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Acute toxicity and antidiabetic effect of the ethyl acetate fraction of *Commelina diffusa* Burm.f. on the high-fat diet and streptozotocin-induced type 2 diabetic mice

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Abstract: Pharmacological studies proved that *Commelina diffusa* Burm.f. performs various biological activities. Nevertheless, the scientific evidence supporting the hypoglycemic activities of this medicinal plant is insufficient. Thus, this study aims to assess the acute toxicity and the antidiabetic activity of the ethyl acetate fraction of *Commelina diffusa* (CD.EAF) in type 2 diabetic mice model induced by a high-fat diet and streptozotocin injection. The oral acute toxicity assessment was conducted following Lorke's method. The *in vivo* study was conducted by feeding Swiss male mice with a high-fat diet for 8 weeks and giving them a single intraperitoneal injection of streptozotocin at 100mg/kg. When the experimental mice model was successfully induced, the CD.EAF at 100mg/kg/day and 300mg/kg/day doses were orally administered to animals for 14 days. After the treatment period, the repeated daily administration of the CD.EAF at both tested doses exposed significant antihyperglycemic activities in comparison with the untreated diabetic group ($p<0.05$). However, it did not affect the serum lipid levels of mice. Besides, there were significant ameliorations in the histopathological images of the liver and pancreas in mice treated with the CD.EAF. Our findings suggested that the CD.EAF might be a potential agent for drug development to prevent and treat type 2 diabetes.

Keywords: *Commelina diffusa*, acute toxicity, antidiabetic, high-fat diet, streptozotocin.

INTRODUCTION

Type 2 diabetes mellitus is responsible for over 90% of all diabetic patients, becoming a serious public health problem (Alam *et al.*, 2021). The pathophysiology of this disease is primarily described by insulin resistance and a progressive decline in insulin-secreting β-cell function of the pancreas. Due to insulin resistance, in the early stages, β-cells compensate and increase insulin secretion in the blood. If insulin resistance persists or worsens, β-cells will not secrete enough insulin and clinical type 2 diabetes will appear (Eizirik *et al.*, 2020). Currently, there are different groups of oral antihyperglycemic medications, including biguanides, insulin secretagogues, thiazolidinediones, dipeptidyl peptidase-4, sodium-glucose co-transporter-2 and α-glucosidase inhibitors (Blahova *et al.*, 2021). However, severe adverse effects, low efficacy, deficiency of target specificity, poor solubility and low permeability are the main drawbacks of these conventional agents (Padhi *et al.*, 2020). Therefore, developing hypoglycemic drugs derived from medicinal plants with fewer side effects is a top priority.

Commelina, which is by far the largest genus of the *Commelinaceae* family, contains about 205-215 species with a worldwide distribution (Pellegrini and Forzza, 2017). It is proven that some species in this genus including *Commelina communis* and *Commelina benghalensis* demonstrate insulin-mimetic and antihyperglycemic activities (Shibano *et al.*, 2008; Dagne *et al.*, 2024). These results suggest that *Commelina diffusa* (*C. diffusa*), which is one of the plant species in the *Commelina* genus, may also have a hypoglycemic property. However, the scientific research to support the antidiabetic effect of this plant is scarce. According to our preliminary investigations, among other solvent fractions, ethyl acetate fraction of *C. diffusa* (CD.EAF) showed the most potent α-glucosidase and α-amylase inhibition activities with IC_{50} values of 21.88±2.47µg/mL and 25.46±1.32µg/mL, respectively when compared to standard agent (IC_{50} values of 135.73±1.79µg/mL and 83.33±2.02µg/mL, accordingly). α-glucosidase and α-amylase involve the hydrolysis process of converting carbohydrates into smaller glucose molecules; as a result, inhibiting these two enzymes will slow down glucose formation and reduce glucose absorption into the blood (Vu *et al.*, 2023). On that basis, the present study aimed to assess the antihyperglycemic activity of the CD.EAF against mice with type 2 diabetes induced by a high-fat diet and streptozotocin.

MATERIALS AND METHODS

Plant preparation

The whole plant of *C. diffusa* was obtained from Nam Dinh province, the Northeast of Vietnam and was

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authenticated by Hanoi University of Pharmacy, Hanoi, Vietnam. The voucher specimen is kept at the University of Medicine and Pharmacy, Vietnam National University. The raw plants were air-dried and minced to afford a rough powder. The dry powder of plant material (5kg) was macerated three times in 15L of methanol at room temperature within 24 hours and then filtered. The solvent was evaporated under a vacuum. The total methanol extract (520g) was consecutively fractionated with *n*-hexane, ethyl acetate, and water. The ethyl acetate extract was concentrated using a rotary evaporator to obtain 23,4g residue.

Experimental animals

Healthy Swiss male mice with an average weight of 25± 2g were obtained from the National Institute of Hygiene and Epidemiology of Vietnam. All mice were kept individually in polycarbonate cages under a light-controlled environment (12h light/dark cycles) at room temperature and were offered pelleted food and water *ad libitum*. The animals were sustained for a quarantine period of seven days to acclimate to the laboratory conditions before the experiment. All experimental procedures were approved by the Ethics Committee of the University of Medicine and Pharmacy, Vietnam National University with a permission number of UMP/062022, and complied with the international regulations for animal research.

Acute toxicity assay

The oral acute toxicity of the CD.EAF was examined according to the method described by Lorke (1983). Briefly, the mice were randomly divided into five groups, with 10 mice in each group and fasted overnight. Each group was orally administered with testing samples at ascending doses of 125, 250, 500, 1000 and 2000mg/kg. The general condition, behavior signs of harmfulness, and mortality in each batch were strictly monitored within 72 hours to determine the median lethal dose (LD_{50}) values. Animals that survived for 72 hours were further observed for seven days for any signs of delayed toxicity.

In vivo experimental procedure

The *in vivo* study was conducted on an experimental mice model with diabetes produced by a high-fat diet and streptozotocin and divided into two stages with some modifications to the previously reported methods (Rivera-Ramírez *et al.*, 2011; Yang *et al.*, 2022). In the first stage, after the acclimation period, mice were randomly assigned into five groups (I, II, III, IV and V) of 10 mice each. Mice from group I were fed a normal-fat diet (NFD) (28.05% protein, 12.14% fat and 59.81% carbohydrate), while mice from remain groups had a high-fat diet (HFD) (18.23% protein, 42.89% fat, and 38.88% carbohydrate) for 8 weeks. Fructose (Daesang Corporation, Korea) solution (55%) was added to the food of mice on the high-fat diet. Hyperglycemic mice were obtained by injecting a single intraperitoneal (ip) of 100mg/kg of streptozotocin (STZ), that was dissolved in 0.1M sodium citrate buffer (pH 4.5). At the same time, mice from group I were administered citrate buffer solution as vehicle control. Blood samples to evaluate glycemia were taken by cutting 1mm from the tip of the tails. Blood glucose value was determined by a commercial glucometer (On-Call EZ II, ACON Biotech, USA) and expressed as mmol/L. Three days after STZ injection, the mice exhibiting glycemia levels equal to or greater than 10mmol/L were included in the study as stable diabetic animals. In the second stage, the experimental mice groups were administered orally once daily for 14 days with the following treatment schedule:

Group I (normal control): NFD + distilled water
Group II (diabetic control): HFD + STZ (100mg/kg, ip) + distilled water
Group III (positive control): HFD + STZ (100mg/kg, ip) + gliclazide (80mg/kg/day)
Group IV (treated group): HFD + STZ (100mg/kg, ip) + CD.EAF (100mg/kg/day)
Group V (treated group): HFD + STZ (100mg/kg, ip) + CD.EAF (300mg/kg/day)

To determine the antihyperglycemic activity of the CD.EAF, after overnight fasting, the fasting blood glucose level was evaluated just before treatment (day 0) and then at the 7 and 14 days post-treatment.

To evaluate the influence of the CD.EAF on the lipid profile of STZ-induced diabetic mice, on completion of the 14 days of treatment, the overnight fasted animals were anesthetized with halothane and sacrificed. The blood samples were collected by cardiac puncture into sterilized heparin tubes. The serum was separated from the blood by centrifuging at 3000rpm for 15 minutes. The high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), total cholesterol (TC), and triglyceride (TG) were examined using the respective commercial diagnostic kit (Erba, Germany). Besides, the livers and pancreas of mice in each group were taken for histopathology evaluations.

STATISTICAL ANALYSIS

Research data were expressed as X ± SD (X: Mean values, SD: Standard deviation). The SPSS 23.0 software used the independent sample t-test and ANOVA (one-way analysis of variance) tests to analyze the differences between tested groups. $p<0.05$ were considered as statistically significant.

RESULTS

Acute toxicity of CD.EAF

As seen from table 1, there was no mortality in all mice groups that were treated with the CD.EAF at doses from

125mg/kg to 2000mg/kg. When using a maximum dose of 2000mg/kg, mice were still healthy, ate, excreted, and moved naturally. After the testing period, CD.EAF did not generate any visible toxicity in mice. Therefore, the LD_{50} index of CD.EAF was thus above 2000mg/kg.

Effects of HFD on body weight of mice

After eight weeks duration, the body weight of mice in the group fed with NFD increased remarkably ($p<0.05$), while a dramatic rise was seen in that of mice groups fed with HFD ($p<0.001$) compared to those before studying (table 2). Furthermore, at all evaluation time points, the body weight of mice groups treated with HFD was significantly higher than that of the group treated with NFD ($p<0.001$).

Effects of HFD on serum glucose level

After 72 hours of STZ injection, the serum glucose concentrations of HFD-treated mice groups exhibited a dramatic development ($p<0.001$) when compared to the time points "Before studying" and "After 8 weeks" (table 3). Meanwhile, there was no significant change in the serum glucose values of the NFD-treated mice group at all time points ($p>0.05$). Furthermore, the concentrations of serum glucose in HFD-treated mice increased significantly as compared to those of NFD-treated mice after 8 weeks of treatment ($p<0.01$) and after 72 hours of injection ($p<0.001$), respectively.

Effect of CD.EAF on blood glucose levels

Based on the results presented in table 4, diabetic mice had significant increases in blood glucose levels when compared with normoglycemic mice ($p<0.001$). After the treatment period, the repeated daily administration of the CD.EAF at both 100mg/kg/day and 300mg/kg/day doses exposed significant antihyperglycemic activities in comparison with the untreated diabetic group (group II) ($p<0.05$). Similarly, the blood glucose concentrations of the mice group treated with the standard drug (gliclazide 80mg/kg/day) were reduced markedly compared with those at the beginning of the testing period ($p<0.05$). Besides, there was no significant difference in blood glucose concentrations between mice groups treated with the CD.EAF (groups IV and V) and gliclazide (group III).

Effect of CD.EAF on blood lipid levels

As shown in table 5, the diabetic animals had remarkably increased concentrations of serum lipid indexes compared to the normal animals ($p<0.05$). No significant difference was observed in the lipid profile between the untreated diabetic mice (group II) and CD.EAF-treated mice (groups IV and V) ($p>0.05$). Therefore, the CD.EAF in both 100mg/kg/day and 300mg/kg/day doses had no effect on the blood lipid levels of STZ-treated mice. In the group treated with gliclazide (group III), there were downward trends in all lipid parameters when compared with group II; however, no significant difference was observed ($p>0.05$).

Histopathological of pancreas and liver

As given in fig. 1, the livers of mice in group II (diabetic control group) showed severe hepatic fatty degeneration with necrosis zones and several inflammatory cells in central venules. While the administration of CD.EAF at 100mg/kg/day and 300mg/kg/day doses (groups IV and V) improved significantly histopathological images of livers as compared with the diabetic control group, no clear effect was seen in the glicazide-treated group (group III). Regarding histopathological examination of the pancreas, the islets of Langerhans of the mice groups were treated with the CD.EAF and gliclazide demonstrated a remarkable recovery compared to those of the diabetic control group (fig. 2).

DISCUSSION

Studies of acute toxicity of medicinal plants aim to find and establish any adverse effects that may occur, their significance, and their dose-dependence responses, including mortality. LD_{50} (the dose that causes death to 50% of the tested animal population) is a major parameter in determining the toxic characteristics of pharmacological agents (Emmanuel *et al.*, 2023). Our present study revealed that the administration of CD.EAF at the highest tested dose (2000mg/kg) using Lorke's method failed to generate any sign of visible toxicity or mortality in mice.

Hence, the LD_{50} value of the CD.EAF was higher than 2000mg/kg, which is supposed to be relatively safe. This finding was consistent with that of previous acute toxicity assessment of the methanolic extract of *C. diffusa*. In particular, although reducing physical activities significantly from 0.5h up to 4h, the mice treated with a 2000mg/kg extract dose were subsequently normal, and the LD_{50} value was thus higher than 2000mg/kg (Sultana *et al.*, 2018).

STZ is an antineoplastic agent that is widely used to produce not only insulin-dependent diabetes mellitus (type 1) but also non-insulin-dependent diabetes mellitus (type 2) in experimental animal models (Ghasemi and Jeddi, 2023). This might be due to the two pathways of diabetes development, which depend on the dosage used of STZ. Generally, STZ penetrates the pancreatic β cells through the binding to the GLUT2 glucose transporter receptor caused by the similarity in its chemical structure with glucose. The privileged accumulation of the chemical in β cells involves the high selective cytotoxicity of STZ to β cells (Kottaisamy *et al.*, 2021). At high doses, STZ damages β cells by alkylating DNA and depleting the nicotinamide adenine dinucleotide level.

Table 1: Acute toxicity of CD.EAF

Doses (mg/kg)	Mortality	Toxicity observed
125	0/10	None
250	0/10	None
500	0/10	None
1000	0/10	None
2000	0/10	None

Table 2: Effects of HFD on body weight of mice

Time points	Body weight (g)	
	Group I: NFD	Groups II, III, IV, V: HFD
Before studying	25.86 ± 2.64	25.34 ± 2.20
After 4 weeks	28.65 ± 2.12*	41.02 ± 6.98$^{***###}$
After 6 weeks	29.78 ± 3.26*	43.86 ± 9.21$^{***###}$
After 8 weeks	34.03 ± 3.31*	49.01 ± 8.84$^{***###}$

$^{***}p<0.05$ and $p<0.001$ compared with the time point "Before studying", $^{###}p<0.001$ compared with group I.

Table 3: Effects of HFD on blood glucose levels

Time points	Blood glucose levels (mmol/L)		*p* (compared with group I)
	Group I: NFD	Groups II, III, IV, V: HFD	
Before studying	5.61 ± 0.69	5.79 ± 0.82	>0.05
After 8 weeks	5.23 ± 0.54	6.41 ± 1.29*	<0.01
72 hours after injection	5.83 ± 0.74	17.68 ± 4.51$^{***###}$	<0.001

$^{***}p<0.05$ and $p<0.001$ compared with the time point "Before studying", $^{###}p<0.001$ compared with the time point "After 8 weeks".

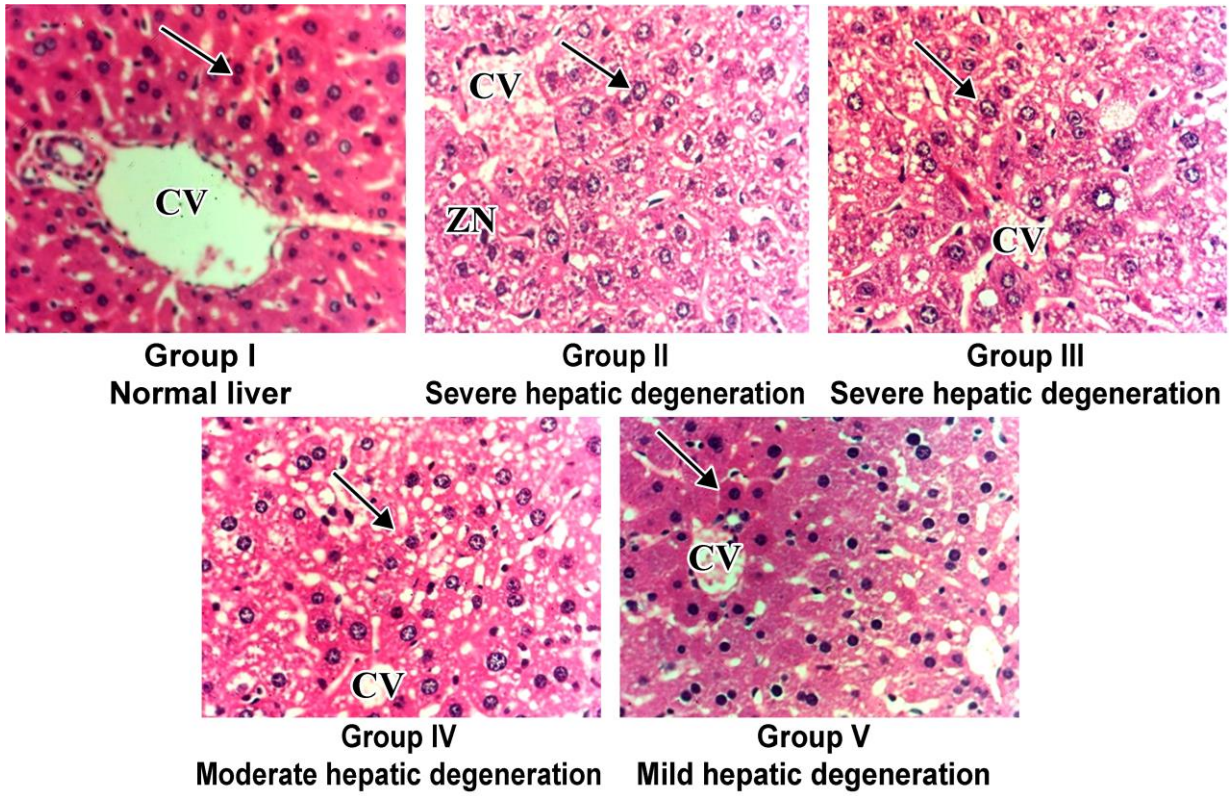

Fig. 1: Effects of the CD.EAF on the liver histology of the treated mice (HE x 400). The black arrows indicate hepatocytes. ZN: Zonal necrosis, CV: Central venule.

Table 4: Effect of CD.EAF on serum glucose concentrations

Groups	Serum glucose concentrations (mmol/L)		
	Day 0	Day 7	Day 14
Group I	5.31 ± 0.71	5.38 ± 0.56	4.94 ± 0.33
Group II	16.01 ± 4.96***	15.74 ± 5.01***	15.81 ± 4.21***
Group III	17.32 ± 2.11***	13.50 ± 4.12***	12.68 ± 3.20***#
Group IV	16.78 ± 3.15***	15.84 ± 3.36***	12.88 ± 3.23***#
Group V	17.16 ± 5.16***	14.48 ± 2.72***	12.62 ± 3.16***#

***p<0.001 compared with group I, #p<0.05 compared with group II.

Table 5: Effect of CD.EAF on lipid profile

Groups	Serum lipid levels (mmol/L)			
	HDL-C	LDL-C	TC	TG
Group I	0.39 ± 0.06	0.79 ± 0.20	1.54 ± 0.23	0.92 ± 0.18
Group II	0.54 ± 0.06***	1.44 ± 0.36***	2.43 ± 0.48***	1.12 ± 0.15*
Group III	0.48 ± 0.04***	1.20 ± 0.15***	2.10 ± 0.21***	0.98 ± 0.18
Group IV	0.42 ± 0.06	1.43 ± 0.32***	2.32 ± 0.26***	1.12 ± 0.16*
Group V	0.46 ± 0.08*	1.61 ± 0.32***	2.55 ± 0.52***	1.09 ± 0.30

***p<0.05 and p<0.001 compared with group I (normal control group).

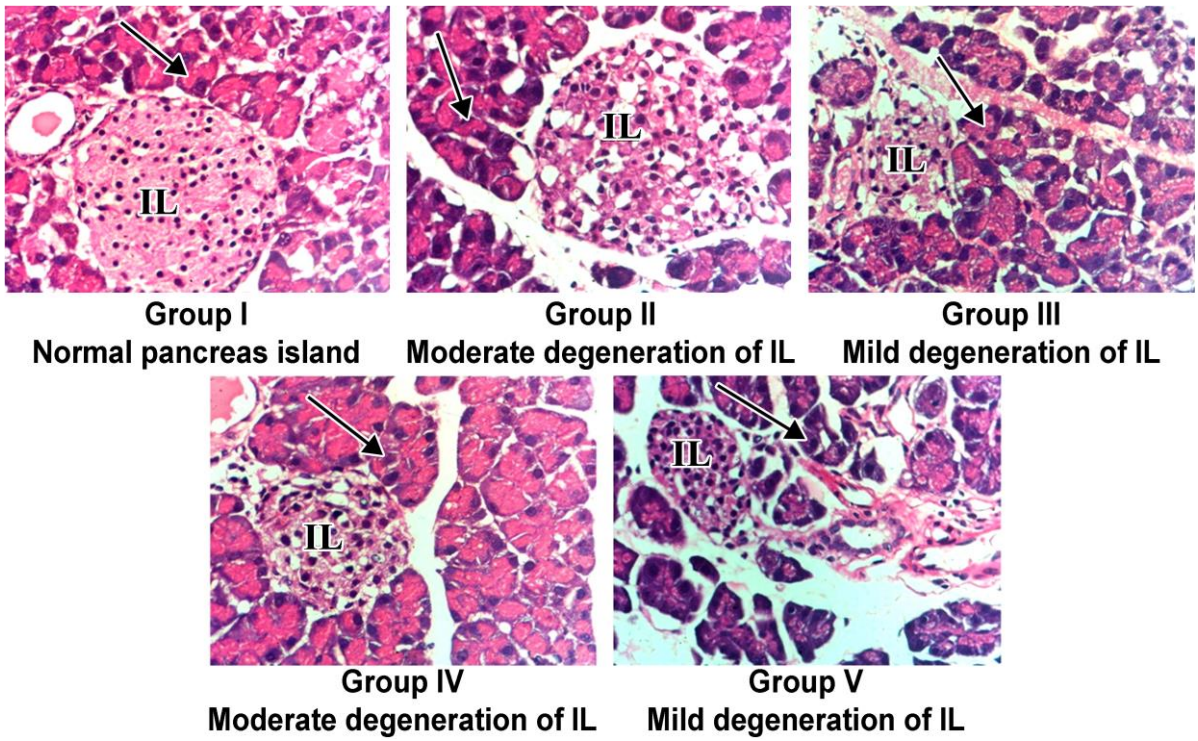

Fig. 2: Effects of the CD.EAF on the pancreas histology of the treated mice (HE x 400). The black arrows indicate acinar cells. IL: Islet of Langerhans.

At low doses, the β cell destruction is related to the inflammatory infiltration of autoimmune T lymphocytes in the pancreatic islets due to the reactions of immune and inflammatory, which are probably created by the ability of STZ to generate glutamic acid decarboxylase autoantigens (Zhu, 2022). The experimental model of type 2 diabetes mice induced by a single intraperitoneal injection of 100mg/kg of STZ was described by Hayashi *et al.* (2006). Besides, obesity is proven to elevate permanent plasma-free fatty acid concentrations and, therefore, the muscle reduces the glucose uptake caused by the predominant utilization of lipids. It is the main factor for insulin resistance, which is responsible for the development of type 2 diabetes (Verma and Hussain, 2017). Hence, this study used STZ combined with a high-fat diet for inducing the mice model with type 2 diabetes. After 8

weeks of treating HFD, the body weight of mice improved significantly by 79.2% in comparison with that at the beginning of treatment. Apart from the body weight, the serum glucose concentrations of HFD-fed mice increased markedly compared with those of NFD-fed mice after the eight-week treatment period. Furthermore, after 72 hours of STZ injection, there was a dramatic rise in the blood glucose values of HFD-treated mice groups, which were about three times higher than those at the beginning of the treatment. As a result, it can be assumed that the combination of HFD and STZ injection led to the successful induction of type 2-like diabetes in tested mice.

Our result indicated that the daily oral administration of gliclazide at doses of 80mg/kg/day and the CD.EAF at two tested doses for 14 days could significantly decrease the serum glucose values of high-fed diet and STZ-induced diabetic mice in comparison with the normal mice. The antidiabetic effects of the CD.EAF might be due to the interaction of its main components with enzymes involved in important cellular activities including α-glucosidase and α-amylase (Vu *et al.*, 2023). Regarding lipid parameters, no significant change was observed in the concentrations of lipid indexes between groups treated with the CD.EAF and the model group. However, previous research showed that the administration of 200mg/kg and 400mg/kg doses of ethanolic extract of *C. diffusa* could enhance the HDL-C level and lower the LDL-C, TC and TG levels in doxorubicin-induced cardiomyopathy rats (Sule *et al.*, 2017). The disparity from our current results may be due to the difference in experimental animal models, tested conditions, part of the plant used, and type of extraction solvent.

Diabetes mellitus and obesity are known to have degenerative effects on the liver and kidney through different pathways (Daryabor *et al.*, 2020). In our study, the result of the histopathological evaluation indicated that a significant improvement was observed in both the liver and pancreas structures in the mice treated with the CD.EAF after 2 weeks of the experiment. Therefore, these activities are supposed to contribute to the hypoglycemic effect of the CD.EAF.

CONCLUSION

In conclusion, the CD.EAF was relatively safe with an LD_{50} value of over 2000mg/kg. The oral administration of the CD.EAF at both 100mg/kg/day and 300mg/kg/day doses had a significant antihyperglycemic effect by reducing the blood glucose levels and improving the hepatocytes and the islets injuries on type 2 diabetic mice induced by a high-fat diet and streptozotocin. Our findings provide a scientific basis for use and a premise for the development of healthcare products based on the hypoglycemic activity of the CD.EAF.

CONFLICT OF INTEREST

No competing interest was declared by the authors.

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