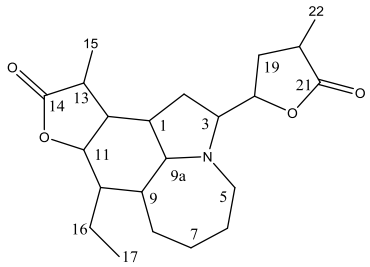
PHYTOCHEMICAL SCREENING AND EVALUATION OF SOME BIOLOGICAL ACTIVITIES OF THE ROOTS OF *STEMONA TUBEROSA* LOUR. (THAMYA)

Yi Yi Win[1], Zin Thu Khaing[2], Yin Yin Htwe[3], Saw Hla Myint[4]

Abstract

The present study investigates some biological effects: *in vitro* antioxidant, *in vitro* α-amylase inhibitory activity, *in vivo* acute toxicity, *in vivo* anti-diabetic activity of the crude extracts and isolated compounds of *Stemona tuberosa* Lour. root. *In vitro* antioxidant activity using DPPH radical scavenging assay, with ascorbic acid as positive control, showed the ethanolic extract possessed excellent antioxidant property (IC_{50}=1.56 μg/mL). No toxic behaviour was observed up to 5000 mg/ kg oral dose of water and ethanol extracts on mice for two weeks treatment following the *in vivo* acute toxicity OECD guideline. The *in vivo* antidiabetic activity, with metformin, oral hypoglycemic agent, as a positive control, against alloxan-induced diabetic mice; and the *in vitro* antidiabetic activity, with acarbose, antihyperglycemic agent, as a positive control, for the inhibitory effect on α-amylase enzyme, was employed to determine the antidiabetic activity. The ethanolic extract of *S. tuberosa* root was found to be exhibited potent antidiabetic activity *in vitro* (IC_{50} values; 80.98 μg/mL for α-amylase activity) as well as *in vivo* (41.29±0.05 % maximum reduction (R)) in blood glucose level. By column and thin layer chromatography, tuberostemonine **J** (**1**) was isolated from active ethanol fraction and identified by modern spectroscopy. Moreover, tuberostemonine **J** (**1**) (41.62 ± 0.01 % R *in vivo*) exhibited similar antidiabetic activity ($p < 0.01$) but good proliferation of beta islet cells of Langerhans and acinar cells in pancreas of diabetic mice when compared to the positive control, metformin (41.87 ± 0.05 % R *in vivo*).



1 (tuberostemonine **J**)

Keywords: *Stemona tuberosa*, blood glucose, antidiabetic, antioxidant, tuberostemonine **J**

Introduction

The herb Radix Stemonae, known as 'Bai-Bu' in Traditional Chinese Medicine, is derived from the root of *Stemona tuberosa* Lour (Stemonaceae family). It is often used as an antitussive drug to treat respiratory disorders, e.g. bronchitis, pertussis and tuberculosis, and also as an anthelmintic agent for domestic animals (Zang Tingmo, 1977). The crude extract of this plant was found to have anti-bacterial, anti-fungal, anti-viral, antitussive, insecticidal and neuroprotective activities (Xu *et al*., 1996). The alkaloids; tuberostemonines from this species were reported to show inhibitory activity on the excitatory transmission at the crayfish

[1] Dr, Associate Professor, Department of Chemistry, Dawei University
[2] Dr, Lecturer, Department of Chemistry, University of Yangon
[3] Dr, Lecturer, Department of Chemistry, Mawlamyine University
[4] Dr, Part-time Professor, Department of Chemistry, University of Yangon

neuromuscular junction. The prominent clinical and pharmacological properties of this plant has prompted many phytochemical studies, and over a dozen stemona alkaloids isolated from this herb of different places of origin can be structurally classified into three categories: (i) stenine-type, e.g. tuberostemonine, (ii) stemoamide-type, e.g. stemoamide, and (iii) tuberostemospironine-type, e.g. tuberostemospironine (Jaing *et al*., 2002). To date, however, there have been no reports on the anti-diabetic constituents of the plant (Figure 1). Therefore, we aimed at investigating the anti-diabetic activity and some constituents possessing such activity in the present study.

(a) (b)

Figure 1 Photographs showing (a) *S. tuberosa* plant and (b) roots

Materials and Methods

Sample Collection of Preparation

Roots of *S. tuberosa* used in this study were collected from Gangaw Township, Magway Region. The collected sample was washed with water, chopped into small pieces using a stainless knife and air dried to a constant weight at room temperature for one month. The dried sample was subsequently milled into coarse powder using a grinding mill and then stored in an airtight plastic container for the experimental works.

Animal Materials

Both sexes of BALB/c mice (20-25 g) were obtained from Animal Section from Parasitology Research Division, Department of Medical Research, Yangon.

Reagents

α-amylase (Jiangsu Boli Bioproducts Co., Ltd), deionized water, disodium hydrogen phosphate, sodium dihydrogen orthophosphate, acarbose (Bayer Pharma AG, Kaiser-Whilheim-Allee, Leverkusen, Germany) and metformin (Denk Pharma Gmbtt Co., Ltd Munchen, Germany), alloxan monohydrate (Titan Biotech Ltd., India), DM sensor blood glucometer (Taiwan), sucrose (BDH), starch, iodine, ethanol, 2,2-diphenyl-1-picry-hydrazyl (DPPH) and Vitamin C were used.

Preparation of Crude Extracts

The dried powdered root (300 g) was percolated with 95% ethanol (1000 mL) for one week and filtered. This procedure was repeated for three times. The combined filtrates were evaporated under reduced pressure by means of a rotatory evaporator. Consequently, 95%

ethanol soluble extract was obtained. Water extract of three samples was prepared by boiling 300 g of sample with 1000 mL of distilled water for 6 h and filtered. It was repeated three times and the filtrates were combined followed by heating on water bath and sand bath to give water extract. Each extract was stored in refrigerator for screening of biological activities.

Screening of *In Vitro* Antioxidant Activity

(a) Preparation of DPPH solutions

DPPH (2.364 mg) was thoroughly dissolved in EtOH (100 mL). This solution was freshly prepared in the brown coloured reagent bottle and stored in the fridge for no longer than 24 h. Each tested samples (2 mg) and 10 mL of EtOH were thoroughly mixed by shaker. The mixture solution was filtered and the stock solution was obtained. By adding with EtOH, the tested sample solutions with different concentrations of 10, 5, 2.5, 1.25 and 0.625 μg/mL were prepared from the stock solution.

(b) Determination of *in vitro* antioxidant activity

The effect on DPPH radical was determined using the method by Marinova and Batchvarov (2011). The control solution was prepared by mixing 1.5 mL of 60 M DPPH solution and 1.5 mL of EtOH using shaker. The tested sample solution was also prepared by mixing thoroughly 1.5 mL of 60 M DPPH solution and 1.5 mL of each sample solution. The mixture solutions were allowed to stand at room temperature for 30 min. Then, the absorbance of these solutions was measured at 517 nm on a UV-7504 UV-visible spectrophotometer. Absorbance measurements were done in triplicate for each concentration and then mean values so obtained were used to calculate percent inhibition of oxidation by the following equation.

$$\% \text{ Inhibition} = \frac{A_{Control} - A_{Sample}}{A_{Control}} \times 100$$

where, $A_{Control}$ = absorbance of control solution

A_{Sample} = absorbance of tested sample solution.

The 50 % antioxidant inhibition concentration (IC_{50}) of tested samples and positive control were determined by linear regressive excel programme.

Screening of *In Vitro* α-Amylase Inhibition

Starch iodine assay

Alpha-amylase inhibitory activity was measured *in vitro* by hydrolysis of starch in the presence of α-amylase enzyme. This process was quantified by using iodine, which gave blue colour with starch. The reduced intensity of blue colour indicates the enzyme-induced hydrolysis of starch to monosaccharides. If the extracts possessed α-amylase inhibitory activity, the intensity of blue colour would be more. In other words, the intensity of blue colour in test sample was directly proportional to α-amylase inhibition activity (Ganapaty *et al.,* 2013). In this assay, 10 μL of α-amylase solution (0.3 U/mL where U means μmol/min, where μmol refers to the amount of substrate conversion) was mixed with 390 μL of phosphate buffer solution (40 mM containing 0.006 M NaCl, pH 7.0) containing different concentrations (100, 60, 40, 20 μg/mL) of tested samples and the positive control, acarbose. After incubation at 37 °C for 10 min, 10 μL of

starch (1 %) was added to the mixture and it was reincubated for 30 min. Then, 0.1 mL of 1 % iodine solution was added to this mixture. After adding 5 mL of distilled water, the absorbance was measured at 565 nm in the UV spectrophotometer. The absorbance of control, without test sample, and blank, without starch, were recorded under the same conditions. The inhibition of enzyme activity was calculated by using the formula,

$$\% \text{ inhibition of enzyme activity} = \frac{A_{Control} - A_{Sample}}{A_{Control}} \times 100$$

where, $A_{Control}$ = absorbance of the control (without sample)

A_{Sample} = absorbance of the tested sample

The concentration of tested samples and positive control which inhibited the hydrolysis of starch by 50 % (IC_{50}) were determined by linear regressive excel programme.

Preliminary Phytochemical Tests

A 1 g each of Thamya roots powder was subjected to the test of alkaloids, α-amino acids, carbohydrates, cyanogenic glycosides, flavonoids, glycosides, organic acids, phenolic compounds, reducing sugars, saponins, starch, steroids, tannins and terpenoids as the preliminary phytochemical test according to reported methods (CCRUM, 1977, Marini-Bettolo *et al*., 1981 and M-Tin-Wa, 1972).

Extraction and Isolation of Phytoconstituents

The air-dried roots powder (500 g) of *S. tuberosa* were extracted with 95 % ethanol (3× 1 L) at room temperature. The combined filtrates were evaporated under reduced pressure by means of a rotatory evaporator to afford an EtOH extract (18 g) which was then acidified with dilute HCl (4 %) to pH 1-2 and partitioned with CH_2Cl_2. The aqueous part was then basified with aqueous NH_3 (28 %) to pH 9-10 and extracted with CH_2Cl_2 to afford crude alkaloids. The crude alkaloids (5 g) were separated by silica gel column (ϕ 10 cm; 70-230 mesh), using gradient mixtures of n-hexane-acetone from 20:1 to 1:2 as mobile phases, affording six fractions. The fraction F3 (2.4 g) was subjected to silica gel CC (ϕ 3 cm; 230-400 mesh) and eluted with gradient solvent systems of n-hexane-CH_2Cl_2 (5:1 → 1:1) to yield compound **1** (20 mg, 0.04 % w/w). These isolated compounds were characterized by their physicochemical properties: melting point, R_f value and identified by modern spectroscopic methods: FT IR, ^{1}H NMR, ^{13}C NMR, ^{1}H ^{1}H COSY, HSQC, HMBC and ESI-MS.

Acute Toxicity Assay

The *in vivo* acute toxicity of ethanol and water extracts of *S. tuberosa* was investigated, according to the OECD Guidelines for the Testing of Chemicals 423, with two doses (5000 and 2000 mg/kg) on BALB/c mice model. A total of 50 healthy mice (both sexes, four to six weeks old, body weight 20-25 g) were grouped to five groups (Group IA for control; only distilled water, Groups IIB and IIC for 2000 mg/kg; and Groups IIIB and IIIC for 5000 mg/kg oral dose of ethanol and water extracts) of 10 mice each. The mice fasted overnight and given only water were used for the experiment. The animals were observed for the first six hours continuously for mortality and behavioral changes, if any, and then every 24 h for one month. The mice were observed from possible manifestation of physical signs of toxicity such as struggle, decreased

motor activity, decreased body/limb tones, decreased respiration and finally death. Records on the number of deaths observed were taken in each group.

Screening of *In Vivo* Antidiabetic (Hypoglycemic) Effect

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 (Yanarday and Colak, 1998). The mice were maintained on 5 % glucose solution for next 24 h to prevent hypoglycemia. Five days later, blood samples were drawn from tail vein and glucose levels were determined to confirm the development of diabetes (250 mg/dL and above). The diabetic mice were divided into six groups, each containing five animals and treated as follows:

Group I : Normal control mice

Group II : Alloxan-induced diabetic mice with 150 mg/kg body dose, Diabetic control

Group III : Alloxan-induced diabetic mice treated with 150 mg/kg body weight dose of ethanol extract

Group IV : Alloxan- induced diabetic mice treated with 150 mg/kg body weight dose of water extract

Group V : Alloxan-induced diabetic mice treated with 15 mg/kg body weight dose of compound **1**

Group VI : Alloxan-induced diabetic mice treated with 150 mg/kg body weight dose of standard drug metformin

Blood Sampling

Blood samples were collected from the tail vein just prior to and 1 h, 2 h, 3 h and 4 h after drug administration for acute study. The blood samples from each mouse was analyzed for blood glucose content at 1 h, 2 h, 3 h and 4 h subsequently using DM sensor blood glucometer and percent reduction in blood glucose level was calculated by the following equation.

$$\text{Reduction (\%)} = \frac{\text{FGBL} - \text{blood glucose level}}{\text{FGBL}} \times 100$$

where, FBGL = fasting blood glucose level at 0h

The results are expressed as mean ± standard error mean, the significance of various treatments was calculated by one-way ANOVA using SPSS software version 16 and were considered statistically significant when $p < 0.01$.

Histopathology of Pancreas

The pancreas from normal control, diabetic control and maximum drug dose treated animals were blotted free of mucus. The tissues were washed in normal saline, cut into the desired size and fixed in 10 % formalin for 24 h. After fixation, tissues were dehydrated and embedded in paraffin. Sections of tissue were cut in a microtome to 5 mm in thickness, mounted on slides, stained with Hematoxylin and Eosin for photographic observations by the pathologist from DMR, Lower Myanmar.

Results and Discussion

Preliminary phytochemical tests indicated the presence of alkaloids, α-amino acids, carbohydrates, flavonoids, glycosides, organic acids, phenolic compounds, reducing sugars, saponins, starch, steroids, tannins and terpenoids. However cyanogenic glycosides were not detected in the roots of Thamya. The presence of alkaloids, flavonoids and phenolic compounds in *S. tuberosa* indicates the antioxidant activity of that plant.

In vitro antioxidant activity by DPPH free radical scavenging assay

The presence of phenolic compounds like polyphenols, flavonoids, tannins, and terpenes in plant extracts shows significant antioxidant effect due to their free radical scavenging activity (Rahman and Moon, 2007). Free radicals such as hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) and superoxide anion radical are often generated by various biological oxidation reactions. These oxidative mediators can lead to the damage of important biomolecules such as proteins, nucleic acid, and lipids. The antioxidant activities of ethanolic and water extracts; and compound **1** of *S. tuberosa* roots were measured as DPPH free radical scavenging activity and exhibited significant variations (Table 1 and Figure 2). The antioxidant (radical scavenging) activity of the ethanolic extract (IC_{50} = 1.50 μg/mL) was significantly higher than that of the water extract (IC_{50} = 3.95 μg/mL) of *S. tuberosa* roots. Moreover, compound **1** (IC_{50} = 1.06 μg/mL) from ethanolic *S. tuberosa* roots extract showed a greater radical scavenging activity than the standard ascorbic acid (IC_{50} = 1.21μg/mL).

Table 1 Radical Scavenging Activity (Percent Inhibition and IC_{50} Values) of Crude Extracts of *S. tuberosa* Roots and Standard Ascorbic Acid

Samples	% Inhibition (Mean ± SD)In Different Concentration (μg/mL)						IC_{50} (μg/mL)
	0.625	**1.25**	**2.5**	**5**	**10**	**20**	
EtOH extract	32.01 ±2.58	40.80 ±0.32	51.14 ±1.29	57.53 ±1.93	62.78 ±2.26	71.68 ±1.29	1.50
H_2O extract	26.11 ±0.30	35.17 ±1.21	44.66 ±1.51	53.85 ±0.60	57.91 ±0.91	63.68 ±1.21	3.95
Compound **1**	45.05 ±0.32	52.11 ±0.11	60.23 ±0.45	70.45 ±1.32	79.17 ±1.51	85.01 ±0.98	1.06
Ascorbic acid*	47.81 ±0.88	54.18 ±1.32	61.09 ±0.22	67.5 ±0.44	71.71 ±1.54	79.21 ±2.43	1.21

Data are expressed as means of triplicate determination ± standard deviation (SD). *****- Standard

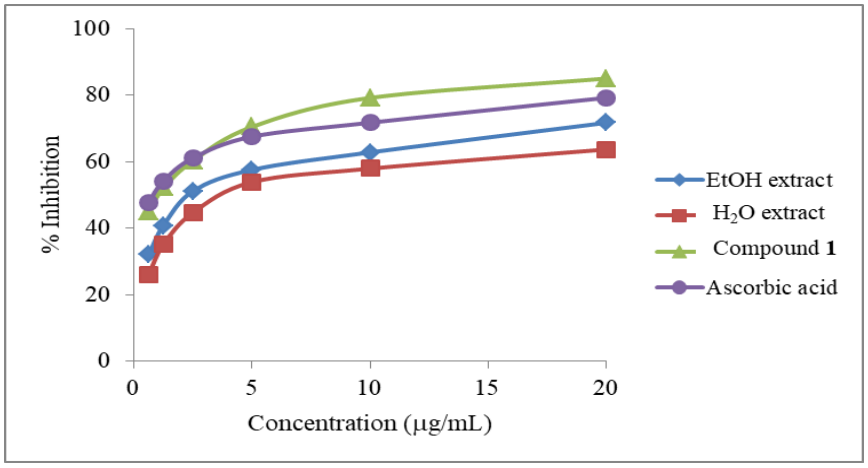


Figure 2 A plot of % radical scavenging activity versus concentration (µg/mL) of crude extracts and compound **1** from roots of *S. tuberosa* and ascorbic acid

In vitro anti-diabetic activity by α-amylase inhibition

In vitro anti-diabetic activity was carried out on the ethanol and water extracts of *S. tuberosa* roots by using alpha amylase inhibitory assays. The α-amylase is a key enzyme in our digestive system which catalyzes the degradation of dietary starch *viz*., maltose to glucose in the small intestine before absorption. Degradation of this dietary starch results in rapid increase in glucose levels, and leads to elevated post prandial hyperglycemia (Abhijit *et al*., 2014). Inhibition of alpha amylase can lead to reduction in post prandial hyperglycemia (Raman *et al*., 2012). Hence, retardation of starch digestion by inhibition of α-amylase enzyme would play a key role in the treatment of diabetes.

In this study, the alcohol and watery extracts, and compound **1** of *S. tuberosa* were evaluated for their inhibitory effect on α-amylase enzyme by *in-vitro* method. The alcohol extract, water extract, compound **1** of *S. tuberosa* (at a concentration of 100 µg/mL) and acarbose exhibited 67.26, 49.26,79.61 and 74.03 µg/mL for α-amylase inhibitory activity (Table 2 and Figure 3); respectively. Acarbose, a synthetic drug has the ability to inhibit alpha amylase was used as a standard for the assay. The α-amylase reduces the rate of starch breakdown, leading to lowered blood glucose levels and leads to minimum absorption of monosaccharides. Furthermore, it was evident that compounds like glycosides, proteins, alkaloids, flavonoids, saponins, tannins, and steroids has inhibitory action on α-amylase. The ethanolic extract and compound **1** of *S. tuberosa*, each has therefore shown best enzyme inhibitory activity with IC_{50} values, 80.98 and 59.25 µg/mL on α-amylase (Table 2 and Figure 3), which were comparable with that of acarbose (64.12 µg/mL).

Table 2 α-Amylase Inhibition % and IC_{50} Values of Crude Extracts and Isolated Compound 1 of *S. tuberosa* Root Compared with Acarbose

Sample	% Inhibition (mean ± SD) in different concentration (μg/mL)					IC_{50} (μg/mL)
	20	40	60	80	100	
EtOH extract	16.10 ± 1.38	22.71±0.89	32.59±1.95	56.11 ± 0.91	67.26 ±0.61	80.98
H_2O extract	6.75 ± 0.69	10.35±1.84	23.44±1.06	39.82 ± 1.53	49.26 ±1.76	101.48
Compound **1**	17.85 ± 0.73	30.30±1.01	50.76±1.07	70.09 ± 0.85	79.61 ±0.90	59.25
Acarbose*	18.98 ± 0.66	32.32±0.21	45.86±0.73	65.96 ± 1.11	74.12 ±0.03	64.12

Data are expressed as means of triplicate determination ± standard deviation (SD).
*= positive control

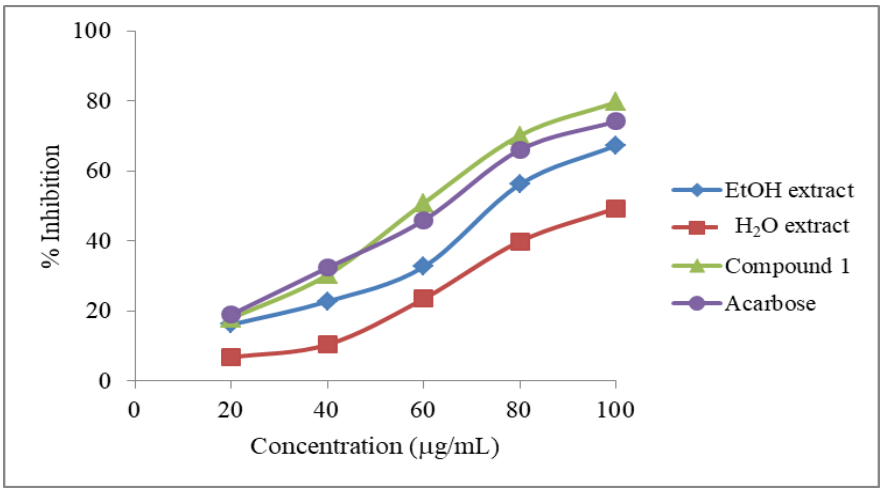


Figure 3 A plot of % inhibition of α-amylase against various concentrations of crude extracts and isolated compound **1** of *S.tuberosa* roots

In vivo acute toxicity assay

Acute toxicity screenings of ethanol and water extracts were done with two oral doses, 2000 and 5000 mg/kg body weight on each group of albino mice. The condition of mice groups were recorded after fourteen days' administration. No lethality of the mice was observed up to the day fourteen of administration. Animals of each group were also still alive and did not show any visible symptoms of toxicity like struggle, respiratory distress, decreased body/limb tone and death (Table 3). Even with up to the dose of 5000 mg/kg body weight administration, there is no lethality at the day fourteen.

Table 3 Acute Toxicity Effect of Crudes Extracts of *S. tuberosa* Roots on Albino Mice Model

No.	Group	Dosage (mg/kg)	No. of Death	% of Death at 14 Days
1	IA	-	Nil	0
2	IIB	2000	Nil	0
3	IIIB	5000	Nil	0
4	IIC	2000	Nil	0
5	IIIC	5000	Nil	0

Each group contains 10 no. of mice.
A = control, B = ethanol extract and C = water extract

In vivo hypoglycemic activity of *S.tuberosa*

Diabetes mellitus is one of the metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion. *In vivo* antidiabetic activity was determined by using alloxan-induced diabetic mice model. Alloxan caused permanent destruction of pancreatic beta cell and induced diabetes for mice. As control, determination of normal fasting blood glucose level was carried out and it was found to be 100 ± 3.39 mg/dL, showing within the normal range. In this study, mean glucose level of the mice treated with alloxan was found to be 100.00 ± 3.39 mg/dL to 253.00 ± 21.25 mg/dL. The results showed that the animals used in these experiments were in diabetic stage and hyperglycemia was developed 72 h after alloxan injection (Yanarday and Colak, 1998). In diabetes control group; without treatment, the mean blood glucose concentration was found to raise from 100.00 ± 3.39 mg/dL to 253. ± 21.25 mg/dL at 72 h, 279.00 ± 22.25 mg/dL at 73 h, 275.00 ± 29.25 mg/dL at 74 h, 275.00 ± 24.50 mg/dL at 75 h and 269.00 ± 22.50 mg/dL at 76 h. The alloxan-induced diabetic mice were treated with metformin (150 mg/kg b.wt), EtOH and H_2O extracts (150 mg/kg b.wt) and isolated compounds from EtOH extract, and compound **1** (15 mg/kg b.wt).

Table 4 Antidiabetic activity of *S. tuberosa* roots extracts and compounds during acute study

Treatment	Blood Glucose Concentration (mg/dL)				
	0 h	1 h	2 h	3 h	4 h
Normal	100.00 ±3.39	101.00 ± 7.35	100.00 ± 5.00	100.67 ± 8.37	100.67 ± 4.03
Alloxan (150 mg/kg)	253.00±21.25	279.00 ±22.25	275.00 ±29.25	275.00 ±24.50	269.00 ± 22.50
EtOH 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)
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)
*Metformin (150 mg/kg)	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)
Compound **1** (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)

R = Reduction, P < 0.05,* = Antidiabetic drug, Data are represented as mean ± SEM (n = 3).

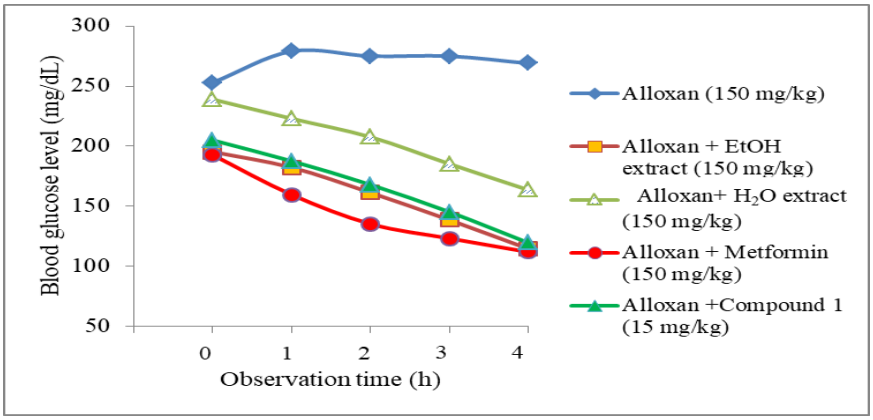
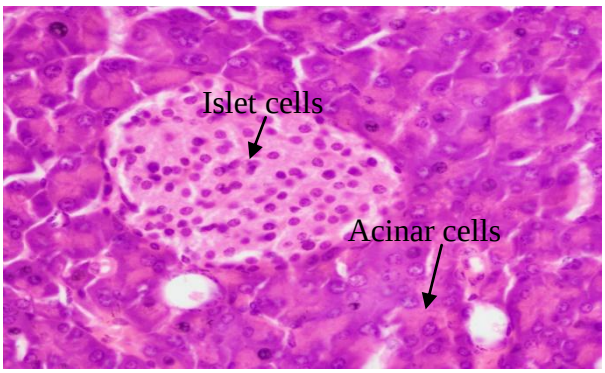


Figure 4 The effect of ethanol, water extracts and compound **1** of *S. tuberosa* roots and metformin on blood glucose levels of alloxan-induced diabetic mice

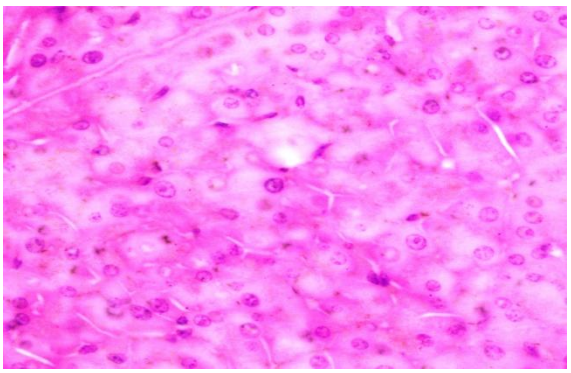
After samples injection, the blood glucose levels were dramatically reduced (Table 4 and Figure 4). Concerning with duration of action, the hypoglycemic effect of tested sample lasted for 4 h. So, all of the test samples showed significant percent reduction of blood glucose level (% R) at 4 h after treatment with samples where $p < 0.01$. The % reduction of blood glucose level in mice produced after administration at 4 h with standard metformin, EtOH and H_2O extracts; compound **1** of roots of *S. tuberosa* were compared in Figure 4. The % reduction of blood glucose levels were compound **1** (41.62%) followed by EtOH extract (41.29 %), H_2O extract (21.52 %). But that of blood glucose level of EtOH extract and compound **1** was not significantly different from standard metformin (41.87%), Therefore, EtOH extract of roots of *S. tuberosa* and compound **1** have significant hypoglycemic effect against alloxan induced diabetic albino mice.

Histopathological examination of pancreas

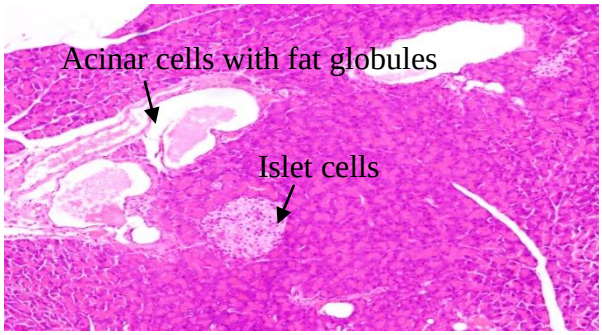
The histopathological examination on pancreatic islet was performed 7 days after alloxan administration by Hematoxylin and Eosin stain method and recorded by using light-microscope with specific images. The images of histopathological of pancreas obtained from mice of each group were described in Figures 5 (a-f). From the histopathological result, the pancreas of metformin treated diabetic mice (c) showed proliferation of beta islets cells and accumulation of fat globules within acinar cells in pancreas but the pancreas of diabetic untreated mice (b) showed destruction of pancreatic beta islets cell comparing with normal pancreatic tissue of normal mice (a). The pancreatic islets cell of the H_2O extracted diabetic mice (d) showed apart of regeneration of ill-defined acinar cells with absence of nucleus. The EtOH extract treated diabetic mice (e) showed regeneration of group of nucleated beta islets cells with ill-defined margin and necrosis of acinar cells in pancreas. In additions, the histological observation of pancreatic cells of mice treated with compound **1** treated diabetic mice (f) showed good proliferation of beta islets cells; two to three groups among the well-defined pyramidal shaped acinar cell and no accumulation of fat globules within acinar cells in pancreas compared with histopathological information of metformin treated diabetic mice (c). The signs of regeneration of beta cells stimulated potentiation of insulin secretion from beta cells of islets of Langerhans and so decreasing of blood glucose level. Nevertheless, the extracts of EtOH and H_2O as well as compound **1** induced regeneration of the islets responsible for the increase in the serum insulin. Therefore the ethanol extract of *S. tuberosa* root and compound **1** showed protective activity in reactive oxygen species (ROS) induce damage tissues.



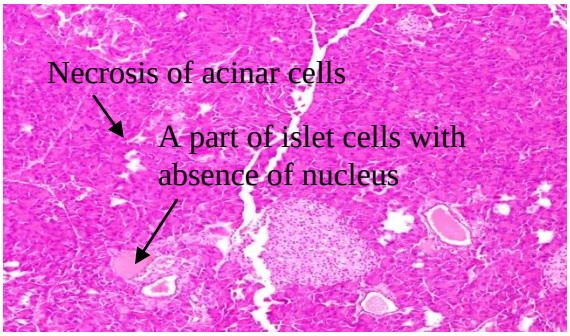
(a) Normal pancreatic tissue of normal



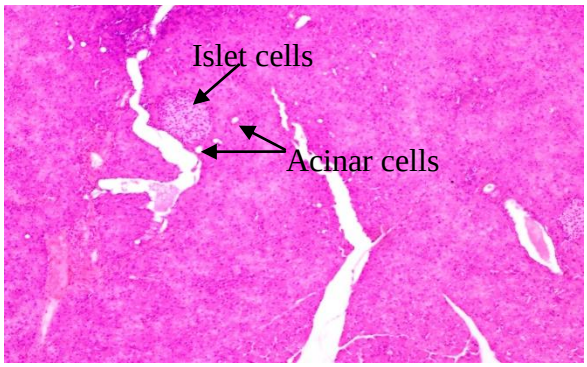
(b) Destructive pancreatic tissue of alloxan-induced diabetic mice



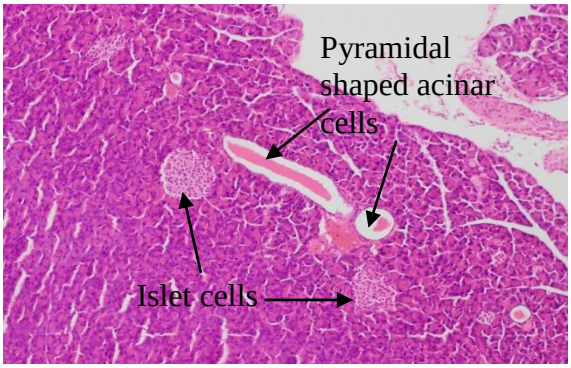
(c) Regeneration of beta islets cells among acinar cell with duct epithelial shape but accumulation of fat globules in acinar cell of pancreatic tissue of alloxan-induced diabetic mice with metformin



(d) A part of regeneration of beta islets cells with absence of nucleus and necrosis of some acinar cell of pancreatic tissue of alloxan-induced diabetic mice with H_2O extract



(e) Regeneration of beta islets cells among acinar cell of pancreatic tissue of alloxan-induced diabetic mice with EtOH extract



(f) Good proliferation of beta islets cells among the well-defined pyramidal shaped acinar cell of pancreatic tissue of alloxan-induced diabetic mice with compound **1**

Figure 5 (a-f) Histological examinations of pancreatic tissue of alloxan-induced diabetic mice after 7 days' treatment of antidiabetic drugs; metformin, H_2O, EtOH and compound **1** [magnification × 40]

Identification of Organic Compound 1

Compound **1** was obtained as a colorless prism. Its molecular formula was established as $C_{22}H_{33}NO_4$ by the positive molecular ion peak at m/z 376.2 $[M+H]^+$ in its ESI MS (Figure 12) with seven degrees of unsaturation. The IR band at 1762 cm^{-1} (Figure 6) and ^{13}C NMR data at δ_C 179.2 and 179.4 suggested the presence of two γ-glactone rings (Figure 8). In the ^1H NMR spectrum (Figure 7), a primary methyl group appeared at δ_H 0.96 (3H, t, J = 7.4 Hz, H-17) and the secondary methyl groups were observed at δ_H 1.61 (3H, d, J = 7.3 Hz, H-22) and 1.24 (3H, d, J = 7.3 Hz, H-15). Two low field germinal protons appeared at δ_H 2.36 and 3.30 (2H, m, H-5). Two protons at the oxygenated carbons were shown at δ_H 4.52 (1H, m, H-11) and 4.31 (1H, m, H-11). The ^{13}C NMR and ^1H-^{13}C HMQC spectra (Figures 8 and 10) showed 22 skeletal carbon signals including three methyls, seven methylenes, ten methines, and two carbonyls. The above characteristic data suggested that compound **1** is agreed structurally to tuberostemonine **J** (Chung *et al*., 2003). The ^1H-^1H COSY correlations of H-11→H-12→H-13→H15 and H-18→H-19→H-20→H3-22 with two oxomethines [δ_H 4.52 (H-11)/δ_C 80.5 (C-11) and 4.38 (H-18)/ 78.9 (C-11)] as starting points (Figure 9) and the HMBC correlations of

H13-15/C-14 and H5-20/C-21 provided evidence for two α-methyl-γ-lactone rings (Figure 11). The ^{1}H-^{1}H COSY spectrum of compound **1** exhibited the correlation of H-5→H-6→H-7→H-8→H-9→H-9a and H-9a→H-1→H2→H-3 involving typical three low field protons attached to the N-atom at [δ_H 2.36 and 3.30 (each H-5), 3.43 (H-3), and 3.05 (H-9a)], indicating the presence of the nitrogen fused perhydroazaazulene ring (Yun-Seo *et al*., 2014). Additional ^{1}H-^{1}H COSY correlations of H-11→H-10→H2-16→H3-17 and the ^{1}H-^{13}C HMBC correlations of H-3/C-1, H-3/C-9a and H-3/C-19 allowed to confirm the connectivities of the above subgroups (Figures 9 and 11). Thus, compound **1** was determined as a known stemona type alkaloid, namely, tuberostemonine **J** in Figure 13.

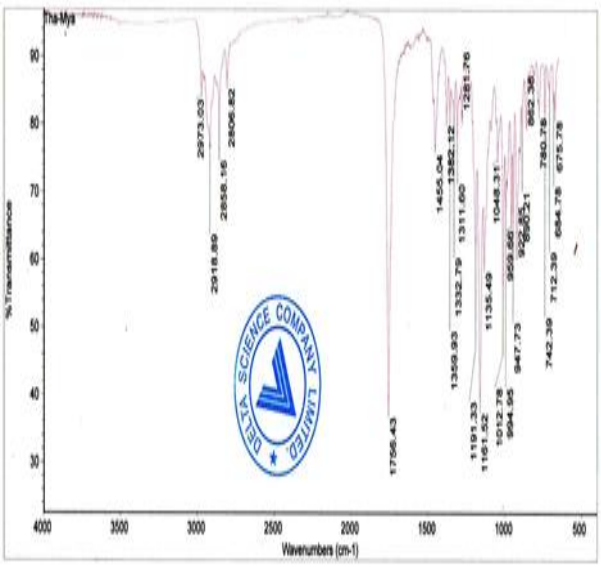


Figure 6 FT IR spectrum of compound **1**

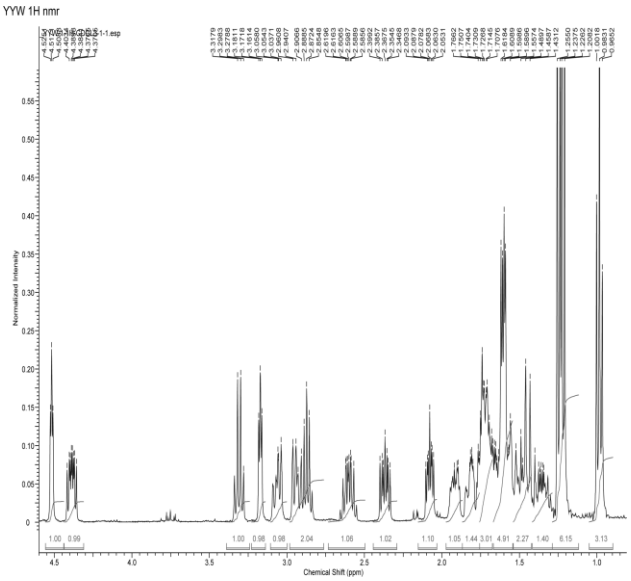


Figure 7 ^{1}H NMR (600 MHz, $CDCl_3$) spectrum of compound **1**

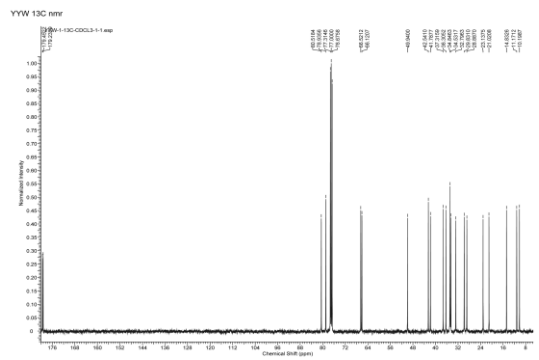


Figure 8 ^{13}C NMR (150 MHz, $CDCl_3$) spectrum of compound 1

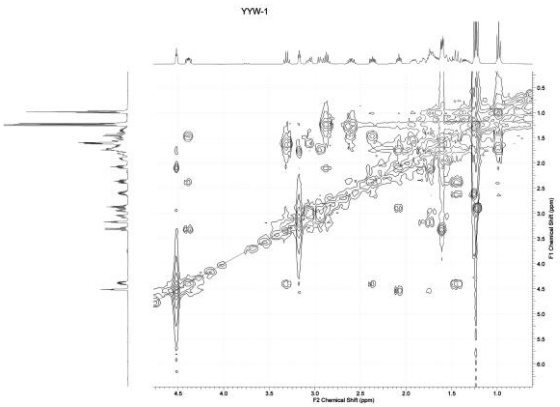


Figure 9 ^{1}H-^{1}H COSY (600 MHz, $CDCl_3$) spectrum of compound 1

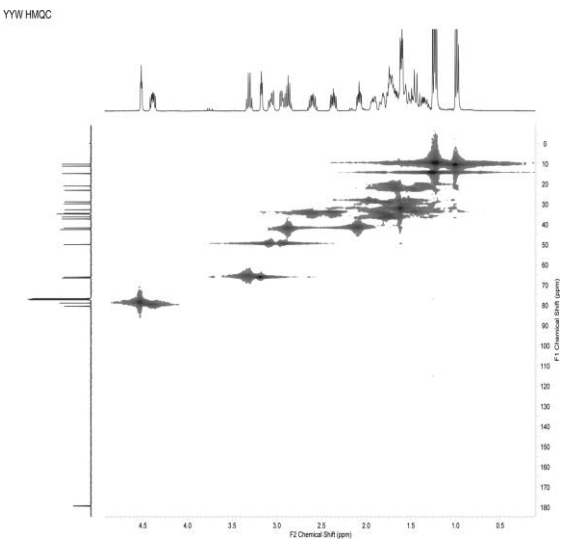


Figure 10 ^{1}H-^{13}C HMQC (600 MHz,$CDCl_3$) spectrum of compound 1

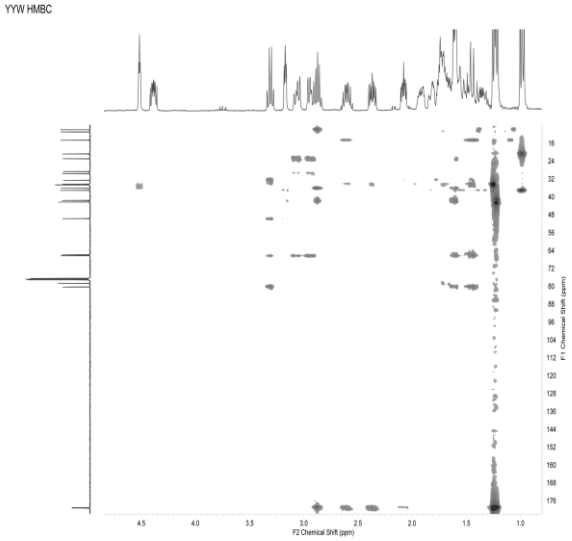


Figure 11 ^{1}H-^{13}C HMBC (600 MHz, $CDCl_3$) spectrum of compound 1

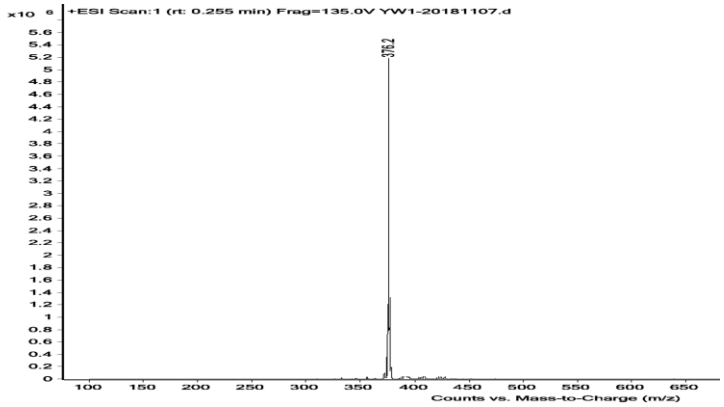


Figure 12 ESI MS spectrum of compound 1

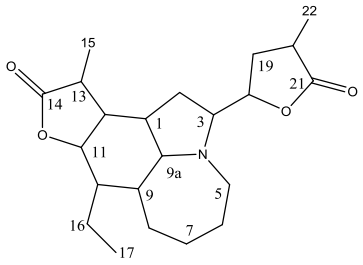


Figure 13 Chemical structure of tuberostemonine J

Compound **1** (Tuberostemonine **J**): A colorless prism crystallized from hexane/EtOAc, mp: 180-182 ^{0}C.; IR (νcm^{-1}) : 2924($\nu_{C\text{-}H}$ asym), 1762 ($\nu_{C=O}$), 1456 ($\delta_{C\text{-}H}$), 1167 ($\nu_{C\text{-}N}$), 1015 ($\nu_{C\text{-}O}$); ESI-MS m/z (% intensity): 376 $[M+H]^{+}$ (100), ^{1}H NMR (6 00 MHz, $CDC1_3$) δ_H (ppm): 1.80 (3H, t, J=7.5 Hz, H-17), 1.24 (3H, d, J=7.5 Hz, H-15), 1.61 (3H, d, J=7.5 Hz, H-22), 1.40-2.07 (15H, H-1, 2H-2, 2H-6, 2H-7, 2H-8, H-9, H-10, H-12, 2H-16, H-19), 3.17(1H, m, H-19), 2.59 (1H, m, H-20), 2.87 (1H, m, H-13), 2.36 and 3.30 (each 1H, m, 2H-5), 3.43 and 3.05 (2H, m, H-3 and H-9a), 4.38 (1H, m, H-18), 4.52 (1H, t, H-11); ^{13}C NMR (150 MHz, $CDC1_3$) δ_C (ppm): 10.2 (C-17), 11.2 (C-15), 14.8 (C-22), 21.0 (C-16), 23.1 (C-7), 28.9 (C-6), 29.8 (C-2), 32.7 (C-8), 34.4 (C-19), 34.5 (C-9), 34.8 (C-10), 36.3 (C-12), 37.3 (C-20), 41.8 (C-13), 42.5 (C-1), 49.4 (C-5), 66.1 (C-3), 66.5 (C-9a), 78.9 (C-11), 80.5 (C-18), 179.2 (C-14), 179.4 (C-21).

Conclusion

This research revealed some biological properties, antioxidant and antidiabetic effects, of the *S. tuberosa* roots extracts, and tuberostemonine **J**. In fact, the present research showed that the extracts of the roots possess not only antioxidant but also hypoglycemic effects. Moreover, tuberostemonine **J** is more potent than vitamin C in antioxidative property as well as more excellent than the antidiabetic drug metformin in proliferation of beta islets cells among the well-defined pyramidal shaped acinar cell of pancreatic tissue of alloxan-induced diabetic mice. Therefore it is hoped that the research findings will contribute to some extent to the search for the antioxidative and antihyperglycemic agent of plant origin and also to the development of the role of Myanmar traditional medicinal formulation, especially in the treatment of oxidative related diseases and diabetes.

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