

Antihyperglycemic activity of functional drinks based on Java Tea (*Orthosiphon aristatus*) in streptozotocin induced diabetic mice

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Abstract

Diabetes is a group of diseases marked by high levels of blood glucose resulting from defects in insulin production, insulin action, or both. Diabetes can lead to serious complications and premature death. Antioxidant compounds in functional drinks such as flavonoid may offer some protection against the early stage of diabetic mellitus and the development of complications. The objective of this study was to investigate the antihyperglycemic effects of functional drinks formulations containing different varieties of Java Tea on streptozotocin induced diabetic mice. The results indicated that the administration of functional drinks in diabetic mice restrain the increase of blood glucose and further inhibit the rate of pancreatic beta cells damage. HPLC analysis shows that the bioactive compounds in the extract ingredient are sinensetin, 6-gingerol, 8-gingerol, 10-gingerol, 6-shogaol, curcumin, desmethoxycurcumin, brazilin, hesperidin and naringin. Consequently, functional drinks based on Java Tea is considered to be a promising functional drink for preventing and treating diabetes.

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Introduction

Diabetes mellitus is a serious disease that if not handled properly will cause some complications and premature mortality. It is a metabolic disease characterised by high-blood glucose levels resulting from defects in insulin secretion, insulin action or both (Ortiz-Andrade *et al.*, 2005). Type 1 diabetes is the consequence of an autoimmune-mediated destruction of pancreatic β -cells, leading to insulin deficiency (Turina *et al.*, 2006). The prevalence of diabetes for all age-groups worldwide was estimated to be 2.8% and 4.4% in 2000 and 2030, respectively. The total number of people with diabetes is projected to rise from 171 million in 2000 to 366 million in 2030. Indonesia is ranked as the 4th largest number of diabetic patients in the world. The total number of people with diabetes in Indonesia is projected to rise from 8.4 million in 2000 to 21.3 million in 2030 (Wild *et al.*, 2004). This shows how fast the number of people with DM is increasing in Indonesia. If it is not handled properly, it can reduce the qualities of life, which will subsequently hamper the development of Indonesia in order to create a healthy and prosperous society.

This phenomenon shows that consumers nowadays have the tendency to go back to nature, including the use of hypoglycemic medications. There are many species of medicinal plants that have

been used to treat various symptoms of diabetes and some of these plants have been scientifically proven to have the antihyperglycemic ability. An option in the utilization of medicinal plants is to formulate them into herbal-based functional beverages.

Research conducted by Wijaya (2010), showed that functional drink based on Java Tea can improve the absorption of glucose by cells in the diaphragm of mice *ex vivo*. The beverages containing *Zingiber officinale* extracts and *Citrus aurantifolia* juice was capable in increasing the absorption of glucose in diaphragm cells, indicating that the beverage have the potential in lowering high blood glucose levels.

Several studies had shown that the plants extracts used singly in the beverage formula possess antihyperglycemic activity (Sriplang *et al.*, 2007; Moon *et al.*, 1990; Sekiya *et al.*, 2004; Meghana, 2007; Ibrahim, 2008). However, there has been no recent studies on the antihyperglycemic ability of a formulated beverage containing the mixture of different plant extract. In this study, various formulations of functional beverages containing mixtures of different extracts were tested on diabetic mice induced with multiple low-dose streptozotocin.

The information on the functional drink capabilities based on Java Tea as antihyperglycemic in diabetic mice have great potential to help people with diabetes in controlling their blood glucose levels.

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Materials and Methods

Plant materials

Orthosiphon aristatus was obtained from Balitro Garden in West Java, Indonesia, meanwhile *Zingiber officinale*, *Citrus aurantifolia*, *Citrus histryx* and *Caesalpinia sappan* were from the local market at Bogor, Indonesia. *Curcuma xanthorriza* was from Biopharmaca Research Center Garden. All plants were identified and deposited at Biopharmaca Research Center, Bogor Agricultural University, Indonesia.

Animals

Sixty six male ddY mice weighing 30 ± 5 g used in this study were obtained from the National Agencies of Food and Drug Control, Indonesia. The mice were housed in colony cages (tree mice per cage) at an ambient temperature of $25 \pm 2^\circ\text{C}$ with alternating 12 h cycles of light and dark. Mice had free access to standard food and water ad libitum for 1 week to adjust them to the environment. The Principles of Laboratory Animal Care were followed throughout the duration of experiment and instruction given by the Animal Care ethical committee, Research & Development of Health Institution, Indonesian Health Ministry, was followed regarding injection and other treatment of the experiment.

Preparation of functional drinks

In this research, there were 4 types of beverages. F1 formula beverages consists of extracts from the purple-flower *O. aristatus*, *C. sappan*, *Z. officinale*, *C. xanthorriza* and *C. aurantifolia* juice. F2 formula beverages consists of extracts from the white-flower *O. aristatus*, *C. sappan*, *Z. officinale*, *C. xanthorriza* and *C. aurantifolia* juice. F3 formula beverages consists of extracts from the purple-flower *O. aristatus*, *C. sappan*, *Z. officinale*, *C. xanthorriza*, *C. aurantifolia* juice, *C. histryx* juice. F4 formula beverages consists of extracts from the white-flower *O. aristatus*, *C. sappan*, *Z. officinale*, *C. xanthorriza*, *C. aurantifolia* juice and *C. histryx* juice. The four different formulation were topped up with CMC, sucralose and water.

Experimental design

The research consisted of several stages. The first phase was characterizing the extract ingredients for their total phenol content (Strycharz and Shetty, 2002), antioxidant activity (Kubo et al., 2002; Molyneux, 2004), phytochemical analysis (Harborne, 1987) and levels of bioactive compounds such as sinensetin (Sigma Aldrich, St. Louis, MO, USA)

(Akowuah et al., 2004), curcumin (Sigma Aldrich, St. Louis, MO, USA) (Almeida et al., 2005), gingerol and shogaol (Sigma Aldrich, St. Louis, MO, USA) (Lee et al., 2007), brazilin (Sigma Aldrich, St. Louis, MO, USA) (Batubara et al., 2010), hesperidin and naringin (Sigma Aldrich, St. Louis, MO, USA) according to the methods of Abeysinghe et al. (2007).

The second phase of the research were to study the effect of different beverages formulation on the glucose utilization by isolated mice hemidiaphragm (Sabu and Subburaju, 2002), the total phenol content and antioxidant activity.

The third phase of the research was to determine the concentration of the formulated drink which was antihyperlicemic in normal mice which had a momentary hyperglycemic induced by glucose solution as much as 1 g/kg BW according to the method described by Suarsana (2009). Thirty-six mice, were classified into 6 groups (n = 6). Group I (negative control, treatment with distilled water 0.52 ml/20 g BW), Group II (positive control hyperglycemic, treatment with glucose solution of 1 g/kg), Group III (hyperglycemic mice, treatment with beverage sample as much as 0.52 ml sample/20 g BW with a concentration of $1 \times$ formula drinks), Group IV (hyperglycemic mice and treatment with beverage samples by 0.52 ml/20 g BW with a concentration of $4 \times$ formula drinks), Group V (hyperglycemic mice and treatment with beverage samples by 0.52 ml/20 g BW with a concentration of $16 \times$ formula drinks) and Group VI (hyperglycemic mice and treatment with insulin Actrapid®). Provision of samples and glucose solution were done after the mice were fasted for 16 hours. Measurement of glucose levels were performed at 0, 30, 60, 120, and 180 minutes after treatment. Blood glucose content was measured using a commercial glucometer (One Touch Ultra, USA).

In the fourth phase of the research, the antihyperglycemic activity from selected formula drink concentration is tested in-vivo on diabetic mice which was induced with multiple low-dose streptozotocins (Sigma Aldrich, St. Louis, MO, USA) according to the methods of Wu and Huan (2008). Thirty male mice were used and grouped into 6 groups (n = 5). Group I (diabetic positive control, diabetic mice with treatment of distilled water), Group II (diabetic mice treated with samples of beverage formula as much as 0.52 ml/20 g BW), Group III (diabetic mice treated with samples of beverage formula without ginger as much as 0.52 ml/20 g BW), Group IV (diabetic mice treated with insulin), Group V (normal mice treated with samples of beverage formula as much as 0.52 ml/20 g BW) and Group VI (negative control, normal

mice treated with distilled water). Measurement of glucose levels were conducted every 5 days and by the end of the research a histopath analysis of the pancreas is conducted, according to the methods of Beesley (1995) and Kiernan (1990).

Testing of fasting plasma glucose level

Fasting PG level was measured every five days from the animals of all these groups. Blood was collected from tip of the tail vein and fasting PG level was measured using single touch glucometer according to the methods of Atkin *et al.* (1991). The results were expressed in terms of milligram per deciliter of blood.

Histopathological study

A portion of pancreatic tissue was dissected out and fixed in 10% buffered neutral formal saline and processed. After fixation, tissues were embedded in paraffin. Fixed tissues were cut at 5 μ m and stained with hematoxylin-eosin and immunohistochemical. The section were examined under lighth microscope and photomicrographs were taken according to the methods of Kiernan (1990) and Beesley (1995).

Statistical and analysis of data

Statistical analyses were performed using one way analysis of variance (ANOVA) followed by Duncan's Test using SPSS version 17. The limit of statistical significance was set at $p < 0.05$.

Result and Discussion

Extracts characteristic

Characterization of extracts were carried out to standardize the extract ingredients, which was extremely useful in determining the quality of extract in order to obtain a standardized formula drinks. The results obtained from analyzing the characteristics of the extract ingredients are presented in Table 1. Extract ingredients with better total antioxidant activity and phenol content as shown in Table 1, were chosen to formulate the functional beverage which have optimum antihyperglycemic activity.

Content of bioactive compounds in the extract

Based on the results of previous researches, the distinguishing compound of each extract which is suspected as the active compound are sinensetin (*O. aristatus*), brazilin (*C. sappan*), gingerol (*Z. officinale*), curcumin (*C. xanthorrhiza*), hesperidin and naringin (*C. aurantifolia* & *C. histryx*) (Sriplang *et al.*, 2007; Moon *et al.*, 1990; Sekiya *et al.*, 2004; Meghana, 2007; Ibrahim, 2008). According to

Table 1. Extract characteristics

Extract Types	Antioxidant Activity (ppm AEAC)	Total of Phenol (ppm GAE/g)
Purple-Flower <i>O. aristatus</i>	556,21 \pm 71,892 ^{bc}	748,150 \pm 1,514 ^a
White-Flower <i>O. aristatus</i>	666,892 \pm 9,216 ^b	787,633 \pm 5,449 ^a
<i>C. sappan</i>	1055,222 \pm 7,857 ^a	727,375 \pm 65,637 ^a
<i>C. xanthorrhiza</i>	386,222 \pm 82,496 ^d	465,636 \pm 1,026 ^b
<i>Z. officinale</i>	529,556 \pm 7,071 ^c	232,626 \pm 35,557 ^d
<i>C. histryx</i>	294,556 \pm 56,569 ^{de}	353,761 \pm 7,133 ^c
<i>C. aurantifolia</i>	194,0 \pm 60,497 ^e	272,089 \pm 33,148 ^d

Note: Numbers followed by the same letter in the same column showed that is not significant different at 5% alpha.

Sriplang *et al.* (2007), the water extract of Java Tea of 0.5 g/kg and 1.0 g/kg body weight of rats was significantly effective in lowering blood plasma glucose levels and increasing HDL plasma. Brazilin contained in a sappan wood extracts can significantly reduce blood plasma glucose levels in diabetic rats, with no increase in insulin levels. In addition, there is an increase in glycogen synthesis, glycolysis, and oxidation of glucose in diabetic animal muscle (Moon *et al.*, 1990). *C. xanthorrhiza* extract contains demethoxycurcumin and curcumin compounds which are categorized in the curcuminoids group. Curcumin had the activity to protect pancreatic β cells against oxidative damage from streptozotocin induction (Meghana, 2007). Curcumin can also inhibit hepatic glucose production (Fujiwara *et al.*, 2008). Fresh ginger extract has the ability to lower blood glucose levels in diabetic rats (Sharma and Sukla, 1977). Antidiabetic ability of ginger is due to its role in affecting serotonin receptor (5-hydroxytryptamin (5-HT)) in glycemic control, in which ginger has anti-serotonin activity and thus increase insulin secretion (Akhani *et al.*, 2004; Heimes *et al.*, 2009). Sekiya *et al.* (2004) also reported that gingerol in ginger extracts can improve insulin sensitivity to glucose in order to improve the state of hyperglycemia. Both these compounds can improve the conditions of hyperlipidemia and hyperglycemia in animals with type 2 diabetes by regulating the partial fatty acid and cholesterol metabolism, and affect expression of genes for enzymes of glucose metabolism (Jung *et al.*, 2006). Hesperidin has antihyperglycemic activity that can inhibit the occurrence of complications in the brains of diabetic mice (Ibrahim, 2008).

The results showed that the extract ingredients used in this study contain those bioactive compounds such as shown in Table 2. There is a difference between the content of bioactive compounds extracted from *C. histryx* with *C. aurantifolia*. *C. histryx* extract contained hesperidin and naringin compound, whereas *C. aurantifolia* extract contained only hesperidin compound. The results obtained by HPLC analysis showed the concentration of bioactive compounds in the extract (Table 2). The data obtained

Table 2. Bioactive compound content in each extract

Extract	Phytochemical Compound	Bioactive Compound	Results of HPLC Analysis (mg/ml)
<i>O. aristatus</i>	Alcaloid, Flavonoid, Tannin, Saponin, Triterpenoid, Hydroquinon	Sinensetin	0.0236
<i>C. sappan</i>	Flavonoid, Tannin, Saponin, Triterpenoid, Hydroquinon	Brazilin	1.3203
<i>C. xanthorrhiza</i>	Alcaloid, Flavonoid, Tannin, Triterpenoid, Hydroquinon	Curcumin Demethoxy curcumin	0.0765 0.0137
<i>Z. officinale</i>	Alcaloid, Flavonoid, Tannin, Triterpenoid, Hydroquinon	6-Gingerol 8-Gingerol 10-Gingerol	1.0190 0.2003 0.4723
<i>C. histrix</i>	Alcaloid, Flavonoid, Tannin, Steroid	6-Shogaol Hesperidin Naringin	0.0670 0.0924 0.0096
<i>C. aurantifolia</i>	Flavonoid, Tannin, Steroid	Hesperidin	0.0263

Table 3. Effect of beverages on glucose uptake by isolated mice hemidiaphragm, antioxidant activity and total phenol content

Formula Beverages	Glucose uptake (μg glucose / g diaphragm)	Antioxidant activity (ppm AEAC/ml)	Total phenol content (ppm GAE/ml)
F1	33,87 \pm 17,31 ^b	587,042 \pm 23,596 ^b	397,713 \pm 33,095 ^a
F2	58,07 \pm 23,06 ^a	733,292 \pm 5,907 ^a	474,184 \pm 74,020 ^a
F3	30,57 \pm 25,28 ^b	545,032 \pm 12,564 ^b	394,871 \pm 32,014 ^a
F4	54,81 \pm 23,88 ^a	726,818 \pm 19,285 ^a	440,157 \pm 55,836 ^a

Note: Numbers followed by the same letter in the same column showed that is not significant different at 5% alpha.

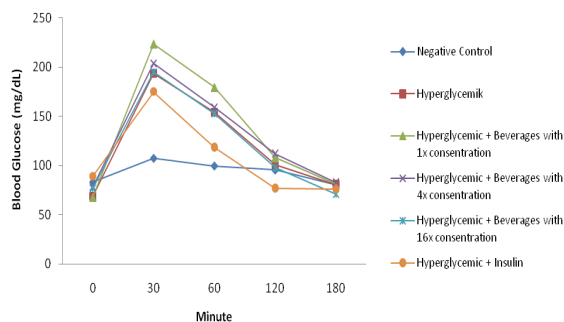


Figure 1. Blood glucose level respond during 180 minutes of observation

can be used to standardize the extract ingredients in the beverage formula.

Effect of different formula beverages on glucose utilization by isolated mice hemidiaphragm and antioxidant activity

In this research, there were 4 types of beverages. The formulas are, F1, F2, F3 and F4. The difference in the beverages formulation cause differences in the physiological active ability (Table 4). F2 and F4 beverage formulas had more potency in increasing the glucose uptake by isolate hemidiaphragm cells compared to the F1 and F3 beverage formulas. The same thing goes for the antioxidant activity. Yet, the total phenol of the four samples weren't significantly different. This showed that glucose absorbance by diaphragm cells *ex-vivo* had strong correlation with the beverage antioxidants activity.

Momentary hyperglycemic activity

This test was conducted to obtain the selected beverage concentrations for further *in vivo* test. Based on observation, in the first 30 and 60 minutes after treatment, provision of F4 formula beverages

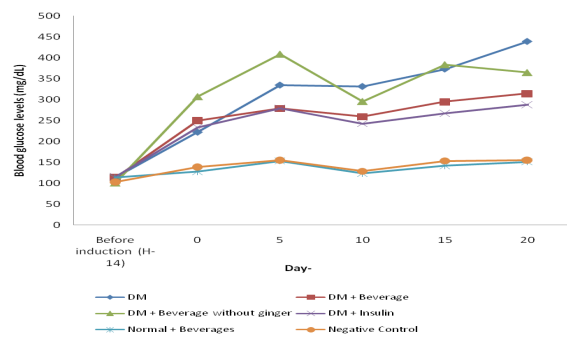


Figure 2. Blood glucose change during research

with 1 \times and 4 \times concentrations increase blood glucose level, which was back to normal after 180 minutes. This is possibly caused by the inadequacy of antioxidant activity and total phenol content from both concentrations to restrain the increase of blood glucose level. Provisions of beverages with 16 \times concentrations reduce the blood glucose level at the 120th minute. Hence, the beverage with 16 \times concentration can increase the glucose uptake into the cells.

Antihyperglycemic activity on diabetic mice

Antihyperglycemic activity testing was conducted on multiple low dose streptozotocin induced diabetic mice for 20 days by measuring the blood glucose levels every 5 days (Figure 2). Multiple low-dose streptozotocin (MLDSTZ)-induced diabetes, one of typical animal models of insulin dependent diabetes mellitus (IDDM), develops in conjunction with a cellular infiltration of the pancreatic islets (Rossini, 1977) and cell-mediated immune mechanisms are involved in the pathogenesis of this model of diabetes mellitus (Rossini, 1985).

Blood glucose levels during observation is varied, caused by the differences in each immunity level against streptozotocin which caused differences in the early stage of (Kim *et al.*, 2006). Observation result showed that beverage with 16 \times concentrations of total ingredients was able to restrain the increase of blood glucose level in diabetic mice, while it didn't cause a decrease of blood glucose level in the normal mice. Beverages with the addition of ginger extract had a steadier hyperglycemic ability because it was able to lower the blood glucose level for 20 days during the study period. It is postulated that gingerol from ginger extract could increase the insulin sensitivity upon glucose, and improve the hyperglycemia condition (Sekiya *et al.*, 2004). Wijaya (2010), also reported that ginger extract can increase the glucose uptake by isolate hemidiaphragm cells.

Effect on langerhans and β cell morphology

Streptozotocin enters the β cell via a glucose

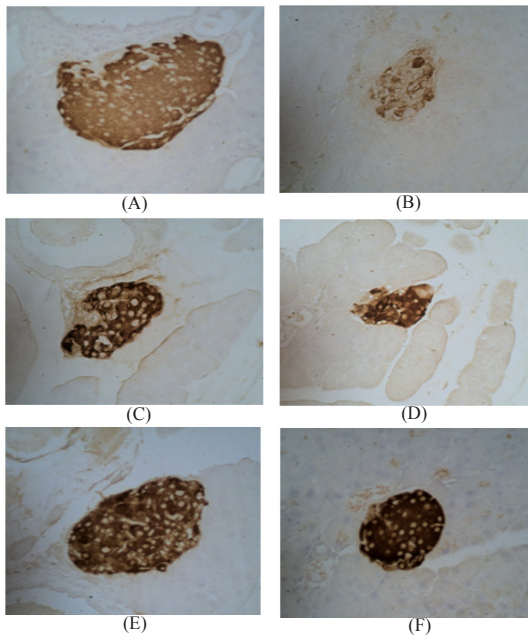


Figure 3. β cells in Langerhan with antiinsulin antibody immunohistochemical staining at magnification 400x. (A) Normal; (B) Diabetic; (C) Diabetic + Beverage; (D) Diabetic + Beverage without ginger; (E) Diabetic + Insulin; (F) Normal + Beverage

transporter (GLUT2) and causes alkylation of DNA. DNA damage induces activation of poly ADP-ribosylation, a process that is more important for the diabetogenicity of streptozotocin than DNA damage itself. Poly ADP-ribosylation leads to depletion of cellular NAD^+ and ATP. Enhanced ATP dephosphorylation after streptozotocin treatment supplies a substrate for xanthine oxidase resulting in the formation of superoxide radicals. Consequently, hydrogen peroxide and hydroxyl radicals are also generated. Furthermore, streptozotocin liberates toxic amounts of nitric oxide that inhibits aconitase activity and participates in DNA damage. As a result of the streptozotocin action, β cells undergo the destruction by necrosis (Szkuldeski, 2001).

Based on histopath observation result by HE staining, the provision of java tea-based functional beverage in normal mice didn't affect the morphology of Langerhans. Its cells had the normal form and proportion, not different from the Langerhans cells of normal mice which are treated with distilled water. For the diabetic mice group, the damage are obvious as the Langerhans cells size decrease (atrophy), with similar observations for the acinar pancreas, observed under the same field of view of the preparations, and there is no islet of Langerhans.

The beverage provision to diabetic mice was able to restrain further damage on islet of Langerhans. Langerhans in diabetic mice, which were given beverage with or without the addition of ginger extract or insulin, has a larger amount and larger size

of Langerhans when compared to positive control mice (diabetic), although the amount is still less than the amount of Langerhans from normal mice group. In the diabetic mice group treated with insulin, an improvement of the Langerhans were observed.

Pancreatic β cells damage specifically observed based on its positive reaction against the staining using anti insulin immunohistochemical method which is marked by the forming of brown colour (Figure 3). Immunohistochem staining results shows that the diabetic mice group had a very severe β cells damage because the brown area and its color intensity is significantly different with the β cells form normal mice group ($p < 0.05$). Observation results from pancreas histopath in the diabetic mice group which was provided with java tea-based functional drink, with or without ginger extract or insulin can hamper further damage on β cells.

Pancreatic β -cells express low levels of antioxidant enzymes and do not up-regulate these enzymes upon exposure to high concentration of glucose. Thus, increased ROS production with low antioxidant defenses could result in ROS accumulation and oxidative stress in β -cells. Elevated ROS affects the function and survival of β -cells through direct oxidization of cellular macromolecules such as DNA and lipids, and activation of cellular stress-sensitive signaling pathways (Wu *et al.*, 2004).

Hyperglycemia condition will lead to increased demand for insulin to lower blood glucose levels, resulting in β cell exhaustion and initiate the degenerative process (Hayden *et al.*, 2007). Increasing insulin production due to chronic hyperglycemia causes oxidative stress and result in β cell dysfunction, followed by reduction of β cell mass (Marchetti *et al.*, 2007). The antioxidant defense system represents a complex network with interactions, synergy and specific tasks for a given antioxidant. The efficiency of this defense mechanism is altered in diabetes and, therefore, the ineffective scavenging of free radicals may play a crucial role in determining tissue damage (Polidori *et al.*, 2001).

These beverages are capable in inhibiting the rate of pancreatic β cell damage, because the beverage with $16\times$ concentrations of total ingredients can improve blood glucose absorption into cells as the gingerol compounds can improve insulin sensitivity upon glucose, so that the insulin is use more effectively, helping to reduce the occurrence of β cell damage. Phenolic compounds in the beverages can help prevent β cell damage due to oxidative stress caused by accumulation of radical compounds from STZ induction. Phenolic compounds have antioxidant activity with the ability to donate hydroxyl group to

form radical compounds that are relatively more stable.

Conclusion

The F4 formula beverages had the potential to increase glucose utilization by diaphragm cells, having a higher level of antioxidant activity and total phenol content. Beverages with 16× concentration of total ingredient which was formulated with the addition of ginger extract had a better antihyperglycemic activity on diabetic mice because it can lower blood glucose level in a more stable way, also restraining further damage of β cells. The active compounds in these formulas might be sinensetin, curcumin, brazilin, gingerol, shogaol, hesperidin, and naringin.

A further testing is needed to comprehend the supplementary mechanism in the body such as, its mechanism upon PPARs in increasing insulin sensitivity, insulin receptor, and glucose transporter in cell membranes, hepatic glucose production. Also the mechanism regarding enzymes which are functional as oxidant resistance in the body. Clinical testing is also needed in the diabetic patients to obtain a more representative scientific proof. Besides, the stability testing of the beverage during process and storage in a pilot or industry scales is also needed in order to maintain the quality of the drink.

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