



Amelioration of antioxidant potential, toxicity, and antihyperglycemic activity of *Hippophae salicifolia* D. Don leaf extracts in alloxan-induced diabetic rats

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Abstract

Efficacy of several plant extracts in the clinical research for modulating oxidative stress correlated with diabetes mellitus (DM) is well documented. In the present study, we investigated the in vitro antioxidant activity, toxicity, and anti-diabetic activity of methanolic extract of *Hippophae salicifolia* leaves in normal and alloxan-induced diabetic wistar rats. *H. salicifolia* leaves were found to be rich in antioxidants. The acute toxicity test of methanolic extract of *H. salicifolia* leaves revealed that the median lethal dose (LD₅₀) was found to be 3.92 g/kg body weight in mice. Administration of *H. salicifolia* leaves at 200 mg/kg and 400 mg/kg in alloxan-induced diabetic rats illustrated significant reduction (22% and 39%, respectively) in fasting blood glucose compared to diabetic control. Both the doses were found to be effective when compared to diabetic rats. The *Hippophae*-treated diabetic rats showed significant increase in the endogenous antioxidant enzymes, superoxide dismutase (50% and 74%, respectively), glutathione peroxidase (57% and 41%, respectively) and decrease in malondialdehyde (33% and 15%, respectively) levels. These results suggested that the methanolic leaf extract of *H. salicifolia* enhanced the antioxidant defence against reactive oxygen species produced under hyperglycaemic conditions.

Keywords *Hippophae salicifolia* · Antioxidants · Diabetes · Toxicology · Flavonoids · Polyphenols · Sea buckthorn

Introduction

Diabetes is a multi-aetiology disorder that can be characterized by long-term presence of excess amounts of glucose in the blood called chronic hyperglycemia. This might be

possibly because of either no action of insulin or impaired insulin secretion or both (Kharroubi and Darwish 2015). The other symptoms that accompany this hyperglycemia are polyuria, polydipsia, ketonemia, ketonuria, and ketoacidosis. (American Diabetes Association 2014). Incidence of diabetes is increasing at a very tremendous rate and has turned into a major health burden in the recent years with an anticipated number of 591.9 million diabetic adults by 2035 (King et al. 1998). The reasons for the high prevalence of diabetes are increasing urbanization, nutritional changes, sedentary lifestyles, obesity, and slow development of health systems (Guariguata et al. 2014).

A number of pharmacotherapies like biguanides, alpha glucose inhibitors, thiazolidinediones, lipase inhibitors, etc., are readily available nowadays (McLellan et al. 2014). Although these medicines help in managing the disease, there is still a dearth of a safe and effective drug (Rawat et al. 2013).

In the present scenario, the treatment used for diabetes mellitus consists mainly of synthetic medicines that act as anti-hyperglycemic drugs like insulin, sulfonylureas (1st generation: glibenclamide second generation: glipizide,

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glimperide, gliclazide), dipeptidyl peptidase-4 inhibitors (alogliptin, linagliptin, saxagliptin, sitagliptin, vildagliptin), sodium-glucose co-transporter 2 (SGLT2) inhibitors (canagliflozin), etc., (Dardano et al. 2014). Out of all these available artificial drugs, intermediate acting insulin, glibenclamide, and metformin are the accepted medicines by almost all the countries (Bazargani et al. 2014). Synthetic drugs help in managing the disease but there is no drug available which can completely eradicate the root cause of diabetes mellitus (Rawat et al. 2013). So traditional medicines with anti-diabetic properties are being considered for treatment as they have fewer side effects (Usha et al. 2017, Dey et al. 2018). Different countries have their own indigenous system of medicine and so does India, whose indigenous systems include Siddha, Ayurveda, Unani and Allopathy (Perumal and Ponnampalam 2007). The usage of herbal medicine throughout the world has been on the rise from the past few years (Mazzari and Prieto 2014). Herbal medicine can be seen as a combination therapy as numerous phytochemicals and bioactivities are found in the plant, that may bring about an anti-diabetic effect. These plant-based alternative medicines have several ways of action like regulation of insulin resistance, regulation of β -cell function, regulation of GLP-1 homeostasis, and regulation of glucose absorption in the gut. Their safety and efficacy in producing multiple target action has made herbal medicines a potential source of treatment for type 2 diabetes (Chang et al. 2013).

Hippophae (Sea buckthorn) is an actinorhizal plant that belongs to Elaeagnaceae family. The delicious and nutritious orange-coloured berries are one of the edible crude herbs that have been in use in Tibetan and Mongolian traditional medicines for a long time (Saikia and Handique 2013). There are altogether four species of *Hippophae* namely, *Hippophae rhamnoides* L., *Hippophae salicifolia* D. Don, *Hippophae tibetiana* Schlecht, and *Hippophae neurocarpa* S.W. Liu et T.N. He. *H. salicifolia* restricted to the mountains of Himalaya possess characteristics between that of a shrub and a tree (Goyal et al. 2013). Healing wounds, preventing ulcer formation, antioxidants (Goyal et al. 2011), anti-atherogenic, anti-platelet aggregation activity are the main properties described, which justifies *H. salicifolia* as a boon for the nutraceutical industries (Ramu et al. 2014). Besides, it also possesses carotenoids, flavonoids, and vitamins (A, B, C, E and K), that can prevent oxidative stress (Usha et al. 2014). Middha et al (2013) reported by in silico approach that glycerophospholipids, phytosterols, and zeaxanthin esters found in *H. salicifolia* hold huge potential for anti-diabetic action and hypothesized their role in reducing diabetic level (Middha et al. 2013). Therefore, there is a need to validate the protective effects of *H. salicifolia* leaves using in vivo system. Our study here seeks to unveil the hidden possibilities of diabetes treatment in this enthralling plant called *H. salicifolia* on pancreatic regions in alloxan-induced diabetic rats.

Materials and methods

Preparation of plant extract

Hippophae salicifolia D. Don leaves were collected from the forests of Lachen valley of North Sikkim between 27°46'31" and 27°42' 30" north latitude and 88°30'00" and 88°35'00" east latitude during May 2014. The plant was authenticated by a plant taxonomist and voucher specimen is deposited at the Sikkim State Council of Science and Technology, Gangtok, Sikkim, India, under voucher number SSCST/HSM/001. These leaves were air-dried and ground into a coarse powder. fifty grams of the powdered sample was dissolved in 400 mL methanol and refluxed for 4 h. The mixture was filtered through a Whatman filter paper and was evaporated under pressure at 50 °C to constant weight and stored at 4 °C until required. The leaf extract was dissolved in double-distilled water (DDW) in desired concentrations prior to use (Middha et al. 2011).

Determination of extraction yield

The yield of evaporated extract based on dry weight basis was calculated using the following equation: yield (g/100 g of dry plant material) = $(W1 \times 100)/W2$, Where W1 and W2 were the weight of the extract after the solvent evaporation and the weight of the dry plant material, respectively.

Determination of total phenolic content

The total soluble phenol in the extract was determined by using Folin–Ciocalteu reagent according to the method of Singleton and Rossi (1965). The absorbance of the developed blue color was read at 760 nm. Gallic acid was used as a standard. The concentration of total phenolic compounds in gallic acid was determined in milligram of gallic acid equivalent (GAE) using an equation obtained from the standard gallic acid graph.

Determination of total flavonoid content

The total flavonoid content of the extract was determined using aluminium chloride (AlCl₃) according to a modified method of Goyal and his team (2010) using quercetin as a standard in milligram QE. The absorbance was measured at 510 nm.

In vitro antioxidant properties (DPPH method)

The antioxidant activity of the extracts and standard was assessed on the basis of the radical scavenging effect of the

stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical. The absorbance of each solution was determined at 517 nm using Thermo UV1-vis spectrophotometer (Goyal et al. 2010). The corresponding blank reading was also taken and the remaining DPPH was calculated using the following formula:

$$\text{Percentage scavenging} = A_0 - A_1/A_0 \times 100,$$

where A_0 is the absorbance of the control and A_1 is the absorbance in the presence of the sample. The actual decrease in absorption induced by the test extract was compared with the positive control. The IC_{50} value was calculated using the dose inhibition curve.

In vivo studies

Animal maintenance

Male Wistar albino rats, weighting 200–250 g and aged 4 months, were obtained from Indian Institute of Science, Bangalore, India. These animals were housed in a group of four in polypropylene, polycarbonate, and stainless steel cages and acclimatized to the standard environmental conditions at the animal house of MLACW, Bengaluru (temperature $23 \text{ }^\circ\text{C} \pm 2 \text{ }^\circ\text{C}$ and relative humidity of $68\% \pm 1\%$, lighting of 350 lx and 12 h light/dark cycle) for 7 days prior to the actual experiment. These animals were supplied with standard diet and had all time access to water ad libitum during the entire experiment (Middha et al. 2015). Cages (size 421 mm \times 290 mm \times 190 mm with a gap of 7 mm between wires) and corn cob paddy were changed every alternate day to make sure that animals are dry and clean as per the National Institutes of Health guidelines (NIH 2010). Health status of animals was observed daily and no adverse incidents were spotted. This study was approved and cleared by Maharani Lakshmi Ammanni College Internal Animal Ethical Committee (1368/ac/10/CPCSEA/BT-SKM/06), Bengaluru, India.

Acute toxicity test

Swiss albino mice (25–30 g) of either sex were divided into five groups of ten each and housed five per cage. Animals made to fast overnight were used for the study. However, free access to water was provided prior to experiment. *H. salicifolia* leaf (HSL) extract at different dose levels (5.0–9.0 g/kg BW/mL) was supplemented orally once to experimental groups and mice were observed for 24 h, morbidity was recorded. Median lethal dose LD_{50} was established as per Middha et al. (2015).

The acute toxicity (LD_{50}) was calculated using the formula:

$$LD_{50} = LDy - \sum (Dd \times Md)/n.$$

where LDy = highest dose and n = number of animals per group ($n = 10$), Dd = dose difference, Md = mean dead.

Diabetic study groups

After acclimatization, these animals were subjected to a starvation period of 16 h for inducing the diabetes condition in them and were administered with alloxan monohydrate (150 mg/kg IP). Animals with fasting blood glucose value with 9.7 mmol/L or above were considered as hyperglycemic and used for further experimentation. Once diabetes was induced in the animals, they were randomized into six groups as given below. Group I (normal control group) received 1 mL saline (NC), group II control diabetic rats remained untreated (DC), group III diabetic rats received the extract at the concentration of 200 mg/kg of body weight (BW) (LHSL), group IV diabetic rats received the extract at the concentration of 400 mg/kg BW (HHSL), group V diabetic rats received glibenclamide (600 μ g/kg BW) in saline solution daily using an intragastric tube for 45 days (DG), group VI diabetic rats received protamine zinc insulin suspension intra-peritoneally (2 units/kg BW) (Insugen, Biocon, Bangalore, India) for 45 days (DI). The procedure of weighing the amount of feed and volume of water was continued for 45 days after initiation of the drug administration along with drawing of blood from the tail vein end cutting method every week and blood glucose level was estimated by Ames One Touch Glucometer (Accu Check, Roche, Germany) (Middha et al. 2011, 2015).

Tissue preparation

At the end of trial period, experimental animals were anesthetized with ketamine:xylazine cocktail (80–120 mg/kg: 10–16 mg/kg IP) and euthanized in a CO_2 chamber (Conlee et al. 2005). Pancreas was removed, cleaned, and washed in ice-cold saline. Then, weighed and homogenized using 50 mM phosphate buffer pH (7.0). The homogenate was centrifuged at 600 g for 15 min at $4 \text{ }^\circ\text{C}$ (RV/FM, superspin, plastocraft, India). The supernatant was collected and used for enzymatic antioxidant analysis.

Enzymatic antioxidant assays

Studied enzymatic antioxidants involve, SOD (Superoxide dismutase), and GPx (Glutathione peroxidase).

Glutathione peroxidase (GPx 1.11.1.9)

GPx activity was measured at $37 \text{ }^\circ\text{C}$ by the method of Flohe and Gunzler (1984). The reaction mixture consisted of 500 μ L of phosphate buffer, 100 μ L of 0.01 M reduced glutathione (GSH), 100 μ L of 1.5 mM NADPH, and 100 μ L of glutathione reductase (GR) (0.24 U). 100 μ L of tissue extract was added to the reaction mixture and incubated at $37 \text{ }^\circ\text{C}$ for 10 min. 50 μ L of 12 mM t-butyl hydroperoxide was added to

450 μL of reaction mixture and absorbance was measured at 340 nm for 180 s in a spectrophotometer (ELICO, BL 192, INDIA). The molar absorptivity of $6.22 \times 10^3 \text{ M/cm}$ was used to determine enzyme activity. One unit of activity is equal to 1 mM NADPH oxidized per minute per milligram protein.

Superoxide dismutase (SOD, EC 1.15.1.1)

SOD activity was determined at room temperature (RT) according to the method of Misra and Fridovich (1972). Tissue extract (100 μL) was added to 880 μL carbonate buffer of 0.05 M, pH (10.2), 0.1 mM EDTA. 20 μL of 30 mM epinephrine in 0.05% acetic acid was added to the mixture and the change in absorbance was recorded for 4 min at 480 nm in a spectrophotometer. The amount of enzyme that results in 50% inhibition of epinephrine auto-oxidation is defined as one unit.

Lipid peroxidation (MDA)

Malondialdehyde (MDA) content was analyzed by the method of Ohkawa et al. (1979) using 1, 1, 3, 3-tetramethoxypropane (TMP) as the standard. Usually for testing MDA production, thiobarbituric acid-reactive substances (TBARS), a spectrophotometric assay that quantifies a chromogen produced by the reaction of MDA with thiobarbituric acid is used. MDA is often used as a marker for ROS-mediated lipid peroxidation. The final TBA–MDA (malondialdehyde) chromogen was measured at 532 nm and the concentration was expressed in terms of n moles of MDA per milligram protein using standard graph.

Statistical analysis

Data are presented as mean \pm standard error mean (SEM) ($n=6$). Differences were evaluated by one-way analysis of variance (ANOVA) test completed by a Tukey test. Differences were considered significant at $p < 0.05$.

Results and discussion

Extraction yield

The yield of evaporated extracts based on dry weight basis was found to be 8.5% w/w.

Total phenol and flavonoid contents

The total phenol and flavonoid content of methanol extracts was found to be $199 \pm 3.2 \text{ QE mg/g}$ and $90.63 \pm 6.14 \text{ GAE mg/g}$. Several studies have reported polyphenols for their

antioxidant activity (Goyal et al. 2013, 2017; Middha et al. 2015, 2016). Foti (2007) reviewed the antioxidant ability of polyphenol to forage free radicals and various reactive oxygen species, such as singlet oxygen and hydrogen peroxide to be eliminated from the cells to sustain healthy metabolism.

Our results are in accordance with Goyal et al. (2011, 2013) who also showed that *H. salicifolia* (HS) is found to be rich in phenols and flavonoids. Arimboor et al. (2008) reported gallic acid, vanillic acid, and salicylic acid in *Hippophae* leaves using RP-HPLC–DAD method. Gallic acid, a flavonoid was reported to scavenge and help in preventing different diseases like cancer development and inflammation (Choubey et al. 2015). Therefore, it might be indicated by the result shown in this study that some of its pharmacological properties could be because of these valuable compounds.

Free radical scavenging (DPPH method)

The antioxidants donate hydrogen atom to stabilize DPPH radicals (Middha et al. 2012; Goyal et al. 2013). In this manuscript, results show that DPPH radical scavenging capabilities of HSL (61%) were higher than ascorbic acid (53.7%) at 80 $\mu\text{g/mL}$ (Supplementary Fig. 1). This study shows that HSL extract has good radical scavenging capability and can be used as a radical scavenger, possibly as a prime antioxidant. Part of the anti-oxidative activity may be due to phenolic or flavonoid compounds or might be the synergistic effect of multiple compounds that are encountered in *H. salicifolia* (Middha et al. 2013).

In vivo studies

Diabetes is a common and rapidly spreading globally and is characterized mainly by hyperglycemia (American Diabetes Association 2014). In the present study, alloxan was used to induce diabetic condition in the experimental animals. Alloxan damages the β -cells of the islets of Langerhans irreversibly, thus inhibiting the release of insulin. It also causes oxidative stress in the rats, it is injected to (Sun et al. 2008).

Acute toxicity test

The acute toxicity test of HSL revealed that the median lethal dose (LD_{50}) was found to be 3.92 g/kg BW in mice and thus can be considered to be relatively safe as per Loomis and Hayes (1996) LD_{50} Scale. In general, the smaller the LD_{50} value, the more toxic the chemical is (Raj et al. 2013; Goyal et al. 2017).

Effect of *H. salicifolia* leaf extract on glucose level

Antihyperglycemic activity of HSL in comparison to glibenclamide and insulin was estimated by measuring blood glucose level at different intervals and by measuring the activity of different antioxidant enzyme defences from oxidative stress. Fasting blood glucose (FBG) levels were within the range of 60–100 mg/dL in all the groups before the administration of alloxan. The level of blood glucose in normal and experimental groups is shown in Table 1. Intraperitoneal alloxan administration leads to a significant increase ($p < 0.05$) in the blood glucose level in diabetic groups when compared to the normal control group. These changes were seen to have significantly ($p < 0.05$) reversed to normal levels on administration of glibenclamide and HSL at 200 and 400 mg/kg body weight (low as well as high levels). The highest reversal to normal glucose level was seen in DI (62%) followed by DG (44%), HHSL (39%) and the LHSL (22%) group. However, the changes were not significant between HS supplemented groups.

Ahmed et al. (2014) also evaluated potential anti-diabetic effects of HS on blood glucose and antioxidant levels in pancreas. Cao et al. (Cao et al. 2003) reported that the flavonoids from the seed and fruit residue of *H. rhamnoides* L. exhibited hypoglycemic effect. The probable means by which HS brings down the glycemia may be by potentiating insulin outcome in plasma by increasing either the pancreatic secretion of insulin from β -cells of islets of Langerhans or its release from the bound form (Parmar and Kar 2007; Middha et al. 2012, 2016). Plant antioxidants are capable of repairing and regenerating pancreatic β -cells. Glyburide (glibenclamide) is a second generation sulfonylurea drug used for treating type 2 diabetes (Zharikova et al. 2009). The drug glibenclamide has been reported to improve the

Table 1 Changes in glucose levels of normal and experimental diabetic rats

Groups	Initial	Final
NC	94.21 \pm 1.27	95.83 \pm 01.64 ^a
DC	394.35 \pm 10.88	318.3 \pm 10.71 ^b
LHSL	304.21 \pm 32.73	235.8 \pm 16.41 ^c
HHSL	310.71 \pm 6.45	187.8 \pm 19.47 ^{de}
DG	312.89 \pm 1.25	174.0 \pm 12.75 ^e
DI	307.38 \pm 1.33	115.0 \pm 02.75 ^a

Values are mean \pm S.E of six animals/group

NC normal control, DC diabetic control, LHSL low *H. salicifolia* (200 mg/kg body weight), HHSL high *H. salicifolia* (400 mg/kg body weight), DG diabetic rats treated with glibenclamide, DI diabetic rats treated with insulin

Significance between the group is represented in lower case. Those not sharing the same letters are significantly different at $p < 0.05$

architectural form of the islets of Langerhans (Asgary et al. 2012).

Effect of *H. salicifolia* leaf extract on the different pancreatic antioxidant enzymes

Antioxidants are a category of substances which inhibit or delay oxidative processes, while getting oxidized themselves. The mechanisms for preventing antioxidant activity are: (1) chain-breaking mechanism whereby the antioxidant gives an electron to the free radical present in the system and (2) involves removal of reactive oxygen species (ROS) and reactive nitrogen species (RNS) initiators by terminating the chain-initiating catalysts (Usha et al. 2014; Middha et al. 2015).

Figure 1 shows the SOD activity in the pancreas. Alloxan treatment significantly ($p < 0.05$) reduced the SOD activity in diabetic group compared to the normal control group. The SOD level was elevated considerably by administering standard drugs like glibenclamide (76%) and insulin (81%) while moderate increase in case of low and high dosages of HSL (50% and 74%) was inferred. The changes were found to be statistically significant ($p < 0.05$) as compared to diabetic control group.

The activity of GPx in the pancreas on different groups is depicted in Fig. 2. As a result of the oxidative stress due to diabetes induction, the level of GPx was significantly decreased ($p < 0.05$). On orally administering the drugs, it is seen that the pattern followed by GPx activity increase (57%

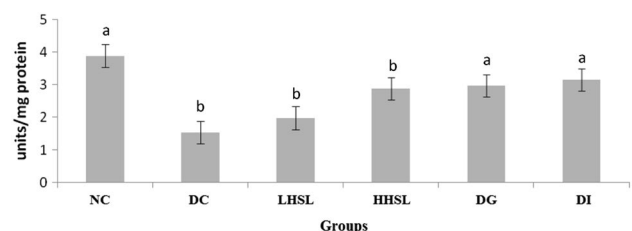


Fig. 1 Superoxide dismutase activity in pancreas of normal and experimental diabetic rats

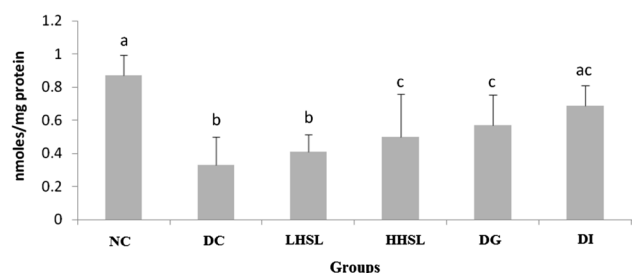


Fig. 2 Glutathione peroxidase activity in pancreas of normal and experimental diabetic rats

and 41%) is similar to that of SOD increase as compared to diabetes control experimental groups. However, the changes were not significant between HSL supplemented groups.

In their study, Sharma and his team (2011) showed *Hippophae* species can reduce hyperglycemia exhibited by STZ-diabetic rats thereby demonstrating its anti-hyperglycemic activity. However there are only a few studies on *Hippophae* in alloxan-treated diabetic rats (Wang et al. 2011). *H. salicifolia* endowed with various antioxidants that can prevent oxidative stress, vitamins (A, B, C, E and K), carotenoids, flavonoids, glycerophospholipids, phytosterols, zeaxanthin esters, etc., and so holds huge potential for anti-diabetic action (Usha et al. 2014; Guliyev et al. 2004) and our studies indicated for the same. Diabetes is seen to build up oxidative stress due to decreased action of the antioxidant enzymes and increased oxygen free radical. It is also seen that one-time injection of alloxan led to a reduction in the pancreatic SOD and GPx activity (Shah and Khan 2014). Present study shows that not only did the activities of SOD and GPx decrease in the pancreas but there was an increase in the levels of MDA in the alloxan-treated rats (Goyal et al. 2017).

In this study, we observed a significant increase in the concentration of MDA, a secondary product of LPO, in pancreas of alloxan-treated animals. Figure 3 portrays the pancreatic activity of MDA on the experimental groups. To test MDA production, thiobarbituric acid-reactive substances abbreviated as TBARS, a spectrophotometric assay that quantifies a chromogen produced by the reaction of MDA with thiobarbituric acid was used. These TBARS are increased significantly ($p < 0.05$) by the administration of alloxan when compared to normal control group. After administering the drugs, the MDA level descends lowest for insulin, thus there is a comparable decrease for HHSL and DG, followed by LHSL by 48%, 33%, 30%, and 15%, respectively. Significant reduction in the MDA level in the pancreas of HS-treated rats and boost in the antioxidant enzymes activities specify an adaptive means in reaction to oxidative stress by extinguishing the free radicals. Our results are in accordance with Sharma et al. (2011), wherein supplementation of *Hippophae* sp. restored the MDA levels

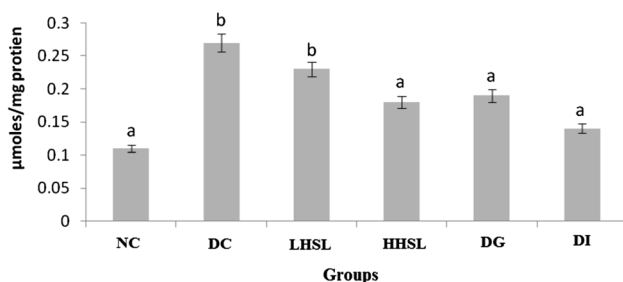


Fig. 3 Changes in MDA content in pancreas of normal and experimental diabetic rats

in pancreas. Some of the current studies have revealed that *Hippophae* sp. can prevent LPO and attenuate antioxidant properties (Cao et al. 2003; Geetha et al. 2003; Zheng et al. 2012; Goyal et al. 2013). While oxidative stress influences the cellular reliability only when antioxidant defence mechanisms are not able to handle the free radical generations then an antioxidant supplementation could boost up the detox mechanism.

Commercially available preparation of *Hippophae* showed maximum resistance, the levels of biochemical parameters such as MDA, reactive oxygen species (ROS), SOD, and glutathione (GSH) in blood samples revealed that *Hippophae* is rich in antioxidants and has adaptogenic potential (Sharma et al. 2011). The effect of a high dose of *H. salicifolia* extract on elevating SOD and GPx levels, decreasing lipid peroxidation, and decreasing blood glucose levels was comparable to that observed for the anti-diabetic drug, glibenclamide. The report from the research literature (Usha et al. 2014) and the current study reveals that *H. salicifolia* has anti-diabetic and antioxidant potential. In this research we found that by treatment with *H. salicifolia* leaves, diabetic rats could have their blood glucose levels returned to near normal.

Conclusions

To conclude, these findings demonstrate that *H. salicifolia* is rich in dietary antioxidants and has anti-diabetic effects on the alloxan-administered diabetic rats. Supplementation of HSL prevented or reversed the changes associated with diabetes. These findings maintain the role of dietetic antioxidant supplementation in the deterrence of diabetes-induced oxidative stress and recommend a potential remedial benefit to decline diabetic complications associated with oxidative stress. As per our knowledge, this report could be the first one on the anti-hyperglycemic activity of *H. salicifolia* leaves using diabetic animal model. However, compound-based mechanism studies are needed for the better understanding of the extract by which it modulates the oxidative stress in endocrine system due to diabetes. There are requirement of studies to test and verify the effect of *H. salicifolia* on diabetes patients.

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Author contribution TU and AKG performed the experiments, analyzed the data and prepared the manuscript. BCB procured and prepared the sample and wrote a part of the manuscript. SKM conceived the idea, designed and coordinated the experiments, improved the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in the studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

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