



Antidiabetic Effect of *Kappaphycusalvarezii* Extracts on Streptozotocin-Induced Type II Diabetic ICR Mice

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Abstract

The importance of marine algae as potentially prolific sources of functional ingredients has been well known. It is due to their valuable health beneficial effects and might represent a lead in the development of new pharmaceutical agents. *Kappaphycusalvarezii* (Rhodophyceae), marine red algae which mainly harvested in east Malaysia and an important resource for production of carrageenan. Diabetes mellitus is a heterogeneous metabolic disorder affecting 2–5% of the adult population in developed countries. Worldwide prevalence figures give an estimate of 130 million people in 2000 and 300 million in 2025, perhaps, it might increase to 552 million by 2030. Edible marine macroalgae are tested around the world for their ability and potential in anti-diabetic effects. Therefore, this study aim to investigate the Antidiabetic effect of *K. alvarezii* extracts at doses of 100 mg/kg and 200 mg/kg on Streptozotocin-induced type II diabetic ICR mice. Changes in plasma glucose level and body weight in mice treated with extracts before and after treatments were evaluated. Pancreas and blood from mice was obtained separately and followed by different assays in order to investigate the effect of *K. alvarezii* on diabetes-induced mice. Pancreas were grounded into fine powder in liquid nitrogen and used for RNA extraction by R&A-Blue™ Total Extraction Kit. Real-time PCR was taken part to study the gene expression of the Insulin-related gene. Blood serum were separated from the blood and tested for the renal function. After the treatment in a period of 8 weeks, *K. alvarezii* treated diabetic mice experienced progressive plasma glucose reduction, although it is not significantly. Furthermore, 100 mg/kg and 200 mg/kg *K. alvarezii* had up regulated the insulin mRNA expression in the late phase of RT-qPCR. Normal renal function was found in the results of blood test. In conclusion, 100 mg/kg and 200 mg/kg *K. alvarezii* did not significantly showed the Antidiabetic effect, however, pancreas is not the only part of the body which playing a role in plasma glucose regulation. The plasma glucose reduction from the diabetic mice probably regulated from the other organ such as renal and liver. Thus, further investigation shall be continued in order to specifically identify the mechanism.

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Introduction

Marine macroalgae, also commonly known as seaweeds are well known with their wealth of bioactive compounds particularly the polysaccharide and thus, provide great biological active resources [1]. Seaweeds are considered as one of the great sources of nutrition as they contain various types of vitamins which includes vitamin A, B₁, B₁₂, C, D, E, riboflavin, niacin, pantothenic acid and folic acid [1]. In addition, seaweeds are also found to be rich in minerals like Ca, P, Na and K as well as various amino acids that are essential for life [2]. Compounds isolated from marine macroalgae have demonstrated various biological activities, such as antibacterial activity, antioxidant potential, anti-inflammatory properties, anti-coagulant activity, anti-viral activity and apoptotic activity [2]. The consumption of edible seaweeds as human food source has started in the distant past, long before the advent of biotechnology [3]. As results, seaweed-derived compounds have important application in a range of products in food, pharmaceuticals and cosmetics.

There are three major groups of seaweeds namely brown (Phaeophyta), red (Rhodophyta), and green (Chlorophyta). *Kappaphycusalvarezii*, the sample of this study, is marine red seaweed mainly harvested in east Malaysia, Sabah. It is a good source of kappa-carrageenan which widely used as a thickening and gelling agent in many industries, mainly food industry [1]. It is 50 m in height and its habitat is usually on the reefs and shallow lagoons. *K. alvarezii* rich of dietary fibers, vitamin C, α-tocopherol, minerals, fatty acid and protein [4].

Diabetes mellitus, commonly referred to as diabetes, is a group of chronic metabolic diseases characterized by elevated blood glucose levels, a condition termed hyperglycemia [5]. Insulin is

Table 1: Anti-diabetic treatments by various concentrations of *K. alvarezii* extracts and experimental controls in different groups of mice.

Group 1	Non-diabetic micewith 0.1 ml distilled water.
Group 2	Diabetic positive control mice with 100 mg/kgb.wt. tolbutamide.
Group 3	Diabetic negative control mice with 0.1 ml distilled water.
Group 4	Diabetic mice treated with 100 mg/kgb.wt. of <i>K. alvarezii</i> extracts.
Group 5	Diabetic mice treated with 200 mg/kgb.wt. of <i>K. alvarezii</i> extracts.

Table 2: Mouse-specific primers used in RT-qPCR reaction for insulin I and insulin II.

Primer Name	Oligonucleotide Sequences
β -actin	Sense 5'-CCAGGGTGTGATGGTGGGAATG-3' Antisense 5'-CGCACGATTTCCCTCTCAGCTG-3'
insulin I	Sense 5'-TAACCCAGCCCTTAGTGACCAGCTATAA-3' Antisense 5'-AAAGTTTTATTGATGAGAGGGTGGGGC-3'
insulin II	Sense 5'-CCCTGCTGGCCCTGCTCTT-3' Antisense 5'-AGGCTGAAGTCACTGCT-3'

one of the key hormones that regulates glucose, and diabetes results when the body is unable to use insulin effectively or does not produce enough insulin [5]. Overtime, chronic disease like diabetes can lead to development of complications, such as cardiovascular disease, blindness, kidney damage, and nerve damage [5]. These complications cause considerable medical, emotional, and economic burdens. In fact, the larger costs of diabetes are due to the premature death and disability caused by its complications.

Diabetes prevalence is increasing worldwide today and affects a large segment of the population, thus, diabetes is emerging as one of the most serious health problems facing the world today. Moreover, diabetes is placing increasing demands and economic burden on our society and healthcare systems. It is estimated that diabetes affects 246 million adults worldwide and will affect 380 million adults by 2025 [5]. In the United States alone, 23.6 million people of all ages or 7.8% of the population, are estimated to have diabetes [5]. Many patients with diabetes have not yet been diagnosed, so they are not receiving effective treatment. The prevalence of diabetes in Malaysia is approaching 21% of the population of all ages which is about 1 out of 5 Malaysian will have diabetes and half of it are undiagnosed [6].

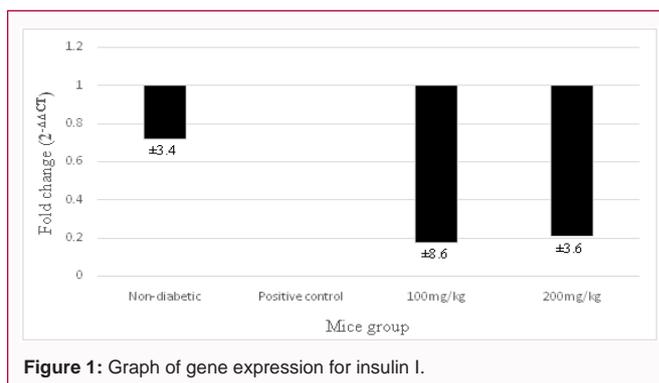
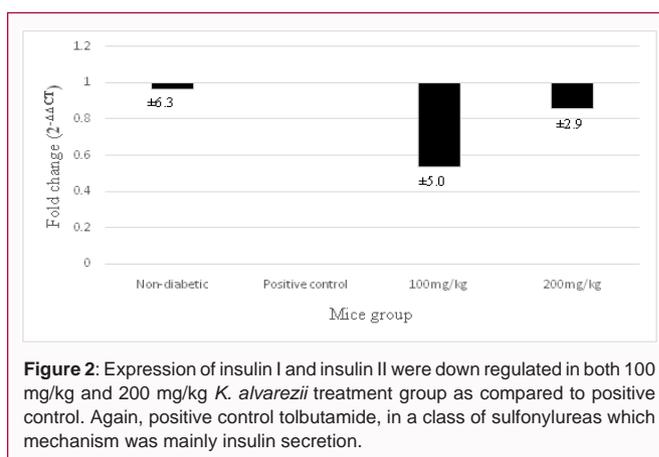
In a modern lifestyle, health become ignorance to people and rate of disease has increasing. Diabetes is now one of the most common disease all around the world. However, diabetes is a manageable disease despite its considerable long term effects. Early and aggressive therapy, with patients and multiple healthcare providers working together as a team, can reduce the impact of diabetes and prevent complications. Therefore, a food like seaweeds which can easily obtained and low cost from the market is now highly recommended to have potential against hyperglycaemia, and benefit the society. Edible marine macroalgae are well studied for its Antidiabetic potential, however, there are still limited investigation of the Antidiabetic properties of *K. alvarezii*.

Materials and Methods

Chemical reagents Liquid nitrogen, methanol, potassium dihydrogen orthophosphate, potassium chloride, disodium hydrogen phosphate, sodium chloride, streptozotocin, tolbutamide, xylazine, ketamine, R&A-BLUE™ Total RNA Extraction Kit, chloroform, isopropanol, ethanol, Oligonucleotide primers:Ins I & Ins II, *i*-Green™ One Step qRT-PCR Kit.

Preparation of *Kappaphycus alvarezii* extracts

A total of 10g of *K. alvarezii* was weighed and first washed 3 times

**Figure 1:** Graph of gene expression for insulin I.**Figure 2:** Expression of insulin I and insulin II were down regulated in both 100 mg/kg and 200 mg/kg *K. alvarezii* treatment group as compared to positive control. Again, positive control tolbutamide, in a class of sulfonylureas which mechanism was mainly insulin secretion.

with tap water to remove salt, epiphytes, and sand attached to the surface, and then carefully rinsed with fresh water. Thereafter, the seaweed samples were lyophilized and homogenized by using mortar and pestle while being freeze-dried using liquid nitrogen. Grounded *K. alvarezii* seaweed was transferred into a 250 ml conical flask and 150 ml of 70% methanol was added to it. The conical flask was then placed on shaker for 24 hours of incubation on 200 rpm shaker. After the incubation period, the seaweed crude extract was filtered using filter paper and filtrate was collected in a round-bottom flask. Filtrate was then concentrated using rotary evaporator at the temperature of 45°C to 50°C, and 30 g of extract per 200 g of powdered *K. alvarezii* was obtained. The collected crude extract was freeze dried and stored in 4°C chiller for storage. The powdered *K. alvarezii* extract were then used in the animal experiment.

Diabetes induction

Before carrying out diabetes induction, seven-week-old male ICR mice were acclimatized for two weeks' time. After that, diabetes induction in mice was carried out by single intraperitoneal injection of Streptozotocin (STZ) at a dose of 50 mg/kg b.wt. In addition to that, an overnight fasting with at least 12 hrs was carried out before STZ injection. After 72 hours (3rd day) post Streptozotocin induction, mice weight was measured using weighing balance. At the same time, approximately 0.1ml or less of blood sample was collected from the tail vein of non-fasted mice using vein puncture method in order to confirm the success of diabetes induction. The determination of postprandial glucose level was done by GOD/POD (glucose oxidase peroxidase) method using Accu-Check[®] Advantage II glucometer. Mice with glucose levels above 11.1 mmol/L (200 mg/dL) were selected for the anti-diabetic study.

Anti-diabetic treatment with *K. alvarezii* extracts. Before the

Table 3: Mean and standard deviation of mice blood glucose level (mmol/L) from week 0 to week 8 of *K. alvarezii* extracts treatment.

		Non-diabetic	Positive control	Negative control	100 mg/kg <i>K. alvarezii</i> crude extracts	200 mg/kg <i>K. alvarezii</i> crude extracts
Mean \pm standard deviation, mmol/L	Week 0	6.7 \pm 1.7	18.8 \pm 4.2	17.1 \pm 4.9	20.0 \pm 6.2	23.5 \pm 6.1
	Week 1	7.5 \pm 1.1	18.1 \pm 1.7	17.6 \pm 6.6	19.6 \pm 4.3	24.2 \pm 3.4
	Week 2	7.4 \pm 0.7	15.5 \pm 1.8	17.2 \pm 5.4	20.8 \pm 6.1	23.6 \pm 1.8
	Week 3	7.5 \pm 0.7	15.9 \pm 4.7	15.9 \pm 3.7	21.0 \pm 5.5	23.5 \pm 1.9
	Week 4	7.3 \pm 0.9	17.5 \pm 5.4	15.9 \pm 2.9	21.9 \pm 2.4	24.3 \pm 1.8
	Week 5	7.3 \pm 1.3	17.4 \pm 6.3	16.3 \pm 4.2	23.0 \pm 1.7	28.8 \pm 5.1
	Week 6	7.1 \pm 1.4	16.3 \pm 5.5	17.3 \pm 6.1	22.5 \pm 1.4	26.6 \pm 5.6
	Week 7	6.9 \pm 1.2	13.7 \pm 5.5	18.9 \pm 5.4	21.0 \pm 4.3	21.0 \pm 4.3
Week 8	7.7 \pm 1.0	10.5 \pm 3.3	19.5 \pm 6.0	20.5 \pm 5.8	19.0 \pm 3.8	

Table 4: Mean percentage of mice blood glucose level (%) from week 0 to week 8 of *K. alvarezii* extracts treatment.

		Non-diabetic	Positive control	Negative control	100 mg/kg <i>K. alvarezii</i> crude extracts	200 mg/kg <i>K. alvarezii</i> crude extracts
Mean \pm standard deviation, %	Week 0	100.0	100.0	100.0	100.0	100.0
	Week 1	114.3 \pm 17.6	99.2 \pm 15.7	105.4 \pm 29.0	101.5 \pm 20.9	108.2 \pm 22.7
	Week 2	113.0 \pm 19.1	86.6 \pm 16.8	101.1 \pm 23.7	102.2 \pm 29.6	104.8 \pm 17.1
	Week 3	114.6 \pm 19.0	90.7 \pm 17.9	94.8 \pm 16.3	106.3 \pm 26.7	104.4 \pm 19.2
	Week 4	111.9 \pm 14.8	99.7 \pm 20.6	95.0 \pm 12.0	115.4 \pm 21.7	108.7 \pm 16.4
	Week 5	110.1 \pm 11.8	100.2 \pm 21.4	96.1 \pm 18.5	124.6 \pm 18.3	130.5 \pm 29.2
	Week 6	106.9 \pm 15.0	93.8 \pm 17.0	102.1 \pm 28.1	119.6 \pm 16.8	114.5 \pm 26.4
	Week 7	111.0 \pm 10.5	78.7 \pm 13.9	112.9 \pm 20.4	108.1 \pm 15.3	90.8 \pm 18.1
Week 8	117.3 \pm 14.1	59.0 \pm 10.1	116.6 \pm 26.3	102.9 \pm 20.7	83.1 \pm 16.0	

anti-diabetic treatment, blood glucose level and body weight were measured and labeled as week 0. Using oral gavage method, mice were treated everyday for 8 continuous weeks. Mice were separated into 5 groups according to the study design (N=5) in Table 3,4. The plasma glucose level and body weight were taken once every week approximately one hour after the anti-diabetic oral treatment. The above study design will be maintained for all the experiments in this study. Statistical analyses on body weight and plasma glucose level were compared between before treatment (week 0) and after treatment (week 8) using paired sample t-test, whereby $P < 0.05$ was considered as statistically significant. In addition, the final body weight and plasma glucose level on week 8 were compared between groups using one-way analysis of variance (ANOVA) and Tukey's post hoc analysis, whereby $P < 0.05$ was considered as statistically significant.

Total RNA extraction

RNA was isolated accordingly to the protocol of R&A-Blue™ Total Extraction Kit. Extracted pancreas tissues were homogenized into fine powder with pestle and mortar in 1mL of R&A-Blue™ lysis reagent. After homogenization, samples with lysis reagent were transferred into 1.5 ml micro centrifuge tubes and subjected to centrifugation at 13,000 rpm for 10 mins at 4°C. Next, the cleared homogenate solution was transferred into a new tube. A total of 200 μ L of chloroform was added into each tube containing lysate, followed by being vigorously vortexed for 15 secs. Lysate was centrifuged at 13,000 rpm for 10 mins at 4°C to separate the mixture into 3 phases, a blue organic phase at the bottom, midlayer and colourless upper aqueous phase containing RNA. The top aqueous layer was carefully transferred into a new tube without touching the midlayer. After that, 400 μ L of isopropanol was added and mixed well by inverting the tube 6 to 7 times for RNA

precipitation. Centrifugation at 13,000 rpm, for 10 mins at 4°C was carried out and supernatant was removed without disturbing the RNA pellet. Next, 1 ml of 75% ethanol was added and mixed well by inverting the tube 4-5 times. Mixture was then centrifuged for 1 min at room temperature and supernatant was discarded. The remaining RNA pellet was left to dry at room temperature. RNA pellet was not allowed to dry completely as this will reduce its solubility. Next, RNA was dissolved using 50 μ L of RNA se free water. Absorbances of RNA at 260 nm and 280 nm, and RNA concentration were determined using Eppendorf BioPhotometer.

The mRNA expression levels of insulin I and II were determined using *i-Green™* One Step qRT-PCR kit. A total of 0.2 μ g RNA from each pancreas tissues were reverse-transcribed and amplified using one-step qRT-PCR according to manufacturer's instruction. Samples were run in triplicates and all assays were performed using Step One Real-time PCR System (Applied Biosystems). The mRNA of housekeeping gene, β -actin was assayed and then normalized to total RNA measurements for each sample. The mRNA content was calculated for each sample relative to β -actin by using equation $2^{-\Delta\Delta CT}$. Statistical significance was analyzed by one-way analysis of variance (ANOVA) and Tukey's post hoc analysis, whereby $P < 0.05$ was considered statistically significant. The following oligonucleotide primers specific for mouse insulin I, insulin II and β -actin were recorded in Table 2.

Results and Discussion

In this study, random plasma glucose is used to measure and determine the blood glucose level of the mice. The average blood glucose level and percentage changes of mice recorded from week 0 to week 8 were shown in Table 3 and Table 4, respectively.

From Table 3 and Table 4, treatment of 100 mg/kg and 200 mg/kg of *K. alvarezii* crude extracts did not show the same efficacy in reducing the blood glucose level in 8 weeks' time as positive control. However, treatment of 200 mg/kg alone did numerically show the effect in reducing the blood glucose level especially started after week 5, it is not significant though. Type 2 diabetes mellitus is a chronic disease as it strongly shown in the blood glucose level in negative control, the increasing trends of blood glucose level after 4th week. On the other hand, treatment of 100 mg/kg shown decreasing in blood glucose level started from 5th week. Therefore, treatment of 100 mg/kg and 200 mg/kg of *K. alvarezii* crude extracts have shown the trends that decreasing the blood glucose level when compared to negative control, not as strong as positive control though. This could be explained by the mechanism of how positive control regulate the blood glucose level in the body. The Positive control, Tolbutamide classified as sulfonylureas in a range of antidiabetic drugs. Sulfonylureas capable of reducing blood glucose levels by stimulating functioning beta-cells in the pancreas to release more insulin, irrespective of glucose levels [7,8]. Hence, sulfonylureas also know insulin secretagogues. This increase is apparent during both the first and second phase insulin responses [7].

The process that stimulates insulin secretion normally begins when glucose enters pancreatic beta-cells. Glucose is then metabolized for energy, and a compound called adenosine triphosphate (ATP) is produced in the process. After this point, ATP causes potassium channels on the beta cell membrane to close, then the closure of potassium channels triggers the opening of calcium channels on the beta cell membrane, which increases the flow of calcium into the cell. Next, the high levels of calcium in the cell trigger the release of insulin from beta cells [8]. Sulfonylureas stimulate insulin secretion by binding to SUR/KIR6.2 receptors on pancreatic beta cells [7,8]. This causes ATP-dependent potassium channels to close and calcium channels to open, resulting in increased insulin secretion. In general, sulfonylureas are established with the strong and fast efficacy even compared to others anti-diabetic drugs. However, there are also some important limitations due to the mechanism of sulfonylureas in terms of the efficacy. Patients must have functioning beta cells for sulfonylureas to be effective and subsequently patients will lose their responsiveness to sulfonylureas due to the non-stop exhaustion of beta cells gradually. This phenomenon known as secondary failure [8,9]. Therefore, glucose lowering effects of *K. alvarezii* should not be denied at this stage by just claiming that it is not as efficacious as positive control. There is possibility that crude extracts of *K. alvarezii* could have comparable or better efficacy in lowering blood glucose levels in a long run like other anti-diabetic agents for instance. Furthermore, in order to attain and maintain glucose control as diabetes is a progressive disease, therapy of combination antidiabetic agents is required [10]. Combination 2 drugs of different classes may sometimes be used as initial pharmacotherapy when there is marked hyperglycemia [10]. In real time PCR analysis, results from Figure 1 and Figure 2 shown that gene expression of insulin I and insulin II were down regulated in both 100 mg/kg and 200 mg/kg *K. alvarezii* treatment group as compared to positive control. Again, positive control tolbutamide, in a class of sulfonylureas which mechanism was mainly insulin secretion outperformed the rest of the *K. alvarezii* treatment group. The expression of insulin gene in positive control seem to be more as compared to non-diabetic control group in the results. This clearly explained the well-known side effect of sulfonylureas termed hypoglycemia because sulfonylureas stimulate

insulin secretion whether or not glucose is present. Although the insulin secretion effect of *K. alvarezii* crude extracts was not as significant as positive control, the mechanisms of glucose lowering effect of *K. alvarezii* crude extracts which discussed earlier was still remains unknown. It could be one or more of the mechanisms which show the improvement in any of the eight metabolic defects that contribute to type 2 diabetes rather than acts on the pancreatic beta cell alone whereby this study only focus on the gene expression of insulin I and II from the beta cells. Insulin resistance in muscle, and liver and beta cell failure represent the core pathophysiological defects in type 2 diabetes [11]. It now is recognized that the beta cell failure occurs much earlier and is more severe than previous thought. In addition to muscle, liver, and beta cell, the fat cell which accelerated lipolysis, gastrointestinal tract, alpha cell, kidney which increased glucose reabsorption, and brain all play important roles in the development of glucose intolerance in type 2 diabetic individuals [8]. Collectively, these eight players comprise the ominous octet and dictate that multiple drugs used in combination will be required to correct the multiple pathophysiological defects. Secondly, treatment should be based on reversal of known pathophysiological abnormalities and not simply on reducing the HbA1c. Lastly, therapy must be started early to prevent or slow the progressive beta-cell failure that is already established in patients with impaired glucose tolerance [8]. Therefore, once again that the treatment on type 2 diabetes can be acted on a several targeted site instead of just focus on pancreas.

Conclusion

In this study, it is yet to prove that *K. alvarezii* has significant antidiabetic effects but the potential could not be written off. The treatment of 200 mg/kg alone did numerically shown the effect in reducing the blood glucose level especially started after week 5. In real time PCR analysis, results shown that gene expression of insulin I and insulin II were down regulated in both 100 mg/kg and 200 mg/kg *K. alvarezii* treatment group as compared to positive control. Although the insulin secretion effect of *K. alvarezii* crude extracts was not as significant as positive control, the mechanisms of glucose lowering effect of *K. alvarezii* crude extracts which discussed earlier was still remains unknown. In order to find out the better way in treating type 2 diabetes, a greater understanding about glucose regulation in the human body and diabetes pathophysiology playing an important role.

For future studies, PCR analysis on the other hormones that help to regulate the glucose in the body such as GLP-1 and GIP from stomach, glucagon from pancreas. Key organs that play a part in regulating the glucose also should be targeting for further investigation. For example, liver, adipose fats, and kidney. *K. alvarezii* has great potential in pharmaceutical and good for human health even though some of the substances in *K. alvarezii* are still remains unknown and it is cheaper in cost comparing to those medications that currently available in the market. Consequently, crude extracts from the red seaweeds, *K. alvarezii* have shown potential glucose lowering effect and it will be worth to further investigate in order to benefit the type 2 diabetes patients, in terms of health and burden of cost.

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