of the potential for karamunting fraction in inhibiting complications of kidney problems in people with diabetes mellitus. The karamunting fraction can improve the directional glucose regulation so that there is an inhibition of AGEs deposition in the renal tissue. It can inhibit inflammation and the inflammatory cascade played by TNF- α expression.

Figure 1 is a histopathological picture, as well as cross-check of the results of biochemical and molecular markers on the cellular image. Figure 1 shows that administration of karamunting fraction can prevent AGEs deposition in renal tissue, which in turn will inhibit inflammation and prevent further damage to the kidneys.

Table 3 shows that each karamunting water fraction is rich in flavonoids with very high antioxidant potential. Flavonoids are the primary and secondary metabolites believed to play a role in inhibiting inflammation and oxidants due to the accumulation of AGEs.

Table 1.
Effect of karamunting extract on glucose regulation

Groups	Fasting blood glucose [mg/dl]		HbA1c [μ g/ml]		Insulin [IU/ml]	
	Day 1	Day 30	Day 1	Day 30	Day 1	Day 30
1	89.9 \pm 6.87	87.8 \pm 5.37	3.9 \pm 0.17	3.8 \pm 0.27	188.2 \pm 15.32	188.1 \pm 16.65
2	389.9 \pm 26.71	399.9 \pm 28.27	12.8 \pm 0.82	12.9 \pm 0.65	32.8 \pm 0.11	32.9 \pm 0.13
3	382.2 \pm 21.13	181.3 \pm 11.27*	12.5 \pm 0.76	7.8 \pm 0.23*	32.4 \pm 0.21	123.1 \pm 10.12*
4	384.3 \pm 23.21	299.2 \pm 24.57*	12.3 \pm 0.43	10.1 \pm 0.95*	32.3 \pm 0.12	87.8 \pm 4.65*
5	383.2 \pm 27.97	197.3 \pm 16.47*	12.9 \pm 0.68	7.9 \pm 0.44*	32.1 \pm 0.22	124.3 \pm 10.12*
6	386.6 \pm 26.57	169.9 \pm 10.22*	12.6 \pm 0.77	6.9 \pm 0.33*	32.3 \pm 0.23	132.9 \pm 11.22

*ANOVA, post hoc Bonferroni, $p < 0,05$ vs group 2; group 1: non-diabetic rat; group 2: streptozocin-induced diabetes without treatment; group 3: streptozocin-induced diabetes with glibenclamide treatment (5 mg/kg BW); group 4: streptozocin-induced diabetes rats with karamunting fraction (10 mg/kg BW); group 5: streptozocin-induced diabetes rats with karamunting fraction (20 mg/kg BW); group 6: streptozocin-induced diabetes rats with karamunting fraction (40 mg/kg BW);

Table 2.
Effect of karamunting extract on AGEs and TNF- α

Groups	AGEs [pg/ml]	TNF- α [pg/ml]
1	17.7 \pm 1.37	89.9 \pm 7.76
2	989.9 \pm 58.74	1221.4 \pm 87.65
3	198.3 \pm 16.23*	453.6 \pm 32.23*
4	249.6 \pm 21.27*	567.8 \pm 43.15*
5	193.3 \pm 15.57*	447.2 \pm 40.41*
6	169.9 \pm 13.92*	398.8 \pm 34.93*

*ANOVA, post hoc Bonferroni, $p < 0,05$ vs group 2; group 1: non-diabetic rat; group 2: streptozocin-induced diabetes without treatment; group 3: streptozocin-induced diabetes with glibenclamide treatment (5 mg/kg BW); group 4: streptozocin-induced diabetes rats with karamunting fraction (10 mg/kg BW); group 5: streptozocin-induced diabetes rats with karamunting fraction (20 mg/kg BW); group 6: streptozocin-induced diabetes rats with karamunting fraction (40 mg/kg BW)

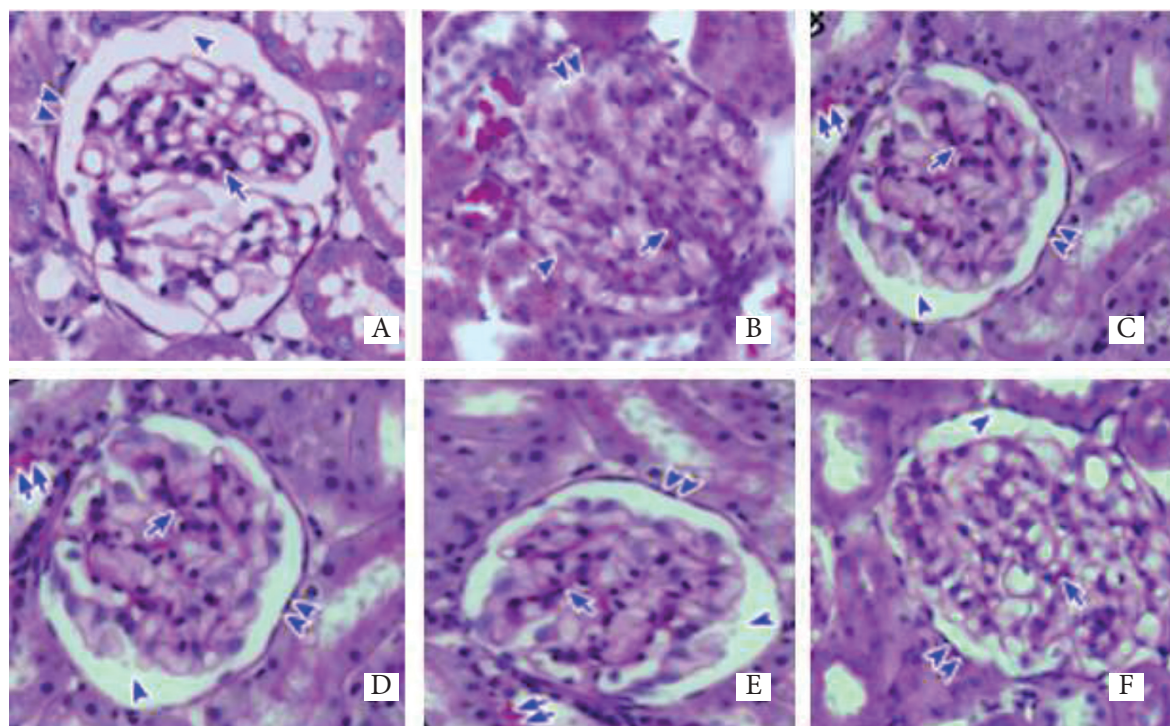


Figure 1.

Histological overview of kidney tissue. PAS Staining. A: group 1, B: group 2, C: group 3, D: group 4, E: group 5, F: group 6. Glycogen deposition (double arrows), Bowman's capsule (double arrowheads), Bowman's space (arrowhead), mesangial area (arrow). 400 x magnification

Table 3.

Phytochemical test of karamunting fraction

Test material	saponin	alkaloid	triterpenoid	steroid	flavonoid
Karamunting fraction	+	+	+	+	+++

Notes: +++ = high; ++ = moderately; + = trace

DISCUSSION

Karamunting water fraction is a part of further exploration carried out to isolate the presence of polar compounds in the karamunting extract. The karamunting water fraction shows that the flavonoid content is relatively high and is believed to be the primary and secondary metabolite that plays a role in improving glucose regulation and inhibition of AGEs deposition in kidney tissue. Karamunting is rich in flavonoids with antioxidant potential.

Diabetes mellitus is a clinical disorder with severe oxidative stress due to chronic stress conditions on cells [1, 5]. Excess glucose intake will lead to overproduction of insulin in pancreatic β cells. Pancreatic β cells will be stimulated to quick production of large amounts of insulin. This condition causes a lot of misfolded insulin protein which will lead to the accumulation of misfolded insulin protein in the endoplasmic reticulum. This condition causes cell stress which leads to a cascade of oxidative stress [7, 11]. Oxidative stress on pancreatic β cells will cause the activation of the inflammatory cascade, which will lead to the activation of death receptors and cause the death of pancreatic β cells [12-13]. This condition will lead to insulin deficiency which will worsen glucose regulation [14-16]. The absence of insulin will cause the accumulation of glucose outside the cells in blood vessels and tissues [17-19]. This condition causes a systemic glycosylation process in all organs, especially in kidneys as a blood filtering organ. This glycosylation process causes the accumulation of AGEs products, which are the reaction products between glucose and proteins, lipids and nucleic acids [18-20].

The accumulation of AGEs in the kidney, especially in the glomerulus, causes the activation of an inflammatory transcription factor that will trigger the activation of the proinflammatory cytokine, TNF- α [21-24]. Activation of inflammation in kidneys leads to kidney damage [25-27]. This damage causes decrease the function of kidneys and a role as a filtration organ [28]. Karamunting acts as an antioxidant that will be able to improve oxidative stress conditions in diabetes mellitus [29, 30]. This study proves the ability of karamunting

to enhance the regulation of blood glucose, insulin and HbA1c. Good glucose regulation ability will reduce the production of AGEs which will cause the inhibition of AGEs accumulation in tissue [18, 19]. The decrease in AGEs will cause a reduction in the production of proinflammatory cytokines which will lead to repair of damage and deterioration of cell function.

CONCLUSION

Karamunting fraction can improve blood glucose regulation so that it can prevent further damage and complications of kidney organs in the condition of diabetes mellitus in rat.

Conflict of interest: Authors declare no conflict of interest.

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