ISOLATION AND IDENTIFICATION OF CATECHIN FROM *DIOSCOREA BULBIFERA* L. TUBERS AND SCREENING ON HYPOGLYCEMIC ACTIVITY

Kalya Cho[1], Yi Yi Win[2], Hnin Hnin Aye[3], Aye Win Oo[4]

Abstract

The key process in the development of highly effective and safe herbal medicine is isolation and identification of pharmacologically active compounds from medicinal plants and evaluation of their biological activities. In the present work, catechin, a flavanol was isolated from the ethyl acetate soluble portion of the 70 % ethanol extract from *D. bulbifera* tubers. The isolated catechin was characterized by using UV, FT IR, ^{1}H NMR, ^{13}C NMR, HSQC, COSY and HMBC spectroscopic methods. The hypoglycemic effects of pet ether, ethyl acetate and water soluble portions of the hydroalcoholic extract and isolated catechin were evaluated on alloxan induced diabetic mice. The percent reduction of blood glucose level (% R) was significant after 4 h administration of tested sample. % R of pet ether, ethyl acetate and water soluble portions, isolated catechin and antidiabetic drug, metformin were found to be 28.83 %, 41.29 %, 31.52 %, 41.62 % and 41.87 %, respectively. % R of ethyl acetate portion and isolated catechin were nearly the same as % R of metformin. The histological examination of pancreatic islet on tested mice was performed by staining with Haematoxylin and Eosin and it was found that ethyl acetate and water soluble portions as well as isolated catechin enhanced the regeneration of the alloxan damaged islet cells.

Keywords : *D. bulbifera*, catechin, alloxan, hypoglycemic effects.

Introduction

Phytopharmaceuticals play an essential role in medicine. Medicinal plants offer a great opportunity to discovered new natural therapeutic compounds. Some of these compounds may have beneficial effects on glucose homeostasis in diabetic patients without causing any undesirable effects (Mrabti *et al*., 2018). Type 2 diabetes is one of the most common metabolic diseases and characterized by hyperglycemia due to defects in insulin secretion, action or both (Zhang *et al*., 2014). The search for improved and safe natural anti diabetic agents is underway and the World Health Organization has also recommended the development of herbal medicine in this concern (Ghosh *et al*., 2012).

D. bulbifera (family Dioscoreaceae) possesses profound therapeutic potential and found throughout the tropical and sub-tropical regions of Myanmar. *D. bulbifera* tubers have been shown to contain substantial amounts of phenolic compounds and hence these tubers could be a good source of dietary antioxidant (Theerasin and Baker, 2009). Furthermore, *D. bulbifera* tubers have been used traditionally to treat diabetes due to lower glycemic index in diabetes mellitus (Williams, 2013). The current study aimed to isolate and identify one of the phenolic compounds, catechin from tubers of *D. bulbifera* and to evaluate its hypoglucemic activity on alloxan induced diabetic mice.

[1] Dr, Lecturer, Department of Chemistry, Mawlamyine University
[2] Dr, Associate Professor, Department of Chemistry, Dawei University
[3] Dr, Professor (Retd.), Department of Chemistry, Pyay University
[4] Dr, Deputy Director, Laboratory Animals Services Division, Department of Medical Research (Lower Myanmar)

Materials and Methods

Plant Material

D. bulbifera tubers were collected from Mawlamyine University Campus and scientific name of this plant was identified at Botany Department, Mawlamyine University. The collected tubers were naturally dried in the shade at room temperature and ground into coarse powder.

Preparation of crude extracts

Dried powder sample (500 g) was maceraed with 70 % ethanol (1000 mL) for one week and filtered. The filtrated was evaporated by mean of a rotatory evaporator. The hydroalcoholic extract was further separated into pet ether, ethyl acetate and water soluble fractions by successive partition method (Tiwari *et al.*, 2011).

Isolation and Identification of Phenolic Compound

The ethyl acetate soluble fraction was subjected through column chromatography over silica gel using n-hexane: ethyl acetate as gradient elution to yield five sub-fractions. From the fourth fraction (n-hexane: EtOAc, 1:2 v/v), catechin was isolated as solid material. The isolated compound was characterized by UV, IR and NMR spectroscopy using UV-Vis spectrophotometer (UV-1800, SHIMADZU, Japan), IR-Tracer (SHIMADZU, Japan) and Bruker Ultra shield Advance II 400 MHz NMR spectrometer. Correlation spectroscopy of nuclear magnetic resonance such as heteronuclear single-quantum coherence spectroscopy (HSQC) and Hetronuclear multiple-bond coherence spectroscopy (HMBC) were also used.

Experimental Animals

Albino mice of both sexes weighing 25-30 g bred at Laboratory Animal Services Division, Department of Medical Research (DMR), Yangon were used. Rearing up of animals and their upkeep in the experimental period conformed to the norms of Institutional Animals Ethics Committee (IAEC) and ethical guideline for investigations of experimental pain in conscious animals (Zimmerman, 1983).

Acute Toxicity Study

The acute toxicity study of 70 % ethanol extract was done according to the Organization for Economic Co-operation and Development (OECD, 2008) guideline No.423. Female albino mice, weighing 25-30 g, were administered with hydroalcoholic extract at 2000 mg/kg dose and 5000 mg/kg dose. Upon administration, the tested animals were closely observed individually during the first 4 h and then daily for 14 days.

Evaluation of Hypoglycemic Activity

Hypoglycemic effect of hydroalcoholic extract portions and isolated catechin was determined by alloxan induced diabetic mice and blood glucose levels were determined by DM sensor blood glucometer.

Induction of diabetes

Male albino mice were made diabetic by a single dose of intraperitoneal (IP) injection of 150 mg/kg body weight of alloxan monohydrate in sterile normal saline. The blood glucose level was checked at 72 h after alloxan injection. Blood sample was drawn from tail vein and glucose level was determined to confirm the development of diabetes (190 mg/dL and above).

Preparation of sample solutions

Firstly 1 % DMSO solution was prepared by mixing of 5 μL of DMSO and 5 mL of distilled water. Each extract and isolated compounds was separately dissolved in 1 % DMSO solution as follows.

(i) 150 mg/kg body weight dose of each extract,

(ii) 150 mg/kg body weight dose of standard drug metformin, and

(iii)15 mg/kg body weight dose of isolated catechin.

Experimental procedure

The diabetic mice were divided into six groups, each containing three mice and treated as follows;

Group I : Normal control mice,

Group II : Diabetic mice administrated with 5 mL/kg of saline water,

Group III : Diabetic mice treated with 150 mg/kg body weight dose of PE soluble fraction,

Group IV : Diabetic mice treated with 150 mg/kg body weight dose of EtOAc soluble fraction,

Group V : Diabetic mice treated with 150 mg/kg body weight dose of H_2O soluble fraction,

Group VI : Diabetic mice treated with 15 mg/kg body weight dose of isolated catechin , and

Group VII : Diabetic mice treated with 150 mg/kg body weight dose of Metformin.

Blood samples were collected from the tail vein and blood glucose levels were recorded after 1, 2, 3 and 4 h of administration using glucometer kit. The percent reduction in blood glucose level was calculated by the following equation.

$$\text{Percent reduction} = \frac{\text{FBG} - \text{blood glucose level}}{\text{FBG}} \times 100$$

where, FBG = fasting blood glucose level

Statistical Analysis

The results are expressed as mean S.E.M. The significant of various treatment was calculated (Student's t-test) using SPSS and were considered statistically significant when $p < 0.05$.

Histological Examination on Pancreas

On the seven days after experiment, each mouse from group I to VII was treated with chloroform and the pencreas were removed. Fixation was done by using 10 % formalin. After fixation, the pancreas was embedded in molten paraffin wax and the paraffin block was cut to make slides. Slides were thoroughly washed with water and stained by Haematoxylin and Eosin stain. Then pancreatic islets were examined under microscope (with the magnification 40 x). Histological examination was done by pathologist from DMR, Lower Myanmar.

Results and Discussion

Extraction of Phenolic Compounds

The solvent system, 30 : 70 % (water : ethanol) solvent ratio, was used to extract phenolic compounds from tubers of *D. bulbifera* (Demir *et al*., 2015).

Isolation and Identification of Catechin

Catechin was isolated from ethyl acetate soluble fraction of hydroalcoholic extract by using column chromatographic method and purified by recrystalization with hot water. The melting point of isolated catechin is 240-241 °C. Photographs of isolated catechin and its TLC chromatogram are shown in Figure 1.

Solvent system = $CHCl_3$:MeOH, 4:1
Spraying reagent = 10 % NaOH
R_f = 0.47

Figure 1 Photographs of the isolated catechin and its TLC chromatogram

The UV spectrum of the isolated catechin (Figure 2) shows absorption band at 280 nm ($\pi \rightarrow \pi^*$ transition) in MeOH. In the presence of NaOH, the main band 280 nm shifted to 298 nm (bathochromic shift) due to the presence of free phenolic -OH group. The FT IR spectrum of isolated catechin (Figure 3) shows a broad band around 3400-3200 cm^{-1} due to -O-H stretching in phenol. The absorption bands at 2934 cm^{-1} and 2853cm^{-1} were due to the stretching of saturated C-H group and C= C stretching of aromatic ring appeared at 1627 cm^{-1} 1251 cm^{-1}.

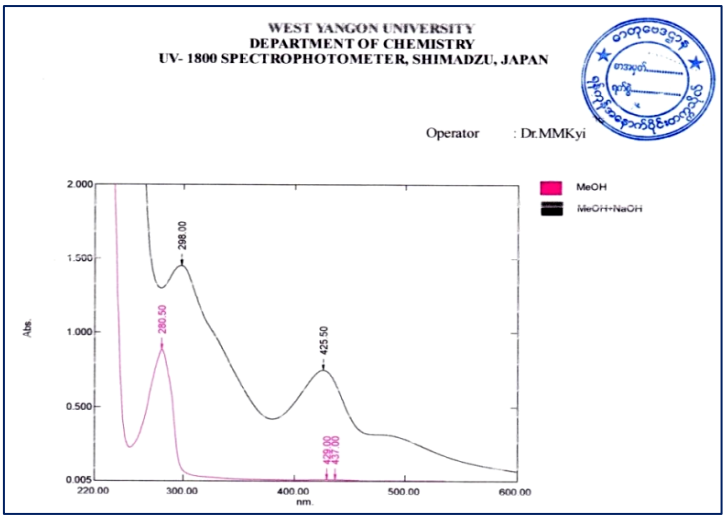


Figure 2 UV spectra of the isolated catechin from tubers of *D. bulbifera* in MeOH and MeOH + NaOH

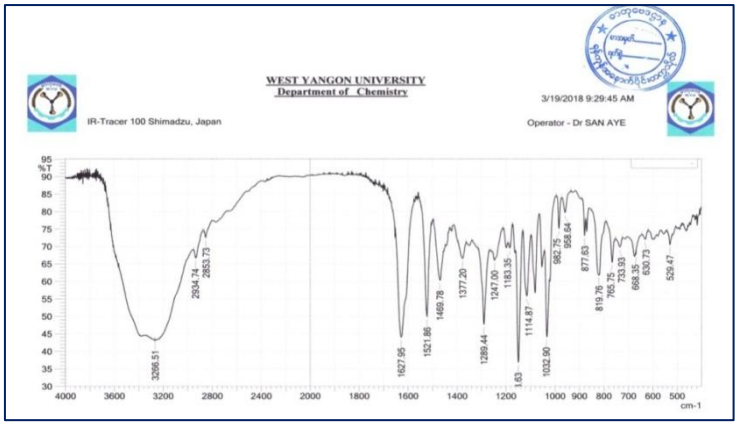


Figure 3 FT IR spectrum of the isolated catechin

The ^{13}C NMR spectrum (Figure 4) shows 15 peaks corresponding to 15 carbons, including one methylene, seven methines and seven quantanary carbons. ^{1}H NMR spectrum (Figure 5) shows nine signals. On the basis of HMQC spectrum (Figure 6), each proton correlated with corresponding carbon. The position of the H-2 chemical shift (δ_H 4.56 ppm) suggested that the flavan structure possessed the correct trans 2-3 sterochemistry. The ^{1}H-^{1}H COSY (Figure 7) shows coupling between H-3 and H-2 and also between H-3 and H-4. These positions were further confirmed by long range coupling observed in HMBC spectrum (Figure 8). The NMR data (Table 1) show signal typical of catechin and it is comparable to the reported ^{1}H and ^{13}C NMR data of catechin (Dong *et al*., 2003).

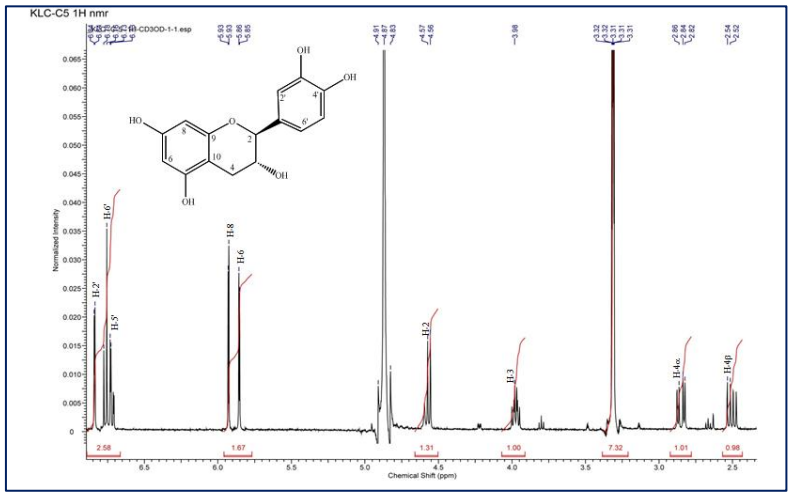


Figure 4 ^{1}H NMR spectrum of the isolated catechin (400 MHz, CD_3OD)

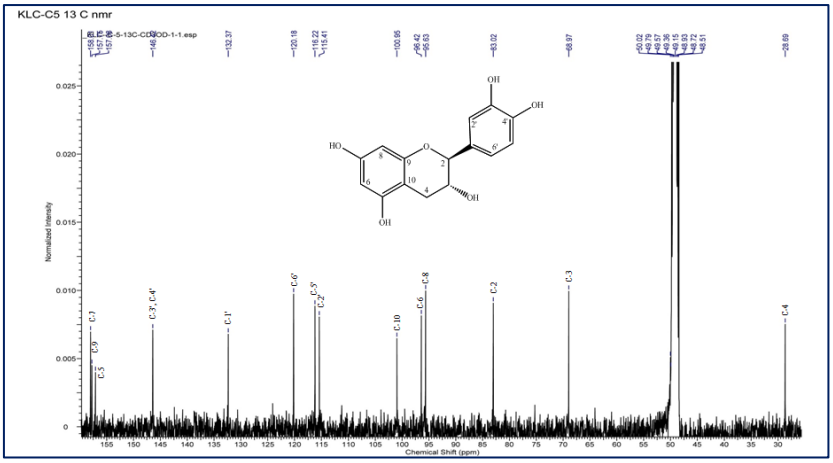


Figure 5 ^{13}C NMR spectrum of the isolated catechin (100 MHz, CD_3OD)

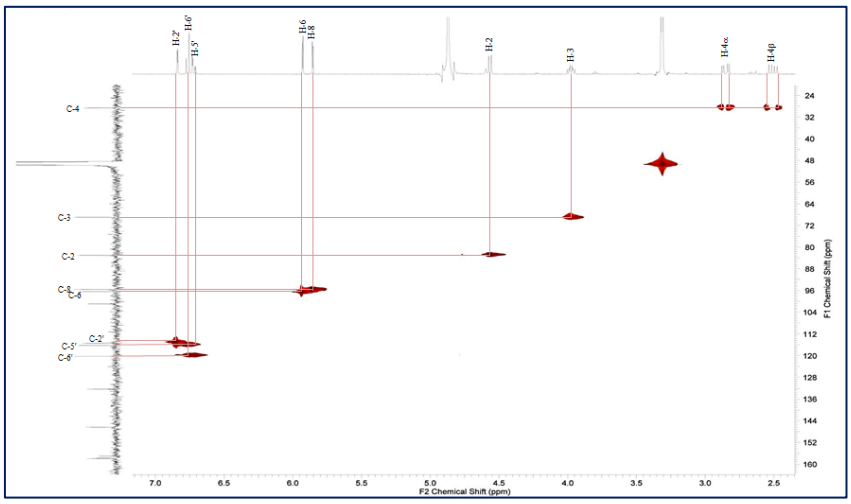


Figure 6 HSQC spectrum of the isolated catechin (400 MHz, CD_3OD)

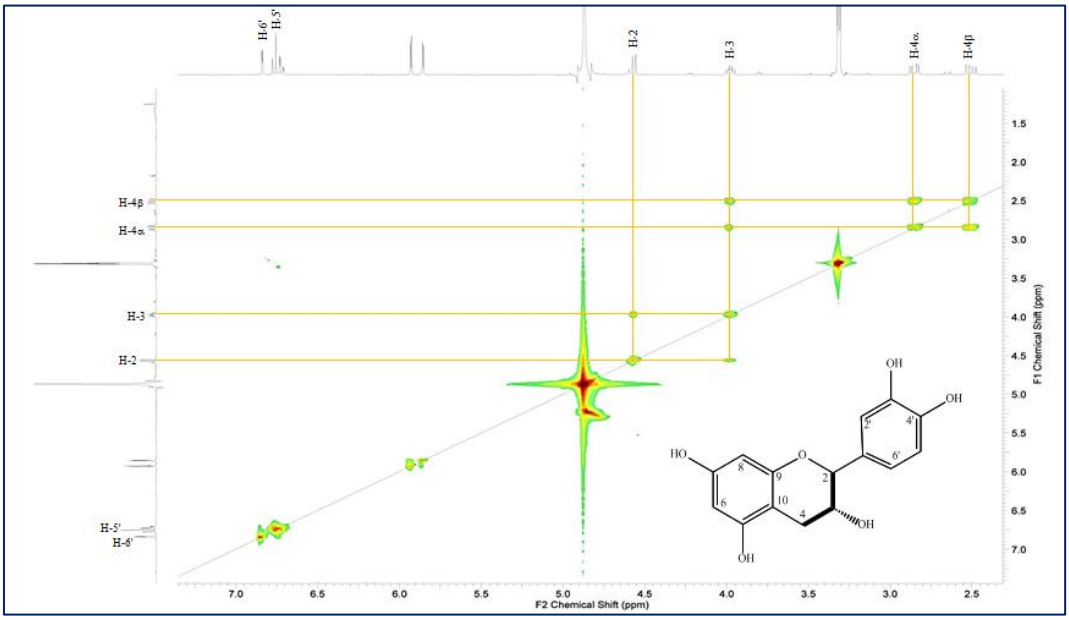


Figure 7 ^{1}H-^{1}H COSY spectrum of the isolated catechin (400 MHz, CD_3OD)

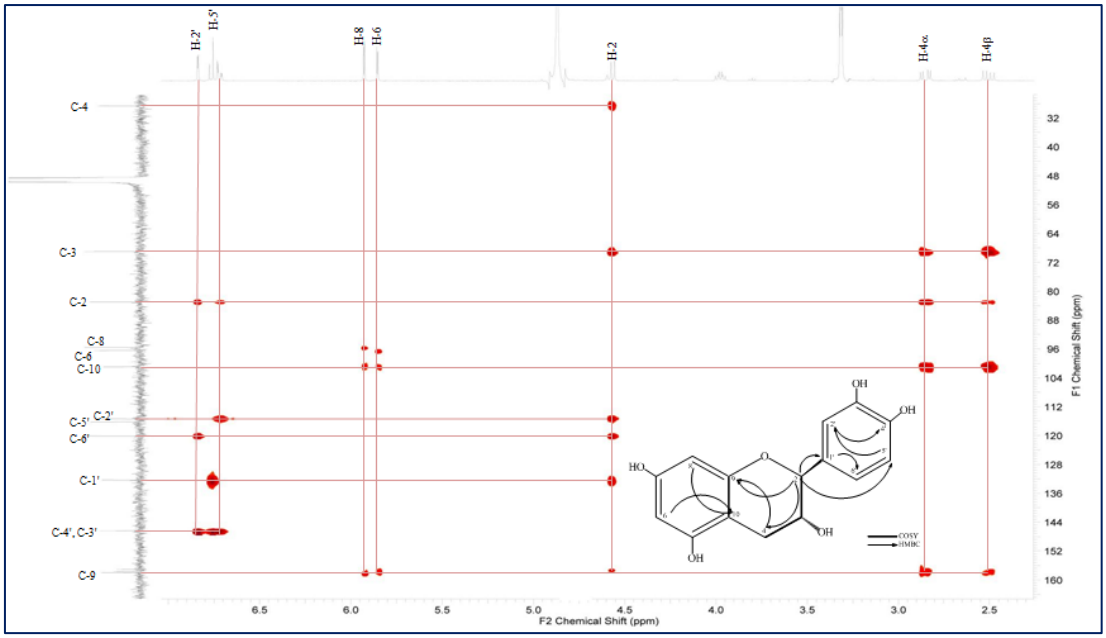


Figure 8 HMBC spectrum of the isolated catechin (400 MHz, CD_3OD)

Table 1 ^{1}H NMR, ^{13}C NMR, ^{1}H-^{1}H COSY and HMBC Data of the Isolated Catechin and Reported Data

Position	C-type	Isolated Catechin in CD_3OD		*-Catechin in CD_3OD		^{1}H-^{1}HCOSY	^{1}H-^{13}C HMBC
		$\delta_{H\ (ppm)}$	$\delta_{C\ (ppm)}$	$\delta_{H\ (ppm)}$	$\delta_{C\ (ppm)}$		
2	O–CH	4.56 (d, 1H, d, J = 8 Hz)	83.0	4.57 (d, 1H, J= 7.6 Hz)	82.3	H-2↔ H-3	H-2→C-4, C-3, C-1' C-5', C-9
3	HO–CH	3.99 (1H, m)	68.8	4.01(1H,ddd,J=5.4, 8.2, 7.6 Hz)	68.3	-	-
4	CH_2	2.85 (1H, dd, J = 6, 16 Hz)	28.7	2.88 (1H, dd, J = 5.4, 16.1 H_z)	28.2	H-4↔ H-3	H-4→ C-3,C-2, C-9, C-10
		2.51 (1H, dd, J = 8, 16 Hz)		2.52 (1H, dd, J = 8,16.1 H_z)			
5	HO–C=C	-	157.7	-	156.5	-	
6	C=CH	5.93 (1H, d, J= 2 Hz)	96.4	5.95 (1H, d, J = 1.8 H_z)	96.2	-	-
7	HO–C=C	-	158.0	-	157.0	-	H-6→ C-10
8	C=CH	5.86 (1H, d, J=2 Hz)	95.6	5.88 (1H, d, J = 1.8 H_z)	95.4	-	-
9	C=C–O	-	157.6	-	156.3	-	H-8→ C-10
10	C=C	-	100.9	-	100.7	-	-
1'	C=C	-	132.3	-	131.6	-	-
2'	C=CH	6.84 (1H, d, J=2 Hz)	115.4	6.83 (1H, d, J = 1.8H_z)	115.0	-	-
3'	HO–C=C	-	146.2	-	145.6	-	H-2'→ C-3', C-4'
4'	HO–C=C	-	146.3	-	145.7	-	-
5'	C=CH	6.78 (1H, d, J = 8 Hz)	116.2	6.77 (1H, d, J = 8 H_2)	116.0	-	-
6'	C=CH	6.73 (1H, dd, J = 2, 8 Hz)	120.1	6.72 (1H, dd, J = 1.8, 8 H_2)	119.9	-	H-5'→C-2,C-2' ,C-4'
							H-6'→ C-1'

* Dong *et al.*, 2003

Acute Toxicity Study

The female mice treated with the limit doses of 2000 mg/kg and 5000 mg/kg 70 % ethanol extract did not show any drug induced physical signs of toxicity and no drug related death occurred throughout the study period. There was no mortality after acute treatment up to 5000 mg/kg dose within 14 days, thus proving the extract being safe for use.

Hypoglycemic Activity

The hypoglycemic effect of *D. bulbifera* tubers was also studied by using alloxan induced diabetic mice. The mice developed diabetes after 72 h alloxan injection were administrated with the pet ether, ethyl acetate and water soluble fractions (150 mg/kg b.wt dose) and the isolated catechin (15 mg/kg dose). The blood glucose levels were significantly reduced after 4 h administration of each sample ($p < 0.005$). The percent reduction of blood glucose levels at 1 h, 2 h, 3 h and 4 h after treated with each fraction, the isolated catechin and metformin are shown in Table 3 and Figures 9 and 10. When compared with control group (treated with metformin), ethyl acetate soluble fraction and isolated catechin has similar hypoglycemic effect.

Table 3 Blood Glucose Levels in Alloxan Induced Diabetic Mice after Treating with PE, EtOAc and H_2O Extracts from Tubers of *D. bulbifera*, Isolated Catechin and Antidiabetic Drug Metformin [Data are represented as mean ± SEM (n = 3)]

Group No.	Treatment	Blood Glucose Concentration (mg/dL)				
		0 h	1 h	2 h	3 h	4 h
I	Normal	100.00 ± 3.39	101.00 ± 7.35	100.00 ± 5.00	100.67 ± 8.37	95.67 ± 4.03
II	Alloxan (150 mg/kg)	253.00 ± 21.25	279.00 ± 22.25	275.00 ± 29.25	275.00 ± 24.50	269.00 ± 22.50
III	PE extract (150 mg/kg)	239.33 ± 25.60	222.00 ± 24.30 (7.24 %R)	208.00 ± 27.10 (13.09 % R)	191.00 ± 26.90 (20.19 % R)	170.33 ± 27.80 *(28.83 % R)
IV	EtOAc extract (150 mg/kg)	195.33 ± 14.80	182.33 ± 16.70 (6.66 % R)	161.33 ± 16.10 (17.41 % R)	138.67 ± 18.80 (29.01 % R)	114.67 ± 20.30 *(41.29 % R)
V	H_2O extract (150 mg/kg)	239.00 ± 19.20	223.00 ± 12.60 (6.69 % R)	207.67 ± 15.80 (13.11 % R)	185.33 ± 18.00 (22.45 % R)	163.67 ± 20.80 *(31.52 % R)
VI	*Metformin (150 mg/kg) (Control)	192.67 ± 11.61	159.67 ± 11.18 (17.21 % R)	135.33 ± 16.95 (29.76 % R)	123.33 ± 15.67 (35.08 % R)	112.00 ± 14.14 *(41.87 % R)
VII	Isolated Catechin (15 mg/kg)	205.00 ± 21.60	187.67 ± 17.80 (8.45 % R)	168.00 ± 26.90 (18.05 % R)	145.33 ± 17.80 (29.11 % R)	119.67 ± 24.70 *(41.62 % R)

SEM = Standard Error Mean, R = Reduction, *P < 0.05 * = Antidiabetic drug
0 h = Before treatment 1 h – 4 h = After sample injection

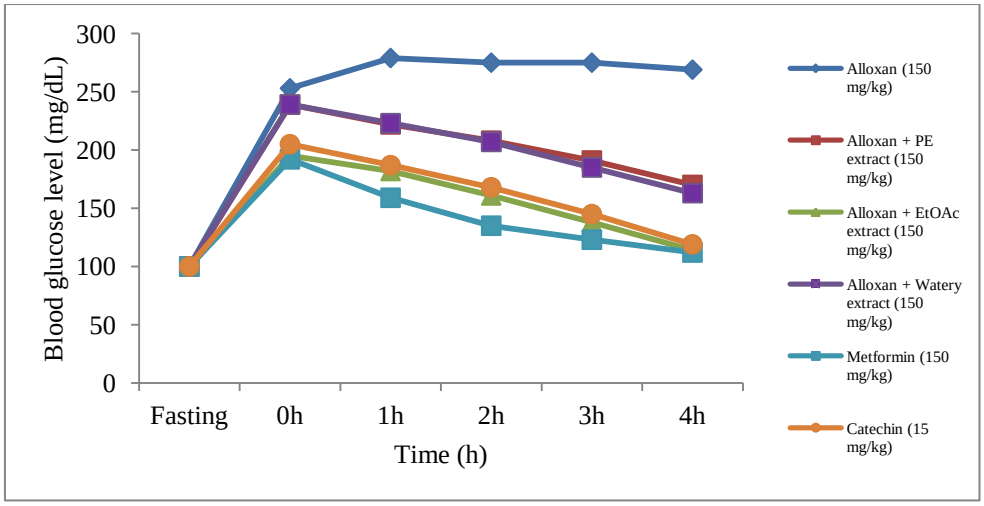


Figure 9 Effect of PE, EtOAc and H_2O extracts of tubers of *D. bulbifera*, isolated chatechin and metformin on blood glucose levels of alloxan induced diabetic mice at different times

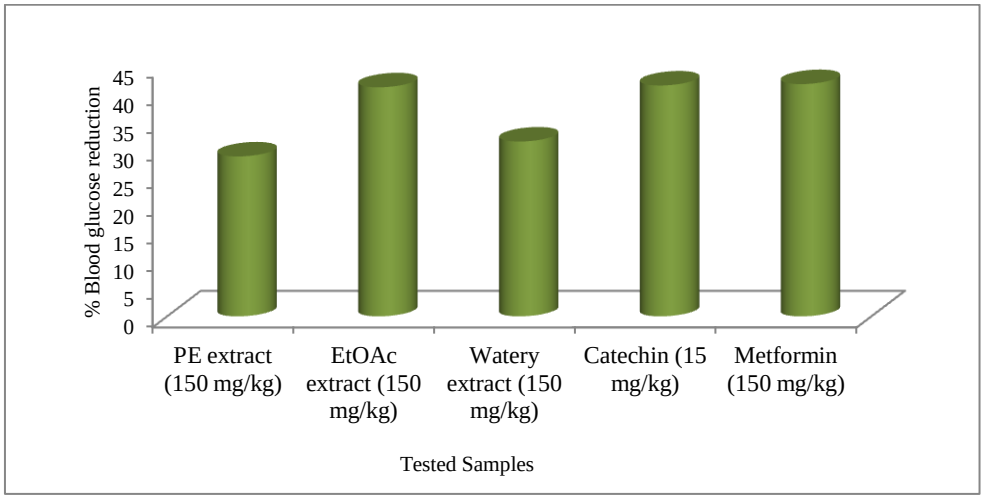
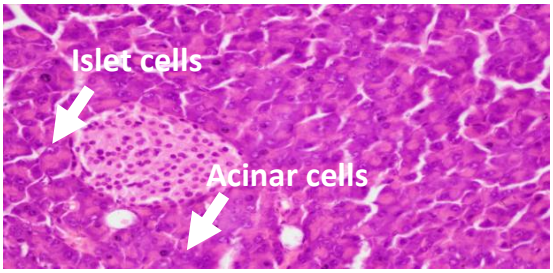


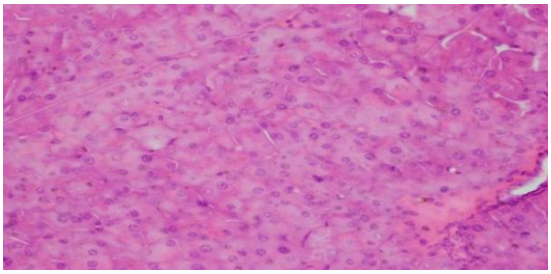
Figure 10 % Reduction of blood glucose level after administration of metformin, crude extracts and isolated catechin from tubers of *D. bulbifera*

Histological Examination of Pancreatic Cells

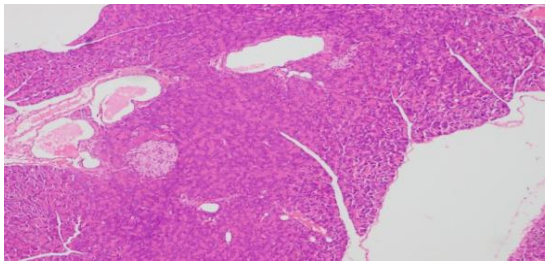
Histological observation of pancreatic cells was performed after 7 days administration with tested sample. The histological images obtained from mice of each group are described in Figure 11. Signs of regeneration of beta cell stimulated potentiation of insulin secretion from β cells of islets and decrease blood glucose level. The EtOAc extract and the isolated catechin induced regeneration of the islet cells. In addition these activities also showed the possible mechanism of action of *D. bulbifera* tubers for its hypoglycemic effect due to the stimulation of insulin secretion from the remnant β cells or regeneration of β cell.



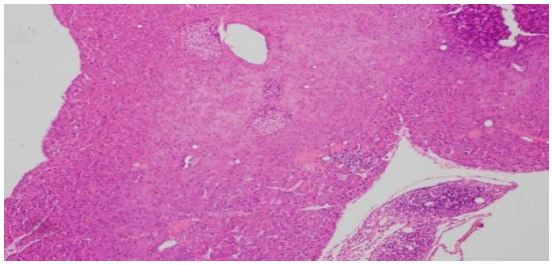
(a) Normal control mouse



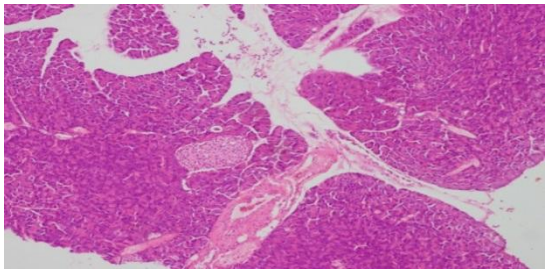
(b) Alloxan induced diabetic mouse



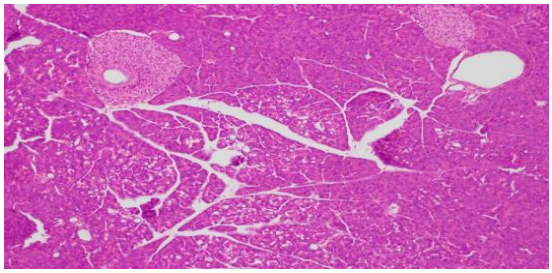
(c) Diabetic mouse treated with metformin



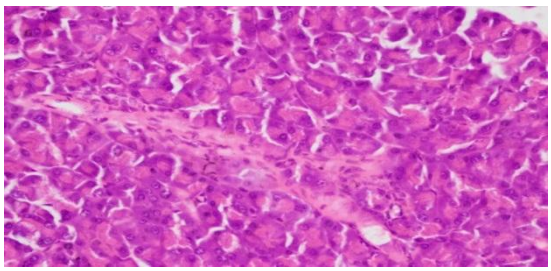
(d) Diabetic mouse treated with PE extract



(e) Diabetic mouse treated with EtOAc extract



(f) Diabetic mouse treated with H_2O extract



(j) Diabetic mouse treated with isolated catechin

Figure 11 Histological examination of pancreas after 7 days experiment [magnification × 40]

Conclusion

The present study deals with isolation and identification of catechin from *D. bulbifera* tubers and screening on hypoglycemic activity.

Extraction of phenolic compounds from *D. bulbifera* tubers was done by using 70 % ethanol and the resulting extract was divided into pet ether, ethyl acetate and water soluble portions. Catechin was isolated from ethyl acetate portion by column chromatography and its structure was identified by modern spectroscopic methods. The hypoglycemic effect of pet ether, ethylacetate and water soluble portions and isolated catechin was evaluated on alloxan induced diabetic mice model. The optimum hypoglycemic effect of extracts (150 mg/kg b.wt dose) and isolated catechin (15 mg/kg b.wt dose) was clear after 4 h administration where $p < 0.05$. The

percent reduction of blood glucose levels were found to be 28.83 % for pet ether fraction, 41.29 % of ethyl acetate fraction, 31.52 % for water fraction and 41.62 % for isolated catechin. So, hypoglycemic effect of ethyl acetate fraction and isolated catechin was similar to that of antidiabetic drug metformin (% reduction of blood glucose level 41.87). Histological examination of pancreatic tissue of mice from each group was done after 7 days experiment and it was found that regeneration process occurred in β islets cells and production of secretion from these cells.

Therefore, the finding from the present work will contribute the scientific validation for traditional use of *D. bulbifera* as remedy in the areas concerned with diabetes mellitus.

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