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RESEARCH ARTICLE

Immunotoxicity screening and immunomodulatory effects of 1-(4-hydroxytridecyloxy) pentadecan-4-ol isolated from *Boerhavia erecta* L., Nyctaginaceae, in BALB/c mice

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ABSTRACT: The aim of the present study was to screen immunotoxicity and assess *in vivo* immunomodulatory activity of an active fraction of *Boerhavia erecta* (BE) having 1-(4-hydroxytridecyloxy) pentadecan-4-ol. A total of 60 female BALB/c mice were randomly divided into six experimental groups (n = 10), Group 1 (vehicle control), Group 2 (250 mg/kg), Group 3 (500 mg/kg), Group 4 (1000 mg/kg), Group 5 (vehicle recovery) and Group 6 (high dose recovery). Clinical laboratory investigations (hematology, biochemistry), immunoglobulins (IgA, IgE & IgG), histopathology, gross pathology, necropsy were performed on day 28 for the first 4 groups and on day 56 in recovery groups (5 and 6). For immunomodulatory studies, plaque forming cell response to check humoral immunity and Sheep Red iBlood Corpuscles (SRBC) to cellular immunity was evaluated. Immunotoxicity study results were normal with no statistically or clinically significant abnormal changes between the tested and recovery groups. BE extract with active fraction of the isolated compound does not have immunotoxic effects but exhibited immunosuppression specific to cell-mediated immunity in female BALB/c mice.

Keywords: Humoral immunity; cell-mediated immunity; immunotoxicity; immunomodulatory; immunosuppressive

INTRODUCTION

Boerhavia erecta L (BE), a powerful anti-inflammatory herb distributed predominantly in the tropical and sub-tropical areas of the world has been paid less attention for many decades inspite of its potential therapeutic activity against arthritis and diabetes. Quite a good research data is available on the roots and stem parts of *B. diffusa*, a close member of *B. erecta*, establishing its potent antidiabetic (Rajeswari *et al*., 2010; Singh *et al*., 2005) and immunosuppressive activities (Karri *et al*., 2017). Our *in vitro* studies have confirmed the results scientifically on *B. erecta*'s anti-inflammatory and antidiabetic activities, hitherto unreported. In this context, we have isolated a novel and biologically active compound, 1-(4-hydroxytridecyloxy) pentadecan-4-ol, from chloroform fraction of hydro-alcoholic extract of root and stem parts of *Boerhavia erecta* L. (Nyctaginaceae). This new compound demonstrated positive results on immunomodulatory activity *in vitro* (In Press). However, it forms a basic and imperative step to screen immunotoxic activity, if any, prior to establishing its immunomodulatory activity *in vivo*. Earlier, acute, sub-acute toxicity studies on aqueous BE extracts were reported on Wistar rats (Lagarto *et al*., 2011) but no data exist on immunotoxicity studies. The present study on screening immunotoxicity and evaluating immunomodulatory effects in the animal model for the active fraction of this herb is one of its kind.

In view of the remarkable similarities between treatments of Rheumatoid Arthritis (RA) and Type 1 Diabetes Mellitus (T1D) and associated long term adverse effects of pharmaceutical drugs in the treatment of such chronic metabolic disorders, it has become significant to explore immunomodulatory activities *in vivo* through herbals. For induction of either RA (Baddack *et al*., 2013) or T1D (Zou *et al*., 2008), BALB/c mice are always preferred over Wistar rats. Further, to correlate at least two autoimmune disorders in a single animal model, our proposed hypotheses to subsequent future animal studies, the present immunotoxicity studies have been conducted exclusively on BALB/c mice.

MATERIALS AND METHODS

2.1 Animals:

In the immunotoxicity study, specific pathogen free female conventionally in-housed random bred BALB/c mice, weighing 20 (±3) g were used. As the subsequent efficacy studies were planned on BALB/c mice, other rodent species (Wistar rats) were not used in any of the toxicity studies. BALB/c mice aged 8 to 10 weeks were housed, air conditioned with adequate fresh air supply under standard laboratory conditions, room temperature at 20.2 – 23.5 °C and relative humidity 58-64% with 12 h fluorescent light and 12 h dark cycle. Animals were fed with pelleted food (Provimi's Nutrilab rodent feed) and potable drinking water supplied ad libitum while housing them in polypropylene cages throughout the acclimatization (5 days) and experimental periods.

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2.2 Ethics:

Acute and immunotoxicity studies were conducted as per the recommendation of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines for laboratory animal facilities published in the gazette of India, December 15th 1998. Animal studies were prospectively reviewed and approved by the Institutional Animal Ethics Committee, Bangalore; vide approval reference # RR/IAEC/03-2015.

2.3 Chemicals:

Dexamethasone and Freund's complete adjuvant were purchased from Sigma (St. Louis, MO), HiMedia Laboratories Pvt. Ltd. (Mumbai) provided stains of Hematoxylin.

2.4 Test Samples:

Chloroform fraction containing 1-(4-hydroxytridecyloxy) pentadecan-4-ol, isolated from hydro-alcoholic extract of root and stem parts of *Boerhavia erecta* L, were the test samples at 3 different doses (250, 500 and 1000 mg/kg body weight) and the animals were dosed per respective body weights.

2.5 Acute toxicity study:

In the acute toxicity study, a sighting or observational study (step I) was initiated to select pertinent dose in one female mice. Because of the unavailability of sufficient toxicological data, a starting dose of 300 mg/kg body weight (b. wt.) was selected for main study as per the sequential dose selection flow chart of Organization for Economic Co-operation and Development (OECD) 420 guideline. No clinical signs of toxicity or mortality were observed in step-I and therefore another single female mouse was selected for sighting study step - II randomly that weighed 2000 mg/kg b. wt. where again clinical signs of toxicity and mortality were not observed. For any clinical signs and mortality prior to conducting the next dose, a 24 h observation period was allowed for each sighting study.

As there were no external signs of toxicity or mortality in step-II, four female mice (nulliparous and non-pregnant) were considered for the main study. The test sample 10% (w/v) was administered as a single oral aqueous solution of 2000 mg/kg b.wt. For any signs of mortality, animals were observed at regular intervals of 30-40 min, 1 h, 2 h, 3 h and 4 h with a buffer period of ± 10 min on day 1 after dosing and once daily for the remaining 14 days of experimental period. Individual animal body weights were recorded on Day 1, Day 7 and on Day 14. All observations were recorded on gross pathology and histology examinations.

2.6 Immunotoxicity study:

2.6.1 Design of the study

Animals were divided into six groups consisting of 10 female BALB/c mice in each group (G1, G2, G3, G4, G5, G6). First four groups served as main groups (G1-G4), while the last two are recovery groups (G5 & G6). Except G1, all the group animals were treated for 28 days (repeated dose), whereas (G5, control recovery - distilled water) and (G6, high dose recovery) were not treated for another 28 days. G5 & G6 groups were to assess the reversal of toxic effects of test drug, if any. On a daily basis, all animals were observed for morbidity and mortality. Physical examinations like tremors, convulsions, and palpebral closure, other observations like salivation, lacrimation, eye prominence, red tears, muscle tone and respiratory character; Open field observations like backing, grooming, gait, arousal and sensory observations like righting reflex, pupil response; Grip strength-forelimb and hind limb, hind limb foot play and rectal temperature were examined. Each dose was prepared fresh daily before oral administration. All dosing procedures were performed approximately same time once every day (±1 h); daily body weights were recorded prior to the dosing. Body (rectal) temperature was measured using digital thermometer (Shenzhen Life Technologies Corporation, China) to minimize the stress response, once per week during the treatment period.

2.6.2 Dosing schedule:

Three test doses of chloroform fraction were selected based on the No Observed Adverse Effect Level (NOAEL) during single dose acute toxicity study. Based on initial value of 2000 mg/kg b. wt. in the previous study, three doses were proposed for *in vivo* immunotoxicity study: (i) 1000 mg/kg b. wt., high dose; (ii) 500 mg/kg b. wt., medium dose; and (iii) 250 mg/kg b. wt., low dose.

2.6.3 Food consumption, body weight, relative organ weights:

Food consumption was measured at weekly intervals. Prior to blood collection, all animals were subjected to fasting condition overnight (water allowed). All mice were observed for morbidity and mortality once daily. Individual body weights were recorded and were subsequently euthanized by intraperitoneal injection of pentobarbital sodium (40 mg/kg b.wt.). Blood samples were collected on day 28 from main group of animals whereas it was on day 56 from the recovery groups. The liver, heart, kidney and spleen were then excised and weighed after blotting. Relative organ weights were calculated as the ratio of organ weight (mg) to the body weight (g).

2.6.4 Clinical laboratory investigations:

During pre-treatment, blood sampling was from the retro-orbital plexus whereas at sacrifice it was from the abdominal aorta. Blood was collected into EDTA for hematology and lithium heparinized tubes for clinical chemistry. Hematology was performed using a Sysmex XT 1800iv haematology analyzer. Plasma was separated from the blood using a refrigerated centrifuge (4000 rpm at 4 °C) and analysed for clinical chemistry using an Rx Daytona (Randox) automatic analyzer.

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2.6.5 Gross Necropsy and Histopathology:

On day 29 (G1-G4) and at the end of the 28-day recovery period (G5 & G6), the animals were left overnight in the fasting condition with mild water intake, anaesthetized, weighed and were subjected to detailed necropsy. The liver, kidney and spleen tissues were collected from each animal and placed in 10% buffered formalin for processing. Subsequently, the sections were embedded in paraffin and the tissues were stained with Heamatoxylin Eosin. Following this, histopathological evaluations were performed on organs of animals from all the groups.

2.6.6 Elisa measurement of the Immunoglobulins (IgA, IgM and IgG):

The ELISA reaction was performed as per standard protocol, post sensitizing with 2 µg/100µL well of the antigen preparation in polystyrene microplates (Hemobag), the microplates were developed. With an automatic reader the microplate absorbances were read at 492 nm (Loveren *et al*., 1991; Ladics *et al*., 1995; Arrriba *et al*., 2002).

2.7 *In vitro* immunomodulatory activity:

2.7.1 Humoral immunity

Cell response assay to sheep RBCs was assessed (Talwar *et al*., 1992) for determining the test sample/compound's humoral immunity. Briefly, from four group of another set of mice (G1 - 250 mg/kg b. wt., G2 - 500mg/kg b. wt., G3 - 1000mg/kg b. wt. & G4 - Dexamethasone control 10mg/kg b.wt.), with each group consisting of five male and female mice were used for the assay and dosed orally at 10% (w/v). The animals were immunized with 5% sheep RBC through i.v on the 24th day of treatment. The animals were euthanized towards end of the treatment period; spleens were homogenized, total number of lymphocytes per spleen was counted and incubated with 2 mL of 1:10 guinea pig complement. The number of plaques (clear zones) per spleen was calculated as a measure of humoral immunity.

2.7.2 Cell mediated immunity:

Cell mediated immunity measured by delayed type hypersensitivity (DTH) response was assessed as per protocol (Higuchi *et al*., 1983), with some modifications. On similar lines of plaque forming cell assay, four group of another set of mice in four groups were used in the DTH study. Mice were sensitized by a subcutaneous (SC) injection with 50 µL of SRBC (10^8 cells) on day 18 of the exposure period (or day 2 for dexamethasone control group). After 10 days, these sensitized mice were challenged by injecting 50 µL of SRBC (10^8 cells) into their right hind footpad. By using a pressure sensitive micrometer screw gauge (Mitutoyo, Kawasaki, Japan) swelling was measured 24 and 48 h post-challenge in the right hind footpad.

2.8 Statistical analysis:

Differences between control and the treatment groups were assessed by one-way analysis of variance followed by Dunnett's t-test. The results were expressed as Mean ± SD. All comparisons were evaluated at 5% ($p<0.05$) level.

3. RESULTS

3.1 Acute toxicity study:

None of the animals had clinical signs of toxicity or deaths with the doses tested. No changes in body weights observed throughout the experimental period at all the doses tested (Table 1). No gross pathological changes were noticed. No observed adverse effect levels (NOAEL) and mortalities were found at the highest dose tested (2000 mg/kg body weight) at 10 % (w/v) strength in acute toxicity study.

3.2 Immunotoxicity study:

3.2.1 Animal feed consumption (g), body weight (g) and relative organ weights:

There were no abnormal clinical signs of toxicity or deaths observed in any of the treatment or control group animals during the course of the study. No statistically different body weight changes between the control and treatment groups were observed during the course of the study. However, the gain in body weight during the course of the study was comparable in all groups (Table 2). This could be due to increase in the age of the animals as evident from recovery group animal weights (G5 & G6). No noticeable variations in the feed consumption between the control and test compound treated groups were observed during the course of the study and also in the recovery period. Relative organ weights of liver, kidney, heart and spleen indicated neither a clinical nor a statistical significant change between the animal groups throughout the study (Table 3).

3.2.2 Hematology:

There were no major differences in the hematology (Fig. 1) parameters evaluated between the treated group animals. However, on day 28, there was a reduction in white blood cell and platelet count in G3 group (500 mg/kg b.wt.) animals. This reduction was not statistically significant when compared to respective control recovery groups on day 28. During the experimental period of 28 days, no drug related deaths were observed and all animals survived. Blood parameters such as total erythrocyte count (TEC), packed cell volume (PCV) and hemoglobin (Hb) count had no significant difference between the groups.

3.2.3 Biochemistry:

Total bilirubin and serum creatinine values increased from baseline (day 0) to day 28 amongst all the groups including the normal control (G1) and recovery control groups (G5 & G6) demonstrating that it is not abnormal rise due to test compound. There was a sudden dip in total protein count in G4 group (highest dose) on Day 14, however, a similar decrease was also observed in control recovery groups (G5 & G6). As the values were normal on day 28 between any of the treatment groups and control recovery groups on day 56, the sudden dip on day

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14 could be incidental and not related to the test drug. Besides these, there were no major differences in any other biochemistry (Fig. 2) parameters like Alkaline Phosphatase (ALP), Serum Glutamic Oxaloacetic Transaminase (SGOT), Serum Glutamic Pyruvic Transaminase (SGPT) between the treated group animals.

3.2.4 Histopathology:

Histopathological examination of liver, kidney and spleen did not reveal any significant changes. Lesions observed in control and high dose groups animals (1000 mg/kg/day) were comparable and appeared to be incidental and spontaneous in nature and hence are believed not to be related to the test item (Fig. 3).

3.2.5 Immunoglobulin (IgA, igM and igG):

Values of three different types of immunoglobulins suggested that overall there were no major changes between different groups. However, in IgA type alone there was a statistical significant change in the value at the lowest dose tested. Whereas, the medium and high test dose groups did not show any such significant variations. Interestingly, both control recovery (G5) and high dose recovery group (G6) animals demonstrated an increase in the IgA values at 33.21± 0.28 and 34.38 ± 0.29 respectively (Fig. 4). The values are expressed in mg/ml. These ELISA procedures were performed in triplicate and the values are mean of experimental results.

3.3 *In vitro* immunomodulatory activity:

3.3.1 Humoral immunity:

Humoral immunity was measured by hemolytic plaque (antibody) forming cell assay (PFC) following challenge with antigen. PFC is a proven method for the evaluation of immunosuppression. Splenic lymphocytes and the plaque (antibody) forming cells in all the test compound treated groups were similar to that of the control group indicating that the compound did not increase the antibody forming cells in the spleen (Fig. 5a).

3.3.2 Cell mediated immunity:

The delayed-type hypersensitivity (DTH) response is considered as an effector measure of cell mediated immunity. It is characterized by visible local inflammatory reaction with erythema, edema and swelling in the vehicle control mice. In the DTH assay the mean skin thickness (mm) in high dose test drug group males was 1.8±0.13, 1.9±0.05 and 2.0±0.08 at 24, 48 and 72 h post challenge and the corresponding scores in the control group were 1.5±0.09, 1.8±0.03 and 1.7±0.07mm respectively. Similar observations were found in the high dose female and their corresponding control group animal values indicating that the test drug may have a role in cell mediated immune response at the tested high dosage level (Fig. 5b & c).

Clinical observations (results not shown) were directly proportional to the test dose administered. At low dose, there was mild edema but no swelling. At high dose, mild edema and erythema was observed. The DTH response, measured as percent increase in paw thickness at a given time point, decreased in the medium test dose mice after 24 h, but was non-significant ($p>0.05$). Whereas in high test dose and Dexamethasone (DXM) treated mice suppressive responses were noted both clinically and statistically. In the standard DXM-treated mice, DTH responses were suppressed significantly at 48 h post challenge in the medium and high test dose groups than at 24 h challenge.

4. DISCUSSION:

Immunomodulatory activities of *Boerhavia diffusa* were established scientifically (Manu *et al*., 2009). However, in *Boerhavia erecta*, a very close family member of *B. diffusa*, no *in vivo* data exists on its immunotoxicity or immunomodulatory effects. In our study, even the highest test dose (1000 mg/kg/day b. wt.) has shown no marked changes in food consumption, body and key organs weight. There were neither clinically significant nor statistically significant changes in laboratory parameters. Decrease in leucocyte and platelet count in the lowest test dose group animals could be incidental. Significant reduction in the total protein count in the highest test dose group animals was comparable to that of control and recovery groups. High or low blood protein is not a specific disease or condition in itself. From the hematology and biochemical parameters, it can be concluded that the test drug is completely safe at various doses. Our results are complementary to the study on low acute toxicity (LD_{50} values of 2148 mg/kg b. wt.) for *Boerhavia erecta* stem bark reported previously (Hilou *et al*., 2006).

In the histopathology, liver sections of all the group animals had normal architecture with central vein surrounded by hepatocytes which show mild degree fatty change (collection of fat vacuoles inside the hepatocytes). Portal triad was normal with no necrosis seen. In kidney section, normal appearing glomeruli, tubules, collecting ducts and pelviureteric junction were seen with no necrosis. Spleen being one of the vital immune organs, the sections consisted of red and white pulp, sinusoids were normal with hematopoietic elements observed.

Each type of antibody adheres to specific foreign substance so that the immune system can destroy them. IgE antibody levels are often high during allergies, IgD antibodies are found in small amounts in the tissues that line the belly or chest and their mechanism of action is unclear. Therefore, out of five immunoglobulin /antibody types, only three sub-types (IgA, IgG, IgM) were considered in the study. No significant alternations found in any of the three immunoglobulins even in the presence of the test drug at all three dosages. The antibodies were neither overexpressed nor inhibited when stimulated with an antigen at the doses tested, and this signifies that humoral immunity remains unperturbed.

T-dependent immunogen is used to determine drug effect on the immune response (Dean *et al*., 2001). Results of the present plaque forming assay clearly demonstrated that the test drug has no effects on humoral immunity; this is re-confirmed when none of the three antibody (Ig) types were altered in the presence of test drug even at the highest dose. However, there is an evidence of suppression of cell

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mediated immunity of test drug in BALB/c animals at doses of 1000 mg/kg b. wt. (Fig. 5). Effects of oral exposure of BE extract on T-cell function was further assessed through measurement of DTH response. Significant reduction in DTH reaction to SRBC, a T-cell dependent antigen, indicates reductions in cell-mediated immunity via TH1 effector cells. This study demonstrated that the chosen herb has immunosuppressive effects comparable to our previous report on *in vitro* study and can hence be attributed to the new compound.

5. CONCLUSION

In conclusion, our results have indicated that there is no direct immunotoxic effect of chloroform fraction of root and stem parts of hydro-alcoholic extract of *B erecta* in BALB/c mice. With significant inhibition of T-cell-mediated response at the high (i.e., 1000 mg/kg b. wt.) dose tested, it is evident that sub-acute oral BE exposure does not suppress humoral immune responses. Together with these results, it is considered that the suppression of cellular immune responses by BE extract are due to direct effects of the isolated compound (1-(4-hydroxytridecyloxy) pentadecan-4-ol) against TH cells. The present study confirms BE is not immunotoxic and at the same time, it suppresses cell mediated immunity.

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Table 1: Individual animal body weight (g) in single dose acute toxicity study

Study Type	Dose (mg/kg)	Concentration (mg/mL)	Animal	Body weight (g) on days		
				1	7	14
Sighting Study – Step – I	300	15	F	30	34	39
Sighting Study – Step – II	2000	100	F	32	35	40
Main Study	2000	100	F	33	36	42
			F	30	35	41
			F	32	37	42
			F	34	37	43

Step I sighting/observational study followed by Step II and then Main study demonstrated that the single dose/day of test compound had no impact on animal body weight when administered orally for 14 consecutive days

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Table 2: Animal feed consumption (g) and body weight (g) in immunotoxicity study

Group	Parameter	Day 7		Day 14		Day 21		Day 28		Day 42		Day 56	
		Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
Control	FC	3.5±0.31	3.2±0.12	3.8±0.45	3.6±0.30	4.2±0.19	3.8±0.27	4.5±0.38	4.3±0.25	-	-	-	-
	BW	19.33±2.1	18.33±2.5	20.67±1.5	19.00±1.9	21.33±2.2	19.33±2.4	22.33±2.3	20.67±2.8				
250 mg/kg	FC	3.6±0.32	3.1±0.32	3.9±0.32	3.7±0.32	4.4±0.32	4. ± 0.32	4.7±0.32	4.4±0.32	-	-	-	-
	BW	18.67±2.3	17.33±2.3	19.67±2.0	21.23±2.1	20.33±2.4	19.33±2.2	22.33±2.5	21.67±2.9				
500 mg/kg	FC	3.8±0.32	3.3±0.32	4.0±0.32	3.9±0.32	4.5 ± 0.32	4.0±0.32	4.2±0.32	4.1±0.32	-	-	-	-
	BW	19.00±2.1	18.67±2.1	20.33±2.1	21.00±2.1	21.33±2.1	20.67±2.1	22.33±2.1	22.00±2.1				
1000 mg/kg	FC	3.5±0.32	3.4±0.32	3.7±0.32	4.0±0.32	4.2±0.32	3.9±0.32	4.1±0.32	4.0±0.32	-	-	-	-
	BW	19.33±2.4	18.00±2.6	20.67±2.6	19.13±2.4	21.33±2.4	19.67±2.3	22.33±2.1	21.67±2.5				
Control - recovery	FC	3.4±0.32	3.5±0.32	3.7±0.32	3.6±0.32	4.2±0.32	3.8±0.32	4.5±0.32	4.3±0.32	4.2±0.32	3.8±0.32	4.5±0.32	4.3±0.32
	BW	20.00±2.2	19.33±2.6	21.67±2.4	19.00±2.9	22.33±2.3	20.33±2.7	23.00±2.1	20.33±2.4	23.33±2.3	21.67±2.5	24.00±2.6	22.33±2.8
1000 mg/kg - Recovery	FC	3.9±0.32	3.6±0.32	4.3±0.32	4.1±0.32	4.2±0.32	4.2±0.32	4.6±0.32	4.2±0.32	4.2±0.32	4.2±0.32	4.6±0.32	4.2±0.32
	BW	20.33±2.2	18.33±2.4	20.67±2.6	19.00±2.3	21.33±2.7	19.33±2.2	21.67±2.5	20.00±2.8	22.00±2.1	20.33±2.6	22.67±2.3	21.00±2.5

Different doses of test compound administered orally for 28 days in 4 groups. No clinically or statistically significant changes observed in feed consumption or body weight in immunotoxicity study for 28 days. Two recovery groups one that received high dose of test compound for initial 28 days and other one was normal control when left untreated for additional 28 days also demonstrated no abnormal changes in feed consumption or body weight until Day 56. Data represent mean ± S.D values of feed consumption (FC) and body weight (BW)

Table 3: Relative organ weight [a] in immunotoxicity study

Parameter	Group	Control	250 img/kg	500 img/kg	1000 img/kg	Control i-iRecovery	1000 img/kg i-iRecovery
Liver	Male	45.06 ± 1.23	47.28 ± 1.37	43.44 ± 1.24	48.60 ± 1.27	44.05 ± 1.43	49.51 ± 1.25
	Female	38.90 ± 0.98	40.02 ± 0.77	42.44 ± 0.88	46.55 ± 0.83	43.66 ± 0.88	47.77 ± 0.93
Kidney	Male	10.99 ± 0.45	12.14 ± 0.35	12.07 ± 0.73	11.26 ± 0.79	12.59 ± 0.77	12.17 ± 0.72
	Female	10.75 ± 0.38	11.45 ± 0.41	11.66 ± 0.65	12.45 ± 0.51	12.71 ± 0.39	11.67 ± 0.37
Heart	Male	4.16 ± 0.28	4.02 ± 0.21	4.10 ± 0.21	4.18 ± 0.24	4.20 ± 0.33	4.17 ± 0.30
	Female	4.83 ± 0.11	4.88 ± 0.09	3.95 ± 0.07	3.87 ± 0.10	4.00 ± 0.14	3.99 ± 0.13
Spleen	Male	3.26 ± 0.32	3.20 ± 0.38	4.20 ± 0.16	3.77 ± 0.28	3.96 ± 0.31	4.13 ± 0.26
	Female	3.98 ± 0.24	3.99 ± 0.17	4.14 ± 0.27	3.95 ± 0.12	4.14 ± 0.28	3.84 ± 0.10

No clinical or statistical significant changes in weights of essential organs at any of the test doses when compared to respective controls.

[a]Relative organ weight was calculated as the organ weight divided by the body weight (reported in milligrams [organ weight] per gram [body weight]).Values represented as mean ± S.D.

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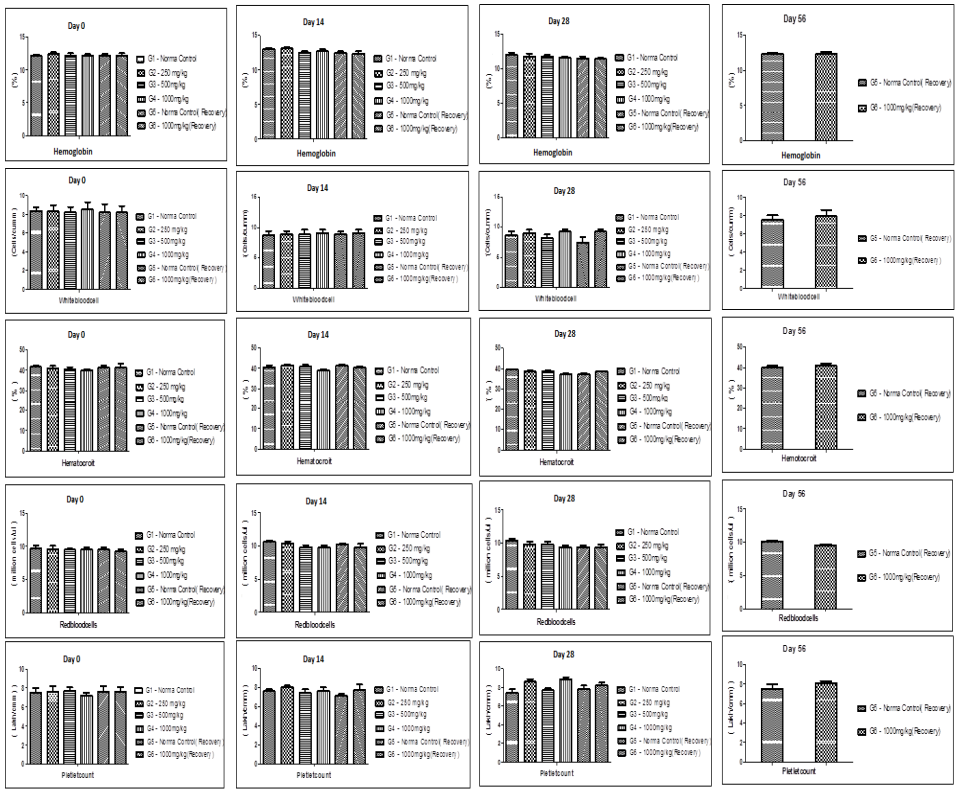
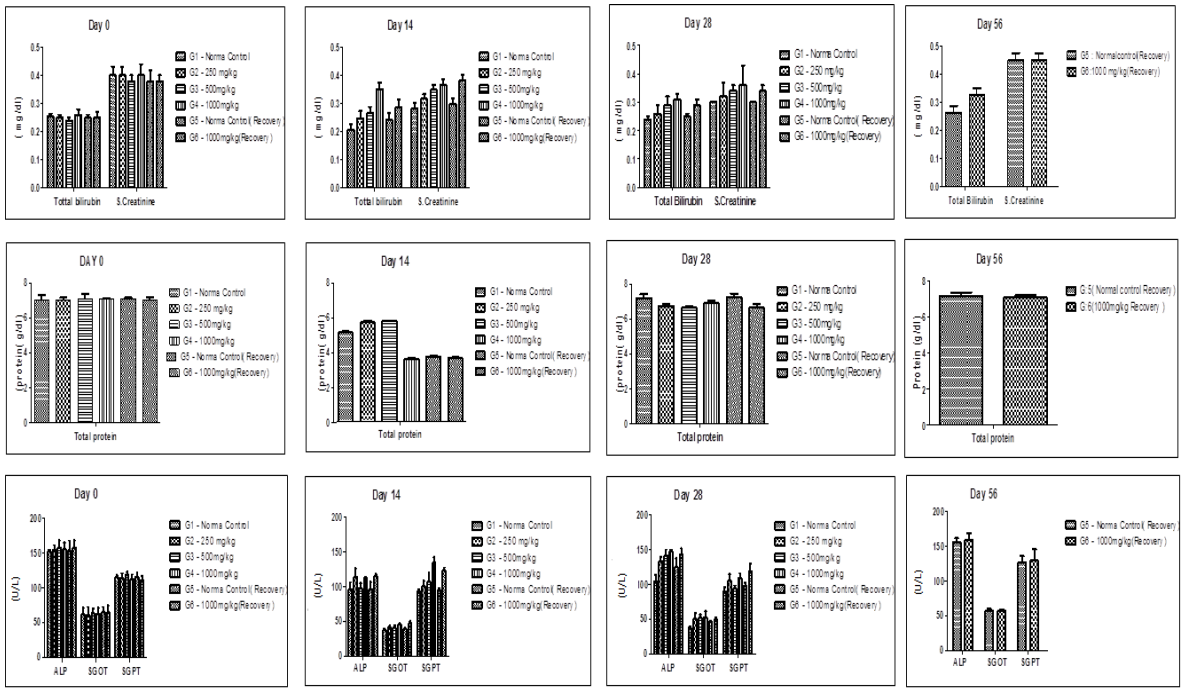
Fig. 1: Hematology data of Immunotoxicity study



Animal data on Day 0, 14, 28. Data on Day 56 recovery (Group 5 & 6) is also represented. No statistically significant changes.

Fig. 2: Biochemistry data of Immunotoxicity study



Animal data on Day 0, 14, 28. Data on Day 56 recovery (Group 5 & 6) is also represented. No statistically significant changes.

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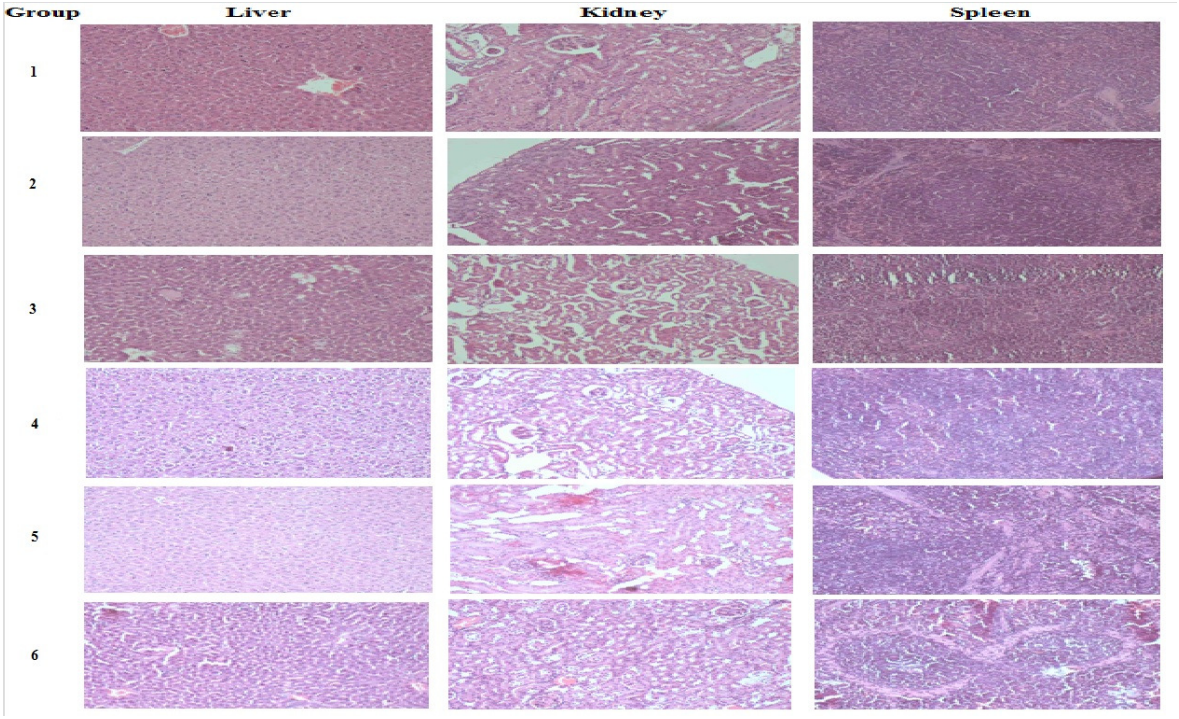
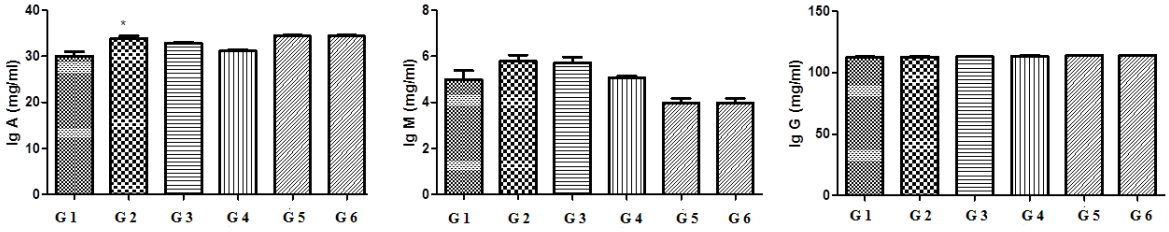
Fig. 3: Histopathology data of Immunotoxicity study



Liver - Normal architecture seen with central vein surrounded by hepatocytes which are normal. Portal triad is normal. No necrosis seen in all the 6 group animals. Kidney - Normal architecture of kidney is maintained. Normal glomeruli, tubules, collecting ducts and pelviureteric junction seen in all the animal groups tested. Spleen - Normal architecture of spleen consisting of red and white pulp is observed. Sinusoids are normal. Few hematopoietic elements are seen and no necrosis seen.

Fig. 4. Immunoglobulins (IgA, IgG and IgM) data of Immunotoxicity study



Dynamics of three sub-types of immunoglobulins across all six groups of animals, no statistically significant changes observed suggesting no immunotoxicity.

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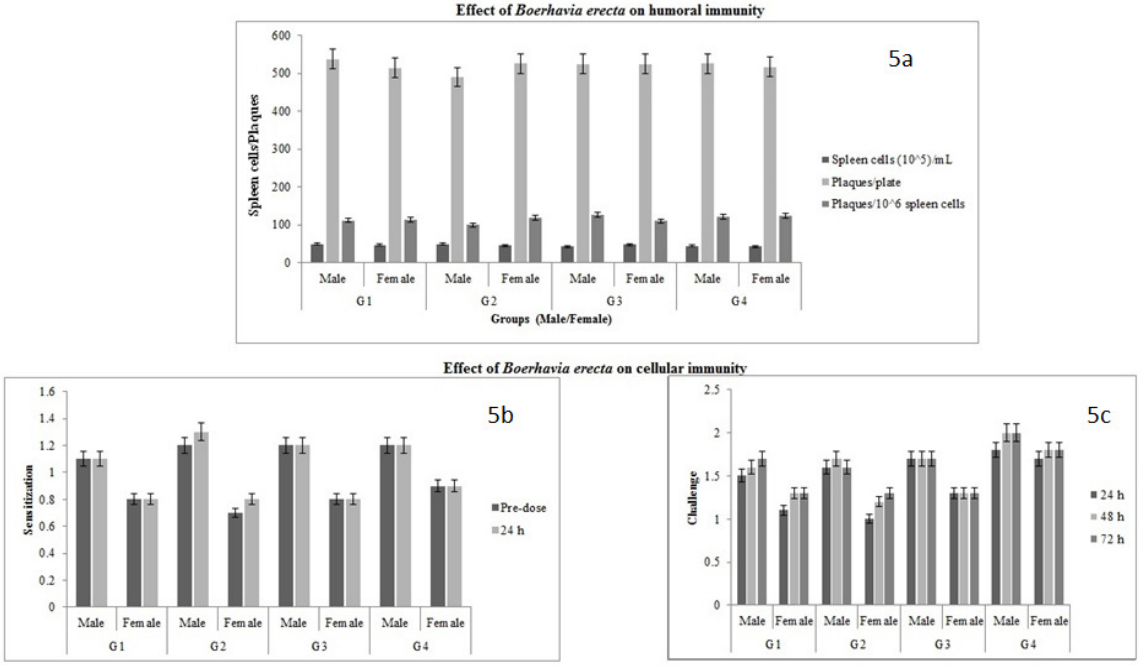
Fig. 5. Immunomodulatory activity of the active fraction of *B erecta*



Results after 28 days of treatment period, humoral (5a) immunity and cell mediated immunity (5b & 5c).

5a reflects plaque forming assay showing no effects on humoral immunity, 5b reflects immune sensitization pre-dose and 24 h post dose across 4 groups of animals after 28 days of treatment. 5c demonstrates immune challenge at different time intervals. From 48 h onwards both medium and high test groups (G2 & G3) demonstrated reduction in DTH reaction and high test dose group reached significance indicating that the test drug reduces cell-mediated immunity when compared to dexamethasone control group (G4).

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CONFLICTS OF INTEREST
"The authors declare no conflict of interest".