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In vitro cytotoxicity and *in vivo* acute toxicity of selected polysaccharide hydrogels as pharmaceutical excipients

Kulkarni GT*, Gowthamarajan K, Raghu C, Ashok G and Vijayan P

J. S. S. College of Pharmacy, Rocklands, Ootacamund – 643 001, The Nilgiris, India

SUMMARY

Polysaccharide hydrogels constitute a structurally diverse class of biological macromolecules with a wide range of physicochemical properties. They also constitute important members of the family of industrial water-soluble polymers. They find application in Pharmacy as binders, disintegrants, suspending, emulsifying and sustaining agents. According to the International Pharmaceutical Excipients Council (IPEC), an excipient must have an established safety profile. Hence, in the present study, *in vitro* cytotoxicity on Vero and HEp-2 cell lines, and *in vivo* acute toxicity in rats were carried out to establish the safety of polysaccharide hydrogels from the seeds of *Plantago ovata* and *Ocimum basilicum*. The *in vitro* cytotoxicity was determined by MTT and SRB assays. In the *in vivo* acute toxicity, the effects of three different doses of hydrogels (100, 200 and 400 mg/kg body weight) on food and water intake, body weight, biochemical and hematological parameters were studied. The results of *in vitro* did not show any cytotoxicity on both the cell lines used. In the *in vivo* acute toxicity, the hydrogels did not show any toxic symptoms in all three dose levels. This establishes the safety of the selected hydrogels. Hence, they can be used as excipients in pharmaceutical dosage forms.

Key words: Cytotoxicity; Acute toxicity; Hydrogel; Polysaccharide; *Ocimum basilicum*; *Plantago ovata*

INTRODUCTION

Polysaccharide hydrogels constitute a structurally diverse class of biological macromolecules with a wide range of physicochemical properties. They also constitute important members of the family of industrial water-soluble polymers (Franz, 1985; Franz, 1989). They find application in Pharmacy as binders (Kulkarni *et al.*, 2002), disintegrants (Srinivas *et al.*, 2003), suspending agents (Ibezim *et al.*, 2000), emulsifying agents (Verma *et al.*, 2003) and sustaining agents (Baveja *et al.*, 1989). The establishment of hydrogels as excipients needs safety evaluation. According to the International Pharmaceutical Excipients Council (IPEC) definition, an excipient is defined as any substance, other than the active drug or prodrug that has been appropriately evaluated for safety and is included in a dosage form or delivery system to aid in the manufacture of dosage form and to protect, support or enhance stability and bioavailability of the drug or active ingredient (Blecher, 1995; Wheatly, 2000). According to the definition, an excipient must have an established safety profile. Hence, in the present study, *in vitro* cytotoxicity on Vero and HEp-2 cell lines, and *in vivo* acute toxicity in rats were carried out to establish the safety of polysaccharide hydrogels from the seeds of *Plantago ovata* and

*Correspondence: Kulkarni GT, Department of Pharmaceutics, J.S.S. College of Pharmacy, Rocklands, Ootacamund - 643 001, The Nilgiris, India. Tel: +91-423-2443393 (O); +91-423-2447240 (R); E-mail: gtkulkarni2003@yahoo.co.in

Ocimum basilicum.

MATERIALS AND METHODS

Materials

The seeds of *P. ovata* and *O. basilicum* were collected from places in and around Ootacamund during October and November 2000. The authentication of the plant materials was carried out by The Survey of Medicinal plants and Collection Unit, Ootacamund, where voucher specimens are preserved. The reagents used for cytotoxicity and the biochemical kits used for the estimation of biochemical parameters were purchased from Sigma and Merck. All other reagents were of AR grade.

Isolation of hydrogels

The seeds (100 g) were soaked in water (500 ml) for 24 h, boiled for 1 h and stirred for 2 h using a mechanical stirrer to release the polysaccharide into water. The material was squeezed in a muslin bag to remove the marc. To the filtrate, equal volume of acetone was added to precipitate the hydrogel, which was separated by centrifugation (1000 rpm for 45 min), dried at 50°C, powdered, passed through sieve no. 80, and stored in desiccator until further use. The yields of hydrogels from *P. ovata* and *O. basilicum* were 28 and 24%, respectively.

In vitro cytotoxicity

Preparation of sample solutions

The hydrogels were dissolved in DMSO separately and the volume was made up to 10 ml with DMEM/RPMI medium to obtain a stock solution of 1 mg/ml concentration and stored at -20°C until use.

Cell lines and culture medium

Vero and HEp-2 cell cultures used in these experiments were obtained from National Center for Cell Sciences, Pune and Pasteur Institute of India, Coonoor. Stock cells of Vero and HEp-2 cell lines were cultured in RPMI-1640 and DMEM supplemented with 10% inactivated sheep serum, penicillin (100 IU/ml), streptomycin (100 μg/ml) and amphotericin B (5 μg/ml) in a humidified atmosphere of 5% CO_2 at 37°C until confluent. The cells were dissociated with 0.2% trypsin, 0.02% EDTA, in PBS. The stock cultures were grown in 110 ml flat bottles and all experiments were carried out in 96 well microtiter plates, where the cell population was adjusted to 10,000 cells per well.

Cytotoxicity

The cytotoxicity assay was carried out as described below. 0.1 ml of cell suspension containing 10,000 cells was seeded to each well of a 96 well microtiter plate (Nunc and Tarson) and fresh medium containing different concentrations of the hydrogels was added after 24 h seeding. Control cells were incubated without the test compound and with DMSO (solvent). The very little percentage of DMSO present in the wells (maximal 0.2%) was proved not to affect the experiment. The microtiter plates were incubated at 37°C in a humidified incubator with 5% CO_2 for a period of 3 days. Twelve wells were used for each concentration. Morphological changes were examined using inverted microscope. The cells were observed at different time intervals after incubation in the presence or absence of hydrogels. Cellular viability was determined by using the standard MTT assay (Francis and Rita, 1986; Ke *et al.*, 1999) and SRB assay (Skehan *et al.*, 1990) from the treated culture of four wells of each concentration.

MTT assay

MTT assay is based on the reduction of the soluble MTT into a blue purple formazan product mainly by mitochondrial reductase activity inside living cells. The number of viable cells is proportional to the extent of formazan production. After incubation, the solutions in four wells of each concentration were discarded and 50 μl of a solution of 2 mg/ml of MTT (Sigma, St. Louis, MO, USA) in DMEM (without phenol red) was added and the cultures were incubated for an additional 3 hours at 37°C.

The supernatant was removed and the cells were dissolved in propanol (100 μl/well) and kept aside for 10 minutes at room temperature. The plate was read on a microtiter plate reader (Bio-Rad, Model 550) at a wavelength of 540 nm and the mean absorbance from four wells was recorded. Mean absorbance taken from cells grown in the absence of the test compound was taken as 100% cell survival (control). The percentage inhibition was calculated using the following formula.

Growth Inhibition %

$$=100 - \left[\frac{\text{Absorbance of sample}}{\text{Absorbance of control}} \times 100\right]$$

The percentage inhibition was plotted against concentration and the CTC_{50} (concentration required to reduce viability by 50%) value for each cell line was calculated.

SRB assay

SRB is a bright pink aminoxanthene dye with two sulfonic groups. Under mild acidic conditions, SRB binds to protein basic amino acid residues in trichloroacetic acid fixed cells to provide a sensitive index of cellular protein content that is linear over a cell density range of at least two order of magnitude. After incubation, the solutions in the wells were flicked off and 100 μl of different concentrations of hydrogels were added to the cells and further incubated at 37°C for 3 days in 5% CO_2 atmosphere. The microscopic examinations were carried out and observations were recorded every 24 h. After 72 h, 50% trichloroacetic acid (25 μl) was added to the wells so that a thin layer was formed over the hydrogel dilutions to form an overall concentration of 10% and the plates were incubated for 1 h at 4°C. The supernatant was then removed and the cells were washed with water, air-dried and stained with SRB for 30 min. The unbound dye was removed by washing with 1% acetic acid and the plates were air-dried. Tris base (10 mM, 100 μl) was then added to wells to solubilize the dye, the plates were vigorously shaken for 5 minutes and the absorbance was measured using microtiter plate reader at 540 nm. The percentage growth inhibition was calculated using the same formula as given under MTT assay. The percentage inhibition was plotted against concentration and the CTC_{50} for each cell line was calculated.

***In vivo* acute toxicity**

The acute toxicity was designed to find out the probable target organ of the testing material and its specific toxic effects on the organ (Suresh Kumar *et al.*, 2000). Any toxic symptoms produced by the isolated hydrogels were screened by acute toxicity in rats (Institutional Ethical Committee Approval No. JSSCP/IAEC/Ph.Ceutics/Ph.D/03/2003-2004).

Adult albino Wistar rats of either sex weighing between 150-200 g were selected. The animals were housed in the animal house in-group of six animals each in clean acrylic cages. The bedding materials of the cages were changed every day. The animals were maintained under 12 h day and night cycle with temperature ranging between 24-26±2°C. The animals were fed with the commercial animal feed pellets supplied by M/s Hindustan Lever Ltd., Bangalore, India. The animals were allowed free access to food and water at all the times during the duration of the study.

The rats were divided into seven groups having six animals in each group. Group-I animals received distilled water and served as control. Animals of Group II, III and IV received the hydrogel from *P. Ovata* at a dose level of 100, 200 and 400 mg/kg of body weight. Group V, VI and VII received the hydrogel from *O. basilicum* at a dose level of 100, 200 and 400 mg/kg of body weight. The doses were administered orally once a day for 28 days.

The animals were observed daily for any toxic symptoms, mortality, body weight, and food and water consumption. Hematological parameters such as haemoglobin content, RBC count, WBC count, and biochemical parameters such as aspirate aminotransferase (ASAT), alanine aminotransferase

(ALAT), alkaline phosphatase, total serum protein, total albumin, total urea, total creatinine and total cholesterol were measured on 0^{th}, 14^{th} and 28^{th} days, using EColine diagnostic kits (Merck India).

Statistical methods

Comparison was made between control and treated groups. The means of different groups were compared and level of significance was calculated using Students *t*-test (Software: SPSS version-10.0.5, SPSS Inc., USA). Mean differences were considered significant at $P<0.01$ and $P<0.001$.

RESULTS AND DISCUSSION

In vitro cytotoxicity using Vero and HEp-2 cell lines, the hydrogel *from P. ovata* exhibited CTC_{50} values of 556.83±18.56 and 583.63±19.32 μg/ml on Vero cell line using MTT and SRB assays, respectively. The CTC_{50} values for HEp-2 cell line were 476.93± 13.28 and 428.79±12.06 μg/ml, respectively, using MTT and SRB assays. The hydrogel from *O. basilicum* exhibited CTC_{50} values of 769.39±23.19 and 697.81±24.52 μg/ml by MTT and SRB assays, respectively, on Vero cell line, and 631.36±18.94 and 653.94±21.33 μg/ml by MTT and SRB assays, respectively, on HEp-2 cell line. The selected hydrogels did not show any significant cytotoxicity on normal as well as cancer cell lines and hence, they can be considered as non-toxic.

During the *in vivo* acute toxicity, there were no toxic symptoms and mortality over a period of 28 days. This confirmed the non-toxicity of the selected hydrogels. There was no significant increase or decrease in food intake, water consumption (data not given), and body weight (Table 1), during the test period, which indicated normal physiological functioning.

The observations made from heamatological parameters, namely, haemoglobin content, RBC, WBC and platelet counts, also did not show any significant alterations with all the selected parameters, over a period of 28 days, with three different doses of hydrogels. The results are shown in Table 2 and 3.

To test the functioning of liver and kidney, different biochemical parameters were estimated using Ecoline diagnostic kits and analyzed by Merck Microlab-200 autoanalyzer. The observations made from the biochemical parameters over a test period of 28 days are shown in Tables 4 to 7. No significant changes were observed with all the selected parameters during the test period, with all the three dose levels of both the hydrogels used. Hence, the selected hydrogels from *Plantago ovata* and *Ocimum basilicum* did not show any toxic symptoms even at the highest dose. This establishes the safety of hydrogels. Hence, they can be used as excipients in pharmaceutical dosage forms.

Table 1. Effect of *P. ovata* and *O. basilicum* hydrogels on mean body weight on 28 days oral toxicity in rats

Group No.	Treatment and dose (mg/kg)	Body weight on			Increase in body weight (%)
		0^{th} day	14^{th} day	28^{th} day	
I	Control	164.58 ± 18.42	18.41 ± 14.82	208.86 ± 19.82	26.90
II	*Plantago ovata* (100)	164.21 ± 21.21	179.83 ± 21.18	209.10 ± 18.26	27.303
III	*Plantago ovata* (200)	166.67 ± 25.45	181.12 ± 20.21	209.12 ± 19.23	25.47
IV	*Plantago ovata* (400)	163.28 ± 21.23	180.23 ± 21.23	208.93 ± 19.86	27.96
V	*Ocimum basilicum* (100)	164.21 ± 21.63	181.43 ± 18.12	206.84 ± 18.21	25.97
VI	*Ocimum basilicum* (200)	163.21 ± 19.83	180.91 ± 19.21	207.12 ± 19.13	27.71
VII	*Ocimum basilicum* (400)	162.18 ± 18.19	180.16 ± 19.26	207.23 ± 18.29	27.78
	P- value	NS	NS	NS	NS

Acute toxicity was carried in rats over a period of 28 days. Values are mean ± SD; n=6 in each group. NS represents non-significant *P* value when compared with control group.

Table 2. Effect of *P. ovata* hydrogel on hematological parameters in rats

Group No. and Dose	Hemoglobin (g %)			RBC ($X10^6$/cmm)			Platelets ($X10^5$/cmm)			Total WBC ($X10^3$/cmm)		
	0^{th} day	14^{th} day	28^{th} day	0^{th} day	14^{th} day	28^{th} day	0^{th} day	14^{th} day	28^{th} day	0^{th} day	14^{th} day	28^{th} day
I Control	13.25 ± 2.52	13.52 ± 2.41	13.41 ± 2.40	6.75 ± 1.30	6.49 ± 1.37	6.53 ± 1.42	3.31 ± 0.52	3.37 ± 0.53	3.63 ± 0.59	7.64 ± 1.16	7.75 ± 1.42	7.76 ± 1.49
II 100 mg/kg	13.38 ± 2.40	13.89 ± 2.47	13.11 ± 2.25	6.70 ± 1.10	6.76 ± 1.32	6.89 ± 1.29	3.52 ± 0.56	3.56 ± 0.63	3.79 ± 0.58	7.47 ± 1.15	7.89 ± 1.57	7.55 ± 1.36
III 200 mg/kg	13.45 ±2.42	13.27 ± 2.35	13.45 ± 2.45	6.72 ± 1.29	7.32 ±1.20	6.89 ± 1.37	3.39 ± 0.54	3.25 ± 0.50	3.61 ± 0.58	7.52 ± 1.27	7.65 ± 1.24	7.60 ± 1.40
IV 400 mg/kg	13.12 ± 2.39	13.19 ± 2.35	13.12 ± 2.49	6.79 ± 1.37	6.88 ± 1.30	6.75 ± 1.40	3.71 ± 0.65	3.55 ± 0.57	3.81 ± 0.63	7.99 ± 1.14	8.02 ± 1.17	8.07 ± 3.10
P- value		NS			NS			NS			NS	

Acute toxicity was carried out in rats for a period of 28 days. Values are mean ± SD; n = 6 in each group. NS represents non-significant *P* value when compared with control group.

Table 3. Effect of *O. basilicum* hydrogel on hematological parameters in rats

Group No. and Dose	Hemoglobin (g%)			RBC ($X10^6$/cmm)			Platelets ($X10^5$/cmm)			Total WBC ($X10^3$/cmm)		
	0^{th} day	14^{th} day	28^{th} day	0^{th} day	14^{th} day	28^{th} day	0^{th} day	14^{th} day	28^{th} day	0^{th} day	14^{th} day	28^{th} day
I Control	13.25 ± 2.52	13.52 ± 2.41	13.41 ± 2.40	6.75 ± 1.30	6.49 ± 1.37	6.53 ± 1.42	3.31 ± 0.52	3.37 ± 0.53	3.63 ± 0.59	7.64 ± 1.16	7.75 ± 1.42	7.76 ± 1.49
II 100 mg/kg	13.24 ± 2.39	13.88 ± 2.48	13.11 ± 2.24	6.62 ± 1.30	6.77 ± 1.11	6.95 ± 1.32	3.51 ± 0.57	3.52 ± 0.56	3.69 ± 0.63	7.51 ± 1.16	7.90 ± 1.57	7.51 ± 1.40
III 200 mg/kg	13.74 ± 2.46	13.35 ± 2.41	13.97 ± 2.23	6.75 ± 1.35	7.31 ± 1.22	6.78 ± 1.43	3.32 ± 0.55	3.22 ± 0.53	3.52 ± 0.57	7.55 ± 1.40	7.60 ± 1.39	7.57 ± 1.38
IV 400 mg/kg	13.14 ± 2.52	13.22 ± 2.34	13.12 ± 2.31	6.79 ± 1.44	6.85 ± 1.37	6.72 ± 1.53	3.67 ± 0.61	3.56 ± 0.59	3.82 ± 0.62	7.95 ± 1.12	8.00 ± 1.14	8.01 ± 3.07
P- value		NS			NS			NS			NS	

Acute toxicity was carried in rats over a period of 28 days. Values are mean ± SD; n = 6 in each group. NS represents non-significant *P* value when compared with control group.

Table 4. Effect of *P. ovata* hydrogel on biochemical parameters in rats

Group No. and Dose	ASAT (IU/L)			ALAT (IU/L)			ALP (IU/L)			Total Protein (g/l)		
	0^{th} day	14^{th} day	28^{th} day	0^{th} day	14^{th} day	28^{th} day	0^{th} day	14^{th} day	28^{th} day	0^{th} day	14^{th} day	28^{th} day
I Control	82.84 ± 10.81	84.89 ± 12.38	83.89 ± 13.43	62.83 ± 8.84	64.69 ± 8.69	63.98 ± 8.28	468.23 ± 23.48	471.12 ± 20.14	472.18 ± 26.81	5.18 ± 1.24	6.69 ± 1.17	6.88 ± 1.25
II 100 mg/kg	86.84 ± 12.05	85.83 ± 13.14	87.86 ± 12.84	62.04 ± 10.82	61.48 ± 9.96	64.31 ± 8.89	493.19 ± 24.13	495.20 ± 21.48	501.23 ± 28.93	6.12 ± 1.12	7.16 ± 1.17	7.02 ± 1.28
III 200 mg/kg	83.83 ± 13.84	84.23 ± 14.42	85.67 ± 16.18	62.84 ± 12.38	61.98 ± 12.34	60.81 ± 10.23	476.5 ± 24.19	475.61 ± 22.16	480.30 ± 24.13	6.94 ± 1.13	6.89 ± 1.31	6.67 ± 1.33
IV 400 mg/kg	82.84 ± 13.47	83.18 ± 13.68	84.28 ± 14.61	60.05 ± 13.16	60.68 ± 14.12	61.19 ± 13.12	488.14 ± 19.24	489.30 ± 18.23	492.80 ± 23.16	5.83 ± 1.02	6.01 ± 1.33	6.23 ± 1.33
P-value		NS			<0.05			NS			NS	

Acute toxicity was carried in rats over a period of 28 days. Values are mean ± SD; n = 6 in each group. NS represents non-significant *P* value when compared with control group.

Table 5. Effect of *P. ovata* hydrogel on biochemical parameters in rats

Group No. and Dose	Albumin (g/l)			Urea (mg/dl)			Creatinine (mg/dl)			Cholesterol (mg/dl)		
	0^{th} day	14^{th} day	28^{th} day	0^{th} day	14^{th} day	28^{th} day	0^{th} day	14^{th} day	28^{th} day	0^{th} day	14^{th} day	28^{th} day
I Control	3.52 ± 0.61	3.68 ± 0.58	3.79 ± 0.41	29.7 ± 4.90	32.14 ± 5.00	30 ± 5.50	0.22 ± 0.02	0.25 ± 0.03	0.24 ± 0.03	76.38 ± 4.30	75.31 ± 4.20	73.81 ± 5.01
II 100 mg/kg	3.64 ± 0.55	3.81 ± 0.61	3.89 ± 0.44	29.5 ± 5.50	32.04 ± 5.10	33.0 ± 5.00	0.23 ± 0.03	0.22 ± 0.04	0.24 ± 0.03	76.19 ± 4.23	76.42 ± 4.32	76.43 ± 4.78
III 200 mg/kg	3.84 ± 0.62	3.73 ± 0.56	3.98 ± 0.70	26.5 ± 4.19	29.18 ± 4.72	30.06 ± 4.19	0.23 ± 0.04	0.023 ± 0.04	0.23 ± 0.03	71.68 ± 4.23	72.10 ± 3.89	74.98 ± 3.96
IV 400 mg/kg	3.8 ± 0.71	3.83 ± 0.61	3.97 ± 0.62	31.67 ± 4.70	30.16 ± 4.92	29.50 ± 4.72	0.24 ± 0.04	0.27 ± 0.03	0.25 ± 0.04	69.46 ± 4.01	70.12 ± 4.26	73.48 ± 3.50
P-value		NS			NS			NS			<0.05	

Acute toxicity was carried in rats over a period of 28 days. Values are mean ± SD; n = 6 in each group. NS represents non-significant *P* value when compared with control group.

Table 6. Effect of *O. basilicum* hydrogel on biochemical parameters in rats

Group No. and Dose	ASAT (IU/L)			ALAT (IU/L)			ALP (IU/L)			Total Protein (g/l)		
	0^{th} day	14^{th} day	28^{th} day	0^{th} day	14^{th} day	28^{th} day	0^{th} day	14^{th} day	28^{th} day	0^{th} day	14^{th} day	28^{th} day
I Control	82.84 ± 10.81	84.89 ± 12.38	83.89 ± 13.43	62.83 ± 8.84	64.69 ± 8.69	63.98 ± 8.28	468.23 ± 23.48	471.12 ± 20.14	472.18 ± 26.81	5.18 ± 1.24	6.69 ± 1.17	6.88 ± 1.25
II 100 mg/kg	85.89 ± 10.86	88.58 ± 12.08	86.89 ± 12.12	63.29 ± 9.83	66.16 ± 8.89	66.92 ± 8.72	468.92 ± 23.46	469.92 ± 20.84	473.81 ± 26.61	5.63 ± 1.21	6.64 ± 1.17	7.22 ± 1.25
III 200 mg/kg	86.23 ± 12.05	84.50 ± 13.12	86.29 ± 11.68	63.86 ± 8.89	64.12 ± 8.29	64.39 ± 7.76	468.19 ± 23.39	477.16 ± 21.42	476.1 ± 24.46	6.92 ± 1.12	7.16 ± 1.17	7.02 ± 1.28
IV 400 mg/kg	83.46 ± 12.84	84.12 ± 12.42	88.39 ± 14.29	66.82 ± 8.68	66.81 ± 4.68	68.34 ± 7.23	472.39 ± 23.69	473.12 ± 22.34	478.40 ± 21.28	6.14 ± 1.31	6.89 ± 1.21	6.09 ± 1.26
P-value		NS			NS			<0.05			NS	

Acute toxicity was carried in rats over a period of 28 days. Values are mean ± SD; n = 6 in each group. NS represents non-significant *P* value when compared with control group.

Table 7. Effect of *O. basilicum* hydrogel on biochemical parameters in rats

Group No. and Dose	Albumin (g/l)			Urea (mg/dl)			Creatinine (mg/dl)			Cholesterol (mg/dl)		
	0^{th} day	14^{th} day	28^{th} day	0^{th} day	14^{th} day	28^{th} day	0^{th} day	14^{th} day	28^{th} day	0^{th} day	14^{th} day	28^{th} day
I Control	3.52 ± 0.61	3.68 ± 0.58	3.79 ± 0.41	29.70 ± 4.90	32.14 ± 5.00	30.00 ± 5.50	0.22 ± 0.02	0.25 ± 0.03	0.24 ± 0.03	76.38 ± 4.30	75.31 ± 4.20	73.81 ± 5.01
II 100 mg/kg	3.12 ± 0.61	3.92 ± 0.55	3.81 ± 0.53	27.91 ± 4.62	29.86 ± 4.19	27.92 ± 4.19	0.22 ± 0.04	0.24 ± 0.04	0.23 ± 0.03	73.19 ± 4.09	73.48 ± 4.26	74.60 ± 4.78
III 200 mg/kg	4.01 ± 0.62	3.88 ± 0.51	4.11 ± 0.55	29.86 ± 4.89	30.14 ± 4.38	32.14 ± 4.92	0.21 ± 0.04	.0.20 ± 0.03	0.22 ± 0.04	69.89 ± 4.39	72.1 ± 4.34	73.41 ± 4.14
IV 400 mg/kg	3.92 ± 0.71	4.00 ± 0.61	4.82 ± 0.02	28.39 ± 4.69	32.89 ± 4.70	30.92 ± 4.72	0.22 ± 0.03	0.23 ± 0.04	0.24 ± 0.04	71.26 ± 4.01	75.4 ± 4.26	74.61 ± 4.38
P-value		NS			NS			NS			<0.05	

Acute toxicity was carried in rats over a period of 28 days. Values are mean ± SD; n = 6 in each group. NS represents non-significant *P* value when compared with control group.

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