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Evaluation of *in vitro* α-amylase inhibitory activity and antidiabetic effect of *Myrica salicifolia* in streptozotocin-induced diabetic mice

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Abstract: The study was aimed to evaluate *in vitro* antioxidant, α-amylase inhibitory and *in vivo* antidiabetic activities of *Myrica salicifolia* root extracts. The powdered roots of *M. salicifolia* were extracted with 80% methanol and then dried. The dried extract was further fractionated into chloroform, ethyl acetate, butanol and aqueous fractions. The phytochemical screening of the crude extract was performed using standard chemical identification tests. The antioxidant activity of the extracts was determined by *in vitro* method using 2,2-diphenyl-1-picrylhydrazyl (DPPH) as radical scavenging reagent. The *in vitro* α-amylase inhibitory activity was performed using the chromogenic3,5-dinitrosalicylic (DNSA) method. The antidiabetic activity of *M. salicifolia* root crude extract (200, 400 and 600 mg/kg) and fractions (400 mg/kg) were evaluated in normal, glucose loaded hyperglycemic and streptozotocin (STZ)-induced diabetic mice. The crude root extract of *M. salicifolia* showed strong DPPH radical scavenging activity (IC_{50} = 4.54µg/ml) which was comparable with the standard antioxidant, ascorbic acid. In α-amylase inhibitory activity, the crude extract and butanol fraction showed highest enzyme inhibition. In the antidiabetic activity, daily administration of the crude extract, aqueous and butanol fractions for fifteen days showed highest significant reduction in fasting blood glucose level (BGL) compared to diabetic control in STZ-induced diabetic mice model. The root extract and fractions of *M. salicifolia* exhibited significant antihyperglycemic, α-amylase inhibitory and antioxidant activity with no sign of toxicity. The antidiabetic effect of the plant could be due to the synergistic effect of various classes of constituents present in the root part of the plant.

Keywords: α-amylase, antidiabetic, antioxidant, DPPH, *Myrica salicifolia,* streptozotocin.

INTRODUCTION

Diabetes mellitus (DM) is a chronic metabolic disorder caused by defects in insulin secretion, insulin action, or both (WHO, 1999). In 2017, about 424 million adults were living with diabetes globally and it is expected to reach 531.6 million people affected by DM by 2045 (International Diabetes Federation, 2017). This indicates that the rapid progression of the disease as compared to 1980 estimate, which was 108 million (WHO, 2016). The increasing frequency of this disorder is more likely to affect low-income countries due to lack of adequate and affordable interventions (Marx, 2002; Fekadu *et al*., 2019).

To date, numerous therapeutic approaches are available for the treatment of DM, including the increasing insulin secretion in the body, restricting utilization of dietary starch and lipid in the body, and treatment with conventional antidiabetic drugs (Birari and Bhutani, 2017). The currently available antidiabetic medications are usually connected with side effects, such as hypoglycemia, weight increase and cardiovascular complications. Also, some of the antidiabetic drugs are not affordable and in some countries even people have no access to these medicines (Meshesha *et al*., 2020). Therefore, search for new low cost medications which have lesser side effect and novel mechanism of action are need of the time. The most promising source for potential antidiabetic agents could be exploring knowledge of traditionally used medicinal plants. Phytochemicals play a pivotal role in controlling glycaemia, preventing the glycosylation of biomolecules, and related pathologies (Srinivasan and Muruganathan, 2016). World Health Organization (WHO) suggests use of herbs and herbal products as an alternative remedy for diabetes and should be investigated to provide novel leads for the development of new antidiabetic drugs (WHO, 1980) that can be used as alternative or complementary to current allopathic medications (Halberstein, 2005). However, many of medicinal plants claiming to treat diabetes lack valid scientific sanctity.

Myrica salicifolia (family, Myricaceae) is an aromatic resinous shrub mentioned in Pedra-hume-ca'a plants (Njung *et al*., 2002; Kariuki *et al*., 2014). The medicinal

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plants known as "Pedra-hume-ca'a" or insulin plants have been used by empirical African and Brazilian culture for diabetes related disorders (Vareda *et al*., 2014). *M. salicifolia* is distributed in Southeast Africa, Ethiopia and Saudi Arabia (Kariuki *et al*., 2014; Silva *et al*., 2015). In folkloric medicine it is sued for gastrointestinal disorder, bilharzia, malaria, chest congestion, pneumonia, diarrhea, nervous disorder, hypertension, respiratory disease, headache, pain, and inflammation (Maara, 2014; Silva *et al*., 2015). In Ethiopia and Tanzania, the roots of *M. salicifolia* are used to treat diabetes related ailments (Lulekal *et al*., 2014). Pharmacological studies reported on *M. salicifolia* include analgesic (Njung *et al*., 2002) and antibacterial (Kariuki *et al*., 2014) properties. Till date, the antidiabetic activity of the roots of *M. salicifolia* has not been studied, therefore, exploring the hypoglycemic activity and potential mechanism of action of this plant may shed light in the future to control the symptoms, modify the natural history of diabetes and also potentially could prevent or cure the disease. Moreover, it may also provide the foundation for safety and efficacy as well as basic research to pave the way for a new therapeutic option for diabetes and its complications. Therefore, the present study was aimed to evaluate the root extracts of *M. salicifolia* for its *in vivo* antidiabetic effect and *in vitro* antioxidant and α-amylase inhibitory activity.

MATERIALS AND METHODS

Chemicals and drugs

Streptozotocin (STZ), glibenclamide (Alfa Aesar, Great Britain), 1,1-Diphenyl-2-picrylhydrazyl radical (DPPH), ascorbic acid, potassium sodium tartrate and dinitro salicylic acid (DNSA) (Sigma-Aldrich, Germany), acarbose tablet and amylase (Pacific Pharmaceuticals Ltd., India), glucose standard strip/kits and ACCU-CHEK Active glucometer (Roche, Germany), ethyl, chloroform (Labort fine Chem Pvt. Ltd, India), *n*-butanol (Faiz Chemical Pvt. Ltd, France), and methanol (Amaira Petro Chem Pvt. Ltd, France). All other chemicals and reagents used were of analytical grade.

Collections, identification and preparation of plant materials

The roots of *M. salicifolia* were collected from Genfoquch, from North part Gondar town, Amhara Region of Ethiopia in December 2018. The plant was authenticated by Abiyu Enyew (botanist), Department of Biology, College of Natural Sciences, University of Gondar; where a specimen (voucher no. YK001) has been deposited at the Herbarium. The roots of the plant were washed with distilled water (DW) and dried under the shade. The plant material was then pulverized and crushed to powder for extraction.

Extraction and fractionation

The powdered roots of *M. salicifolia* (700 g) were macerated with 80% methanol (v/v) for 72 h with occasional shaking and filtered through Whatman No.1 filter paper. The mark was re-macerated two times following the above steps and filtrates were combined, concentrated *in vacuo* using rotary evaporator at 40^{o}C. The obtained dried extract (reddish brown color, 186 g, 26.57% w/w) was then stored in refrigerator (at 2-4°C) for further use.

The crude extract (30g) was further fractionated following the method of Otsuka and co-workers (Amgen and PreLabs, 2010). The crude extract was dissolved in 200 mL distilled water and fractionated with successively chloroform (200mL x 3), ethyl acetate (200mL x 3), and *n*-butanol (200mL x 3). The chloroform extract appeared milky whitish (2.5g, 8.33% w/w), ethyl acetate, canary yellow (2g, 6.67% w/w), reddish *n*-butanol (3g, 10% w/w) and aqueous (20.5g, 68.33% w/w).

Experimental animals

Swiss albino mice (M/F, weighing 20-30 g and age of 8-12 weeks) were purchased from Ethiopian Public Health Institute, Addis Ababa. Mice were acclimatized to the laboratory conditions for a week with access to the standard pellet diet and water *ad libitum*. The experiments involving animals were undertaken according to the US guideline Care and Use of Laboratory Animals) (Otsuka, 2006). Ethical approval for study on animals was obtained from Health Research Ethics Review Committee, College of Health Sciences, Mekelle University (Approval number – 1018/2017). After the experiments, mice were scarified by cervical dislocation and disposed safely.

Preliminary phytochemical screening

M. salicifolia root extract was screened using various chemical tests to confirm the presence or absence of different class of phytoconstituents using the methods described by Trease and Evans (1989) and Edeoga *et al.*, (2005).

Acute oral toxicity test

Five female, Swiss albino mice (non-pregnant, nulliparous, 20-30g, age of 8-12 weeks) were used for the limit test to determine the acute toxicity of the plant extract as recommended by OECD (Organization for Economic Co-operation and Development) [2008]. Initially, one mouse fasted for 3-4 h was orally given the extract (2000 mg/kg) dissolved in distilled water and observed strictly for any sign of toxicity for first 4h and then for 24 h. After survival of the first mouse, the other four mice (fasted for 3-4 h) were also given the extract at the same dose and were observed for physical and behavioral sign of toxicity up to 14 days.

DPPH radical scavenging activity

The *in vitro* antioxidant activity of the extract was evaluated by the DPPH radical scavenging method (Manssouri *et al*., 2020). Different concentrations (1000, 500, 250, 125, 62.5 µg/ ml) of the extract and standard compound (Ascorbic acid) were prepared in methanol. From each concentration of the extract and standard solutions, 30µL was mixed with 3 ml of DPPH solution (0.004% DPPH in MeOH) of and incubated for 30 min in dark. The absorbance of the each concentration was measured in triplicate at 517 nm using spectrophotometer (JENWAY6404) at and percent inhibition activity was calculated. The DPPH radical scavenging activity (RSA) of the samples was calculated using the following formula:

$$\% \text{ RSA} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

In vitro α-amylase inhibitory activity

The inhibitory property of the extract against the α-amylase enzyme was evaluated following the chromogenic 3,5-dinitrosalicylic acid (DNSA) method of Miller (1959). The test samples at concentrations 100, 50 and 10 µg/ml (crude extract and fractions) were incubated for 10 min at 37°C with 0.05 M sodium phosphate buffer (pH 6.9), and 50 µl. After pre-incubation of the test samples, 1% starch solution (500 µl, w/v) was added in each test samples and further incubated for 15 min at 37°C. Thereafter, 1.0 ml DNSA reagent was added to the above incubated test mixture to terminate the reaction and then boiled in water bath for 5 min. The reaction mixture was cooled at room temperature and the absorbance was measured at 540 nm by Spectrophotometer. Acarbose was used as standard inhibitor and assayed in the same method described above. The IC_{50} value was calculated by using regression analysis of the standard curve. The inhibitory property of the test samples was calculated using the following formula:

$$\% \text{ Inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

Induction of experimental diabetes

Overnight (12-14 h) fasted male Swiss albino mice were weighed and injected intraperitoneally with a single dose of freshly prepared STZ (150 mg/kg) in buffer solution (0.1 mole/L sodium citrate and 0.1 mole/L citric acid, pH 4.5) (Etuk, 2010). The mice were given access to feed and water after 30 min of the STZ injection. The mice were given 5% dextrose solution after 6 h of STZ injection, which continued for the next 24h (Liang *et al*., 2013). After 7 days of the STZ injection, the induction of diabetes in mice was confirmed by measuring their BGL. Mice with the BGL greater than 200 mg/dl (Yadav *et al*., 2018) were selected for antidiabetic activity.

Animal grouping and selection of doses

The animals were randomly assigned into different groups of six animals per group to perform oral glucose tolerance test (OGTT), hypoglycemic and antihyperglycemic activity. All the substances were given orally by using oral gavage

The doses for the antidiabetic study were selected according to OECD guideline (OECD, 2008). On the basis of acute toxicity study 1/10 (200 mg/kg), 1/5 (400 mg/kg) of the medium lethal dose (LD_{50}) and the dose 600 mg/kg of the extract was selected from the previous study of the related species exhibited the antidiabetic activity at this dose (Vareda *et al*., 2014). Glibenclamide (5 mg/kg) was used as standard antidiabetic drug for the study (Gebremeskel *et al*., 2020).

Hypoglycemic test in normal mice

The overnight fasted mice having normal glucose level were randomly divided into 5 groups containing six animals each. The normal control group (Group I) received DW (10 ml/kg) and the positive control group (Group II) received the glibenclamide at a dose 5 mg/kg. The test groups (Group III, IV and V) received *M. salicifolia* extract at the dose of 200, 400 and 600 mg/kg, respectively. The blood was collected from tail of the mice to determine the BGL at 0, 1, 2, 3 and 4 h post-treatment. One-touch glucometer (ACCU-CHEK-Active) and standard glucose strips was used to measure BGL in triplicate, and the average value was considered for measurement.

Oral glucose tolerance test (GTT)

Overnight fasted mice were grouped randomly into five groups with six mice in each group. The normal control group (Group-I) mice received DW (10 ml/kg) and positive control (Group-II) received the standard drug glibenclamide (5 mg/kg). The test groups (Group III, IV and V) received crude extract of *M. salicifolia* at 200, 400 and 600 mg/kg, respectively.

After 30 min of the treatment, mice were given orally 2 g/kg glucose solution (Kumar *et al*., 2013). The BGL level was determined immediately before and after treatment at 30, 60, 120 and 180 min (Toma *et al*., 2015) of glucose challenge. Blood samples were collected from the tail and BGL was determined in triplicate, using standard glucose strips and glucometer.

Anti-hyperglycemic activity of M. salicifolia extract in diabetic mice

In this experiment, 36 mice (6 normal and 30 diabetic mice) were used and divided into six groups (n=6). Group I (Normal control) and Group II (Diabetic control) mice received DW (10 ml/kg, P.O.) for 15 days. Group III-diabetic mice received glibenclamide (5 mg/kg P.O.) for 15 days; Group IV-VI diabetic mice were given the root extract of *M. salicifolia* extract at doses of 200, 400 and

Table 1: Effect of *Myrica salicifolia* extract (MSE) on fasting blood glucose level (BGL) in normal mice

Groups	BGL (mg/dl) in different time intervals (h)				
	0	1	2	3	4
Normal control	127.00 ± 4.79	130.67 ± 6.04	131.83 ± 8.42	127.83 ± 4.83	125.83 ± 4.34
GL-5	125.67 ± 4.40	119.33 ± 17.89	85.50 ± 6.23 $^{a\,1,\,b\,3}$	75.00 ± 7.41 $^{a\,2,\,b\,3}$	71.33 ± 4.84 $^{a\,2,\,b\,3}$
MSE-200	131.17 ± 4.41	110.33 ± 3.23 $^{a\,1}$	104.17 ± 2.49 $^{a\,2,\,b\,1}$	97.50 ± 5.21 $^{a\,3,\,b\,2}$	96.67 ± 5.98 $^{a\,3,\,b\,3}$
MSE-400	128.83 ± 3.59	114.83 ± 3.07 $^{a\,1}$	105.50 ± 3.06 $^{a\,3,\,b\,1}$	87.33 ± 3.16 $^{a\,3,\,b\,3}$	76.83 ± 3.41 $^{a\,3,\,b\,3}$
MSE-600	129.33 ± 7.71	137.50 ± 5.46	120.83 ± 5.06	120.00 ± 5.27	114.00 ± 4.44

Values are expressed in mean ± SEM; n = 6; Normal control = Distilled water (10 ml/kg, p.o.); GL-5 = glibenclamide (5 mg/kg, p.o.); MSE-200 = *Myrica salicifolia* extract (200 mg/kg, p.o.), MSE-400 = *Myrica salicifolia* extract (400 mg/kg, p.o.); MSE-600 = *Myrica Salicifolia* extract (600 mg/kg, p.o.); [a] compared with fasting BGL (t = 0 h); [b] compared with DW group. *P* values were analyzed using One-way ANOVA followed by post hoc Tukey's test.: [1]$p < 0.05$, [2] $p < 0.01$, [3] $p < 0.001$.

Table 2: Effect of *Myrica salicifolia* extract (MSE) on oral glucose tolerance tests (GTT) in normal mice

Groups	BGL (mg/dl) in different time intervals (min)				
	0	30	60	120	180
Normal Control	132.17 ± 4.42	260.00 ± 9.65	232.50 ± 9.84	196.83 ± 8.06	131.17 ± 5.77
GL-5	143.67 ± 8.22	174.83 ± 22.74 **	102.33 ± 11.51 ***	89.50 ± 2.73 ***	81.67 ± 4.88 ***
MSE-200	130.50 ± 5.93	204.83 ± 10.68 *	164.17 ± 14.48 **	147.00 ± 15.30 **	105.00 ± 8.01 *
MSE-400	135.00 ± 9.92	194.50 ± 8.29 *	164.50 ± 6.50 **	136.33 ± 5.03 ***	100.83 ± 5.39 **
MSE-600	135.07 ±15.39	197.33 ± 5.53 *	163.50 ± 9.41 **	136.50 ± 6.99 ***	88.17 ± 4.90 ***

Values are expressed in mean ± SEM; n = 6; Normal control = Distilled water (10 ml/kg, p.o.); GL-5 = glibenclamide (5 mg/kg, p.o.); MSE-200 = *Myrica salicifolia* extract (200 mg/kg, p.o.), MSE-400 = *Myrica salicifolia* extract (400 mg/kg, p.o.); MSE-600 = *Myrica Salicifolia* extract (600 mg/kg, p.o.); *P* values were analyzed using One-way ANOVA followed by post hoc Tukey's test: $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ as compared with control.

Table 3: Effect of *Myrica salicifolia* extract (MSE) on fasting blood glucose level (BGL) in normal and diabetic mice

Groups	BGL (mg/dl) at different time intervals			
	Day 0	5th day	10th day	15th day
Normal Control	160.00 ± 14.40 $^{b\,1}$	147.67 ± 9.15 $^{b\,3}$	187.50 ± 6.93 $^{b\,3}$	157.50 ± 4.74 $^{b\,3}$
Diabetic Control	380.17 ± 44.45 $^{a\,1}$	465.17 ± 46.33 $^{a\,3}$	473.33 ± 44.21 $^{a\,3}$	454.00 ± 51.26 $^{a\,3}$
Diabetic + GL-5	388.50 ± 45.60 $^{a\,1}$	288.83 ± 28.47 $^{a\,1,\,b\,1}$	214.67 ± 38.80 $^{b\,3}$	119.67 ± 28.07 $^{b\,3}$
Diabetic + MSE-200	384.17 ± 49.77 $^{a\,1}$	386.17 ± 47.57 $^{a\,3}$	238.33 ± 42.07 $^{b\,2}$	207.17 ± 48.87 $^{b\,3}$
Diabetic + MSE-400	378.50 ± 37.41 $^{a\,1}$	319.83 ± 26.69 $^{a\,2,\,b\,1}$	235.83 ± 41.07 $^{b\,3}$	171.67 ± 41.76 $^{b\,3}$
Diabetic + MSE-600	385.50 ± 37.26 $^{a\,1}$	306.33 ± 20.84 $^{a\,1,\,b\,1}$	221.33 ± 29.79 $^{b\,3}$	128.67 ± 17.91 $^{b\,3}$

Results are expressed in Mean ± SEM, n = 6, Normal control and diabetic control = Distilled water (10 ml/kg, p.o.), GL-5 = glibenclamide (5 mg/kg, p.o.); MSE-200 = *Myrica salicifolia* extract (200 mg/kg, p.o.), MSE-400 = *Myrica salicifolia* extract (400 mg/kg, p.o.); MSE-600 = *Myrica Salicifolia* extract (600 mg/kg, p.o.); [a] compared with normal control, [b] compared with diabetic control, *P* values were analyzed using One-way ANOVA followed by post hoc Tukey's test: [1] $p < 0.05$, [2] $p < 0.01$, [3] $p < 0.001$

Table 4: Effect of *Myrica salicifolia* extract (MSE) on body weight in normal and diabetic mice

Groups	Body weight (gm) change during treatment period			
	Day 0	Day 5	Day 10	Day 15
Normal control	25.43 ± 0.86	28.35 ± 0.88	29.67 ± 0.81	30.83 ± 0.88 ^{b}
Diabetic control	26.91 ± 1.36	26.63 ± 1.22	25.38 ± 1.26	24.48 ± 1.20 ^{a}
Diabetic + GL-5	27.83 ± 1.64	26.45 ± 1.74	27.11 ± 1.90	27.30 ± 1.75
Diabetic + MSE-200	26.20 ± 1.66	24.62 ± 1.60	25.50 ± 1.56	26.05 ± 1.47
Diabetic + MSE-400	27.40 ± 1.11	26.10 ± 1.09	26.58 ± 1.01	27.11 ± 1.06
Diabetic + MSE-600	27.03 ± 2.17	24.85 ± 2.06	25.87 ± 2.01	26.70 ± 1.10

Results are expressed in Mean ± SEM, n =6, Normal control and diabetic control = Distilled water (10 ml/kg, p.o.), GL-5 = glibenclamide (5 mg/kg, p.o.); MSE-200 = *Myrica salicifolia* extract (200 mg/kg, p.o.), MSE-400 = *Myrica salicifolia* extract (400 mg/kg, p.o.); MSE-600 = *Myrica Salicifolia* extract (600 mg/kg, p.o.); *P* values were analyzed using One-way ANOVA followed by post hoc Tukey's test: [a] when $p < 0.05$ verses compared normal control, [b] when $p < 0.05$ versus diabetic control.

Table 5: Effect of different solvent fractions of *Myrica salicifolia* on fasting blood glucose level (BGL) in normal and diabetic mice

Groups	BGL (mg/dl) in different time intervals (days)			
	Day 0	Day 5	Day 10	Day 15
Normal control	160.67 ± 7.08 [b 1]	154.00 ± 9.02 [b 3]	144.17 ± 17.43 [b 3]	134.50 ± 5.32 [b 3]
Diabetic control	354.83 ± 43.13 [a 1]	416.17 ± 32.83 [a 3]	441.33 ± 42.46 [a 3]	458.83 ± 29.46 [a 3]
Diabetic + GL-5	367.67 ± 36.02 [a 1]	234.33 ± 30.91 [b 2]	209.33 ± 39.04 [b 2]	133.00 ± 17.79 [b 3]
Diabetic + AQF-400	362.80 ± 45.53 [a 1]	256.40 ± 18.73 [b 1]	230.80 ± 41.76 [b 1]	209.00 ± 27.86 [b 3]
Diabetic + CHF-400	362.33 ± 41.39 [a 1]	370.17 ± 56.93 [a 3]	352.50 ± 54.50 [a 1]	299.50 ± 29.34 [a 1, b 1]
Diabetic + BUF-400	346.83 ± 49.38 [a 1]	257.67 ± 8.58 [b 1]	242.17 ± 31.19 [b 1]	166.00 ± 28.54 [b 3]
Diabetic + EAT-400	361.83 ± 37.01 [a 1]	392.50 ± 46.89 [a 3]	386.17 ± 64.37 [a 1]	370.00 ± 53.35 [a 3]

Results are expressed in Mean ± SEM, n = 6, Normal control and diabetic control = Distilled water (10 ml/kg, p.o.) GL-5 = glibenclamide (5 mg/kg, p.o.), AQF-400 = aqueous fraction (400 mg/kg, p.o.), CHF-400= chloroform fraction (400 mg/kg, p.o.), BUF-400= butanol fraction (400mg/kg, p.o.), EAT-400= ethyl acetate (400 mg/kg, p.o.), [a] compared with normal control, [b] compared with diabetic control, *P* values were analyzed using One-way ANOVA followed by post hoc Tukey's test: [1]$p<0.05$, [2]$p< 0.01$, [3]$p<0.001$

Table 6: Effect of different solvent fractions of *Myrica salicifolia* on body weight in normal and diabetic mice

Groups	Body weight change during treatment (g)			
	Day 0	Day 5	Day 10	Day 15
Normal control	27.23 ± 1.23	28.75 ± 1.13	31.15 ± 1.11 [b]	32.41 ± 1.08 [b]
Diabetic Control	23.66 ± 0.79	22.76 ± 0.47	22.58 ± 0.19 [a]	22.10 ± 0.73 [a]
Diabetic + GL-5	24.21 ± 1.39	23.65 ± 1.52	24.13 ± 1.54	24.95 ± 1.67
Diabetic + AQF-400	24.58 ± 2.68	21.82 ± 2.37	20.94 ± 2.00 [a]	21.26 ± 1.68 [a]
Diabetic + CHF-400	26.05 ± 1.76	26.06 ± 2.10	26.61 ± 2.28	26.62 ± 2.16
Diabetic + BUF-400	26.31 ± 2.22	24.65 ± 2.42	24.70 ± 2.59	26.76 ± 2.61
Diabetic + EAT-400	23.91 ± 1.55	24.88 ± 1.92	24.88 ± 2.19	25.15 ± 1.26

Results are expressed in Mean ± SEM, n = 6, Normal control and diabetic control = Distilled water (10 ml/kg, p.o.), GL-5 = glibenclamide (5 mg/kg, p.o.), AQF-400 = aqueous fraction (400 mg/kg, p.o.), CHF-400 = chloroform fraction (400 mg/kg, p.o.), BUF-400 = butanol fraction (400 mg/kg, p.o.), EAT-400 = ethyl acetate (400 mg/kg, p.o.); *P* values were analyzed using One-way ANOVA followed by post hoc Tukey's test: [a] when $p<0.05$ verses compared normal control, [b] when $p < 0.05$ versus diabetic control

600 mg/kg, respectively P.O. daily for 15 days. Blood samples were collected from the tail of mice on 0, 5th, 10th and 15th day (Kumar *et al*., 2008) to estimate fasting BGL. Blood glucose of each mouse was measured with an auto-analyzer glucometer (ACCU-CHEK-Active) and their body weight also determined at the same time.

Anti-hyperglycemic activity of fractions of M. salicifolia in diabetic mice

To evaluate anti-hyperglycemic effect of *M. salicifolia* fractions, a total of 42 mice (six normal and 36 STZ-induced diabetic mice) were divided into seven groups with six mice each. Group-I (normal control) and group II (diabetic control) were given only DW (10 ml/kg). Group III served as a positive control and were given glibenclamide (5 mg/kg), while group IV-VII served as test groups were treated with fractions (chloroform, ethyl acetate, *n*-butanol and aqueous fraction) of extract at a dose of 400 mg/kg. All fractions were given to diabetic mice by suspending in DW and administered orally for 15 days. Body weight and fasting BGL was measured at 0, 5th, 10th and 15th day. Fasting BGL was measured with an auto-analyzer glucometer (ACCU-CHEK-Active).

STATISTICAL ANALYSIS

The results are expressed as mean ± standard error of means (SEM). Data was analyzed with SPSS version 21.0. One-way analysis of variance (ANOVA) was used to determine the differences between treated and untreated groups. Tukey's post-hoc multiple comparisons method was used to determine the source of significant differences where appropriate. The results were considered significant when $p < 0.05$.

RESULTS

Preliminary phytochemical screening

The crude root extract of *M. salicifolia* analyzed qualitatively by different chemical tests revealed the presence of polyphenols, steroids, flavonoids, tannins, terpenoids, phlobatanins, and saponins.

Acute oral toxicity test

The crude root extract of *M. salicifolia* showed no physical and physiological sign of toxicity in mice. All mice found physically active and no death was observed during two weeks of follow up period. Thus, LD_{50} value

indicates that the plant extract is safe at 2000 mg/kg dose level.

Antioxidant activity

The crude root extract of *M. salicifolia* showed strong DPPH radical scavenging activity with IC_{50} similar to that of the standard antioxidant, ascorbic acid as shown in fig 1.

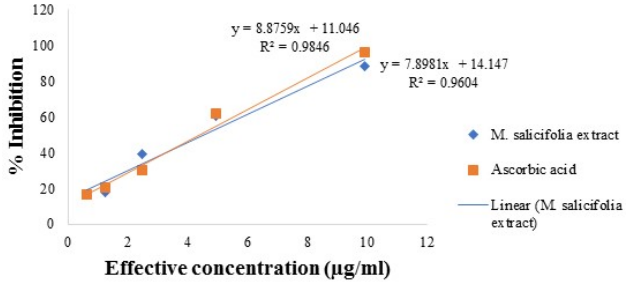

Fig. 1: Percentage inhibition of DPPH radical by *Myrica salicifolia* root extracts in comparison with ascorbic acid (a reference compound).

In vitro α-amylase inhibition study

In this study, the crude extract and solvent fractions demonstrated inhibitory effects on the a-amylase activity. The butanol fraction showed highest inhibitory activity followed by crude extract compared to positive control (fig. 2).

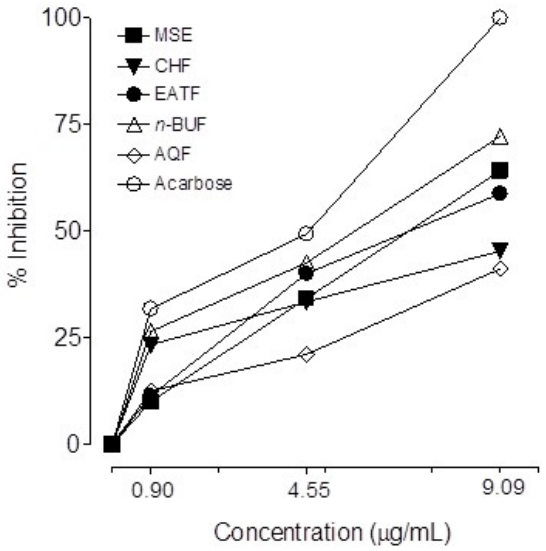

Fig. 2: *α*-amylase inhibitory effects of *Myrica salicifolia* extract (MSE) and solvent fractions

MSE = *Myrica salicifolia* extract, CHF = chloroform fraction, EATF = ethyl acetate fraction, n-BUF = n-butanol fraction, AQF = aqueous fraction

Hypoglycemic effect of M. salicifolia extract in normal mice

As can be seen from the table 1, the fasting BGL of normal control group was remained between 125 to 132 mg/dL whereas pretreatment with *M. salicifolia* at doses of 200 and 400mg/Kg significantly reduced the fasting BGL. Furthermore, pretreatment with glibenclamide significantly reduced the fasting BGL starting after 2h of the administration. The glibenclamide and extract continuously decreased BGL in mice till the end of experiment.

Effect of M. salicifolia extract on oral glucose tolerance tests (GTT) in normal mice

In this experiment, we observed that after glucose load, BGL rises to a maximum at 30 minutes in mice and remained high over the next 90 min then declined to near basal level at 180 min, in the control group. The crude extract significantly decreased the BGL in non-diabetic mice at 30 min compared to untreated non-diabetic mice. This suppression effect in fasting BGL arrived at a higher level at 180 min (table 2).

Antihyperglycemic activity of M. salicifolia extract in diabetic mice

Diabetic mice showed significant differences in fasting BGL compared to normal control mice ($p < 0.05$) at different time interval. Oral administration of *M. salicifolia* crude extract significantly reduced fasting BGL as compared to diabetic control group at higher dose. The positive control group treated with glibenclamide showed highest BGL lowering effect in diabetic mice (table 3).

Effect of M. salicifolia extract on body weight of diabetic mice

As shown in the table 4, the normal and diabetic mice showed change in body weight with time. The diabetic control group continued to lose weight until the end of the study period whereas, the extracts treated groups and glibenclamide treated positive control group revealed improvement in body weight throughout the study period.

Antihyperglycemic activity of fractions of M. salicifolia extract in diabetic mice

In this study, comparison with diabetic control, the aqueous, and butanol fraction as well as glibenclamide treated groups showed significant reduction in the fasting BGL starting from day five till the end of the treatment period (table 5). The chloroform fraction showed delayed but significant antihyperglycemic effect on the 15th day of the treatment whereas the ethyl acetate fraction failed to reduce the hyperglycemia. The positive control (glibenclamide) group showed the highest percentage reduction in fasting BGL followed by Butanol fraction as most potent among the fractions.

Effect of fractions of M. salicifolia extract on body weight of diabetic mice

As shown in table 6, the treatments with different fractions and glibenclamide resulted in improvement in body weight while continued weight loss was observed in the diabetic control group till end of the study period.

DISCUSSION

The present study was designed to determine the antidiabetic and *α*-amylase inhibitory activity of methanolic root extract of *M. salicifolia* and its fractions. Also, the crude extract of the root was examined for its antioxidant properties, safety and type of chemical constituents.

In this study, to assess hypoglycemic effect of the plant in normoglycemic mice, the methanolic extract at doses of 200 and 400 mg/kg caused a significant hypoglycemic effect after second hour of post-treatment. Interestingly, the fasting BGL reduction revealed by 400 mg/kg of the extract was comparable with the positive control (glibenclamide). Glibenclamide's hypoglycemic effect is due to its ability to stimulate insulin secretion from the pancreatic *β*-cells and to inhibit glucagon secretion (Ramkumar *et al*., 2011; Gebreyohannis *et al*., 2013). In accordance with this finding, previous study has demonstrated that phytoconstituents like flavonoid and tannins found to cause hypoglycemia through stimulation of insulin secretion from pancreatic *β*-cells (Al-Ishaq *et al*., 2019). The phytochemical screening of the crude extract revealed the presence of flavonoids, tannins and polyphenols which seem to be corroborates with Vareda *et al* (2014) research which found phenolic acids and flavonoids as the main constituents of *Myrica bella* leaf extracts; therefore, the hypoglycemic effect of the plant extract could be due to presence of the polyphenolic constituents that may act singularly or synergistically to either stimulate insulin release or reveals insulin-like effect. Surprisingly, the crude extract at higher dose failed to show the hypoglycemic effect in normoglycemic mice. This result match those observed in other plant species like *Murraya koenigii* (Kesari *et al*., 2005), *Cynodon dactylon* (Singh *et al*., 2007), *Otostegia integrifolia* (Shewamene *et al*., 2015) and *Caylusea abyssinica* (Tamiru *et al*., 2012). The reason for less hypoglycemic properties at high dose of the extract is not clear but some studies speculatethat the existence of both hypoglycemic and hyperglycemic compounds in the plant extract may raise blood glucose as they get increased in concentration at a higher dose (Tamiru *et al*., 2012; Shewamene *et al*., 2015).

Further, the current study found a significant enhancement in glucose tolerance in glucose loaded hyperglycemic mice which reflect a better glucose utilization capacity of mice treated with the extract and glibenclamide. The capability of BGL suppression in oral glucose tolerance test (GTT) can be reasoned as the extract was able to show insulin stimulation effect (Lenzen, 2008; Nagmoti *et al*., 2015) or could be due to the extracts ability to prevent glucose absorption from the stomach, promote peripheral glucose utilization, and initiate glycogenic process (Gupta *et al*., 2012). Inhibition of carbohydrate-hydrolyzing enzymes such as *α*-amylase, and *α*-glucosidase from the gastrointestinal tract is a considerable approach to decrease postprandial hyperglycemia (Taher *et al*., 2016). The results of this study indicate that a moderate *α*-amylase inhibition capacity of the methanolic root extract of *M. salicifolia* and its fractions which is consistent with findings of previous studies indicating the *α*-amylase inhibitory effect of polyphenols (Tundis *et al*., 2010), saponin (Nafiu and Ashafa, 2017), terpenoid (Sales *et al*., 2012) present in these plants. The post-prandial hyperglycemia restoration activity shown by *M. salicifolia* extract could be partly associated to the presence of these active constituents which may have role in preventing the occurrence of diabetic and related complications due to an elevation of postprandial glucose and therefore it could be used as effective therapy with minimal side effects.

The principal goal in the treatment of DM is to optimize both short-term and long-term BGL within tolerable limits (Emordi *et al*., 2016). In this study, the crude extract and its fractions significantly reduced the fasting BGL in diabetic mice compared to diabetic control. This finding is in agreement with Vareda *et al* (2014) finding which showed the antidiabetic activity of a related species (*M. bella*) leaf extract. In the current study, the butanol fraction compared to other fractions showed the highest hypoglycemic effect. This observed increase in fasting BGL reduction could suggest that the active constituents are polar in nature (Kamran *et al*., 2016). The fractions which showed moderate hypoglycemic activity could be reasoned due to inherent inability of compounds in these fractions to reduce hyperglycemia or the active compounds in this fraction may exist in small amount (Ali *et al*., 2012).

Flavonoids are known to have different pharmacological effect on various mammalian cell systems (Kikuchi *et al*., 2019). In a related study, flavonoids were found to lower BGL in animal studies (Tundis *et al*., 2010; Vareda *et al*., 2014). Phenolic compounds have been reported as effective antihyperglycemic agents (Gebreyohannis *et al*., 2013). Flavonoids such as quercetin, myricetin (Liu *et al*., 2005; Vessal *et al*., 2003), kaempferol glycosides and kaempferol-containing plant extracts (Arunachalam and Parimelazhagan, 2014) have exhibited that the antidiabetic activities in STZ-induced diabetic animal model. These active compounds have previously been isolated and characterized from genus *Myrica* (Nguyen *et al*., 2010; Spínola *et al*., 2014; Silva *et al*., 2015) and thus such active constituents in *M. salicifolia* may be envisaged. Flavonoids exhibit antidiabetic activity due to their ability to regenerate pancreatic β-cells (Gebreyohannis *et al*., 2013; Al-Ishaq *et al*., 2019); tannins and phenolic compounds exhibit antidiabetic effect through decreasing the absorption of nutrients and food intake, initiation of β-cells regeneration, directly act on adipose tissue, and insulin signaling pathway in

hepatocytes (Taher *et al*., 2016). In accordance with these mechanisms, the potential effect of the extract and the active fractions could be due to stimulation of insulin secretion from undamaged β-cells of islets or regenerated β-cells, enhanced transport of glucose to the peripheral tissues, and reduction of hepatic gluconeogenesis and glycogenolysis.

STZ-induced DM is allied with the severe loss in body weight (Ramkumar *et al*., 2011; Nagmoti *et al*., 2015) and this also accords with present study. The scarcity of carbohydrate in the body of diabetic mice results into utilization proteins for energy source could contribute in reducing the body weight (Ramkumar *et al*., 2011). Hence, in untreated diabetic mice, the excessive catabolism of protein at the time of insulin insufficiency cause muscle wasting and weight loss (Nguyen *et al*., 2010; Liang *et al*., 2013). In contrast with this, diabetic mice treated with different doses of *M. salicifolia* root extract and its fractions found to cause improvement in body weight. A possible explanation for this might be that reversal of proteolysis, gluconeogenesis, and glycogenolysis (Ahmed *et al*., 2014; Kumar *et al*., 2014).

The biochemical abnormality like non-enzymatic glycation, glucose oxidation and lipid peroxidation results in the increase of free radical formation in diabetes. Oxidative stress plays a key role in the pathogenesis and complications of diabetes (Ullah *et al*., 2016). Another important finding was that the crude root extract of *M. salicifolia* root scavenged the DPPH radical similar to that of ascorbic acid, used as standard antioxidant. Phenolic and flavonoids are strongly correlated with antioxidant activity (Ali *et al*., 2015) and have the potential to treat both type 1 and type 2 diabetes (Ullah *et al*., 2016). Free radical scavenging and lipid peroxidation effect of rutin, a flavonoid glycoside and suggested it to prevent STZ-induced oxidative stress and therefore protect β-cells (Spínola *et al*., 2014). The intake of flavonoids is found to reduce the oxidation of excess lipids in blood circulation that resulted from the generation of ROS (Vareda *et al*., 2014). Therefore, the potent antioxidant property shown by *M. salicifolia* could be one of the mechanisms responsible its antidiabetic activity.

CONCLUSIONS

The chemical analysis revealed presence of different classes of phenolic compounds which together with other constituents could be responsible for the antidiabetic activity. The potent antioxidant and moderate *α*-amylase inhibitory activity shows the extracts potential to control diabetes and related complications due to oxidative stress and an elevation of postprandial glucose. This study has demonstrated that root extract, its *n*-butanol and aqueous fractions have significant hypoglycemic activities in normal and diabetic mice, and good glucose handling effect in glucose loaded non-diabetic mice. Moreover, the root extracts of *M. salicifolia* possesses a strong antidiabetic activity and good safety profile. The possible mechanisms of antidiabetic properties of the plant extract and the active fractions are multidimensional and could be due to stimulation of insulin secretion from undamaged *β*-cells of islets or regenerated *β*-cells, inhibition of glucose absorption, and antioxidant effect. Taken together, these results justify its traditional use for the treatment of diabetic-related disorders. Further studies on the crude and active fraction are recommended to confirm the above potential mechanism and the constituents responsible for the effect.

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