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Evaluation of Safety and Immunotoxicity of Immunol, A Polyherbal Formulation in Rats

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Abstract: Immunol, a polyherbal formulation was evaluated for its safety and effect on the immune system of rats. The rats were administered a single oral dose of 20 mL kg^{-1} b.wt. while others were given repeated doses of 1, 5 and 10 mL kg^{-1} b.wt., orally for 28 days. Immunol treatment did not elicit any abnormalities on the body weight gain, food consumption, neurological assessment, hematology and clinical chemistry parameters, organ weights, gross organ pathology and histopathology and humoral immunity, but it enhanced the cell mediated immunity. It was concluded that Immunol was safe at single oral dose of 20 mL kg^{-1} b.wt. and repeated dose of 10 mL kg^{-1} b.wt. in rats for 28 days.

Key words: Toxicity, neurological assessment, humoral immunity, cell mediated immunity, immunomodulation, dinitrochlorbenzene

INTRODUCTION

In recent years there has been an upsurge in the clinical use of indigenous drugs. Polyherbal preparations, originally used in the traditional systems of medicine, are now being investigated and effectively tried in a variety of pathophysiological states (Shah *et al*., 1997). Ayurveda is one of the traditional systems of medicine practiced in India and Sri Lanka and can be traced back to 6000 B.C (Samhita, 1949). Great emphasis has been placed on research of herbal formulations which can be helpful in the management of stress related disorders. One of the main approaches in Ayurvedic medicine is to increase the body's natural resistance to disease/stress causing agents rather than directly neutralizing the agent itself. This has been achieved by using extracts of various plant materials known as Rasayana [Rejuvenation] (Pallabi *et al*., 1998). Side effects and higher expenses often associated with allopathic drugs have provoked the need for research into drugs, which are cost effective and devoid of side effects, especially those belonging to the traditional systems of medicine like Ayurveda, Homeopathy, Unani, Siddha etc. Emphasis is also laid on the integration of indigenous healthcare systems with medical practices (Ziauddin *et al*., 1996).

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Despite their growing popularity as naturally safe with pharmacologically active principles (Mabeku *et al*., 2007), very little information is available on the safety of herbal products. Several plants extracts or formulations were investigated for toxicity or safety. Malathi and Gomaz (2008) evaluated methanolic extract of *Tylophora asthmatica* (META) for safety in rats and concluded that META is safe to rats at dose of 50 to 200 mg kg^{-1} b.wt. and did not cause any detrimental effects. Joshua *et al*. (2008) tested five polyherbal formulations for acute oral toxic effects in wistar rats and suggested that there were no treatment-related effects up to 5000 mg kg^{-1} b.wt. Aqueous extract of *Crptolepis sanguinolenta* roots, possessing anti-malarial activity did not cause any toxic effects to rats (Ansah *et al*., 2008). A study of the active extract of *Garcinia parnifolia* Miq. stem bark was safe and the estimated LD_{50} was more than 8000 mg kg^{-1} b.wt. (Tjokrosonto *et al*., 2008). Aqueous suspension of *Hyphaene thebaica* L. roots was reported as hepatoxic and nephrotoxic in rats by Zanna *et al*. (2008). The toxic effects of feeding *Rhanterium epapposum* aerial parts to wistar rats was first reported by Younis and Adam (2008). The dose levels of 100 and 200 g kg^{-1} of *Rhanterium epapposum* aerial parts in diet induced hepatonephropathy, hematologic and serobiochemical alterations. Ethanolic extract of *Ageratum conyzoides* daily oral doses of 500 and 1000 mg kg^{-1} weight administered for a one month period did not show any toxic effect in rats and this coupled with the high LD_{50} value of 10100 mg kg^{-1} b.wt. confirm that *A. conyzoides* is safe for use in ethnomedicine (Igboasoiyi *et al*., 2007). Many of the herbal/polyherbal medicines are available over the counter and are used without medical prescription/supervision, there is a need to scientifically evaluate efficacy and toxicity/safety of these preparations. Immunol is a polyherbal formulation containing extracts of *Tinospora cordifolia*, *Withania somnifera*, *Boerhaavaia diffusa*, *Asparagus racemosus*, *Trigonella foenum-graecum*, *Terminalia chebula* and *Tylophora asthamatica*. Immunol is presently used in dogs as a vaccination primer for better immune response, as an adjunct to antimicrobial therapy in dermal, ocular, otic and recurrent chronic infections and as a follow-up with antibiotics in acute bacterial infections and co-treatment and also as a follow-up with corticosteroids and antineoplastic therapies. The information on the toxicity and immunotoxicity potential of most monoherbal/polyherbal formulations is lacking in published literature and hence the present investigation has been undertaken to evaluate the toxicity of a polyherbal formulation with special emphasis to its immunomodulatory effect.

MATERIALS AND METHODS

Experimental Period

The research project was performed at the Department of Safety Assessment, Advinus Therapeutics, Bangalore during February to April 2007. The research facility is certified for Good Laboratory Practice (GLP) compliance by the Monitoring Authorities of Germany (BfR), The Netherlands (W and V inspectie) and the National GLP Compliance Monitoring Authority of India and is accredited for animal care and use by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International. The experimental project was approved by Institutional Animal Ethics Committee (IAEC).

Animals

Wistar rats, in-house bred at Toxicology Department of Advinus Therapeutics Private Limited, Peenya Industrial area, Bangalore-560 058, were used in the experiments. Animals of 8 to 10 weeks old were randomly selected and were acclimatized to experimental conditions

for 5 days. Animals were housed under standard laboratory conditions, air conditioned with 12-15 filtered fresh air changes/hour, environmental temperature 17-23°C, relative humidity 30-70%, with 12 h fluorescent light (6.00 am to 6.00 pm) and 12 h dark cycle. Rats were housed individually in standard polypropylene cages (size: approximately L 410 x B 280 x H 140 mm), with stainless steel top grill having facilities for pellet food (Tetragon Chemie Ltd., Bangalore, India) and Aquaguard filtered cum-purified water.

Test Material

Immunol® (Batch No. H276006 ; Manufacture date: August 2006 ; Expiry date: August 2009) manufactured by The Himalaya Drug Company, Makali, Bangalore, India, was the test product used for the experiment. Each milliliter of Immunol contains extracts of *Tinospora cordifolia* (14.5 mg), *Withania somnifera* (7 mg), *Boerhaavaia diffusa* (7 mg), *Asparagus racemosus* (7 mg), *Trigonella foenum-graecum* (4.8 mg), *Terminalia chebula* (3.5 mg) and *Tylophora asthamatica* (4.8 mg).

Design of the Experiment

The experiment was conducted in two parts, A. Single Dose Acute toxicity study and B. Repeated dose toxicity study with Immunotoxicity screening.

Part. A. Single Dose Acute Toxicity Study

The objective of this acute oral toxicity study was to assess the toxicological profile and to determine the Maximum Tolerable Dose (MTD) of Immunol when administered by a single oral gavage. The results of this experiment aided the selection of doses for the repeated dose studies.

Five male and five females rats were orally administered single dose of 20 mL kg^{-1} Immunol. The animals were observed for clinical signs and mortality daily throughout the observation period of 14 days. All animals were observed for changes in skin and fur, eyes, mucous membrane, musculature or skeletal, CNS, respiratory, cardiovascular and gastrointestinal symptoms. The individual body weights were recorded on day 1 prior to the administration of the dose and on days 7 and 15 (at sacrifice). At the end of the 15 day observation period, all the surviving animals were euthanised under isoflurane anaesthesia and subjected to detailed necropsy on day 15.

Part. B. Repeated Dose (28 Day) Subchronic Toxicity with Immunotoxicity Assessment in Rats

Four groups of rats consisting of 15 males and 15 female rats were used (five/sex/group each were used as satellite group for assessment of humoral and cell mediated immunity). These groups were considered as control (G1-10 mL kg^{-1} distilled water), low dose (G2-1 mL kg^{-1}), mid dose (G3-5 mL kg^{-1}) and high dose (G4-10 mL kg^{-1}) groups. Two groups of animals, control recovery (G1R: Distilled water recovery) and high dose recovery (G4R) was maintained for 14 day no-treatment period, to assess the reversal of toxic effects, if any.

All the animals were examined for clinical signs, morbidity and mortality once daily throughout the experimental period. In addition a thorough veterinary examination was conducted for all the animals once a week. Detailed neurological examination was performed during the 4th week of the treatment period and at the end of recovery period. Neurological assessment parameters included; a: Home cage observations-convulsions, tremors and palpebral (eyelid) closure, b: Handling observations-ease of removal from the cage, ease of handling animal in hand, lacrimation, salivation, piloerection, muscle tone, eye prominence,

chromodacryorrhea (red tears), palpebral (eyelid) closure and respiratory character, c: Open field observation- mobility, backing, grooming, gait, arousal, convulsions and tremors, d: Sensory observation- response to nociceptive stimuli, startle response (auditory response), touch response (tactile response) and pupil response (visual response) and righting reflex, e: Neuromuscular observations- grip strength-forelimb and hind limb, motor activity and hind limb foot splay and f: Physiological observations-rectal temperature.

Clinical Laboratory Investigations

Blood was collected from the retro-orbital plexus pre-treatment and from the abdominal aorta at sacrifice. An aliquot of blood was collected in tubes containing 3.8% sodium citrate solution for determination of prothrombin time and the remaining blood was collected into EDTA and lithium heparinized tubes for haematology and clinical chemistry respectively.

Complete blood cell count was performed using ADVIA 2120 Haematology System (Bayer HealthCare LLC, USA). Prothrombin time was assessed using STart-4 coagulation analyser (Diagnostica Stago, France). Plasma was separated in a refrigerated centrifuge and analysed in Roche/ Hitachi 902 Automated Analyser (Hitachi Science Systems Ltd, Japan), using Diagnostic kits (Roche Diagsnostics GmbH, Germany). Urine was analysed for specific gravity, leukocytes, nitrite, pH, albumin (expressed as proteins), glucose, Acetone (expressed as ketone bodies), urobilinogen, bilirubin and erythrocytes, using Combur 10 test-U strips using Urilux R Reflectance Photometer (Roche Diagnostics GmbH., GERMANY). Urine was subjected to microscopic examination for sediments such as crystals, epithelial cells and cast.

Determination of B-Lymphocyte Function

To determine the effect of Immunol on humoral immunity, the Plaque forming cell response of splenic lymphocytes to sheep erythrocytes was assessed using the method described by Talwar and Gupta (1992). Five males and five females selected from each group were used for Plaque forming cell assay. The rats were immunized by intravenous injection of 5% Sheep RBC (males: 2 mL, females: 1.5 mL) on the 24th day of treatment. At the end of the treatment period (day 28), the animals were euthanized, spleens collected from each animal, homogenized and suspended in chilled Hank's Balanced Salt Solution and the number of lymphocytes per spleen were counted and 0.25 mL of spleen cell suspension was incubated with 0.1 mL of 20% SRBC suspension in 2.0 mL of agarose in petriplates. After the agar set, the plates were incubated at 37±1°C for 1 h in humidified atmosphere. After incubation, 2 mL of 1:10 guinea pig complement was added to each plate and the plates were again incubated in the incubator for 30 min. After the 2nd incubation, the complement from each plate was poured out and the number of plaques (clear zones) per spleen were calculated and taken as a measure of humoral immunity.

Determination of T-Lymphocyte Function

To determine the effect of Immunol on cell mediated immunity, delayed hypersensitivity to 2,4-dinitrochlorbenzene (DNCB) was assessed using the Delayed Type Hypersensitivity (DTH) skin test described by Rajan *et al.* (1981). Five males and five females selected from each group were used DTH assay. On day 14 an area of 3 cm in diameter was marked on the left flank of the animal using a metallic ring. The hair on the marked site was clipped using an electrical hair clipper. On day 15 primary sensitization was done using 0.4 mL of 2% DNCB in acetone applied drop by drop on the marked area keeping the metallic ring on the site marked. The solution was allowed to evaporate quickly by blowing gently during each application. After 14 days of the primary sensitization a challenge dose of 0.25 mL of 2%

DNCB was applied at the same site. The skin thickness was measured before and after primary sensitization and also at 24 and 48 h after challenge. The increase in the skin thickness was taken as a measure of delayed hypersensitivity to DNCB.

Gross Necropsy, Organ Weights and Histopathology

On day 29 and at the end of the 14-day recovery period, the animals were fasted overnight (water allowed), anaesthetised, weighed and were subjected to detailed necropsy. Thirty five tissues were collected from each animal and preserved in 10% buffered neutral formalin for further processing for histopathology. Liver, adrenals, kidneys, gonads, brain, epididymides, thymus, spleen and heart were weighed and organ weights as percentage of body weights were determined. Histopathological evaluation was performed on the preserved organs and tissues of 5 male and 5 female rats from control and high dose groups. The tissues were processed for routine paraffin embedding and 5 micron sections were stained with Mayer's Haematoxylin Eosin stain.

Statistical Analysis

All the grouped data were statistically evaluated with SYSTAT Statistical software package version 12.0. The results were expressed as Mean±SD. Differences between control and the treatment groups were assessed by hypothesis testing methods included one-way analysis of variance followed by Dunnett's t-test. All analyses and comparisons were evaluated at 5% ($p<0.05$) level.

RESULTS

Single Dose Acute Oral Toxicity in Rats

Administration of Immunol at the dose volume of 20 mL kg^{-1} orally as a single dose did not induce any clinical signs, morbidity and mortality. Immunol treatment did not affect the growth. The mean body weight gains in males and females were 9±1.10 and 8±2.44 g at day 8 and 20±4.15 and 18±5.34 g at day 15, respectively (Table 1). There were no gross pathological organ changes. Based on the results of this study it was concluded that the Maximum Tolerable Dose (MTD) of Immunol was more than 20 mL kg^{-1} b.wt. and this formed the basis for the dose fixation for further repeated dose experiments.

Repeated Dose (28 Day) Subchronic Oral Toxicity with Immunotoxicity Assessment in Rats

Immunol at doses of 1.0, 5.0 and 10.0 mL kg^{-1} for 28 days did not elicit any clinical signs of toxicity or mortality with no effect on the Functional Observation Batteries (FOBs) parameters at any of the doses in both sexes indicating the safety of Immunol upon repeated administration in rats.

Body Weight and Food Consumption

Immunol at any of the doses did not show any deleterious effects on the body weight throughout the treatment (and recovery) period/s in both sexes (Table 2). The body weights

Table 1: Effect of single dose Immunol on the body weights of rats

		Body weight (g)			Body weight gain (g)	
Group and dose	Sex	Predose	Day 8	Day 15	Day 8	Day 15
G1 20 mL kg^{-1} b.wt.	Male	264±4.72	273±4.87	283±5.27	9±1.10	20±4.15
	Female	191±6.83	200±6.52	209±4.97	8±2.44	18±5.34

in all groups increased steadily from the initial weight to the end of the experiment. The mean net body weight gains in the control, low, mid and high dose Immunol treated groups over the 28 days treatment period were: Males : 54, 48, 61 and 54 g; Females : 31, 34, 35 and 41 g, respectively. The mean net body weight gains in the control and high dose treated recovery groups over the 28 days treatment + 14 days recovery period were: Males: 98 and 73 g; Females: 47 and 51 g, respectively.

The food consumption was comparable in all groups throughout the treatment (and recovery) period/s in both sexes (Table 3). The mean food conversion efficiency (FCE) in the control, low, mid and high dose Immunol treated groups over the 28 days treatment period were: Males : 58.0, 52.9, 64.8 and 57.6%; Females : 45.2, 49.0, 49.6 and 56.2%, respectively. The mean FCE in the control and high dose treated recovery groups over the 28 days treatment + 14 days recovery period were: Males: 68.9 and 52.8%; Females: 44.9 and 49.7%, respectively. The FCE was significantly lower in the high dose recovery males in comparison with the control recovery group. Immunol caused a dose related trend in the increase in FCE in the females, but not in the males.

Table 2: Effect of immunol on the body weights of rats upon administration of immunol for 28 days

	Body weight (g) at week							Net body weight gain (g) at group
Groups	Pre-dose	1	2	3	4	5	6	termination
Males								
G1	252±14.36	273±18.28	281±19.65	291±16.62	306±26.50			54±12.89
G2	259±10.13	285±12.79	290±19.34	306±22.69	307±27.07			48±21.62
G3	260±2.61	278±7.62	293±8.08	311±14.98	320±20.84			61±17.34
G4	277±28.52	298±26.18	312±29.33	331±31.57	331±27.56			54±22.51
G1R	267±12.56	282±17.07	294±18.11	313±19.51	332±19.66	349±22.38	365±24.27	98±12.34
G4R	269±13.22	278±14.88	291±16.51	305±16.91	319±17.17	331±14.79	342±17.16	73±6.12*
Females								
G1	185±16.28	199±18.51	207±17.77	214±19.68	216±18.01			31±4.50
G2	185±7.50	204±8.56	205±10.73	213±8.70	220±12.44			34±5.54
G3	176±4.60	190±9.67	194±8.94	209±7.29	211±7.83			35±3.54
G4	179±4.16	200±5.41	200±5.50	209±5.54	220±7.07			41±6.47*
G1R	193±23.28	204±28.46	212±27.72	219±30.08	228±27.75	235±25.78	240±26.02	47±3.43
G4R	193±13.61	201±15.09	212±16.99	222±18.80	228±19.92	235±20.78	243±22.22	51±8.38

Values are Mean±SD. *p≤0.05 vs control or control recovery group

Table 3: Effect of immunol on the food consumption of rats upon administration of immunol for 28 days

	Food consumption (g/rat/day) at week						
Groups	1	2	3	4	5	6	Food conversion efficiency
Males							
G1	23.0±1.30	22.7±1.33	23.0±0.98	23.6±1.15			58.0±13.8
G2	23.4±1.58	23.2±1.54	22.5±1.83	22.8±2.22			52.9±27.1
G3	23.6±1.30	23.6±0.76	23.2±0.62	23.9±1.25			64.8±22.3
G4	23.2±1.49	24.0±1.55	23.5±1.94	24.3±2.15			57.6±27.6
G1R	22.9±0.80	24.0±1.40	24.3±1.65	25.0±1.94	22.4±1.89	23.3±1.58	68.9±10.0
G4R	22.6±1.74	23.1±1.19	23.3±0.72	23.9±0.72	23.1±1.32	23.0±0.32	52.8±4.8*
Females							
G1	16.7±1.23	16.7±0.61	17.1±0.86	17.2±0.72			45.2±7.3
G2	17.2±0.50	17.9±0.53	17.3±0.55	17.8±0.44			49.0±8.7
G3	18.1±0.68	17.9±0.84	17.2±0.86	17.8±1.30			49.6±5.1
G4	18.0±1.10	17.8±1.04	17.9±0.86	18.6±0.65			56.2±9.3
G1R	17.3±1.12	16.5±0.82	17.8±0.59	17.7±0.84	17.5±0.71	17.5±0.50	44.9±3.4
G4R	16.4±0.70	16.9±0.94	16.6±0.91*	16.8±0.61	17.6±0.51	17.4±0.63	49.7±8.7

Values are Mean±SD. *p≤0.05 vs. control recovery group

Haematology

There were a few changes such as: decreased leucocyte count in G3 group males (5 mL kg^{-1}), decreased leucocyte count, increased MCV and decreased MCHC values, increased neutrophil and decreased lymphocyte counts in G4 group males (10 mL kg^{-1}) when compared to the control group. There was also a tendency for a marginal reduction in RBCs and Hb (not statistically significant) (Table 4).

In female rats haematological parameters did not differ significantly in the Immunol treated groups when compared to control group except for decreased Hct in G2 (1 mL kg^{-1}) group, decreased MCHC concentration and increased Platelet count in G4 (10 mL kg^{-1}) group. Similar to the males, there was also a tendency for a marginal reduction in RBCs and Hb and an increase in MCV (not statistically significant). In the recovery group animals, there were no alterations in the haematology except for a slight increase in prothombin time. The results indicated that Immunol has no toxic effects on the haemopoietic system as the observed changes in haematological parameters were considered incidental and not-related to Immunol treatment or adaptive findings and hence are of no toxicological significance.

Table 4: Effect of immunol on the hematology upon administration for 28 days

	Groups					
Parameters	G1	G2	G3	G4	G1R	G4R
Males						
Leucocytes (G L^{-1})	5.55±0.56	5.02±0.70	4.34±0.19*	4.45±0.86*	4.46±2.06	4.16±0.62
Erythrocytes (T L^{-1})	8.81±0.12	8.67±0.23	8.37±0.54	8.36±0.40	9.10±0.46	8.87±0.20
Hemoglobin (g L^{-1})	161±6.10	156.0±7.96	154±7.60	153±7.09	162±6.76	160±1.82
Hematocrit (l L^{-1})	0.47±0.02	0.46±0.02	0.45±0.03	0.48±0.03	0.50±0.03	0.48±0.03
Mean corpuscular volume Fl	53.5±1.61	53.3±2.78	53.8±0.62	57.6±1.12*	54.6±2.20	54.6±1.69
Mean corpuscular hemoglobin (pg)	18.3±0.56	18.0±1.36	18.4±0.78	18.4±0.81	17.8±0.63	18.1±0.41
Mean corpuscular hemoglobin conc (g L^{-1})	342±6.10	337±10.78	342±14.37	319±10.65*	326±8.96	332±16.32
Platelet (G L^{-1})	845±71.26	896±82.35	788±88.55	846±81.11	933±346.0	955±76.96
Prothrombin time (sec)	16.1±1.22	16.5±1.18	17.4±0.99	18.1±1.83	16.0±1.11	16.3±0.54
Neutrophil (%)	20.8±3.11	23.7±5.41	16.9±5.39	31.5±9.79*	18.2±7.37	17.9±4.93
Lymphocyte (%)	75.6±3.57	72.4±6.07	78.6±5.35	63.4±11.35*	77.9±8.23	78.5±5.17
Monocyte (%)	1.9±0.47	2.0±0.76	1.9±0.40	2.9±1.77	1.4±0.24	1.5±0.47
Eosinophil (%)	1.2±0.33	1.4±0.53	2.1±0.47	1.7±1.04	2.0±0.77	1.6±0.42
Large unstained cell (%)	0.2±0.07	0.3±0.17	0.3±0.15	0.3±0.15	0.3±0.07	0.3±0.08
Basophil (%)	0.2±0.16	0.2±0.05	0.2±0.09	0.2±0.11	0.2±0.12	0.2±0.05
Reticulocyte (%)	2.58±0.26	2.85±0.42	2.39±0.47	2.51±0.44	2.83±0.29	2.61±0.23
Females						
Leucocytes (G L^{-1})	4.66±0.81	4.97±1.13	4.80±1.24	4.90±0.95	4.94±1.45	4.99±1.12
Erythrocytes (T L^{-1})	8.38±0.48	7.83±0.37	8.07±0.34	8.14±0.39	8.11±0.38	8.17±0.21
Hemoglobin (g L^{-1})	158±9.61	149±6.19	149±3.63	149±6.52	151±6.91	150±8.85
Hematocrit (l L^{-1})	0.47±0.03	0.43±0.01*	0.46±0.02	0.48±0.02	0.47±0.01	0.45±0.02
Mean corpuscular volume Fl	55.7±1.61	54.7±1.46	57.3±1.98	58.4±1.39	57.4±1.91	55.3±1.93
Mean corpuscular hemoglobin (pg)	18.9±0.38	19.0±0.28	18.5±0.41	18.3±0.57	18.6±0.98	18.4±0.72
Mean corpuscular hemoglobin conc. (g L^{-1})	340±14.59	347±8.58	323±11.69	314±13.20*	324±8.28	333±16.61
Platelet (G L^{-1})	824±78.87	847±87.35	847±66.04	970±54.30*	949±196.9	1055±78.90
Prothrombin time (sec)	16.2±1.05	14.6±0.96	15.6±1.81	15.8±1.23	15.4±0.70	16.3±0.25*
Neutrophil (%)	14.9±4.46	17.8±6.37	16.5±3.42	14.6±4.96	20.3±3.48	24.5±5.29
Lymphocyte (%)	80.2±5.28	78.1±7.06	79.5±4.08	80.7±5.36	74.8±4.86	70.5±5.43
Monocyte (%)	1.9±0.72	1.6±0.38	1.8±0.41	2.1±0.53	1.9±0.73	2.0±0.41
Eosinophil (%)	2.5±1.19	1.9±0.86	1.8±0.81	2.1±1.05	2.8±1.44	2.6±0.27
Large unstained cell (%)	0.3±0.11	0.4±0.22	0.2±0.07	0.2±0.04	0.1±0.11	0.2±0.11
Basophil (%)	0.2±0.13	0.1±0.11	0.2±0.08	0.2±0.04	0.2±0.15	0.2±0.15
Reticulocyte (%)	2.16±0.49	2.26±0.33	2.41±0.19	2.72±0.93	3.34±0.27	2.80±0.94

Values are Mean±SD. *$p \leq 0.05$ vs. control or control recovery group

Clinical Chemistry

Immunol administration for 28 days did not affect the biochemical parameters in males and females at all dose levels as compared to the control (Table 5). However, in females there were slight decrease of sodium in G2 group, trend of dose related increase in the ALP values in the G3 and G4 groups and increase of chloride concentration in G4 group. All the immunol treated groups males exhibited slightly lower Albumin/globulin ratio. In the recovery group males, slight decreases in the cholesterol and triglycerides levels were observed. These changes are marginal and the values are within in the normal range of variation, hence these changes were considered incidental and were considered to be of no biological relevance, hence were not attributed to Immunol treatment.

Urinalysis

The urine specific gravity ranged from 1.010 to 1.020 and the pH was 8 to 9. Incidences of leukocytes, nitrite, proteins, ketone bodies and urobilinogen were observed in control and Immunol treated groups. These incidences are considered as normal observations in rats as per the in-house laboratory reference data. There was no incidence of bilirubin or erythrocytes in any of the group. Urine microscopy did not reveal any significant pathological findings.

Table 5: Effect of immunol on the clinical chemistry upon administration for 28 days

	Groups					
Parameters	G1	G2	G3	G4	G1R	G4R
Males						
Glucose (mmol L^{-1})	7.72±1.38	6.90±0.31	6.58±0.86	7.19±1.38	7.97±1.51	9.35±0.51
Urea Nitrogen (mmol L^{-1})	1.98±0.34	2.20±0.28	2.12±0.40	2.43±0.42	3.21±0.53	3.10±0.47
Total Protein (g L^{-1})	61.7±1.97	64.8±3.09	63.2±2.64	65.5±5.14	61.1±2.23	61.4±1.10
Aspartate amino transferase (U L^{-1})	61±11.30	57±7.01	54±5.08	70±19.51	81±24.68	55±8.17
Alanine amino transferase (U L^{-1})	30±6.76	24±3.46	21±3.00	25±5.12	25±7.73	23±2.95
Alkaine phoshatase (U L^{-1})	91±8.04	81±5.13	76±12.01	90±39.85	80±16.35	76±9.77
Gamma glutamyl transpeptidase (U L^{-1})	0±0.45	0±0.00	0±0.45	1±0.89	0±0.00	0±0.00
Total Bilirubin (mmol L^{-1})	2.25±0.52	1.89±0.58	2.11±0.32	2.09±0.52	2.63±0.38	2.79±0.42
Albumin (A) (g L^{-1})	40.5±1.68	39.4±1.65	40.0±1.40	41.2±5.62	40.3±1.45	41.2±0.61
Globulin (G) (g L^{-1})	21.2±0.58	25.4±3.77	23.2±1.34	24.3±2.04	20.8±1.01	20.2±0.89
A/G ratio	1.9±0.08	1.6±0.23	1.7±0.05	1.7±0.30	1.9±0.05	2.0±0.11
Cholesterol (mmol L^{-1})	1.61±0.23	1.53±0.17	1.35±0.25	1.50±0.28	2.21±0.20	1.90±0.16*
Triglycerides (mmol L^{-1})	0.87±0.33	0.83±0.16	0.77±0.16	0.61±0.08	1.27±0.30	0.77±0.16*
Sodium (mEq L^{-1})	142±13.17	158±10.38	148±1.91	143±12.96	130±6.72	135±3.76
Potassium (mEq L^{-1})	5.08±0.30	5.63±0.34	5.48±0.42	5.26±0.60	5.62±2.34	3.54±0.50
Chloride (mmol L^{-1})	112±21.27	92±10.69	101±1.60	152±77.33	110±4.92	111±2.22
Females						
Glucose (mmol L^{-1})	6.07±0.82	6.19±0.52	5.48±1.04	6.02±0.78	9.12±1.30	7.95±1.84
Urea Nitrogen (mmol L^{-1})	2.66±0.59	2.36±0.34	2.42±0.29	3.28±0.57	3.00±0.42	2.42±0.40
Total Protein (g L^{-1})	68.5±3.96	67.4±3.91	67.7±2.86	71.1±1.37	63.2±4.08	69.9±5.63
Aspartate amino transferase (U L^{-1})	51±6.53	48±2.51	56±10.29	62±8.25	73±19.03	63±10.59
Alanine amino transferase (U L^{-1})	17±3.19	17±4.87	19±1.92	22±3.24	25±5.22	23±2.74
Alkaine phoshatase (U L^{-1})	34±7.89	36±4.93	44±5.93*	48±5.50*	55±16.05	45±15.81
Gamma glutamyl transpeptidase (U L^{-1})	0±0.45	0±0.00	0±0.55	1±0.55	0±0.00	0±0.00
Total Bilirubin (mmol L^{-1})	2.23±0.40	1.94±0.50	1.82±0.30	2.55±0.73	2.63±0.63	3.02±0.34
Albumin (A) (g L^{-1})	44.9±2.42	44.9±3.06	45.3±1.90	46.0±1.03	43.4±4.27	48.4±4.20
Globulin (G) (g L^{-1})	23.6±2.31	22.5±0.98	22.4±2.14	25.1±1.72	19.8±1.52	21.5±1.70
A/G ratio	1.9±0.15	2.0±0.08	2.0±0.21	1.9±0.18	2.2±0.33	2.2±0.15
Cholesterol (mmol L^{-1})	1.42±0.29	1.24±0.10	1.34±0.24	1.50±0.18	1.83±0.35	1.70±0.38
Triglycerides (mmol L^{-1})	0.54±0.10	0.55±0.19	0.41±0.06	0.43±0.05	0.77±0.46	0.68±0.37
Sodium (mEq L^{-1})	160±7.69	145±1.43*	151±6.90	154±9.25	137±3.70	132±14.89
Potassium (mEq L^{-1})	5.85±0.46	5.18±0.13	6.38±2.24	5.47±0.29	4.05±0.35	3.60±0.72
Chloride (mmol L^{-1})	89±9.45	104±3.29	101±10.03	120±19.01*	108±3.12	113±10.22

Values are Mean±SD. *$p \leq 0.05$ vs. control or control recovery group

Gross Pathology, Organ Weights and Organ Weight to Body Weight Ratios and Histopathology

At necropsy, treated groups did not show any gross pathological lesions in any of the organs and no effect on organ weights and their ratios in both the sexes with no significant histopathological or morphological alterations observed in the Immunol treated groups in comparison with the untreated control.

Immunotoxicity Assessment of Immunol upon Administration to Rats for 28 Days

To assess the immunotoxicity potential of Immunol, the parameters such as mortality, body weight, clinical signs, haematology, clinical chemistry profiles, histopathologic examinations of immune associated organs and tissues and organ body weight ratio measurement including thymus and spleen were considered. Immunol treatment for 28 days at any of the doses did not induce any clinical signs such as rales, nasal discharge indicative of respiratory infection or no mortaliy and did not decrease the body weights. Immunol treatment did not affect the total leucocyte count, differential leucocyte count, haematocrit, platelet count, albumin, total proteins and albumin/globulin ratios. Hence, there was no indication of Immunol inducing any immunomodulation, either immunosuppression or immunopotentiation. Immunol treatment did not affect the organ weights or the ratios of thymus and spleen. Adrenal glands, bone marrow, kidney, lung, spleen, thymus, thyroids, mesenteric and axillary lymph nodes showed normal appearance with no pathological or morphological alterations. This indicated that Immunol treatment did not cause histopathological alteration in the immune mediated organs and did not cause immunosuppression or immunopotentiation.

Plaque Forming Cell Assay (PFC)

The hemolytic plaque forming cell assay (PFC) is a measure of the ability to produce humoral immunity following challenge with antigen. PFC assay has been found to be a powerful predictor of immunosuppression. Splenic lymhocytes and the plaque (antibody) forming cells (Table 6) in the all the Immunol treated groups were similar to that of control group indicating that Immunol did not increase the antibody forming cells in the spleen.

Delayed Type Hypersensitivity (DTH) Assay

The delayed type hypersensitivity assay represents an effector function of cell-mediated immunity. The DTH assay assesses cellular proliferation and infiltration in to the site of an active cellular immune reaction, following challenge with an antigen and has been found to be a powerful predictor of immunosuppression. The challenged skin response measurement in high dose Immunol treated male and female showed a significantly increased DTH response when compared to the control group. The mean skin thickness (mm) in high dose Immunol treated group males was 1.8±0.09, 2.0±0.09 mm and 2.0±0.14 mm at 24, 48 and 72 h post challenge and the corresponding scores in the control group were 1.5±0.05,

Table 6: Effect of Immunol on the humoral immunity (plaque forming cell response of splenic lymphocytes to sheep erythrocytes)

	Males			Females		
Groups	Spleen cells ($\times 10^5$) mL^{-1}	No. of plaques/plate	Plaque/10^6 spleen cells	Spleen cells ($\times 10^5$) mL^{-1}	No. of plaques/plate	Plaque/10^6 spleen cells
G1	49±7.5	538±35.8	112±18.4	47±9.0	514±17.0	113±18.8
G2	49±7.4	489±41.6	100±11.9	45±3.8	526±25.7	118±7.3
G3	42±2.0	525±21.4	126±9.1	48±4.2	525±21.2	110±10.5
G4	44±3.2	526±12.9	121±7.5	42±4.2	517±21.2	124±10.5

Table 7: Effect of Immunol on the cellular immunity (delayed hypersensitivity to dinitrochlorbenzene)

	Skin thickness (mm)				
	Sensitization		Challenge		
Groups	Pre-dose	24 h	24 h	48 h	72 h
Males					
G1	1.1±0.05	1.1±0.05	1.5±0.05	1.6±0.13	1.7±0.11
G2	1.2±0.05	1.3±0.15	1.6±0.23	1.7±0.28	1.6±0.15
G3	1.2±0.18	1.2±0.18	1.7±0.15	1.7±0.15	1.7±0.13
G4	1.2±0.07	1.2±0.07	1.8±0.09*	2.0±0.09*	2.0±0.14*
Females					
G1	0.8±0.06	0.8±0.06	1.1±0.19	1.3±0.16	1.3±0.16
G2	0.7±0.06	0.8±0.06	1.0±0.16	1.2±0.14	1.3±0.11
G3	0.8±0.04	0.8±0.04	1.3±0.04	1.3±0.04	1.3±0.08
G4	0.9±0.08	0.9±0.08	1.7±0.11*	1.8±0.00*	1.8±0.00*

Values are Mean±SD. *p×0.05 vs. control group

1.6±0.13 and 1.7±0.11 mm respectively. In high dose females, the mean skin thickness was 1.7±0.11, 1.8±0.00 mm and 1.8±0.00 mm at 24, 48 and 72 h post challenge and the corresponding scores in the control group were 1.1±0.19, 1.3±0.16 and 1.3±0.16 mm, respectively (Table 7). This indicates that Immunol may have a role in cell mediated immune responses at the high dose tested.

DISCUSSION

The present study was performed to assess the toxicity potential of Immunol, a polyherbal formulation in rats. A single dose of 20 mL kg^{-1} body weight of immunol did not induce any clinical sign, mortality, reduction in body weight and gross pathological changes. It shows that the active principles in Immunol are quite safe and may be generally regarded as Safe (GRAS). This is in line with the results obtained by Joshua *et al.* (2008) when they studied the acute oral toxicity potential of a single dose each of five polyherbal preparations in Wistar rats. In all cases, there were no treatment related adverse effects. On the contrary, administration of the pure alkaloid of *Tylophora asthamatica* (one of the constituent of Immunol), suspended in peanut oil and given in single doses (12-100 mg kg^{-1}) by gavage, to male rats caused inactivity, respiratory distress, salivation, nasal discharge and diarrhoea. The oral LD_{50} value of the alkaloid was 35.32 mg kg^{-1} (Malathi and Gomaz, 2008). Medicinal plants are natural resources yielding valuable phytochemical products which are often used in the treatment of various diseases. Crude extracts of these medicinal plants are used in most of the Ayurvedic preparations. The active principles of these medicinal plants are highly efficacious or toxic. This study shows that efficacy or toxicity of the extracts of polyherbal formulations differs from the active principle of a single plant.

Immunol administration at any of the tested doses did not induce any clinical signs, did not affect body weight gain and food consumption and appeared to have a beneficial effect on FCE especially in females. There were no abnormalities in neurological assessment, no adverse effects on haematology, clinical chemistry and urinalysis parameters, no gross or histopathological changes. However, in both sexes, there was a tendency for a marginal reduction in hemoglobin (Hb) and Red Blood Cells (RBC). The Mean Corpuscular Volume (MCV) was also higher with concomitant reduction in Mean Corpuscular Hemoglobin Concentration (MCHC). This appears to be a homeostatic regenerative response during which the higher MCV could be due to higher influx of larger young RBCs and the lower MCHC may be due to these larger cells which have a lower Hb concentration.

Liver being the main detoxification organ of mammals and kidney the major excretory organ, these are very susceptible to the toxicities by drugs. The results from the present study indicate that liver specific enzymes AST, ALT, ALP and bilirubin did not increase drastically. Synthesis of serum proteins (albumin) was not affected. In many liver disorders, serum globulin may rise to such a level so as to maintain normal or increased total protein concentration, even when there is severe albumin depletion. Decreased albumin and elevated globulins in serum indicate hepatocellular damage, jaundice or liver diseases. Similarly, a high level of cholesterol is found in obstructive jaundice or chronic hepatitis of any type. In the present study, no such incidences were seen. Renal parameters were also evaluated in the present study. Kidney eliminates waste products of metabolism from the body. In renal failure, waste products particularly nitrogenous substances like non-protein nitrogen, urea and uric acid accumulate. Creatinine is the least variable nitrogenous constituent of blood. Creatinine is increased in early nephritis and in chronic hemorrhagic nephritis with uremia. In the present study, Immunol did not alter either the urea nitrogen or the creatinine values at any of the doses tested. Aphale *et al*. (1998) after assessing the safety of combination of ginseng and ashvagandha in rats with 90 day oral administration concluded that food consumption, body weight, clinical chemistry and haematology investigation did not reveal any abnormalities. Brain, heart, lung, liver, spleen, kidneys, stomach, testes and ovaries were normal on gross and histopathological examination. Malathi and Gomaz (2008) have reported that treatment of methanolic extract of 300 mg kg^{-1} *Tylophora asthmatica* to rats for 15 days resulted in significant elevation of ALT, ALP and bilirubin values, decrease in total protein, albumin, globulin and A/G ratio, but no changes in the AST values. The extent of liver injury is associated with increased serum levels of AST and ALT. These injuries may be acute or chronic, reversible or irreversible. Of the two enzymes, ALT is thought to be more specific to hepatic injury because it is mainly present in the cytosol of the liver cells and is in low concentrations elsewhere.

Immunol treatment did not alter the total leucocyte count, differential leucocyte count, haematocrit, platelet count, albumin, total proteins and albumin/globulin ratios, organ weights or the ratios of thymus and spleen or the histology of adrenal glands, bone marrow, spleen, thymus, mesenteric and axillary lymph nodes. These findings indicate that Immunol did not cause any immunomodulation, either immunosuppression or immunopotentiation mediated through haemopoietic system or immune-mediated organ system in eunormal rats and also implies that Immunol is quite safe upon repeated treatments and is suitable for sustainable longer treatment periods in immunocompromised individuals. The results of the present study were in disagreement with findings of Daswani and Radha Yegnanarayan (2002) who found an increase in Plaque Forming Cells (PFC) when a polyherbal formulation (Septilin) containing *Balsamodendron mukul*, Shankha bhasma, Maharasnadi quath, *Tinospora cordifolia*, *Rubia cardifolia*, *Emblica officinalis*, *Moringa pterygopserma* and *Glycyrrhiza glabra* was administered to albino mice. The number of lymphocytes per spleen was not altered by either dose of septilin, however the number of plaque forming cells per spleen which is a measure of humoral immunity was increased by both the doses 1.5 and 3.0 g kg^{-1} of septilin. They concluded that Septilin has dual effects on immune system, with lower doses showing greater stimulant and higher doses showing predominantly suppressive effects. Another study by Chattarjee (2001), who studied the effect of Immu-21, a herbal formulation (composing of equal parts of the standardized extracts of *Ocimum sanctum*, *Emblica officinale*, *Tinospora cordifolia* and *Withania somnifera*) observed dose dependent increase in plaque forming cells in mice at the doses of 25, 50 and 100 mg kg^{-1} Immu-21. The difference in the findings of humoral immune response (plaque forming cells)

of the present study and the reported studies may be due to difference in the formulations used and/or the species of animals used in the experiments.

Cell mediated immune response was measured by delayed type hypersensitivity using Dinitrochlorbenzene as the sensitizing agent and the skin thickness was measured at induction and challenge. Immunol at high dose showed significantly increased skin thickness, suggestive of having a role in enhancing cell mediated immune response. Similar results were reported by Sharma and Ray (1997) with septilin in mice. On the contrary, Daswani and Yegnanarayan (2002) have reported that septilin; a polyherbal formulation suppressed the delayed hypersensitivity response to oxazalone. The suppression of DTH response was attributed to anti-inflammatory effect of septilin. Lakadawala *et al*. (1988) have reported that rohitukine, a compound isolated from a plant *Dysoxylum binectariferum* showed anti-inflammatory effects in models of acute inflammation but actually enhanced delayed hypersensitivity to oxazalone. Thus, based on this finding it seems that septilin had inhibitory effect on the cell mediated immunity.

CONCLUSIONS

Immunol administration to wistar rats either a single dose or repeated doses did not cause adverse effects. Immunol has dual effects on the immune system as the humoral immunity was unaffected while the cellular immunity was enhanced on administration of the high dose of 10 mL kg^{-1} for 28 days.

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