Hypoglycemic activity of brown seaweed (*Sargassum plagiophyllum*) kombucha bioactive compound in rat model type-2 of diabetes

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Abstract. Hyperglycemia is a condition of increased blood glucose with out of normal limits criteria associated with diabetes mellitus. Uncontrolled hyperglycemia is one of the causes of complications in diabetes mellitus sufferers. *Sargassum plagiophyllum* is one of brown seaweed that contains many bioactive compounds which are useful as hypoglycemic agents. The kombucha fermentation method is used to reduce structural complexity, thereby increasing the bioavailability of bioactives. This study aims to evaluate the administration of *S. plagiophyllum* kombucha (SK) to the blood profile of type 2 diabetes mellitus (DM2) rats treated with a 21% high-fructose diet (HFD) with normal feed. *S. plagiophyllum* was obtained from Talango, Sumenep, Madura Island, East Java Province. 2-month-old male *Rattus norvegicus* was used as a model animal. The study consisted of 5 groups, including negative control, positive control (DM2), DM2 + SK 1.5 mL/250 g bw once a day, DM2 + SK 1.5 mL/250 g bw twice a day, and DM2 + SK 1, 5 mL/250 g bw three times a day. Animal model research was carried out for 2 months. The study's conclusion shows that *S. plagiophyllum* kombucha has bioactive compounds as hypoglycemic agents and contributes to improving body weight, fasting blood glucose (FBG), lipid profiles, insulin, and MDA in type-2 diabetes rats model.

1 Introduction

Diabetes mellitus is a health symptom characterized by high blood glucose levels caused by insulin secretion dysfunction or other biological dysfunction. Diabetes is one of the most common metabolic diseases in the world today [1]. Chronic hyperglycemia with criteria of continuously increasing blood glucose levels can cause microvascular and macrovascular complications. Microvascular complications include small blood vessels, namely capillaries, which can result in nephropathy, retinopathy, neuropathy, and microangiopathy. Macrovascular complications include the narrowing of large arteries and veins due to atherosclerosis, which results in cerebrovascular, cardiovascular, or peripheral arterial disease [2]. Insulin resistance is a condition of reduced responsiveness in tissues with insulin

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targets. This results in increased blood sugar levels, if it occurs continuously it can cause chronic hyperinsulinemia to β-cell failure [3]. Hyperglycemic conditions produce free radicals in the body, which can cause oxidative damage in body cells [4]. Excessive oxidative stress conditions can cause complications and even damage [5].

External factors in the form of poor diet and food consumption can cause diseases such as hypertension, metabolic abnormalities, hyperinsulinemia, insulin resistance, and hypertriglyceridemia [6]. One of the factors contributing to obesity and type-2 diabetes is the frequent intake of beverages with added sugar [7]. One of the sweeteners often used in food processing is fructose. Consumption of fructose can cause hyperglycemia, insulin resistance, and hypertriglyceridemia. The mechanism is by disrupting the glucose metabolism pathway and its absorption due to excess fructose. Disrupted metabolism causes an increase in the rate of lipogenesis and triacylglycerol synthesis through high concentrations of glycerol molecules and acyl from fructose catabolism, which results in insulin resistance [8].

A person with diabetes will normally consume drugs to maintain blood glucose stability, which can cause side effects. Natural ingredients can be used as an alternative with the criteria of having active compounds that can inhibit enzymes that work on glucose and work as antioxidants with the ability to capture free radical [9]. One of the natural ingredients that has active compounds is brown seaweed, namely *Sargassum* sp., known to contain polyphenols, phlorotannins, gallic acid, phloroglucinol, plastoquinones, and ellagic acid that can activate insulin secretion and inhibit enzymes that degrading carbohydrate. act as hypoglycemic agents. With these properties, *Sargassum* sp. can be used as raw material for antihyperglycemic products [10].

Kombucha is a fermentation refreshing health drink made from sugared tea infusion. Fermented by a symbiotic consortium of yeast species and acetic acid bacteria. Various types of yeast (e.g. *Pichia*, *Candida*, *Zygosaccharomyces*, *Brettanomyces*, and *Saccharomyces* species) and *Acetobacter xylinum* have been identified in kombucha fermentation [11]. In diabetic animal models, kombucha tea can be utilized as a treatment for dyslipidemia and hyperglycemia. Known mechanisms of action include decreased damage to pancreatic B-cells, increased insulin synthesis, decreased glucose absorption from the digestive tract, and enhanced glucose uptake by cells [12]. There are several studies that have examined brown seaweed kombucha, but the analysis is still limited to its physico-chemical characteristics [13], [14]. The utilization of *Sargassum* sp. with the kombucha tea fermentation technique is expected to be a diversification of seaweed products and functional foods with attributes as hypoglycemic agents.

2 Materials and methods

2.1 Materials

Brown seaweed (*Sargassum plagiophyllum*) obtained from Talango, Sumenep, Madura Island, East Java, Indonesia. Kombucha starter, sucrose, and fructose were obtained commercially. The selection of brown seaweed as a raw material for making kombucha is because it contains most active secondary metabolites such as phlorotannin, fucoidans, alginic acid, and fucoxanthine which have anti-inflammatory and antioxidant activities [15]. Especially phlorotannin which is abundant in *Sargassum* sp. is one type of secondary metabolite that can work as a hypoglycemic agent [16].

2.2 Kombucha preparation

Fresh *S. plagiophyllum* is washed until clean and then dried using an oven. Once dry, it is ground using a blender. Making kombucha begins by preparing seaweed, sugar, and water with a ratio of 1:10:100, referring to [17], then boiled for 15 minutes, strained, and placed in a sterile glass container. After reaching room temperature, add kombucha starter as much as 10% (v/v). Wrap a sterile towel over the container and secure it firmly. Fermentation runs for 14 days at room temperature.

2.3 LC-MS/MS analysis

The harvested kombucha was then analyzed for its metabolite compound content using LC-MS/MS [18]. The specification of the tools is ultra-performance liquid chromatography (UPLC) apparatus (LC: Acquity UPLC® H-Class System, Waters, USA) with pairing a mass spectrometer (Xevo G2). -S QTof, Waters, USA). The column used is C18 (1.8 µm 2.1x100 mm, ACQUITY UPLC® HSS, Waters, USA), running at room temperature of 25°C and column temperature of 50°C. Water + 5 mM ammonium formic acid (A) and acetonitrile + 0.05% formic acid (B) are the mobile phases of the LC-MS analysis. The injection volume was 5 µL, which was first filtered through a 0.2 µm syringe filter, and the flow rate was set to 0.2 mL/min (step gradient) for 23 minutes. Mass spectrometry (MS) mass range is 50–1200 m/z with source and desolvation temperatures of 100 and 350°C employing electrospray ionization (ESI) in positive mode. Furthermore, 0 L/hr and 793 L/hr cone and desolvation gas flow rates, respectively, were employed, and the collision energy ranged from 4 to 60 eV. Masslynx software version 4.1 and the ChemSpider website were used for data analysis and interpretation of control instruments.

2.4 Animal experiment

Animal experiment testing was conducted using male Wistar rats (*Rattus norvegicus*) aged 2-3 months weighing ±150 grams. The animal experiment has a proper permit issued by the Institutional Ethics Committee of Animal Care and Use, Brawijaya University, Malang, Indonesia (No: 159-KEP-UB-2023. The experimental animal research began with acclimatization for 7 days before the treatment was carried out. The rats were grouped into five groups with five repetitions and randomized as follows: negative control (N), normal; positive control (P), fructose 21% drink *ad libitum*; dose 1 (SK1), fructose 21% drink *ad libitum* and SK at a dose of 1.5 mL/250 g BW once in a day, dose 2 (SK2), fructose 21% drink *ad libitum* and SK at a dose of 1.5 mL/250 g BW twice in a day, dose 3 (SK3), fructose 21% drink *ad libitum* and SK at a dose of 1.5 mL/250 g BW three times in a day. The cage used was a single type, with a room temperature of 25°C in a controlled room with 12 hours light: 12 hours dark cycle. During maintenance, the rat was weighted periodically to determine the dose of SK. In addition, it was also to observe the benefits of the drink on the weight of the rat. The administration of *S. plagiophyllum* kombucha doses was carried out for 2 months. After 2 months, the rats were induced with low-dose *streptozotocin* (STZ) 20 mg/kg in all groups except the negative control group (N). Furthermore, a week of response adjustment time was given. Then, the blood glucose of each mouse was measured. After that, the surgical process was carried out with the preparation of rats induced with ketamine HCl, which functions as anaesthesia. This animal experience research scheme is a modification of the research from [19].

2.5 Animal analysis

The characteristics of the experimental animals measured included body weight, fasting blood glucose (FBG), lipid profiles (cholesterol, triglyceride, and high-density lipoprotein (HDL) cholesterol levels), insulin, and malondialdehyde (MDA). The body weight of rats was measured with a digital scale once a week. FBG was measured using *Autocheck* glucometer tools. Lipid profiles were measured using the Horiba C200 automatic analyzer from blood serum. Insulin used the Rat Insulin ELISA Kit Elabscience, Cat. No. E-EL-R3034. MDA was analyzed using a sample of rat liver.

2.6 Statistical analysis

The data obtained from the research were then analyzed using analysis of variance (ANOVA), with further testing using the LSD test at $p < 0.05$.

3. Results and discussions

3.1. Compound identity of *S. plagiophyllum* kombucha

LC-MS/MS analysis results show that *S. plagiophyllum* kombucha has 31 identified compounds. The potential compounds identified are ten compounds. The grouping is based on the group of each compound, including the ester, alkaloid, flavonoid, organic, amino acid, steroid, and phenolic groups.

Table 1. Potential bioactive compound of *S. plagiophyllum* kombucha.

	Rt (min)	Measured Mass (m/z)	Formula	Compound	Compound Group
1	1,19	365,11	$C_{14}H_{20}O_{11}$	2,3,4,5-Tetra-O-acetylhexonic acid	Ester
2	4,13	195,09	$C_8H_{10}N_4O_2$	Caffeine	Alkaloid
3	4,37	565,16	$C_{26}H_{28}O_{14}$	Apiin	Flavonoid
4	11,82	387,25	$C_{24}H_{34}O_4$	Medroxyprogesterone 17-acetate	Steroid
5	12,94	522,38	$C_{30}H_{51}NO_6$	2,2'-{[3,5-Bis(decyloxy)phenyl]imino}diacetic acid	Organic
6	13,25	611,29	$C_{35}H_{38}N_4O_6$	Manidipine	Organic
7	14,14	515,32	$C_{23}H_{42}N_6O_7$	N2-Acetyl-L-glutaminyl-N-[(3S,4S)-1-{[(2S)-1-amino-3-methyl-1-oxo-2-butanyl]amino}-3-hydroxy-6-methyl-1-oxo-4-heptanyl]-L-alaninamide	Amino Acid
8	14,28	639,41	$C_{42}H_{54}O_5$	(3β)-Cholest-5-en-3-yl [(6-oxo-6H-benzo[c]chromen-3-yl)oxy]acetate	Steroid
9	14,41	541,34	$C_{25}H_{44}N_6O_7$	(8S,11R,12S)-N12-Hydroxy-11-isobutyl-N8-[2-(4-methyl-1-piperazinyl)-2-oxoethyl]-2,10-dioxo-1-oxa-3,9-diazacyclopentadecane-8,12-dicarboxamide	Alkaloid
10	14,52	607,26	$C_{34}H_{38}O_{10}$	(3S)-3-[(2E)-3-Carboxy-2-buten-1-yl]-7-hydroxy-4-methoxy-1,1,8,8,9-pentamethyl-11-(3-methyl-2-buten-1-yl)-6-oxo-3,6,8,9-tetrahydro-1H-difuro[3,2-b:3',4'-h]xanthene-3-carboxylic acid	Phenolic

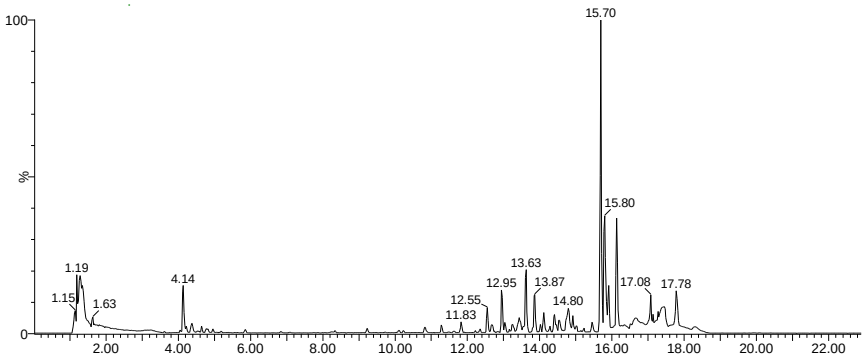

Fig 1. LC-MS/MS peaks of *S. plagiophyllum* kombucha.

Based on the results of the compounds obtained in SK show that the compounds contained therein are diverse. In the diversity of these compounds, several groups of compounds have the potential to be used as hypoglycemic agents, mainly phenolic, flavonoid, and alkaloid groups as bioactive compound groups. Phytochemical screening shows that *Sargassum* sp. has various bioactive compounds, such as phenolics, flavonoids, saponins, terpenoids, and phlorotannins, depending on the type of solvent used in the extraction process [20]. Bioactive components obtained from at least 800 types of plants have the potential for antidiabetic activities. Bioactive compounds have various mechanisms, such as inhibition of stimulation of enzymatic activity or protein expression. The most effective group of active natural compounds as antidiabetics are polyphenols, flavonoids, and saponins [21].

3.2. Animal body weight

Periodic weighing of experimental animals was carried out to determine the development and effects of SK on animal weight. The results showed that the lowest weight difference between before and after treatment from all groups was in group SK3 or the treatment of SK three times a day. This was different from group P, which had DM conditions without SK administration and an average final weight reaching 270 g. Excessive fructose consumption has an impact on the potential for obesity and metabolic syndrome. Hyperglycemia is one part of metabolic syndrome, which is the beginning of type-2 diabetes [22]. Treatment SK3 had the lowest difference because SK may contain ingredients with a working mechanism as an antiobesity. Fibre plays an antiobesity role by suppressing energy intake through at least three different pathways. The first is the removal of nutrients and calories from the available diet. Secondly, fibre makes people chew more, which reduces consumption by encouraging the flow of gastric juice and saliva. It causes the stomach to expand and fullness levels to rise. The third way is that the fibre makes the small intestine less effective in absorbing nutrients. The bulking and viscosity characteristics of dietary fibre are primarily in charge of affecting satiety and satiation because they lengthen the time and effort required for mastication, which in turn slows down the rate of ingestion and produces a sense of fullness [23]. Fucoxanthin found in *Sargassum* sp. has been shown to considerably lower plasmatic and hepatic triglyceride concentrations. Additionally, it has been shown to positively effect enzymes that regulate cholesterol, including acyl-coenzyme A and 3-hydroxy-3-methylglutaryl coenzyme A reductase [24].

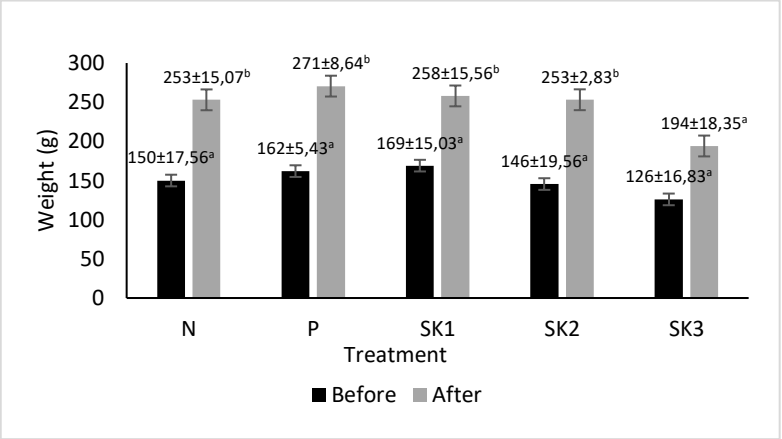

Fig 2. Effect of the kombucha administration on rats body weight difference between before and after treatment.

3.3. Fasting blood glucose (FBG)

The results obtained from the FBG analysis were that treatment P obtained a significant value ($p < 0.05$) on blood glucose levels between before and after STZ induction. Treatment N had the lowest level compared to the others because it did not receive STZ/normal model induction. Treatments SK1, SK2, and SK3 obtained insignificant values. This is possible because SK has a role as a hypoglycemic agent that works as a preventive measure in the occurrence of type-2 diabetes mellitus. As a natural nutraceutical to prevent diabetes, phenol has the ability to inhibit human salivary alpha-amylase. Polysaccharides, proteins, lipids, polyphenols, and pigments are only a few of the bioactive compounds found in brown seaweeds, and they may all have health benefits [25]. Sulfated polysaccharide fucoidan, which is also present in many species of brown algae, has been shown to reduce blood glucose, glycated haemoglobin and glucagon-like peptide-1 in patients with type-2 diabetes, and alpha-glucosidase activity [26]. Brown seaweed phlorotannin has been shown to inhibit alpha-glucosidase activity, guard against oxidative stress caused by glucotoxicity and diabetes [27]. From the various contents contained in SK, it is known to have benefits for DM2 with each mechanism, both inhibiting and free radicals scavenging.

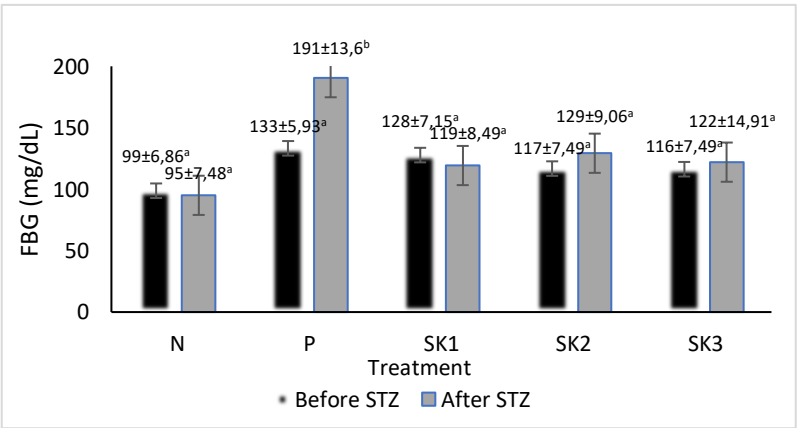

Fig 3. Effect of the kombucha administration on fasting blood glucose (FBG) levels in rats. Different notes mean a significant difference ($p < 0,05$) between before and after induced STZ.

3.4. Lipid profiles

The results of the analysis of lipid profiles in the blood of rats, including cholesterol, triglycerides, and HDL, showed that the profile values of normal rats (N) with DM rats (P) had significant values ($p < 0.05$) in each test parameter. Each treatment value P2 to P4 showed insignificant and relatively normal values in cholesterol analysis. However, in triglyceride analysis, treatments SK1 to SK3 showed relatively high values when compared to normal controls. In HDL analysis, treatments SK1 to SK3 showed relatively high values but did not approach the values of control rats. Bioactive compounds in *Sargassum* sp., like saponin, will bind to bile acids and enhance the excretion of bile acids in the faeces and neutral sterols, which can lower blood plasma cholesterol. Tannins lower cholesterol levels by interacting with intestinal epithelial cells' mucosal proteins to prevent fat from being absorbed in the intestine. And steroids are phytosterols that the body produces to reduce cholesterol levels by preventing the absorption of cholesterol [19]. Phenolic substances improve lipid profiles by reducing the auto-oxidation rate and giving lipid radicals (R*, ROO*) a hydrogen atom donation, transforming them into a more stable state. It has been shown that the oxidation of fatty acids is inhibited or prevented when lipids are supplemented with a low dose of primary antioxidants [12]. In increasing HDL, the mechanism of phenolic is helping metabolisms produce energy, supporting fat metabolisms, raising bile acid excretions, and preventing total cholesterol absorption by binding cholesterol carriers as they cross brush border membranes, phenolic compounds can also raise HDL-c. As a result, lecithin cholesterol acyl transferase (LCAT) activity is increased and lipoprotein synthesis is decreased. LCAT is an enzyme involved in HDL-c metabolisms that transforms free cholesterol into cholesterol esters [28].

Table 2. Effect of the kombucha administration on lipid profiles serum levels in rats.

Treatment	Cholesterol (mg/dL)	Trygliseride (mg/dL)	HDL (mg/dL)
N (Normal)	56.00 ± 7.8 a	44.00 ± 5.34 a	42.80 ± 3.11 c
P (DM)	84.00 ± 7.4 b	150.75 ± 26.49 e	17.30 ± 1.81 a
SK1 (DM + 1x SK)	67.00 ± 13.3 ab	93.50 ± 13.90 bc	21.90 ± 2.58 ab
SK2 (DM + 2x SK)	50.00 ± 5.9 a	97.25 ± 28.01 bd	21.54 ± 3.20 ab
SK3 (DM + 3x SK)	56.50 ± 5.0 a	82.75 ± 14.11 b	24.23 ± 0.63 b

3.5. Insulin

The results of insulin analysis using the ELISA technique obtained the lowest value in the DM2 rat treatment (P). The highest value was obtained when SK was administered once a day (SK1). The results obtained in the SK1 treatment can be said to be the best treatment because the value is higher than the normal control and is significant ($p<0.05$). Diabetes mellitus is a long-term metabolic disease marked by high fasting blood glucose (FBG) levels brought on by abnormalities in the secretion, action, and combination of insulin. The varied condition known as type 2 diabetes mellitus is marked by a progressive reduction in insulin activity, which is followed by the suppression of pancreatic cells to make up for the failure of insulin resistance cells. One of the main causes of type 2 diabetes and a key factor in the aetiology of cardiovascular disorders is insulin resistance [29]. Insulin binds to cell surface receptors and subsequently activates the intrinsic kinase of the insulin receptor, which causes the insulin receptors to autophosphorylate. In a dose-dependent manner, Sargassum sp. may promote glycogen synthesis in the liver and muscles, likely due to the insulin-stirring effect on glycogen synthesis and the suppressive effect on hepatic glucose production. The body's blood glucose balance mostly depended on the liver and muscles, which were important insulin target organs [30].

Table 3. Effect of the kombucha administration on insulin levels in rats.

Treatment	Insulin (µIU/mL)
N (Normal)	3.04 ± 0.32 bcd
P (DM)	2.21 ± 0.05 a
SK1 (DM + 1x SK)	3.29 ± 0.13 d
SK2 (DM + 2x SK)	2.46 ± 0.45 a
SK3 (DM + 3x SK)	3.12 ± 0.21 cd

3.6. Malondialdehide (MDA)

Malondialdehyde analysis was conducted to determine the oxidative stress level in experimental animals. The results showed that the SK3 treatment had a significant value ($p < 0.05$) against the normal rat treatment. This can explain why the intensity of SK administration has a real effect on MDA levels in the rat's body. Meanwhile, DM rats with P1 treatment had the highest value, indicating high oxidative stress in rats. MDA is used to check the extent of lipid peroxidation in the liver. *Sargassum* sp. shows possesses effective antioxidant activities in vivo with lowering levels of MDA and may be an important potentiator for contributing to its immunoprotective effect [31]. The capacity of phenolic compounds to donate electrons or hydrogen atoms from the -OH group, which renders them unstable, is assumed to be the cause of their strong antioxidant properties. Nevertheless, electrons are stabilized for their desired qualities by the benzene ring's resonance with them [12]. This increases the benefits of kombucha for the body's antioxidant enzyme system, which lessens the negative consequences of diabetes mellitus [32].

Table 4. Effect of the kombucha administration on MDA levels in rats.

Treatment	MDA (µM)
N (Normal)	0.36 ± 0.12 c
P (DM)	0.60 ± 0.12 d
SK1 (DM + 1x SK)	0.28 ± 0.03 abc
SK2 (DM + 2x SK)	0.31 ± 0.09 bc
SK3 (DM + 3x SK)	0.13 ± 0.04 a

4 Conclusions

Research results with three different doses of *Sargassum* Kombucha showed good results on the characteristics of rat weight and blood and organ profiles. The bioactives contained in the kombucha drink have a mechanism for its impact as a hypoglycemic agent. Although some characteristics that have been analyzed do not produce significant values, but they can answer the benefits of *Sargassum* Kombucha on rats with type 2 diabetes models.

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