Available online on 15.12.2019 at http://jddtonline.info

Journal of Drug Delivery and Therapeutics

Open Access to Pharmaceutical and Medical Research

Open Access Research Article

In Vivo and *In Vitro* Antidiabetic Activity of Hydroalcoholic Extract of *Dactylorhiza Hatagirea* Roots: An Evaluation of Possible Phytoconstituents

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ABSTRACT

Diabetes mellitus is one of the most common endocrine disorder which causes microvascular and macrovascular complications. Several synthetic drugs used to treat diabetes have adverse effects on prolonged usage. This has given the impetus to the search for alternative medicines with no or less side effects. The plants of *Orchidaceae* family have displayed antimicrobial, anti-inflammatory, antioxidant, anticancer and antidiabetic activity. However, their antidiabetic properties are yet to be explored. This study sought to evaluate the antidiabetic potentials of hydroalcoholic extract of root of *Dactylorhiza hatagirea* (*D. hatagirea*) in diabetic rats. Qualitative analysis of various phytochemical constituents and quantitative analysis of total flavonoids were determined by the well-known test protocol available in the literature and quercetin was detected in hydroalcoholic extract of root of *D. hatagirea* under study by using RP-HPLC analysis. Percentage inhibition of α-amylase activity by *D. hatagirea* root extract was estimated with acarbose as the positive control, *D. hatagirea* extract show dose dependent activity. IC_{50} value of acarbose and extract was found to 35.33 and 224.45 µg/ml respectively. Diabetes was induced in wistar albino rats by administration of alloxan monohydrate (120 mg/kg; *i.p.*). The of hydroalcoholic extract of *D. hatagirea* at a dose of 100 and 200 mg/kg body weight was administrated at a single dose per day to diabetes-induced rats for a period of 15 day. The effect of hydroalcoholic extract of *D. hatagirea* root on blood glucose, total cholesterol (TR), triglycerides (TG), total protein and body weight were measured in the diabetic rats. The effect of hydroalcoholic extract of *D. hatagirea* root elicited significant reduction in blood glucose (p<0.001), (p<0.01), lipid parameters (TC, TG, total protein) (p<0.01) and significantly increased body weight at the dose of 200 mg/kg when compared with the diabetic-induced control. For future studies, phytochemcials responsible for various activities can be isolated and modified for pharmacological purpose.This study, therefore, justifies the use of the plant in the treatment of diabetes mellitus.

Keywords: *Dactylorhiza hatagirea,* Phytochemical profile, Quercetin, RP-HPLC, *In Vivo* and *In Vitro* Antidiabetic Activity

Article Info: Received 10 Oct 2019; Review Completed 19 Nov 2019; Accepted 28 Nov 2019; Available online 15 Dec 2019

Cite this article as:

Choukarya R, Choursia A, Rathi J, *In Vivo* and *In Vitro* Antidiabetic Activity of Hydroalcoholic Extract of *Dactylorhiza Hatagirea* Roots: An Evaluation of Possible Phytoconstituents, Journal of Drug Delivery and Therapeutics. 2019; 9(6-s):76-81 http://dx.doi.org/10.22270/jddt.v9i6-s.3752

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INTRODUCTION

Diabetes is characterized by hyperglycemia, altered lipids, carbohydrates and proteins metabolism which affect the patient quality of life in terms of social, psychological well-being as well as physical ill health[1,2]. Two forms of diabetes (Types 1 and 2) differ in their pathogenesis, but both have hyperglycemia as a common hall mark. In type 2 diabetes, hyperglycemia caused due to impairment in insulin secretion combined with or without impairment of insulin action[3]. The World Health Organization reported that worldwide global population is in the midst of a diabetes epidemic. The people in Southeast Asia and Western Pacific are being under greater risk and the majority of patients have type 2 diabetes. Insulin resistance typically precedes the onset of type 2 diabetes and is commonly accompanied by other cardiovascular risk factors such as dyslipidemia, hypertension and prothrombotic factors[4].Diabetes-related cardiovascular complications occur due to altered lipoprotein metabolism-mediated atherosclerosis and diabetics are 2 to 4 times more likely to suffer from stroke[5]. Although different classes of drugs are available to control type 2 diabetes, still it is a challenging task to bring a better molecule which is devoid of undesirable adverse effects than existing drugs. In Indian traditional medicine systems, the number of medicinal plants has been used since ancient time to effectively treat diabetes[6]. Multiple mechanisms, due to many phytoconstituents were documented for the antidiabetic activity of medicinal plants. Therefore, documenting the efficacy of antidiabetic medicinal plants has been increased and their characterizations of chemical constituents are focused in drug discovery programmes to bring a better lead molecule to treat diabetes[7]. The genus *Dactylorhiza* (Orchidaceae) is represented by approximately

75 species, distributed in most parts of Northern temperate zone. *D. hatagirea* (D. Don) Soo is a perennial orchid, native to the Himalayan region[8]. This species is distributed in India, Pakistan, Afghanistan, Nepal, Tibet and Bhutan. In India, it is distributed in Jammu and Kashmir[9], Uttarakhand[10-12] and Himachal Pradesh[13]. *D. hatagirea* is also a high-value medicinal orchid used in Ayurveda, Siddha and Unani medicine[14]. It is widely used to cure various diseases including dysentery, diarrhea, chronic fever, cough, stomachache, wounds, cuts, burns, fractures, and general weakness. The rhizomes of *D. hatagirea* contain bitter substance, glucoside mucilage, albumen, starch, a trace of volatile oil and ash[15]. Chemically, dactylorhins A to E, dactyloses A and B, lipids, and so on are found as major constituents. The tubers of this species yield a high quality salep which is used as a tonic to increase immunity (immunomodulator) and as a vajikaran drug (aphrodisiac); it increases sexual performance as well as serving as a nervine tonic[16]. Recently herbal health beverages have also been developed from this species[17]. Tubers of this species are supposed as a potent medicine in Leucorrhea in Traditional System of Medicine (TSM). Hence, there is a need for a more effective drug with lower side effects. Therefore, the present study was aimed to investigate in *vitro* and vivo antidiabetic activity of hydroalcoholic extracts of *D. hatagirea* in type 2 diabetic rats. The phytoconstituents (biomarkers) present in extract were characterized by high-performance liquid chromatography (HPLC) analysis.

MATERIALS AND METHODS

Collection of plant material

Fresh root plant material of *D. hatagirea* was collected from local area of Bhopal (M.P.) in the month of August, 2019. The plant material were washed thoroughly with normal tap water followed by sterile distill water. Then plant material was dried under shaded condition at room temperature. Fresh plant materials of *D. hatagirea* were crushed to powder using grinding machine. Powder was stored at 4°C in tight air container bottle.

Chemical reagents

Alloxan (Central Drug House Pvt. Ltd., India), Glibenclamide tablets (Daonil; Aventis Pharma. Ltd., India) were procured from the authorized distributor of the company. All the chemicals used in this study were obtained from Hi Media Laboratories Pvt. Ltd. (Mumbai, India), Sigma Aldrich Chemical Co. (Milwaukee, WI, USA), SD Fine-Chem Chem. Ltd. (Mumbai, India) and SRL Pvt. Ltd. (Mumbai, India). Quercetin was kindly provided by Scan Research Laboratories, Bhopal (India). Methanol and acetonitrile were of HPLC grade and purchased from Merck Ltd, New Delhi, India. All other chemicals and solvents used were of HPLC and analytical grade.

Extraction procedure

The maceration method was followed for the extraction. 42 gm powder of dried plant material was added into 100 ml of 70 % methanol in an Erlenmeyer flask (250 ml capacity) and resulting mixture was vortexed well. The maceration process was carried out in shaker incubator at with 50 rpm for 48-72 hrs. After this process, the extracts were filtered dried extract was stored in refrigerator for their future use in phytochemical analysis.

Qualitative phytochemical analysis of plant extract

The *D. hatagirea* extracts obtained was subjected to the preliminary phytochemical analysis following standard methods by Khandelwal and Kokate[18,19] . The extract was screened to identify the presence or absence of various active principles like phenolic compounds, carbohydrates, flavonoids, glycosides, saponins, alkaloids, fats or fixed oils, protein and amino acid and tannins.

Total flavonoids determination

The total flavonoid content was determined using the method of Olufunmiso *et al* [20]. 1 ml of 2% $AlCl_3$ methanolic solution was added to 3 ml of extract or standard and allowed to stand for 15 min at room temperature; the absorbance of the reaction mixture was measured at 420 nm using UV/visible spectrophotometer The content of flavonoids was calculated using standard graph of quercetin and the results were expressed as quercetin equivalent (mg/100mg).

Quantification of flavonoid compounds by HPLC technique

For HPLC investigation of flavonoid compounds the hydroalcoholic extracts of *D. hatagirea* root under study were used as a preliminary assessment of various compounds. The HPLC apparatus used for analysis was composed of a waters equipped with a UV dual detector and generated data were analyzed using Waters Ace software. For chromatographic separation Thermo C18 column (250 X 4.6 mm, 5µm) was applied. The chromatographic analysis was performed at ambient temperature on a RP-C18 analytical column with a mobile phase composed of Acetonitrile: Methanol (50:50 v/v) and was isocratically eluted at a flow rate of 1 ml/ min. A small sample volume of 20 µl was used for each sample run, being injected into the HPLC system. The chromatogram was monitored with UV detection at a wavelength of 256 nm. Sample volume (20 µl) and analysis time was 10min for both, standards and samples used for analysis. A quercetin was used as standards. A thermospectronic model of Labindia 3000+UV/VIS Spectrophotometer with 1cm. matched quartz cells were used for determination of λmax. The sample solution was chromatographed and a concentration of quercetin in extract sample was found out using regression equation.

In vitro anti diabetic activity of extract

Inhibition of alpha amylase enzyme

A total of 500 µl of test samples and standard drug (10-50µg/ml) were added to 500 µl of 0.20 mM phosphate buffer (pH 6.9) containing α-amylase (0.5mg/ml) solution and were incubated at 25°C for 10 min. After these, 500 µl of a 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9) was added to each tube. The reaction mixtures were then incubated at 25°C for 10 min. The reaction was stopped with 1.0 ml of 3, 5 dinitrosalicylic acid colour reagent. The test tubes were then incubated in a boiling water bath for 5 min, cooled to room temperature. The reaction mixture was then diluted after adding 10 ml distilled water and absorbance was measured at 540 nm. Control represent 100% enzyme activity and were conducted in similar way by replacing extract with vehicle.

In vivo anti diabetic activity of extract

Animals

Wistar rats (180-230 g) were group housed (n= 6) under a standard 12 h light/dark cycle and controlled conditions of temperature and humidity (25±2°C, 55-65%). Rats received standard rodent chow and water *ad libitum*. Rats were acclimatized to laboratory conditions for 7 days before carrying out the experiments. All the experiments were carried in a noise-free room between 08.00 to 15.00 h. Separate group (n=6) of rats was used for each set of

experiments. The animal studies were approved by the Institutional Animal Ethics Committee (IAEC), constituted for the purpose of control and supervision of experimental animals by Ministry of Environment and Forests, Government of India, New Delhi, India.

Acute oral toxicity

Acute toxicity study of the prepared extracts was carried out according to the Organization for Economic Co-Operation and Development (OECD) Guidelines-423[21] the animals were fasted for 4 h, but allowed free access to water throughout. As per the OECD recommendations, the starting dose level should be that which is most likely to produce mortality in some of the dosed animals; and when there is no information available on a substance to be tested in this regard; for animal welfare reasons, The dose level to be used as the starting dose is selected from one of three fixed levels 50, 100,150, 300 and 2000 mg/kg body weight orally for 4 days of six groups of rats (n=6). Acute toxicity was determined as per reported method[22].

Induction of experimental diabetes in rats

After fasting, diabetes was induced by a single intraperitoneal injection of 120 mg/kg body weight of 'Alloxan monohydrate' in distilled water. The animals were allowed to drink 5% glucose solution overnight to overcome the drug-induced hypoglycaemia. These animals were tested for diabetes after 15 days and animals with blood glucose (fasting) were selected for experimentation[23].

Experimental protocol

Five groups of rats were employed in the present study and each group contains six animals, as follows

Group I: Rats served as normal-control and received the vehicle (0.5 ml distilled water/day/rat)

Group II: Rats served as diabetic-control and received the vehicle (0.5 ml distilled water/day/rat)

Group III: Rats (diabetic) were administered *Dactylorhiza hatagirea* (100 mg/kg p.o.) for 15 days.

Group IV: Rats (diabetic) were administered *Dactylorhiza hatagirea* (200 mg/kg p.o.) for 15 days.

Group V: Rats (diabetic) were administered Glibenclamide (600µg/kg p.o.) for 15 days.

Bioassay

On 15th day of treatment, blood samples were collected by retro-orbital plexus puncture method under mild ether anesthesia and serum was separated by centrifugation. Serum glucose, total protein, cholesterol (TC) and total triglyceride (TG) levels were evaluated using a commercial kit[24]. Body weights of rats were taken before and after treatment[25].

Statistical analysis

Results were expressed as mean ± SEM. Data were analyzed with one way ANOVA for the comparison between groups, followed by Tukey as a post hoc test.

RESULTS AND DISCUSSIONS

The crude extracts so obtained after maceration extraction process was concentrated on water bath by evaporation the solvents completely to obtain the actual yield of extraction. The yield of hydroalcoholic extracts was found to be 8.556 %. The results of qualitative phytochemical analysis of the crude powder of root of *D. hatagirea* were shown in Table 1. Hydroalcoholic extracts of *D. hatagirea* showed the presence of flavonoids, carbohydrate and saponins. The content of total flavonoid compounds (TFC) was expressed as mg/100mg of quercetin equivalent of dry extract sample using the equation obtained from the calibration curve: **$Y = 0.06X+0.019$, $R^2= 0.999$,** where X is the quercetin equivalent (QE) and Y is the absorbance Table 2.

Table 1 Results of phytochemical screening of *D. hatagirea*

S. No.	Phytochemicals	Tests	Observation	Inference
1.	Alkaloids	Iodine Test	No blue colour	-
		Wagner's Test	No reddish brown precipitate	-
		Dragendorff's Tests	No orange brown precipitate	-
2.	Flavonoids	NaOH Tests	Colourless	+
		Shinoda Tests	Colourless	-
3.	Glycosides	Keller-Kiliani Test	No Bluish green colour	-
4.	Phenols	Phenol Tests	No Blue colour	-
5.	Saponins	Foam Test	Layer of foam	+
6.	Tannins	Gelatin Test	No white precipitate	-
7.	Carbohydrates	Molisch's test	No violet colour	+
		Fehling's test	Reddish orange precipitate	-
8.	Proteins	Millon's test	No white precipitate	-

Table 2 Estimation of total flavonoid content

S. No.	Solvents→ Bioactive compound↓	Hydroalcoholic Extract (*Dactylorhiza hatagirea*)
1.	Total flavonoid (Quercetin equivalent (QE) mg/100mg)	0.866

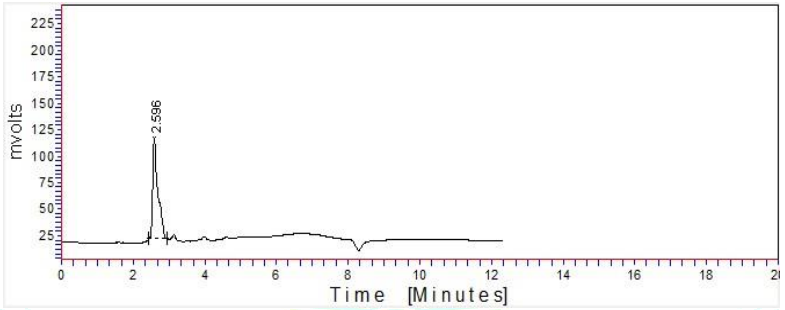
Flavonoids compounds are secondary metabolites in plants which play an immensely important role in human health and nutrition. The HPLC chromatogram of standard quercetin and hydroalcoholic extract are shown in Fig. 1 and the values are expressed in ppm. The retention time for standard and extract was found to be 2.596 min and 2.42min respectively. Characteristics parameters for standard quercetin was given in table 3 and results of quantitative estimation of quercetin in hydroalcoholic seed extract were given in table 4. Percentage inhibition of α-amylase activity by *D. hatagirea* root extract was estimated with acarbose as the positive control. *D. hatagirea* extract show dose dependent activity. IC_{50} value of acarbose and extract was found to 35.33 and 224.45 µg/ml respectively Table 5.

Table 3 Characteristics of the analytical method derived from the standard calibration curve

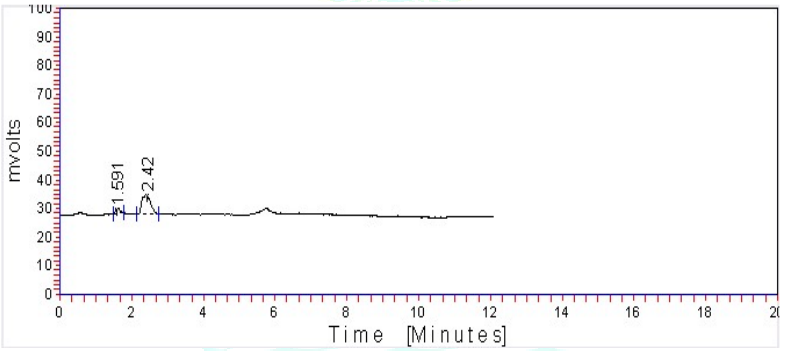
Compound	Linearity range µg/ml	Correlation co-efficient	Slope	Intercept
Quercetin	5-25	0.999	94.39	-30.43

Table 4 Quantitative estimation of quercetin in hydroalcoholic extract

S. No.	Extract	RT	Area	% Assay
1.	***Dactylorhiza hatagirea***	2.42	546.88	0.1421



(A)



(B)

Figure 1 Chromatogram of (A) Standard Quercetin (B) Hydroalcoholic Extract of *Dactylorhiza hatagirea*

Table 5: *In vitro* antidiabetic activity of *D. hatagirea* root extract analyzed by α-amylase inhibition assay and comparison with standard drug acarbose

S. No	Acarbose		*Dactylorhiza hatagirea*
	Conc.	% Inhibition	% Inhibition
1.	100	51.190	39.417
2.	200	70.105	45.502
3.	300	74.206	57.010
4.	400	85.185	66.137
5.	500	88.756	84.391
IC_{50} (µg/ml)		35.33	224.45

It is well known fact that alloxan monohydrate induces diabetes mellitus in rats by selective necrotic action on the beta cells of pancreas leading to insulin deficiency. Insulin deficiency leads to various metabolic aberrations in animals like increased blood glucose level, increased levels of cholesterol and triglyceride and decreased protein content[26]. As expected in alloxan treated rats, there was significant increase in blood glucose, cholesterol (TC) and triglyceride (TG) levels. The diabetic animals showed significant decrease in blood glucose level after 15 days treatment.

Moreover it also decreased the levels of cholesterol (TC) and triglyceride (TG) increased by alloxan treatment. Alloxan treatment of the rats has showed the loss in body weight as compared to normal rats. However, the hydroalcoholic extract was more effective and results are comparable with that of reference drug, gliblenclamide. Hence, we can say that presence of flavonoid in the ethyl acetate fraction may be responsible for antidiabetic activity. As shown in Table 6 Blood glucose level of animals in all groups was recorded at 0, 8th and 15th day. Progressive decrease in blood glucose level was found in all treatment groups during study. At the end of experiment Glibenclamide 600µg/kg, *D. hatagirea* 100 and 200 mg/kg/p.o. (130.50; 150.30 and 139.40) treated group blood glucose level was decrease significantly ($p<0.01$) at 15thdays, respectively.

Table 6 Effect of hydroalcoholic extract of *D. hatagirea* treatment on blood glucose (mg/dl) in normal and diabetic rats

Group	Treatment	Blood glucose (mg/dl)		
		Days 0	Days 8	Days 15
I	Normal	90.17± 5.50	98.00 ± 5.50	101.30 ± 5.50
II	Diabetic Control	270.00 ± 10.35	280.00± 10.11#	284.75± 10.12#
III	Diabetic + *Dactylorhiza hatagirea* (100 mg/kg)	250.00 ± 4.50	150.30± 4.50***	125.20 ± 4.50***
IV	Diabetic + *Dactylorhiza hatagirea* (200 mg/kg)	245.00 ± 5.00	139.40 ± 5.10***	117.80 ± 5.0***
V	Diabetic + Glibenclamide (600µg/kg)	240.00 ± 4.55	130.50± 4.50***	112.70 ± 4.50***

Values are expressed as mean±S.E.M (n = 6).Values are statistically significant at # $p<0.001$ vs. normal group; *P < 0.001, **P < 0.01vs diabetic control group (Two-way ANOVA test).

As shown in Table 7 Glibenclamide 600µg/kg, *D. hatagirea* 100 and 200 mg/kg/p.o. treated group biochemical parameters like TC, TG level was decrease significantly ($p<0.01$) and total protein level was increase as compared to diabetic control group. As represented in Table 8 body weights of animals in all groups were performed at the initial and end of the study. Body weight of animals was significantly ($p<0.05$) maintained in all treatment groups (Glibenclamide 600µg/kg, *D. hatagirea* 100 and 200 mg/kg/p.o. 205, 195, 200) during study as compared to control group (160).

Table 7 Effect of hydroalcoholic extract of *D. hatagirea* treatment on biochemical parameters in normal and diabetic rats

Group	Treatment	TC (mg/dL)	TG (mg/dL)	Total protein(g/dl)
I	Normal	90.00 ± 5.00	80.00 ± 5.00	8.00 ± 0.45
II	Diabetic Control	180.0 ± 6.00	153.0 ± 6.00	5.00 ± 0.50
III	Diabetic + *Dactylorhiza hatagirea* (100 mg/kg)	109.6 ± 5.55**	95.50 ± 5.50*	7.90 ± 0.30**
IV	Diabetic + *Dactylorhiza hatagirea* (200 mg/kg)	105.1 ± 5.10**	92.50 ± 5.56*	8.35 ± 0.45**
V	Diabetic + Glibenclamide (600µg/kg)	101.1 ± 5.10**	89.20± 5.22*	8.75 ± 0.25**

Table 8 Effects of hydroalcoholic extract of *D. hatagirea* on body weight

Group	Treatment	Initial weight (gm)	Final weight (gm)
I	Normal	160.00 ± 9.00	180.10 ± 9.06
II	Diabetic Control	170.00 ± 8.40	160.00 ± 8.40
III	Diabetic + *Dactylorhiza hatagirea* (100 mg/kg)	165.00 ± 10.00	195.00 ± 7.32
IV	Diabetic + *Dactylorhiza hatagirea* (200 mg/kg)	165.00 ± 9.20	200.00 ± 8.42
V	Diabetic + Glibenclamide (600µg/kg)	160.00 ± 7.80	205.00 ± 8.16

CONCLUSION

From the present study, it is concluded that *D. hatagirea* may be useful in treating diabetes mellitus with no visible signs or symptoms of toxicity in normal rats indicating a high margin of safety. The hydroalcoholic extracts of *D. hatagirea* have indicated high level of anti-diabetic activity. The extracts exhibited anti-hyperglycemic activity comparable to that of a standard anti-diabetic drug, glibenclamide. The traditional use of *D. hatagirea* to treat diabetes is supported by laboratory results from this study, suggesting a need to isolate and evaluate active constituents responsible for the exhibited biological activity.

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