Antidiabetic Potential of Brassica Oleracea Var. Italica in Type 2 Diabetic Sprague Dawley (sd) Rats

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Research Article

Antidiabetic Potential of *Brassica Oleracea* Var. Italica in Type 2 Diabetic Sprague Dawley (sd) Rats

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ABSTRACT

Now-a-days functional foods are considered as the most convenient means for the prevention and management of chronic diseases with minimal or no side effects. *Brassica oleracea* var. Italica (family Brassiceae) is a popular dietary vegetable eaten all over the world. It has been reported to exhibit antimicrobial and anticancer properties but antidiabetic activities are not yet extensively explored. The present study was designed to investigate the antidiabetic potential of *Brassica oleracea* var. Italica in type 2 diabetic Sprague-Dawley (SD) rats. Type 2 diabetes mellitus (T2DM) was induced in the SD rats with high fat diet and injecting a low dose (35 mg/kg) of streptozocin. Diabetic rats were treated with *Brassica oleracea* var. italica extracts at a dose of 200, 400 and 800 mg/kg body weight for 28 days. Metformin (250 mg/kg body weight) was used as a standard antidiabetic drug. Fasting blood glucose (FBG), oral glucose tolerance, glycated haemoglobin, serum insulin and Hemoglobin were determined from the serum by using standard kits. After 28th day, daily administration of Brassica oleracea var. Italica in diabetic treated SD rats showed improvement in body weight, water and food intake as compared to diabetic control rats. out of the three different doses viz., 200, 400 and 800 mg/kg body weight, the highest dose (800 mg/kg) caused a significant attenuation in the blood glucose at 180 min in Streptozotocin (STZ) induced diabetic rats when compared to the diabetic control group ($P<0.001$). The study demonstrated that ethanolic extract of *Brassica oleracea* extract have potential antidiabetic activities. Thus Brassica oleracea as vegetable or its extract can be useful to control hyperglycemia.

Keywords: *Brassica oleracea* var. Italica, Type 2 diabetes, Sprague Dawley rats, Hyperglycemia

INTRODUCTION

Ancient Egyptians recognized the diabetes 3500 years ago. One of the first clinical descriptions was by Aretaeus, who practiced in Cappadocia around 120 AD[1]. Diabetes is now cited as a global epidemic and is intertwined with the obesity epidemic. Diabetes mellitus (DM) is a metabolic disorder that affects carbohydrate, fat and protein metabolism. Globally, as of 2010, an estimated 285 million people had diabetes, with type 2 making up about 90% of the cases[2]. The World Health Organization estimates an increase from 171 million in 2000 to 366 million in 2030[3]. Approximately 70% of this growth is predicted to occur in the developing world and will increasingly affect people aged younger than 65 years who are still in the productive stages of their life cycle. The increase in the younger aged group being diagnosed with DM Type 2 poses an economic threat over and above the more direct diseases to the public[4]. T2DM, the most common type of diabetes, is characterized by impaired insulin sensitivity and secretion. Unlike T1DM, persons with T2DM retain a certain production of insulin although insufficient to keep the blood glucose within normal range. T2DM progresses slowly and as a result people can have the disease for years without knowing it. Treatment of T2DM starts with oral medication that increases insulin sensitivity but as the disease progresses it is often necessary to treat with insulin injections. The incidence rate increases with age with a cumulative incidence rate by age 70 of 11 %. The major risk factors which influence the developing T2DM are obesity and a family history of the disease[5]. In type 2 diabetes, the body is able to produce insulin but either this is not sufficient or the body is unable to respond to its effects, leading to a build-up of glucose in the blood. Many people with type 2 diabetes remain unaware of their illness for a long time because symptoms may take years to appear or be recognized, during which time the body is being damaged by excess blood glucose. They are often diagnosed only when complications of diabetes have already developed. Although the reasons for developing type 2 diabetes are still not known, there are several important risk factors. These include obesity, poor diet, physical inactivity, advancing age, family history of diabetes, ethnicity and high blood glucose during pregnancy affecting the unborn child In contrast to people with type 1 diabetes, the majority of those with type 2

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diabetes usually do not require daily doses of insulin to survive. Many people are able to manage their condition through a healthy diet and increased physical activity or oral medication. However, if they are unable to regulate their blood glucose levels, they may be prescribed insulin. The number of people with type 2 diabetes is growing rapidly worldwide. This rise is associated with economic development, ageing populations, increasing urbanization, dietary changes, reduced physical activity, and changes in other lifestyle patterns[3]. The oral antihyperglycemic agents currently used in clinical practice have characteristic profiles of serious side effects[6].This leads to increasing demand for herbal products because of their effectiveness, minimal side effects in clinical experience and low cost[7].Therefore search for safe and more effective agents has continued to be an important area of active research. *Brassica oleraceae var. Italica (*Broccoli) has antimicrobial, and anticancer activities and has become established as an important human food crop plant used because of its large food reserves[8,9]. Thus the significance of the current study is to find out the antidiabetic activity of the *Brassica oleraceae* var. Italica. We hypothesis that the *Brassica oleraceae* var. Italica will potentially reduce hyperglycemia with good safety profile in diabetes. The outcome of the study may significantly contribute to the effective management of the diabetes.

MATERIALS AND METHODS

Drug, chemicals and reagents

Streptozotocin was supplied by Sigma-Aldrich (M) Sdn. Bhd. A-07-11, Empire Office, Empire Subang Jalan SS16/1, SS16, and 47500 Subang Jaya-Selangor Darul Ehsan Malaysia. Glucose assay kit (Sigma Diagnostics, INC., St Louis, MO, USA) and Plasma insulin (Awareness Technologies, USA) levels were determined by the ELISA method. Accu-chek (Roche Diagnostics USA) was used for glucose tolerance test. All the other chemicals and solvents used were of analytical grade.

Instruments

Several validated instruments have been used in the current research. Buchi rotary evaporator R-144, Buchi water bath B-480 (Buchi Labortechnik AG, Switzerland). Chemical hood (Pacific Vinitex Pte Ltd, Singapore) -86°C Freezer (Forma Scientific), -20°C & 4°C ACMA Refrigerator, Analytical balance (Precisa 40SM-200A, Swiss), Beckman Avanti™ J-25I Centrifuge (Fullerton, CA, USA), Beckman JA 25-25 Rotor (Fullerton, CA, USA), ELX 800 Microplate Readers (Bio-Tek Instruments Inc., USA), RA 50 biochemical analyzer (Bayer ltd), UV-1601 Spectrophotometer (Shimadzu, Japan), Water Incubator (Everbloom Medical & Scientific Pte, Ltd., Singapore).

Animal model

Selection of animals

The animals used for in vivo experiments were Sprague Dawley (SD) male rats weighing 150-200 gm), age ranging 6-12 week.

Maintenance of experimental rats

All the animals were kept in the animal house of the Department of Pharmacology, faculty of pharmacy, Lincoln University College, Malaysia. The animals were kept in plastic cages (34×47×18cm3) at animal house, in an air conditioned environment with six animals in each cage and maintained at room temperature of 25C°±2C with relative humidity (60 ± 10%) under 12 hrs night and light cycle. Paddy husk was used as bedding material and changed twice a week. The animals were kept on overnight fasting before every experiment. The animals used for the experiment were approved by the Animal Ethics committee of the Lincoln University College, Malaysia.

Collection of the plant material

The whole plants of *Brassica oleracea* var. Italica was collected in the month of June, 2012 from the cultivated field in Selangor, Malaysia. The plant was authenticated by *Ms. Tan Ai Lee* at Forest Research Institute Malaysia (FRIM) and a voucher specimen herbarium with number (SBID: 018/12) was deposited at the Faculty of Pharmacy, Lincoln University College, Malaysia.

Phytochemical screening

A small portion of the hydroethanolic extracts of *Brassica oleracea var. Italica* was subjected to the phytochemical test using *Trease and Harbourne* methods to test for the presence of alkaloids, tannins, reducing sugars, saponins, terpenoids, phenols, flavonoids and anthraquionones[10].

Acute and sub-acute toxicity study of Brassica oleracea var. Italica extract

Acute toxicity study

The acute toxicity was evaluated according to OECD guidelines 423[11] on SD rats (150-200 gms)[12] with a limit test dose of 4000 mg/kg. All the animals were kept at overnight fasting before to every experiment with free excess to water. The animals were divided into four groups, each comprising 5 animals. The 1st group served as a negative control, while 2nd, 3rdand 4th was considered as tested groups received Brassica oleracea var. Italica extract (dissolved in normal saline) at dose of 300 mg/kg, 2000 mg/kg and 4000 mg/kg. Before dose administration, the body weight of each animal was determined and the dose was calculated according to the body weight. The animals were observed for any toxic effect for first 4-hrs after the treatment period. Further animals were investigated for a period of 3 days for any toxic effect. Behavioral changes and other parameters such as body weight, urinations, food intake, water intake, respiration, convulsion, tremor, temperature, constipations, changes in eye and skin colors etc.

Sub- acute toxicity study

The oral sub- acute toxicity study was carried out according to OECD guideline 407[13]. Adult healthy SD rats (150-200gms) of each sex were divided into 3 groups of 5 animals each and were placed under standard conditions. Group I was consider as control and the other two groups which was considers as tested groups received the plant extract at a dose of 400 and 8000 mg/kg. During the study body weight of each animal were evaluated respectively for 28 consecutive days

Induction of type 2 diabetes (T2DM)

Table 1: Body weight, water and food intakes in HFD-STZ-diabetic rats before and after oral treatment with control, B. *oleraceae* var. Italica and Metformin.

Treatment Group	Body weight (g) (mean ± SEM)		Water (ml/rat/day) (mean ± SEM)		Food (g/rat/day) (mean ± SEM)	
	Before	After #	Before	After #	Before	After #
Diabetic Control	224 ± 5	237± 11(7)	176± 21	186±16 (6)	42± 4	60±5(43)
B.oleracea var Italica Extract	231 ± 11	253±25 (9)	155± 12	178±15(14)	45± 3*	46±4 **(2)
Metformin	226± 8	247± 14(9)	163± 17	167±16 (2)	42± 2*	43±3* *(2)

**P<0.01 compared with vehicle-treated rats (two-way ANOVA)

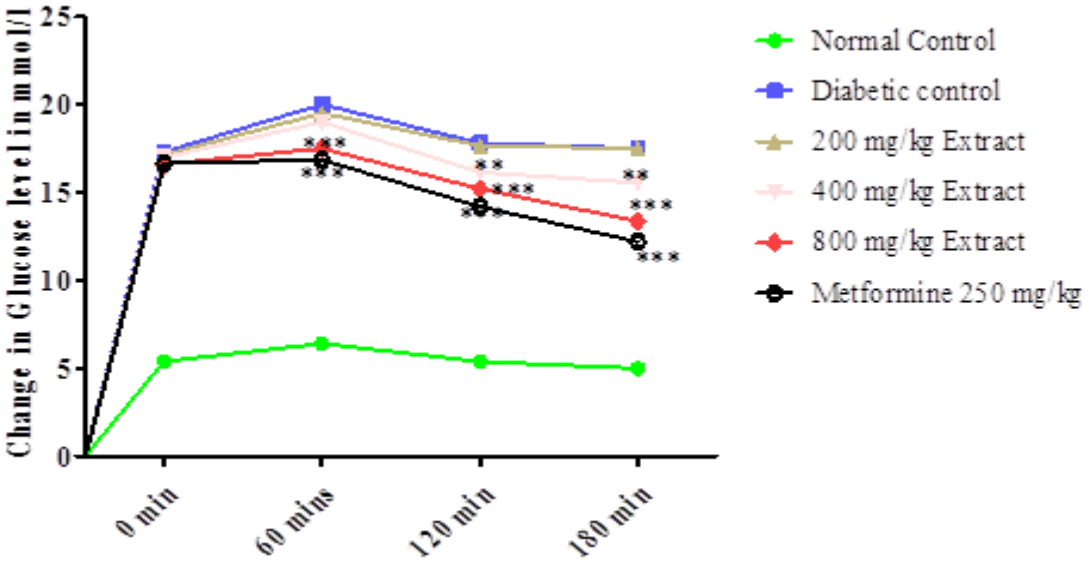

Figure 1: Effects of ethanolic extract of B. oleracea on OGTT in HFD-STZ- diabetic rate.

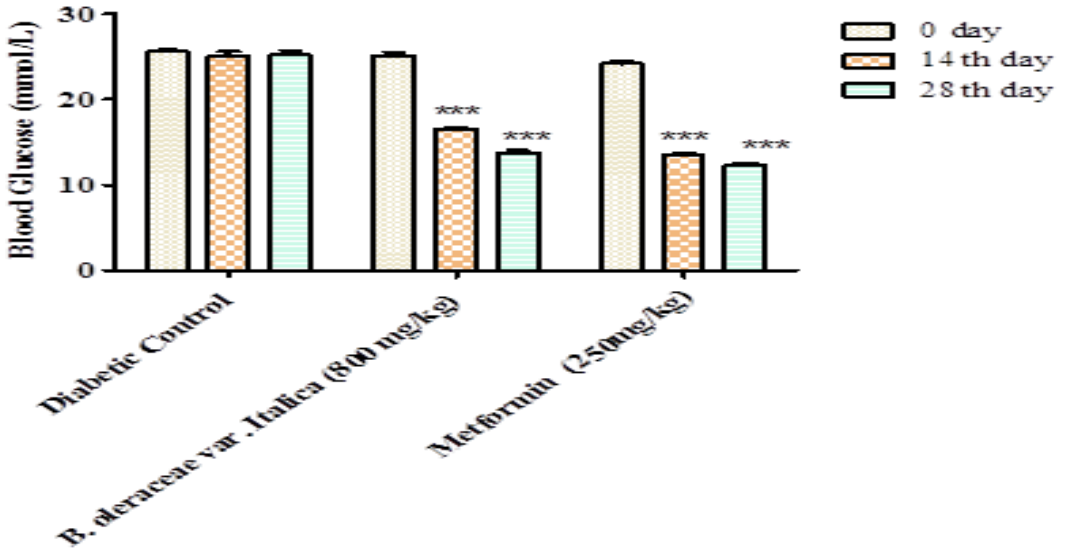

Figure 2: Effects of the B. oleracea var Italica on FBG levels in HFD-STZ- diabetic rats.

Male rats were fed high fat diet (HFD) consisting of 20% fat, 46% carbohydrate and 20% protein (w/w) (Glen Forrest Stock Feeders, WA, Australia). After 4 weeks on HFD, animals were administered streptozotocin (STZ, 35 mg/kg) intraperitoneally. Diabetes in the rats was identified by measuring fasting serum glucose concentration 72-h after injection of STZ. Rats with a serum glucose level above 17.0 mmol/l were selected for experiment[14,15].

Preparation of plant extract

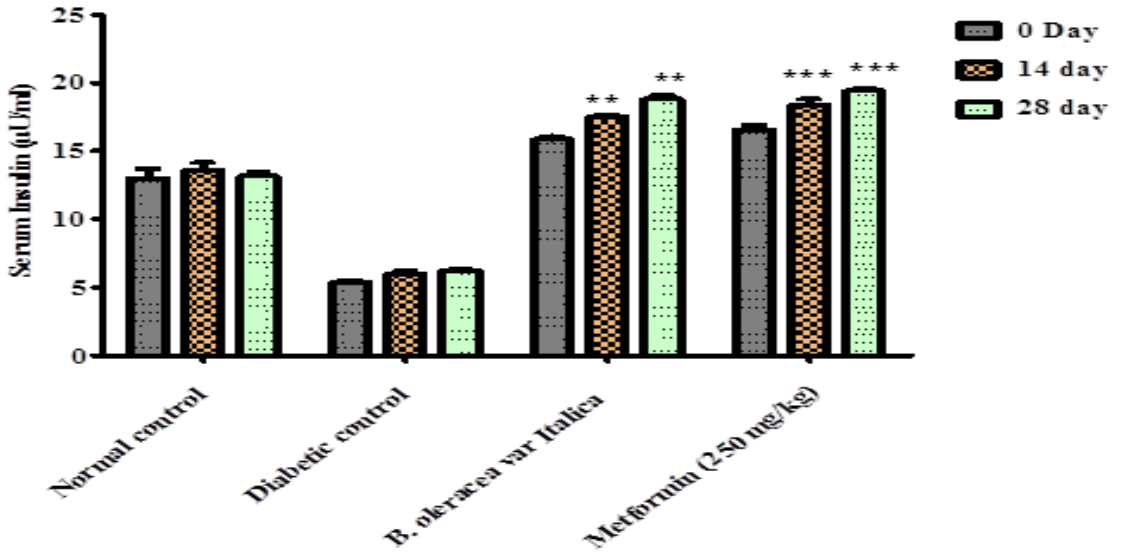

Figure 3: Effects of B. oleracea var Italica on the serum insulin levels in HED-STZ- diabetic rats.

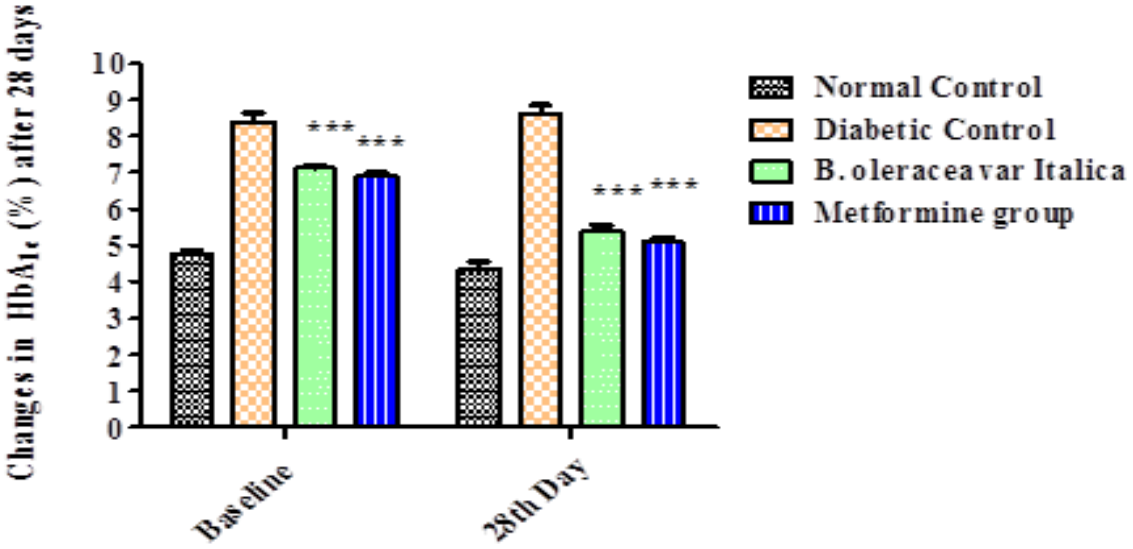

Figure 4: Effect of B. oleracea var italica extract in the %Level of Glycostylated Hemoglobin HFD-STZ- diabet

After identification of the plant *Brassica oleracea* var. italica from Forest Research Institute Malaysia (FRIM) was then washed with running water to decontaminate from dust particles. After washing with water, the plants were covered with cloth and dried in shade for 20 days at room temperature. After shade drying, the plants were grinded through blender and converted into coarse of powder. The powder 200 gm was extracted by continuous hot extraction using the soxhlet apparatus at a temperature of 78°C for 48hr using 95% ethanol. The extract was then concentrated under reduce pressure through rotary evaporator. The extracts were collected and preserved in a desiccator until used for further studies Stored in an air tight sterilized glass container in cool dry place. Weighed the dry extract and percentage yields were determined by following equation:

Percentage yield

$$= \frac{\text{Weight of dry extract} \times 100}{\text{Weight of powder taken}}$$

The dry extract was placed in desicator in order to avoid moisture and for further pharmacological studied

Experimental design

A total of 36 rats (30 diabetic surviving rats and 6 normal rats) were divided in to 6 groups of 6 animals each and given the following treatment orally using an intragastric tube for the period of 28 days.

Group I: Normal control was given distilled water only

Group II: Diabetic control was given distilled water only

Group III: Diabetic rats were given 200 mg/kg of B. oleraceae var. Italica extract

Group IV: Diabetic rats were given 400 mg/kg of B. oleraceae var. Italica extract

Group V: Diabetic rats were given 800 mg/kg of B. oleraceae var. Italica extract

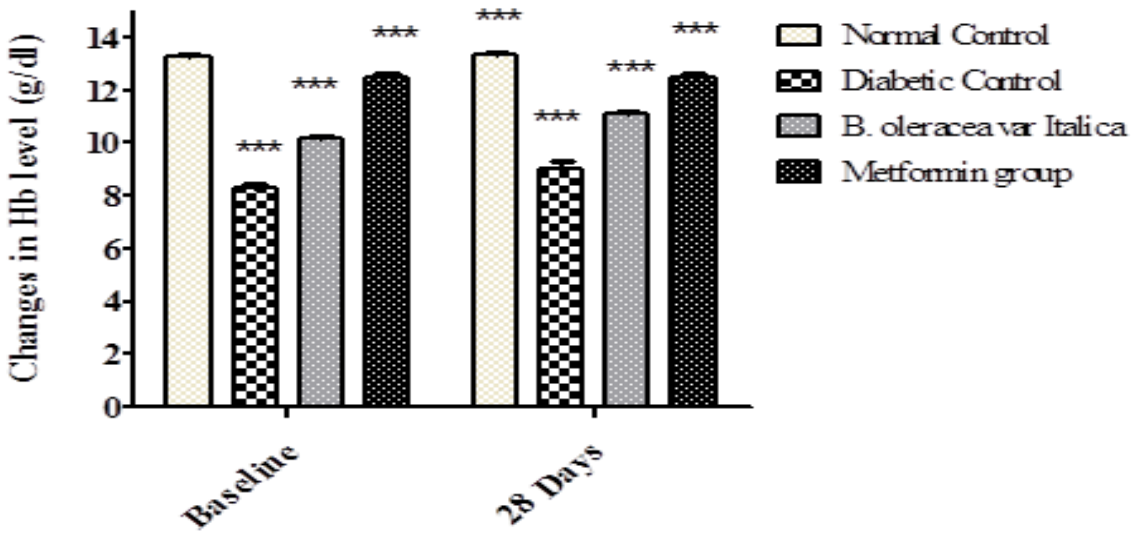

Figure 5: Wffect of B. leracea var Italica extract in the level of hemoglobin in HFD-STZ- diabetic rate

Group VI: Diabetic rats were given Std. antidiabetic drug (Metformin 250mg/kg)

Daily administration of B. oleraceae var. Italica in HFD-STZ induced diabetic SD rats

The HFD fed-STZ-diabetic rats (blood glucose ≥ 17.0 mmol/L) were divided on day zero into six groups (each with 6 rats). The FBG level was measured on day zero at 9.00 am. Distilled water and *B. oleraceae* var. Italica extracts were then administered orally twice a day at 9.00 am and 9.00 pm to diabetic control, treatment and metformin groups respectively for 28 days Body weight, food and water intakes were monitored every day between 9.00 and 10.00 am for 28 days. On the morning of the 29th day, after overnight fasting, the rats were decapitated and the blood was collected for estimation of the biochemical parameters.

Oral glucose tolerance test (OGTT)

An oral glucose tolerance test (OGTT) was used to determine the ability of the body to metabolize and clear glucose out of the blood stream. As shown in the figure 1, HFD-STZ-diabetic rats were fasted for 14 hrs before OGTT. Distilled water (Control) and ethanolic extract of *B. oleraceae* var. Italica at a dose of 200, 400 and 800 mg/kg body weight and the reference drug, metformin, at a dose of 250 mg/kg body weight were orally administered to groups of 6 rats each. Thirty minutes later, glucose (3 g/kg) was orally administered[17] to each rat with a feeding syringe. Blood samples were collected from the tail vein by tail milking at – 30 min (just before the administration of distilled water, fractions of *B. oleraceae* var. Italica and metformin in respective groups), 0 (just before the oral administration of glucose), 30, 60, 120, and 180 min after glucose load for the assay of glucose using glucometer (Accu-chek, Roche Diagnostics, USA).

Biochemical parameters estimation

The animals were anesthetized by using ketamine and xylazine and blood was collected by cardiac puncture. The animals were then euthanized by using over dose of ketamine and xylazine injection. The plasma was used for the fasting blood glucose determination and serum was then separated by centrifugation and was either assayed immediately or stored at −20°C. Blood was collected into dry centrifuge tubes and allowed to stand for 30 min at 37°C. The clear serum was separated at 2500 rpm for 10 min. and was used for the estimation of Serum Insulin and HbA1c using semi automated bioanalyser with specific kits for each measurement. Whole blood was used for hemoglobin determination[16].

Data analysis of the study

The results are presented as means ± SEM. The statistical methods used to analyze the data in this study were unpaired Student's t-test (two-tailed) and two-way analysis of variance (TWO-WAY ANOVA) using SPSS version 22.0. P values < 0.05 were considered to be statistically significant.

RESULTS

Effect of Brassica oleracea var. Italica extract on OGTT

Antidiabetic effects of an ethanolic extracts of *Brassica oleracea* var. Italica extract was evaluated in STZ-induced type 2 diabetic SD rats at a doses of 200, 400 and 800 and the effect was compared with metformin used as a standard drug. For Oral Glucose Tolerance Test, the blood samples were analyzed for glucose content at 0, 60, 120 and 180 minutes, respectively. The blood sugar levels of *B. oleraceae* var. Italica treated groups were compared with the diabetic and normal control and the effects were dose-dependent. The blood glucose levels of the normal rats reached a peak at 60 min after the oral administration of glucose and gradually decreased to pre-glucose load level. Of the three different doses viz., 200 mg, 400 mg and 800 mg/kg body weight the highest dose (800 mg/kg) caused a significant attenuation in the blood glucose at 180 min compared to the diabetic control group ($P<0.001$). In the diabetic rats, FBG levels were 4-5 times

higher than that of the normal SD rats. No significant attenuation was observed in the rats administered 200 mg of *B. oleraceae* var. Italica /kg, even at 180 minutes. However, *B. oleraceae* var. Italica at a dose of 400 mg/kg caused a significant attenuation (P<0.01) in the blood glucose only at 120 min and 180 min, when compared to the diabetic control treated group. Metformin (250 mg/kg) caused significant attenuation at 60 min (P<0.001), 120 min (P<0.001) and 180 min (P<0.001) when compared to the control group. Of the three doses of *B. oleraceae* var. Italica tested, the highest dose (800 mg/kg) appeared to be most effective in improving glucose tolerance (P<0.001). Hence this dose was selected for the 28 days study. The graph represents the mean changes in blood glucose concentration over 60 min level in vehicle, ethanolic extract of B. oleraceae var. Italica and metformin treated (250 mg/kg) diabetic rats. The bars represent SEM (n = 6).

***P<0.001 Metformin-treated group vs diabetic control -treated group (TWO-WAY ANOVA).

**P<0.01 ethanolic extract of B. oleraceae var. Italica -treated group vs diabetic control treated group (TWO-WAY ANOVA).

Effect of Brassica oleracea var. Italica extract on mean body weight, food and water intake

The change in mean body weight, food and water intakes in *B. oleraceae* var. Italica treated group were 9%, 14 % and 2% respectively when compared to the day 0 values. On the other hand, the change in mean body weight, food and water intakes in diabetic control group were 7%, 6% and 43% whereas in metformin group was 9%, 2% and 2% when compared to the day 0 values. The water intake in *B. oleraceae* var. Italica -treated group were much lower than the diabetic control group (Table 25).

Effect of Brassica oleracea var. Italica extract on Fasting blood glucose

B.oleracea var. Italica caused a significant (P<0.001) dose-dependent hypoglycemic effect after daily oral administration of 800 mg/kg body weight for 28 days compared to the control treated control group (Fig. 2). The FBG levels were measured on day 0, day 14, and day 28 at 9.00 a.m. after a 14-hour fast in the diabetic control (distilled water), B. oleraceae var. Italica (800 mg/kg) and metformin (250 mg/kg)-treated HFD-fed STZ-diabetic rats. Columns represent the mean ± SEM (n = 6).

**P<0.001 compared with the diabetic untreated rats (TWO-WAY ANOVA).

Effect of Brassica oleracea var. Italica extract on serum insulin, HbA1c and hemoglobin levels

B. oleraceae var. Italica administration significantly (P<0.01) increased the serum insulin level and decreases the HbA1c (P<0.001) in HFD STZ induced type 2 diabetic rats (Fig.3 and Fig. 4) on day 14 and 28. *B. oleraceae* var. Italica administration also significantly (P<0.001) increases the hemoglobin level when compared to the control groups.

The serum insulin levels were measured on day 0, day 14, and day 28 at 9.00 a.m. after a 14-hour fast in the normal Control (distilled water), *B. oleraceae var. Italica* (800 mg/kg) and Metformin (250 mg/kg)-treated HFD-fed STZ-diabetic rats.

Columns represent the mean ± SEM (n = 6).

***P<0.001 compared to normal control group

**P<0.001 compared to normal control group

The Level of Glycosylated Hemoglobin were measured on day 0 and 28 after a 14-hour fast in the Control (distilled water), B. oleraceae var. Italica (800 mg/kg) and Metformin (250 mg/kg)-treated HFD-fed STZ-diabetic rats.

Columns represent the mean ± SEM (n = 6).

***P<0.001 when compare with the normal group (TWO-WAY ANOVA).

The Hemoglobin Level was measured on day 0 and 28 after a 14-hour fast in the Control (distilled water), *B. oleraceae* var. Italica (800 mg/kg) and Metformin (250 mg/kg)-treated HFD-fed STZ-diabetic rats (Fig.5)

Columns represent the mean ± SEM (n = 6).

***P<0.001 when compare with the normal group. (TWO-WAY ANOVA)

DISCUSSION

The uses of medicinal plants as a source of drugs in primary health care have become popular universally, particularly in developing countries as a safe because of natural source. The bioactive compound isolated from herbal plants are believe to be harmless without any side effect to health, and thus is widely used as Over-The-Counter (OTC) medication[18]. Plant origin drugs are known to play a vital role in the management various chronic diseases and have received a great preference by researcher as alternatives source to allopathic pharmaceutical drugs in recent times[19]. The herbal products today symbolize safety in contrast to the synthetics that are regarded as unsafe to human and environment. Malaysia has plenty of medicinal and more than 1 300 medicinal plant species are found in Peninsular Malaysia alone and the use of medicinal plants as a source of drugs by Malaysian has always been some thousands of year ago[20]. However, there is a lack of proven scientific studies on the toxicity and adverse effect of these treatments. The present research stated that the ethanol leaf extract of *Brassica oleracea* var. Italica possesses definite hypoglycemic properties in STZ-diabetic rats after 28 days of treatment.

The HFD-STZ-induced diabetic rat is one of the animal models of human non-insulin-dependent diabetes mellitus (NIDDM) or type 2 diabetes mellitus[21]. As in human T2DM, diet has a great influence on the development of overt diabetes as well as hypertension, hyperlipidaemia, and eventually nephropathy in experimental model[22]. From the OGTT data, it is clear that administration of ethanolic extracts of Brassica oleracea at the dose 800 mg/kg effectively prevented the increase in serum glucose level (P>0.001) without causing a hypoglycemia as efficiently as the reference drug metformin (P>0.001). This result confirms the reduction of intestinal glucose transporter and is similar to the finding of Mehrabi et.al. 2007[23]. The present investigation also indicates the effectiveness of ethanolic extracts in decreasing the blood

glucose levels in normal and STZ induced diabetic rats (P>0.001). Such antidiabetic activity of Brassica oleracea may be through the stimulation of surviving β-cells to release more insulin. Diabetic rats treated with the Brassica oleracea extracts regains body weight as compared to the diabetic control rats, which may be due to its effect in controlling muscle wasting. The STZ treated animals reported a decrease in hepatic glycogen content which may be due to an increased glucose-6-phosphatase activity and/or decreased hexokinase activity. STZ treatment induces weight loss related to diabetes severity. The reduction of body weight might be due to the low utilization of uptake blood sugar in cell. The elevation of serum insulin in STZ-diabetic rats could either be due to the insulinotropic substances present in the fractions, which induce the intact functional β-cells to produce insulin, or the protection of the functional β-cells from further deterioration so that they remain active and produce insulin. Similarly the extracts of *Medicago sativa*[24], *Eucalyptus globulus*[25] and *Sambucus nigra*[26] have been shown to possess insulin-releasing action both in vitro and in vivo. Since insulin inhibits the activity of Glc-6-Pase in the liver of STZ-diabetic rats and controls HGP, the insulinotropic effect of ethanolic extract might play a crucial role in the control of hyperglycemia in both STZ and HFD-STZ-diabetic rats. HbA1C levels were elevated in diabetic rats and the extent of this increase was directly proportional to FBG levels[27]. Koenig et al., 1976 also reported a 16% increase in HbA1C levels in diabetic patients. Total hemoglobin decreased in the diabetic group, possibly due to the increased formation of HbA1C. This result was well correlated with an earlier report of decreased hemoglobin levels in experimentally diabetic rats[28]. The increase in hemoglobin levels in animals receiving extract may have been due to the decreased blood glucose levels. In this context, several medicinal plants have also been reported to have the ability to reduce HbA1C levels in diabetic rats[29].

CONCLUSION

In conclusion the antidiabetic effects of an ethanolic extracts of Brassica oleracea var. Italica plant evaluated in STZ-induced type 2 diabetic SD rats at a doses of 800 mg/kg possesses a definite antihyperglycemic properties in HFD-STZ- type 2 diabetic rats after 28 days of treatment. However the hypoglycemic mechanism of *Brassica oleracea* var. Italica extract remains unclear and further studies are required to elucidate cellular and molecular mechanisms. *Brassica oleracea* var. Italica can further be analyzed using modern liquid chromatographic techniques coupled with mass spectrometry (LC-MS), nuclear magnetic resonance spectroscopy (LC-NMR), ultra-violet spectroscopy (LC-UV) and infrared spectroscopy (LC-IR) for the identification and isolation of novel anti-diabetic component(s) and can be analyzed on pancreatic insulin content in STZ-induced diabetic rats.

Competing interest

The authors declare that they have no competing interests.

Author's contributions

Experimental design: Mashood Ahmad Shah, Md. Moklesur Rahman Sarker. Performed the experiments: Mashood Ahmad Shah. Analyzed the data: Mashood Ahmad Shah, Md. Moklesur Rahman Sarker and Md. Gousudddin. Paper writing: Mashood Ahmad Shah. Manuscript review: Mashood Ahmad Shah, Md. Moklesur Rahman Sarker. Interpreted the data: Mashood Ahmad Shah, Md. Moklesur Rahman Sarker. Data acquisition: Mashood Ahmad Shah, Md. Moklesur Rahman Sarker and Md. Gousudddin. Overall supervision and critical comments: Md. Moklesur Rahman Sarker. All authors read and approved the final manuscript

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