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IN VITRO CYTOTOXIC, *IN VITRO* AND *IN VIVO* ANTIDIABETIC ACTIVITY OF *ROYLEA CINERIA*

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ABSTRACT

Roylea cineria belong to family Lamiaceae is an evergreen, erect or scandent aromatic shrub found at 2000-5000 ft high. It is commonly known as Karui. *In vitro* cytotoxic activity of ethanol extract was carried out against different cell lines SK-Mel 41, U-87 MG, Hela, MDA-MBA-231 cell line and LC_{50} value was determined was found to be 131.8 µg/mL, 275.4 µg/mL, 302.0 µg/mL, respectively. *In vitro* anti-diabetic potential of EtOH extract (UB-1), petroleum ether extract (UB-2), ethyl acetate extract (UB-3), MeOH extract (UB-4) was tested, which showed maximum of 65.7%, 39.0%, 42.9% and 61.3% PTP-1B% inhibition, respectively at 100 µM concentration. IC_{50} value for UB-1 and UB-4 was found to be 7.5 µm and 9.1 µM, respectively. *In vivo* anti-diabetic potential of EtOH extract (UB-1), petroleum ether extract (UB-2), ethyl acetate (UB-3), MeOH extract (UB-4) were tested for lowering the blood glucose level of streptozotocin-induced diabetic rats. UB-2, UB-3 and UB-4, which showed decline in blood glucose level to the tune of 23.4% ($p < 0.01$), 18.2% ($p < 0.01$) and 20.7% ($p < 0.01$) during 0-24 hr, respectively at 100 mg/Kg oral dose.

Key words: Cytotoxic, Antidiabetic, *In vivo*, *In vitro*, *Roylea cineria*.

INTRODUCTION

Roylea cineria (also known as *Roylea elegans*) belong to family Lamiaceae is an evergreen, erect or scandent aromatic shrub found at 2000-5000 ft high[1]. It is commonly known as Karui, Titpatti and Patkarru[2]. It is locally used to cure ailments such as fever, malaria, skin diseases and diabetes[3]. An infusion of the leaves is drunk for continuous produced by boiling, and in Kumaon the same preparation used as a bitter tonic and febrifuge[4]. It is a shrub 1.8 m, pleasantly aromatic stems including older woody parts, densely greyish tomentose with a covering of adpressed and some spreading eglandular hairs. Leaves 2-4 × 0.7-3 cm ovate, crenate to almost lobed, truncate or broadly cuneate, acute with few scattered hairs on adaxial side, below with numerous eglandular spreading hairs mainly on veins and densely gland dotted. It is distributed in NW India, Himalaya to Nepal[5]. This herb is traditionally used for cure of several diseases. The objective of present study is to validate *in vitro* cytotoxic activity and in vitro and *in vivo* anti-diabetic activity of this plant.

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EXPERIMENTAL

Roylea cineria aerial parts were collected from Tehri Garhwal in August 2013 and identified for authenticity at Department of Botany HNB Garhwal University Srinagar Garhwal (U.K.) and a voucher specimen was deposited in Department herbarium. The air and shade dried powder of *Roylea cineria* was exhaustively extracted with 95% EtOH. Extract was concentrated using rotaevaporator afforded dark brown residue, which was further fractionated using petroleum ether, ethyl acetate followed by MeOH and these fractions were concentrated by vacumn evaporator.

Cell viability assay preparation

Cells were grown in a 96-well plate in Delbecco's minimum essential medium (DMEM) supplemented with 10% fetal bovine serum and antibiotic (Streptomycin, Penicillin-G, Amphotericin). About 1 mL of each cell suspension type (10^5 cells/mL) was seeded in each well and incubated at 37°C for 48 hr in 5% CO_2 for the formation of confluent monolayer. The monolayer of cells in the plate was exposed to various dilutions of ethanol extract.

The cell viability was measured using MTT assay (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide) with MTT (5 mg/mL) and DMSO. This tetrazolium salt is metabolically reduced by viable cells to yield a blue insoluble Formazan product measured at 540 nm spectrophotometrically. Controls were maintained throughout the experiment (untreated wells as control) the assay was performed in triplicate for each of the concentration. The mean of the cell viability values was compared to the control to determine the effect of extract on cells and % cell viability was plotted against concentration values.

IC_{50}, the concentration of extract required to inhibit 50% cell growth, was determined by plotting a graph of Log (concentration of extract) *vs* % cell inhibition, a line drawn from 50% value on the *Y* axis meeting the curve and interpolated to the *X* axis. The X-axis value gave Log (concentration of extract). The antilog of that value gave IC_{50} value. Percentage inhibition of extract against all cell lines was calculated using the following formula:

$$\% \text{ Cell survival} = (A_t - A_b) \times 100/(A_c - A_b)$$

Where A_t = Absorbance of Test,

A_b = Absorbance of blank (Media) and

A_c = Absorbance of control (cells)

% Cell inhibition = 100 − % cell survival.

In vitro Anti-diabetic activity using PTP-1B assay

Plant fractions EtOH extract (UB-1), petroleum ether extract (UB-2), ethyl acetate extract (UB-3), and MeOH extract (UB-4) were evaluated for its *in vitro* inhibitory activity against Protein Tyrosine Phosphatase-1B using colorimetric, non-radiaoactive PTP-1B tyrosine phosphatase drug discovery kit – BML-AK 822 from Enzo life Sciences, USA. PTP-1B enzyme inhibitory activity of extracts was evaluated using human recombinant PTP-1B enzyme provided in the kit at 10 µM concentration taking sodium orthovanadate and suramin as a control. Other components of the kit include substrate (IR5 insulin receptor residues), biomol red (phosphate determining reagent), assay buffer, suramin (PTP-1B inhibitor) and calibration standards. Assay was done according to the manufacturer's protocol, in brief the reaction was carried out in 96 well flat bottomed micro titer plates by the addition of assay buffer, solution of test extracts and diluted PTP1B enzyme. Enzyme reaction was initiated by addition of 50 µL of warmed 2x substrate;

then incubating the plate at 30°C for 30 min. After incubation for 30 min, reaction was terminated by addition of 25 µL of biomol red reagent and mixed thoroughly by repeated pipetting.

Test samples were dissolved in dimethyl sulfoxide (DMSO). Dilution of other components were accordingly as instruction in the manual provided in the kit. PTP-1B phosphatase acting on phosphopeptide substrate, release phosphate. The detection of free phosphate released is based on classic malachite green assay. After adding biomol red to reaction wells after 30 min of incubation as described earlier, the plate was incubated for another 20 min to develop the color. Absorbance was recorded at 620 nm on a microplate reader. The percentage inhibition of PTP-1B enzyme by test compound was calculated based on activity in the control tube (without inhibitor) taking as 100% from three independent set of experiments. The concentration of dimethyl sulfoxide (DMSO) in the test well (1.0%) has no demonstrable effect on PTP-1B enzyme activity.

In vivo anti-diabetic activity

Plant fractions EtOH extract (UB-1), petroleum ether extract (UB-2), ethyl acetate extract (UB-3), and MeOH extract (UB-4) were evaluated for its in vivo inhibitory activity. Male albino rats of Sprague-Dawley strain (8 to 10 weeks of age, body weight 160 ± 20 g) were selected for this study. Streptozotocin (Sigma, USA) was dissolved 100 mM citrate buffer pH 4.5 and calculated amount of fresh solution was injected to overnight fasted rats (60 mg/Kg) intraperitonically. Fasting blood glucose level was checked 48 hr later by glucometer using glucose strips and animals showing blood glucose values over 270 mg/dL were selected and divided into groups of five animals each.

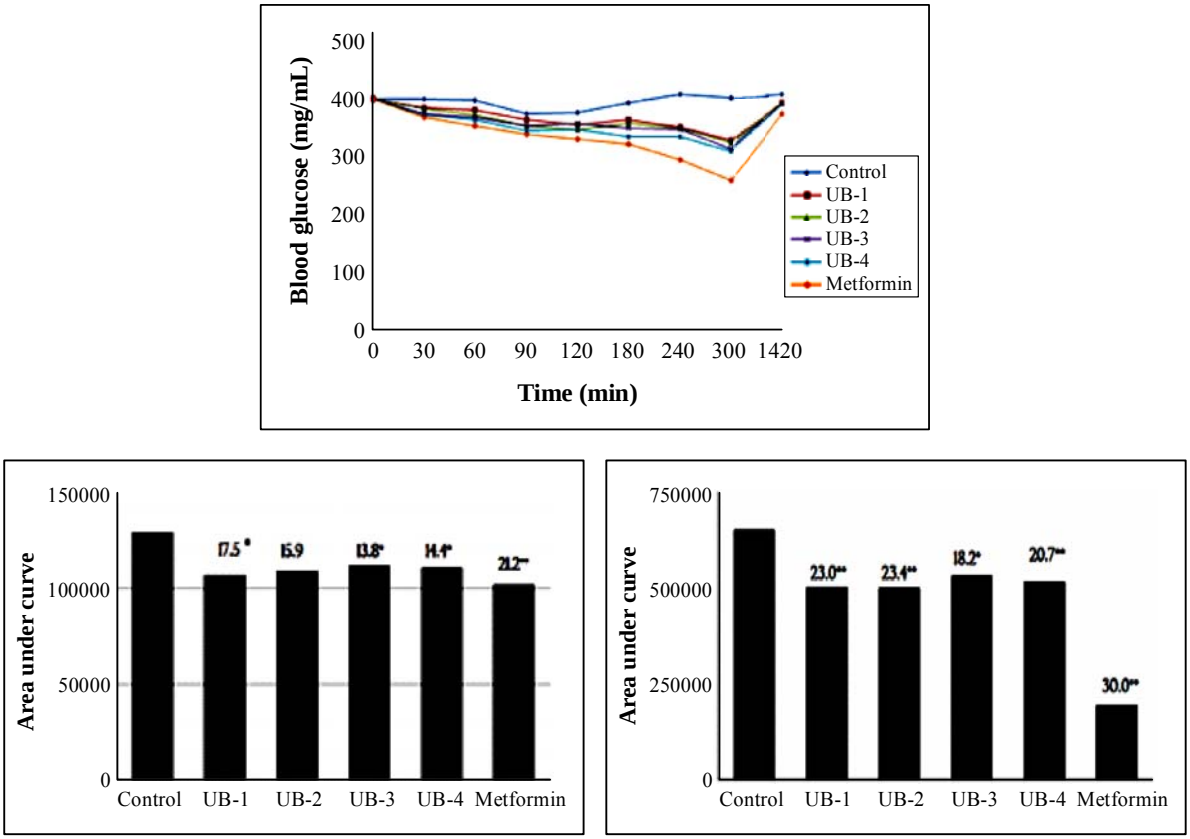

Fig. 1: Effect of fractions EtOH extract (UB-1), petroleum ether extract (UB-2), ethyl acetate (UB-3), MeOH extract (UB -4) and metformin (100 mg/Kg) on blood glucose levels of streptozotocin induced diabetic rats

Rats of experimental groups were administered suspension of standard drug and desired test fractions i.e. UB-1 to UB-4 orally (made in 1.0% gum acacia) at a dose of 100 mg/Kg body weight. Animals of control group were given an equal amount of 1% gum acacia. The blood glucose level of each animal was determined just before the administration of standard anti-diabetic drug and test samples (0 min) and thereafter at 30, 60, 90, 120, 180, 240, 300 and 1440 min. Food but not water was withdrawn from the cages during 0-300 min. The average lowering in blood glucose level between 0-300 min and 0 to 1440 min was calculated by plotting the blood glucose level on y-axis and time on x-axis and determined the area under curve (AUC) (Fig. 1). Comparing the AUC of experimental group with that of control group determined the percent lowering of blood glucose level during the period. Statistical analysis was made by Dunnett's test (Prism Software).

RESULTS AND DISCUSSION

(a) *In vitro* cytotoxic activity

Cancer is the most common and fatal disease responsible for 2-3% of deaths recorded worldwide annually. About 60% of anticancer drugs used nowadays are obtained from natural sources[6]. Medicinal plants can be a promising source of novel chemotherapeutic agents including cancer. Isolation of vincristine and vinblastine from *Catharanthus roseus*, have provided a clue for it. In addition, synthesis of topotecan and irinotecan derived from campotothecin (*Camptotheca*) further provided evidence that plant derived compounds if not effective as a drug can be converted to an effective agent[7]. A large number of plant species have been screened through bioassays in search of novel plant based anticancer drugs[8]. Bioactivity guided isolation is an important strategy for discovery of potent anticancer agents[9]. Roylea cinerea plant parts like leaves and flower have great therapeutic potential in Indian system of medicine[10]. Present investigation was aimed to assess the cytotoxic potential of ethanol extract of *Roylea cinerea*.

Crude ethanolic extract of *Roylea cinerea* against SK-MEL 2 cell line. (Table 1) showed % cell cytotoxicity of 2.43% at 10 µg/mL of concentration, which increased to 52.55% at 320 µg/mL concentration with IC_{50} value of (antilog 2.12 = 131.8 µg/mL) (Fig. 2). Ethanolic extract subjected against BE (2) C neuroblastoma cell line. (Table 2) showed maximum % cell cytotoxicity of 2.97% at 10 µg/mL and 54.8% at 320 µg/mL with IC_{50} (antilog 2.44 = 275.4 µg/mL) (Fig. 3). Ethanolic extract against U87MG human neuronale glioblastoma astrozytom cell line (Table 3) showed 1.11% cell cytotoxicity at 10 µg/mL concentration and 52.22% cell cytotoxicity at 320 µg/mL with IC_{50} value (antilog 2.48 = 302.0 µg/mL) of µg/mL concentration (Fig. 4). Ethanolic extract against against MDA-MB 231 breast cancer cell line (Table 4) showed 1.68% cell cytotoxicity at 10 µg/mL concentration and 30.08% cell cytotoxicity at 320 µg/mL. Its IC_{50} value was not calculated.

Table 1: Cytotoxic activity of ethanolic extract against SK-MEL 2 cell line

Concentration	Log_{10} concen.	O. D. Average	% Cell viablity	S. D.	% Cell cytotoxicity
Control	–	0.495	100	3.12	0
10 µg/mL	1	0.483	97.57	1.19	2.43
40 µg/mL	1.60	0.421	85.05	2.05	14.95
80 µg/mL	1.90	0.335	67.67	1.02	32.33
160 µg/mL	2.20	0.285	57.57	1.38	42.43
240 µg/mL	2.38	0.268	54.14	3.23	45.86
320 µg/mL	2.50	0.236	47.67	2.44	52.33

Table 2: Cytotoxic activity of ethanolic extract against BE(2)C neuroblastoma cell line.

Concentration	Log_{10} Concen.	O. D. Average	% Cell viablity	S.D.	% Cell cytotoxicity
Control	-	0.573	100	2.34	0
10 µg/mL	1	0.556	97.03	2.53	2.97
40 µg/mL	1.60	0.509	88.83	3.41	11.17
80 µg/mL	1.90	0.426	74.34	2.67	25.66
160 µg/mL	2.20	0.338	58.98	3.36	41.02
240 µg/mL	2.38	0.279	48.69	3.72	51.31
320 µg/mL	2.50	0.259	45.20	3.03	54.80

Table 3: Cytotoxic activity of ethanolic extract against U87 MG human neuronale glioblastoma astrozytom cell line

Concentration	Log 10 concen.	O. D. Average	% Cell viability	S.D.	% Cell cytotoxicity
Control	-	0.632	100	3.21	0
10 µg/mL	1	0.624	98.89	4.12	1.11
40 µg/mL	1.6	0.545	86.31	3.43	13.69
80 µg/mL	1.9	0.452	71.51	2.89	28.49
160 µg/mL	2.2	0.403	63.81	3.81	36.19
240 µg/mL	2.38	0.345	54.58	3.53	45.42
320 µg/mL	2.5	0.302	49.22	4.02	52.22

Table 4: Cytotoxic activity of ethanolic extract against MDA-MB 231 breast cancer cell line

Concentration	Log_{10} concen.	O. D. Average	% Cell viablity	S.D.	% Cell cytotoxicity
Control	-	0.731	100	3.03	0
10 µg/mL	1	0.718	98.32	3.56	1.68
40 µg/mL	1.60	0.689	94.37	4.22	5.63
80 µg/mL	1.90	0.634	86.82	3.83	13.18
160 µg/mL	2.20	0.582	79.63	2.89	20.37
240 µg/mL	2.38	0.543	74.32	3.68	25.68
320 µg/mL	2.50	0.511	69.92	4.13	30.08

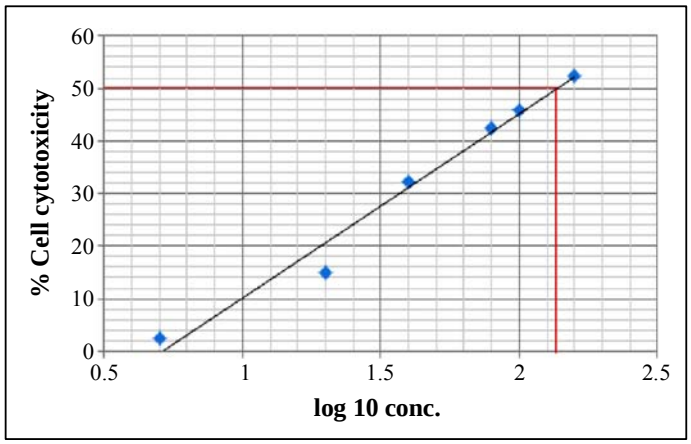

Fig. 2: Calculation of IC_{50} value (antilog 2.12 = 131.8 µg/mL) for Sk-Mel 2 cell line

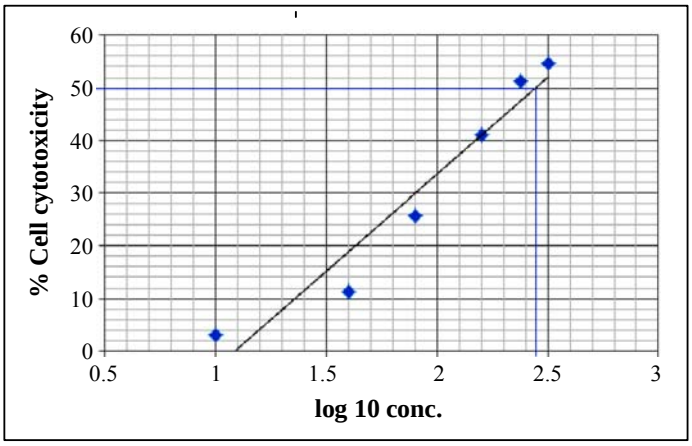

Fig. 3: Calculation of IC_{50} value (antilog 2.44 = 275.4 µg/mL) for BE (2) C neuroblastoma cell line

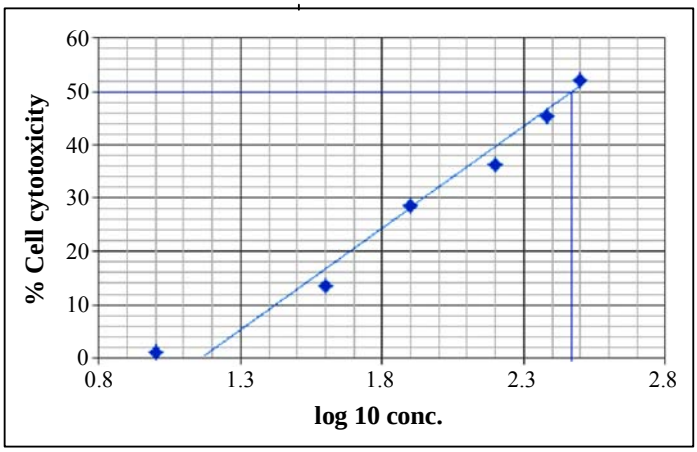

Fig. 4: Calculation of IC_{50} value (antilog 2.48 = 302.0 µg/mL) for U87MG Human neuronale glioblastoma astrozytom cell line

In vitro anti-diabetic activity

In an attempt to evaluate the *in-vitro* anti-diabetic potential EtOH extract (UB-1), petroleum ether extract (UB-2), ethyl acetate (UB-3), and MeOH extract (UB-4) were selected and tested using *in vitro* PTP-

1B inhibitory assay. Fractions UB-1, UB-2, UB-3 and UB-4 showed 52.9%, 20.0%, 18.7% and 53.2% of PTP1B% inhibition, respectively as compared to sodium orthovanadate and suramin controls having 71.24 and 76.9% inhibition at 10 µM concentration. Fractions UB-1, UB-2, UB-3 and UB-4 showed maximum of 65.7%, 39.0%, 42.9% and 61.3% PTP1B% inhibition, respectively at 100 µM concentrations (Table 5). Hence these fractions were taken with the goals of identifying active pure compounds responsible for these potencies. Isolation and structure elucidation of these active principles is in progress.

Table 5: *In vitro* anti-diabetic activity of EtOH extract (UB-1), Petroleum ether extract (UB-2), Ethyl acetate (UB-3) and MeOH extract (UB-4)

Compound code	PTP1B % inhibition			IC_{50} (µM)
	Compound/Inhibitor conc.			
	10 µM	50 µM	100 µM	
UB-1	-52.9	-62.2	-65.7	7.5
UB-2	-20.0	-21.5	-39.0	Not done
UB-3	-18.7	-21.0	-42.9	Not done
UB-4	-53.2	-56.3	-61.3	9.1
Sodium orthovanadate	-71.24			
Suramin	-76.9			

In vivo anti-diabetic activity

Fig. 1 shows the effect of fractions UB-1, UB-2, UB-3 and UB-4 on decline in blood glucose level of streptozotocin-induced diabetic rats. Metformin was taken as positive standard. It is evident from the results that all the fractions except UB-3, showed significant decline in blood glucose level of streptozotocin-induced diabetic rats. Among these four fractions, UB-1 showed maximum decline i.e. 17.5% ($p < 0.05$) during 0-5 hr and 23.0% ($p < 0.01$) during 0-24 hr, respectively followed by other fractions i.e. UB-2 and UB-4 to the tune of 15.9% ($p < 0.05$) and 14.4% ($p < 0.05$) during 0-5 hr; however, UB-2 and UB-4 to the tune of 15.9% ($p < 0.05$) and 14.4% ($p < 0.05$) during 0-4 hr; UB-2, UB-3 and UB-4 showed decline in blood glucose levels to the tune of 23.4 % ($p < 0.01$), 18.2% ($p < 0.01$) and 20.7% ($p < 0.01$) during 0-24 hr, respectively on streptozotocin-induced diabetic rats at 100 mg/Kg oral dose. The standard drug metformin showed significant decline to the tune of 21.2% ($p < 0.01$) during 0-5 hr and around 30.0 % ($p < 0.01$) during 0-24 hr, respectively.

CONCLUSION

In vitro cytotoxic activity, *In vitro* and *In vivo* anti-diabetic activity studied for the first time in this plant species from region of Srinagar Garhwal. Ethanol extract has been observed for considerable cytotoxic activity against SK-Mel cell line, so attempt should be made for activity guided isolation and characterization of chemical constituents and thereafter, activity study of isolated pure active principles, which can be a step ahead in search of new cytotoxic agents from plants. *In-vitro* anti-diabetic potential IC_{50} value was calculated for EtOH extract (UB-1), and MeOH extract (UB-4), which was found to be 7.5 µm and 9.1 µM, respectively, which itself indicate that plant have anti-diabetic potential, which was also observed in the *in vivo* experiments, where there was a decline in blood glucose level of streptozotocin–induced diabetic rats. Hence, these fractions were taken with the goals of identifying active phytoconstituents responsible for these potencies. Isolation and structure elucidation of these active phytoconstituents is in progress.

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