



Antidiabetic Activity of Methanol Extract and Fractions of *Thymus schimperii* Ronniger Leaves in Normal and Streptozotocin Induce Diabetic Mice

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ABSTRACT

To study the anti-diabetic effects of the crude leaves extract and fractions of *Thymus schimperii* Ronniger in normal and streptozotocin induced diabetic mice. The crude extract and the fractions were screened for antidiabetic activities in streptozotocin induced diabetic mice. A normoglycemic mice model and oral glucose tolerance test were also undertaken to assess the hypoglycemic and antihyperglycemic effect of the crude extract in normoglycemic and glucose loaded mice, respectively. The methanolic crude extract has significantly reduced blood glucose level in streptozotocin induced diabetic mice at all given doses compared to the negative control and the percentage reduction observed was in a dose dependent manner i.e. [250 mg/kg (14.76±6.1%), 500 mg/kg (25.12±11.5%) and 750 mg/kg (27.15±10.0%)]. The crude methanol extract was devoid of hypoglycemic effect in normoglycemic mice but significantly reduced post prandial hyperglycemia starting from 1 h post glucose administration. Among the fractions, higher percentage reduction was recorded in the n-butanol fraction at a dose level of 500 mg/kg (36±7.3%) compared to 250 mg/kg (22.2±4.3%). The aqueous fraction 250 mg/kg and 500 mg/kg also reduced the blood glucose level by 17.6±6.0% and 18.4±5.0%, respectively. This study revealed that methanol extract as well as butanol and aqueous fractions of *T schimperii* possess anti-diabetic activity.

Conflicts of Interest: Declared None

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Keywords

Thymus schimperii Ronniger,
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INTRODUCTION

Diabetes Mellitus is manifested by persistent hyperglycaemia and other complications including the development of retinopathy, nephropathy, heart attack, stroke and peripheral arterial disease [1]. The current approaches to the management of diabetes mellitus include administration of insulin and/or oral anti-diabetic drugs together with lifestyle modification and dietary therapy [2]. However, these drugs are associated with many adverse reactions including gastrointestinal disturbance, blood dyscrasia, hypersensitivity reactions, and lactic acidosis [3].

Besides, a recent study indicated that up to 2.5% and 17.5% of sulphonyl urea treated patients experience major and minor hypoglycemia, respectively [4]. Body weight increases of 1 to 5 kg are also common with both sulphonylurea and thiazolidindiones therapy. Hence, there exists a huge need, among the scientific community, to develop new anti-diabetic drugs. With this regard, it's believed that medicinal plants could be potential sources of anti-diabetic drugs.

Thymus schimperii Ronniger (Fam. Lamiaceae) is a

perennial herb, locally known as Tosign in Amharic and is endemically found in Ethiopia. An ethnobotanical study reported that *T. schimperi* has been traditionally used for the treatment of diabetes in Ethiopia [5]. So far, the plant was pharmacologically evaluated for its diuretic, anti-hypertensive effect [6] and antimicrobial effect [7]. Besides, another study showed the blood glucose lowering effects of the leaves in alloxan-induced diabetes mice model [8]. The aim of the present study was to investigate the antidiabetic effects of the methanol leaves extract and its fractions (ethyl acetate, n-butanol and aqueous) in streptozotocin induced diabetic mice model. The herbal extract was also evaluated for the hypoglycemic nature and post prandial glucose lowering potential in normal and glucose loaded mice, respectively.

MATERIALS AND METHODS

Animals

Swiss albino mice of either sex weighing 20-30g were used with the approval of Research Ethics Committee of the College of Health Sciences, Mekelle University (ERC 1028/2017). Animals were kept in plastic cages, acclimatized to the laboratory condition at room temperature under naturally illuminated environment of a 12 h light: 12 h dark cycle. Animals were fed a standard pellet and water was given *ad libitum*.

Plant materials and preparation of extracts and solvent fractions

Thymus schimperi Ronniger (Lamiaceae) were collected from Gudoberet, North Shoa Zone, Amhara region, Ethiopia. The plant was authenticated and the voucher specimen was deposited at the National Herbarium, Department of Biology, College of Natural Sciences, Addis Ababa University, Ethiopia (AS001/2016).

The plant leaves were dried under shade and crushed to coarse powder using electrical grinder. The powdered leaves (800g) were extracted with 80% methanol by maceration method with a continuous shaking in orbital shaker for 6 h at room temperature. The residue was removed by filtration and the process was continued for three times till the marc changes to colorless. A rotary evaporator was used to dry the combined filtrate at 40°C under reduced pressure. The approximate yield of methanol extract was 14.63%. The extract was administered orally after dissolving in distilled water. The leaves extract of *T. schimperi* was screened for the presence of phytochemicals and showed positive result for alkaloids, phenolic compounds, flavonoids, saponins, terpenoid and tannins. 30g of methanol leaves extract was further fractionated using solvents in increasing polarity: n-hexane, ethyl acetate and n-butanol. Thus the obtained fractions were kept in glass bottles and stored in a refrigerator for future investigation.

Acute oral toxicity test

Five female albino mice weighing 20-25 g were fasted for 4 h and orally administered 2000 mg/kg b.w. of the

extract. Mice were observed continuously for the first 4 h and then periodically up to 24 h for toxic manifestations like: drowsiness, restlessness, writhing, convulsion, piloerection and mortality if any and the observation was continued for two weeks [9].

Induction of diabetes in mice

Diabetes was induced in mice as per the methods previously described [10, 11]. Swiss albino mice were allowed to fast for 8 hours and a single dose (150 mg/kg i.p.) of streptozotocin dissolved in 0.1 M sodium citrate buffer was injected to them. Blood glucose level of each animal was determined after 72 hours and animals with a fasting blood glucose range above 200 mg/dl were selected for the study. These diabetic mice were divided into five groups, each containing six animals (n=6): Group II, (untreated mice) given 0.1 ml distilled water, Group III, diabetic mice given 0.66 mg/kg glibenclamide, Groups IV, V and VI were treated with 250, 500 and 750 mg/kg of methanol extract of *Thymus schimperi* leaves respectively. Group I (normal control) received 0.1 ml distilled water. Treatments were given orally daily for fifteen days and their body weight and blood glucose levels were measured at day 0, 5, 10 and 15.

Evaluation of hypoglycemic activity in normoglycemic mice

Hypoglycemic effect of methanol extract of *Thymus schimperi* leaves was evaluated on five groups of normal mice (n=6) fasted for 8 hours but allowed free access to water [11]. Group I (normal control) received (0.1 ml *p.o.*) distilled water, Group II, received glibenclamide (0.66 mg/kg *p.o.*), Groups III, IV and V were treated with 250, 500 and 750 mg/kg of methanol extract of *Thymus schimperi* leaves respectively. Blood glucose levels were assessed at 0, ½ h, 1 h, 2 h, and 3h intervals by using a One Touch Glucometer (Prodigy Autocode, USA).

Oral glucose tolerance test

This test was undertaken based on the previously described method with little modification [12]. Mice were fasted for 8 hours and divided into six groups (n=6). First two groups were received 0.1 ml distilled water orally. Group III, treated with standard drug glibenclamide (0.66 mg/kg, *p.o.*), Groups IV, V and VI were treated with 250, 500 and 750 mg/kg of methanol extract of *Thymus schimperi* leaves respectively. Glucose solution (2.5 g/kg, *p.o.*) was administered orally to each group 30 minutes prior to the administration of plant extract. Blood glucose levels were estimated at 0, ½ h, 1 h, 2 h, and 3h of post glucose administration using One Touch Glucometer.

Evaluation of antihyperglycemic effect of fractions in streptozotocin induced diabetic mice

Animals were divided into nine groups (n=6) and the fractions of *Thymus schimperi* leaves extract were given orally. Group I, normal control received 0.1 ml distilled water, Group II served as diabetic control and took 0.1 ml

distilled water, Group III, positive control treated with glibenclamide (0.66 mg/kg) daily and each group of IV to IX were treated with two doses of each fraction (250 and 500 mg/kg) of ethyl acetate, n-butanol and aqueous, respectively. The animals were then observed for 15 days by measuring their body weight and blood glucose levels at day 0, 5, 10 and 15 [13].

Statistical analysis

Statistical analysis was done using one-way analysis of variance (ANOVA) followed by the Tukey post hoc test. P value <0.05 was considered statistically significant. Data analysis was performed using SPSS software package Version 20.0.

RESULTS

Acute oral toxicity study

In the acute toxicity study, the methanol extract of *T. schimperi* leaves did not produce significant changes in behavior nor caused mortality up to a dose of 2000 mg/kg b.w. in female mice during the first 24 h as well as in the following 14 days of observation.

Hypoglycemic effect in normal mice

The blood glucose levels of the glibenclamide treated group showed statistically significant difference compared to the normal control group and the extract treated groups ($p < 0.05$) (Table 1). The extract treated groups did not show any significant differences within 3 hours of treatment ($p > 0.05$), whereas the positive control group showed statistically significant variation after 3 hour of administration ($p < 0.05$).

Oral glucose tolerance test in mice

All extract treated groups significantly reduced the raised blood glucose starting 1 h post administration till 3 h,

compared to the diabetic control group. Glibenclamide treated group showed the highest significant ($p < 0.05$) reduction during this period (Table 2). The percentage reduction of blood glucose levels for 250, 500 and 750 mg/kg doses of extract at post 3 h administration of the extract compared with the blood glucose levels obtained at 30 minute post glucose administration of each group were 56.4, 49.16 and 46.98 %, respectively (Table 2).

Effects on fasting blood glucose level of streptozotocin induced diabetic mice

The fasting blood glucose levels of streptozotocin induced diabetic mice were increased (355.33 ± 32.55) when compared to the normal control (160.00 ± 14.40) (Table 3; Fig. 1). Besides, the fasting blood glucose levels were significantly reduced ($p < 0.05$) at day 10 and 15 in diabetic mice treated with 500 mg/kg and 750 mg/kg methanol extract of *T. schimperi* where as those treated with 250 mg/kg showed a significant reduction only at day 15 (Table 3). The finding also revealed that the extracts showed a dose dependent percent reduction in fasting blood glucose levels: 14.76% (250 mg/kg), 25.12% (500 mg/kg) and 27.15% (750 mg/kg) on day 15 (Fig. 1).

The result of this study also showed that the aqueous fraction produced a significant reduction of fasting blood glucose levels at doses of 250 mg/kg and 500 mg/kg at day 15 and the butanol fraction showed a significant reduction in the fasting blood glucose levels at days 10 and 15 (Table 4). The peak percentage reduction (33.85%) was observed in the group treated with 500 mg/kg butanol fraction followed by the aqueous fraction at a dose of 500 mg/kg (19.04%). Aqueous fraction at dose 250 mg/kg reduced blood glucose by 17.49% as well (Fig. 2).

Effects on body weight of streptozotocin induced diabetic mice

Table 1. Hypoglycemic effect of the crude leaf extract of *Thymus schimperi* in normoglycemic mice

Treatment groups	BGL (mg/dl) during the treatment period				
	0 h	½ h	1 h	2 h	3 h
Normal control	145.40±8.57	144.80±9.74	154.20±7.65	152.60±9.37	151.60±5.59
GB 0.66 mg/kg	163.80±9.85	95.80±5.44*	94.40±4.99*	88.60±3.17*	88.40±1.63*
TS250 mg/kg	172.20±3.37	175.60±10.03	164.60±10.87	167.00±9.38	163.40±6.97
TS500 mg/kg	175.00±14.70	173.40±16.51	164.20±13.99	171.40±6.09	171.20±5.00
TS750 mg/kg	166.00±4.34	159.00±5.36	154.80±2.43	150.40±3.44	150.00±6.38

Results are expressed in Mean ± S.E.M and all data were analyzed using ANOVA followed by Turkey's test for multiple comparisons for means. n = 6, * when $p < 0.05$ versus normal control (TS= *Thymus schimperi*, GB= Glibenclamide, BGL = Blood glucose level)

Table 2. The effect of the crude leaves extract of *Thymus schimperi* in glucose loaded mice

Groups	BGL (mg/dl) during the treatment period				
	0 h	½ h	1 h	2 h	3 h
Normal control	149.20±7.10	151.00±10.45	154.40±7.85	154.80±6.23	152.00±5.67
Diabetic control	161.20±8.05	289.40±11.46	228.40±18.43	216.40±16.50	207.00±13.39
GB 0.66 mg/kg	161.80±8.11	264.60±6.95	144.20±8.59*	97.60±2.62*	94.40±3.89*
TS250 mg/kg	176.00±6.40	289.40±11.47	184.20±6.46*	135.00±11.94*	126.20±10.58*
TS500 mg/kg	159.80±10.76	286.80±5.30	180.40±12.31*	156.00±8.89*	145.80±10.05*
TS750 mg/kg	151.20±10.90	291.60±13.73	180.40±5.53*	158.40±6.18*	154.60±5.68*

Results are expressed in Mean ± S.E.M and all data were analyzed using ANOVA followed by Turkey's test for multiple comparisons for means. n = 6, * when $p < 0.05$ versus negative control (TS= *Thymus schimperi*, GB= Glibenclamide, BGL = Blood glucose level)

Table 3. Effects of the crude extract of leaves of *Thymus schimperi* on BGL (mg/dl) in STZ induced diabetic mice

Treatment groups	BGL (mg/dl) during the treatment period			
	Day 0	Day 5	Day 10	Day 15
Normal Control	160.00±14.40	151.17±9.23	169.50±7.58	156.50±4.20
Diabetic control	355.33±32.55	368.00±24.63	393.17±28.50	397.50.83±29.52
GB 0.66 mg/kg	326.00±45.01	225.50±28.21**	207.67±28.49**	174.83±28.82**
TS 250mg/kg	337.50±32.61	324.83±19.15	304.50±12.27	287.67±20.76**
TS 500mg/kg	363.67±41.73	331.33±37.17	284.33±42.20**	272.33±41.85**
TS 750mg/kg	345.00±39.58	286.17±34.04	264.67±29.39**	251.33±34.67**

Results are expressed in Mean ± S.E.M and all data were analyzed using ANOVA followed by Turkey's test for multiple comparisons for means. n = 6, *when $p < 0.05$ versus Diabetic control and ** $p < 0.01$ (TS= *Thymus schimperi*, GB= Glibenclamide, BGL = Blood glucose level)

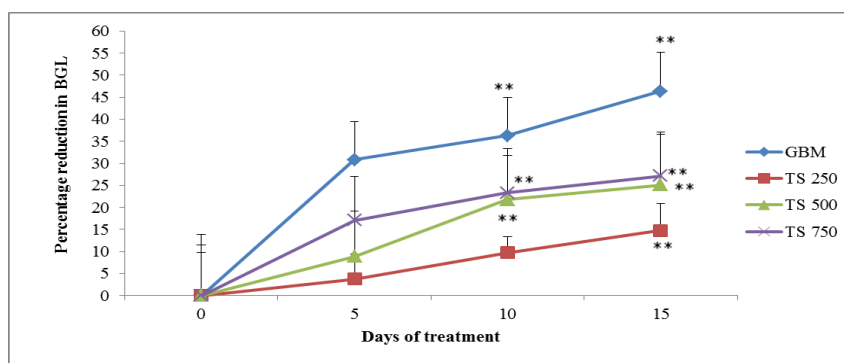


Figure 1. Effects of methanol extract of leaves of *Thymus schimperi* (250, 500, & 750 mg/kg, *p.o*) on percent reduction of BGL in STZ induced diabetic mice. Results are expressed in Mean ± S.E.M and all data were analyzed using ANOVA followed by Turkey's test for multiple comparisons for means. n = 6, * when $p < 0.05$ versus Diabetic control and ** $p < 0.01$ (TS= *Thymus schimperi*, GBM= Glibenclamide, BGL=Blood glucose level)

In this study, the negative control showed 13% loss in body weight and the normal control group showed a 24.67 % increase during the treatment period (Fig. 3). The body weight of diabetic mice receiving glibenclamide showed a 15.21% improvement in their body weight. A dose dependent body weight increment was observed in diabetic mice treated with the different doses of the extract (250, 500 and 750mg/kg): 9.79%, 9.99 % and 11.67 %, respectively (Figure 3). Similarly, a dose dependent body weight increment was also observed in diabetic mice treated with n-butanol fraction i.e. 17.24% (250 mg/kg) and 18.31% (500 mg/kg) (Fig. 4).

DISCUSSION

Diabetes mellitus is one of the rapidly growing endocrine

disorders with major complications affecting populations living throughout the world [14]. The pathophysiological mechanisms are being scrutinized and the knowledge on heterogeneity and complexity of this disease is being advanced. Accordingly, the search for more appropriate therapy is also being under way. In line with that, traditional medicines are used substantially by diabetic patients across the globe [15] and medicinal plants have been identified to be a target for scientists to come up with newer and better therapeutic options in the future. Streptozotocin (STZ) was used to induce diabetes in the current study. The toxic effect of the chemical is with its metabolic products and the free radicals generated, which finally destroy the pancreatic β -cells by alkylating DNA, impairing mitochondrial system and inhibiting O-GlcNAcase. It is a nitric oxide (NO) donor

Table 4. Effects of fractions of leaves of *Thymus schimperi* in fasting BGL (mg/dl) of STZ induced diabetic mice

Groups	BGL (mg/dl) during the treatment period			
	Day 0	Day 5	Day10	Day 15
Normal control	160.60±17.62	163.60±18.50	199.60±10.87	161.60±2.90
Diabetic control	393.20±32.59	388.40±28.91	402.66±30.93	398.20±26.16
Positive control GB0.66 mg/kg	333.00±18.31	235.80±11.61**	208.00±34.45**	161.60±23.40**
Ethyl acetate fraction	250mg/kg 347.00±30.51	325.20±32.02	324.60±35.76	329.20±23.65
500mg/kg	354.20±18.10	334.80±7.30	330.60±21.89	331.20±19.78
n-butanol fraction	250mg/kg 337.80±20.64	306.40±30.22	273.00±23.34**	266.20±17.67**
500mg/kg	384.60±38.47	321.00±34.30	275.40±33.66**	254.40±30.77**
Aqueous fraction	250mg/kg 355.60±32.96	335.00±42.21	320.80±26.02	293.40±20.38**
500mg/kg	357.20±24.86	319.60±20.16	312.80±27.23	289.20±20.86**

Results are expressed in Mean ± S.E.M and all data were analyzed using ANOVA followed by Turkey's test for multiple comparisons for means. n = 6, * when $p < 0.05$ versus Diabetic control and ** $p < 0.01$ (GB= Glibenclamide, BGL = Blood glucose level)

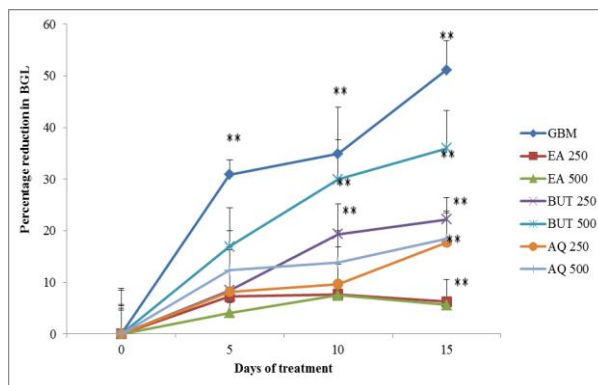


Figure 2. Effects of fractions of leaves of *Thymus schimperi* in fasting blood glucose level of STZ induced diabetic mice. Results are expressed in Mean \pm S.E.M and all data were analyzed using ANOVA followed by Turkey's test for multiple comparisons for means. $n=6$, * when $p<0.05$ versus Diabetic control and ** $p<0.01$. (GBM= Glibenclamide, EA 250/500 = ethyl acetate fractions 250/500mg/kg, BUT 250/500= n-butanol fractions 250/500 mg/kg, AQ 250/500 = aqueous fractions 250/500mg/kg).

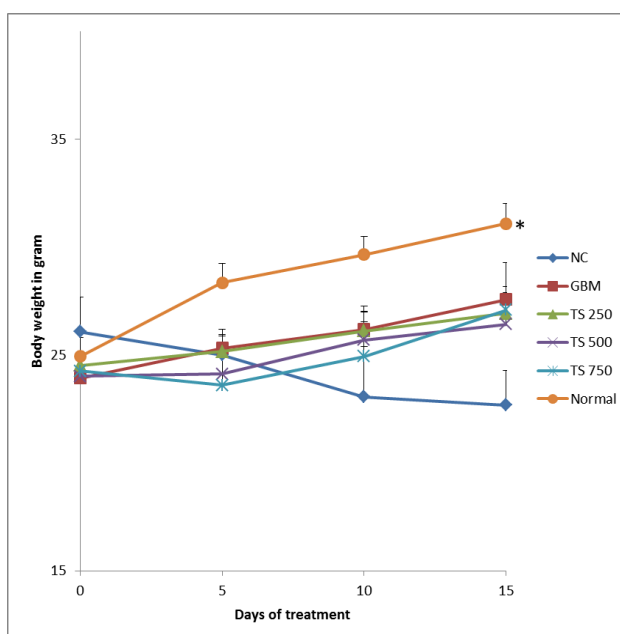


Figure 3. The effect of methanol extract of leaves of *Thymus schimperi* (250, 500, & 750 mg/kg, *p.o*) on body weight of diabetic mice. Results are expressed in Mean \pm S.E.M and all data were analyzed using ANOVA followed by Turkey's test for multiple comparisons for means. $n=6$, * when $p<0.05$ versus Negative control (NC= Negative Control, TS= *Thymus schimperi*, GBM= Glibenclamide)

and it's indicated that NO contributes for the degeneration of the Langerhans islets beta cells [16]. In the present study, STZ induced persistent hyperglycemia and brings about a significant weight loss in experimental mice and it was consistent with many other studies. The loss in body weight is due to the fact that polyphagic condition and loss of weight tends to promote excessive catabolism of fats and tissue proteins as an alternative energy source because of unavailability of carbohydrates [17]. In this study, oral administration of *T schimperi* leaves crude extract and fractions consecutively for two weeks to diabetic mice improved body weight of mice, which is an implication of a relatively good control of the diabetic condition. The effect of the extract on fasting blood glucose levels (FBGL) revealed that the extract showed a time dependent decrease in blood glucose levels: 14.76% (250 mg/kg), 25.12% (500

mg/kg) and 27.15% (750 mg/kg). With regard to the fractions, a significant blood glucose reduction in both dose levels was observed with the n-butanol and aqueous fraction. This might be due to the ability of the two solvents to leach out the required antidiabetic phytoconstituents from the extract. Peak value observed by n-butanol fraction as compared to the aqueous fraction can be explained by its more content of the antidiabetic phytochemicals.

The reduction in blood glucose levels of ethyl acetate fraction treated mice was not significant ($p>0.05$) at a dose of 250 mg/kg and 500 mg/kg and this might be the absence or insignificant content of the active phytochemicals responsible for antidiabetic effect in the ethyl acetate fraction. Therefore, it is plausible to state that the polar phytochemicals in the extracts of *T schimperi* are responsible for its antihyperglycemic effect.

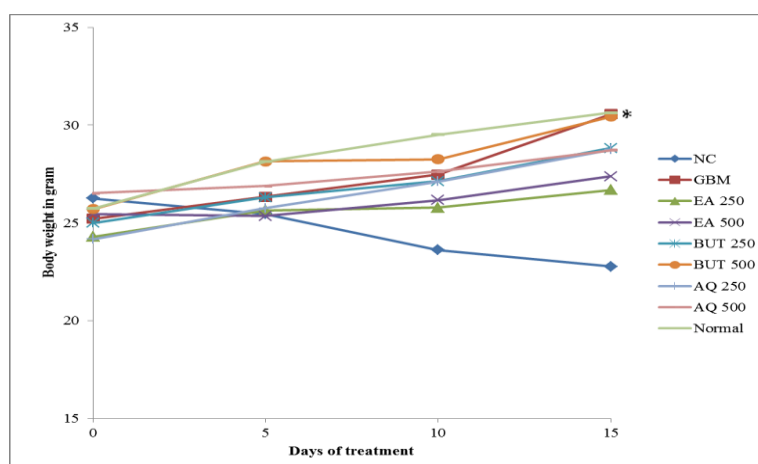


Figure 4. The effect of fractions of leaves of *Thymus schimperii* on body weight of diabetic mice. Results are expressed in Mean \pm S.E.M and all data were analyzed using ANOVA followed by Turkey's test for multiple comparisons for means. $n=6$. * when $p < 0.05$ versus Negative control (NC= Negative Control, GBM= Glibenclamide, EA 250/500 = ethyl acetate fractions 250/500mg/kg, BUT 250/500= n-butanol fractions 250/500 mg/kg, AQ 250/500 = aqueous fractions 250/500mg/kg).

The current finding indicated that, the leaves extract of *T schimperii* showed antihyperglycemic effect on STZ induced diabetic mice without causing hypoglycemia on normoglycemic mice unlike that of the standard drug glibenclamide. Hence, it is reasonably assumed that the mechanism of action of the extracts is different from the standard drug. The mechanism probably resembles those of insulin sensitizers that include promoting tissue glucose uptake, reducing hepatic glucose output, interfering with glucose metabolism and there by producing an antihyperglycemic effect without hypoglycemia [18].

The phytochemical analysis of this plant indicated the presence of alkaloids, polyphenols, flavonoids, tannins, saponins and terpenoids. Formerly, it was reported that polyphenols, flavonoids, alkaloids, terpenoids from different plant origin demonstrated anti-diabetic effects in animal model of diabetes [19]. Flavonoids are implicated in diabetes treatment by reducing the aldose reductase, regenerating the pancreatic cells, enhancing insulin release, increasing calcium ion uptake [20], delaying the gastric emptying rate and reducing active transport of glucose across intestinal brush border membrane [21]. Various studies also reported about their potential powerful antioxidant properties [22, 23]. On the other side, previous studies done on saponins have demonstrated a protective effect on pancreatic islet cells and increase insulin secretion [24]. Finally, alkaloids had also been found to have anti-diabetic effect [20]. These findings of the secondary metabolites might be implicated for the antidiabetic activity of *T schimperii*.

Oral glucose tolerance test (OGTT) measures the body's ability to use glucose, the body's main source of energy and is used to diagnose prediabetes and diabetes [25]. In the current study, glucose loaded mice showed a significant increase in the blood glucose levels as compared to the normal mice since it is attributed to increased hepatic gluconeogenesis and abnormal utilization of glucose by tissues. Mice treated with glibenclamide and all dose levels

of the extract had shown a significant reduction in blood glucose levels starting from 1 h post glucose administration. This result is consistent with other findings in that some plants have the ability to improve oral glucose intolerance. For example, aqueous extract of *Justicia Schimperiana* leaves [26], *Moringa oleifera* Lam [27], and *Caylusea abyssinica* Fresen [28] were found to have an ameliorating effect for glucose intolerance. The significant reduction of blood glucose levels in hyperglycemic mice treated with the three doses of the extract could suggest that there exists an improved glucose homeostasis and the normal homeostasis mechanism might be augmented by the phytochemicals present in the extract.

One of the effective strategies to control diabetes is to inhibit the activity of enzymes like α amylase enzyme and α -glucosidase, which are involved in breaking down of starch molecule in to glucose and maltose [29]. Thus, inhibitors of α -amylase and α -glucosidase delay the metabolism of carbohydrate in the small intestine and decrease the postprandial blood glucose levels in diabetic patients [30]. According to a recent study, polar extracts of *T schimperii* exhibited highest percentage of α -amylase inhibition activity in *in vitro* model [31]. This finding supports our current study and helps to claim that the antihyperglycemic effect of the plant extract could be due to an extra pancreatic mechanism though this requires further investigations. A similar species, *Thymus serpyllum* also demonstrated 50% inhibitory effect on α -glucosidase enzyme in an *in vitro* investigation which could also have a clue to our current investigation [32]. Furthermore, it has been demonstrated that treatment with antioxidants improves impaired insulin-mediated glucose uptake in animal models [13]. Also, a study showed that the major phenolic components in thyme extracts, particularly thymol and carvacrol, exerted higher antioxidant activity than the well-known butylated hydroxytoluene and α - tocopherol [33]. With these prior studies, it is possible to suggest that the antihyperglycemic

activity of the extracts of *T. schimperi* in STZ induced diabetes might be due to the improvement of insulin resistance against oxidative stress.

CONCLUSION

This study revealed that the methanol leaves extract, and n-butanol and aqueous fractions possess antihyperglycemic effect in diabetic mice model justifying the local use of *T. schimperi* leaves in the treatment of diabetes.

Ethics approval and consent to participant

All animal procedures and experimental protocols were approved by the Research Ethics Committee of the College of Health Sciences, Mekelle University (with protocol number ERC 1028/2017).

Availability of data and materials

Corresponding author can provide all the datasets used in this particular study upon request. The plant material is freely available at the National Herbarium, Department of Biology, College of Natural Sciences, Addis Ababa University, Ethiopia.

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CONFLICTS OF INTEREST

The author(s) declare(s) that there is no conflict of interest regarding the publication of this article

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