An Evaluation of Antioxidant and Antidiabetic Potential of *Cynometra cauliflora* (Nam-nam, Fabaceae)

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ABSTRACT Oxidative damage of biomolecules is implicated in the pathogenesis of various chronic diseases including diabetes. This has led to intensive investigation aimed at reducing the extent of such oxidative injury. *Cynometra cauliflora* or normally known as "Nam-nam" is a native of Malaysia, grown mainly in northern peninsular Malaysia and possesses many medicinal values in treating several diseases and for health care maintenance. However, antidiabetic and antioxidative potential of *C. cauliflora* have not been fully investigated. Therefore, the present study was aimed to evaluate the antioxidative, hypolipidemic and hypoglycemic potentials of *C. Cauliflora* extract against alloxan induced diabetes rats. Treatment of rats with alloxan resulted in a significant increase ($P<0.05$) level of blood glucose, total cholesterol and low density lipoprotein. On the other hand, oxidative stress was noticed in pancreatic tissue as evidenced by a significant decrease in glutathione level, catalase activity, and also significant increase in malondialdehyde when compared to normal saline control group. Pancreases were examined by hematoxylin and eosin staining. Additionally, serum biochemistry and oxidative stress markers were consistent with the pancreatic histopathological studies. Treatment of diabetic rats with *C. Cauliflora* extract significantly prevented these alterations and attenuated alloxan-induced oxidative stress. The results of the present study indicated that the hypolipidemic and hypoglycemic potentials of *C. Cauliflora* might be ascribable to its antioxidant and free radical scavenging properties. Thus, it concluded that *C. Cauliflora* may be helpful in the prevention of diabetic complications associated with oxidative stress.

KEYWORDS: *Cynometra cauliflora*; Nam-nam; Antidiabetic; Antioxidant activity; Oxidative stress

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INTRODUCTION

Malaysia blessed to possess unique biological diversity and also to be rich in medicinal plants. Notwithstanding that, only about 300 species that are native to this country have been consumed, undergone clinical studies, and documented. According to the World Health Organization (WHO), in developing countries, about 80% of the human population in developing countries continue to use traditional medicine for their primary health care needs. This trend has captured the attention of many researchers to focus on the scientific evaluation of local medicinal plants.

C. cauliflora is an underutilized fruit usually grown in orchards or gardens around houses (Ikram *et al.*, 2009). *C. cauliflora*, also known as nam-nam was believed to be native to Malaysia (Mudiana and Darmayanti, 2010). This plant categorized in the genus cynometra in the kingdom plantae. *C.cauliflora*, a rare species, is a member of the fabaceae family and cultivated along the east coast of Peninsular Malaysia. The recorded height of *C. cauliflora* spans from 3 metres to 15 metres. This fruit found in the form of kidney-shaped pods (Mudiana and Darmayanti, 2010). The *C.cauliflora* tree gets its local name because of the shrubby fruit and brownish green colour of the fruit's skin. Old folks believe that this plant can treat high blood pressure and diabetes.

Diabetes mellitus, commonly known as diabetes, is a metabolic disease characterised by hyperglycaemia (Varvarovska et al., 2004) that reportedly affects 5% of the world population (Ndiaye *et al.*, 2008). According to survey result from National Health Morbidity Survey III (NHMS

III), Zanariah *et al.*, 2008, the prevalence rate in Malaysia has risen much faster than expected. WHO predicted that in the year 2030, Malaysia would have a total of 2.48 million people with diabetes (Zanariah et al., 2008).Diabetes can affect the eyes, kidney and nervous system, and it has also been attributed as a contributor to cardiovascular diseases (Pushparaj, 2004). Diabetes occurs when the pancreas does not produce enough insulin or does not properly use insulin that has been produced (WHO).

This investigation is to determine the in vivo efficiency of *C. cauliflora* against alloxan-mediated changes in the levels of blood glucose, lipid profiles, and serum transaminases. Also to assess the in vivo efficiency of *C. cauliflora* against the alloxan-mediated manifestation of oxidative stress in the pancreas by measuring malondialdehyde (MDA) concentration, the end product of lipid peroxidation and assessing total antioxidant power such as reduced glutathione and antioxidant enzymes.

METHODOLOGY

Plant materials

Cynometra cauliflora samples leaves were collected from Kota Bharu, Kelantan. The plant material was identified by botanist Mr. Johnny Gisil from Institut Biologi Tropika dan Pemuliharaan (IBTP), Universiti Malaysia Sabah (UMS).

Animals

Male adult Sprague-Dawley rats weighing between 50g to 300g were obtained from the animal house of Institut Penyelidikan Bioteknologi, Universiti Malaysia Sabah. All the animals were acclimatized for one week in room temperature under a 12 hours dark- light cycle before undergoing the experiment. The animals were housed in IVC cages and all animals were provided standard animal feeds and drinking water ad libitum. The animal well maintained under animal ethic committee regulations (UMS/IP7.5/M3/4-2012).

Induction of diabetes

Alloxan monohydrate (Alloxan; Sigma Co., St.Louis, USA) was used for inducing diabetes in the rats. Alloxan was dissolved in normal saline (A.Y.Kumar et al., 2011). After an overnight fast with free access to water, Sprague Dawley rats were injected intravenously with 125mg/kg body weight of Alloxan. Rats in the control group were injected with the same volume of normal saline.

Experimental design induction of diabetes

After the induction of diabetes in the rats, all rats were randomized into six groups consisting of five in each group (n=5). All animals fasted for 16 hours before undergoing the treatment. The experiment was conducted for 14 days. Body weight and blood glucose levels of each group of rats were measured weekly. All animals were anaesthetized before being sacrificed at day 15, 24 hours after their respective daily dosages.

Group 1: Normal rats administered with normal saline
Group 2: Normal rats administered with *C.cauliflora* (200mg/kg)
Group 3: Diabetic rats administered with normal saline
Group 4: Diabetic rats administered with *C.cauliflora* (50 mg/kg)
Group 5: Diabetic rats administered with *C.cauliflora* (100 mg/kg)
Group 6: Diabetic rats administered with *C.cauliflora* (200 mg/kg)

Determination of blood glucose levels

The blood glucose levels were determined by the glucose oxidase method using a reflective glucometer (Accu-check, Roche). The blood samples were collected from the tail vein.

Killing Method and blood collection

Before the onset treatment and after treatment, all treated animals were weighted using physical balance (Sartorius, 6202-1S). The animals were anesthetized under light ether and sacrificed after their last doses by cervical dislocation on the 15th day. Their pancreases were dissected out quickly and cleaned of extraneous material and immediately perfused with ice cold saline (0.85% w/v, sodium chloride) and stored at -80℃ for biochemical and histopathological investigations. The blood sample of each animal was collected from cardiac punctures after cervical dislocation before their heart beats stop. The blood samples were collected in separated BD Vacutainer® Blood Collection Tubes. The blood samples were centrifuged at 1375 x g for 20 min and the serum was separated.

Post Mitochondrial Supernatant (PMS) preparation

The pancreases of each animal of every group were washed immediately in ice-cold saline (0.85% w/v, sodium chloride). They were then homogenized in a chilled phosphate buffer (0.1 M, pH 7.4) containing KCI (1.17% w/v, potassium chloride) using a homogenizer (Polytron PT 1200E). The homogenate sample was centrifuged. The supernatant obtained was kept in -80^{0}C before further analysis.

Biochemistry analysis

Determination of reduced glutathione (GSH)

Glutathione reduced was determined by the method of Jollow et al (1974). The yellow colour developed was read immediately at 412 nm on spectrophotometer. The result was expressed as micromoles of GSH/ gram of tissues by using molar extinction coefficient of 13.6 x 10^3 M^{-1} cm^{-1} to calculate the specific activity.

Determination of Lipid Peroxidation (LPO)

Lipid peroxidation was measured according to the method of Buege & Aust (1978), as described by Iqbal et al (1999), by measuring the rate of production of thiobarbituturic acid reactive substance of TBARS, which is expressed as malondialdehyde equivalents (MDA). The absorbance was measured at 535 nm using a spectrophotometer. The results were expressed as amount of MDA formed in each of the samples by using a molar extinction coefficient of 1.56 x 10^5 M^{-1} cm^{-1} to calculate the specific activity.

Determination of Catalase (CAT) Activity

Catalase activity was done following the method prescribed by Claiborne (1985) and modified by Iqbal et al (1998).The reaction was initiated by the addition of PMS. The changes in absorbance were recorded at 240 nm for every 30 second for 3 min using a spectrophotometer and the enzyme activity was calculated as nmol H_2O_2 consumed /min /mg protein by using molar extinction coefficient of 6.4 x 10^3 M^{-1} cm^{-1}.

Serum Biochemistry

Determination of Serum Alanine Aminotransferase (ALT)

Serum Alanine Aminotransferase was determined based on the absorbance of hydrazones of α-ketoglutarate and sodium pyruvate in an alkaline medium. The absorbance of the solution was measured at 510 nm using a spectrophotometer. The calibration curve of sodium pyruvate as standard was plotted at concentrations of 20,40,60,80 and 100.

Determination of Serum Aspartate Aminotransferase (AST)

Serum Aspartate Aminotransferase was determined based on the absorbance of hydrazones of α-ketoglutarate and sodium pyruvate in an alkaline medium with convertion of L-aspartate to α-ketoglutarate to9 form oxaloacetate and L-glutamate. The absorbance of the solution was measured at 510 nm using a spectrophotometer. The calibration curve of sodium pyruvate as standard was plotted at concentrations of 20,40,60,80 and 100.

Lipid Profile

Total Cholesterol (TC)

Serum concentration of total cholesterol was measured using Chod-Pap method as described in instruction provided with the kit (BIOLABO, France).

High Density Lipoprotein Cholesterol (HDL)

Serum concentration High Density Lipoprotein Cholesterol of was measured using direct method as described in instruction provided with the kit (BIOLABO, France).

Low Density Lipoprotein Cholesterol (LDL)

Serum concentration Low Density Lipoprotein Cholesterol of was measured using direct method as described in instruction provided with the kit (BIOLABO, France).

Histopathological Examination

Histopathological examination is important especially for pancreas tissues to evaluate the harmful effect of alloxan monohydrate. The process involves fixing of tissue specimens in 10% neutral buffered formalin solution, preparing the blocks in paraffin, microtome sections 5µm thick and staining with Haematoxylin and Eosin stain (H&E). The sections of pancreas were examined under microscope.

Statistical Analysis

The results were expressed as mean ± SD. Significant differences among experimental groups were analysed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison using SPSS statistical analysis software. A p-value < is regarded as significant.

RESULT AND DISCUSSION

Blood glucose levels were determined at day -4 for pre-treatment until day 14 of treatment. Presented result in Table 1 showed that blood glucose levels for six groups of the experimental rats. Blood glucose levels three day after induction with alloxan monohydrate were significant ($P < 0.05$) increasing in all diabetic rats as compared to normal control rats.

In this study showed the diabetic induced group significant hyperglycemia compared to the control group after alloxan injection on day 3. The increasing blood glucose level in diabetic induced rats is caused by that alloxan can cause diabetes by rapid depletion of beta cells by DNA alkylation and accumulation of cytotoxic free radicals (Indradevi *et al.*, 2012). Pancreatic beta cells are particularly vulnerable to harmful effects by reactive oxygen species because of their low expression of the antioxidant enzymes as compared to other tissues (Swathi *et al.*, 2014; Zhang and Brunk, 1995)

The present study, three different dosages of *C. cauliflora* aqueous extract at the concentration 50 mg/kg, 100 mg/kg and 200 mg/kg of body weight showed significantly decreased ($p<0.05$) in blood glucose level in alloxan induced diabetic rats compared to control group. This may due to bioactive

compounds that reported possessing as an antidiabetic effect. From the analysis, it could be suggested that glucose lowering activity by *C.cauliflora* aqueous extract is due to bioactive compounds that inhibit or suppress the free radical by alloxan in diabetic rats. However, normal rats administered with 200 mg/kg plant extract did not show any significant changes compared to control groups. This can be a positive indicator because the blood glucose in the normal person is unaffected by the plant extract. In contrast, blood glucose level of the untreated diabetic rats remained elevated throughout the experimental period, hence, this was evidence for the believed of traditional claim regarding *C.cauliflora* for its antidiabetic effect.

Table 1. Effect of *C.cauliflora* on blood glucose levels

Group	Day – 4 (mmol/L)	Day 0 (mmol/L)	Day 3 (mmol/L)	Day 7 (mmol/L)	Day 14 (mmol/L)
Normal control	6.12 ±0.43	4.0± 0.27	5.98±0.21	5.30±0.74	6.46±0.24
Diabetic rat control	4.52±0.74*	4.06±0.54*	18.8±4.59*	19.00±7.19*	21.23±4.24*
Diabetic rat treated with *C.cauliflora* (50 mg/kg of b.w.t)	6.62±0.39**	5.70±0.25**	17.90±7.59**	17.06±4.15**	16.83±8.12**
Diabetic rat treated with *C.cauliflora* (100 mg/kg of b.w.t)	5.64±0.43**	4.13±0.23**	19.5±2.13**	16.85±3.7**	16.10±2.39**
Diabetic rat treated with *C.cauliflora* (200 mg/kg of b.w.t)	5.86±0.45**	5.44±0.46**	18.38±5.56**	10.73±3.08**	6.38±1.66**
Normal rat treated with *C.cauliflora* (200 mg/kg of b.w.t)	5.24±0.19**	4.86±0.29**	6.32±0.17**	5.28±0.26**	5.06±0.35**

Data are expressed as mean ± SEM. *Significant value at P<0.05 compare to the saline treated control saline.
** Significant value at P< 0.05 compare to the diabetic control.

Lipid peroxidation (LPO) is established indicator of oxidative stress in cell and tissues. Lipid peroxidation is where the process of free radicals takes electrons from the lipids in cell membranes. Malondialdehyde (MDA) is a secondary product that generates naturally during the process of lipid peroxidation (Ayala *et al.*, 2014; Marnett, 1999).

In the present study, the tissue lipid peroxidation in diabetic rats was significant increased compared to control group. This is might due to high glucose stress in diabetes which enhanced production of reactive oxygen species that react with polyunsaturated fatty acids in the cell membrane that leading to increasing of MDA level (Rajasekaran *et al.*, 2005; Ramesh and Saralakumari, 2012; Swathi *et al.*, 2014; Alkhamees, 2013). In this recent study, the results showed the significantly decreased lipid peroxidation in the group of plant administered with the different dosage that almost attained to normal level compared to the untreated diabetic group. This suggesting that *C. cauliflora* could improve the pathologic condition of diabetes by inhibiting lipid peroxidation in diabetic rats. Additionally, this finding may due to decreased oxidative load and also may due to the presence of various antioxidant compounds such as phenolic compounds that well known could trap the initiating radicals or propagation peroxyl radicals and ultimately break the lipid peroxidation chain reaction (Koh, 2011; Makinwa *et al.*, 2013).

Reduced Glutathione also known as glutathione is an important endogenous antioxidant that plays a central role of co-ordinating the body's antioxidants defence process (Jollow et al., 1974; Indradevi et al., 2014). The catalase is an enzyme ubiquitous in almost all prokaryotic and eukaryotic cells and also protected the cell from oxidative stress by hydrogen peroxide and hydroxyl radical (Swathi el at., 2014). In the present study, untreated diabetic rats showed significantly decreased GSH level in pancreas tissues compared to control. According to the study by Indradevi et al., (2012), stated the decrease in the GSH level represents increased utilization to make the stable condition of free radicals. On the contrary, administration of *C.cauliflora* extract at a different dosage to alloxan-induced rats significant attained near to normal level compared to normal rats and untreated diabetic group.

In this study, the levels of catalase were found decreased in diabetic control, this may result from inactivation by glycation of the enzyme, which has been reported earlier studies to occur in diabetes (Sozmen et al., 2001; Rajasekaran et al., 2005). On the contrary, treatment with plant extract showed recovered the enzyme activities toward normal in a different dosage. Thereby, the finding demonstrated that *C.cauliflora* has the potential to counter back the oxidative damage in alloxan-induced diabetic rats with increase the antioxidant enzyme activities. In addition, CAT in the pancreas of non-diabetic administered with plant extract has been observed and showed no significant changes.

Table 2. Effects of *C.cauliflora* aqueous extract on pancreatic GSH, MDA levels and serum AST & ALT levels.
Note: all values are expressed mean ± SEM

Treatment group	GSH (µmol / g tissue)	MDA formation (nmol MDA / g tissue)	AST enzyme activity (µmol / min / dL)	ALT enzyme activity (µmol / min / dL)
Control	4.22 ± 0.37	39.26 ± 0.129	37.89 ± 0.14	44.85 ± 0.19
Diabetic rat control	3.81 ± 0.12*	59.35 ± 0.316*	103.16 ± 0.11*	123.85 ± 0.56*
Diabetic rat treated with *C.cauliflora* (50 mg/kg of b.w.t)	4.01 ±0.16**	44.48 ± 0.142**	89.47 ± 0.25**	82.30 ± 0.27**
Diabetic rats treated with *C.cauliflora* (100 mg/kg of b.w.t)	4.11 ± 0.31**	44.09 ± 0.163**	69.47 ± 0.38**	63.61 ± 0.25**
Diabetic rat treated with *C.cauliflora* (200 mg/kg of b.w.t)	4.15 ± 0.705**	41.50 ± 0.185**	48.42 ± 0.70**	57.77 ±0.63**
Normal rat treated with *C.cauliflora* (200 mg/kg of b.w.t)	4.20 ±0.309**	39.27 ± 0.228**	38.37 ± 0.38**	45.62 ± 0.96**

* Significant value at $P < 0.05$ compared to normal saline treated control
** Significant value at $P < 0.05$ compared to Diabetic control.

Serum Alanine Aminotransferase (ALT) is an enzyme that usually occupy in cell tissues and serum, notably in the liver. ALT released into the serum as an indicator of the liver function injury. The concentration of ALT in serum may increase due to acute damage of liver (Hsueh *et al*., 2011).

Therefore, this study to show the effect of *C.cauliflora* extract on serum alanine aminotransferase and serum Aspartate aminotransferase presented in Table 2. Serum Aspartate Aminotransferase (AST) is an enzyme found notably in the liver and also found in blood, pancreas and other organs (Huang., 2006). In the present study showed that significantly increased activities of serum ALT and AST in diabetic group control compared to the control group. High level of ALT and AST in blood stream indicates the alloxan-induced effect in tissues damage. Therefore, the increasing activities of ALT and AST in blood serum may be due to the leakage of these enzymes from damage tissues into the blood stream (Navarro et al., 1993; El-Demerdash et al., 2005). On the other hand, the treatment the alloxan-induced diabetic rats with difference dosage showed the reduction in the enzyme ALT and AST activities in blood serum compared to the untreated diabetic group. In addition, normal rats administered 200mg/kg of plant extract did not show any changes in ALT and AST.

Effects of *C.cauliflora* aqueous extract on serum lipid profile was shown in Table 3. Alterations of lipid profile in diabetic conditions are very common. In diabetic condition, due to the blood glucose is not utilized by tissues; the fatty acid from adipose tissues is generated for energy purpose, and excess of fatty acids gathered up in the liver are converted to triglyceride (Das *et al.*, 2012; Indradevi *et al.*, 2012). From the result of this study, it was observed a significant increase in the concentration of total cholesterol and LDL-cholesterol, while HDL-cholesterol was low level in the diabetic control group compared to control group. The increased level of total cholesterol and LDL-cholesterol and decrease in HDL-cholesterol denotes in high risk of cardiovascular diseases associated with diabetic (Akinnuga *et al.*, 2013). In addition, present study elevated serum total cholesterol and LDL-cholesterol level in treated diabetic rats with different dosage of plant extract were brought down, notably in diabetes induced treated with 200 mg/kg of body weight. Moreover, HDL-cholesterol in diabetic-induced rats treated with plant extract showed a significant increase compared to diabetic control. Furthermore, normal rats treated with 200 mg/kg plant extract did not show any significant changes compared to control group.

Table 3. Effects of *C.cauliflora* aqueous extract on pancreatic catalase and serum lipid profile
Note: all values are expressed mean ± SEM

Treatment group	CAT (U H_2O_2 consumed/ min/mg protein)	Total cholesterol (mg/dL)	HDL cholesterol (mg/dL)	LDL cholesterol (mg/dL)
Control	30.62 ± 0.25	92.86 ± 0.25	33.67 ± 0.36	52.12 ± 0.28
Diabetic rat control	7.15 ± 0.37*	110.54 ± 0.56*	21.13 ± 0.50*	137.11 ± 0.26*
Diabetic rat treated with *C.cauliflora* (50 mg/kg of b.w.t)	11.29 ± 0.61**	106.37 ± 0.38**	30.71 ± 0.33**	96.06 ± 0.14**
Diabetic rats treated with *C.cauliflora* (100 mg/kg of b.w.t)	11.61 ± 0.96**	95.47 ± 0.24**	30.96 ± 0.43**	90.89± 0.10**
Diabetic rat treated with *C.cauliflora* (200 mg/kg of b.w.t)	15.58±0.145**	91.44 ± 0.31**	33.25 ± 0.32**	79.05 ± 0.12**
Normal rat treated with *C.cauliflora* (200 mg/kg of b.w.t)	35.39±0.286**	92.96 ± 0.11**	33.60 ± 0.13**	54.96 ± 0.023**

* Significant value at $P < 0.05$ compared to normal saline treated control
** Significant value at $P < 0.05$ compared to Diabetic control.

In the present study, the histological observations showed that pancreatic islets from control group displayed normal histological features as shown in Figure 1. Histopathological examination showed the morphological changes, including infiltration of inflammatory cells, fibrosis, necrosis and severity of insulinitis in the diabetic control group compared to control group as shown in Figure 2. Insulinitis present with heavy lymphocytic infiltration in and around the islet. Moreover, the disorganized of an islet of Langerhans and diminution with a paucity of acini cells were revealed in this group and a small amount of haemorrhage lesion was observed as shown in figure 2. In the present work, histological finding in diabetic-induced rats treated with 50 mg/kg body weight of plant extract treated group showed improvement the previous degenerative changes compared to diabetic control group as shown in Figure 3. Moreover, some of the scanty cells were seen in the islet are β-cells. The islets present a scanty number of interlobular necrotic lesions. The necrosis and fibrosis were negligible. The number of insulinitis is gradually reduced in the area of islets cell. The present study in diabetic-induced and treated with 100 mg/kg plant extract showed an interesting finding which was the presence of cellular connections between some duct and the islets cells as shown in Figure 4. This observation explained by Rehab et al. (2012) reported those islets stem cells exist in the pancreas are located in or near the pancreatic duct. In the present work, the administered *C.cauliflora* extract with 200 mg/kg of body weight on diabetic rats showed no significant changes in cells compared to positive control group as shown in Figure 5. However, treatment of plant extract alone at dose of 200 mg/kg of body weight showed normal histological features as shown in Figure 6.

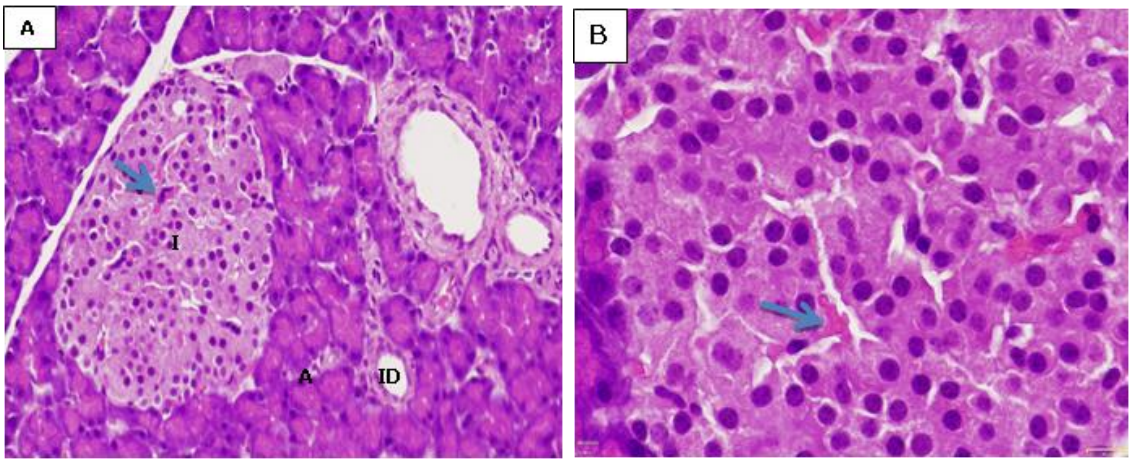

Figure 1. A photomicrograph of rat pancreatic tissue of the control group. (A) Magnification 20X. (B) Magnification 80X. I= islets of Langerhans; A = acini cell; ID= interlobular duct

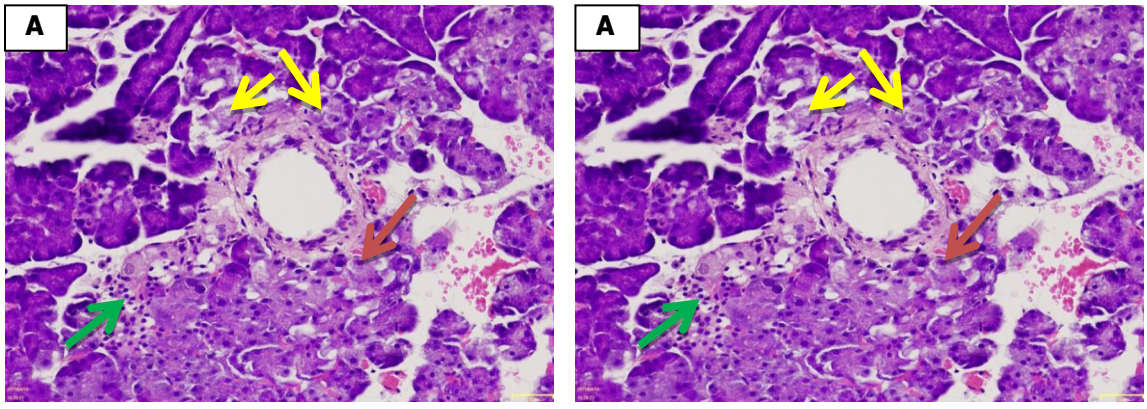

Figure 2. A photomicrograph of rat pancreatic tissue of the diabetic control group. (A) Magnification 20X. (B) Magnification 80X

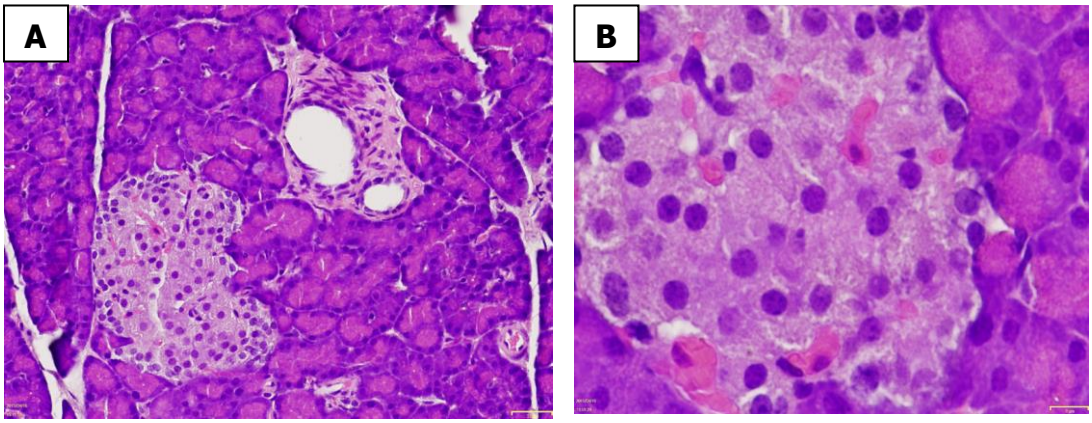

Figure 3. A photomicrograph of rat pancreatic tissue of the alloxan-induce diabetic treated with 50mg/kg body weight plant extract group. (A) Magnification 20X. (B) Magnification 80X

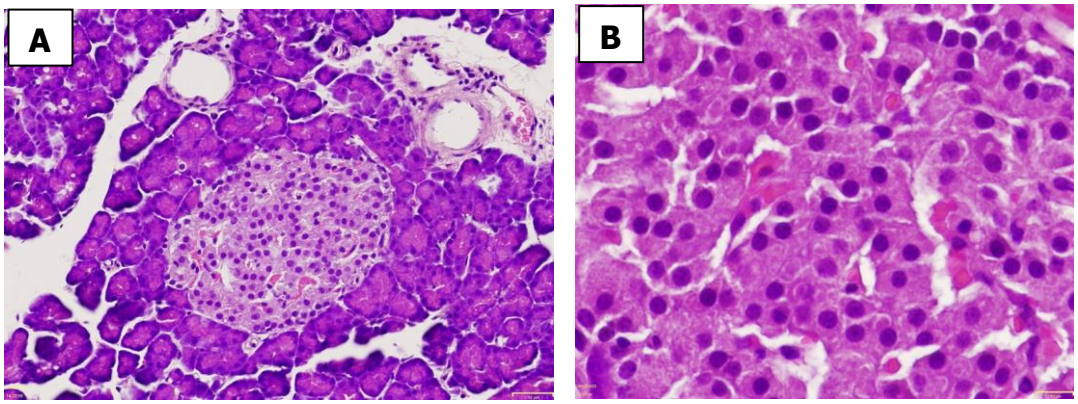

Figure 4. A photomicrograph of rat pancreatic tissue of the alloxan-induce diabetic treated with 100mg/kg body weight plant extract group. (A) Magnification 20X. (B) Magnification 80X.
CT= connective tissue

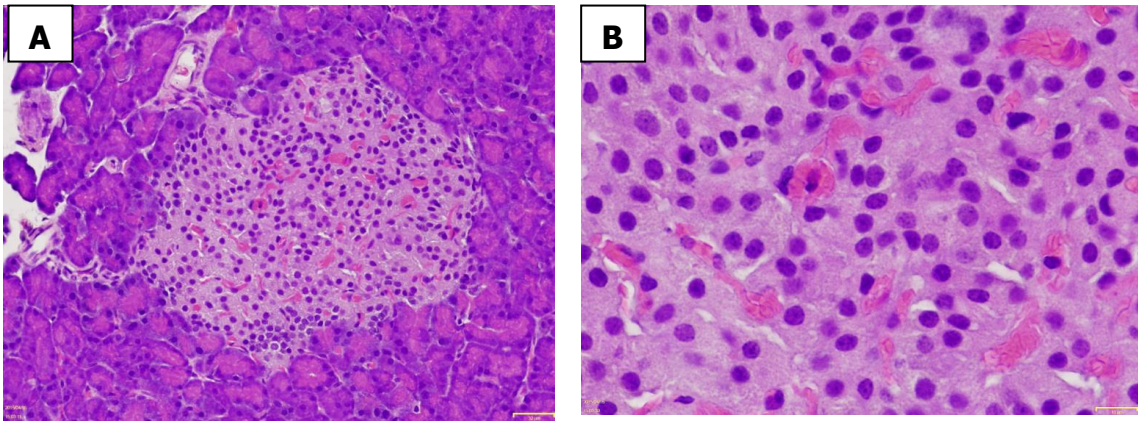

Figure 5. A photomicrograph of rat pancreatic tissue of the alloxan-induce diabetic treated with 200 mg/kg body weight plant extract group. (A) Magnification 20X. (B) Magnification 80X

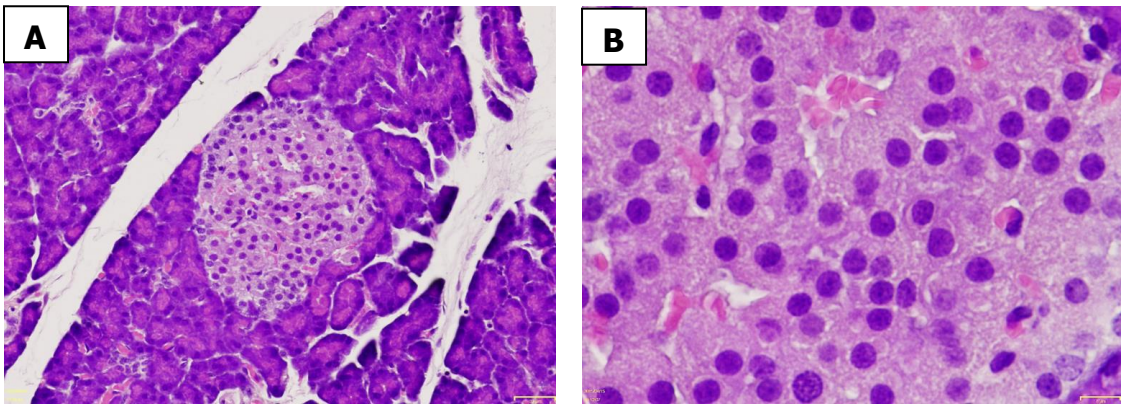

Figure 6. A photomicrograph of rat pancreatic tissue of normal rats treated with 200 mg/kg body weight plant extract group. (A) Magnification 20X. (B) Magnification 80X

CONCLUSION

In conclusion, the results of the present study indicated that the hypolipidemic and hypoglcemic potentials of *C. cauliflora* might be ascribable to its antioxidant and free radical scavenging properties. Thus, it concluded that *C. cauliflora* may be helpful in the prevention of diabetic complications associated with oxidative stress. This study could serve as a constructive reference for future exploitation of *C. cauliflora* to treat diabetes and for human consumption.

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