Original Research Article

Evaluations of Antioxidant and antidiabetic activities of *Hygrophila schulli* (Buch.-Ham.) leaf extract in alloxan-induced mice

Abstract

Background: *Hygrophila schulli* is a medicinal plant that has traditionally been used to treat a wide range of chronic diseases. The goal of this experiment was to seek the antioxidant and antidiabetic properties of the methanolic extract of *H. schulli* leaf (MEHL) by *in vitro* and *in vivo* study.

Results: MEHL also showed significant scavenging activity in DPPH and ABTS free radical scavenging assay (IC 50 value is 105.80 µg/mL and 27.47 µg/mL respectively). In the α-amylase inhibition assay, MEHL at a concentration of 120 g/mL inhibited α-amylase activity by 23.13%, furthermore, at 100 g/mL concentration of MEHL, 55.62% inhibition of α-glucosidase activity was detected. MEHL-treated diabetic mice exhibited a significant drop in blood glucose levels of 12.66%-33.45% from the 5th to the 21st day in the in vivo assay at a concentration of 200 mg/kg body weight. MEHL decreased the activity of serum SGPT and SGOT significantly in diabetic mice compared to that of the diabetic control group. In diabetic mice, the extract also showed a remedial effect on TG, TC, LDL, HDL and VLDL levels compared to that of untreated mice.

Conclusion: The current study discovered that *H. schulli* leaf extract is a natural source of antioxidants, has substantial antidiabetic effects, restores the lipid profile parameters in diabetic mice, and could be used as an alternative therapy for diabetes management.

KEYWORDS:*Hygrophila schulli* (Buch-Ham), Antioxidant activity, Antidiabetic effect, Alloxan, Lipid profile.

1. Introduction

Nowadays, diabetes mellitus (DM) is considered one of the most common health problems in the world (1). It is a metabolic disorder featured by chronic hyperglycemia with disturbances of carbohydrate, fat, and protein metabolism resulting from abnormalities in insulin secretion, insulin action, or both (2). According to the International Diabetic Federation (IDF) Atlas 2021, the estimated global prevalence of diabetes was 537 million in adults (20–79 years). This number is predicted to rise to 643 million by 2030 and 783 million by 2045 with a continued upward trend (3).

DM is known to cause hyperlipidemia through various metabolic derangements, which is found in about 40% of diabetic patients and diminishing antioxidant defense mechanism through the process of chronic oxidative stress due to hyperglycemia, which in turn causes defective insulin gene expression and insulin secretion as well as increased apoptosis this is the fact linking diabetes mellitus with oxidative stress (4,5). Hyperglycemia-induced metabolic dysfunction may be caused by reactive oxygen species (ROS) produced in the mitochondrial electron transport chain (6). ROS are physiological metabolites formed as a result of respiration in aerobic organisms but their excessive levels have been linked to the onset of diseases such as cancer, stroke and diabetes (7). Therefore, the search for the discovery of antioxidant and antidiabetic agents from plant sources is an important strategy required to combat the widespread nature of this condition. This is because the present synthetic drugs have many drawbacks ranging from limited efficacy and several side effects such as hypoglycemia, weight gain and chronic tissue damage (8).

Traditional herbal medicines have an advantage in the prevention and treatment of diabetes, as they have fewer side effects (9,10). According to the World Health Organization, it was found that approximately 80% of modern civilizations rely on the substance of plants. Herbs generate bioactive compounds, including antioxidants that react to free radicals and suppress microbial growth in other species of the environment (11). Medicinal plants play a leading role in resisting or regulating diseases due to their constituents of antioxidant properties usually associated with various polyphenol compounds (12). According to a previous study, plants are the major sources of different drugs, and almost 800 plants may process antidiabetic activity (13–16). Many plants are already being used for the treatment of DM in traditional medicine (15–17)

Hygrophila schulli (Buch-Ham) is one of the traditionally used medicinal plants for the treatment of dropsy jaundice and urinogenital diseases and is widely distributed in Indo-China, Nepal, Tropical Africa and Bangladesh (18). Previous studies show that leaf and root extracts of *H. schulli* showed antibacterial activity against various bacterial strains (19). Besides the seed extract of *H. schulli* possessed significant anti-nociceptive and anti-inflammatory activities in a rodent model (20). This plant also possesses antitumor (21,22) and hepatoprotective activity (23). There is no available scientific data regarding the pharmacological properties of *H. schulli* (Buch.-Ham.) leaf.Hence, this study aimed to assess the *in vitro*antioxidantandantidiabeticand also *in vivo*antidiabeticactivity of *H. schulli* (Buch.-Ham.) leaf extract.

2. Methods

2.1 Chemicals and reagents

Sodium phosphate, sulphuric acid, potassium phosphate dibasic, potassium phosphate monobasic, ammonium molybdate, potassium ferricyanide [$K_3Fe(CN)_6$], trichloroacetic acid (TCA), ferric chloride ($FeCl_3$), 2, 2-diphenyl-1-picryl-hydrazyl (DPPH●),2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)diammonium salt (ABTS●), potassium persulfate (K2S2O7), and ascorbic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol was purchased from E-Mark (Germany). All the chemicals used in this study were of analytical grade.

2.2 Collection of plant materials and extractions

The plant, *Hygrophila schulli* (Buch.-Ham.) was collected from Motihar, Rajshahi-6205, Bangladesh, in February 2022 and authenticated by the renowned taxonomist (Voucher no. AA109). Leaves were washed thoroughly with water to remove adhering dirt and shed dried at 25-28° C. Then the plant components were crushed into coarse powder by a milling machine (FFC-15, China) and kept at room temperature for later use. About 100 gm of dried powder of *H. schulli* leaves was taken in a clean round-bottomed bottle, added 400 ml of methanol, and left for seven days at room temperature with occasional

shaking and stirring.The mixture was filtered through Whatman No. 1 filter paper, and the filtrate was collected. Methanol was then evaporated using a rotary evaporator at 39°C to generate methanolic extract of *H. schulli* Leaf (MEHL). The concentrated extract was kept in storage vials at 4°C for future experimental use.

2.3 Determination of total antioxidant activity

The total antioxidant activity of the MEHL was estimated by the method of Prieto et al.(24) with some modifications**.** In brief, 0.3 mL of leaf extract at different concentrations was mixed with 3 mL of reaction mixture containing 0.8 M sulphuric acid, 14 mM sodium phosphate, and 0.4% ammonium molybdate and incubated at 95°C for 10 minutes. After the samples had cooled to room temperature, the absorbance of the aqueous solution of each was measured at 695 nm against a blank. Ascorbic acid was used as standard.

2.4 Determination of ferric-reducing antioxidant activity

The reducing power of methanolic extracts was determined according to the method of Oyaizu et al.(25)with slight modifications. Different concentrations of 0.25 mL of extract were added with 0.625 mL potassium buffer (0.2 M, pH 6.7) and 0.625 mL of 1% potassium ferricyanide [$K_3Fe(CN)_6$]. The mixture was incubated at 50^{o}C for 20 min. After incubation, 0.625 mL of 10% trichloroacetic acid solution was added to each tube and the mixture was centrifuged at 3000 rpm for 10 min. Clear supernatant (1.8 mL) was taken and mixed with an equal amount of distilled water, 0.36 mL of ferric chloride (0.1% w/v) solution was added and absorbance was recorded at 700 nm. Here ascorbic acid was used as the reference standard.

2.5 DPPH free radical scavenging assay

Free radical scavenging activity of MEHL was determined against 2, 2- diphenyl-1-picryl-hydrazyl (Sigma-Aldrich) radical using the method described byCheel et al.(26). In brief, 1 mL of MEHL solution

in methanol and ascorbic acid were mixed with 3 mL of DPPH solution (0.1 mM) in methanol. The mixture was allowed to stand for 30 minutes and the absorbance was measured at 517nm. The free radical scavenging activity of each sample was calculated by using the following formula:

DPPH Radical scavenging rate (%) = $[\{A_0 - A\}/ A_0] \times 100$

Where "A_0"(control) was the absorbance of DPPH blank solution, and "A" was the final absorbance of the tested sample after 30 minutes of incubation.

2.6 ABTS free radical scavenging assay

The ABTS method was used according to Re et al. (27) with slight modifications. ABTS was dissolved in distilled water at a final concentration of 7mM and mixed with 2.45mM potassium persulfate and the mixture was left to stand in the dark at room temperature for 12–16 hours before use. For this experiment, ABTS solution was diluted with water to an absorbance of 0.70±0.02 at 734nm. Then 1mL of various concentrations of the sample was mixed with 3.0 mL of ABTS solution and vortexed. The absorbance was measured at 734 nm after 6 minutes of incubation. Ascorbic Acid was used as positive control. The scavenging rate was calculated using the formula:

ABTS radical scavenging rate (%) = $[\{A_0 - A\}/ A_0] \times 100$

Where "A_0"(control) was the absorbance of ABTS blank solution, and "A" was the final absorbance of the tested sample.

2.7 α-amylase inhibition assay

Screening for α-amylase inhibition by extract was carried out according to the method described by Wang, Y et al. (28) with slight modifications. Different concentrations of 50µLextracts were added to 25 µL of α-amylase solution (45 units/mL) containing 0.02 M sodium phosphate buffer (pH 6.9 with 6 mMNaCl) and were mixed and incubated at 25°C for 10 minutes. After incubation, 50 µL of 1% starch solution in 0.02 M sodium phosphate buffer was added to each tube. The reaction mixtures were further

incubated at 25°C for 10 minutes. Then the reaction was stopped with 50 µL of 3M HCl and subsequently 30 µL of a mixture solution of 5 mM I_2 and 5 mM potassium iodide was added to each test tube as color developing reagent. The absorbance of each test tube was measured at 620 nm and the α-amylase inhibitory activity was calculated as follows:

% inhibition of α-amylase = $[(A_{cont} - A_{samp})/A_{cont}] \times 100$

Where A_{samp} was defined as the absorbance of the sample and A_{cont} was the absorbance of the control.

2.8 α-glucosidase inhibition assay

The α-glucosidase inhibition assaywas performed using a method from Schmidt et al (29) method with slight modification. In brief, in a 96-well microtiter microplate, each well was added with 50 µL of extract at various concentrations and 90 µL of 0.1M sodium phosphate buffer (SPB) pH containing 0.02% sodium azide. A solution of 80 µL of α glucosidase (2.0 U/ml) in SPB was added in each well and the mixture was incubated at 28° C for 10 minutes. Acarbose was used as a positive control. After the incubation, 20 µL of pNPG (dissolved in SPB) was mixed into the solution to initiate the reaction. The rate of pNPG conversion to p-nitrophenol was determined by the measurement of absorbance of p-nitrophenol at 405 nm using a Multiscan FC microplate photometer (Thermo Fisher Scientific, Waltham,MA, USA).

The percentage of alpha-glucosidase inhibition was calculated by the following equation:

% of α glucosidase inhibition= {(Absorbance of Blank – Absorbance of sample)/Absorbance of blank}×100

2.9 Animal care

Swiss albino mice weighing 22-25 gm were collected from the Department of Pharmacy, Jahangirnagar University for experimental study. They were acclimated to the animal house (temperature 25 ± 2°C; humidity 55 ± 5% with 12 h light/ dark cycle), fed with commercial pellets and had free access to water. Food intake was withdrawn before 16-18 hours to start the experiments. The experimental protocol was

approved by the Institutional Animal, Medical Ethics, Bio-Safety and Bio-Security Committee (IAMEBBC) for Experimentations on Animal, Human, Microbes, and Living Natural Sources, Memo No: 249(35)/320/IAMEBBC/IBSc. Institute of Biological Sciences, Bangladesh.

2.10 Induction of diabetes

In overnight fasting mice, diabetes was induced by a single intraperitoneal injection of alloxan (80 mg/kg body weight) in a 0.1M sodium citrate buffer (pH 4.5). The age-matched control mice received an equivalent amount of citrate buffer. After alloxan was administered, food and liquid intake were closely monitored regularly. A portable glucometer (CERA-CHEK, Korea) was used to check the mice's tail vein blood 48 hours after receiving alloxan to determine the development of hyperglycemia. Mice with fasting blood glucose levels ≥ 11.0 mmol/L were considered diabetic mice.

2.11 Experimental design

After 1 week of the acclimation period, the mice were randomly divided into five groups. Each group consisted of five rats (n=5), and they were treated for four weeks as follows:

Group-1 (Normal control): Mice fed with standard pellet diet and water.

Group-2 (Diabetic control): Diabetic mice without treatment.

Group-3 (Positive control): Diabetic mice were treated with glibenclamide at a dose of 5 mg/kg BW (30,31).

Group-4 (MEHL 100 mg/kg BW): The diabetic mice were treated with MEHL at a dose of 100 mg/kg BW for 21 days(32)

Group-5 (MEHL 200 mg/kg BW): The diabetic mice treated with MEHL at a dose of 200 mg/kg BW for 21 days (32)

2.12 Collection of blood

Blood samples were collected from the tail vein on days 1, 5, 10, 15, and 21 in a fasting state using 26 G needle and syringe. After an overnight fasting condition, mice were anesthetized with diethyl ether and sacrificed at the end of the experiment's 21-day duration. Then blood was collected from the artery of the heart. The serum was separated by allowing blood samples at a temperature of 25 ℃ for 10 minutes, followed by centrifuging at 3000 rpm for 20 minutes. After that, the serum was collected and stored in a plastic vial at -80^{o}C until further experiments were performed.

2.13 Measurement of biochemical parameters

Parameters ofserum lipid profile such as total cholesterol (TC), triglyceride (TG), high-density lipoprotein (HDL), low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL) were analyzed using commercially available kits (Ultracare diagnostic, Egypt). Serum SGPT and SGOT levels were also estimated using commercially available kits (Ultracare Diagnostic, Egypt). Hitachi 7180 automatic analyzer (Hitachi, Tokyo, Japan) was used to estimate these biochemical parameters.

2.14 Statistical analysis

Statistical analysis was done with one-way analysis of variance (ANOVA) using GraphPad Prism 8. Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1 Total antioxidant capacity

Figure 1 shows the total antioxidant potentials of *H. schulli* leaf extract and ascorbic acid, evaluated from their ability to reduce Mo (VI) to Mo (V) and the subsequent formation of a green phosphate/Mo (V) complex at acidic pH, MEHL showed a gradual increase in absorbance with the increasing concentration of extract (Fig. 1). The antioxidant capacity of this extract might be attributed to its chemical composition and phenolic content.

3.2 Ferric reducing antioxidant activity

Figure 2 shows the reducing capacity of methanolic extract of *H. schulli* leaf compared to ascorbic acid. Authors previously discovered a direct relationship between antioxidant activity and the reducing power of specific plant extracts zhu (33). The presence of reductones which have been revealed to exert an antioxidant effect by breaking the free radical chain and donating a hydrogen atom is often associated with the presence of reducing characteristics. This findings on the reducing capacity of the investigated extracts indicate that it is likely to play a significant role in the observed antioxidant effect.

3.3 DPPH radical scavenging activity

DPPH is a stable free radical that can be converted into a stable diamagnetic molecule by accepting an electron or hydrogen radical (34). The extract is able to reduce the radical DPPH to the yellow-colored diphenylpicrylhydrazine. The scavenging effect of MEHL and standard with the DPPH radical at the dose of 25 µg/ml are 96.09% and 47.07% respectively. From Fig. 3 We found a dose-response relationship in the DPPH radical scavenging activity; the activity increased as the concentration of MEHL increased. The IC_{50} value for ascorbic acid is 25.782 µg/mL whereas for MEHL IC_{50} is 105.80 µg/mL.

3.4 ABTS free radical scavenging activity

ABTS• is far more reactive than DPPH, and unlike DPPH reactions, which need the transfer of a hydrogen atom, ABTS• reactions require the transfer of an electron. In figure 4 ascorbic acid revealed 98.45% ABTS• radical scavenging activityat a concentration of 20 µg/mL whereas MEHL Showed 35.271% scavenging activityat the same concentration. The IC_{50} value for ascorbic acid is 9.121 µg/mLon the other hand for MEHL IC_{50}value is 27.47 µg/mL.

3.5 α-amylase inhibitory assay

α-amylase inhibitory activity of MEHL is shown in (Figure 5). This assay showed a dose-dependent increase in percentage inhibitory activity against α-amylase enzyme. The percent of inhibition of α-amylase activity was 23.13±1.63% for MEHL at a concentration of 120 µg/mL whereas at the same concentration,acarbose showed 50.17±2.22% inhibition activity.

3.6 α-glucosidase inhibition assay

α-glucosidase inhibitory activity of *H. schulli* leaf extract is shown in (Figure 6). In this assay, there was a dose-dependent increase in percentage inhibitory activity against the α-glucosidase enzyme. The percent of inhibition of leaf at 100 µg/mL was 55.62% and standard acarbose had 80.79% of α-glucosidase inhibition activity at the same concentration. Although the antidiabetic activities of *H. schulli*leaf were relatively low compared to standard acarbose, it might be a good alternative regarding the adverse side effects of synthetic compounds.

3.7 Effects of MEHL on blood glucose level in alloxan-inducedswiss albino

In the*in vivo* assay, MEHL was administered orally to evaluate changes in the blood glucose level of diabetic mice. The plant extract produced significant changes in the blood glucose level of alloxan-induced diabetic mice (Figure 7). Plant extract administered subject showed a significant reduction of blood glucose level in both concentrations of 100mg/kg and 200 mg/kg body weight in diabetic mice. This level of reduction was as near as the glibenclamide-administered subject. From the5th to 21stdays, MEHL at both doses (100 mg/kg and 200 mg/kg body weight) lowered the glucose level by 9.65% - 27.36% and 12.66% - 33.45%, while glibenclamide lowered the blood glucose level by 14.54% - 51.43% compared to the diabetic control group.

3.8 Effects of MEHL on the parameters of lipid profile

Figure 8 shows the effect of MEHL on serum TC, TG, HDL, LDL and VLDL levels in the experimental mice. In the experiment, we found a significant increase in the serum level of TC, TG, LDL and VLDL,

and a decreased level of serum HDL in diabetic mice compared to that of normal control mice. Supplementation of MEHL demonstrated a significant reduction of TC by 10.02% in MEHL 100mg/KG BW and 17.57% in MEHL 200mg/KG BW compared to the DC mice (Figure 8). MEHL 100mg/KG BW and MEHL 200mg/KG BW supplements reduced serum TG 15.78% - 21.02%, LDL 30.37% - 46.16% and VLDL 15.78% - 21.04% compared to the DC mice whereas there is an increase in the serum level of HDL 38.25% - 42.54% compared to the DC mice. Glibenclamide (5 mg/kg body weight) treated mice showed a reduction of TC by 30.34%, TG by32.52%, LDL by 67.82%, VLDL by 32.52% and an increase in HDL by 47.02%.

3.9 Effects of MEHL on serum SGPT and SGOT

Figure 9 shows the effects of MEHL supplementation on the activity of serum SGPT and SGOT in diabetic mice. There was a significant ($P< 0.001$) increase in SGPT and SGOT levels (Figure 9) in diabetic mice than that of normal control. Compared to diabetic control mice, MEHL decreased the activity of serum SGPT by 16.65% (100 mg/kg BW) and 26.75% (200 mg/kg BW), whereas glibenclamide reduced the activity of serum SGPT by 35.86%. The reduction of SGOT activity with the treatment of MEHL was 15.14% (100 mg/kg BW) and 19.25% (200 mg/kg BW), whereas glibenclamide lowered SGOT activity was 30.85%.

4. Discussion

Diabetes mellitus is one of the most common chronic diseases and is associated with hyperlipidemia and comorbidities such as obesity and hypertension (35). Many new bioactive phytochemicals isolated from plants having hypoglycemic and antihyperglycemic effects show the same antidiabetic activity and are sometimes even more potent than already known oral hypoglycemic agents (36,37). Therefore, finding new antidiabetic compounds with fewer adverse effects is very important. The present study investigated the *in vitro* and *in vivo*antidiabetic and antioxidant activity of methanolic crude extract of *H. schulli* (Buch.-Ham.) leaf in alloxan-induced diabetic mice.

All data of this study showed that MEHL contains significant antioxidant potential and possesses *in vitro* and *in vivo*antidiabetic and antioxidant activity. The most common characteristic of DM is increased blood glucose levels due to lack of insulin or insulin resistance (2). MEHL supplementation also reduced blood glucose levels significantly in diabetic mice compared to that of diabetic control mice; which is almost similar to the glibenclamide-treated diabetic rats (Figure 7). These results were consistent with separate findings of MEHL ingredients in some previous studies. (38–40); therefore our present study supports the results of the previous studies.

Hyperlipidemia is common in DM because DM affects lipid metabolism. In DM elevated level of TG, TC, LDL, and VLDL and decreased level of HDL is found, these anomalies leadto the development of cardiovascular diseases also(41,42)

Significantly decreased levels of serum TC, TG, LDL and VLDL, and increased levels of HDL in MEHL-treated diabetic mice compared to diabetic control mice were noted in this study. These data represent possible potential effects of MEHL to reduce or prevent DM-associatedcomplications in lipid metabolism.

Chronic DM also leads to liver damage by affecting the metabolism of lipids, carbohydrates and proteins, which can further progress to non-alcoholic steatohepatitis, liver cirrhosis, and finally hepatocellular carcinomas promoting oxidative stress inflammatory response (43). Liver damage causes increased activity of liver function enzymes, such as SGPT and SGOT in patients with DM. MEHL treatment for 21 days significantly reverted the levels of SGPT and SGOT in diabetic mice compared to that of diabetic control mice.

5. Conclusion

This research work suggests the effects of MEHL in controlling the complications of diabetes as well as alleviating the development of cardiovascular diseases. Moreover, MEHL eases liver damage from

chronic diabetes. Therefore, this study suggests antioxidant potential and possible beneficial effects of MEHL for the treatment of diabetes mellitus and diabetes mellitus-associated complications in the future.

7. Abbreviations

Not applicable

Declarations

Ethics approval and consent to participate

The experimental protocol was approved by the Institutional Animal, Medical Ethics, Bio-Safety and Bio-Security Committee (IAMEBBC) for Experimentations on Animal, Human, Microbes, and Living Natural Sources, Memo No: 249(35)/320/IAMEBBC/IBSc. Institute of Biological Sciences, University of Rajshahi, Bangladesh.

Consent for publication

Not applicable.

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7. Figure Legend

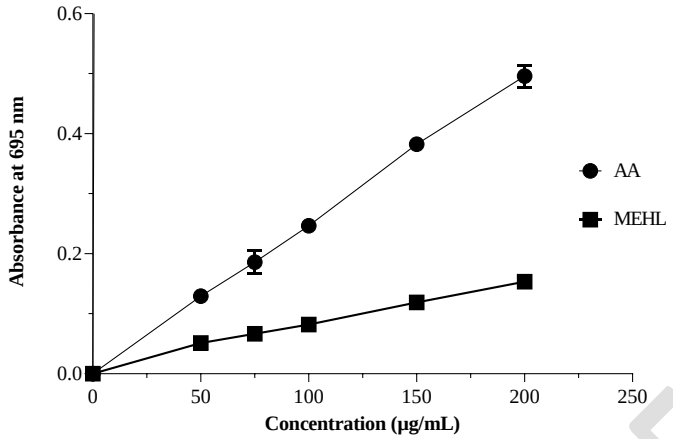

Figure 1: Total antioxidant capacity of MEHLand ascorbic acid (AA). Results are expressed as mean±SD of three parallel measurements.

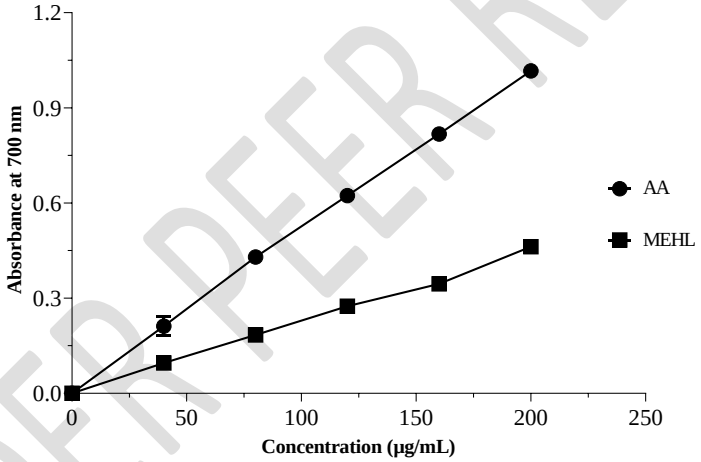

Figure 2: Ferric-reducing antioxidant activity of MEHLand ascorbic acid. Results are expressed as mean±SD of three parallel measurements.

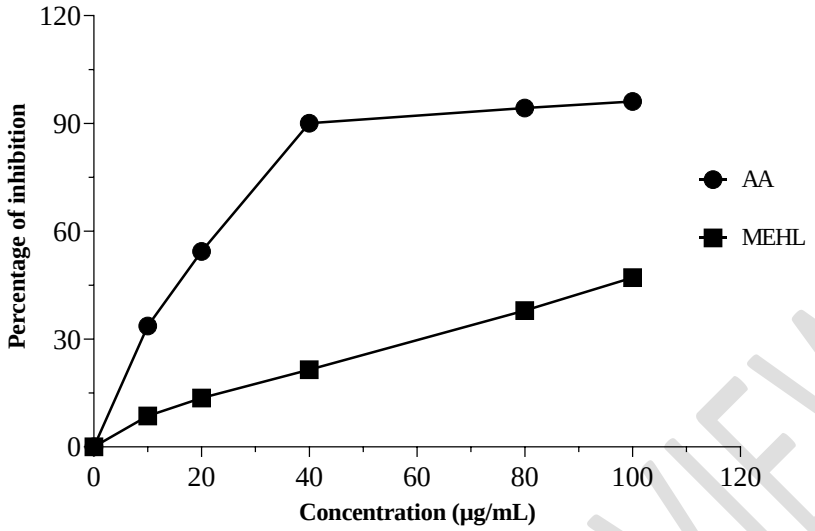

Figure 3: Scavenging activity of MEHLand ascorbic acid against DPPH radical. Results are expressed as mean±SD of three parallel measurements.

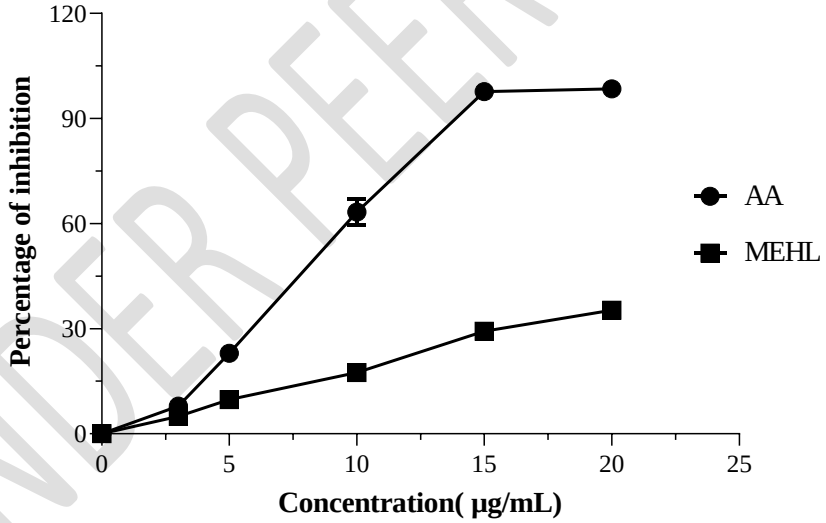

Figure 4: Scavenging activity of MEHLand ascorbic acid against ABTS radical. Results are expressed as mean±SD of three parallel measurements.

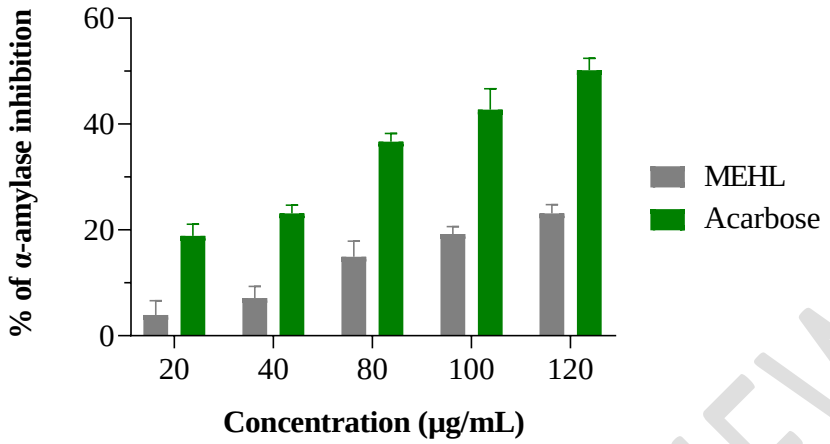

Figure 5: α-amylase inhibition activity of MEHL. Results are expressed as mean±SD of three parallel measurements.

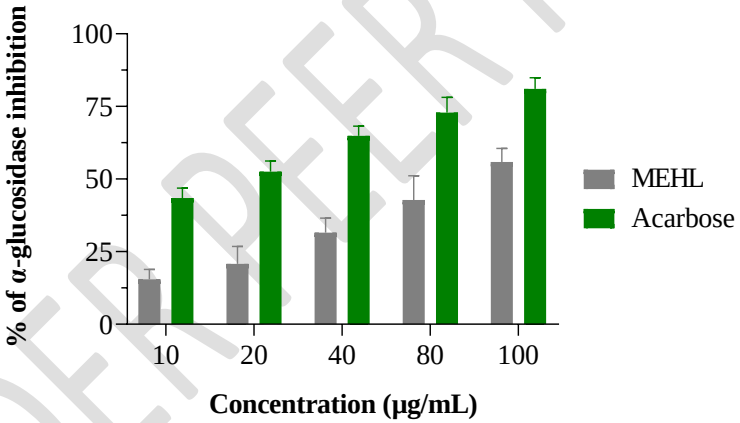

Figure 6: α-glucosidase inhibition activity of MEHL. Results are expressed as mean±SD of three parallel measurements.

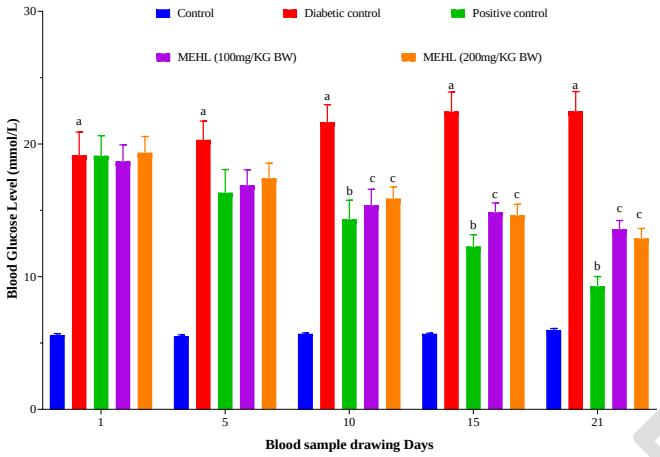

Figure 7: Change of blood glucose level after methanolic extract of *H. schulli*Leaf (MEHL) treatment in alloxan-induced diabetic mice.

[All data are expressed as mean ± SEM (n; number of mice = 5). Here "a" indicates $p < 0.0001$ vs normal control mice, "b" indicates $p < 0.001$ vs diabetic control mice and "c" indicates $p < 0.01$ vs diabetic control mice]

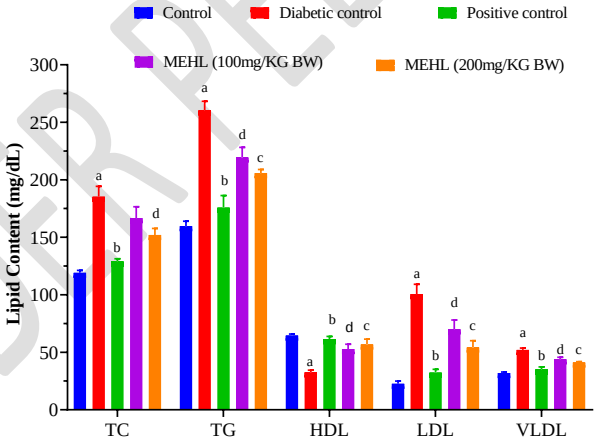

Figure 8: Effects of Methanol extract of *H. schulli*leaf (MEHL) on lipid profile of diabetic mice after 21 days of treatment.

[All data are expressed as mean ± SEM (n; number of mice = 5). Here "a" indicates $p < 0.0001$ vs normal control mice and "b" indicates $p < 0.001$ vs diabetic control mice, "c" indicates $p < 0.01$ vs diabetic control mice, "d" indicates $p < 0.05$ vs diabetic control mice]

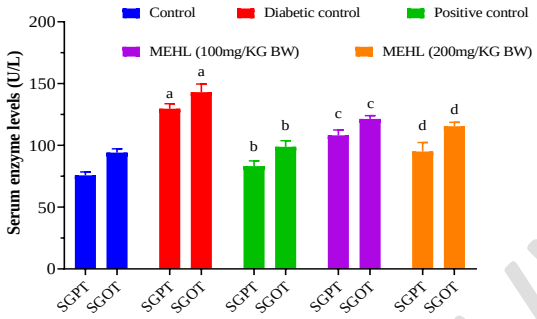

Figure 9: Effect of *H. schulli* leaf (MEHL) on serum SGPT and SGOT in diabetic mice after 21 days of treatment.

[All data are expressed as mean ± SEM (n; number of mice = 5). Here "a" indicates $p < 0.0001$ vs normal control mice, "b" indicates $p < 0.001$ vs diabetic control mice and "c" indicates $p < 0.05$ vs diabetic control mice, "d" indicates $p < 0.01$ vs diabetic control mice]