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Research Article

ANTI-OXIDANT AND ANTI-DIABETIC ACTIVITIES OF ETHANOLIC EXTRACT OF *Primula denticulata* FLOWERS

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ABSTRACT

The present investigation was carried out to focus on the anti-oxidant and anti-diabetic effect of ethanolic extract of flowers of *Primula denticulata* by DPPH, ferrous chelating, reducing power assay and streptozotocin induced diabetes respectively. Dose selection was made on the basis of acute oral toxicity study (100mg/kg, 200mg/kg, 400mg/kg bodyweight) as per OECD guidelines 423. The blood glucose levels were measured by using blood glucose measuring strips based on glucose-oxidase method. The extract showed significant anti-oxidant and anti-diabetic activity when compared with standard drug.

Keywords: *Primula denticulata,* anti-oxidant and anti-diabetic activity.

INTRODUCTION

Diabetes mellitus is a chronic disease caused by deficiency in production of insulin by the pancreas, or by the ineffectiveness of the insulin produced. Due to diabetes numerous complications occurs such as retinopathy, neuropathy and peripheral vascular insufficiencies (Chehade *et al*., 2000). It is considered as one of the five leading causes of death in the world. (Ahmed *et al*., 2010)Traditional plants are used throughout the world for the treatment of diabetes. The World Health Organization has also recommended the evaluation of traditional plant treatments for diabetes (Day, 1998).Oxidative stress which is defined as an imbalance between the generation of oxidants and antioxidant defense capacity of the body is suggested as a mechanism underlying diabetes and diabetic complications like many other diseases (Atalay *et al* .,2002). There is also evidence that higher glucose concentration causes depression on natural antioxidant defense agents such as glutathione or vitamin C (Gumieniczek *et al*., 2002). The increase in the levels of reactive oxygen species and free radicals cause damage in the biological structures such as cell wall, genetic material and enzymes. These oxidants also cause micro-vascular and macrovascular complications, cardiovascular diseases, kidney and nerve damage (Aydın *et al*., 2001).

P. denticulata belongs to the family Primulaceae is an easy-growing, rumbustious species originating from meadows and the light woodland of the Himalayas. It is also called as drumstick primula. *Primula denticulat*a grows best in a partly shaded spot with soil that does not dry out in summer. *Primula* species have medicinal properties and used as anti-inflammatory, bactericidal, diuretic, antitumour and *hepatoprotective*. (Tokalov *et al*., 2004) *Primula denticulata* contains triterpene glycosides denticin and denticulatin and flavanoid. (Ahmad *et al*., 1990). As there is no scientific data available regarding anti-diabetic activity of plant, the aim of the study was to investigate anti- diabetic effect of ethanolic extract by streptozotocin induced diabeties in rats, as well as to evaluate the anti-oxidant effect of ethanolic extract by three different models DPPH, ferrous chelating and reducing power assay.

MATERIAL AND METHODS

Collection and authentification of plant material

P. denticulata was authenticated at Department of Botany H.N.B Garhwal University, Srinagar Garhwal, Uttarakhand by

taxonomist Dr. J. K. Tewari. The flowers of *Primula denticulata* were collected from Rudraprayag, Garhwal, Uttarakhand, India during July-August 2010.

Extraction of plant material

The flowers of *P. denticulata* was collected and dried by means of a natural sundry. The dried plant material was powdered and subjected to hot extraction by means of soxhlet apparatus using 95% ethanol. The solvent from total extract was distilled off and concentrate was evaporated on water bath to syrupy consistency and then evaporated to dryness.

Preliminary phytochemical screening

The qualitative chemical tests of ethanolic extract of *P. denticulata* were carried out by using standard procedure to determine the presence of various phytochemicals (Kokate, 2005),

***In-vitro* antioxidant studies**

DPPH method

The stable 1, 1-diphenyl-2-picryl hydrazyl radical (DPPH) was used for determination of free radical-scavenging activity of the extracts. On accepting hydrogen from a corresponding donor (antioxidant), its solutions lose the characteristic deep purple (λmax 515-517nm) colour. DPPH is very popular for the study of natural antioxidants.Antioxidants reacts with DPPH, which is reduced to the DPPHH and as consequence the absorbance's decreased from the DPPH radical to the DPPH-H form. The degree of discoloration indicates the scavenging potential of the antioxidant compounds or extracts in terms of hydrogen donating ability. 75μM DPPH solutions in methanol were freshly prepared. Different concentrations of extract and standard ascorbic acid were added to the equal volume of methanolic solution of DPPH. After 30min at room temperature, the absorbance was recorded at 517nm. A control reaction was carried out without the test sample. Radical scavenging activity was calculated by the following formula. % Inhibition=$(A_0-A_1)/ A_0$ x100 where A_0 is the absorbance of control and A_1 is the absorbance of extract or standard. IC_{50} values denote the concentration of sample, which is required to scavenge 50% of DPPH free radicals. IC_{50} value was determined by linear regression analysis from the graph plotted between % inhibition and the different concentrations of *P. denticulata* extracts as well as standarad. (Curcic *et al.,* 2012).

Ferrous chelating activity

On 1mL of each ethanolic extract (200-1000μg/mL) and standard BHT dilutions (100-400μg/mL) was added to a solution of 2mM ferrous chloride (0.2mL). The reaction was initiated by addition of 5mM ferrozine (0.4mL). The total volume was adjusted to 4mL with ethanol. Then, the reaction mixtures were shaken vigorously and kept at room temperature for 10min. Absorbance of all the solutions were measured spectrophoto-metrically at 562nm (Robu *et al*., 2012). Ferrous chelating activity was calculated by the following formula. % ferrous chelation=$(A_0-A_1)/A_0$x100 where A_0 is the absorbance of control and A_1 is the absorbance of extract or standard.

Reducing power Method

Substances, which have reduction potential, react with potassium ferricyanide (Fe3+) to form potassium ferrocyanide (Fe2+), which then reacts with ferric chloride to form ferric ferrous complex that has an absorption maximum at 700nm. 1mL of each ethanolic extract (100-400μg/mL), standard ascorbic acid dilutions (20-100 μg/mL) and control sample (1mL distilled water) was mixed with 2.5mL phosphate buffer solution (pH 6.6) and 2.5mL potassium ferricyanide (1%). The final reaction mixtures were properly mixed and incubated at 50ºC for 20min. After incubation, the reaction mixtures were rapidly cooled and mixed with 2.5mL of 10% tri chloroacetic acid. All solutions were centrifuged at 3000rpm for 10min. About 2.5mL of the supernatant was taken from each reaction mixture and 2.5mL distilled water and 0.5mL of ferric chloride (0.1%) was added, mixed well and allowed to stand for 10min. The absorbance was measured at 700nm (Maisarah *et al*., 2013).

***In vivo* Antidiabetic Activity**

Acute toxicity testing

Acute toxicity was performed for all extract according to the acute toxic classic method (as per OECD guidelines 423).

Table I. Preliminary phytochemical investigation *of P. denticulata*

S.No	Tests	Inference
1	Alkaloids	
	a) Dragendorff's test	–
	b) Hager's test	–
2	Carbohydrates	
	a) Benedict's test	+
	b) Fehling's test	+
	c) Molisch's test	+
3	Flavanoids	
	a) Shinoda's test	+
4	Triterpenoids	
	a) Liebermann -Burchard's test	+
5	Proteins	
	a) Biuret test	+
	b) Million's test	+
6	Steroids	
	a) Liebermann -Burchard's test	+
	b) Salkowaski reaction	+

Female albino rats were used and the animals were kept fasting for overnight providing only water, after which the extract was administered orally at the dose of 300mg/kg and observed for 24h. If the mortality was observed in two out of three animals, then the dose administered was assigned as toxic dose. If the mortality was observed in one animal, then the same dose was repeated to confirm the toxic dose. If mortality was not observed, the procedure was repeated for further higher dose i.e. 2000mg/kg. One tenth of maximum dose of the extract tested for acute toxicity was selected as middle dose i.e. 200mg/kg and double and half of middle dose was selected for higher and lower dose i.e. 400, 100mg/kg.

Experimental induction of diabetes

The rats were injected intraperitonially with streptozotocin 55 mg/kg b.w. dissolved in sterile citrate buffer (1M, pH 4.5). (Aslan *et al*., 2000, Aslan *et al*., 2003, and Nilufer *et al*.,2006) After 72h treatment blood glucose levels of all the rats were determined and rats with blood glucose level above 250mg/dL were selected for the acute study. The animals were randomly divided into six groups after the induction of diabetes. Group I contained normal animals and served as normal control. Group II served as diabetic control. Groups I and II received vehicle1% CMC during the experiment, while the Group III received the reference standard drug glimeperide (0.1mg/kg) and groups from IV to VI received the extract of (100mg/kg, 200mg/kg and 400mg/kg) respectively. Blood samples were collected just prior to 1, 3, 5 and 7h after drug administration.

RESULTS AND DISCUSSION

Phytochemical analysis of crude extract revealed the presence of carbohydrates, flavanoids, triterpenoids, proteins and steroids (Table I). The ethanolic extract of flowers of *P. denticulata* shows significant anti-oxidant activity by DPPH method, Reducing power assay and Ferrous chelating method. The IC_{50} values of extract and ascorbic acid are found to be 127.68 µg/ mL and 6.727 µg/ mL respectively by DPPH method (Table II). The IC_{50} values of extract and ascorbic acid are found to be 221.5µg/mL and 43.7µg/mL respectively by reducing power assay method (Table III). The IC_{50} values of extract and BHT are found to be 527.5µg/mL and 165.63µg/mL respectively by ferrous chelating method (Table IV).

This study showed no mortality up to the dose of 2000mg/kg body weight. So, the extract is found to be safe for the experiment.

Table II. Antioxidant activity of ethanolic extract of flower of *Primula denticulata* by DPPH method.

S. No.	EXTRACT		ASCORBIC ACID	
	Concentration (µg/mL)	% Inhibition	Concentration (µg/mL)	% Inhibition
1	100	43.95	2	13.22
2	200	62.55	4	23.42
3	300	75.32	6	43.24
4	400	85.69	8	59.04
5			10	80.22
		IC_{50} 127.68µg/mL		IC_{50} 6.727µg/ mL

Table III. Antioxidant activity of ethanolic extract of flower of *Primula denticulata* by reducing power assay.

S. No.	EXTRACT		ASCORBIC ACID	
	Concentration (µg/mL)	Absorbance	Concentration (µg/mL)	Absorbance
1	100	0.251	20	0.282
2	200	0.497	40	0.48
3	300	0.674	60	0.672
4	400	0.884	80	0.884
5			100	1.12
		IC 50 221.5 µg/mL		IC 50 43.7 µg/mL

Table IV. Antioxidant activity of ethanolic extract of flower of *Primula denticulata* by ferrous chelating method.

S. No.	EXTRACT		ASCORBIC ACID	
	Concentration (µg/mL)	% Inhibition	Concentration (µg/mL)	% Inhibition
1	200	31.24	100	44.12
2	400	44.5	200	52.24
3	600	55.36	300	64.26
4	800	67.18	400	82.21
5	1000	72.55		
		IC 50 527.5µg/mL		IC 50 165.63µg/mL

The dose 100mg/kg, 200mg/kg and 400mg/kg body weight were selected for the model streptozotocin induced diabetes in rats. The effect of the treatment with ethanolic extract and glibenclamide on blood glucose concentration in normal fasted and diabetic rats (Tablet V) n After 7th hour of experiment blood glucose level was found to be 254.69, 310.91, 306.62 and 293.59mg/dL in the diabetic rats treated with glibenclamide and plant ethanolic extract (100, 200 and 400mg/kg body weight) respectively.

In this study, the plant extract, up to the highest dose (2000mg/kg, p.o.) used in the acute toxicity test, did not cause any death or acute toxicity symptoms in the rats. The LD50, therefore, may be greater than 4000mg/kg (p.o.). This relatively high LD50 shows that the plant extract is non-toxic and/or safe in rats for the study. However, this study did not ascertain the doses used by the practitioners for such treatments. Streptozotocin shows diabetogenic properties by pancreatic beta cell destruction, beta cells normally regulate blood glucose levels

Table V. Effect of ethanolic extract of the flowers of *Primula denticulata* on blood glucose level in streptozotocin induced diabetic rats (mg/dl) (Acute Study)

Group	Treatment	Blood glucose level mg/dl (±SEM)				
		0h	1h	3h	5h	7h
1.	Normal	95.5 ±0.24	114.12 ±0.42	112.26 ±0.55	115.72 ±0.31	104.29 ±0.44
2.	Diabetic control	290.81 ±1.21	327.21 ±0.64	306.29 ±0.45	292.62 ±0.35	314.49 ±0.29
3.	Diabetic + glibenclamide (10mg/kg)	298.72 ±0.53	290.50* ±0.56	279.43** ±0.30	270.34** ±0.98	254.69** ±0.88
4.	Diabetic + extract (100mg/kg)	325.67 ±0.85	320.47 ±0.63	315.81 ±0.14	314.71 ±0.43	310.91 ±0.71
5.	Diabetic + extract (200mg/kg)	316.31 ±0.29	312.26 ±0 .57	311.34 ±0.54	308.64* ±0.67	306.62* ±0.66
6.	Diabetic + extract (400mg/kg)	305.44 ±0.22	301.51 ± 0.21	298.1* ±0.45	294.62* ±0.54	293.59* ±0.66

*****p<0.05, **p<0.01 significant compared to diabetic control

by producing the hormone insulin. Streptozotocin has been widely used to induce diabetes in experimental animals (Junod *et al*., 1969). STZ also induces oxidative stress or relative overload of oxidants i.e. reactive oxygen species (Wright *et al*., 1999). *P.denticulata* flower extract shows very significant invitro anti-oxidant activity.Various studies have shown that diabetes is associated with increased formation of free radicals and a decrease in antioxidant potential. The anti-oxidant and antidiabetic activity may be due to the presence of flavanoids and triterpenoid saponins. (Tokalov *et al*., 2004 and Ahmad *et al*.,1990)

CONCLUSION

The results revealed that *P.denticulata* possess significant antidiabetic and anti-oxidant activity by streptozotocin induced diabetic rats and DPPH method, reducing power assay and ferrous chelating method respectively. Further studies are necessary to elucidate the mechanism of action at cellular levels and components present it.

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