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THE EFFECT OF AQUEOUS EXTRACT OF *SMILAX PERFOLIATA* ON BLOOD GLUCOSE, LIPIDS AND SELECTED HEPATIC ENZYMES OF ALLOXAN-INDUCED DIABETIC MICE.

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ABSTRACT

The antidiabetic properties of aqueous extract of *Smilax perfoliata* (Smilacaceae) leaves on alloxan-induced diabetic mice is reported. The hypoglycemic activity, antihyperglycemic activity and Oral Glucose Tolerance Tests (OGTT) demonstrated that the extract possesses blood glucose lowering potential. Further, the extract treated diabetic mice displayed an overall improvement in the lipid profile and liver enzymes (AST and ALT) activity. *Smilax perfoliata* extract also exhibited DPPH radical scavenging activity *in vitro*. Total polyphenols, flavonoids, protein and carbohydrate content were assayed and found to be 63.45 mg GAE/g dry weight, 34.06 mg Rutin equivalents/g dry weight, 0.39 % and 40.68 %, respectively. The results from the experiments conducted show that aqueous extract of *Smilax perfoliata* displays potent antidiabetic and hypolipidemic activity and merit its inclusion as a natural product with therapeutic potential.

KEYWORDS: ***Smilax perfoliata*, antidiabetic, antihyperglycemic, hypolipidemic, AST, ALT.**

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INTRODUCTION

Diabetes mellitus is a group of disorders of heterogeneous etiology, characterized by chronic hyperglycemia and other metabolic abnormalities leading to macrovascular and microvascular complications[1,2]. Diabetes can be of various types, but according to the World Health Organization, the type named Type 2 includes the common major form of diabetes which results from defect(s) in insulin secretion, almost always with a major contribution from insulin resistance[3]. The disease affects many organs of the body and associated anomalies get worse with disease progression. Prolonged hyperglycemia can have deleterious effects and has been liked to oxidative stress or the accumulation of free radicals such as Reactive Oxygen Species (ROS)[4,5]. The increased production and/or ineffective scavenging of ROS play a critical role and therefore probable that antioxidants may contribute to the alleviation of diabetic complications[6]. Diabetes has been associated with a number of diseases and lifestyle conditions and one of the strongest links is to obesity. Obesity is a strong factor leading to diabetes and the risk of developing diabetes is doubled with a family history of diabetes[7]. Diabetes is also now considered to be a part of a group of disorders called as metabolic syndrome whose features, among others, include elevated triglyceride levels, low HDL (High Density Lipoprotein) cholesterol levels and insulin resistance[8]. It is also known that LDL (Low Density Lipoprotein) cholesterol oxidation increases in states of oxidative stress, and oxidized LDL is considered predictive of incident type 2 diabetes mellitus[9,10]. Further, increased serum levels of triglycerides and liver enzymes Aspartate transaminase (AST), (also called serum glutamic oxaloacetic transaminase or SGOT) and Alanine transaminase (ALT) (also called serum glutamic pyruvic transaminase or SGPT) are indicators of diabetes[8,11].

Natural products have been a contributing source of lead molecules in drug discovery, especially with the emerging modern tools of chemistry and biology[12,13]. Plants have formed the basis for traditional medicine systems for thousands of years in countries such as China and India[12]. Plants represent the richest source of inspiration for the identification of novel scaffold structures that can serve as the basis for drug design[12,15]. Plants with antidiabetic properties, in particular are generating a lot of interest primarily because the number of diabetics worldwide are fast reaching epidemic proportions, with the total number of people with diabetes projected to rise from 171 million in 2000 to 366 million in 2030[16]. The current available therapeutic options for non–insulin-dependent diabetes mellitus including various oral antidiabetic agents such as sulfonylureas, biguanides, α-glucosidase inhibitors, and glinides, with associated adverse effects have prompted the search for more effective and safer hypoglycemic agents[17]. According to Bailey and Day, traditional antidiabetic plants might provide a useful source of new oral hypoglycemic compounds for development as pharmaceutical entities, or as simple dietary adjuncts to existing therapies[18]. India is known for its plethora of medicinal plants, many of which have antidiabetic potential. Some examples of plants found in India which are reported to possess hypoglycemic and/or antihyperglycemic properties include *Momordica charantia*, *Ficus bengalensis, Flemingia macrophylla, Potentilla fulgens L., Ixeris gracilis, Osbeckia chinensis* L., *Albizzia lebbek*, *Curcuma amada, Coriandrum sativum* and *Gymnopetalum cochinchinensis*[19-28]. Further, as plants are rich sources of antioxidants, especially flavonoids and polyphenols[29,30], they may be useful in the search for newer and better drugs to manage diabetes and its complications. Various studies on the ethnobotanicity[31,32] and chemical constituents[33] of *S. perfoliata* have been described, with rutin (a flavonoid) being reported as one of the constituents in the EtOAc (ethyl acetate) portion of the 95% ethanolic extract of the plant's rhizoma. In this paper, the therapeutic potential of the aqueous extract of the leaves of *Smilax perfoliata*, a plant which is found in many parts of India, including Meghalaya, is reported.

MATERIALS AND METHODS

(i) Chemicals

Alloxan monohydrate and 1,1-diphenyl-2-picryl-hydrazil (DPPH·), were procured from Sigma Co., USA. Kits for AST and ALT assays were obtained from Crest Biosystems, India. Glucometer for blood glucose level determination as well as glucostix used were from SD check, India; glibenclamide from Aventis Pharma Ltd.; insulin from Torrent Pharmaceuticals Ltd. and metformin from USV Ltd. Kits for total cholesterol, HDL cholesterol, LDL cholesterol and triglyceride estimation were obtained from Span Diagnostics, India; while other chemicals used were of analytical grade.

(ii) Test animals

Healthy, adult female Swiss albino mice weighing 20-30 grams were used for the study. The mice were properly maintained on a 12 hour light/dark cycle under ambient conditions of temperature (22 °C) and fed balanced mice feed obtained from Pranav Agro Limited, New Delhi. All experiments were performed according to Institutional guidelines.

(iii)Preparation of diabetic mice

Diabetes was induced by the administration of alloxan monohydrate to mice according to Syiem *et al*[22]. Mice with two- to three-fold increase in blood sugar levels were considered diabetic and used for further tests.

(iv) Plant material

Smilax perfoliata leaves were obtained from Mawdiangdiang area of East Khasi Hills district of Meghalaya. The specimens were submitted and identified (Voucher No: NEHU-11918) by Dr. P.B. Gurung Curator Herbarium, Department of Botany, NEHU, Shillong, Meghalaya.

(v) Plant extraction

Leaves of *S. perfoliata* were separated, weighed, washed and dried in the shade. The dried leaves were finely powdered and the powdered mass was weighed and repeatedly extracted with 10 times the volume of solvent (distilled water) for 24 hours. Following filtration, the filtrate was evaporated to dryness in a Heto lyolab 3000 lyophilyzer and the dried mass obtained used for the required studies. All extracts were transferred into clean air-tight containers and stored at -20°C until used for experiments.

(vi) Preparation of extracts for use in experiments:

Prior to use for experiments, the appropriate amount of extract was weighed and dissolved in distilled water to give the desired concentration and used for experiments.

(vii) Hypoglycemic and antihyperglycemic studies

Normoglycemic mice were divided into six groups (Table 1). Each group comprised of a minimum of six mice. The mice were starved overnight but given water *ad libitum* prior to performing the experiment. To the test groups, varying doses of 250, 450, 650, 850 and 1000 mg/kg body weight (b.w.) of SP (*Smilax perfoliata)* extract were administered via intraperitoneal (i.p.) injection. The control group received only distilled water, being the vehicle used for dissolving the extract. Blood glucose level was monitored at different time intervals up to 24 hours following the extract administration. After determination of blood glucose level at 6 hours, food was given to the mice and blood glucose level was monitored again at 24 hours.

Table 1
Normoglyclemic mice grouped for hypoglycemic studies.

Serial Number	Group	Administered with
1	Control	Distilled water
2	Test (1)	SP Extract, 250 mg/kg body weight
3	Test (2)	SP Extract, 450 mg/kg body weight
4	Test (3)	SP Extract, 650 mg/kg body weight
5	Test (4)	SP Extract, 850 mg/kg body weight
6	Test (5)	SP Extract, 1000 mg/kg body weight

For the antihyperglycemic studies, alloxan-induced diabetic mice were administered (i.p.) a single dose of 250 mg/kg b. w. of the extract. This was the optimum dose determined in the normoglycemic studies. Blood glucose level was measured at varying time intervals for 24 hours.

(viii) Oral glucose tolerance test (OGTT) in normoglycemic and alloxan-induced diabetic mice

The oral glucose tolerance test and collection of blood for determination of blood glucose level were performed according to the protocol used earlier[22] with slight modification: glucose load of 3000 mg/kg b.w. was given. Mice were fed after measuring blood glucose level at 120 minutes and blood glucose level was monitored again at 1440 minutes (24 hrs). Insulin, glibenclamide and metformin were the standard drugs used in the present study[22].

(ix) In vitro assays for total polyphenol, flavonoid, protein and carbohydrate content

Total polyphenol content, expressed as mg GAE/g dry weight (milligram gallic acid equivalents per gram dry weight) in the various plant extracts was assayed using Folin-Ciocalteau reagent according to Singleton, Orthofer and Lamuela Raventos[34]. Flavonoid estimation was followed according to the protocol of Lamaison and Carnat[35] and the flavonoid content was expressed as mg Rutin equivalents/g dry weight. Carbohydrates were estimated using anthrone reagent[36] while Bradford's dye binding method[37] was employed for the quantification of proteins. Both were expressed as percentage (%).

(x) DPPH radical scavenging activity of plant extracts

Free radical scavenging activity of the plant extracts was ascertained by the ability of the extracts to scavenge the DPPH radical, according to the method of Brand-Williams *et. al.*[38]. Various concentrations of the plant extract were reacted with DPPH radicals in methanol. The mixture was mixed vigorously and incubated for 30 minutes at room temperature, after which the absorbance of the test samples and control were then recorded at λ517 nm. The radical scavenging activity was expressed using the IC_{50} value which corresponds to amount of antioxidants necessary to decrease the initial DPPH absorbance by 50%.

(ix) Assessment of lipid profile and activities of liver enzymes Aspartate transaminase (AST) and Alanine transaminase (ALT)

For the elucidation of hypolipidemic properties and the effect of the extract on the liver enzymes AST and ALT; total cholesterol, HDL and LDL cholesterol, triglycerides, AST and ALT levels were determined using commercially available kits. In all cases, manufacturer's instructions were followed. For each set of experiments, alloxan-induced diabetic mice were grouped into three groups, each group comprising of a minimum of 6 mice. In addition, another group comprising of normoglycemic mice treated with distilled water served as the normal control and used as a reference group for representing values of the enzymes and lipids (total cholesterol, HDL and LDL cholesterol, triglycerides) in normoglycemic conditions. The route of administration (once daily injections) was i.p. in all cases. The details of the pattern of grouping is shown in table 2.

Table 2
Mice groupings for studying lipid profile and enzyme assays.

Group	Administered with
1-Normoglycemic	Distilled water
2-Diabetic	Distilled water
3-Diabetic	250 mg/kg b.w. SP extract
4-Diabetic	10 mg/kg b.w. glibenclamide

After the treatment period of 12 days[39,40], mice were sacrificed by cervical dislocation under local anesthesia and blood collected by retro orbital puncture. The serum obtained was then used for the various assays. AST and ALT enzyme activity were reported as U/L where one unit (U/L) is defined as the amount of enzyme that catalyzes the transformation of one micromole of substrate per minute under specified conditions.

STATISTICAL ANALYSIS

Student's't'-test was used for determining the levels of significance between the normal, diabetic control and the diabetic treated groups. Results are expressed as mean ± standard error of mean (S.E.M.).

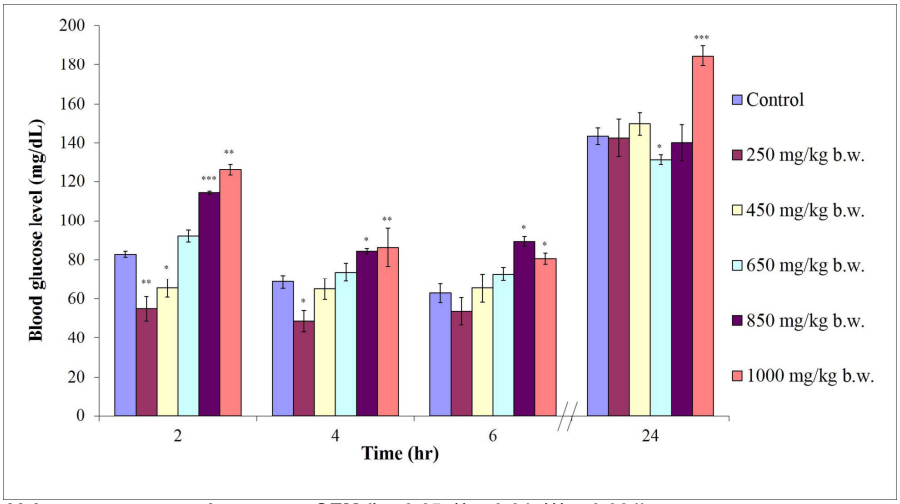
RESULTS

1. Hypoglycemic activity

The aqueous extract of *S. perfoliata* exhibited mild hypoglycemic activity (Figure 1). Although different doses were employed for the experiment, the response was not dose dependent. The extract exhibited hypoglycemic activity at the lower doses of 250 (33%, $p<0.01$) and 450 (29%, $p<0.05$) mg/kg b.w. at 2 hours after administration of extract. The other doses (650, 850 and 1000 mg/kg b.w.) failed to exert any hypoglycemic effect when compared with the control group. The dose of 250 mg/kg b.w. continued to demonstrate glucose lowering activity at 4 hours after extract administration, decreasing glucose level by 29% ($p<0.05$) while the higher doses could not lower glucose level as efficiently as the dose of 250 mg/kg b.w. at 4 hours following administration of SP extract.

Figure 1

Effect of varying doses (250, 450, 650, 850 and 1000 mg/kg b.w.) of aqueous extract of S. perfoliata on blood glucose of normoglycemic mice at different time intervals.

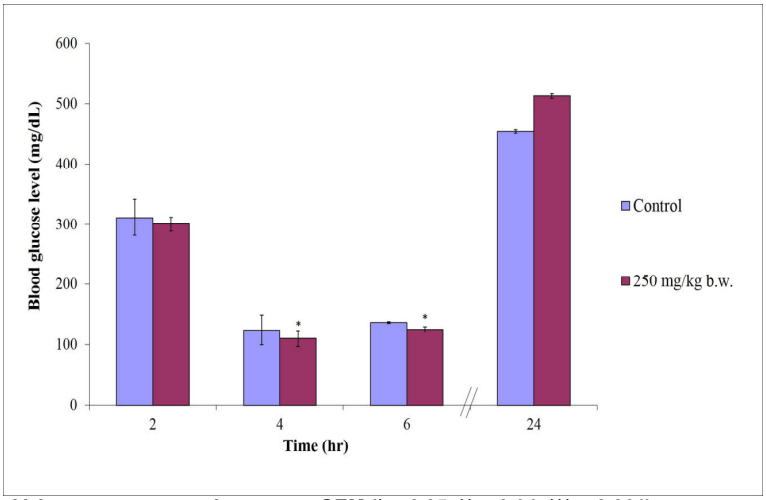


Values are expressed as mean ±SEM (*p<0.05, **p<0.01, ***p<0.001).

2. Antihyperglycemic activity

Based on the hypoglycemic studies, the dose of 250 mg/kg b.w. was taken for the antihyperglycemic studies. The aqueous extract of *S. perfoliata* exhibited antihyperglycemic activity (Figure 2), bringing about a reduction in blood glucose by 11% ($p<0.05$) at 4 hours and 8% ($p<0.05$) at 6 hours after administration of extract.

Figure 2
Effect of aqueous extract of S. perfoliata (250 mg/kg b.w.) on blood glucose of alloxan-induced diabetic mice at different time intervals.

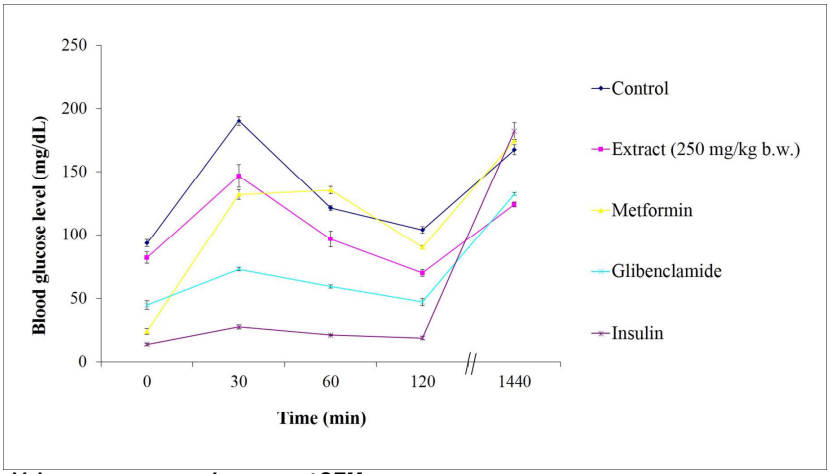


*Values are expressed as mean ±SEM (*p<0.05, **p<0.01, ***p<0.001).*

3. Oral glucose tolerance test (OGTT)

In the normoglycemic OGTT experiment (Figure 3), the test group exhibited better glucose tolerance at 30 minutes after glucose load than the control group. The effect of the extract on glucose load was most pronounced at 120 minutes with a glucose peak suppression of 32% (p<0.001). Significantly, the aqueous extract of *S. perfoliata* showed better glucose lowering activity than the standard biguanide metformin at 60 minutes (20% versus metformin 11%) and 120 minutes after glucose load (32% versus metformin 13%).

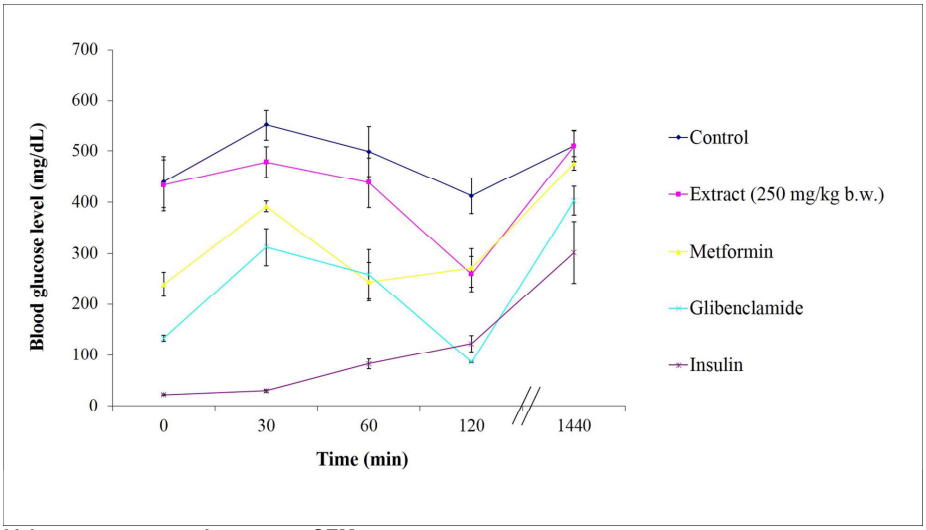
Figure 3
Glucose tolerance in normal mice administered with 250 mg/kg b.w. aqueous extract of S. perfoliata and reference drugs.



Values are expressed as mean ±SEM.

Diabetic mice treated with the extract displayed a better tolerance for glucose as compared to the control group (Figure 4). *S. perfoliata* caused some suppression of the glucose peak (13%, p<0.001) at 30 minutes after glucose load. Inhibition of glucose elevation was similar to that of metformin at 120 minutes after oral glucose load (37%, p<0.01 versus 34%, p<0.01 of metformin). The extract could not, however, lower glucose to levels attained by insulin.

Figure 4
Glucose tolerance in alloxan-induced diabetic mice administered with 250 mg/kg b.w. aqueous extract of S. perfoliata and reference drugs.



Values are expressed as mean ±SEM.

4. Total polyphenol, flavonoid, carbohydrate and protein content

The total polyphenol and flavonoid content in the aqueous extract were found to be 63.45 mg GAE/g dry weight and 34.06 mg Rutin equivalents/g dry weight, respectively. Carbohydrate content was higher than that of protein (40.68 % and 0.39 % respectively) (Table 3).

5. DPPH radical scavenging activity

The extract displayed an IC_{50} of 185.69 µg/ml (Table 3), which reflects its ability to scavenge the DPPH free radical.

Table 3
Total polyphenol, flavonoid, protein and carbohydrate content of aqueous extract of S. perfoliata; and DPPH radical scavenging activity.

Total polyphenol (mg GAE/g dry weight)	63.45 ± 1.33322
Flavonoid (mg Rutin equivalent/g dry weight)	34.06 ± 0.207657
Protein (%)	0.39 ± 0.00012562
Carbohydrate (%)	40.68 ± 0.003123
DPPH radical scavenging (IC_{50}) (µg/ml)	185.69 ± 2.84835776

Results are expressed as mean ±SEM.

6. Effect on lipid profile

The amount of total cholesterol increased in diabetic control compared to the normoglycemic group (Table 4). Both *S. perfoliata* aqueous extract and glibenclamide could significantly lower total cholesterol levels in diabetic mice. HDL cholesterol, which was lowered in diabetic mice when compared to the normal reference group increased in the test group treated with the extract. Results were comparable to those produced by glibenclamide. A significant reduction of 46 % ($p<0.001$) in serum LDL levels was observed in diabetic mice treated with the extract. Triglyceride levels, which had increased dramatically in diabetic mice, were brought down to levels comparable to those of the normoglycemic group in diabetic mice treated with the aqueous extract (61%, $p<0.001$). Significantly, the reduction in triglyceride levels was greater in the extract treated than the glibenclamide (22%, $p<0.05$) treated diabetic group (Table 4).

7. Effect on liver enzymes AST and ALT

Overall, alloxan-induced diabetic mice displayed a higher activity of both liver enzymes (AST and ALT) (Table 4). The aqueous extract of *S. perfoliata* could not produce any significant changes in the activity of AST, but significant reduction in serum ALT activity (43%, $p<0.01$) was observed. The standard reference drug glibenclamide, however, had no significant effect on serum ALT levels.

Table 4
The levels of total cholesterol, HDL and LDL cholesterol, triglycerides, AST and ALT in various groups of mice.

Group	Total cholesterol (mg/dL)	HDL cholesterol (mg/dL)	LDL cholesterol (mg/dL)	Triglycerides (mg/dL)	AST (SGOT) (U/L)	ALT (SGPT) (U/L)
Normoglycemic	72.5	69.5	7.25	45	135	95
Diabetic	134.5	23	89.65	99.75	183	130
Diabetic (extract treated)	96.59[c]	39.75[b]	48.09[c]	38.75[c]	182[d]	73.67[b]
Diabetic (glibenclamide treated)	96[a]	39.75[a]	38.85[b]	78.25[a]	224.34[c]	140.34[d]

Results are expressed as mean ± SEM ([a]p<0.05, [b]p<0.01, [c]p<0.001, [d]p = not significant), compared to the diabetic control group.

DISCUSSION

The aqueous extract of *Smilax perfoliata* displayed very mild hypoglycemic activity which was not observed to be dose or time dependent. The antihyperglycemic and glucose tolerance studies indicate that the aqueous extract of *Smilax perfoliata* possesses antihyperglycemic activity. It may be noted that the dose of 250 mg/kg b.w. did not bring about a significant change in blood glucose level of normoglycemic mice at 6 hours following extract administration, but could alter blood glucose levels in diabetic mice at this same time interval. Many plants are reported to be antihyperglycemic agents by affecting insulin secretion, sensitizing cells to or even mimicking insulin,[41,42] and these results suggest that the aqueous plant extract may possess constituents which can act through any one or a combination of pathways to bring about a reduction in blood glucose level. Notably, while the methanolic extract of *S. perfoliata* has been reported to lower blood glucose level, the studies conducted used normal mice as a model[43]. In the experiments which we had conducted, alloxan-induced diabetic mice were used, which is the commonly accepted model for diabetes study[44]. The finding that the aqueous extract of *Smilax perfoliata* produced significant decrease in serum ALT activity but no changes in serum AST activity is highly noteworthy since higher ALT values are associated with both IGT (impaired glucose tolerance) and diabetes while AST levels are reportedly unrelated to variations in hepatic insulin action[11]. Our findings also indicate that the aqueous extract may have hepatoprotective effects, as elevated levels of ALT are associated with liver injury and diseases linked to insulin resistance like non-alcoholic fatty liver disease[45,46]. Diabetic dyslipidemia, which is associated with type 2 diabetes is characterized by a high concentration of triglycerides and small dense LDL cholesterol, together with a low concentration of HDL cholesterol[47]. People who are insulin resistant have poor ability to store triglycerides[2]. The extract's ability to bring down the levels of triglycerides in diabetic mice are therefore positive indications of its effect on lipid metabolism. Moreover, the aqueous extract of *S. perfoliata* also caused a substantial increase in the level of HDL cholesterol, which is considered as the "good" cholesterol[48]. The extract also displayed the ability to counter the increase in the concentrations of LDL cholesterol seen to be elevated in the diabetic control group. Since the role of free radical scavenging in biological

systems is delegated to antioxidants, the observed ability of the plant extract to scavenge DPPH radicals may be attributed to the presence of antioxidants like polyphenols and flavonoids. Many plants with antidiabetic properties have also been reported to possess antioxidant potential[49-51]. A related finding is that the solubility of polyphenols changes with different solvent systems[52]. The results reported here may thus not be a complete assessment of the total content of polyphenols in the plant, *per se,* but rather its concentration in the aqueous extract of the plant.

CONCLUSION

The aqueous extract of *Smilax perfoliata* exhibited hypoglycemic and antihyperglycemic activity, with effective glucose tolerance improvement capability. Its hypolipidemic and ALT lowering effects complement its glucose lowering property. Further, the ability of the extract to scavenge free radicals are positive indicators of its therapeutic potential.

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