

Effect of *Camellia flava* (Pitard) Sealy flower extract on the degeneration of Islets of Langerhans and insulin resistance in alloxan-induced hyperglycemia model on Swiss albino mice

Huu Lac Thuy Nguyen¹, Thi Thu Phuong Ha¹, Ngoc Phuc Nguyen Nguyen¹,
 Nguyen Hoang Linh Phan¹, Dang Thuy Hien Nguyen¹, Van Dat Truong², Minh Nhut Truong³,
 Thao-My Nguyen-Hoang¹, Thi Nghia Luu¹, Ngoc Phuc Nguyen¹, Thanh Binh Nguyen^{4,5},
 Huynh Nhu Mai¹

¹ Faculty of Pharmacy, University of Medicine and Pharmacy at Ho Chi Minh city, Ho Chi Minh city, 700000, Vietnam

² Ministry of Health, Hanoi, 100000, Vietnam

³ Faculty of Traditional Medicine, University of Medicine and Pharmacy at Ho Chi Minh city, Ho Chi Minh city, 700000, Vietnam

⁴ Cell-Tissue Department, Biomedical Research Center, Pham Ngoc Thach University of Medicine, Ho Chi Minh city, 700000, Vietnam

⁵ Thu Dau Mot University, Binh Duong, 820000, Vietnam

Corresponding author: Huynh Nhu Mai (mhnhu@ump.edu.vn)

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Abstract

Diabetes has always been a matter of concern to health experts as well as the community due to the increasing number of patients with diabetes and the severe consequences it may cause. Many attempts have been made to discover new treatment options for diabetes, and herbal medicines are currently considered to have great potential. This study was conducted to evaluate the effect of *Camellia flava* flower extract on the degeneration of the islets of Langerhans and insulin resistance in an alloxan-induced hyperglycemia model in Swiss albino mice. Hyperglycemic conditions were induced by alloxan (55 mg/kg, i.v.). The animals were then treated with glibenclamide (10 mg/kg, p.o.) and flower extract at doses of 1.09 and 2.19 g/kg, p.o. The results showed that the blood glucose, AUC, HbA1c, and HOMA-IR levels of two groups of mice receiving flower extract were considerably lower than those of the hyperglycemic untreated group ($p < 0.05$). The body weights of these two groups were also lower than the untreated group on the last day of the experiment, though the differences were not significant ($p > 0.05$). However, this was not observed when assessing insulin levels as well as relative organ weights. In biochemical tests, creatinine and AST and ALT concentrations were evaluated. There was no significant variation in creatinine and AST concentrations between the five experimental groups, whereas mice treated with glibenclamide and flower extract at both doses showed a remarkable decline in ALT concentration ($p < 0.05$). The hepatic histomicrographs were consistent with ALT results, while the H&E staining of kidneys showed no difference between groups. Histomicrographs of the pancreas revealed that the treatment groups using glibenclamide and flower extract had larger islets of Langerhans than those of the alloxan-treated group. Based on these results, this study demonstrated that *Camellia flava* flower extract exerted several beneficial effects, including blood sugar level reduction, weight loss promotion, and organ protection, hence making it a new potential herbal medication for the management of diabetes.

Keywords

alloxan, *Camellia flava*, hypoglycemic effect, islets of Langerhans, insulin resistance

Introduction

Diabetes is a chronic disease that occurs when the body can neither synthesize enough insulin nor use insulin effectively (WHO, 2023). The lack of insulin results in sugar not being consumed by the tissues, thus leading to high blood glucose levels. Patients with diabetes often experience many dangerous complications, both chronic and acute, such as macro- and microvascular diseases, diabetic ketoacidosis (DKA), and hyperosmolar hyperglycemic state (HHS). As of 2019, according to the World Health Organization (WHO), there were approximately 422 million people worldwide suffering from diabetes, with the majority coming from low- and middle-income nations (WHO 2023). The incidence of diabetes was 9.3% of the global population in 2019, and this figure is predicted to rise to 10.9% in 2045 (Saeedi et al. 2019). In Vietnam, there were about 5 million people with diabetes in 2021, making up 7.1% of the adult population. The proportion of diabetic patients in Vietnam has nearly doubled over the last 10 years, and it is estimated that one in every 20 Vietnamese adults is living with diabetes (WHO 2016).

Due to this increase in the incidence of diabetes worldwide, there have been many attempts at discovering and developing new medications that help with the treatment of diabetes. According to the American Diabetes Association 2023, there are seven groups of medications approved to be used in curing diabetes, including metformin, iSGLT2 (sodium-glucose transporter 2 inhibitors), GLP-1 RAs (glucagon-like peptide 1 receptor agonists), iDPP-4 (dipeptidyl peptidase 4 inhibitors), thiazolidinediones, sulfonylureas, and insulin. All of the above-mentioned medications have proved to be effective in the management of diabetes, but they also carry some mild to severe side effects such as renal toxicity, heart failure, and obesity (ElSayed et al. 2023). Many herbal medicines or medicinal plants are remarkably considered an alternative therapy to pharmaceutical drugs because of their significant potential in the treatment of diabetes and fewer adverse effects.

Camellia flava (Pitard) Sealy, one golden camellia species belonging to the Theaceae family, is mainly distributed in Asian countries such as China, India, and Vietnam. It has been reported to contain several phytochemical constituents, such as polysaccharides, phenolics, saponins, flavonoids, and so on. These compounds exhibited various pharmacological effects, including antioxidant, antimicrobial, antihyperglycemic, and metabolic balance (Hakoda and Ninh 2001).

With *Camellia flava* being recognized as a potential herbal medicine for diabetes treatment, there is growing interest in investigating the pharmacological effects of this plant. In our preliminary study, we have already evaluated

the acute toxicity and hypoglycemic effect of *Camellia flava* flower extract on 60 mg/kg, i.v., alloxan-induced hyperglycemic mice (Nguyen et al. 2023). The mice were treated with flower extract orally at 4 different doses of 0.22, 0.66, 1.09, and 2.19 g/kg. However, only the mice receiving treatment at doses of 1.09 and 2.19 g/kg showed markedly hypoglycemic signs. Therefore, following this study, we decided to further investigate the effect of *Camellia flava* flower extract at these 2 doses (1.09 and 2.19 g/kg) on the degeneration of the islets of Langerhans and insulin resistance in a hyperglycemia model induced by alloxan 55 mg/kg, i.v., on Swiss albino mice.

Materials and methods

Animals

Male and female Swiss albino mice were provided by the Pasteur Institute in Ho Chi Minh City, Vietnam. The mice had no deformities or abnormal behaviors and weighed about 20.2 ± 2 g. They were fed with standard feed from the Pasteur Institute and water *ad libitum*. Male and female mice were kept separately. These mice were adapted to the conditions of the pharmacology laboratory in the Faculty of Pharmacy, University of Medicine and Pharmacy, Ho Chi Minh City, for 1 week before the experiment. All the experiments were conducted in the Laboratory Animal House, Department of Pharmacology, Faculty of Pharmacy, University of Medicine and Pharmacy at Ho Chi Minh City, according to the approval of the Ethics Committee of the University of Medicine and Pharmacy at Ho Chi Minh City (668/GCN-HDDNCTDV). The protocol of the experiment was approved by the institutional review board of the University of Medicine and Pharmacy at Ho Chi Minh City (Decision: 1081/QD-DHYD).

Plant collection and extraction

Flowers of *Camellia flava* (Pitard) Sealy were provided by Truong Duong Trading Investment Joint Stock Company in Gia Lai province on February 10th, 2022. The materials were then dried and ground into powder. The concentrated extracts were obtained by soaking 1.0 kg of flowers (humidity of 9.6%) with 70% ethanol for a period of 24 h at room temperature and then filtering. Repeat the process three times (with 5, 3, and 2 L of 70% ethanol, respectively). The total filtrate was finally evaporated to obtain the concentrated ethanolic extract. The extraction experiment was carried out in the laboratory of the Department of Analytical Chemistry and Drug Quality Control, Faculty of Pharmacy, University of Medicine and Pharmacy, Ho Chi Minh City.

Determination of total flavonoid content in *Camellia flava* flower extract

The process of determining total flavonoid content was carried out as mentioned elsewhere (Khan et al. 2018). Samples reacted with the AlCl_3 reagent in a basic environment to create a yellow product. It was then taken to measure absorbance at 415 nm. The results were expressed in mg RE/g.

Evaluation of the antioxidant activity of *Camellia flava* flower extract

2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity

The process of evaluating DPPH free radical scavenging activity was carried out according to the study of Kedare et al. (2011) (Kedare and Singh 2011; Truong et al. 2022). The results were expressed using the IC_{50} value ($\mu\text{g/mL}$) and compared with a positive control (ascorbic acid).

Determination of the route of injection for alloxan

Alloxan monohydrate (A7413-25G, Sigma-Aldrich) was dissolved in citrate buffer pH 4.5 (Klebanoff and Greenbaum 1954; Yin et al. 2018; Sekiou et al. 2021) with a concentration of 1% to be used in the experiment. The male and female mice were divided approximately equally into four groups, including:

Control group: distilled water, p.o.

I.P. 150 group: single dose of alloxan (150 mg/kg body weight, i.p.) (Federiuk et al. 2004; Das et al. 2011)

I.V. 55 group: single dose of alloxan injected into the lateral tail vein (55 mg/kg body weight, i.v.) (Tang et al. 2006; Sharma et al. 2015).

The animals were fasted for 12 hours prior to the alloxan injection. The mice were observed throughout the 6 hours after injection for any reaction. When the mice started to show signs of tiredness and shivering, they were

administered 0.1 mL of an aqueous solution of glucose 5% orally (Yashwant Kumar et al. 2011).

Fasting blood glucose levels of the mice were measured on days 1, 7, and 14 after alloxan injection. The mice were also weighed every 2 days, starting on day 3 and ending on day 13. Blood samples were collected from the vein at the tip of the tail to reduce bleeding and stress for the mice (King et al. 2020). The appropriate route of injection for alloxan was determined based on the mortality rate, the percentage of mice with a blood glucose level ≥ 200 mg/dL, and the stability of the blood glucose level throughout the experiment.

Experimental design for the evaluation of the hypoglycemic effect of *Camellia flava* on mice

The animals were fasted for 12 hours prior to injection. The mice were then injected with 1% alloxan intravenously into the lateral tail vein at a dose of 55 mg/kg (day -2) (Fig. 1). They were observed throughout the 6 hours after the injection for any reactions. When the mice started to show signs of tiredness and shivering, they were administered 0.1 mL of an aqueous solution of glucose 5% orally (Yashwant Kumar et al. 2011).

Fasting blood glucose values were measured 3 days after injection (day 1) (Fig. 1). Mice with blood glucose levels ≥ 200 mg/dL were considered hyperglycemic and were used for the experiment. The doses of 1.09 and 2.19 g/kg of *Camellia flava* flower extract were chosen according to the results of the previous pilot study (Nguyen et al. 2023). The male and female mice were randomly divided approximately equally into five groups, including:

Control group: distilled water, p.o.

ALX 55 group: alloxan (55 mg/kg, i.v.)

ALX 55 + GBC group: alloxan (55 mg/kg, i.v.) + glibenclamide (10 mg/kg, p.o.) (Ajabnoor 1990)

ALX 55 + CF 1.09 group: alloxan (55 mg/kg, i.v.) + *Camellia flava* flower extract (1.09 g/kg, p.o.)

ALX 55 + CF 2.19 group: alloxan (55 mg/kg, i.v.) + *Camellia flava* flower extract (2.19 g/kg, p.o.)

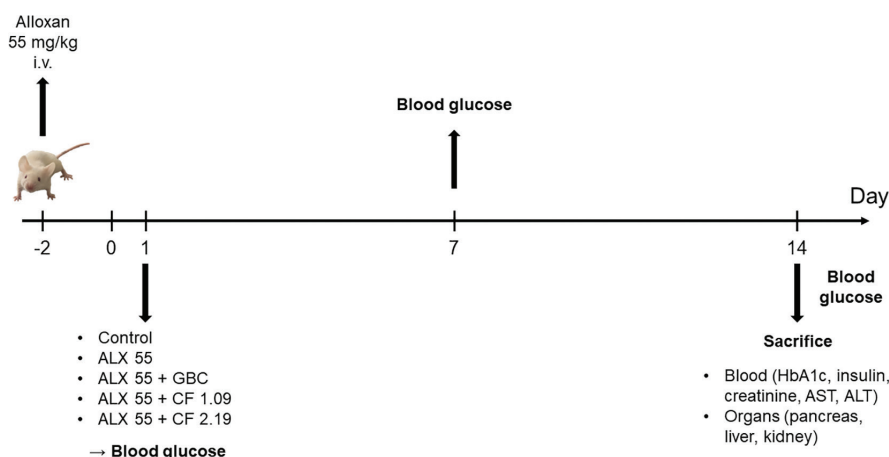


Figure 1. Experimental timeline. ALX = alloxan, GBC = glibenclamide, and CF = *Camellia flava* flower extract.

Evaluation of blood glucose levels

The blood glucose levels of all five groups of mice were assessed on days 1, 7, and 14 after the induction of hyperglycemia (Fig. 1). After overnight fasting, blood samples were collected from the vein at the tip of the tail (King et al. 2020) to measure fasting glucose levels using a GlucoDr. Auto Glucometer (All Medicus Co., Ltd., Korea). The mice were also weighed every 2 days, starting on day 3 and continuing until day 13 of the experiment.

On the 14th day of the experiment, the body weights of the mice were recorded. The animals were then sacrificed, and their blood samples were collected from their hearts. Blood samples were preserved in an EDTA buffer tube to avoid coagulation. Pancreases, livers, and kidneys were collected, weighed, rinsed with 0.9% saline solution, and fixed by being immersed in 10% buffered formalin (Fig. 1).

Evaluation of HbA1c, insulin levels, and insulin resistance

HbA1c assay

The assay of HbA1c was carried out using the NycocardTM HbA1c test (1116813, Abbott, U.S.A.) according to the guidelines of the manufacturer.

Insulin assay

The assay of insulin was carried out using the ARCHITECT i1000SR immunoassay analyzer (Abbott, U.S.A.).

Evaluation of insulin resistance

Insulin resistance was calculated using the homeostatic model assessment and insulin resistance (HOMA-IR) index with the following formula (Antunes et al. 2016; Chu et al. 2022):

$$HOMA-IR = \frac{Glucose\left(\frac{mg}{dL}\right) \times Insulin\left(\frac{ng}{mL}\right)}{405}$$

Evaluation of creatinine levels

The assay of creatinine was carried out using the Creatinine Jaffe reagent (CRCO-0600, ELITech Group, France) according to the guidelines of the manufacturer. Briefly, the reagent and sample were mixed and measured at 505 nm at 37 °C. The creatinine was calculated as follows:

$$\frac{A(sample)}{A(standard)} \times n(standard\ concentration) \times 88.40$$

Unit of creatinine: $\mu\text{mol/L}$

Evaluation of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels

AST assay

The assay of AST was carried out using the AST/GOT 4+1 SL reagent (ASSL-0430, ELITech Group, France) according

to the guidelines of the manufacturer. Briefly, the reagent and sample were mixed, incubated for 1 minute, and measured at 340 nm at 37 °C. The AST was calculated as follows:

$$\frac{\Delta A}{min.} \times 3333$$

Unit of AST: IU/L

ALT assay

The assay of ALT was carried out using the ALT/GPT 4+1 SL reagent (ALSL-0430, ELITech Group, France) according to the guidelines of the manufacturer. Briefly, the reagent and sample were mixed, incubated for 1 minute, and measured at 340 nm at 37 °C. The ALT was calculated as follows:

$$\frac{\Delta A}{min.} \times 3333$$

Unit of ALT: IU/L

Hematoxylin and eosin (H&E) staining

According to a standard protocol, paraffin-embedded specimens were prepared, and 7.0 μm sections were stained with H&E (Sigma-Aldrich, U.S.A.). Pancreatic, hepatic, and renal histopathology were assessed with an Olympus BX40 microscope by an investigator who was blinded to the experimental treatment groups (Huynh et al. 2024a, b; Nguyen-Huu et al. 2024). The areas of islets of Langerhans were calculated using the Java-based image processing program ImageJ (NIH Image, <https://imagej.net/ij/>). The H&E staining was conducted in the Cell-Tissue Department, Biomedical Research Center, Pham Ngoc Thach University of Medicine, Ho Chi Minh City.

Statistical analysis

Data were presented as mean $\pm$ S.D. or S.E.M. (standard deviation or standard error of mean). The statistical analysis was carried out by one-way analysis of variance (ANOVA), followed by a Tukey post-hoc test using GraphPad Prism 9.5 (GraphPad Software Inc., U.S.A.). The differences were considered to be statistically significant at $p < 0.05$.

Results

Total flavonoid content in *Camellia flava* ethanolic flower extract

The total flavonoid content was determined using an aluminum chloride colorimetric assay. Rutin was used as a reference standard, and the total flavonoid contents were expressed as mg RE per gram dry extract using the standard curve equation: $y = 0.0028x + 0.0207$, $R^2 = 0.9997$, where y is absorbance at 415 nm and x is total flavonoid content in golden camellia flower extract. The total flavonoid content in the ethanol flower extract was found to be 333.58 ± 2.69 mg RE/g dry extract (mean $\pm$ S.E.M).

Antioxidant activity of *Camellia flava* flower extract

The antioxidant activity of *Camellia flava* flower extract was evaluated in terms of DPPH free radical scavenging capacity, as shown in Fig. 2. The IC_{50} values of ascorbic acid and *Camellia flava* flower extract were $2.114 \mu\text{g.mL}^{-1}$ (Fig. 2A) and $6.793 \mu\text{g.mL}^{-1}$ (Fig. 2B), respectively. The antioxidant property of *Camellia flava* flower extract was 3.2 times better than that of ascorbic acid.

Effect of routes of administration for alloxan on mice

Blood glucose level

According to Fig. 3A, apart from the control group, the other two groups of mice had their blood glucose levels elevated above 200 mg/dL after one day of alloxan administration. Specifically, mice injected with alloxan intravenously at a dose of 55 mg/kg reached their peak blood glucose level of approximately 400 mg/dL and remained

slightly unchanged until the last day of the experiment. Mice receiving alloxan at 150 mg/kg, i.p. showed a considerable increase in blood glucose levels throughout the 14 days. All the differences were statistically significant compared to the control group ($p < 0.0001$).

Body weight

As shown in Fig. 3B, the body weights of mice in the control and 55 mg/kg, i.v. alloxan-injected groups tended to increase throughout the experiment. It is also revealed that intraperitoneal injection of alloxan at a dose of 150 mg/kg did not produce much effect on the body weight of the mice, as this figure remained rather steady until the end of the experiment. The differences between the three groups were still recorded, though not statistically significant.

Survival/mortality rate

Fig. 3C demonstrates the survival/mortality rate of two groups of mice at the end of the experiment. The survival rates in both 55 mg/kg, i.v., and 150 mg/kg, i.p. groups were similar to each other.

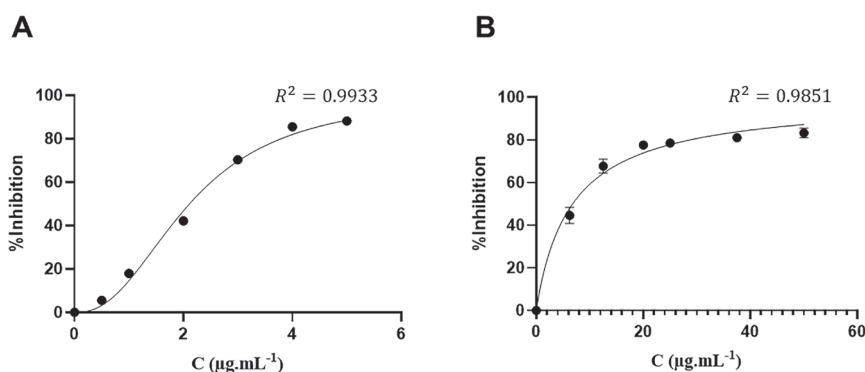


Figure 2. DPPH free radical scavenging activity of the positive control and test samples. **A.** Ascorbic acid; **B.** *Camellia flava* flower extract with 70% ethanol. Data were expressed as mean $\pm$ S.D ($n = 3$).

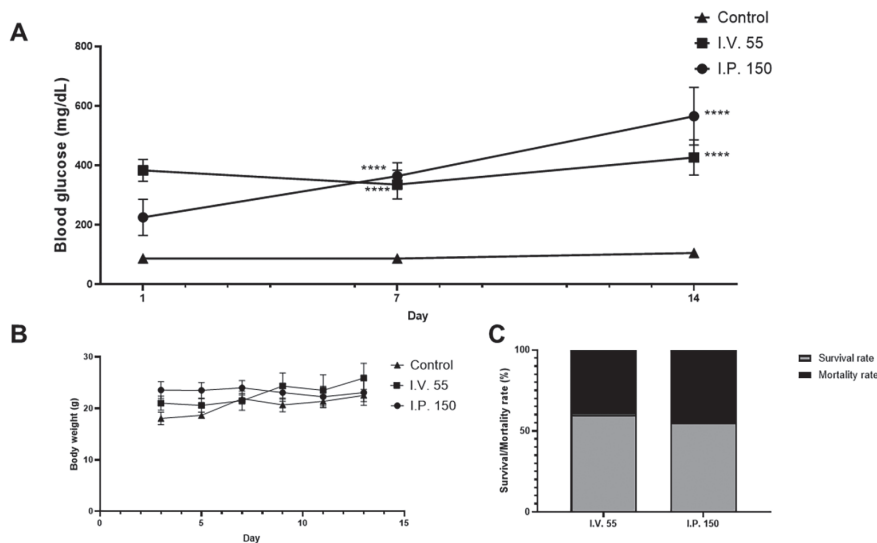


Figure 3. Effect of routes of administration for alloxan with different doses on mice. **A.** Changes in blood glucose level; **B.** Changes in body weight; **C.** Survival/mortality rate. Data were expressed as mean $\pm$ S.E.M, $n = 10$ –15 mice per group (male and female were used approximately equally). Comparisons between the groups were made by one-way analysis of variance (ANOVA), followed by a Tukey post-hoc test. **** $p < 0.0001$ vs. control group. I.V. = intravenous injection. I.P. = intraperitoneal injection.

Effect of *Camellia flava* flower extract on blood glucose levels in alloxan-induced hyperglycemic mice

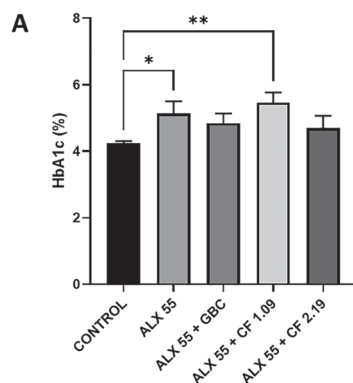
Changes in the blood glucose levels in five groups of mice after 14 days of alloxan-induction were described in Table 1. After 1 day, the blood glucose concentration of the disease group was significantly higher than that of the control group ($p < 0.05$) and remained increasing throughout 2 weeks. The blood glucose levels in three treatment groups using glibenclamide and different doses of flower extract declined remarkably when compared to the untreated group ($p < 0.001$ and $p < 0.0001$, respectively) after 14 days. In addition, two groups treated with flower extract showed a considerable decrease in blood glucose levels in comparison with the glibenclamide-treated group. It is also revealed that the group treated with the higher dose of flower extract (2.19 g/kg) had a lower glycemic index than the one treated with the lower dose (1.09 g/kg).

Table 1. Effect of *Camellia flava* flower extract on blood glucose levels. Data were expressed as mean $\pm$ S.E.M, $n = 10-15$ mice per group (male and female were used approximately equally). Comparisons between the groups were made by one-way analysis of variance (ANOVA), followed by a Tukey post-hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ vs. control group. ### $p < 0.001$, #### $p < 0.0001$ vs. ALX 55 group. ALX = alloxan, GBC = glibenclamide, and CF = *Camellia flava* flower extract.

Group	Blood glucose (mg/dL)		
	Day 1	Day 7	Day 14
Control	78.57 $\pm$ 5.36	100.43 $\pm$ 7.48	106.29 $\pm$ 7.09
ALX 55	408.9 $\pm$ 43.09****	300.38 $\pm$ 54.85**	423.5 $\pm$ 52.71****
ALX 55 + GBC	368.82 $\pm$ 32.59****	257.5 $\pm$ 46.98**	246.6 $\pm$ 27.44****
ALX 55 + CF 1.09	366.07 $\pm$ 24.59****	247.89 $\pm$ 32.56*	139.63 $\pm$ 34.93****
ALX 55 + CF 2.19	400.43 $\pm$ 29.39****	195.00 $\pm$ 31.83	113.4 $\pm$ 12.08****

Effect of *Camellia flava* flower extract on HbA1c, insulin levels, and insulin resistance in alloxan-induced hyperglycemic mice

After the experiment, blood samples were collected to measure HbA1c and insulin levels. According to Fig. 4A,



the HbA1c levels of the disease group and the group treated with flower extract at a dose of 1.09 g/kg increased significantly when compared to the control group ($p < 0.05$ and $p < 0.01$, respectively). The group treated with flower extract at a dose of 2.19 g/kg has yet to demonstrate a protective ability on the HbA1c level; however, the figure showed a decreasing tendency in comparison to the alloxan group, which was similar to the glibenclamide group. Furthermore, no statistically significant difference could be seen in the HbA1c levels of the three treatment groups when compared to the untreated group.

As shown in Fig. 4B, the insulin levels did not differ significantly between all five groups of mice.

According to Table 2, the HOMA-IR index increased significantly in the untreated group in comparison with the control group ($p < 0.05$). On the contrary, three treatment groups using glibenclamide and flower extract at both doses showed a considerable decline in HOMA-IR compared to the untreated group. Specifically, mice receiving the higher dose of flower extract (2.19 g/kg) had the HOMA-IR approximating that of the control group.

Table 2. HOMA-IR index in experimental groups. Data were expressed as mean $\pm$ S.E.M, $n = 10-15$ mice per group (male and female were used approximately equally). Comparisons between the groups were made by one-way analysis of variance (ANOVA), followed by a Tukey post-hoc test. * $p < 0.05$ vs. control group. # $p < 0.05$ vs. ALX 55 group. ALX = alloxan, GBC = glibenclamide, and CF = *Camellia flava* flower extract.

Group	HOMA-IR
Control	0.0159 $\pm$ 0.0027
ALX 55	0.0671 $\pm$ 0.0223*
ALX 55 + GBC	0.0303 $\pm$ 0.0054#
ALX 55 + CF 1.09	0.0201 $\pm$ 0.0097#
ALX 55 + CF 2.19	0.0195 $\pm$ 0.0045#

Effect of *Camellia flava* flower extract on body weights in alloxan-induced hyperglycemic mice

According to Fig. 5, the body weight of the untreated hyperglycemic mice tended to rise throughout the experi-

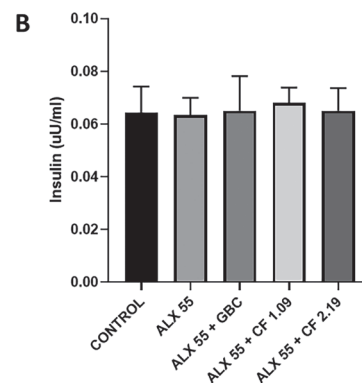


Figure 4. Effect of *Camellia flava* flower extract on HbA1c and insulin levels. **A.** Changes in HbA1c level; **B.** Changes in insulin levels. Data were expressed as mean $\pm$ S.E.M, $n = 10-15$ mice per group (male and female were used approximately equally). Comparisons between the groups were made by one-way analysis of variance (ANOVA), followed by a Tukey post-hoc test. * $p < 0.05$, ** $p < 0.01$ vs. control group. ALX = alloxan, GBC = glibenclamide, and CF = *Camellia flava* flower extract.

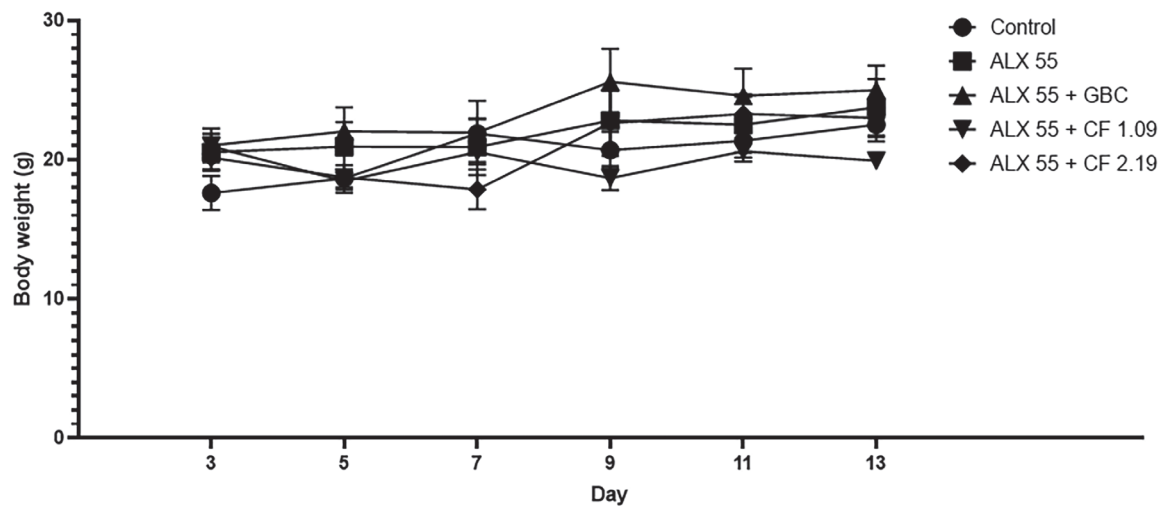


Figure 5. Effect of *Camellia flava* flower extract on body weights. Data were expressed as mean $\pm$ S.E.M, $n = 10$ – 15 mice per group (male and female were used approximately equally). Comparisons between the groups were made by one-way analysis of variance (ANOVA), followed by a Tukey post-hoc test. ALX = alloxan, GBC = glibenclamide, and CF = *Camellia flava* flower extract.

ment. The glibenclamide-treated group showed an increase in body weight when compared to the other groups. Mice receiving flower extract treatment at both doses had lower body weights than those of the untreated group. However, the differences were not statistically significant.

Effect of *Camellia flava* flower extract on biochemical indices in alloxan-induced hyperglycemic mice

After the experiment, blood samples were collected to measure the biochemical indices, including creatinine, AST, and ALT. As shown in Table 3, there was no statistically significant difference in blood creatinine and AST levels between the five groups of mice. On the contrary, considerable differences were observed in the ALT levels in all groups. The alloxan-treated group showed an increase in this index when compared to the control group ($p < 0.05$). In addition, the ALT levels in hyperglycemic mice injected with glibenclamide and flower extract (both 1.09 and 2.19 g/kg) appeared to be remarkably lower in comparison with the alloxan-treated group ($p < 0.05$).

Table 3. Effect of *Camellia flava* flower extract on creatinine, AST, and ALT levels in plasma. Data were expressed as mean $\pm$ S.E.M, $n = 10$ – 15 mice per group (male and female were used approximately equally). Comparisons between the groups were made by one-way analysis of variance (ANOVA), followed by a Tukey post-hoc test. * $p < 0.05$ vs. control group. # $p < 0.05$ vs. ALX 55 group. ALX = alloxan, GBC = glibenclamide, and CF = *Camellia flava* flower extract.

Group	Creatinine ($\mu\text{mol/L}$)	AST (IU/L)	ALT (IU/L)
Control	41.133 $\pm$ 1.056	120.217 $\pm$ 20.056	56.117 $\pm$ 6.991
ALX 55	42.167 $\pm$ 1.037	123.300 $\pm$ 22.418	83.567 $\pm$ 16.137*
ALX 55 + GBC	44.525 $\pm$ 4.419	84.100 $\pm$ 2.241	39.525 $\pm$ 7.726#
ALX 55 + CF 1.09	44.233 $\pm$ 0.437	86.367 $\pm$ 2.691	43.167 $\pm$ 3.335#
ALX 55 + CF 2.19	45.800 $\pm$ 3.169	116.725 $\pm$ 11.603	50.225 $\pm$ 5.757#

Effect of *Camellia flava* flower extract on pancreases in alloxan-induced hyperglycemic mice

As shown in Fig. 6A, the pancreas of the control group showed a normal architecture of islets of Langerhans. In comparison, the alloxan-induced diabetic group suffered from a reduction both in number and size of the islets of Langerhans with signs of diffuse cell necrosis. Fig. 6B demonstrates the comparison of the islets of Langerhans in the other four groups with the alloxan group in terms of area. The results were displayed as percentages (%). It can be seen in Fig. 6B that the area of the islets of Langerhans in the control group was 3.34 times as large as that of the alloxan group. In addition, the treated groups with glibenclamide and flower extract at both doses showed ameliorative effects against the alloxan effect, as shown in the increased size of the islets of Langerhans. Glibenclamide and CF 1.09 groups showed a similar increase in the size of the islets of Langerhans (164% and 192%, respectively). *Camellia flava* flower extract at a dose of 2.19 g/kg appeared to be the most effective in protecting the islets of Langerhans, as it nearly recovered the size of the islets from that of the control group.

Effect of *Camellia flava* flower extract on liver and kidney in alloxan-induced hyperglycemic mice

According to Fig. 7, the liver of the control group showed normal hepatocellular morphology in terms of size and structure, with no signs of necrosis, inflammation, fat accumulation, or any other lesions. By contrast, the untreated and treated hyperglycemic groups all suffered from mild hepatic necrosis, as depicted by the aggregate of abnormal cells. Specifically, the extent of damage in the untreated group was slightly greater than that in the treatment groups.

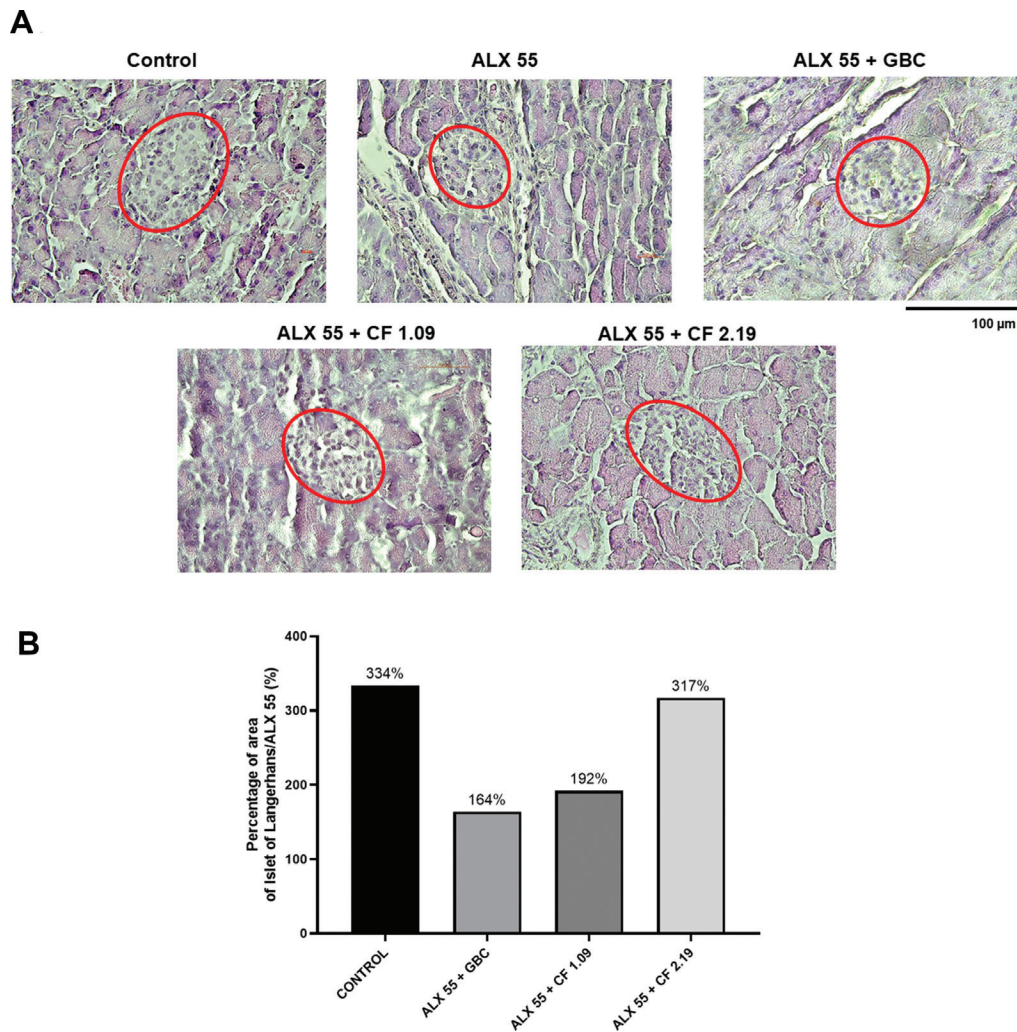


Figure 6. A. Histomicrographs of pancreases of five groups of mice at 40 $\times$ magnification; B. Percentage of area of the islets of Langerhans in other groups compared to the ALX 55 group (n = 3). ALX = alloxan, GBC = glibenclamide, and CF = *Camellia flava* flower extract. Red oval = the islets of Langerhans. Scale bar: 100 μ m.

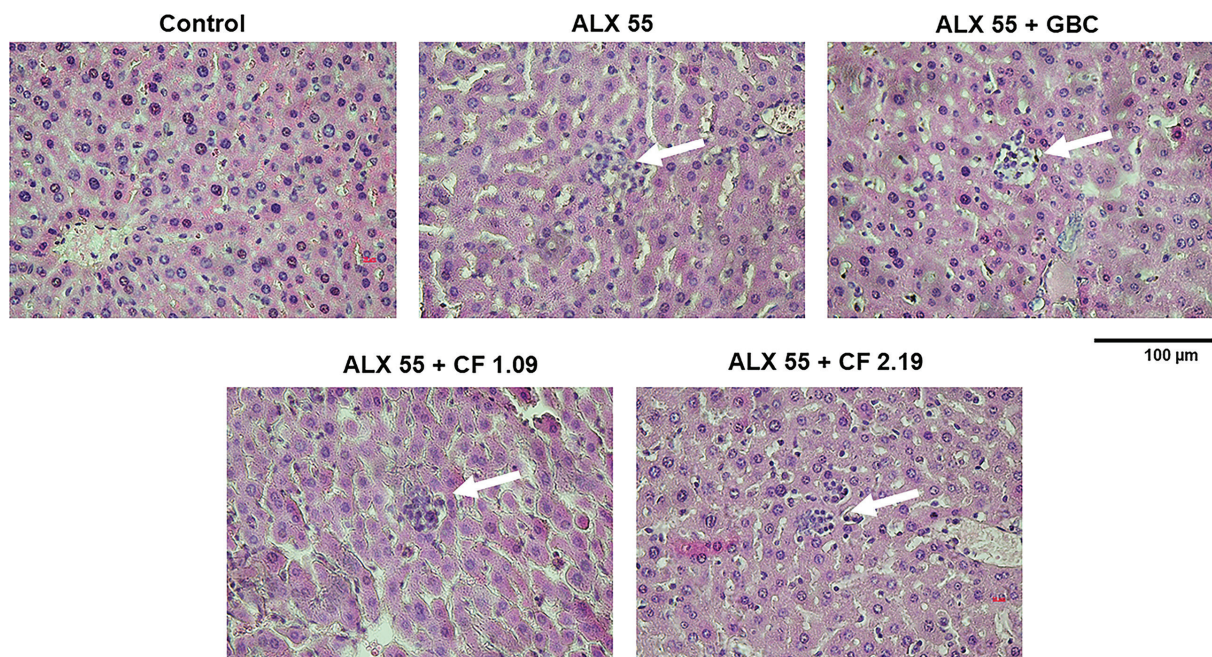


Figure 7. Histomicrographs of the livers of five groups of mice at 40 $\times$ magnification. ALX = alloxan, GBC = glibenclamide, and CF = *Camellia flava* flower extract. White arrow = abnormal cells. Scale bar: 100 μ m.

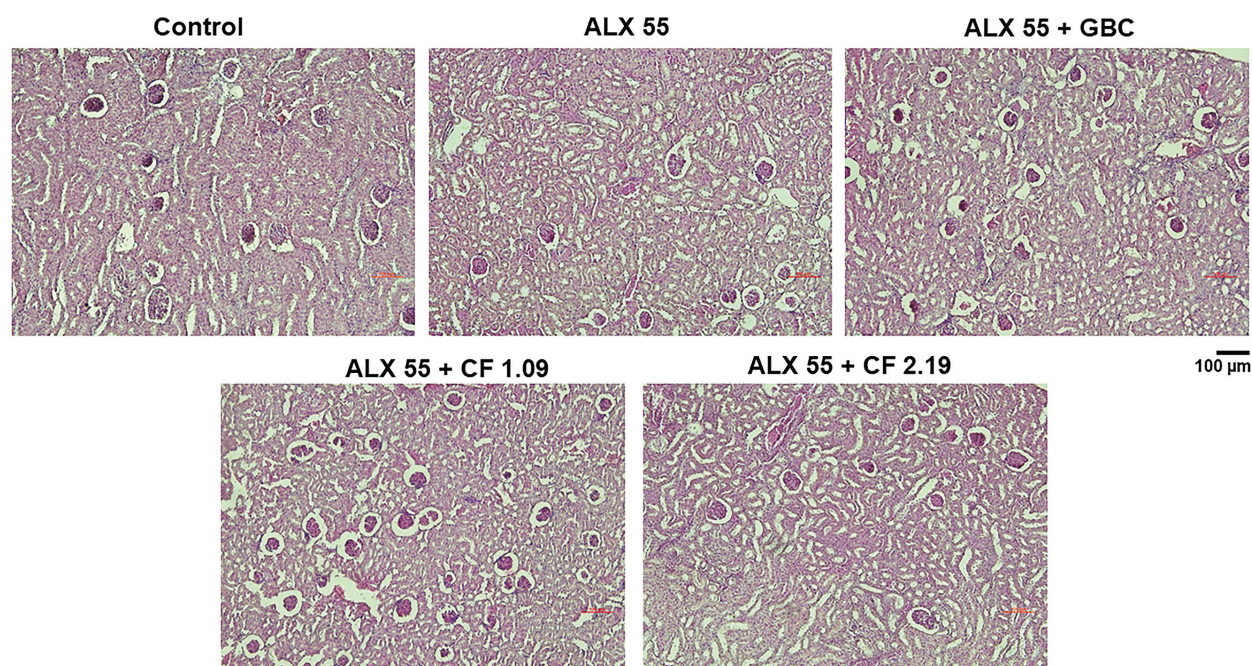


Figure 8. Histomicrographs of the kidneys of five groups of mice at 10× magnification. ALX = alloxan, GBC = glibenclamide, and CF = *Camellia flava* flower extract. Scale bar: 100 μm.

As shown in Fig. 8, the kidneys of four alloxan-injected groups showed normal glomeruli in terms of size and structure in comparison with the control group.

Discussion

Flavonoids are a widely found group of natural compounds with numerous phytochemical constituents that have been successfully extracted, isolated, and structurally identified. Moreover, this group of compounds is primarily responsible for many biological effects such as antitumor, antioxidant, antimicrobial, anti-inflammatory, antihypertensive, anti-depressant, and, notably, hypoglycemic effects (Rana and Gulliya 2019). Several studies have reported the blood glucose-lowering effects and reduction of diabetic complications of flavonoid compounds such as (i) apigenin, baicalein, and catechin (lowering blood glucose through antioxidant processes); (ii) hesperidin and myricetin (improving neuropathic complications); (iii) glycyrrhizin (improving gestational diabetes); (iv) quercetin (reducing complications on the kidneys); (v) kaempferol and puerarin (beneficial for cardiovascular diseases); and (vi) dihydromyricetin (improving cognitive impairment caused by diabetes) (Bai et al. 2019).

Golden camellia is a precious and expensive group of medicinal herbs belonging to the *Camellia* genus, and it is known as the “Queen of Tea” in Vietnam. Unlike green tea (*Camellia sinensis*), which is well studied, there is limited comprehensive research on the chemical composition and pharmacological effects of golden camellia species, particularly *Camellia flava*, both in Vietnam and worldwide (Chaudhary et al. 2023; Wei et al. 2024). *Camellia flava* is a native golden camellia species that thrives mainly in Vietnam (Hakoda and Ninh 2001; Beech et al. 2017; POWO). It has a large biomass and is widely used in the treatment of various

ailments based on folk knowledge. In previous studies, we conducted preliminary research on *Camellia flava*, including plant identification, quantification of certain compound groups, and evaluation of its biological effects, particularly its hypoglycemic effects (Truong et al. 2022). Therefore, we have chosen this particular species for further in-depth research on the mechanisms of its hypoglycemic properties.

The *Camellia flava* species has been used in traditional medicine for its health benefits, and many could be deemed non-toxic according to the GHS classification. Likewise, our previous study recorded no signs of acute toxicity at the dose of 11.75 g/kg body weight of mice (Nguyen et al. 2023). Subsequently, mice were treated with flower extract orally at 4 different doses of 0.22, 0.66, 1.09, and 2.19 g/kg. However, only the mice receiving treatment at doses of 1.09 and 2.19 g/kg showed markedly hypoglycemic signs. Therefore, following this study, we decided to further investigate the effect of *Camellia flava* flower extract at these 2 doses (1.09 and 2.19 g/kg) on the degeneration of the islets of Langerhans and insulin resistance in a hyperglycemia model induced by alloxan 55 mg/kg, i.v., on Swiss albino mice.

The determination of total flavonoid content using an aluminum chloride reagent was a common quantitative method widely applied in flavonoid and herbal medicine research. This reagent reacted with neighboring hydroxyl and/or carbonyl groups in the structure of flavonoids, forming colored complex products with maximum light absorption at a specific wavelength. The quantification results were typically expressed as equivalent amounts of reference substances in amounts of dry material or extract, in terms of rutin and quercetin as two commonly used standard compounds (Shraim et al. 2021).

Rutin is a flavonoid glycoside compound with a flavonol aglycone structure that has been reported to exhibit various pharmacological effects, including anti-inflammatory,

antioxidant, neuroprotective, hepatoprotective, and hypoglycemic activities. Several mechanisms of rutin's blood glucose-lowering effects have been studied and proposed, including reducing carbohydrate absorption in the small intestine, inhibiting gluconeogenesis and enhancing glucose uptake in the tissue, stimulating insulin secretion in pancreatic β -cells, and preventing degeneration of the islets of Langerhans (Ghorbani 2017). Additionally, using modern LC-MS/MS analysis techniques, rutin has been detected in other golden camellia species such as *Camellia euphlebia* and *Camellia nitidissima* (He et al. 2019; Zhang et al. 2020). Therefore, we chose rutin as the reference compound to express the total flavonoid content. The relatively high flavonoid content in the flower extract of *Camellia flava* (333.58 ± 2.69 mg RE/g dry extract) indicated the potential of this golden camellia species to exhibit multiple biological effects, particularly its anti-hyperglycemic effect.

Hyperglycemic conditions in mice could be induced using several methods, including a high-fat diet (Surwit et al. 1988; Winzell and Ahren 2004), streptozotocin (Junod et al. 1967), alloxan (Shaw Dunn and McLetchie 1943), etc. Among these methods, streptozotocin and alloxan are the most preferable diabetogenic agents to be used in laboratories (Szkudelski 2001; Lenzen 2008). Regarding efficacy, both could be utilized for induction of type 1 and type 2 diabetes, and their hyperglycemic effects last for 2–4 weeks or even longer after administration. In addition, alloxan is also more affordable and available than streptozotocin (Ighodaro et al. 2017). As a result, alloxan was chosen for hyperglycemic induction in our study.

Alloxan-induced hyperglycemia models commonly have a mortality rate of 20–90% depending on the dose, route of administration, and speed of injection (Bukhari et al. 2015; Bacevic et al. 2020). In the preliminary study, we induced hyperglycemia in mice by injecting alloxan (60 mg/kg, i.v.) (Nguyen et al. 2023). However, a high mortality rate in mice was recorded at the end of our study (data not shown). Therefore, in this study, we decided to evaluate two more different routes of injection and doses (55 mg/kg, i.v., and 150 mg/kg i.p.), which are commonly used for alloxan (Radenković et al. 2016), to determine the appropriate route that may produce a low mortality rate while maintaining stability in blood glucose levels during the experimental period. When administered intravenously, alloxan is delivered directly into the systemic blood circulation and rapidly achieves high concentrations in the pancreas. This may contribute to the fact that the dosage for intravenous injection of alloxan in mice is normally lower than the intraperitoneal route (Radenković et al. 2016). The 150 mg/kg i.p. group showed a rising tendency in blood glucose level throughout 2 weeks (Fig. 3A), which led to a higher mortality rate compared to the 55 mg/kg i.v. group (Fig. 3C). Therefore, injecting alloxan at 55 mg/kg i.v. produced a high survival rate and percentage of mice that met the criteria, as well as stable blood glucose levels.

As mentioned above, only 2 high doses in 4 doses of *Camellia flava* flower extract (1.09 and 2.19 g/kg) exhibited hypoglycemic effects after 2 weeks of experimentation

(Nguyen et al. 2023). This result aligned with several studies on other medicinal herbs rich in flavonoids, which demonstrated a change in blood glucose level after 2 weeks (Tafesse et al. 2017; Yin et al. 2018). It was also suitable for the alloxan-induced diabetes model, which is often conducted within less than a month (Ma et al. 2015; Tafesse et al. 2017; Yin et al. 2018), as the high rate of mortality is a major drawback of this agent (Ighodaro et al. 2017), especially in the untreated group of animals. This is deemed a limitation in inducing hyperglycemia using chemical compounds. When the long-term effects of hyperglycemia are investigated, genetically engineered mice could be taken into consideration (Brockman et al. 2013; Yao et al. 2014; Wang and Zhu 2016). Notwithstanding, this study was merely pioneering pharmacological research on the hypoglycemic activity of *Camellia flava* flower extract. More thorough investigations into this plant are necessary to evaluate its long-term therapeutic effects.

The ability of *Camellia flava* flower extract to lower blood glucose levels in alloxan-induced hyperglycemic mice may be due to the antioxidant activity of the flavonoid content of *Camellia flava* (Fig. 2B). The antioxidant activity of *Camellia flava* flower extract was evaluated using the DPPH free radical scavenging model with ascorbic acid as a positive control. Ascorbic acid is mostly known for its antioxidant properties and plays a vital role in maintaining several functions of the human body (Gęgotek and Skrzydlewska 2022). Fig. 2 demonstrates the IC_{50} values of ascorbic acid and *Camellia flava* flower extract using 70% ethanol as solvent. Interestingly, the result of the flower extract was merely three times lower than that of ascorbic acid ($2.114 \mu\text{g.mL}^{-1}$ compared to $6.793 \mu\text{g.mL}^{-1}$). We also evaluated the antioxidant effect of this plant extract with 50% and 96% ethanol. The IC_{50} results were $12.26 \mu\text{g.mL}^{-1}$ and $10.19 \mu\text{g.mL}^{-1}$, respectively (Suppl. material 1: fig. S1). It showed that the flower extract using 70% ethanol brought about the highest antioxidant effect among the three solvents. There have been numerous reports on the antioxidant properties of many species in the *Camellia* genus. The study of Piao et al. (2011) showed that the ethanol extract of *Camellia japonica* flower scavenged 49% DPPH free radical at $12.5 \mu\text{g.mL}^{-1}$ (Piao et al. 2011). Therefore, the antioxidant effect of *Camellia flava* flower extract was much higher, which may contribute to the underlying mechanism by protecting pancreatic β -cells from toxic ROS, hence enhancing insulin sensitivity in tissues.

In addition, *Camellia flava* has also been proven to possess α -amylase inhibitory activity *in vitro*, which may support its hypoglycemic effect (Truong et al. 2022). Apart from these suggested mechanisms, the main mechanism of hypoglycemia in *Camellia flava* flower extract still remains unknown. Another well-known plant in the same *Camellia* genus (*Camellia sinensis*) has been reported to have an antihyperglycemic effect with several mechanisms, such as stimulating insulin secretion and serving as a DPP-4 enzyme inhibitor or GLP-1 agonist (Ansari et al. 2022). This may further emphasize the potential of *Camellia flava* as a new antidiabetic drug to be developed in the future.

Alloxan exerts its effect via two mechanisms of action. It has a similar structure to glucose and is therefore selectively transported into the β -cell via the GLUT2 glucose transporter. Subsequently, it generates reactive oxygen species (Radenković et al.), leading to the necrosis of β -cells. In addition, alloxan also inhibits insulin secretion through the inhibition of glucokinase, which results in a high blood glucose level due to hypoinsulinemia (Lenzen 2008). The mice injected with alloxan and remained untreated showed clear signs of hyperglycemia with elevated glucose concentration (Table 1) and diminished the size of the islets of Langerhans (Fig. 6A) when compared to the control group. Regardless of the mechanism of the testing drugs (Banda et al. 2018), glibenclamide is often used as a positive control for alloxan-induced hyperglycemia based on the mechanisms of action of alloxan. Glibenclamide is widely used as it stimulates the secretion of insulin through the closing of ATP-sensitive K^+ channels in the β -cell, leading to the depolarization of the cell, which improves the hypoinsulinemia induced by alloxan (Luzi and Pozza 1997).

The untreated alloxan-injected group showed a considerable increase in HbA1c levels in comparison with the control group, indicating its hyperglycemic effect on mice. No significant difference in HbA1c levels between the glibenclamide and flower extract (1.09 and 2.19 g/kg) groups was observed when compared to the untreated group (Fig. 4A). This may be due to the short period of time during which the experiment was conducted, which was not enough for this index to decrease. The HbA1c level in the mutation mouse model was usually determined at a time point of over 40 days (Membrez et al. 2010; Kim et al. 2014). By contrast, the alloxan-induced hyperglycemic model should measure the HbA1c level at an earlier time point (14 days) due to its high mortality rate. Interestingly, our result showed that HbA1c levels start to increase significantly at 14 days, indicating further investigation may commence at an earlier timepoint for preventing hyperglycemia efficiency.

The insulin levels did not significantly differ between the five groups and still remained high in the untreated group (Fig. 4B). This can be attributed to the fact that even though the destruction of β -cells is irreversible due to the limit of β -cell regeneration from stem cells, they can still be induced to secrete insulin when the blood glucose level increases (Bernard et al. 1999). In addition, the increase in glucose concentration can stimulate insulin secretion in β -cells (Takemoto et al. 2016). This might also contribute to the constancy of insulin levels in the untreated group despite several degenerative changes in pancreases in terms of the size of the islets of Langerhans. Mice in two treatment groups using flower extract had rather larger islets of Langerhans than those of the untreated group (Fig. 6). On the other hand, in our study, the experiment only lasted for more than 2 weeks due to the limitation of the alloxan model (Tafesse et al. 2017; Yin et al. 2018), during which β -cells in the islets of Langerhans still maintained the ability to produce insulin. As a result, the changes in insulin level may not be recorded in the alloxan group

with the limitation of time. Further experiments need to be conducted for a clearer phenomenon.

With the stability of insulin levels being recorded, we continued to evaluate the extent of insulin resistance between five groups of mice using HOMA-IR. HOMA-IR is a commonly used index to assess β -cell function and insulin sensitivity from basal fasting glucose and insulin concentrations (Matthews et al. 1985; Antunes et al. 2016). Unlike clinical trials on humans, pharmacological tests on animals' HOMA-IR were conducted at an early point in time after confirming the status of diabetes/obesity (Strage et al. 2021; Mugni et al. 2023). There was a considerable increase in HOMA-IR in the untreated hyperglycemic group in comparison with the control group (Table 2), indicating a reduction in the insulin response of this group at day 14. This contributed to the rise in blood glucose concentration of the untreated group (Table 1) despite the unchanged insulin level in a short period of time, as there was a decrease in insulin sensitivity leading to tissues not being able to utilize insulin for glucose intake. By contrast, the treatment groups using glibenclamide and flower extract at both doses (1.09 and 2.19 g/kg) showed a remarkable decrease in HOMA-IR when compared to the untreated group and a corresponding improvement in insulin resistance (Table 2). Therefore, *Camellia flava* flower extract exhibited an insulin-sensitivity-enhancing effect. However, it is important to emphasize that the molecular mechanism behind this is still unclear and needs more elucidation.

The body weight of the glibenclamide-treated group appeared to be higher than the other groups (Fig. 5), as glibenclamide belongs to the sulfonylureas, which are associated with weight gain (Cheng and Kashyap 2011). *Camellia flava* flower extract seemed to be more effective than glibenclamide in reducing body weight to some extent, though the differences were not great. The α -amylase inhibitory activity of the flower extract (Truong et al. 2022) might be one of the reasons for this observation, as it plays a role in impeding the process of converting polysaccharides into glucose and thus reducing glucose intake. This result was consistent with the study of Nguyen et al. (2023), which revealed that the flower extract had no effect on raising body weight in hyperglycemic mice (Nguyen et al. 2023). This showed that *Camellia flava* flower extract may intend to decrease body weight, yet the differences were not statistically significant, thus opening the way for new research to investigate its potential for promoting weight loss.

Creatinine is commonly used as a biomarker to help estimate kidney function. Increased creatinine concentration in plasma suggests a higher risk of many renal diseases (Kashani et al. 2020). Mice consuming *Camellia flava* flower extract at both doses (1.09 and 2.19 g/kg) showed no significant variation in plasma creatinine level in comparison with the hyperglycemic untreated group (Table 3). Furthermore, there were no abnormalities in terms of size and structure in the kidneys of all treated groups (Fig. 8). Therefore, *Camellia flava* flower extract was considered safe for kidneys to be used at these two doses for a long time.

Besides creatinine, plasma AST and ALT levels were also evaluated. AST and ALT are two biochemical indices often used in liver function tests to detect hepatic injuries as well as necrosis of hepatocytes. ALT can be found with a high concentration in the cytosol of the hepatocytes, whereas AST is distributed in many organs such as the heart, liver, kidney, or skeletal muscle. As a result, ALT is considered to be more sensitive and specific to the liver than AST. There was a statistically significant difference between the ALT levels between the 5 experimental groups, but this was not observed when evaluating AST (Table 3). The rising tendency of the ALT level of the disease group aligned with its histomicrograph showing liver damage (Fig. 7), which might stem from the necrosis of hepatocytes, which led to the enzyme being released abundantly into the bloodstream. On the contrary, the decline in ALT concentration (Table 3) as well as the protective effect on liver (Fig. 7) of two treatment groups using flower extract was possibly due to the antioxidant activity of *Camellia flava* flower extract, which prevented the hepatocytes from being damaged by ROS and regulated the release of ALT. In addition, mice treated with glibenclamide showed a substantial decrease in ALT level (Table 3) and liver damage shown in its histomicrograph (Fig. 7) compared to an untreated one (Table 3), as glibenclamide not only stimulates β -cells to secrete more insulin but also protects them from the destruction of alloxan (Ao et al. 2008). This further suggests the hepatoprotective effect of this drug when faced with liver diseases.

Importantly, alloxan reduces the size of the islets of Langerhans, which leads to the necrosis and degeneration of pancreatic cells (Muthuraman et al. 2009; Ismail et al. 2020). It is clear that *Camellia flava* flower extract helped enhance this damage by maintaining the size of the islets of Langerhans as well as diminishing the vacuolation and deterioration of cells (Fig. 6). As a result, it showed the effect of recovering insulin secretion and raising insulin sensitivity.

Conclusion

Our research team successfully created a hyperglycemia model in mice by injecting alloxan intravenously at a dose

of 55 mg/kg, with the percentage of mice having blood glucose levels above 200 mg/dL being 60% after 2 weeks. This study also determined that *Camellia flava* flower extract produces many positive outcomes when administered to alloxan-induced hyperglycemic mice, including decreased blood glucose and HbA1c concentrations and improvements in insulin resistance. Additionally, *Camellia flava* flower extract was proved to be safe for kidneys for long-term use and exhibited a hepatoprotective effect in terms of reducing ALT level and minimizing liver damage, as shown in histomicrographs. Last but not least, it exerted ameliorative activity on the degeneration of the islets of Langerhans. Overall, this medicinal plant has great potential for developing into a new medication for the treatment of diabetes.

Competing interests

The authors have declared that no competing interests exist.

CRediT authorship contribution statement

H.L.T.N: First author: Methodology, Supervision, Writing – original draft, Validation; T.T.P.H: Conceptualization, Data curation, Methodology; N.P.N.N: Writing – original draft, Investigation; N.H.L.P: Data curation, Visualization, Formal analysis; D.T.H.N: Methodology, Writing – original draft; V.D.T, M.N.T: Data curation, Investigation, Methodology; T.M.N.H: Writing – original draft, Data curation; T.N.L: Methodology, Animal care; N.P.N: Data curation, Formal analysis, Methodology; T.B.N: H&E staining, Methodology; H.N.M: Corresponding author: Writing – review & editing, Resources, Formal analysis, Supervision.

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Supplementary material 1

Data about AUC values and antioxidant activity of *Camellia flava* flower extract

Authors: Huu Lac Thuy Nguyen, Thi Thu Phuong Ha, Ngoc Phuc Nguyen Nguyen, Nguyen Hoang Linh Phan, Dang Thuy Hien Nguyen, Van Dat Truong, Minh Nhut Truong, Thao-My Nguyen-Hoang, Thi Nghia Luu, Ngoc Phuc Nguyen, Thanh Binh Nguyen, Huynh Nhu Mai

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