The ability of active fraction from *Kappaphycus alvarezii* (Doty) Doty Ex.P.C.Silva to Decrease Glucose Levels inhibit advanced Glycation End-Products (AGEs) Formation and RAGE (AGER) Gene Expression in Diabetic Rats

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Abstract

Background. *Kappaphycus alvarezii* (Doty) Doty ex P.C.Silva is a widely used seaweed that has antioxidant and antiglycation activities. The purpose of this study was to examine the ability of active fraction from *Kappaphycus alvarezii* to decrease glucose level and inhibit glycation process.

Methods. This study used bioassay-guided fractionation through three stages of the extraction, partition, and fractionation processes that were monitored using Thin Layer Chromatography and BSA-Glucose test. Inhibition of glycation was known by calculating percentage of inhibition and IC_{50}. Selected active fraction was used for in vivo tests using 24 Wistar male rats. Measurement of glucose levels used GOD-PAP method, while levels of glycated albumin (GA) and Nε- (carboxymethyl) lysine (CML) were measured using ELISA. Analysis of RAGE gene expression used qPCR.

Results Glycation test showed a significant difference ($p < 0.05$) between all treatments. Chloroform extract showed higher percentage of inhibition (62.4 ± 3.45%) with lower IC_{50} (0.33 ± 0.01 mg/ml) compared to methanol extract (0.52 ± 0.03 mg/ml). Methanol-soluble extracts had a higher percentage of inhibition (51.10 ± 1.64%) with IC_{50} 0.45 ± 0.05 mg/ml compared to methanol-insoluble extract (1.25 ± 0.05 mg/ml). Fraction II had a higher percentage of inhibition (53.37 ± 1.92%) with IC_{50} 0.12 ± 0.01 mg/ml compared to other fractions. Selected active fraction reduced blood glucose by 1.3% and 5.2% and CML levels by 50.6% and 42.4% at concentrations of 0.17 and 0.255 mg/ml in diabetic rats. RAGE gene expression was lower in the diabetic rat groups treated with active fraction compared to untreated diabetic group.

Conclusions The active fraction has ability for reducing blood glucose, antiglycation, or reducing CML levels, and RAGE gene expression.

Background

Glycation, also known as the Maillard reaction, is a slow nonenzymatic reaction, starting by the attachment of glucose or its derivatives to a protein amine group and formation of Schiff base, which is followed by the Amadori rearrangement to form a stable fructosamine residue (ketoamine). Further oxidation and rearrangement of the initial glycation product irreversibly results in an Advanced Glycation End-products (AGEs). This process is often associated with an oxidative phenomenon called "glycoxidation", which occurs when an oxidative reaction affects the initial glycation product [1] During the aging process, AGEs are formed and accumulated in the walls of blood vessels. These processes are increased in patients with hyperglycemia and also in patients with diabetes. The accumulation of AGEs in the walls of blood vessels and different organs is associated with the development of microvascular and macrovascular diseases and other secondary complications of diabetes [2]. Excessive AGEs in tissues can cause various disorders, especially metabolic disorders, such as diabetes and obesity, and can cause tissue damage and clinical complications associated with this disease [3].

Natural products, especially from plants, have been proven to be relatively safe for humans. Consumption of some plant extracts can prevent the formation of AGEs. Compounds with antioxidant capacity, such as polyphenols, have antiglycation effects at physiological concentrations. Plant materials that contain hydrophobic compounds, such as sapogenin, ursolic acid, and oleanolic acid, have antioxidant and hypoglycemic activities. Plants that are rich in triterpenoids and phenolics in general show antioxidant ability and antiglycation effects. Extracts or natural compounds that have antioxidant and antiglycation capabilities have great therapeutic potential for treating diabetes and diabetes complications [4].

One source of exogenous antioxidants and antiglycation is seaweed, i.e., algae. *Kappaphycus alvarezii* (Doty) Doty ex P.C.Silva is one of the largest macroalgae in the tropics with relatively high growth rates compared to other types of seaweed [5]. In Indonesia, seaweed cultivation generally uses the genus Eucheuma [6]. This algae is a potential source of various compounds, such as dietary fiber, vitamin C, α-tocopherol, minerals, fatty acids, and protein. *Kappaphycus alvarezii* (Doty) Doty ex P.C.Silva is a rich source of antioxidants and can significantly prevent tissue damage by stimulating the wound healing process and acting as an anti-inflammatory because it has phenolic compounds and their derivatives, including simple phenols, flavonoids, hydroquinone, triterpenoids, phenyl proponoid, tannins, lignin, and many other substances, and contains aromatic rings and hydroxyl groups, which will determine the destructive power of radical compounds [7, 8].

The use of medicinal plants in the form of crude extracts causes several difficulties, including the amount of plant bioactive compounds that varies according to location and collection season, the presence of bioactive molecules that may become strong poisons when they are consumed in excess, low bioactive compound content that may makes suboptimal doses ineffective, and also their drug properties that can be quickly lost during the storage process. Therefore, it is important to isolate and identify bioactive molecules from a plant extract. Bioassay-guided fractionation from plant extracts linked to chromatographic separation technique can lead to isolation of an active molecule. Fractionation of plant extracts based on biological activity has begun to be developed [9].

Diabetes mellitus research using animal models is very important for understanding various aspects of its pathogenesis and finding new therapeutic methods. Animal diabetes models are very useful in biomedical studies because they can provide new insights into human diabetes research. Most animal models used are rodents due to their small size, short generation intervals, easy availability and economic considerations [10]. Rats were made into hyperglycemia using STZ and NA. In experimental animals, the most prominent diabetogenic chemical that is often used is streptozotocin (STZ) among other substances that also have a diabetes effect (alloxan, vactor, dithizone, and 8-hydroxyquinolone). Alloxan destroys pancreatic beta cells through oxidative stress and, compared to STZ, has lower effectiveness and side effects, including liver and kidney damage. The rat model induced with STZ-NA into type 2 diabetes is based on the protective effect of NA against the β-cytotoxic effects of STZ [11]. [12] examined the effect of administering 100–350 mg / kg NA injected intraperitoneally 15 minutes before administration of 65 mg / kg intravenous STZ and concluded that the most appropriate NA dose was

230 mg / kg. Administration of STZ and 230 mg / kg nicotinamide will result in an increase in glucose concentration of 5–11 mmol / l (90–198 mg / dl).

This study was conducted to explore the active fraction from *Kappaphycus alvarezii* (Doty) Doty ex P.C.Silva, which is responsible for the inhibitory activity of glycation process and to know the effect of giving algae active fraction to the decrease of fasting blood glucose, glycated albumin, and CML levels and decreased of RAGE gene expression.

Methods

Plant Material

Kappaphycus alvarezii (Doty) Doty ex P.C.Silva were obtained from Lombok Tengah, Nusa Tenggara Barat, Indonesia in February 2019. The algae were identified at Biology Laboratory, Faculty of Mathematics and Science, Universitas Mataram, Mataram, NTB, Indonesia with identification sertificate number 03/UN18.7/LB/2019.

Animals

Rats used in this study were from Bogor Life Science and Technology (BLST) Company, Bogor, Indonesia. All animal experiment procedures were approved by Faculty of Medicine, Public Health and Nursing, Gadjah Mada University Ethics Commission, Yogyakarta, Indonesia with Ref. No. : KE / FK / 0564 / EC / 2019.

Instruments And Chemicals

The instruments used were oven (Memmert, Germany), centrifuge (Hitachi 18PR / 5, Automatic high-speed refrigerated centrifuge), UV lamp, spectrofluorometer (Shimadzu RF 6000), high resolution mass spectrometry (HRMS) using Thermo Scientific™ Dionex™ Ultimate 3000 RSLCnano UHPLC coupled with Thermo Scientific™ Q Exactive™ High Resolution Mass Spectrometer. The chemicals used were purchased from Merck. The chemicals were methanol, chloroform, ethyl acetate, ether, silica gel GF254, BSA, sodium azide, glucose, silica plate, benzene, phosphate-buffered saline (PBS), STZ and NA, Glucose GOD-PAP Biolab Reagents, ELISA kit for *glycated albumin*, *Nε-(carboxymethyl) lysine* (CML) (Bioassay Technology Laboratory), RNAprep Pure Kit (Invitrogen), iScript™cDNA Synthesis Kit Cat. No. 1708890, Sso Fast ™ EvaGreen ® Supermix Cat No 172–5200, Eppendorf tube 1.5 mL, qPCR tube 200 µL, tip, RAGE primer, β-actin primer.

Study Design

In this study, according to Federer's formula, (t-1)(n-1) ≥ 15, with t = number of the group (4 groups) and n = number of the rats, the sample size in each group were 6 of Wistar male rats. Twenty-four male Wistar rats, with an average weight of 200 g, aged 8 weeks were acclimated for 1 week in the laboratory. Each rat was placed in an individual cage that had been cleaned. Room temperature ranges from 25–28 °C with a 12-hour lighting circulation. Rats were given standard feed and drink ad libitum

Extraction, Partition, And Fractionation

The thalli of red algae (*Kappaphycus alvarezii* (Doty) Doty ex P.C.Silva) were cut into pieces, sun dried, and powdered. A thousand grams of dry powder was extracted by maceration three times for 24 hours at room temperature using 1 liter of chloroform. Filtering was done using a Buchner funnel. The filtrate was combined and evaporated, so the chloroform extract was obtained. The residue then extracted with methanol, as was done with chloroform, to collect the methanol extract. The success of extraction was monitored by thin layer chromatography (TLC) and antiglycation test using BSA-glucose method.

The active extract (chloroform extract), 28 grams, was then partitioned using methanol, centrifuged at a speed of 5000 rpm for 10 minutes, to obtain two extracts, namely extract containing methanol-soluble compounds and extract containing methanol-insoluble compounds. The success of this partition was monitored using TLC and characterized by the absence or little similarity between the two extracts, then each extract was tested for its antiglycation activity using the BSA-glucose method.

The active extract (3 grams) was then fractionated by liquid chromatography that was modified using vacuum with a stationary phase of silica gel GF254. The mobile phase used was benzene (100%), benzene : ethyl acetate (9 : 1 v/v), benzene : ethyl acetate (8 : 2 v/v), benzene : ethyl acetate (7 : 3 v/v), benzene: ethyl acetate (6 : 4 v/v), benzene : ethyl acetate (5 : 5 v/v), benzene : ethyl acetate (4 : 6 v/v), benzene : ethyl acetate (3 : 7 v/v), benzene : ethyl acetate (2 : 8 v/v), benzene : ethyl acetate (1 : 9 v/v) ethyl acetate (100%), and finally chloroform : methanol (1 : 1 v/v). The eluate obtained was collected and evaporated to dryness. Each fraction was examined by using TLC and the fractions that showed similarity of spots on the chromatogram were combined and evaporated. The combined fractions obtained were weighed first, then used for antiglycation test and TLC.

Compound Analysis With High Resolution Mass Spectrometer

To find out the compounds contained in the active fraction, an analysis was performed using HRMS (Thermo Scientific ™ Dionex ™ UltiMate 3000 RSLC nanoplan UHPLC coupled with Thermo Scientific Q Exactive ™ High Resolution Mass Spectrometer). The mobile phase used was A = Water + 0.1% Formic Acid and B = Acetonitrile + 0.1% Formic Acid. Analytical column used was Hypersil GOLD aQ 50 mm x 1 mm x 1.9 µm with flow of 10 µL/min, injection volume of 5 µL, 30-minute run time, Full MS at 70,000

FWHM Resolution, and data-dependent MS2 at the resolution of 17,500 FWHM. Compound Identification used Thermo Scientific ™ Compound Discoverer Software

In Vitro Glycation Of Bovine Serum Albumin (bsa)

BSA was incubated with glucose in PBS (20 mM, pH 7.4) and extract containing 0.02% sodium azide at 37∘C with a final concentrations of BSA (5 mg/ml), glucose (200 mM), and samples (0.1 to 0.5 mg/ml). The solution was incubated in the dark at 37°C for 6 days. The AGE formation was measured using a spectrofluorometer with an excitation wavelength of 370 nm and an emission wavelength of 450 nm. Percentage of inhibition was calculated as follows: (see Equation 1 in the Supplementary Files)

In Vivo Test

Twenty four male Wistar rats, with an average weight of 200 grams and age of 8 weeks were divided into 4 groups (n = 6 per group). Three experimental groups were induced to become hyperglycemic with NA as much as 230 mg/kg bw intraperitoneally 15 minutes before STZ administration (65 mg/ kg) in citrate buffer (0.1 M, pH 4.5) after fasting overnight. Nondiabetic control rats were injected with citrate buffer (pH 4.5) only. Diabetic rats with fasting blood glucose above 150 mg/dL were selected for study and divided into 3 groups: diabetic rats, diabetic rats treated with 0.17 mg/ml active fraction from red algae, and diabetic rats with a treatment of 0.255 mg / ml active fraction from red algae. Red algae active fraction administration was done every day for 4 weeks. Blood samples were collected from the retro orbital plexus of the rats after fasted for 10 hours using a hematocrit capillary. Rats fasted for 10 hours before the sacrifice procedure. Euthanasia was done using the cervical dislocation method. After the rats died, they were placed on the operating table in the supine position. Then an incision was made in the medial line - the lateral side of the abdominal wall to the peritoneal cavity using scissors. For examination of gene expression, kidney samples were stored in a tube containing RNA later solution and stored at -20 ° C.

Glucose, Glycated Albumin, and Nε-(carboxymethyl) lysine **Levels Test**

Glucose level was determined quantitatively by using the GOD-PAP method using Glucose GOD-PAP Biolab Reagents. Measurement of glucose level was carried out by spectrophotometry at 500 nm. GA and CML levels were measured using ELISA method. OD values at 450 nm wavelength were converted to GA and CML levels using CurveExpert software.

Rage (ager) Gene Expression

Messenger RNA (mRNA) was isolated using RNAprep Pure Kit (Invitrogen) from 0.3 mg of rat kidneys and stored at -80ºC. cDNA was made using iScript ™ cDNA Synthesis Kit with PCR conditions for incubation of 5 minutes at 25ºC, 30 minutes at 42ºC, 5 minutes at 85ºC, and finally at 4ºC, with the number of cycle

was 1 cycle. RAGE gene analysis used SsoFast TM EvaGreen ® Supermix, with a PCR conditions of 30 seconds for enzyme activation at 95ºC, 5 seconds at 95ºC for denaturation, 5 seconds at 55 ºC for annealing, and finally 2–5 seconds/step at 65–95 ºC for melting curve. The number of cycles was 40 cycles using the primer sequence F: 5 'CACCATGCCAGCGGGGAC 3' and R: 5 'AGCTCTGCACGTTCCTCCTCAT 3'

Statistical Analysis

The data obtained were tested using one-way ANOVA parametric statistics and Duncan post hoc analysis, whereby $p < 0.05$ was considered statistically significant. The data before and after treatment for in vivo test were tested using paired t-test per group.

Results

The bioassay-guided fractionation process began by separating polar compounds using methanol and separating non-polar compounds using chloroform solvents. The two extracts were then monitored by bioassay (BSA-glucose) method and the chromatography pattern was also observed. This bioassay-guided fractionation method had been applied to detect the presence of active fraction from *T. diversifolia* (Hemsley) A Gray which has antiplasmodial potential [13]. İt has been widely used for the initial stages in the isolation of compounds from natural materials, such as the compound tagitinin C from *T. diversifolia* [14] and Zerumbone from *Zingiber zerumbet* [15].

The results of chloroform extract and methanol extract glycation tests showed a significant difference ($p < 0.05$). The percentage of inhibition of glycation process in chloroform extract was higher than it was in methanol extract (see Fig. 1.a). Chloroform extract with a concentration of 0.4 mg/ml showed the highest percentage of inhibition among other treatments (62.4 ± 3.45%). In addition, the IC_{50} value of chloroform extract was lower (0.33 ± 0.01 mg/ml) when compared to the IC_{50} value of methanol extract (0.52 ± 0.03 mg/ml).

Chloroform extract has a lower IC_{50} value compared to methanol extract, so it was used for further testing. Chloroform extract was partitioned using methanol as a solvent, producing two extracts from the partition, namely, methanol-soluble and methanol-insoluble extracts. The results of the antiglycation test are shown in Fig. 1.b.

A significant difference between the treatments using methanol-soluble and methanol-insoluble extracts was observed ($p < 0.05$). The highest percentage of inhibition was produced by treatment using extracts that was soluble in methanol at the concentration of 0.5 mg/ml (51.10 ± 1.64%) as seen in Fig. 1.b. IC_{50} values obtained from the two treatment groups showed that methanol-soluble extract had lower IC_{50} (0.45 ± 0.05 mg/ml) compared to methanol-insoluble extract (1.25 ± 0.05 mg/ml).

The methanol-soluble extracts was then fractionated using benzene and ethyl acetate with decreased polarity.The results of the antiglycation test from the fraction obtained are shown in Fig. 1.c, demonstrating a significant difference among different treatments with fractions from red algae ($p < 0.05$). Fraction II treatment at a concentration of 0.15 mg/ml had the highest percentage of inhibition (53.37 ± 1.92%) and the lowest IC_{50} value (0.12 ± 0.01 mg/ml).

To confirm that the separation between polar and non-polar compounds of the extraction process was completed, TLC test was used to monitor (see Fig. 2.a). The TLC results showed that the compounds of chloroform and methanol extracts were completely separated with the difference spots between two group.

The success of partitioning of chloroform extract and fractination of methanol-soluble extracts was confimed with TLC, as shown in Fig. 2.b. and 2.c respectively The spot differences or the absence of the same spots between the extracts indicated the success of the partition and fractionation process. Furthermore, all IC_{50} values treated using Bioassay-guided Fractionation (BSA-Glucose method) are shown in Table 1.

Table 1

IC_{50} values for the antiglication test of all extracts using Bioassay-guided Fractionation.

Extracts	**IC_{50} (mg/ml)**
MeOH	0.52 ± 0.03^{ab}
CHCl3	0.33 ± 0.01^{ab}
MeOH soluble	0.45 ± 0.05^{ab}
MeOH insoluble	1.25 ± 0.05^{c}
Fraction I	0.33 ± 0.05^{ab}
Fraction II	0.12 ± 0.01^{ab}
Fraction III	0.67 ± 0.57^{b}
Fraction IV	-0.22 ± 0.02^{a}
Each value represents the mean ± SD. Different letters indicate significant differences ($p < 0.05$).	

IC_{50} results using all extracts showed a significant difference between treatments ($p < 0.05$). The data in Table 1 shows that fraction II had the lowest IC_{50} compared to other extracts. In this study, fraction II had a higher inhibitory activity because fractions with the same concentration contained more active compounds than extracts. The active compound component contained in the active fraction was also not

as much as that contained in the extract. The difference in polarity of the solvent for extraction and fractionation also affects the inhibitory activity.

In Vivo Test Results

The selected active fraction obtained from the bioassay-guided fractination was then used for the ın vivo test by using rats with hyperglycemia. At the end of the study, rats in each group were in good health with an average weight in each group between 198-281.5 grams. All rats in this study were further analyzed, i.e 6 per group.

1. Glucose Levels

The results of glucose level measurements showed different results before induction and before treatment and after treatment with active fraction of red algae. There was a difference ($p < 0.05$) between treatment before induction and after induction of STZ and NA.

These results showed that treatment with active fraction of red algae did not provide significant results for the decrease of glucose levels ($p > 0.05$). Nevertheless, treatment with active fractions with a concentration of 0.17 mg/ml could reduce glucose levels as much as 1.3%, while treatment with active fractions with a concentration of 0.255 mg/ml could reduce glucose levels by 5.2% (Fig. 3). Treatment in diabetic rats without the administration of active fractions showed an increase in glucose levels of 2.3%. Meanwhile, non-diabetic rats showed a decrease in glucose levels of 15.3%.

2. Glycated Albumin (ga) Levels

The results of paired t-test for GA levels in all treatments did not show any significant difference between the treatments before and after administration of red algae active fraction ($p > 0.05$). The treatment in the diabetic rats group showed 19.6% increase of GA levels. In the group with treatment of 0.17 mg/ml active fraction, GA levels decreased 0.4%. Whereas, treatment with active fraction of 0.255 mg/ml increased GA levels by 7.9% (see Fig. 4).

3. Levels Of Nε- (carboxymethyl) Lysine (cml)

Figure 5 shows that Nε-(carboxymethyl) lysine (CML) levels of all treatments decreased. However, the non-diabetic and diabetic groups did not show significant results ($p > 0.05$). CML levels in both treatment at a concentration of 0.17 and 0.255 mg/ml showed a significant decrease ($p < 0.05$). Giving active fraction of 0.17 mg/ml can reduce CML levels by 50.6%, while giving active fraction of 0.255 mg/ml can reduce CML levels by 42.4%

4. Rage Gene Expression Analysis

The results of RAGE gene expression analysis showed a decrease in RAGE gene expression, both in the group of rats with active fraction of 0.17 mg/ml and the group of rats with active fraction 0.255 mg/ml (Fig. 6)

From Fig. 6, it can be seen that there was an increase in RAGE gene expression of diabetic rats group that was not treated with red algae active fraction by 0.71 times. Meanwhile, two other groups of diabetic rats treated with red algae active fractions of 0.17 mg / ml and 0.255 mg/ml only showed an increase of 0.5 times and 0.51 times respectively.

5. Measurement Results With Hrms

HRMS test results showed that there were 707 compounds in the active fraction of red algae. Some of them have been known to have the ability to reduce glucose levels in the blood and have anti-glycation activity (Table 2).

Table 2
Results of analysis of active fraction content from red algae with antiglycation ability using HRMS

Name	Formula	Molecular Weight	RT [min]	Area (Max.)
(-)-Lupinine	C10 H19 N O	169.1464	22.893	249069.529
Shogaol	C17 H24 O3	276.1721	21.776	5197299.59
Thymol	C10 H14 O	150.1043	3.375	3305904.6
(E)-Ferulic acid	C10 H10 O4	194.0579	17.175	362,404.05
Pyrogallol	C6 H6 O3	126.0316	3.311	702966.266
Putrescine	C4 H12 N2	88.10046	3.845	463575.4833
Pheophorbide A	C35 H36 N4 O5	592.2674	29.898	273975531.7
p-cymene	C10 H14	134.1094	3.393	880859.4327
Kahweol	C20 H26 O3	314.1876	24.869	2482362.849
Cafestol	C20 H28 O3	316.2033	22.996	8339006.62
(E)-p-coumaric acid	C9 H8 O3	164.0473	3.328	803138.4894
Cinnamic acid	C9 H8 O2	148.0524	3.402	3772748.16
Anacardic acid	C22 H36 O3	348.2657	24.644	177735.4029

Discussion

The protocol that is widely used to isolate active ingredients from plants is bioassay-guided fractionation, meaning separation through several extraction steps based on different physicochemical properties by assessing biological activity, followed by subsequent separation and testing processes. The extract was fractionated using chromatography to find the active fraction through a test. Each extraction step is evaluated in the bioassay system and only the active extract is further separated. Therefore, if the fraction that is produced is longer, the inhibitory activity will be higher and IC_{50} value will be lower.

Previous research has proven that there are some plants that have the ability to reduce blood glucose. These plants have the potential to reduce glucose levels in the blood because they contain several bioactive compounds. One of the plants is the red algae *Kappaphycus alvarezii* (Doty) Doty ex P.C.Silva. The active fraction of this plant contains several chemical compounds that are proven to reduce blood glucose levels. Although the reduction was not significant, treatment with active fraction with a concentration of 0.17 mg/ml could reduce glucose levels as much as 1.3%, while treatment with an active fraction with a concentration of 0.255 mg/ml could reduce glucose levels as much as 5.2%. Some potential antidiabetic compounds are shown in Table 2.

Cafestol and kahweol are natural diterpenes. Research conducted by [16] showed a decrease of blood glucose levels in the group of mice treated with kahweol. Kahweol induces AMPK (AMP-activated protein kinase) activation. AMPK regulates glucose metabolism by increasing glycolysis, activating 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase, and suppressing glycogen synthesis through inhibition of glycogen synthase. AMPK increases glucose absorption by increasing the expression of glucose transporter 4 and hexokinase II in skeletal muscle cells [17]. Meanwhile, cafestol showed antidiabetic ability. Cafestol interventions for 10 weeks at low doses (0.4 mg/day) and high doses (1.2 mg/day) significantly reduced blood fasting glucose and glucagon. It also increased insulin secretion and sensitivity in KKAy mice [18]. Another compound with the same activity is anacardic acid [19].

Protein glycation is a complex cascade of steps that starts with the non-catalytic binding of sugar, such as glucose, fructose or its derivatives, to the amino groups of a protein. A rearrangement of functional protein molecules occurs before the glycated protein is produced, followed by the formation of crosslinking of these proteins. In diabetic patients, the formation of AGEs is increased, resulting in damaging effects on vital tissues such as the retina, neurons, nephrons, and heart.

Different therapeutic approaches have been developed to limit the adverse effects of AGEs. First, it is by reducing the rate of absorption of AGEs through an adjusted diet or by reducing the absorption of the AGEs in gastrointestinal tract. Second, the formation of AGEs is stopped in the early stages, due to the presence of inhibitors. Third, it is by administering several molecules that can damage the formed AGEs. Finally, interaction of AGEs with its receptor, RAGE, is interfered either by using several traps or antibodies, thereby avoiding the intracellular cascade and its effects [20].

In vitro test results with the BSA-Glucose method showed the ability of active fraction II from red algae to inhibit the glycation process at a concentration of 0.15 mg/ml. Some of the ingredients in the active fraction of red algae have antiglycation abilities, for example, a phenolic compound, ferulic acid. The antiglication effect of this compound was investigated using the BSA-glucose method. Ferulic acid has electron donating groups in the benzene ring (3-methoxy and 4-hydroxyl) which can form phenoxy radicals. Ferulic acid was inhibited in the formation of AGEs because of its ability to capture free radicals, metal chelating, and trapping carbonyl. Ferulic acid prevents the formation of AGEs by acting as an antioxidant, binding to amino groups, and inhibiting sugar autoxidation and degradation of Maillard Reaction Products (MRP) early [21]. Another compound that works by binding to amino groups in BSA is thymol [22]. The results showed that thymol could inhibit the formation of Amadori products. Another compound used in this study was p-Cymene that can capture Amadori intermediates, which reduce AGEs levels. An alkaloid compound, lupinine [23], has moderate antiglication ability and through the interaction of a number of N with reducing sugars, it cannot bind to the amino groups of protein.

In the in vivo test, there were no differences in GA levels before and after treatment of algae active fraction. However, CML levels dropped significantly, both in the active fraction treatment groups of 0.17 and 0.255 mg/ml. This might be due to the active fraction of algae working in inhibiting the formation of AGEs, in this case CML, from Amadori products produced previously.

Inhibition of AGEs formation through methylglyoxal (MGO) blockage has been demonstrated in studies using shogaol [24] and putrescine [25]. Research conducted [26] showed the ability of shogaol to reduce the formation of CML in a BSA/fructose solution. The antioxidant properties of shogaol prevented fructose and Amadori products from oxidation, which leads to inhibition of AGEs production.

The results of the study [27] showed that pyrogallol had antiglication ability by inhibiting further oxidation of glycated proteins and oxidation of metal-catalyzed glucose that leads to the formation of AGEs. The antiglication activity of pyrogallol showed IC_{50} value of 16 µg/ml, which was much stronger than IC_{50} of aminoguanidine treatment in studies using the HSA-glucose model.

Research [28] showed that pheophorbide A had inhibitory activity on RAGE mRNA expression and inflammatory cytokines that were induced by the presence of AGEs significantly. Pheophorbide A had a protective effect and prevented diabetes complications by inhibiting the formation of AGEs.

p-Coumaric acid is a phenolic compound that is known as an efficient antiglication agent. The action of phenolic compounds on protein glycation is associated with its antioxidant ability to prevent oxidation and the formation of AGEs, which is often referred to as autoxidation [29].

Cinnamic acid and its derivatives are phenolic acids that are known to be effective in reducing blood glucose levels in animal models [30]. Research [31] showed the inhibition of protein glycation activity of cinnamic acid and its derivatives using the BSA-fructose method. Cinnamic acid and its derivatives significantly inhibited the formation of AGEs around 11.96 to 63.36% at a concentration of 1 mM.

Cinnamic acid and its derivatives reduced fructosamine levels, CML formation, and cross-β amyloid levels.

It was previously known that hyperglycemic conditions can increase RAGE synthesis [32]. This is evident from the results obtained in this study that the expression of the RAGE (AGER) gene increased by 0.71 times in the group of diabetic rats that were not treated with active fraction from red algae compared to it was in the non-diabetic control group. The decrease in AGEs levels, namely CML, in this study was followed by a decrease in the expression of the RAGE (AGER) gene. The treatment with active fractions from red algae at concentrations of 0.17 mg/ml and 0.255 mg/ml only showed an increase of 0.5 times and 0.51 times, respectively. Research [33] showed that RAGE was expressed in normal podocytes and increased concentration in diabetic nephropathy. CML is the main AGEs in the renal basal membrane in diabetic nephropathy, and its accumulation involves an increase in regulation of RAGE in podocytes.

Conclusıons

The results of this study indicated that fraction II was the main anti-glycation agent of *Kappaphycus alvarezii* (Doty) Doty ex P.C.Silva with the lowest IC_{50} value (0.12 ± 0.01 mg / ml) and a high percentage of inhibition (53.37 ± 1.92%). The administration of this active fraction in diabetic rats could reduce blood glucose levels as much as 1.3% and 5.2% at concentrations of 0.17 and 0.255 mg/ml, respectively. CML levels showed a decrease of 50.6% and 42.4% at concentrations of 0.17 and 0.255 mg/ml. RAGE gene expression in the diabetic group of rats treated with active fraction from algae was lower than it was in the group of diabetic rats without treatment.

List Of Abbreviations

GOD PAP : Glucose Oxsidase – Peroxidase Aminoantypirin

TLC : Thin-layer chromatography

H_2O_2 : *Hydrogen peroxide*

GA : *Glycated albumin*

CML : Nε- (carboxymethyl) lysine

AGEs : Advanced glycation end products

TNFα : *Tumor necrosis factor α*

RAGE : Receptor for Advanced Glycation End Products

NOX4 : NADPH oxidase 4

NFκB : Nuclear factor-κB

Declarations

Ethics approval and consent to participant

This study used the approval of the Faculty of Medicine, Public Health and Nursing Gadjah Mada University Ethics Commission with Ref. No. : KE / FK / 0564 / EC / 2019

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analysed during the current study available from the author on reasonable request because the data used is not shared data.

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

EY, S and MSHW constructed an idea and hypothesis for research and manuscript. EY, S and MSHW planned the methods to generate hypothesis, and to reach the conclusion. S and MSHW supervised the course of the project and the article. EY, S and MSHW provided financial support, equipment and instruments that are vital for the project , biological materials and reagents. EY, S and MSHW taking responsibility in execution of the experiments, data management and reporting. EY, S and MSHW taking responsibility in logical interpretation and analysis of the results. E taking responsibility for conducting literature search. E taking responsibility in the construction of the entire or a substantial part of the manuscript. EY, S and MSHW reviewed the article before submission not only for spelling and grammar but also for its intellectual content. All authors read and approved the final manuscript.

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Figures

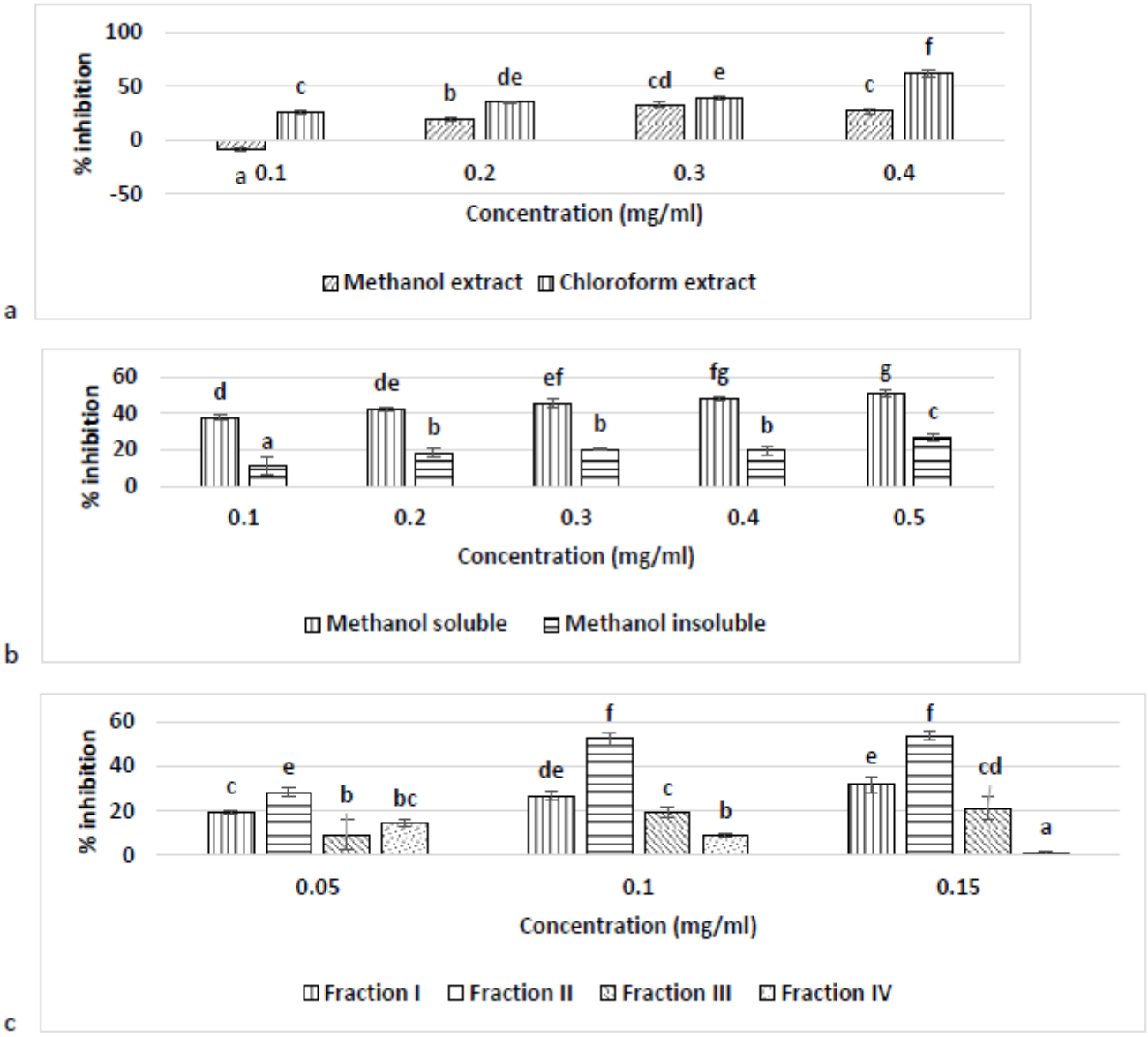

Figure 1

Percentage of inhibition of BSA-glucose test results using Bioassay Guided Fractionation. a. Methanol and chloroform extracts. b. Methanol soluble and insoluble extract. c. Fractination extracts in the formation of fluorescent glycation products. Each value represents the mean ± SD after incubation for 6 days at 37°C. Different letters show significant differences ($p < 0.05$).

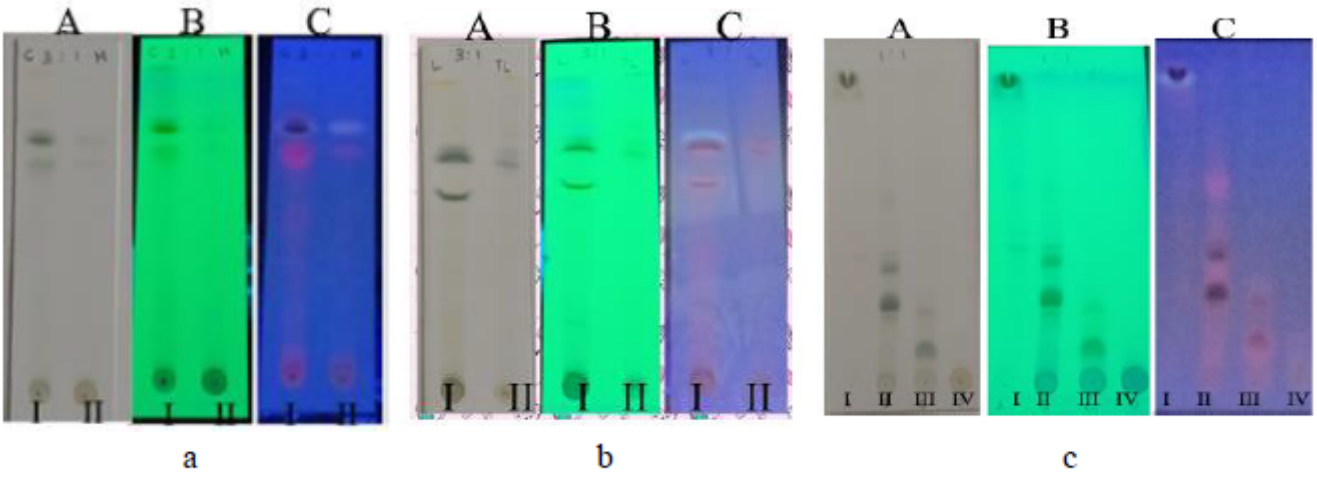

Figure 2

TLC profile of all extracts. a. Methanol extract (I) and chloroform extracts (II). b. methanol-soluble extracts (I) and methanol-insoluble extracts (II). c. Fraction I (I), Fraction II (II), Fraction III (III) and . Fraction IV (IV). A = visible light, B = 254 nm UV light, C = 365 nm UV light. Stationary phase = silica gel 60 F254; Mobile phase = wash benzene: ethyl acetate (3: 1 v / v)

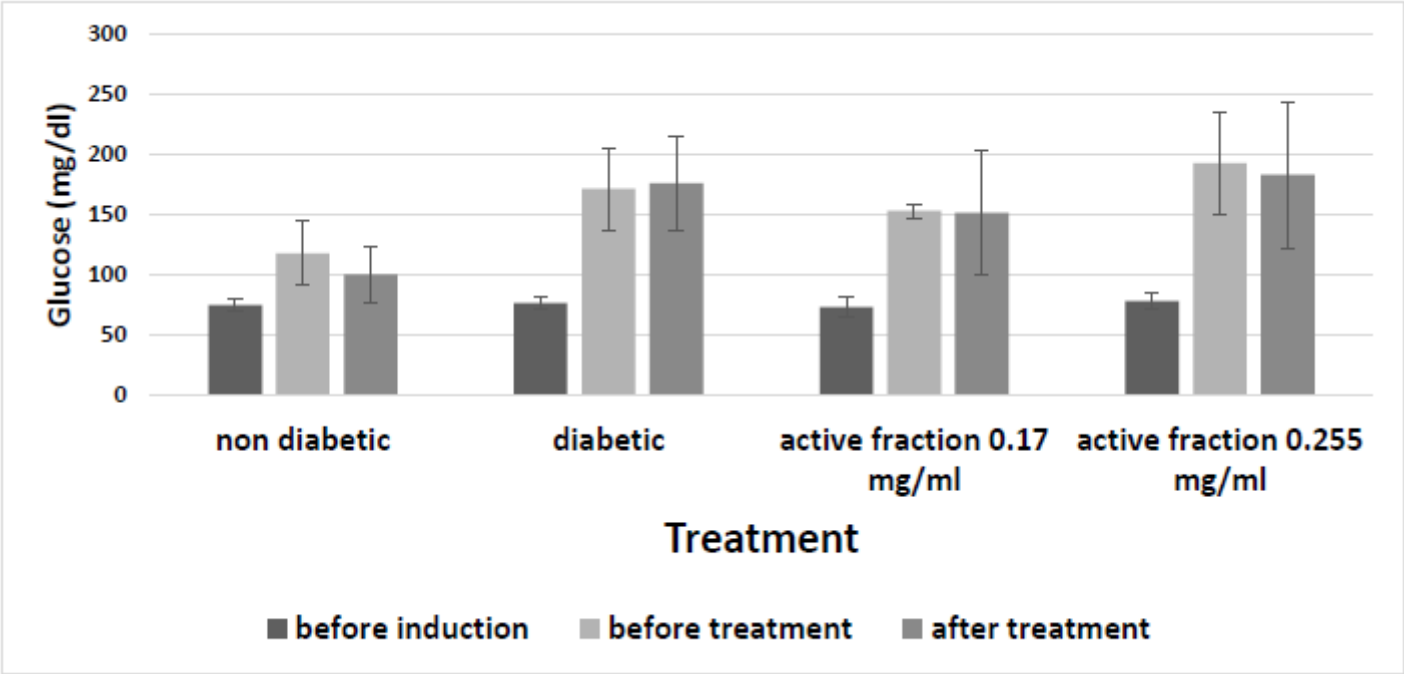

Figure 3

Glucose levels before induction, before and after treatment with active fraction of red algae. Each value represents the mean ± SD of each treatment group.

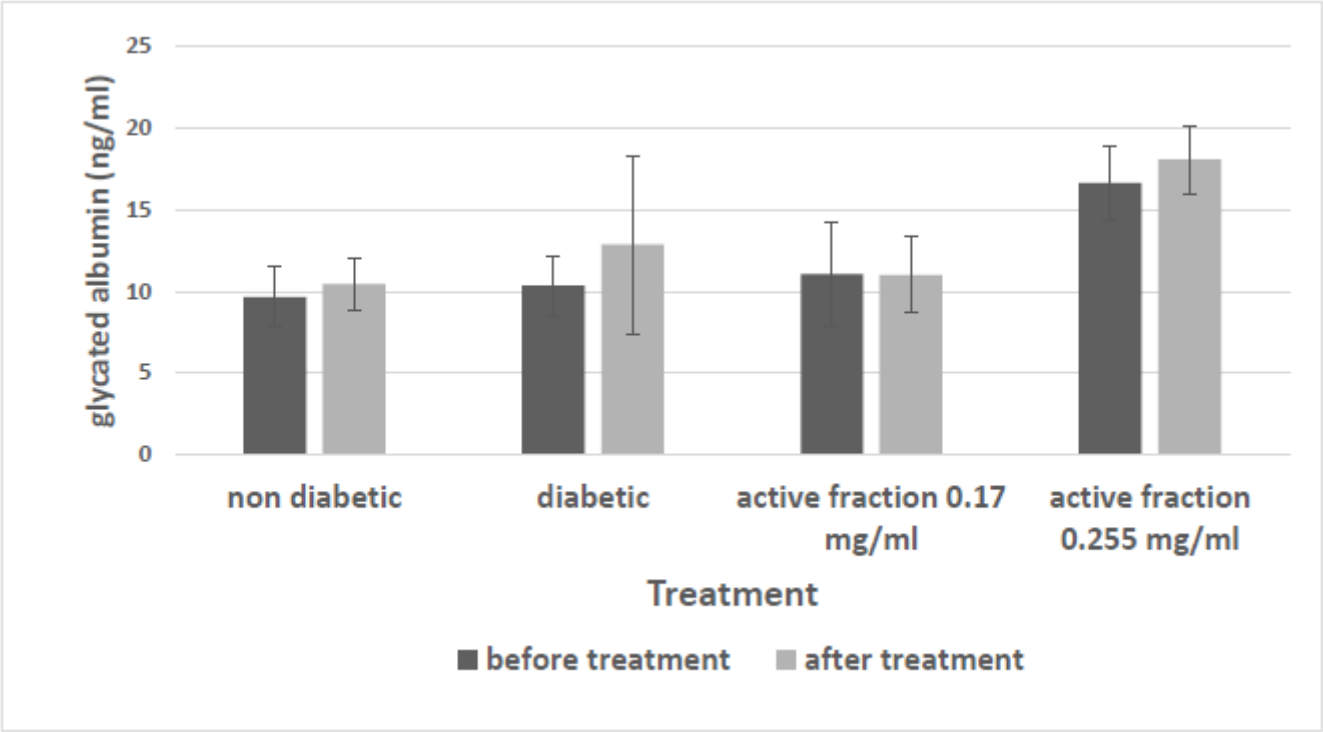

Figure 4

GA levels before and after treatment with the active fraction from red algae. Each value represents the mean ± SD of each treatment group

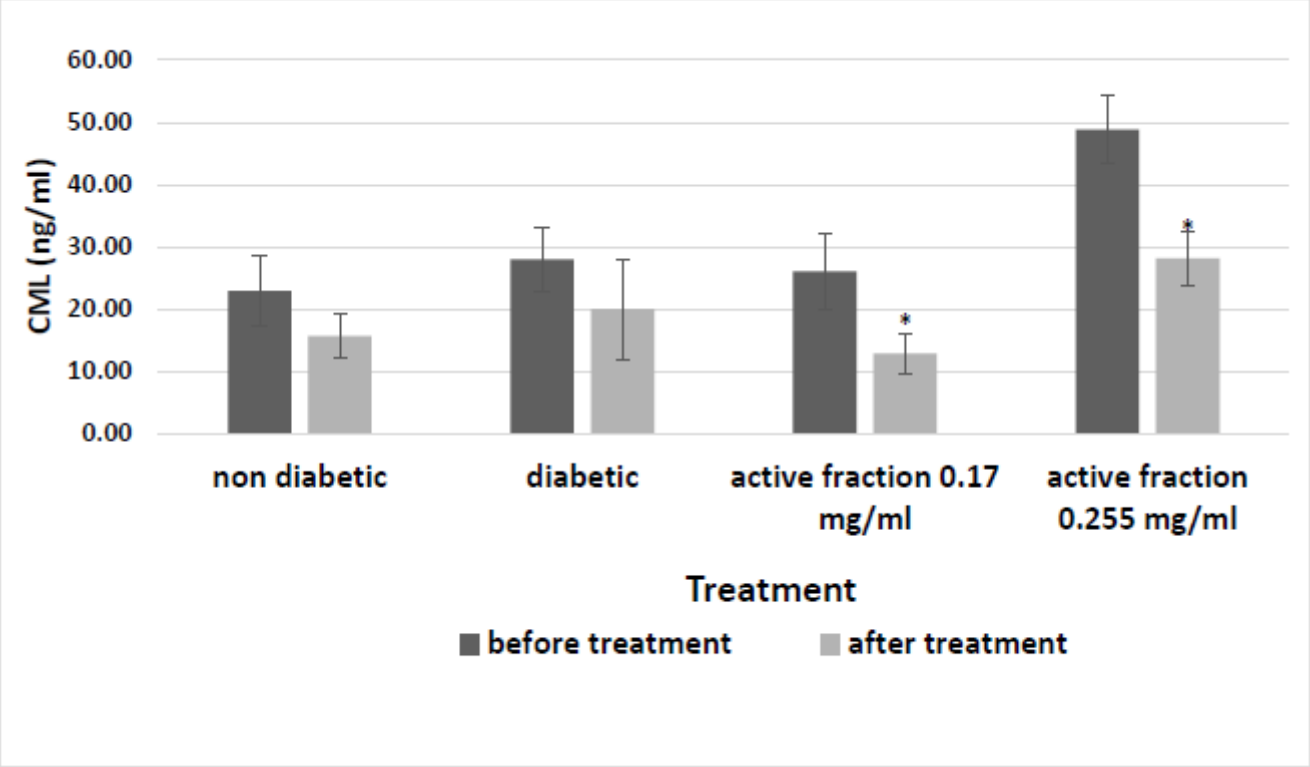

Figure 5

CML levels before and after treatment with active fraction of red algae. Each value represents the mean ± SD of each treatment group. * indicating significant differences ($p < 0.05$) compared to before treatment

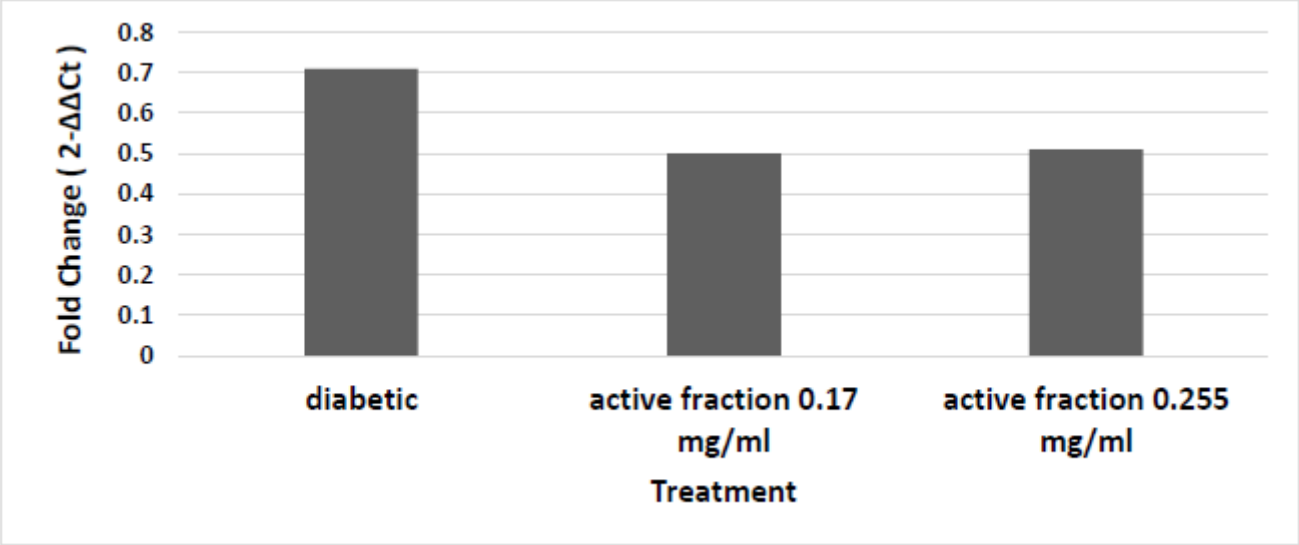

Figure 6

Renal RAGE gene expression

Supplementary Files

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- arrivechecklist.pdf
- Equation1.pdf