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ASSESSMENT OF TOXICOLOGICAL PROFILE OF CELL WALL CONTENTS OF LACTOBACILLUS ACIDOPHILUS (PROBIOTIC) IN WISTAR RATS AND SWISS ALBINO MICE

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ABSTRACT:
The objective of present study was to assess the toxicological profile of the cell wall contents of L.acidophilus by acute (single dose) and subacute (repeated dose) toxicity study. The results of study provide information on target organs, possibilities of accumulation and estimation of No Observed Adverse Effect Level (NOAEL), which can be useful in establishing safety criteria for human exposure. Toxicity studies were carried out in rats and mice of each sex by subcutaneous administration. Acute study was carried out by subcutaneous administration of single high dose of cell wall contents of L.acidophilus obtained from 10^{12} CFU/mL. Subacute toxicity study was carried out by repeated administration of cell wall contents of L. acidophilus obtained from 10^{6}, 10^{9} and 10^{12} CFU/mL for 28 days in each sex of rats and mice. Signs and symptoms of toxicity were observed periodically. Physio-dynamic parameters viz. change in body weight; food intake and water intake were recorded weekly. After completion of study, animals were sacrificed; their hematological and biochemical parameters were estimated and gross morphology with histopathology of vital organs was done. Cell wall contents of L.acidophilus did not show any mortality and signs of toxicity; moreover, no significant changes in hematological, biochemical and histopathological parameters were observed. In conclusion, cell wall contents of L.acidophilus studied at highest dose was found to be nontoxic.

Keywords: Acute and Subacute Toxicity, Cell wall contents, L.acidophilus, Histopathology, Hematological and biochemical parameters.

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INTRODUCTION

Probiotic based microorganisms have been subject of research since long time because of their popularity, effectiveness and safe remedies against variety of diseases and are being used widely as food supplements and nutraceuticals. Probiotics consist of bacteria or yeasts and can be considered as functional foods that can re-colonize and restore the microflora symbiosis of the intestinal tract. Several health benefits associated with the probiotics in various diseases includes inflammatory bowel disease (IBD), colon cancer, rotavirus-associated diarrhoea, H.pylori infection and liver disease1. The rationale behind probiotic use is to elevate the endogenous numbers of beneficial bacterial strains including lactobacillus and bifidobacterium2. This increase will impart the beneficial effects seen by probiotic administration, including an increase in fatty acid production, particularly butyrate, which can provide fuel for enterocytes, prevent pathogenic adherence and production of anti-bacterial substances therefore decrease in luminal pH3. The most commonly used probiotics are Lactobacilli and Bifidobacterium. Examples of Lactobacillus species include L. acidophilus, L. fermentum, L. jhonsonii, L. plantarum, L. rhamnosus and Bifidobacterium species include B. bifidum, B. breves, B. lactis, B. longum4. A set of lactobacillus species were shown to suppress

transcription of IL-1β, TNF-α, NF-κB, as well as the translation of IL-1β and IL-6 in experimental colitis and other digestive disorders in rats5. Lactobacillus species regulate immune responses by enhancing innate immunity & modulating pathogen induced inflammation6. Additionally, cell wall contents (Lipoteichoic acid, Peptidoglycan and techoic acid) of Lactobacilli have been reported to inflammation in animal models of experimental colitis7. Other mechanisms of probiotics include immunomodulation, enhancement of barrier function & anti-microbial activity. It is interesting to note that spores of Lactobacillus acidophilus has been tested in the IBD8 as well as the we have also tested the relevance of inhibition by **"cell wall contents of lactobacillus acidophilus"** in our laboratory using experimental model of colitis by Chauhan and Chorawala9. However, the toxicity study of cell wall contents of such probiotic has never been done. Therefore, we made an attempt to assess the possible health hazards likely to arise from repeated exposure over a relatively limited period of time. The results of this study should provide information on target organs for establishing safety criteria for human exposure.

MATERIALS AND METHODS

Experimental animals: Healthy Male and female Wistar rats weighing 180-220 gm and healthy male and female Swiss albino mice weighing 20-25 gm were used for the present study (Schedule Y, 2005). The experimental protocol (KBIPER/2011/287) of present study was approved by Institutional Animal Ethical Committee under the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India before carrying out the experiment. All animals were housed in polypropylene cage (6 rats per cage) under controlled conditions of temperature (22 ± 2°C), humidity (55 ± 5%) and 12hrs/12hrs light-dark cycle. Animals were acclimatized for one week prior to experiment. Animals had free access to conventional laboratory diet (normal pellet diet) (Pranav Agro, Baroda) and water ad libitum.

Preparation of test item or isolation of cell wall contents of L.acidophilus: The method described by Roberson & Cromatte, 1962 was used with slight modification10. Briefly, 20gm of bacteria (by weighing wet colonies) was suspended in 350ml of hot water (65-680C). To that, 350ml of 90% phenol (65-680c) was added and stirred for 1hr.at 65-680C. Then, it was cooled in an ice bath to 2-80C, or left overnight in refrigerator. Then, it was centrifuged at 6000-7000rpm for 45min. Upper water layer was preserved (hot phase) and residual phenol & interphase was further treated, if required, with equal volume of hot water and preceded as described above. The phenol layer consists of lipids and insoluble residue of cell wall proteins whereas, aqueous phase consist of Lipoteichoic acid, Lipoic acid, Polysaccharides, amino acids, Teichoic acid and Peptidoglycans etc. The aqueous phase was used for treatment purpose.

Study design

Acute toxicity study: The selected animals were randomly divided into two groups containing minimum 10 animals per group, each 5 males and 5 females for rats and mice as summarized in table 1. The animals were fasted overnight and single dose of cell wall contents of L.acidophilus obtained from 1012 CFU/mL was administered subcutaneously to group IIA and IIB of rats and mice. Group IA and IB of rats and mice were served as vehicle control and received WFI subcutaneously. Animals were observed individually after dosing for a total of 14 days to assess any clinical sign of toxicity and mortality.

Table 1: Grouping of Animals for Acute Toxicity Study

Species	Group No.	Sex	Treatments	No. of animals / group
Rat	IA	Male	vehicle, s.c.	05
	IB	Female	vehicle, s.c.	05
	IIA	Male	Cell wall contents of Lactobacillus acidophilus (10^{12} CFU/animal, s.c.)	05
	IIB	Female	Cell wall contents of Lactobacillus acidophilus (10^{12} CFU/animal, s.c.)	05
Mice	IA	Male	vehicle, s.c.	05
	IB	Female	vehicle, s.c.	05
	IIA	Male	Cell wall contents of Lactobacillus acidophilus (10^{12} CFU/animal, s.c.)	05
	IIB	Female	Cell wall contents of Lactobacillus acidophilus (10^{12} CFU/animal, s.c.)	05

Cage side observation: All animals were observed daily twice for clinical signs and mortality, throughout the experimentation period. Symptoms like changes in skin, fur, eyes and mucous membranes, occurrence of secretion and excretions were also examined. Autonomic activity like lacrimation, piloerection, pupil size and unusual respiratory pattern were also monitored. Changes in gait, posture, response to handling, presence of clonic or tonic movements, stereotypes like excessive grooming and repetitive circling or bizarre behavior like self-mutilation, walking backward were also examined.

Changes in body weight, food intake and water intake: Any changes in body weight, food and water intake were recorded weekly.

Gross morphology: At the end of observation period, all the male and female animals from groups I to II were euthanized and gross morphological examination of vital organs (lungs, liver, kidney, heart, spleen, brain, stomach, testis, uterine horn) was performed.

Subacute toxicity study: One day before the initiation of treatment, the selected animals were randomly divided into four different groups containing minimum 12 animals per group, each of 6 males and 6 females for rats and similar for mice as summarized in table 2.

The cell wall contents of L. acidophilus was administered once daily for 28 days by subcutaneous route. Toxic manifestation (diarrhea, tremor, salivation, convulsion, changes in color of eyes, skin or fur, lethargy, sleep etc…), behavioral changes and mortality were monitored daily, while changes in body weight, food intake and water intake were observed weekly. At the end of study period, animals were fasted for 12 hrs and blood samples were collected from all animals under anesthetic ether. The blood samples were collected by cardiac puncture method, transferred into 1.5 mL capacity microcentrifuge tube containing sodium citrate solution as an anti-coagulant and clinically evaluated for hematological and biochemical parameters. After blood collection, all the animals were sacrificed; vital organs (lungs, liver, heart and kidney) were removed, observed for gross morphology, freed of extraneous material, weighed and preserved in 10% buffered formalin for histopathology.

Changes in body weight, food intake and water intake: Body weights were measured before the treatment and weekly thereafter, and on the day of sacrifice. Similarly food and water intake were recorded weekly and on the day of sacrifice.

Hematological studies: All the animals were fasted overnight prior to blood collection. Blood samples were collected by cardiac puncture under anesthetic ether into 1.5 mL capacity microcentrifuge tube containing sodium citrate as an anti-coagulant and clinically evaluated for hematological parameters. After that blood samples were centrifuged at 4000 RPM at 4°C for 10 mins to obtain plasma for biochemical analysis. Various hematological parameters (Hb%, Total RBC, Total WBC and Differential WBC) were determined by standard clinical procedure using automatic hematological analyzer (Roches Integra, 400 Plus, Diagnostic system).

Biochemical studies: Plasma samples obtained after centrifugation were used to estimate biochemical parameters such as glucose, total cholesterol, triglyceride, albumin, total protein, SGPT, SGOT, alkaline phosphatase, total bilirubin, creatinine and urea. Biochemistry was done with commercially available standard kit of Span diagnostic limited, India using an automated biochemical analyzer (Reflotron plus, Roches, USA).

Gross morphology, organ weight and histopathology: After blood collection, all the animals were sacrificed; vital organs (lungs, liver, heart and kidney) were removed, observed for gross morphology, freed of extraneous material, weighed and preserved in 10% buffered formalin for histopathology. Standard histological procedures were followed to observe any microscopic changes in any above mentioned organs.

Organ weight to body weight ratio: Organ weight to body weight ratio was calculated using following formula:

Ratio = organ weight/body weight of animal

STATISTICAL ANALYSIS

Numerical data were expressed as mean ± SEM of six observations. Differences between the groups were analyzed using analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test and student unpaired t-test. Minimum criteria for statistical significant was set at p less than 5% ($p<0.05$) for all the comparisons.

RESULTS

Acute toxicity study: No mortality and morbidity or any signs of behavior changes or toxicity were observed throughout 14 days of study period after single subcutaneous administration of cell wall contents obtained from 10^{12} CFU/mL to rats and mice. Morphological characteristics (fur, eye, skin, nose, tongue) appeared normal. No tremors, convulsion, salivation, lethargy, diarrhoea or unusual behaviors such as self mutilation, walking backward, circling behavior and stereotype behavior were observed; gait and posture, response to handling or sensory stimuli and grip strength were normal. There were no significant changes in body weight, food and water intake between control and treatment groups (not mentioned).

Subacute toxicity study: The animals were healthy with no difference being noted with respect to control group. No significant changes were observed in body weight, food and water intake of repeatedly treated group as compared to vehicle control group (table 3a, 3b, 3c, 3d, 3e, 3f)) and no mortality was observed during entire toxicity study period. The weight of vital organs (lungs, liver, kidney, heart) was not significantly altered by cell wall contents of L.acidophilus as compared to vehicle control group (Table 4a, 4b).

Table 2: Grouping of Animals For Sub-Acute Toxicity Study

Species	Group No.	Sex	Treatments	No. of animals per group
Rat	IA	Male	vehicle, s.c.	06
	IB	Female	vehicle, s.c.	06
	IIA	Male	Cell wall contents of Lactobacillus acidophilus (10^6 CFU/animal, s.c.)	06
	IIB	Female	Cell wall contents of Lactobacillus acidophilus (10^6 CFU/animal, s.c.)	06
	IIIA	Male	Cell wall contents of Lactobacillus acidophilus (10^9 CFU/animal, s.c.)	06
	IIIB	Female	Cell wall contents of Lactobacillus acidophilus (10^9 CFU/animal, s.c.)	06
	IVA	Male	Cell wall contents of Lactobacillus acidophilus (10^{12} CFU/animal, s.c.)	06
	IVB	Female	Cell wall contents of Lactobacillus acidophilus (10^{12} CFU/animal, s.c.)	06
Mice	IA	Male	vehicle, s.c.	06
	IB	Female	vehicle, s.c.	06
	IIA	Male	Cell wall contents of Lactobacillus acidophilus (10^6 CFU/animal, s.c.)	06
	IIB	Female	Cell wall contents of Lactobacillus acidophilus (10^6 CFU/animal, s.c.)	06
	IIIA	Male	Cell wall contents of Lactobacillus acidophilus (10^9 CFU/animal, s.c.)	06
	IIIB	Female	Cell wall contents of Lactobacillus acidophilus (10^9 CFU/animal, s.c.)	06
	IVA	Male	Cell wall contents of Lactobacillus acidophilus (10^{12} CFU/animal, s.c.)	06
	IVB	Female	Cell wall contents of Lactobacillus acidophilus (10^{12} CFU/animal, s.c.)	06

TABLE 3(a): Effect of cell wall contents of L.acidophilus on body weight in rats.

SEX	GROUPS	BODY WEIGHT IN GMS (WEEKLY)				
		DAY 0	DAY 7	DAY 14	DAY 21	DAY 28
MALE	GROUP IA	260.67±7.41	259.67±9.04	257.67±9.01	261.5±8.06	275.5±9.05
	GROUP IIA	270±4.88	259±7.76	258.83±8.08	263.17±8.16	268.33±8.35
	GROUP IIIA	267.83±10.77	263.67±15.46	271.83±14.12	271.33±16.05	275.5±9.05
	GROUP IVA	317.17±4.95	317.33±5.08	324.33±8.33	337.5±8.92	346.83±10.94
FEMALE	GROUP IB	304±10.77	301±10.96	307.67±10.65	324.33±11.96	312.5±12.42
	GROUP IIB	338.67±1.84	319.5±2.06	331.33±5.23	337±5.68	340.33±3.59
	GROUP IIIB	280.17±3.13	275.5±3.33	284±4.64	293.5±3.36	297.5±4.25
	GROUP IVB	251.67±3.57	256±6.59	261.67±6.01	266.17±3.9	270±2.58

Each observation represents value in mean ± SEM, n=6. No significant difference between group I, II, III and IV during entire study period.

TABLE 3(b): Effect of cell wall contents of L.acidophilus on food consumption in rats.

SEX	GROUPS	FOOD CONSUMPTION IN GMS (WEEKLY)				
		DAY 0	DAY 7	DAY 14	DAY 21	DAY 28
MALE	GROUP IA	125	135	132	108	117
	GROUP IIA	136	142	127	127	134
	GROUP IIIA	111	118	101	134	98
	GROUP IVA	142	137	125	149	136
FEMALE	GROUP IB	132	118	121	131	112
	GROUP IIB	127	127	113	105	108
	GROUP IIIB	109	116	114	105	95
	GROUP IVB	118	127	126	136	114

TABLE 3(c): Effect of cell wall contents of L.acidophilus on water intake in rats.

SEX	GROUPS	WATER INTAKE IN mL (WEEKLY)				
		DAY 0	DAY 7	DAY 14	DAY 21	DAY 28
MALE	GROUP IA	86	92	75	104	96
	GROUP IIA	95	112	87	113	92
	GROUP IIIA	104	112	98	107	108
	GROUP IVA	89	91	98	94	96
FEMALE	GROUP IB	78	82	77	90	85
	GROUP IIB	74	78	65	71	81
	GROUP IIIB	85	82	89	92	96
	GROUP IVB	92	98	94	83	92

TABLE 3(d): Effect of cell wall contents of L.acidophilus on body weight in mice.

SEX	GROUPS	BODY WEIGHT IN GMS (WEEKLY)				
		DAY 0	DAY 7	DAY 14	DAY 21	DAY 28
MALE	GROUP IA	36.5±1.48	36.83±1.7	35.67±1.98	36.67±1.63	37±1.32
	GROUP IIA	37.5±2.11	36.83±2.12	35±2.38	36.17±1.92	37.33±1.82
	GROUP IIIA	38.67±1.93	35.67±1.82	38.5±1.67	39.33±1.58	38.67±1.41
	GROUP IVA	45.83±1.3	44.33±0.88	48.5±1.73	48.17±1.19	48.83±1.56
FEMALE	GROUP IB	44.17±0.95	46.83±1.22	45.83±1.62	46±1.48	46.33±1.74
	GROUP IIB	42.17±2.23	38.83±2.32	41.5±2.19	42.83±2.1	42.17±1.85
	GROUP IIIB	35.83±0.83	34.17±0.83	35±1.83	34.17±1.54	35.83±0.83
	GROUP IVB	32.5±2.14	30±2.58	31.67±3.07	34.17±1.54	35.83±0.83

Each observation represents value in mean ± SEM, n=6. No significant difference between group I, II, III and IV during entire study period.

TABLE 3(e): Effect of cell wall contents of L.acidophilus on food consumption in mice.

SEX	GROUPS	FOOD CONSUMPTION IN GMS (WEEKLY)				
		DAY 0	DAY 7	DAY 14	DAY 21	DAY 28
MALE	GROUP IA	35	36	35	35	36
	GROUP IIA	34	34	34	36	36
	GROUP IIIA	40	37	38	37	38
	GROUP IVA	42	45	44	44	43
FEMALE	GROUP IB	35	35	33	36	35
	GROUP IIB	33	34	30	32	34
	GROUP IIIB	37	36	36	36	38
	GROUP IVB	29	29	27	29	30

TABLE 3(f): Effect of cell wall contents of L.acidophilus on water intake in mice.

SEX	GROUPS	WATER INTAKE IN mL (WEEKLY)				
		DAY 0	DAY 7	DAY 14	DAY 21	DAY 28
MALE	GROUP IA	42	37	40	40	39
	GROUP IIA	45	43	43	44	44
	GROUP IIIA	42	38	42	42	43
	GROUP IVA	48	45	48	50	48
FEMALE	GROUP IB	32	29	32	35	35
	GROUP IIB	44	41	44	46	44
	GROUP IIIB	47	50	40	40	39
	GROUP IVB	47	51	51	49	51

TABLE 4(a): Effect of cell wall contents of L.acidophilus on organ weight in rats

SEX	GROUPS	ORGAN WEIGHT IN GMS					
		LIVER	KIDNEY	HEART	SPLEEN	BRAIN	LUNGS
MALE	GROUP IA	6.67±0.17	2.34±0.11	1.26±0.04	1.03±0.05	1.78±0.06	2.18±0.08
	GROUP IIA	6.54±0.18	2.29±0.09	1.22±0.04	1.06±0.05	1.7±0.04	2.33±0.04
	GROUP IIIA	6.76±0.08	2.42±0.04	1.31±0.06	1.02±0.04	1.74±0.05	2.2±0.06
	GROUP IVA	6.48±0.09	2.555±0.1	1.23±0.09	1.04±0.06	1.67±0.13	2.12±0.06
FEMALE	GROUP IB	5.21±0.33	1.53±0.07	0.79±0.05	0.56±0.05	1.38±0.07	1.66±0.09
	GROUP IIB	5.14±0.1	1.48±0.09	0.74±0.02	0.58±0.02	1.42±0.08	1.55±0.08
	GROUP IIIB	5.32±0.26	1.42±0.03	0.78±0.04	0.64±0.03	1.28±0.05	1.65±0.05
	GROUP IVB	5.04±0.29	1.51±0.09	0.71±0.03	0.53±0.04	1.31±0.04	1.57±0.08

Each observation represents value in mean, n=6. No significant difference between group I, II, III and IV during entire study period.

TABLE 4(b): Effect of cell wall contents of L.acidophilus on organ weight in mice

SEX	GROUPS	ORGAN WEIGHT IN GMS					
		LIVER	KIDNEY	HEART	SPLEEN	BRAIN	LUNGS
MALE	GROUP IA	1.78±0.17	0.59±0.05	0.22±0.02	0.12±0.02	0.43±0.01	0.27±0.02
	GROUP IIA	1.53±0.08	0.53±0.04	0.2±0.02	0.12±0.01	0.44±0.02	0.21±0.01
	GROUP IIIA	1.59±0.13	0.52±0.04	0.2±0.01	0.11±0.01	0.41±0.02	0.22±0.01
	GROUP IVA	1.61±0.12	0.58±0.03	0.27±0.02	0.12±0.02	0.38±0.03	0.23±0.01
FEMALE	GROUP IB	1.89±0.05	0.53±0.02	0.23±0.02	0.12±0.02	0.35±0.03	0.27±0.01
	GROUP IIB	1.68±0.07	0.46±0.03	0.23±0.02	0.11±0.01	0.33±0.01	0.25±0.02
	GROUP IIIB	1.72±0.1	0.48±0.03	0.19±0.01	0.12±0.01	0.34±0.03	0.27±0.01
	GROUP IVB	1.84±0.06	0.54±0.04	0.24±0.01	0.11±0.02	0.31±0.01	0.24±0.02

Each observation represents value in mean, n=6. No significant difference between group I, II, III and IV during entire study period.

TABLE 5(a): Effect of cell wall contents of L. acidophilus on Hb %, Total RBC and Total WBC in rats

SEX	GROUPS	HAEMATOLOGICAL PARAMETERS		
		HAEMOGLOBIN (gm/dL)	TOTAL RBC (COUNT x 10^6 /cmm)	TOTAL WBC COUNT (CELLS*10^3/cmm)
MALE	GROUP IA	15±1.04	5.97±0.54	6.53±0.85
	GROUP IIA	16.8±0.37	6.76±0.27	4.79±0.35
	GROUP IIIA	15.3±0.81	6.4±0.67	7.32±0.7
	GROUP IVA	13.6±1.1	6.68±0.53	5.9±0.41
FEMALE	GROUP IB	14.6±1.18	7.02±0.22	5.76±0.46
	GROUP IIB	16.4±0.47	5.84±0.43	5.28±0.36
	GROUP IIIB	17.8±0.69	7.88±0.45	6.84±0.75
	GROUP IVB	14.3±0.74	7.57±0.24	7.49±0.94

Each observation represents value in mean, n=6. No significant difference between group I, II, III and IV during entire study period.

TABLE 5(b): Effect of cell wall contents of L.acidophilus on differential WBC in rats

SEX	GROUPS	DIFFERENTIAL WBC				
		LYMPHOCYTES	MONOCYTES	NEUTROPHILS	EOSINOPHILS	BASOPHILS
MALE	GROUP IA	71.4±3.48	20.4±4.3	7.4±1.1	0.8±0.34	0±0
	GROUP IIA	68.6±4.91	19.8±2.03	11.2±4	0.4±0.22	0±0
	GROUP IIIA	67.2±3.41	21.4±2.66	11±1.26	0.4±0.22	0±0
	GROUP IVA	69.4±2.72	14.6±1.18	15.6±1.93	0.4±0.22	0±0
FEMALE	GROUP IB	60±0.82	18.6±1.1	21±1.15	0.4±0.22	0.2±0.18
	GROUP IIB	66.4±2.98	14.8±1.43	17.6±2.75	1±0	0.2±0.18
	GROUP IIIB	68.33±1.17	22.33±1.58	8±0.63	1.33±0.33	0±0
	GROUP IVB	69.67±2.64	23±3.17	6.17±0.83	1.17±0.31	0±0

Each observation represents value in mean, n=6. No significant difference between group I, II, III and IV during entire study period.

TABLE 5(c): Effect of cell wall contents of L.acidophilus on Hb %, Total RBC and Total WBC in mice

SEX	GROUPS	HAEMATOLOGICAL PARAMETERS		
		HAEMOGLOBIN (gm/dL)	TOTAL RBC (COUNT x 10^6 /cmm)	TOTAL WBC COUNT (CELLS*10^3/cmm)
MALE	GROUP IA	16.4±0.27	7.87±1.2	5.3±0.47
	GROUP IIA	16.4±0.85	7.44±0.76	4.37±0.53
	GROUP IIIA	16.3±0.4	7.24±0.93	7.3±1.08
	GROUP IVA	18.4±1.2	9.79±1.42	6.44±0.47
FEMALE	GROUP IB	17.8±0.97	8.35±0.76	5.82±0.9
	GROUP IIB	17±0.75	7.18±0.53	7.65±0.97
	GROUP IIIB	10.67±1.22	8.49±0.33	7.15.5±0.36
	GROUP IVB	12.67±0.36	7.86±0.23	8.22.17±0.13

Each observation represents value in mean, n=6. No significant difference between group I, II, III and IV during entire study period.

TABLE 5(d): Effect of cell wall contents of L.acidophilus on differential WBC in mice

SEX	GROUPS	DIFFERENTIAL WBC				
		LYMPHOCYTES	MONOCYTES	NEUTROPHILS	EOSINOPHILS	BASOPHILS
MALE	GROUP IA	52.2±5.02	23±3.87	24.8±2.91	0±0	0±0
	GROUP IIA	69.2±4.93	10.8±2.64	19.8±2.4	0.2±0.18	0±0
	GROUP IIIA	67.6±3.7	14.8±3.73	17.6±0.94	0±0	0±0
	GROUP IVA	63.2±2.62	12.6±1.82	24.2±1.54	0±0	0±0
FEMALE	GROUP IB	77.2±3.62	4.8±1.06	17.8±3.06	0.2±0.18	0±0
	GROUP IIB	70.8±3.62	7.4±1.86	21.8±2.26	0±0	0±0
	GROUP IIIB	67.17±2.55	7±1.15	25±1.95	0.83±0.4	0±0
	GROUP IVB	71.67±1.89	6±1.44	21.5±1.38	0.83±0.31	0±0

Each observation represents value in mean, n=6. No significant difference between group I, II, III and IV during entire study period.

TABLE 6(a): Effect of cell wall contents of L.acidophilus on various biochemical parameters in rats

SEX	GROUPS	BIOCHEMICAL PARAMETERS IN SERUM				
		GLUCOSE (mg/dL)	TOTAL CHOLESTEROL (mg/dL)	TRIGLYCERIDE (mg/dL)	ALBUMIN (gm/dL)	TOTAL PROTEIN (gm/dL)
MALE	GROUP IA	76.75±5.38	102.57±2.33	117.05±4.85	3.58±0.17	4.43±0.31
	GROUP IIA	79.35±6.65	82.36±2.75	96.37±3.06	3.46±0.21	3.96±0.25
	GROUP IIIA	69.93±3.73	100.25±1.9	106.94±4.26	3.26±0.19	4.08±0.25
	GROUP IVA	80.81±4.28	89.08±1.56	94.98±3.9	3.21±0.24	4.63±0.41
FEMALE	GROUP IB	81.52±5.77	99.31±3.16	100.23±3.95	2.18±0.34	5.06±0.28
	GROUP IIB	84±6.66	102.07±2.06	102.31±3.86	2.23±0.31	4.34±0.21
	GROUP IIIB	81.85±4.44	104.02±2.42	89.35±2.71	1.96±0.08	4.97±0.17
	GROUP IVB	73.17±4.49	96.86±3.63	112.96±2.55	2.38±0.24	4.42±0.08

Each observation represents value in mean, n=6. No significant difference between group I, II, III and IV during entire study period.

TABLE 6(b): Effect of cell wall contents of L.acidophilus on various biochemical parameters in rats

Sex	Groups	Biochemical parameters in serum					
		SGPT activity (IU/l)	SGOT activity (IU /l)	Alkaline phosphatase (IU /l)	Total bilirubin (mg/dl)	Creatinine (mg/dl)	Urea (mg/dl)
Male	Group Ia	44.9±1.96	29.87±2.38	51.23±7.55	0.53±0.06	0.91±0.16	18.56±1.8
	Group IIa	25.57±1.15	23.87±4.24	45.2±4.62	0.56±0.05	0.93±0.23	17.05±1.74
	Group IIIa	36.97±4.82	35.16±3.12	44.6±4.19	0.53±0.08	0.97±0.14	16.29±1.23
	Group IVa	30.56±3.16	24.15±3.28	30.13±3.19	0.54±0.06	0.6±0.05	16.29±2.23
Female	Group Ib	41.95±5.59	26.02±4.67	64.79±5.93	0.37±0.03	0.66±0.11	16.67±1.52
	Group iib	39.94±7.76	32.88±2.69	65.99±5.04	0.46±0.08	0.67±0.07	12.12±2.25
	Group iiib	21.61±2.73	36.32±3.64	45.8±10.18	0.43±0.07	0.67±0.12	10.61±0.96
	Group ivb	23.18±1.96	27.08±2.09	64.18±4.85	0.47±0.06	0.73±0.12	8.71±1.6

Each observation represents value in mean, n=6. No significant difference between group I, II, III and IV during entire study period.

TABLE 6(c): Effect of cell wall contents of L.acidophilus on various biochemical parameters in mice

SEX	GROUPS	BIOCHEMICAL PARAMETERS IN SERUM				
		GLUCOSE (mg/dL)	TOTAL CHOLESTEROL (mg/dL)	TRIGLYCERIDE (mg/dL)	ALBUMIN (gm/dL)	TOTAL PROTEIN (gm/dL)
MALE	GROUP IA	90.38±5.81	106.36±4.9	106.16±3.67	3.31±0.44	3.69±0.43
	GROUP IIA	72.91±4.93	115.89±6.63	109.23±4.24	2.68±0.32	3.44±0.18
	GROUP IIIA	91.89±7.21	116.8±8.22	115.85±5.8	2.79±0.23	3.57±0.43
	GROUP IVA	70.67±3.66	103.18±4.76	110.98±4.13	3.08±0.41	3.64±0.29
FEMALE	GROUP IB	91.15±7.05	120.16±7.24	115.39±4.5	2.21±0.26	4.28±0.21
	GROUP IIB	86.3±5.13	107.9±4.82	107.38±6.31	3.18±0.37	3.76±0.12
	GROUP IIIB	82.76±7.03	109.17±5.69	109.18±3.13	3.02±0.2	3.29±0.13
	GROUP IVB	81.77±5.29	110.54±4.97	113.18±5.98	3.32±0.27	3.54±0.06

Each observation represents value in mean, n=6. No significant difference between group I, II, III and IV during entire study period

TABLE 6(d): Effect of cell wall contents of L.acidophilus on various biochemical parameters in mice

SEX	GROUPS	BIOCHEMICAL PARAMETERS IN SERUM					
		SGPT ACTIVITY (IU/L)	SGOT ACTIVITY (IU/L)	ALKALINE PHOSPHATASE (IU/L)	TOTAL BILIRUBIN (mg/dL)	CREATININE (mg/dL)	UREA (mg/dL)
MALE	GROUP IA	31.28±6.63	28.61±2.27	57.86±3.49	0.44±0.06	0.48±0.08	15.8±2.88
	GROUP IIA	22.18±6.65	25.67±4.34	45.69±4.71	0.42±0.04	0.43±0.1	13.53±0.91
	GROUP IIIA	34.73±2.28	31.6±10.17	47.01±3.17	0.45±0.1	0.26±0.05	14.02±3.78
	GROUP IVA	36.65±4.84	39.27±1.85	50.87±4.37	0.39±0.06	0.43±0.16	14.67±3.44
FEMALE	GROUP IB	16.85±7.89	32.46±3.16	63.57±1.8	0.38±0.04	0.85±0.1	20.7±2.4
	GROUP IIB	27.89±6.88	27.74±7.16	52±2.52	0.29±0.03	0.78±0.04	22.35±3.29
	GROUP IIIB	18.66±3.74	23.38±3.91	64.16±3.02	0.32±0.04	0.32±0.15	19.91±3.05
	GROUP IVB	20.23±3.15	21.61±3.17	61.61±3.74	0.35±0.03	0.38±0.14	21.39±1.92

Each observation represents value in mean, n=6. No significant difference between group I, II, III and IV during entire study period.

Hematological studies: The effect of repeated dose of subcutaneously administered cell wall contents on hematological parameters is presented in table (5a, 5b, 5c, 5d). Hematological analysis showed no significant changes in test item groups as compared to control groups.

Biochemical studies: The effect of repeated dose of cell wall contents on biochemical markers (glucose, total cholesterol, triglyceride, albumin, total protein, SGPT, SGOT, alkaline phosphatase, total bilirubin, creatinine and urea) is summarized in table (6a, 6b, 6c, 6d). Results show that there were no significant changes in biochemical markers values of treated animals as compared to vehicle control group animals.

Histopathology: No abnormalities were detected in pathological examinations of tissues during microscopic examination of vital organs in comparative histology of tissues of control and test animals. Treatment of cell wall contents of L.acidophilus did not affect the histology of vital organs, viz., lungs, liver, kidney and heart.

DISCUSSION

In acute toxicity study, no mortality was observed at highest dose of cell wall contents of L.acidophilus obtained from 10^{12} CFU/mL after single dose administration in rats and mice. The changes in body weight have been used as a marker of adverse effect of test item[11]. Since no remarkable changes were observed in animal behavior, body weight, food and water intake at highest dose level in treated animals as compared to control groups, it can be inferred that cell wall contents of L. acidophilus is non-toxic at the dose administered. Similar results were also observed in subacute toxicity study. Further, data analyses animals blood parameters can be translated for risk evaluation in human, since changes in hematological system have a higher predictive value for human toxicity[12,13]. Subacute toxicity studies conducted in our laboratory also showed no significant changes in hematological parameters between control and tested item groups. There was a transient increase in total WBC counts. An increase in WBC counts may indicate impact of cell wall contents of L.acidophilus on immune system of treated groups. The results indicate that cell wall contents of L.acidophilus are neither toxic to circulating RBC nor it interferes with their production.

GPT, GOT, albumin and total bilirubin are generally used as markers of liver damage[12,13]. No significant changes were found in level of GPT, GOT, albumin and total bilirubin post cell wall contents administration. Therefore, cell wall contents of L.acidophilus did not provoke any detrimental effect on liver. Moreover, activity of alkaline phosphatase enzymes in addition to levels of creatinine and urea were found normal suggest no toxic effect exerted on repeated administration of cell wall contents of L.acidophilus. The non-toxicity of cell wall contents of L.acidophilus on specific organ was further confirmed by histopathological assessment. Histopathological examination of selected vital organs (lungs, liver, kidney and heart) from both treated and control animals showed normal architecture, suggesting no microscopic changes and morphological disturbances were caused due to subcutaneous administration of cell wall contents of L.acidophilus at all dose levels.

CONCLUSION

The results strongly suggest that the cell wall contents of L.acidophilus is safe and well tolerated at tested subcutaneous doses since no deleterious changes were observed in animal macro-parameters, behavior, hematological and biochemical parameters and histopathology. Further, the isolated cell wall contents of L.acidophilus were found to be nontoxic in acute and

repeated dose toxicity studies. Animal toxicity study along with efficacy studies of cell wall contents of L.acidophilus conducted in our laboratory have shown very encouraging results, suggesting a long term, therapeutic/nutritive potential of cell wall contents of L. acidophilus.

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