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Antidiabetic and antilipidemic effect of *Ilex kaushue* S.Y. Hu (Aquifoliaceae) leaves' extract on streptozotocin-induced diabetic mice

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ABSTRACT

Diabetes disease is a worldwide problem. Until now, there is no effective treatment. Traditional medicine uses *Ilex kaushue* S.Y. Hu (Aquifoliaceae) for supporting the treatment of diabetes and hyperlipidemia. This study was conducted to evaluate the α-glucosidase inhibitory activity and the hypoglycemic and hypolipidemic effect of *I. kaushue* leaves' extract. *Ilex kaushue* leaves were extracted by ethanol 30% with sonication 45 minutes, which was repeated thrice. *Ilex kaushue* leaves' extract was used to evaluate the α-glucosidase inhibitory activity *in vitro* and the hypoglycemic and hypolipidemic effect *in vivo* on streptozotocin-induced diabetic mice (50 mg/kg b.w. i.p.). Our results show that *I. kaushue* leaves' extract had a moderate α-glucosidase inhibitory activity with an IC_{50} value of 147.25 µg/ml and strong hypoglycemic and hypolipidemic effect on diabetic mice. Our data show that *I. kaushue* leaves' extract has the potential antidiabetic and antilipidemic effect.

INTRODUCTION

Diabetes mellitus is characterized by frequent hyperglycemia or impaired insulin secretion for many reasons (Kerner and Bruckel, 2014). Chronic hyperglycemia often leads to a variety of disorders and complications such as organ damage, especially the heart, blood vessels, kidneys, eyes, and nerves (Reusch and Manson, 2017). Dyslipidemia is a common complication in most diabetic diseases (Mooradian, 2009). Dyslipidemia is a disorder related to hypercholesterolemia, hypertriglyceridemia, low-density lipoprotein (LDL), and decreased high-density lipoprotein (HDL). Type 2 diabetes is the most common form of diabetes, accounting for about 80%–90% of all diabetic patients (Williams *et al.*, 2020). There are several different types of oral hypoglycemic agents but these drugs are expensive and have many side effects. In recent years, numerous traditional medicinal plants with antidiabetic effects have been studied. Many researchers have showed that these plants have fewer side effects (Arumugam *et al.*, 2013).

Ilex kaushue S. Y. Hu, also known as bitter tea, is a popular tea in many Asian countries such as Vietnam, China, and Japan. *Ilex kaushue* has a variety of effects according to traditional medicine, including brightening the mind and eyes, detoxifying, reducing cough and sore throat, digestive stimulation, lowering blood pressure and blood sugar, and antibacterial and anti-inflammatory properties (Li *et al.*, 2013). *Ilex kaushue* leaves are also used to treat insomnia, red eyes, headache, and toothache. Phytochemical studies have showed that bitter tea leaves have many bioactive compounds such as saponins, alkaloids, triterpenoids, phenolics, and flavonoids (Li *et al.*, 2012; Tang *et al.*, 2005; Yi *et al.*, 2018). Traditional medicine has used bitter tea as a beverage for reducing blood glucose in diabetes patients, but there is no scientific evidence demonstrating the antidiabetic effect of *I. kaushue* leaves. Therefore, this study was conducted to assess the hypoglycemic and hypolipidemic effects of *I. kaushue* leaves extracted by ethanol 30% on diabetic mice.

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MATERIALS AND METHODS

The leaves of *I. kaushue* S. Y. Hu were cultivated and harvested from Cao Bang province. Samples were identified by the Department of Pharmacognosy and Traditional Pharmacy, Medicine and Pharmacy University, Vietnam National University, Hanoi, Vietnam (UMP-VNU). A voucher specimen has been deposited in the UMP-VNU (UMP 0482020). Leaves were washed and dried at 60°C. Eight hundred grams of dried leaves was extracted by ethanol 30% (4l) with sonication for 45 minutes, which was repeated thrice. The extracts were filtered, combined, and evaporated under low pressure to obtain ethanol extract (97 g), and extraction efficiency was 12.1%.

Reagents included streptozotocin (STZ) (250 mg, Aladdin, China), yeast α-glucosidase, p-nitrophenyl-α-D-glucopyranoside (pNPG), 4-nitrophenol (Sigma), and gliclazide (Diamicron RMR 60 mg). All other chemicals were of analytical grade.

The animals, Swiss albino mice, male sex, healthy, weighing 20 ± 2 g, were purchased from the National Institute of Hygiene and Epidemiology, Vietnam. The mice were kept in cages (10 animals per cage) under standard conditions of humidity (50% $\pm$ 5%), temperature (25°C $\pm$ 2°C), and light (12 hours light/12 hours dark cycle), and were fed with standard food and water *ad libitum*. Before the experiment was carried out, mice were adapted to their laboratory condition within 7 days.

Evaluate the α-glucosidase inhibitory activity

The inhibitory activity of α-glucosidase was determined according to the previously described method with some modifications (Ting *et al.*, 2005). Appropriate dilutions of the samples (50 µl) and 100 µl of α-glucosidase (0.2 U/ml) in 0.1M phosphate buffer (pH 6.8) solution were incubated at 37°C for 15 minutes. Then, 50 µl of 5 mM pNPG in 0.1 M phosphate buffer (pH 6.9) solution was added. The mixtures were incubated at 37°C for 60 minutes. The reaction was interrupted by adding 80 µl Na_2CO_3 0.2M. The absorbance was measured at 410 nm in the spectrophotometer. The reference sample included all other reagents and the enzymes except the test sample. One unit of α-glucosidase activity was defined as the amount of enzyme liberating p-nitrophenol (1.0 µM) per min. The IC_{50} value was defined as the concentration of α-glucosidase inhibitor that inhibited 50% of α-glucosidase activity. Acarbose, a known α-glucosidase inhibitor, was used as a positive control. The α-glucosidase inhibitory activity was expressed as percentage inhibition as follows:

$$\text{Inhibition (\%)} = [(A_{ref} - A_{sample})/A_{ref}] \times 100$$

Evaluation of the hypoglycemic effect of *I. kaushue leaves*' ethanol 30% extract on diabetic mice induced with STZ

Method developed by Bansal *et al.'s* (2012) was followed with some modifications according to our laboratory conditions. Mice were fed with a high-fat diet (containing 35% fat in the form of lard) for 28 days to induce obesity. Mice that were over 35 g were used for the experiment. Then, mice had induced diabetes by injecting three doses of STZ 50 mg/kg/b.w. for 3 consecutive days. STZ was dissolved in 0.01 M citrate buffer, pH 4.3. The physiological control group was injected with citrate buffer.

The blood samples were withdrawn from the mouse tail and blood glucose levels were measured with a portable glucose meter. After 10 days, tail bleeds were carried out and the animals with blood glucose concentrations above 10 mmol/l were considered diabetic (Flodstrom *et al.*, 1999). The procedures for handling and care of animals adhered to the guidelines of the current international laws and policies [National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals], and they were approved by the animal ethics committee at our university (CS.20.01/20-CN-HDDD).

The animals were randomly assigned into 5 groups of 10 mice per cage.

The control group (Group I) was given an i.p. injection of citrate buffer.

STZ-induced group (Group II) was injected with and STZ dose of 50 mg/kg, i.p.

The positive control group (Group III) was injected with STZ (50 mg/kg, i.p.) + gliclazide (dose of 5 mg/kg, b.w.) for 28 days.

Group IV comprised STZ-induced diabetic mice treated with *I. kaushue* leaves extract (100 mg/kg, p.o.), administered orally for 28 days.

Group V comprised STZ-induced diabetic mice treated with *I. kaushue* leaves' extract (200 mg/kg, p.o.), administered orally for 28 days.

All groups were treated daily for 28 days, and blood glucose was monitored in 0, 10, 20, and 28 days.

Evaluation of the hypolipidemic effect of *I. kaushue leaves*' ethanol 30% extract on diabetic mice induced with STZ

After completing the experiment (28 days), mice were fasted for 24 hours prior to sampling. The mice were anesthetized with diethyl ether, using a 1 ml needle to conduct a heart blood draw. After 28 days of treatment, weak samples were collected into tubes containing heparin. The serum was separated by centrifugation at 3,000 rpm at 25°C for 15 minutes and sent to Hanoi Medical University Hospital for biochemical analysis: total cholesterol, HDL cholesterol, and triglycerides.

Statistical analysis

All results were expressed as $X \pm$ SEM (X = average value of each group; SEM: standard error of measurement). Serial measurements were analyzed by one-way analysis of variance with Tukey's *post-hoc* test using Sigma Stat 3.5 program and figures were produced by using Sigma Plot 10.0 program (Systat Software Inc.). The critical significance level α was 0.05 and statistical significance was defined as $p < 0.05$.

RESULTS

α-Glucosidase inhibitory activity of *I. kaushue leaves*' ethanol extract

The inhibitory effect of *I. kaushue* leaves' extract was determined by using pNPG as a substrate and was compared to the effect of the clinically applied α-glucosidase inhibitor, acarbose.

The α-glucosidase inhibitory activity of *I. kaushue* leaves' extract is shown in Figure 1. The results showed that the *I. kaushue* leaves' extract had moderate α-glucosidase inhibitory activity with an IC50 value of 147.25 μg/ml. In this study, we compared the inhibitory α-glucosidase effect of *I. kaushue* leaves' extract with acarbose, an α-glucosidase inhibitor, which has been used in diabetes treatment. Acarbose inhibited α-glucosidase activity with an IC50 value of 156.08 μg/ml. Because the lesser the IC_{50}, the better its inhibitory α-glucosidase activity (Kumar *et al.*, 2011; Thu *et al.*, 2019; Thuy *et al.*, 2020). Then, our results show that *I. kaushue* leaves' extract has a higher α-glucosidase inhibition activity than acarbose.

Hypoglycemic effect of *I. kaushue leaves*' extract on diabetic mice induced with STZ

The effects of *I. kaushue* leaves' extract on blood glucose concentration and mouse body weights are presented in Tables 1 and 2. In Group I (control group), blood glucose levels and body weights were stable during the experiment. In Group II (diabetic group), blood glucose level and body weight had increased significantly when compared to the physiological control group. In Group III (diabetic mice treated with gliclazide), the blood glucose level and body weight significantly reduced when compared to Group II. However, when *I. kaushue* leaves' extract (100 and 200 mg/kg, respectively) was administered, the decrease in blood glucose levels and body weight was significantly alleviated (10.52 ± 1.34 and 8.91 ± 1.12, respectively; $p < 0.05$).
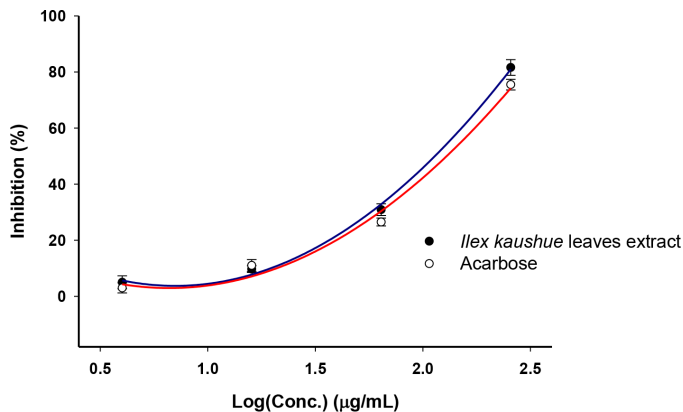

Figure 1. α-Glucosidase inhibitory activity of *I. kaushue* leaves' extract.

These results indicated that *I. kaushue* leaves' extract has a potential antidiabetic effect to prevent and support the treatment of diabetes.

Hypolipidemic effect of *I. kaushue leaves*' extract on mice

Table 3 presents the effects of *I. kaushue* leaves' extract on blood lipid levels of mice after 28 days of treatment. Groups IV and V were treated with *I. kaushue* leaves' extract at a dose of 100 and 200 mg/kg which was able to reduce total cholesterol, triglyceride, and LDL cholesterol levels significantly when compared to Group II ($p < 0.05$). Furthermore, *I. kaushue* leaves' extract was also shown to significantly increase HDL cholesterol levels when compared to Group II ($p < 0.05$).

DISCUSSION

Type 2 diabetes is characterized by insulin resistance and impaired insulin secretion by pancreatic cells. Obesity is characterized by excess or abnormal fat accumulation in the body (Chiang *et al.*, 2011). Fatty acid accumulation is a key factor in increasing insulin resistance. Based on this, many experimental diabetes models have been built with the tendency to create resistance and insulin and/or impair the function of beta-pancreatic cells. Each model has both advantages and disadvantages. In order to evaluate the hypoglycemic and hypolipidemic effects on obese mice with type 2 diabetes, the model of creating obese mice by high-fat diet combined with low-dose STZ of 50 mg/kg injection in mice for three consecutive days was selected.

STZ is a glucosamine nitrosourea compound, which is chemically defined as $C_8H_{15}N_3O_7$. STZ has specific toxicity to insulin-producing β-cells of the pancreas in animals. STZ was found in a *Streptomyces achromogenes* strain in the 1950s. Currently, STZ is being used in medicine to treat some cancers of the islet of the Langerhan pancreas and also used as an effective agent to induce diabetes on experimental animal models (Lukic *et al.*, 1998). The toxic mechanism of STZ could be explained as STZ recognizes and infiltrates β-cells via Glucose transporter 2 (GLUT2) glucose transporter, alkylates, and destroys DNA, eventually leading to cell necrosis (Kwon *et al.*, 1994). Alkylation activity is thought to be due to the activity of the nitrosourea group, especially the O^6 position of the guanine. In addition, STZ also activates ribosylation of poly(adenosine diphosphate) and the release of nitric oxide. As a result, pancreatic cells are destroyed due to necrosis (Kumar *et al.*, 2012).

Table 1. Blood glucose level (mmol/l) in diabetic mice induced with STZ before and after 28 days of treatment using *I. kaushue* leaves' extract.

Groups	Before treatment	Day 10	Day 20	After treatment for 28 days
Group I: control group	5.07 ± 0.66	5.58 ± 1.04	5.79 ± 1.01	5.14 ± 0.91
Group II: STZ + distilled water	14.58 ± 3.21	15.02 ± 2.24#	16.09 ± 2.51#	16.23 ± 2.31#
Group III: STZ + gliclazide	15.83 ± 1.21	13.25 ± 3.32	11.27 ± 2.36*	9.52 ± 2.25*
Group IV: STZ + IKL-100 mg/kg	15.07 ± 1.45	13.87 ± 1.89	12.69 ± 1.35*	10.52 ± 1.34*
Group V: STZ + IKL-200 mg/kg	14.69 ± 2.53	12.67 ± 2.12	11.57 ± 1.82*	8.91 ± 1.12*

IKL: *I. kaushue* leaves' extract.

*Significant differences compared to Group II.

#Significant differences compared to Group I.

Table 2. Bodyweight (g) of diabetic mice induced with STZ before and after 28 days of treatment using *I. kaushue* leaves' extract.

	Before treatment	After treatment for 15 days	After treatment for 28 days	% change
Group I: control group	35.2 ± 0.3	37.7 ± 0.8	38.2 ± 1.7	Increase 8.5
Group II: STZ + distilled water	36.5 ± 2.5	37.9 ± 0.4	42.2 ± 1.1#	Increase 15.6
Group III: STZ + gliclazide	40.3 ± 2.8	38.8 ± 2.1	34.5 ± 3.2*	Decrease 13.5
Group IV: STZ + IKL-100 mg/kg	41.2 ± 2.5	38.4 ± 1.2	36.9 ± 2.9*	Decrease 11.6
Group V: STZ + IKL-200 mg/kg	39.9 ± 1.9	37.3 ± 0.7	33.2 ± 2.2*	Decrease 16.8

IKL: *I. kaushue* leaves' extract.
*Significant differences compared to Group II.
#Significant differences compared to Group I.

Table 3. Hypolipidemic effect of *I. kaushue* leaves' extract on mice.

Group	Total cholesterol (mg/ml)	Triglyceride (mg/ml)	HDL cholesterol (mg/ml)	LDL cholesterol (mg/ml)
Group I: control group	45.78 ± 2.5	60.1 ± 5.2	42.3 ± 3.5	17.5 ± 2.5
Group II: STZ + distilled water	86.7 ± 4.6#	97.9 ± 5.5#	16.1 ± 3.3#	50.2 ± 2.4#
Group III: STZ + gliclazide	65.6 ± 5.5*	73.7 ± 5.4*	31.9 ± 3.4*	29.6 ± 2.9*
Group IV: STZ + IKL-100 mg/kg	74.2 ± 3.7*	81.4 ± 3.1*	29.2 ± 4.3*	43.8 ± 4.1*
Group V: STZ + IKL-200 mg/kg	69.4 ± 4.8*	76.7 ± 5.3*	35.5 ± 3.8*	35.4 ± 3.5*

IKL: *I. kaushue* leaves' extract.

*Significant differences compared to Group II.

#Significant differences compared to Group I.

There are several levels of streptozocin that can cause diabetes. Through reference to the models of previous studies, we choose a low dose of 50 mg/kg (Akbarzadeh *et al.*, 2007; Gajdosik *et al.*, 1999). In this model, the high-fat diet has both obesity and insulin resistance effects, eventually increasing diabetes and increasing the risk of cardiovascular disease. Hence, low-dose STZ produces a human-like model of type 2 diabetes mouse. At the same time, low doses will also reduce complications and increase survival rates in mice. The model is based on the pathogenesis mechanism of diabetes type 2 which has the advantages of low cost, easy implementation, like human, and useful in preclinical evaluation models to treat diabetes (Lenzen, 2008; Zhang *et al.*, 2008). Our results showed that *I. kaushue* leaves' extract has a moderate α-glucosidase inhibitory activity. Moreover, the *I. kaushue* leaves' extract showed a strong hypoglycemia effect equivalent to gliclazide in diabetic mice induced by STZ.

The α-glucosidase is an enzyme located in intestinal cell membranes, concentrated in the small intestine, involved in the absorption of sugar from the digestive system. α-Glucosidase further decomposes disaccharides into simpler sugars, making them available for intestinal absorption. The inhibition of their activity in the digestive tracts of humans is believed to be effective in controlling diabetes because of the diminished glucose absorption. This study investigated the role of the *I. kaushue* leaves' extract in the inhibition of the activities of carbohydrate hydrolyzing enzymes and alleviation of postprandial hyperglycemia. Therefore, these enzyme preparations decrease the absorption of glucose compounds into the blood. Enzyme α-glucosidase inhibitors have been used as drugs to regulate type 2 diabetes such as acarbose, miglitol, and voglibose (Tung *et al.*,

2018). Acarbose is relatively well researched and widely used in Western medicine today. Acarbose is also used as a reference for experiments α-glucosidase inhibitory effects of other compounds (Carrascosa *et al.*, 2001). In this study, acarbose was used as a positive control for the experimental evaluation of α-glucosidase inhibition of *I. kaushue* leaves' extract. In the future, research on the isolation of compounds capable of inhibiting α-glucosidase from *I. kaushue* leaves' extract is necessary.

According to traditional Vietnamese medicine, bitter tea leaves have been used to treat many ailments, including diabetes. Recent studies have also found that *I. kaushue* leaves' extract has many effects such as antioxidant, lipid-lowering, and state control. In *in vitro* research on Caco 2 cells, Wang *et al.* (2008) showed that glucose uptake of Caco 2 cells was inhibited by *I. kaushue* leaf extract (Wang *et al.*, 2008). Song *et al.* (2016) reported that the total extract from *I. kaushue* leaves had a lipid-lowering effect on rats. The study also showed an increase in serum HDL concentrations and a significant reduction in the index of fibrosis in mice after using the extract (Song *et al.*, 2016). *Ilex kaushue* leaf extract at a dose of 50 mg/kg body weight has been shown by Fan *et al.* (2012) to prevent body weight gain, reduce the size of fat cells, and reduce serum triglycerides, cholesterol, LDL cholesterol, fasting glucose levels, and glucose tolerance in rats with the high-fat diet (Fan *et al.*, 2012). Ethanol extraction of *I. kaushue* leaves was also shown to improve total cholesterol parameters, as well as Low density lipoprotein - cholesterol (LDL-C) in rats fed a high-fat diet. Studies by Linh *et al.* (2016) also showed that bitter tea combined with other medicinal plants' extracts is effective against diabetes and hyperlipidemia. Our results are consistent with previous studies and also show that, in mice with type 2

diabetes after 28 days of treatment with *I. kaushue* leaves' extract at doses of 100 and 200 mg/kg, the decrease in blood glucose level was 30.19% and 39.35%, respectively, when compared with the baseline blood glucose group. At a dose of 200 mg/kg, blood glucose concentrations are significantly reduced. It has also been shown to be effective in reducing triglycerides, total cholesterol, LDL cholesterol, and increasing HDL cholesterol levels.

CONCLUSION

In this study, we showed that *I. kaushue* leaves' extract had a moderate α-glucosidase inhibitory activity with an IC_{50} value of 147.25 µg/ml and strong hypoglycemic and hypolipidemic effect on diabetic mice. Our results supported the effectiveness of the *I. kaushue* leaves' extract on the prevention and treatment of diabetes and dyslipidemia. Further studies on the isolation of the bioactive compounds from *I. kaushue* leaves should be conducted to clarify the effects and mechanisms of this medicinal plant.

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AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interests.

ETHICAL APPROVAL

The study protocol was approved by the institutional animal ethics committee at our university (CS.20.01/20-CN-HDDD).

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