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Original Article

Sub-chronic Safety Evaluation of Ayurvedic Immunostimulant Formulation 'Immuforte' in Rats in Reverse Pharmacology

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ABSTRACT

Objective: The present study was undertaken to determine target organ safety of "Immuforte" to establish relationship between dose or exposure and response and also to identify potential parameters for monitoring adverse effects of "Immuforte" in clinical studies, if any. **Materials and Methods:** A total of 40 males and 40 females were randomly assigned to the four groups, namely group I (vehicle control; gum acacia), group II (120 mg/kg BW of Immuforte in gum acacia), group III (360 mg/kg BW of Immuforte in gum acacia), and group IV (600 mg/kg BW of Immuforte in gum acacia) consisting of 10 males and 10 females in each group. Additionally, a recovery group (600 mg/kg BW of Immuforte in gum acacia) containing 5 males and 5 females was included. **Results:** The results showed significant decrease in percent lymphocyte count of high and mid dose groups as compared to control group. The percent neutrophil counts in all the three treated groups of male and female rats were found to be significantly higher than that of control group ($P < 0.05$). In females MCV values in low dose and mid dose were significantly higher as compared to control ($P < 0.05$). The males from low dose group showed significant decrease in total serum protein, globulin, electrolytes, direct bilirubin, creatinine levels, whereas in mid dose group along with albumin, globulin. A significant decrease in AST and cholesterol was observed. In females, significant decrease was observed in total protein and globulin of low dose and mid dose of Immuforte-treated rats ($P < 0.05$). Though few hematological and biochemical parameters were different from control group, no does related response was observed and further, all these values were comparable with historical control data of the colony. Terminal body weight, organ weight, gross, and histopathology did not reveal any toxicity-related any adverse effects. Heavy metal analysis of the blood samples collected from terminally sacrificed animals did not show presence of heavy metals viz. lead (Pb), mercury (Hg), cadmium (Cd), and arsenic (As). **Conclusion:** The results of the present study demonstrated that Immuforte does not cause any observable toxicity at doses used in the study when administered for the period of 90 days and is safe for the human use and thus, Immuforte could be used safely for therapeutic use in humans.

Key words: Antioxidant, ayurveda, immunomodulation, stress, quality control

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INTRODUCTION

Ayurvedic medicine originated in India more than 2000 years ago and relies heavily on herbal medicine products (HMPs).[1] Immuforte is a herbo-mineral ayurvedic product manufactured by Shree Dhootapapeshwar Limited. It will be used as an immuno-stimulant in human. It

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mainly contains Guduchi (*Tinospora cordifolia*) and Ashwagandha (*Withania somnifera*). Guduchi is widely used in ayurvedic medicine for its general tonic, anti-leprotic,[2] anti-pyretic,[3] anti-allergic,[4] anti-inflammatory,[5,6] and anti-diabetic properties.[7-9] It has been reported to benefit the immune system in a variety of ways.[10-12] The experimental works on the alcoholic and aqueous extracts of Guduchi have shown significant immuno-modulatory activity.[13-19] Guduchi activates macrophages, which results in increased GM-CSF (Granulocyte-macrophage colony-stimulating factor), which leads to leukocytosis and improved neutrophil function.[20] Immuforte is also fortified with sufficient quantity of Ashwagandha (*Withania somnifera*), a well-known therapeutic agent of the ayurvedic medicine. Ashwagandha has been used and proved for its effect as immunomodulator, anti-stress,[21,22] antioxidant activity.[23-25] The Ashwagandha has been reported to exhibit its immuno-modulatory activity by mobilization and activation of peritoneal macrophages, phagocytosis, and increased activity of the lysosomal enzymes.[26]

Apart from its main ingredients Guduchi and Ashwagandha, it also contains oxides of minerals like zinc and gold in the form of Jasada Bhasma (Zn) and Suvarna Bhasma (Gold). The ayurvedic system of medicine has mentioned the therapeutic potential of these metallic bhasma, and they are regularly utilized and consumed by large number of Indian population for the therapeutic ailment without any observed side-effects. Jasada Bhasma (incinerated zinc) increases strength and intellect.[27] Suvarna Bhasma (incinerated gold) acts as immunomodulator, aphrodisiac and cardiac stimulant. It increases physical strength, complexion, intellect, and memory. It alleviates disorders caused by all the three vitiated *Doshas* and is used in the management of poisoning.[27] Immuforte also contains Shuddha *Hingula*, which is an ore of mercury. The presence of the heavy metals in the ayurvedic preparations is always a point of concerns for its safety.[28] Therefore, use of these products has been banned in the European countries. The ancient traditional methods for detoxification of the metals as mentioned in the ayurvedic texts has been carried out during the manufacture of Immuforte tablets, which includes multiple heating/cooling cycles along with addition of specific herbs. The present study was undertaken to assess proper detoxification of metals constituents of Immuforte and to determine target organ safety, establish relationship between dose or exposure and response and also to identify potential parameters for monitoring adverse effects in clinical studies.

MATERIALS AND METHODS

Materials

Immuforte tablets of Batch number:-E-19 (Code No. IMFT-092) were obtained from Shree Dhootapapeshwar Limited, Arogya Mandir, Tilak Road, Panvel – 410 206. The formulation in tablet form was stable at room temperature. All the other chemicals, reagents, and buffer solutions were of standard laboratory grade purchased from Sigma Aldrich.

Formulation of one Immuforte tablet is as follows:

Ingredients	
Suvarna (Gold) Bhasma	1.00 mg
Mouktik Pishti	6.10 mg
Shuddha Hingul (Purified *Cinnabar*)	9.15 mg
Marich (*Piper nigrum*)	12.20 mg
Jasad (Zinc) Bhasma	24.39 mg
Navneet (Butter)	7.62 mg
Guduchi (*Tinospora cordifolia*)	1000.00 mg
Ashwagandha (*Withania somnifera*)	1000.00 mg
Nimboo (Lemon) Rasa	30.50 mg
Pimpali (*Piper longum*)	62.50 mg
Excipients	
Gum Acacia	Q.S
DCP	Q.S
Starch	Q.S

Methods

Dose preparation

Dose selection and formulations preparation:
The dose formulations were evaluated at the reported therapeutic dose (low dose), thrice the therapeutic dose (mid dose), and five times the therapeutic dose (high dose).

For administration in rats, each tablet was crushed to powder with the help of mortar and pestle and mixed with 0.02% gum acacia to obtain a uniform suspension for administration to rats. The dose suspensions were freshly prepared daily and used.

Animal care and animal husbandry

The study protocol involving animals was reviewed and approved by the Institutional Animal Ethics Committee (IAEC) with a number NIRRH/IAEC/06-07 dated 27/09/2007 prior to the initiation of the study, and experiments were performed in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experimental Animals (CPCSEA), India.

Healthy adult Holtzman rats of 8 to 10 weeks age were used for the study. The animals were bred on the premises of NIRRH and were housed in polypropylene cages containing autoclaved paddy husk with a maximum of three animals of same sex per cage. All animals were housed in an experimental room maintained at the temperature of 23 ± 1°C, humidity of 55 ± 5%, in a 14 hr light/10 h dark cycle. Throughout the study, the bedding material was changed twice weekly, all animals were provided with

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soy-free, in-house-prepared rat pellets (consisting of crude protein%, fiber%, and nitrogen-free extract) prepared at the institute and *ad libitum* filtered drinking water.

Test methodology

Experimental design

A total of 40 males and 40 females were randomly assigned to the four groups, namely group I (vehicle control; gum acacia), group II (120 mg/kg BW of Immuforte in gum acacia), group III (360 mg/kg BW of Immuforte in gum acacia), and group IV (600 mg/kg BW of Immuforte in gum acacia) consisting of 10 males and 10 females in each group. Additionally, a recovery group (600 mg/kg BW of Immuforte in gum acacia) containing 5 males and 5 females was included

Gr. no.	Groups	Dose mg/kg BW	Number of males	Number of females
I	Control	Vehicle	10	10
II	Low	120 (TD)	10	10
III	Mid	360 (3TD)	10	10
IV	High	600 (5TD)	10	10
V	Satellite/ Recovery	600 (5TD)	5	5

All the animals belonging to control and treatment groups were gavaged with vehicle and test suspensions, respectively, consecutively for 90 days. All animals were sacrificed 24 h after the last dose of administration, except for the recovery group, which was sacrificed 15 days later.

During the treatment period, the animals were daily examined for avert clinical signs, morbidity, and mortality, if any. The body weight and food consumption were recorded weekly throughout the dosing period.

Clinical pathology

Hematology parameters like hemoglobin (Hb; g/dl), packed cell volume (PCV; %), total red blood cell count (RBC), total white blood cell count (WBC), absolute erythrocyte indices, differential WBC count, and blood biochemistry parameters like total protein, albumin, globulin, alanine aminotransferase, aspartate aminotransferase, cholesterol, alkaline phosphatase, glucose, creatinine, urea, uric acid, triglycerides, bilirubin (total and direct), calcium, and phosphorous were carried out.

Gross pathology, organ weight, and histopathology

After completion of dosing period, the animals were euthanized using CO_2 chamber and necropsied for the gross evaluation of the various organs. The necropsy also included careful and consistent dissection of various target organs like heart, liver, spleen, kidneys, intestine and stomach, determination of absolute organ weight, and calculation of organ weight to body weight ratios (Percent Relative Organ Weight). Finally, the dissected tissues were fixed in 10% neutral buffered formalin, processed (Tissue processor Leica ASP300), and embedded (Paraffin Embedder Leica EG1150 H) in paraffin wax. Sections (5 μ) (Fully Automated Rotary Microtome Leica RM2255) of these tissues taken on glass-slides were stained using a combination of hematoxylin-eosin before observing under a microscope for histopathological evaluations.

Heavy metal analysis from blood samples

At the terminal sacrifice, blood samples were collected and analyzed for heavy metal estimation at Shree Dhootapapeshwar Ayurvedic Research Foundation (SDARF) using Atomic absorption spectrophotometer (AAS) for various heavy metals *viz*. lead (Pb), mercury (Hg), cadmium (Cd), and arsenic (As).

Statistical analysis

For all the toxicological evaluations, the results of the treatment groups were compared with those of the control group. Data was expressed as mean ± S.D. and was analyzed by two-tailed Student's *t*-test. Differences were considered significant at $P < 0.05$.

RESULTS

Clinical signs, body weight, and food consumption

The treated animals did not exhibit any treatment-related adverse clinical signs; no significant differences were observed in body weights or food consumption compared with control group [Table 1 and Figure 1].

Clinical pathology

In males, a significant decrease in percent lymphocyte count in mid and high dose groups and increase in

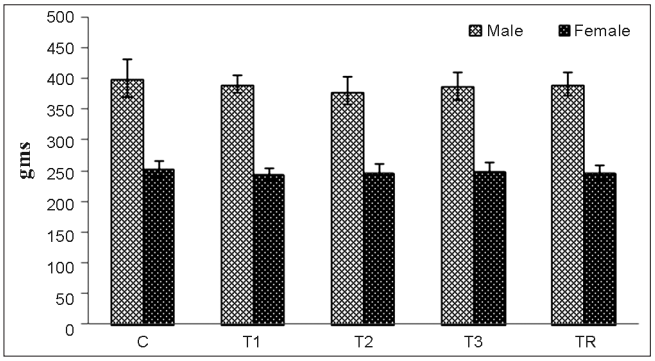


Figure 1: Average terminal body weight. The *x*-axis represents treatment groups and *y*-axis represents weight of the animals. Data represent the mean ± SD (n=10). C=Vehicle control, T1-120 mg/kg BW, T2-360 mg/kg BW, T3-600 mg/kg BW, TR=Recovery 600 mg/kg BW

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Table 1: Mean of weekly feed intake (gms) values (Mean±SD), *n*=10, recovery group *n*=5

Sex	Weeks	Control	Low dose	Mid dose	High dose	Recovery
Males	1st	40.52±7.099	40.11±6.698	37.84±6.688	39.49±6.549	54.83±12.84
	2nd	40.36±8.109	41.25±6.109	38.53±6.665	40.49±5.572	50.77±9.55
	3rd	40.18±6.911	43.29±6.762	39.05±4.210	39.54±7.427	50.90±13.20
	4th	41.71±6.933	42.52±5.286	41.77±6.108	40.31±8.407	55.46±15.19
	5th	38.78±5.542	43.84±6.061	43.65±5.940	44.01±6.570	61.94±14.98
	6th	37.02±5.575	34.63±5.532	41.70±5.286	39.78±4.494	60.34±13.57
	7th	39.10±6.883	34.13±6.669	44.48±7.330	38.40±8.398	57.71±11.40
	8th	45.58±8.798	46.22±6.432	45.61±6.424	44.36±9.509	57.37±10.71
	9th	45.81±7.911	47.24±6.185	44.89±5.714	43.03±7.927	59.09±14.62
	10th	44.21±8.914	45.59±6.052	43.32±8.098	39.60±7.202	56.15±18.53
	11th	45.39±7.765	44.48±5.621	43.35±9.169	39.81±5.233	60.55±19.81
	12th	43.88±8.358	45.14±5.646	44.34±7.889	41.99±6.673	59.49±11.96
	13th	42.45±7.524	44.61±5.125	45.98±6.843	40.48±5.661	62.18±15.79
Female	1st	28.72±3.129	26.94±4.700	28.80±4.119	27.90±4.088	37.17±11.40
	2nd	27.99±4.534	26.93±4.456	30.05±4.061	27.26±3.009	36.41±9.73
	3rd	28.07±4.359	25.80±3.488	30.29±3.999	26.60±3.333	33.52±5.14
	4th	35.50±15.246	28.10±3.143	30.69±4.714	27.36±3.140	33.40±6.25
	5th	26.42±3.821	33.13±4.817	33.92±6.040	32.36±6.207	42.41±8.30
	6th	30.70±5.278	29.68±5.906	40.53±15.487	29.19±3.526	44.91±17.61
	7th	31.63±2.288	31.59±4.593	32.17±5.351	30.98±5.468	39.28±8.01
	8th	32.24±2.407	30.99±5.508	33.30±3.974	32.11±4.943	41.06±7.70
	9th	37.53±12.273	29.08±4.751	32.21±5.488	36.35±11.276	45.62±9.06
	10th	32.86±6.310	29.81±6.940	31.78±5.210	29.36±4.849	39.16±10.21
	11th	32.90±7.654	30.38±5.418	30.45±5.117	30.74±6.174	38.53±11.33
	12th	31.51±3.406	29.38±4.468	29.67±3.896	29.29±3.311	35.10±6.80
	13th	29.56±2.836	28.64±4.021	30.73±3.266	28.18±3.638	37.47±7.65

percent neutrophil count was observed in all treated group compared to control. This finding was same in recovery group [Table 2].

In females, MCV values in low dose (120 mg/kg BW) and mid dose (360 mg/kg BW) were significantly higher as compared to control ($P < 0.05$). There was significant decrease in percent lymphocyte count ($P < 0.05$) and increase in percent neutrophil count ($P < 0.05$) of treated group as compared to control. This finding was same in recovery group.

In males, significant decrease in total protein, globulin, and chloride was observed in low and mid dose groups, significant decrease in direct bilirubin, creatinine, sodium, and potassium was observed in low dose group, albumin, significant decrease in AST, and cholesterol was observed in mid dose group compared to control group. Recovery group was well comparable with the control [Table 3].

In females, significant decrease in total protein and globulin was observed in low dose and mid dose compared to control. In the present investigation, although some hematological and biochemical values were significantly different from that of control, these values were well within the normal range of historical control data.

Gross and histopathology

In males, no significant difference was observed between absolute organ weights of treated groups and recovery group as compared to control group, except for low dose group; liver weight was increased ($P < 0.05$) and adrenal weight was decreased ($P < 0.05$) as compared to control. In females, there was no significant difference in absolute organ weight of treated groups and control group [Table 4].

In males, no significant difference was observed in relative organ weights of treated groups and recovery group as compared to control group, except for low dose; relative weight of liver was increased as compared to control group I. In females, there were no significant differences observed in relative organ weights of treated groups and control group [Table 5].

Post-necropsy revealed no dose-related toxicity lesions; though few incidental findings were seen during histopathology like hyperplasia of bronchial associated lymphoid tissue, emphysema in lungs, simple biliary cyst etc., were observed; these lesions were well comparable to the historical control data [Figure 2].

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Table 2: Average of hematological parameters *significance 95% ($P<0.05$), ↓significantly lower than control ($P<0.05$), ↑significantly higher than control ($P<0.05$), values (Mean±SD), $n=10$, recovery group $n=5$

Sex	Parameter	Control	Low dose	Mid dose	High dose	Recovery
Males	Hemoglobin (g/dl)	15.28±2.894	13.61±4.446	14.86±2.816	16.69±0.564	15.68±0.727
	RBC (X 106/cmm)	9.25±1.811	8.47±2.176	9.15±1.810	10.08±0.394	11.43±0.532
	PCV (%)	47.21±9.354	43.39±11.154	47.33±9.112	51.43±2.737	51.80±2.624
	MCV (pg)	50.88±1.045	51.87±0.838	51.81±1.125	51.01±1.116	48.03±3.522
	MCH (fl)	16.54±0.413	16.51±0.363	16.26±0.565	16.56±0.331	13.65±0.404
	MCHC (g/dl)	32.54±1.449	31.79±1.059	31.40±1.533	32.53±1.205	30.28±0.718
	WBC (X 103/cmm)	12.27±4.336	9.17±4.166	10.93±4.602	11.30±2.122	14.80±2.820
	Lymphocytes (%)	81.88±11.087	72.59±5.523	69.90±4.553*↓	66.34±4.046*↓	66.25±4.500*↓
	Neutrophils (%)	14.74±7.754	26.00±2.944*↑	25.27±6.701*↑	29.90±3.704*↑	32.75±4.349*↑
	Monocytes (%)	2.36±3.541	2.77±1.056	4.02±1.857	3.26±1.794	0.75±0.500
	Platelets (%)	452.11±124.065	562.86±135.347	447.43±123.092	457.86±77.652	370.50±102.754
Females	Hemoglobin (g/dl)	15.64±0.688	14.03±2.859	12.50±5.133	15.73±0.505	15.67±0.709
	RBC (X 106/cmm)	9.42±0.412	7.57±3.141	7.63±3.260	9.38±0.276	8.94±0.347
	PCV (%)	49.20±1.905	45.93±10.211	41.13±17.465	49.20±1.581	52.43±2.888
	MCV (pg)	52.24±1.261	54.97±1.525*↑	54.17±1.230*↑	52.45±0.437	52.57±1.401
	MCH (fl)	16.60±0.212	16.92±0.866	16.80±1.150	16.78±0.075	15.77±0.462
	MCHC (g/dl)	31.82±0.928	30.81±1.496	30.98±1.742	32.00±0.253	30.27±0.950
	WBC (X 103/cmm)	7.59±1.534	5.78±1.565	5.21±2.847	6.55±1.774	9.31±5.050
	Lymphocytes (%)	84.47±8.389	71.12±2.444*↓	66.57±3.524*↓	66.52±2.662*↓	66.40±0.529*↓
	Neutrophils (%)	13.60±6.994	25.56±3.276*↑	31.39±3.552*↑	30.27±2.074*↑	31.67±0.577*↑
	Monocytes (%)	0.78±1.339	2.40±1.841	1.60±0.860	2.80±1.789	1.67±0.577
	Platelets (%)	461.56±30.964	571.90±106.014	404.70±225.38	430.83±68.534	312.33±164.69

MCV=Mean corpuscular volume, MCH= mean corpuscular hemoglobin, MCHC=Mean Corpuscular Hemoglobin Concentration* ↓Decreased, *↑Increased

Table 3: Average of biochemical parameters *significance 95% ($P<0.05$), ↓significantly lower than control ($P<0.05$), ↑significantly higher than control ($P<0.05$), values (Mean±SD), $n=10$, recovery group $n=5$

Sex	Parameter	Control	Low dose	Mid dose	High dose	Recovery
Males	Total protein (g/dl)	8.35±0.959	7.19±0.335*↓	6.77±0.869*↓	7.70±0.660	7. 55±0.197
	Albumin (g/dl)	4.57±0.172	4.29±0.112	4.21±0.412*↓	4.34±0.192	4.28±0.110
	Globulin (g/dl)	3.78±0.920	2.64±0.430*↓	2.56±0.510*↓	3.36±0.687	3.28±0.297
	ALT (IU/L)	160.33±20.162	159.88±13.851	148.13±24.422	157.43±19.484	154.25±3.862
	AST (IU/L)	179.67±41.055	146.13±17.716	127.25±20.769*↓	147.29±11.280	170.50±8.266
	ALP (IU/L)	291.44±72.130	391.38±142.458	247.63±72.334	374.43±140.871	506.00±61.660
	Direct bilirubin (mg/dl)	0.21±0.079	0.10±0.019*↓	0.13±0.058	0.14±0.079	0.08±0.031
	Total bilirubin (mg/dl)	0.36±0.201	0.19±0.036	0.20±0.140	0.21±0.077	0.30±0.021
	Cholesterol (mg/dl)	96.56±11.103	90.25±7.741	81.13±9.643*↓	92.14±5.786	89.75±7.182
	Glucose (mg/dl)	109.78±25.312	110.13±8.509	99.75±14.558	89.86±24.970	110.50±7.594
	Urea (mg/dl)	45.44±17.903	36.88±10.710	40.25±7.066	46.14±13.471	39.00±3.916
	Creatinine (mg/dl)	0.83±0.220	0.57±0.149*↓	0.61±0.162	0.60±0.097	0.68±0.095
	Na (mmol/L)	149.56±2.343	145.85±1.794*↓	147.13±2.002	147.83±2.904	149.70±1.652
	K (mmol/L)	6.88±0.720	6.21±0.407*↓	6.27±0.476	6.46±0.667	6.36±0.432
	Cl (mmol/L)	101.09±1.250	98.05±0.997*↓	99.21±0.620*↓	101.50±1.954	96.83±1.097
Females	Total protein (g/dl)	8.18±0.652	7.43±0.287*↓	7.50±0.301*↓	7.68±0.579	7.74±0.191
	Albumin (g/dl)	4.63±0.139	4.56±0.144	4.53±0.154	4.49±0.216	4.59±0.153
	Globulin (g/dl)	3.55±0.667	2.87±0.274*↓	2.97±0.201*↓	3.07±0.533	3.15±0.133
	ALT (IU/L)	156.20±37.037	134.80±22.429	139.40±39.534	139.75±17.294	129.80±22.387
	AST (IU/L)	145.80±29.772	121.40±23.614	133.80±33.429	136.75±23.777	140.40±22.075
	ALP (IU/L)	243.80±57.584	287.80±86.205	248.20±50.274	240.00±64.103	369.20±55.197
	Direct bilirubin (mg/dl)	0.18±0.071	0.13±0.039	0.16±0.081	0.18±0.074	0.08±0.043
	Bilirubin (mg/dl)	0.38±0.242	0.21±0.071	0.44±0.265	0.22±0.071	0.20±0.080
	Cholesterol (mg/dl)	93.80±29.970	95.70±13.284	100.70±6.343		108.60±13.259
	Glucose (mg/dl)	95.30±20.667	117.70±13.992	113.30±16.111		111.20±8.899

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Table 3: Contd...

Sex	Parameter	Control	Low dose	Mid dose	High dose	Recovery
	Urea (mg/dl)	45.30±12.093	36.10±8.439	35.30±13.524		34.60±2.608
	Creatinine (mg/dl)	0.87±0.189	0.71±0.131	0.71±0.226		0.65±0.062
	Na (mmol/L)	148.58±3.332	148.54±2.937	146.76±2.742		150.00±1.735
	K (mmol/L)	6.39±0.966	6.02±0.523	5.80±0.343		5.89±0.437
	Cl (mmol/L)	100.93±1.363	100.00±1.536	98.53±2.641		99.63±1.950

ALT= Alanine transaminase, AST= aspartate aminotransferase, ALP=Alkaline phosphatase, Na= sodium, K= potassium, Cl= Chlorine

Table 4: Average body weight and absolute organ weight (gms) *Significance 95% (P<0.05), ↓significantly lower than control (P<0.05), ↑significantly higher than control (P<0.05), values (Mean±SD), n=10, recovery group n=5

Sex	Organs	Control	Low dose	Mid dose	High dose	Recovery
Males	Body weight	442.78±23.150	440.63±33.385	435.13±28.628	420.71±10.828	438.25±17.328
	Heart	1.62±0.162	1.65±0.121	1.74±0.194	1.56±0.232	1.60±0.170
	Liver	10.96±0.800	13.47±1.935*↑	12.70±1.009	11.40±1.311	15.17±1.068
	Kidney	2.52±0.213	2.64±0.102	2.57±0.529	2.71±0.373	2.83±0.222
	Adrenal	0.06±0.013	0.04±0.013*↓	0.05±0.008	0.05±0.004	0.05±0.006
	Lung	3.31±1.000	3.82±1.150	3.02±0.393	3.27±0.461	3.63±0.643
	Spleen	0.92±0.269	0.86±0.107	0.81±0.081	0.87±0.080	0.85±0.068
	Brain	2.02±0.101	2.05±0.084	2.05±0.075	1.80±0.311	1.80±0.311
	Testis	3.60±0.614	3.76±0.157	3.58±0.278	3.61±0.216	3.65±0.144
Females	Body weight	269.40±14.607	258.70±8.731	268.90±12.115	267.50±16.954	259.60±10.644
	Heart	1.11±0.127	1.19±0.211	1.15±0.090	1.16±0.133	1.21±0.120
	Liver	6.73±0.684	6.81±0.509	6.95±0.395	6.96±0.453	8.93±0.792
	Kidney	1.66±0.146	1.61±0.110	1.71±0.129	1.66±0.073	1.78±0.129
	Adrenal	0.06±0.008	0.06±0.011	0.06±0.007	0.05±0.009	0.06±0.011
	Lung	2.61±0.518	2.18±0.440	2.62±0.713	2.43±0.451	3.01±0.370
	Spleen	0.66±0.106	0.58±0.091	0.58±0.082	0.64±0.077	0.65±0.046
	Brain	1.84±0.124	1.90±0.071	1.93±0.069	1.85±0.237	1.86±0.078
	Uterus	0.94±0.308	1.07±0.391	0.83±0.300	0.87±0.334	1.33±0.340

Table 5: Average relative organ weight (%) * significance 95% (P<0.05), ↓significantly lower than control (P<0.05), ↑significantly higher than control (P<0.05), values (Mean±SD), n=10, recovery group n=5

Sex	Organ	Control	Low dose	Mid dose	High dose	Recovery
Males	Heart	0.366±0.031	0.375±0.014	0.400±0.047	0.386±0.060	0.364±0.028
	Liver	2.473±0.080	3.093±0.683*↑	2.928±0.293	2.815±0.222	3.459±0.120
	Kidney	0.569±0.036	0.602±0.045	0.594±0.131	0.671±0.096	0.645±0.027
	Adrenal	0.013±0.003	0.008±0.003*↓	0.010±0.002	0.012±0.001	0.010±0.001
	Lung	0.751±0.234	0.881±0.305	0.695±0.079	0.810±0.121	0.832±0.167
	Spleen	0.208±0.060	0.195±0.021	0.187±0.013	0.214±0.012	0.193±0.018
	Brain	0.458±0.034	0.467±0.029	0.474±0.047	0.448±0.091	0.467±0.033
	Testis	0.813±0.119	0.855±0.049	0.823±0.052	0.896±0.068	0.834±0.046
Females	Heart	0.414±0.041	0.459±0.084	0.427±0.040	0.434±0.059	0.47±0.044
	Liver	2.497±0.195	2.631±0.152	2.588±0.144	2.612±0.268	3.44±0.308
	Kidney	0.617±0.032	0.620±0.026	0.636±0.041	0.621±0.028	0.68±0.029
	Adrenal	0.021±0.003	0.021±0.004	0.021±0.003	0.020±0.004	0.02±0.004
	Lung	0.970±0.194	0.841±0.165	0.973±0.256	0.906±0.152	1.16±0.144
	Spleen	0.244±0.030	0.225±0.031	0.214±0.031	0.239±0.028	0.25±0.015
	Brain	0.681±0.034	0.734±0.031	0.718±0.037	0.692±0.098	0.72±0.044
	Uterus	0.348±0.114	0.416±0.160	0.308±0.106	0.328±0.134	0.51±0.123

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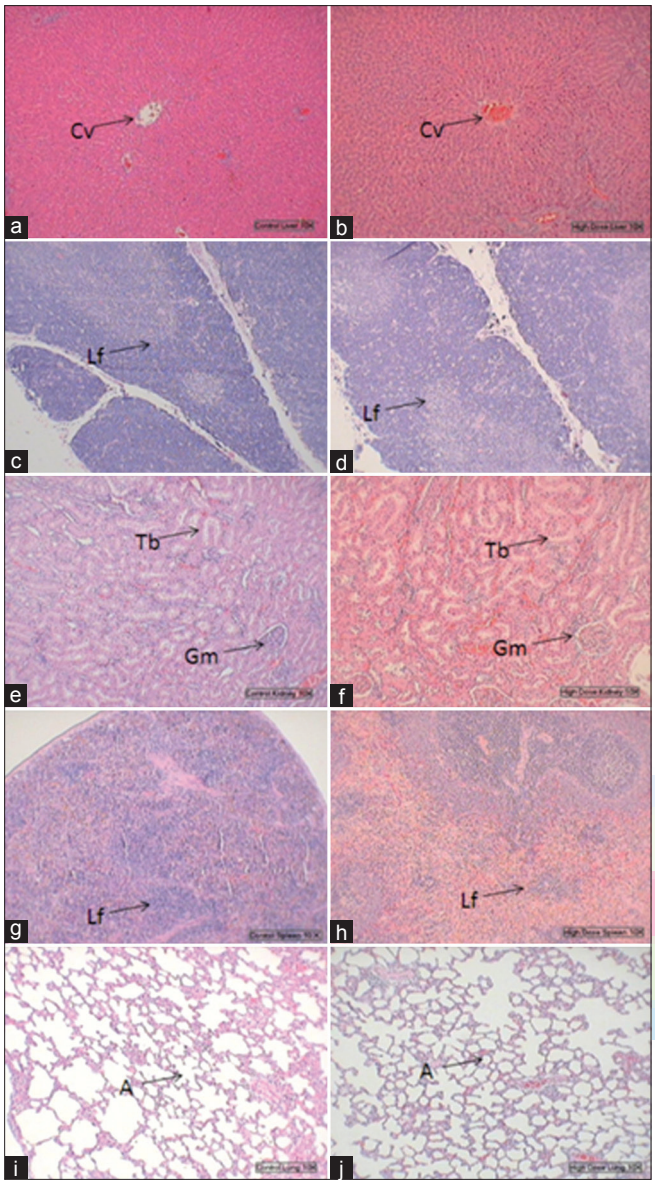


Figure 2: Histomorphology of: (a) liver (Vehicle control), (b) liver (600 mg/kg BW), (c) Thymus (Vehicle control), (d) Thymus (600 mg/kg BW), (e) kidney (Vehicle control), (f) kidney (600 mg/kg BW), (g) spleen (Vehicle control), (h) spleen (600 mg/kg BW), (i) lung (Vehicle control), (j) lung (600 mg/kg BW) The various organ sections were taken at 4-5 μm and stained with hematoxylin and eosin (×10). Cn=Congestion, Cv=Central Vein, Lf=Lymphoid follicle, Gm=Glomerulus, Tb=Tubule, A=Alveoli

Heavy metal analysis from blood samples

Blood samples analyzed on AAS for heavy metals viz. lead (Pb), mercury (Hg), cadmium (Cd), and arsenic (As) did not show any detectable level.

DISCUSSION

The study provided information on the possible toxic effects, indicate target organs and the possibility of accumulation, and a no-observed-adverse-effect level (NOEAL) of exposure, which is used in selecting dose levels for chronic studies and for establishing safety criteria for human exposure. The animals did not exhibit any treatment-related abnormal behavioral signs and symptoms. The observations indicated that long-term administration of the Immuforte had no adverse effects on the general health of the animals.

Amongst the clinical pathology observations, the hematology data is a direct reflection of the possible tissue injury caused by the test compound. For instance, increase in red blood cells reflects overproduction of these elements in response to tissue injury etc.[29] Blood biochemistry parameters constitute yet another sensitive parameter of toxicity evaluation. For instance, changes in blood enzymes may be due to cellular/tissue injury leading to their systemic leakage from intracellular sites or target tissues. Similarly, manifestations of altered electrolyte levels are corollary to toxicity-associated conditions like renal dysfunction, dehydration, anorexia, cardiac toxicity, altered vascular permeability.[29] In the present investigation, although some hematological and biochemical values were significantly different from that of control, all these values were well within the normal range of historical control data of the colony. Secondly, no dose-dependent changes were observed in these parameters, and also recovery group was well comparable to control group, thus the observed difference was due to biological variation and not due to test substance.

Absolute terminal organ weight and percent relative organ weight indicative of test compound caused changes in functioning of target organs, changes in phospholipids metabolism, over- or under- secretion of enzymes and hormones, hyper/hypoplasia, and possible tissue necrosis. There were no significant difference in absolute organ weights of treated group and recovery group as compared to control group. Although there was difference in some organ weights viz., liver and adrenals, these changes were not dose-dependent, so these could be considered as biological variation. Similarly, the gross and histopathology of various target organs like heart, liver, spleen, kidneys, intestine, and stomach, post-necropsy, revealed that the natural architecture of the various organs remained unaffected. Based on the findings of the study, 600 mg/kg was considered as NOAEL for 'Immuforte.' Blood samples analyzed on atomic absorbance spectrometer (AAS) for heavy metals viz., lead (Pb), mercury (Hg), cadmium (Cd), and arsenic (As) did not show any detectable levels in blood.

CONCLUSIONS

It can be concluded that repeated administration of Immuforte for the period of 90 days did not showed any treatment-related adverse effect at the doses used in the

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study. Thus, Immuforte could be used safely for therapeutic use in humans.

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