Research Article

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SAFETY EVALUATION IN ICR MICE OF LINK LIVECARE™: A HEPATOPROTECTIVE POLYHERBAL FORMULATION

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ABSTRACT

Link livecare™ is a polyherbal formulation consisting of fourteen medicinal plants. The hepatoprotective activity of an extract of Link livecare™ against carbon tetrachloride and galactosamine induced hepatotoxicity in mice has been reported previously with an optimal dose of 80 mg/kg body weight. Acute, sub-acute and sub-chronic oral toxicity of Link livecare™ were studied in mice, in accordance with OECD procedures utilizing a range of histological, biochemical and haematological parameters. No toxic effects were observed at a single dose at 2000 mg/kg body weight or with daily doses ranging from 80 mg/kg body weight to 400 mg/kg for periods of fourteen days and ninety days. These results support the safety claims for Link livecare™.

Keywords: Link livecare™, polyherbal hepatoprotective formulation, safety evaluation, acute toxicity, sub-acute toxicity, sub-chronic toxicity.

INTRODUCTION

Link livecare™ (LLC) is a polyherbal formulation consisting of fourteen medicinal plants, *Andrographis paniculata, Eclipta Alba, Phyllanthus amarus, Phyllanthus emblica , Terminalia chebula ,Terminalia bellerica, Tinospora cordifolia, Curcuma longa, Glycyrrhiza glabra, Boerhavia diffusa , Osbeckia octandra, Tephrosia purpurea, Piper longum* and *Vernonia cinerea*. The hepatoprotective activity of LLC against carbon tetrachloride and galactosamine induced hepatotoxicity has been reported previously[1]. There is however no published safety data available on the product.

Single dose (acute toxicity) test and repeated dose (sub-acute and sub-chronic toxicity) tests are some of the commonly performed tests in order to evaluate the safety of a particular substance[2,3]. The acute toxicity test is the first test to be conducted in the evaluation of safety. The aim of the acute toxicity test is to determine the ratio between the lethal dose and the pharmacologically effective dose (the therapeutic index). Repeated dose toxicity studies are carried out to find out the side effects that can arise from repeated administration of a drug and to determine the safe dosages to be used in the initial human clinical trials. Recovery groups in which animals are kept alive for a period of time after the end of drug administration, are also important in determining of any changes related to drug withdrawal.

We report here the results of acute, sub-acute (14-day) and sub-chronic (90-day) oral toxicity studies carried out on LLC in mice, in accordance with OECD guidelines. These results are useful to evaluate the safety of LLC as a hepatoprotective agent.

MATERIALS AND METHODS

Equipment

Serum samples were analyzed by Konelab 20XT auto analyzer. Haematological assessments were carried out using BCC-3000B, DIRU three-part hematology analyzer. Histopathological assessments were carried out using an Olympus CH30 light microscope.

Drugs and Chemicals

The standardized proprietary mixture of powdered plant materials used in the formulation of LLC was provided by Link Natural Products (Pvt) Limited, Sri Lanka. All the chemicals used were of analytical grade.

Preparation of the Extract of the Proprietary Mixture of Plant Materials (LLC plant extract)

The proprietary mixture of plant materials (107.5 g) obtained as a dry powder was extracted by maceration for 24 h with 500 ml of isopropanol: water (70:30 v/v). The residue was macerated twice more with another two portions of 500 ml of isopropanol: water (70:30 v/v). The extracts were combined, filtered and evaporated under reduced pressure at a temperature below 60 ^{0}C to obtain a brown solid (19.1 g). The appropriate amount of the solid was suspended in 0.25% carboxymethyl cellulose (CMC) for oral administration to the animals.

HPLC

HPLC analysis of the LLC plant extract was performed on an Agilent Technologies system 1260 infinity (Quat Pump VL) system fitted InnertSustain® Column (C 18, 5µm 4.6 x 150 mm).

The column temperature was 30 ºC. The mobile phase consisted of 1.0 % (v/v) acetic acid in distilled water (A) and acetonitrile (B). Elution commenced with 60 % of A and 40 % of B. After 6 min, a linear gradient was introduced to bring the composition of the eluant to 0 % of A and 100 % of B at 20 min. Elution was continued for a further 10 min with 0 % of A and 100 % of B. Total run time was 30 min. The flow rate was set equal to 1 ml/min and the injection volume was 10 µl. The eluant was monitored at 254 nm.

Experimental Animals and Housing

Institute of cancer research (ICR) mice (8 -12 weeks) purchased from the Medical Research Institute, Sri Lanka were used. The mice were acclimatized for one week at the animal house of the University of Sri Jayewardenepura before the study. Animals were caged by sex and they were exposed to the natural light and dark cycle of approximately 12 h of light and 12 h of dark. The animals were housed in polypropylene cages with stainless steel grill tops and bedding of cleaned wood shaves. They were fed with a standard diet and water *ad libitum*. The experimental protocol was approved by the Ethics Review Committee of the medical faculty, University of Sri Jayewardenepura (No. 545/11).

Toxicity Studies

Toxicity studies were carried out in accordance with OECD guidelines[4,5].

Acute Oral Toxicity (Limited dose test)

Five female mice were used. The dose level was 2000 mg/kg bw. The dose volume was kept as 1 ml. The mice were fasted 3 h before and after the administration of the extract. Only water was provided during this period. All the animals were observed for mortality and behavioral changes for first 10 min, and at 30 min, 1 h, 2 h, 4 h, and 6 h after dosing and thereafter once a day for 14 days. The body weight of mice was recorded on day 1, day 4, day 7 and day 14. The mice were euthanized after light ether anesthesia on day 15. At necroscopy, organs were collected in 10% formalin for histopathological assessments. The gross features of the internal organs were observed for any pathological changes.

Sub-acute Oral Toxicity study (14 Days)

Twenty-five male mice were used. They were divided into five groups (n = 5). The four test groups received the LLC plant extract orally administered at four different doses (160, 240, 320 and 400 mg/ kg b. w.) daily for a period of 14 days. The control groups of mice received vehicle (0.25% CMC) alone. The mice were observed for mortality and abnormal behavior daily during the 14-day study period. Body weights of treated mice were recorded at the initiation of the study (day 1), day 7 and day 14. The mice were sacrificed on day 15 for necroscopy. The gross features of the internal organs were observed for any pathological changes. Blood was obtained by cardiac puncture and analyzed for liver enzymes, total protein and total bilirubin.

Sub-chronic Oral Toxicity study (90 Days)

A total of hundred ICR mice (fifty from each sex) were used. The animals were separated according to sex randomly divided into six groups. The three test groups and the control group had twenty mice in each (consisting of ten male and ten female mice).The test group animals were administered orally with LLC plant extract (in 0.25% CMC) at the dose levels of 80, 160 and 400 mg/ kg once daily for 90 consecutive days. The control group received 0.25% CMC only. The two recovery groups for control and high dose level, each having five mice per sex, were given the respective treatments for 90 days and were kept for further 28 days. The females used were nulliparous and nonpregnant. The dose volume was kept constant at 1 ml for all dose levels including the control group and the dose volume administered to individual mice was adjusted according to its most recently recorded body weight. The mice were dosed at approximately the same time each day. All animals were observed twice daily for mortality and abnormal clinical signs / behavioral changes during the study period. The body weight and feed consumption data of all animals were recorded on the day of commencement and at weekly intervals throughout the study period.

At the end of the 90-day period, all mice except the recovery groups were fasted overnight and sacrificed under light ether anesthesia. Blood samples were collected by cardiac puncture for haematology (in EDTA containing tubes) and blood biochemistry (in plain tubes). The recovery group mice were treated similarly after a further 28 days.

Blood samples for biochemical analysis (0.75 ml) were centrifuged at 3000 rpm for 15 min. The serum biochemistry parameters were analyzed using an auto analyzer and commercially available test kits. Accordingly, alkaline phosphatase (ALP), aspartate transaminase (AST), alanine transaminase (ALT), total bilirubin (TB), total protein (TP), albumin (ALB), total cholesterol (CHOL), blood glucose (GLU), creatinine (CREAT), blood urea (UREA), inorganic phosphorous (PHOS) and calcium (Ca) were measured.

Hematological parameters were measured using were analyzed using a BCC-3000B, DIRU three-part hematology analyzer. Total white blood cells (WBC), total red blood count (RBC), hemoglobin (Hb), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), percentage of lymphocyte (LYM %), percentage monocyte (MON %) and percentage granulocyte (GRA %) were measured.

Necropsy and Histopathology

At necropsy, the animals were sacrificed to analyze the gross and microscopic features of the internal organs. The liver, kidneys, spleen, lung, brain, heart, uterus, and testes/ovaries were excised and weighed. Relative organ weight to terminal body weight was also calculated. Liver, kidneys, spleen, lung, brain, heart, uterus, and testes/ovaries were also examined macroscopically and fixed in 10% neutral buffered formalin for histopathological examination. Formalin-fixed samples were embedded in paraffin, sectioned, and stained with hematoxylin-eosin for histological analysis. Photographs of sections were taken using an Olympus FSX 100 immunofluorescent microscope.

Statistical analysis

Data of male and female rats were analyzed separately by one-way analysis of variance (ANOVA) followed by two sample t-test using Minitab software version 14. Statistical significance level was set at $p < 0.05$.

RESULTS AND DISCUSSION

As the polyherbal formulation is a complex mixture of many active compounds its identity is established by chromatographic fingerprints. The HPLC fingerprint of the LLC extract used in the toxicology studies is given in Figure 1.

Table 1: Effect of 14-day repeated dose of the extract of LLC in ICR male mice (N = 5)

Parameter	Vehicle control	Treated group (160 mg/kg)	Treated group (240 mg/kg)	Treated group (320 mg/kg)	Treated group (400 mg/kg)
Body Weight (g)	35.2 ± 5.4	39.6 ± 2.6	43.2 ± 5.2	41.2 ± 3.9	41.2 ± 3.9
AST (IU/L)	85 ± 5	75 ± 7	114 ± 10	95 ± 3	97 ± 3
ALT (IU/L)	18 ± 1	21 ± 2	23 ± 2	21 ± 1	22 ± 2
ALP (IU/L)	90 ± 3	83 ± 4	107 ± 5	95 ± 3	87 ± 4
Total Protein (g/dL)	5.9 ± 0.1	6.0 ± 0.2	5.8 ± 0.2	5.9 ± 0.2	5.8 ± 0.1
Total bilirubin (mg/dL)	0.29 ± 0.11	0.28 ± 0.11	0.29 ± 0.10	0.30 ± 0.12	0.27 ± 0.12

Table 2: Group mean feed consumption (g/week) of male and female mice during the 90-day sub-chronic administration of extract of LLC

	Control / recovery	80 mg/kg		160 mg/kg		400 mg/kg		400 mg/kg recovery	
Male	Average	Average	% of control	Average	% of control	Average	% of control	Average	% of control
1	26	37	142	25	96	25	96	25	96
2	26	34	131	22	85	27	104	25	96
3	27	35	130	25	93	25	93	26	96
4	26	36	138	26	100	28	108	24	92
5	27	30	111	24	89	24	89	27	100
6	27	35	130	23	85	27	100	26	96
7	26	35	135	22	85	25	96	24	92
8	25	32	128	24	96	25	100	26	104
9	28	35	125	25	89	24	86	26	93
10	27	36	133	26	96	27	100	27	100
11	28	30	107	26	93	26	93	27	96
12	28	35	125	28	100	28	100	29	104
13	26							29	112
14	25							27	108
15	27							28	104
16	27							27	100
Female									
1	25	22	88	27	108	24	96	24	96
2	24	22	92	26	108	22	92	24	100
3	26	23	88	30	115	26	100	23	88
4	25	23	92	22	88	24	96	22	88
5	23	22	96	23	100	22	96	24	104
6	26	24	92	26	100	27	104	24	92
7	22	22	100	26	118	25	114	25	114
8	24	26	108	22	92	25	104	25	104
9	25	26	104	24	96	27	108	24	96
10	24	22	92	26	108	23	96	25	104
11	23	23	100	24	104	27	117	26	113
12	23	25	109	26	113	29	126	26	113
13	24							25	104
14	25							26	104
15	24							25	104
16	24							24	100

Table 3: Hematological parameters (Mean ± SD) of male and female mice after 90-day sub-chronic administration of the extract of LLC

Parameter	Vehicle	80 mg/kg	160 mg/kg	400 mg/kg	400 mg/kg recovery	Control recovery
Male						
WBC(x10^9/L)	5.5 ± 0.2	4.1 ± 0.8	3.8 ± 0.3	4.0 ± 1.1	4.2 ± 0.7	3.9 ± 0.6
RBC(x10^9/L)	7.7 ± 0.2	7.6 ± 0.5	7.8 ± 0.5	8.7 ± 1. 8	7.4 ± 0.4	7.2 ± 0.4
Hb (g/dL)	12.5 ± 0.3	13.0 ± 0.6	12.6 ± 1.2	14.5 ± 2.6	12.2 ± 0.9	12.8 ± 0.8
HCT(%)	38.4 ± 0.9	38.8 ± 1.5	37.7 ± 4.1	43.2 ± 8.2	35.6 ± 2.5	61.1 ± 5.4*
MCV (fL)	152.6 ±6.9	154.4± 6.4	143.9 ± 6.7	150.1 ± 4.1	144.7 ± 6.3	153 ± 7.5
MCH (pg)	49.3 ± 1.1	51.7 ± 1.5	48.1 ± 1.6	50.3 ± 1.7	49.4 ± 1.5	48.1 ± 1.2
MCHC(g/dL)	97.3 ± 3.1	100.6 ± 2.3	100.3 ± 1.7	100.6 ± 1.6	102.3 ± 2.5	105.2 ± 4.3
PLT(x10^9/L)	1515 ± 159	1147 ± 191	1248 ± 120	1139 ± 270	1210 ± 156	1152 ± 122
LYM(x10^9/L)	5.3 ± 1.9	4.3 ± 1.6	3.5 ± 0.3	3.3 ± 1.0	3.7 ± 0.5	3.5 ± 0.5
MON(x10^9/L)	0.48 ±0.12	0.36 ± 0.13	0.24 ± 0.13	0.36 ± 0.13	0.35 ± 0.14	0.36 ± 0.15
GRA(x10^9/L)	0.12 ± 0.16	0.18 ± 0.26	0.12 ± 0.16	0.30 ± 0.21	0.30 ± 0.12	0.42 ± 0.26
Female						
WBC	3.6 ± 0.7	4.7 ± 1.5	4.2 ± 1.0	4.0 ± 0.8	3.9 ± 0.7	4.1 ± 0.6
RBC(x10^9/L)	7.5 ± 0.5	7.9 ± 0.4	8.5 ± 0.8	8.0 ± 0.3	7.4 ± 0.4	7.7 ± 0.4
Hb (g/dL)	12.8 ± 0.9	13.4 ± 0.7	13.7 ± 1.4	13.6 ± 0.6	13 ± 0.8	12.3 ± 0.6
HCT (%)	38.3 ± 2.9	39.5 ± 2.1	42.3 ± 3.7	39.5 ± 2.5	58.1 ± 5.4*	38.2 ± 3.6
MCV (fL)	154 ± 5	150 ± 3	149 ± 4	150 ± 5	143 ± 9	155 ± 6

MCH (pg)	52 ± 2	51 ± 2	50 ± 2	50 ± 1	49 ± 2	49.4 ± 1.6
MCHC(g/dL)	100 ± 2	101 ± 3	100 ± 3	101 ± 3	101 ± 4	103.3 ± 4.2
PLT($x10^9$/L)	1040 ±326	1089 ±175	1110 ±123	1101 ± 172	1201 ± 122	1143 ± 156
LYM($x10^9$/L)	3.2 ± 0.7	4.3 ± 2.0	3.8 ± 1.2	3.7 ± 0.8	3.5 ± 0.5	3.6 ± 0.8
MON($x10^9$/L)	0.21± 0.14	0.31 ± 0.10	0.38 ± 0.13	0.41 ± 0.20	0.40 ± 0.22	0.41 ± 0.13
GRA($x10^9$/L)	0.12 ±0.15	0.21 ± 0.14	0.30 ± 0.16	0.30 ± 0.10	0.30 ± 0.20	0.36 ± 0.27

*significantly different from vehicle control

Table 4: Biochemical parameters (Mean ± SD) of male and female mice after 90-day sub-chronic administration of the extract of LLC

Parameter	Vehicle control	80 mg/kg	160 mg/kg	400 mg/kg	400 mg/kg recovery	Control recovery
Male						
ALP(IU/L)	112 ± 18	118 ± 14	106 ± 12	110 ± 10	109 ±18	101 ± 31
ALT(IU/L)	21 ± 1	19 ±1	15 ± 5	20 ± 2	35 ± 12	38 ± 17
AST(IU/L)	133 ± 25	150 ± 18	165 ± 5	146 ± 29	143 ± 5	128 ± 23
CHOL (mg/L)	204 ± 4	175 ± 14	197 ± 15	203 ± 7	167 ± 15	163 ± 25
GLU (mg/dL)	107 ± 11	124 ± 14	140 ± 10	137 ± 15	201 ± 30	200 ± 41
CREA (mg/dL)	0.4 ± 0.02	0.4 ± 0.01	0.5 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	0.3 ± 0.08
TB (mg/L)	3.7 ± 0.5	3.5 ± 0.7	3.3 ± 0.1	3.6 ± 0.3	3.3 ± 0.1	3.1 ± 0.7
TP (mg/L)	6 ± 0.2	5.7 ± 0.2	5.7 ± 0.2	5.4 ± 0.1	5.9 ± 0.3	6 ± 0.2
ALB (mg/L)	3.64 ± 0.5	3.5 ± 0 .3	3.6 ± 0.1	3.5 ± 0.1	3.5 ± 0.2	3.4 ± 0.3
UREA (mg/dL)	6.5 ± 0.3	6.2 ± 1	5.7 ± 0.5	6.7 ± 0.4	5.5 ± 0.8	5.3 ± 1.4
PHOS (mg/dL)	26 ± 3	28 ± 1.6	23 ± 2	25 ± 2	26 ± 2	25 ± 1
Ca (mg/dL)	12 ± 1	11 ± 1	11 ± 0	11 ± 0	11 ± 0	12 ± 0.7
Female						
ALP (IU/L)	116 ± 17	115 ± 18	125 ± 11	120± 10	125 ± 15	124 ± 20
ALT (IU/L)	20 ± 3	23 ± 5	28 ± 1	25 ±4	25 ± 3	26 ± 2
AST (IU/L)	162 ± 20	204 ± 63	161 ± 35	131 ± 3	161 ± 3	151 ± 26
CHOL mg/L)	133 ± 6	152 ± 9	142 ± 10	165 ± 32	134 ± 21	125 ± 32
GLU (mg/dL)	118 ± 26	101 ± 18	132 ± 10	113 ± 25	213 ± 45	208 ± 43
CREA (mg/dL)	0.46 ± 0.06	0.43 ±0.01	0.49 ±0.02	0.45 ± 0.02	0.44 ±0.02	0.43 ±0.03
TB (mg/L)	3.2 ± 0.3	3.3 ± 0.3	3.0 ± 0.4	3.0 ± 0.2	3.0 ± 0.2	2.8 ± 0.4
TP (mg/L)	5.6 ± 0.3	5.7 ± 0.2	5.7 ± 0.2	5.7 ± 0.1	5.8 ± 0.1	5.9 ± 0.6
ALB (mg/L)	3.5 ± 0.1	3.7 ± 0.2	3.6 ± 0.1	3.6 ± 0.1	3.6 ± 0.1	3.6 ± 0.2
UREA (mg/dL)	6.2 ± 0.5	5.6 ± 0.3	5.6 ± 0.3	6.4 ± 0.5	6.4 ± 0.5	6.6 ± 0.4
PHOS (mg/dL)	26 ± 1.6	26 ± 1	21 ±2	23 ± 2	23 ± 2	25 ± 2
Ca (mg/dL)	11 ± 1	11 ± 0	11 ± 0	11 ± 0	11 ± 0	11 ± 0.3

Table 5: Absolute (g) and relative organ weights (Mean ± SD) of male and female mice after 90-day sub-chronic administration of the extract of LLC

Organ		Vehicle control	80 mg/kg	160 mg/kg	400 mg/kg	400 mg/kg recovery	Control recovery
Male Body weight		43.6 ± 1.8	41.4 ± 1.5	37.4 ± 0.5	41.8 ± 1.1	43.0 ± 0.7	42.4 ± 3.2
Liver	weight	1.518 ± 0.090	1.558 ± 0.138	1.852 ± 0.293	1.946 ± 0.054	1.998 ± 0.068	1.700 ± 0.248
	% b.w.	3.486 ± 0.262	3.773 ± 0.439	4.950 ± 0.769	4.656 ± 0.119	4.645± 0.083	4.023 ± 0.601
Kidney	weight	0.305 ± 0.059	0.248 ± 0.065	0.220 ± 0.040	0.222 ± 0.042	0.222 ± 0.042	0.242 ± 0.037
	% b.w.	0.703 ± 0.150	0.597 ± 0.145	0.587 ± 0.101	0.524 ± 0.102	0.516 ± 0.098	0.572 ± 0.088
Spleen	weight	0.186 ± 0.055	0.160 ± 0.027	0.182 ± 0.043	0.188 ± 0.042	0.188 ± 0.042	0.148 ± 0.047
	% b.w.	0.429 ± 0.135	0.386 ± 0.065	0.486 ± 0.112	0.445 ± 0.102	0.438 ± 0.100	0.349 ± 0.109
Testis	weight	0.152 ± 0.028	0.140 ± 0.005	0.140 ± 0.010	0.142 ± 0.008	0.152 ± 0.027	0.141 ± 0.009
	% b.w.	0.348 ± 0.058	0.338 ± 0.017	0.374 ± 0.027	0.335 ± 0.014	0.353 ± 0.058	0.334 ± 0.034
Heart	weight	0.188 ± 0.030	0.162 ± 0.042	0.148 ± 0.026	0.162 ± 0.022	0.162 ± 0.022	0.162 ± 0.018
	% b.w.	0.432 ± 0.076	0.391 ± 0.096	0.396 ± 0.067	0.383 ± 0.058	0.377 ± 0.054	0.383 ± 0.046
Brain	weight	0.678 ± 0.029	0.690 ± 0.020	0.660 ± 0.058	0.640 ± 0.053	0.640 ± 0.053	0.678 ± 0.025
	% b.w.	1.559 ± 0.122	1.668 ± 0.074	1.764 ± 0.147	1.513 ± 0.159	1.489 ± 0.126	1.607 ± 0.131
Female							
Body weight		37.3 ± 1.4	37.4 ± 1.0	38.3 ± 1.5	37.9 ± 0.7	40.0 ± 1.4	38.7 ± 0.8
Liver	weight	1.820 ± 0.191	1.740 ± 0.161	1.640 ± 0.170	1.730 ± 0.091	1.760 ± 0.062	1.612 ± 0.175
	% b.w.	4.866 ± 0.469	4.568 ± 0.369	4.287 ± 0.374	4.563 ± 0.235	4.412 ± 0.273	4.217 ± 0.382
Kidney	weight	0.218 ± 0.037	0.238 ± 0.610	0.260 ± 0.066	0.260 ± 0.066	0.301 ± 0.067	0.283 ± 0.074
	% b.w.	0.585 ± 0.088	0.630 ± 0.172	0.683 ± 0.188	0.686 ± 0.176	0.753 ± 0.171	0.744 ± 0.200
Spleen	weight	0.190 ± 0.027	0.194 ± 0.034	0.189 ± 0.036	0.189 ± 0.036	0.190 ± 0.050	0.182 ± 0.049
	% b.w.	0.510 ± 0.077	0.511 ± 0.091	0.495 ± 0.100	0.498 ± 0.089	0.477 ± 0.135	0.476 ± 0.129
Testis	weight	0.221 ± 0.017	0.226 ± 0.027	0.215 ± 0.016	0.211 ± 0.013	0.212 ± 0.013	0.212 ± 0.012
	% b.w.	0.592 ± 0.036	0.595 ± 0.072	0.562 ± 0.039	0.557 ± 0.033	0.531 ± 0.038	0.554 ± 0.025
Heart	weight	0.161 ± 0.020	0.168 ± 0.023	0.176 ± 0.029	0.174 ± 0.029	0.190 ± 0.027	0.185 ± 0.027
	% b.w.	0.431 ± 0.049	0.443 ± 0.068	0.462 ± 0.087	0.459 ± 0.079	0.475 ± 0.067	0.486 ± 0.078
Brain	weight	0.629 ± 0.059	0.635 ± 0.052	0.648 ± 0.059	0.648 ± 0.059	0.684 ± 0.270	0.685 ± 0.024
	% b.w.	1.686 ± 0.140	1.676 ± 0.179	1.698 ± 0.204	1.711 ± 0.168	1.710 ± 0.049	1.796 ± 0.089

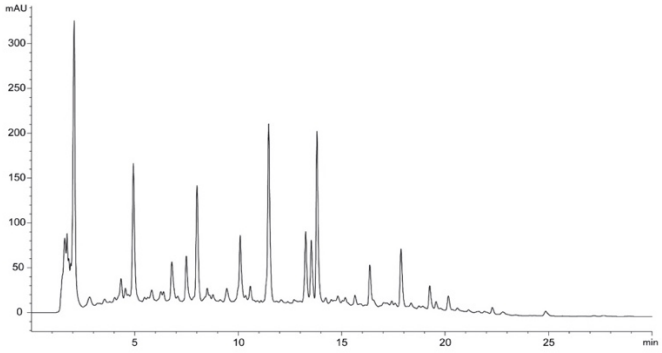

Figure 1: HPLC fingerprint of LLC

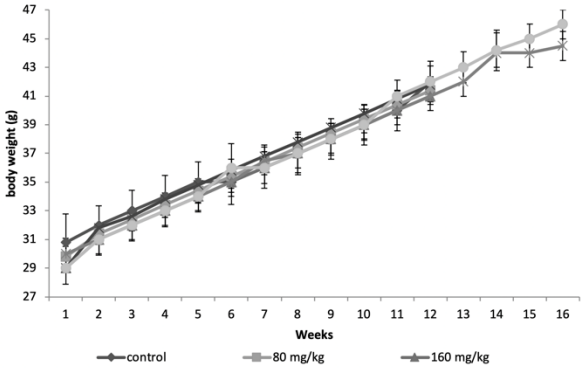

Figure 2: Group mean body weight (mean ± SD) of male ICR mice administered the extract of LLC for 90 days orally.

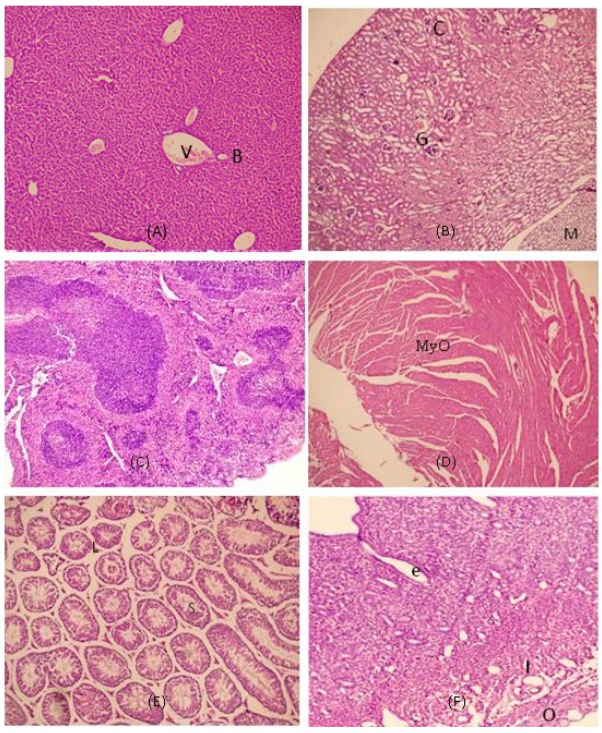

Figure 3: Representative cross sections of a mouse treated with the dose of 400 mg/kg for 90 days. (A) Liver, V-Venule, B-bile ductule, (B) Kidney, C-cortex, M-medulla and G- glomeruli (C) Spleen. (D) Heart, Myo-Myocardium (E) Testis, S-seminiferous tubules and L-Leydig cell (F) Uterus e- endometrium, I-inner and O-outer smooth muscle layer. All sections were stained with H & E (40 x 4.2).

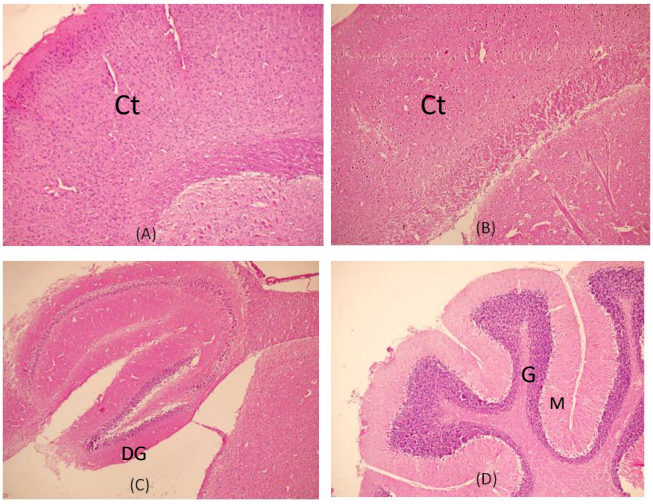

Figure 4: Representative cross sections of a mouse brain treated with the dose of 400 mg/kg for 90 days. (A) mid brain, Ctx- cerebral cortex (40 x 4.2), (B) mid brain (40 x 10) (C) Hippocampus, DG- mouse dentate gyrus (40 x 4.2), (D) Cerebellum, Gr- the granular layer and the molecular layer (40 x 4.2), All sections were stained with H & E.

Acute Oral Toxicity

In a safety evaluation of test substances, acute oral toxicity study is considered as the preliminary step, whereas repeated dose oral toxicity studies are carried out to assess the adverse effects of a substance that may likely to occur from continuous exposure.

The acute oral toxicity of the extract of LLC was studied in mice at a dose of 2000 mg/kg. All animals survived and did not show any sign of toxicity, either immediately after administration of the dose or during the observation period. There was no adverse effect on body weight gain and no gross pathological changes were observed on necropsy. Hence the median lethal dose for extract of LLC is greater than 2000 mg/kg.

Sub-acute Oral Toxicity

A 14-day repeated dose oral toxicity study was conducted to select doses for a more extended 90-day sub-chronic oral toxicity test. It had been shown in a previous study that the optimum hepatoprotective dose in mice was 80 mg/kg[1]. Accordingly, multiples of 2,3,4 and 5 of the optimum doses (160, 240, 320 and 400 mg/kg) were used to study sub-acute oral toxicity of LLC.

All animals survived throughout experimental period. There was no adverse effect on body weight gain and no gross pathological changes were observed on necropsy. There was no significant ($p > 0.5$) difference in the liver enzymes between the test groups and the control group (Table 1). Further, no histopathological changes were observed in the liver tissue. Thus, no hepatotoxicity was observed at five times the therapeutic dose over a period of 14 days.

Sub-chronic Toxicity study (90-Day repeated dose)

Repeated dose toxicity studies are conducted to evaluate the adverse effects of a test substance after prolonged use and are carried out to provide information about the possible health hazards likely to arise from repeated exposure over a relatively limited period of time including information about target organs, the possibilities of cumulative effects, and an estimate of the dose at which there is no observed adverse effect[3].

Based on the result of the 14-day repeated dose study dose levels of 80, 160 and 400 mg/kg b.w. were selected for the sub-chronic oral toxicity study.

No deaths, adverse clinical signs or toxic effects were observed in any of the animals at all dose levels throughout the dosing period of 90 days and post dosing recovery period of 28 days.

In general, there was no dose-associated difference in terms of body weight or food/water consumption other than in the food intake of the male mice treated with 80 mg/kg b.w. which was significantly higher than the other treated and control groups (Table 2). However, the change of food intake did not affect the body weight gain in the animals of the group (Figure 2).

Both hematological and biochemical data reflect the overall health status of an animal and are also related to the general metabolic, adaptive, or toxic processes and target organs associated with exposure to toxic agents. Both hematological and biochemical parameters are necessary to evaluate adverse effects and toxicologic dose–response relationships in non-clinical toxicology testing. The results of our analysis for hematological and biochemical parameters associated with systemic toxic symptoms indicated no adverse changes in either sex of mice after treatment with the highest dosage of LLC extract.

Changes in numbers of RBCs, WBCs, and PLT are used identify the potential hematological effects of a test substance. In addition to changes in number of RBCs, the Hb, HCT and red cell indices—MCV, MCH, MCHC, RDW were also evaluated. Platelet indices—MPV, PDW and total WBC count with relative and absolute WBC differential counts are also important in toxicological evaluation[6]. The total counts of RBCs, WBCs and PLT were not significantly changed in the animals in the present study. It was noted that a component of WBCs, the granulocytes in both males and females fluctuated widely amongst animals within each group leading to large standard deviations and that therefore the differences observed in the average values amongst the groups were not statistically significant.

It was also noted that HCT in the male control recovery group and females in the 400 mg/kg recovery group were significantly increased. However, these deviations were not regarded as toxicologically relevant because they occurred randomly over the

dose range and without showing consistency between sexes. Results of hematological analysis are shown in Table 3.

Biochemical tests performed in toxicological studies give information about metabolism of carbohydrates, lipids and proteins, and the integrity of urinary, hepatobiliary, musculoskeletal, cardiovascular, and gastrointestinal systems, but give less information about the central nervous system toxicity[6]. Treatment related significant changes were not observed in either sex (Table 4). GLU level was found to be raised in the 400 mg/kg recovery groups. However, as there was a corresponding increase in the control recovery groups as well, it cannot be directly related to the treatment. All the other parameters of mice treated with LLC at 400 mg/kg and the recovery groups were found to be comparable with those of the control group at end of the 90 days treatment and recovery period.

Liver and kidney are the major organs involved in the metabolism and elimination of drugs and other foreign compounds[7]. They can be considered as major target organs that suffer systemic adverse reactions following oral administration of drugs. Serum concentrations of blood urea nitrogen and creatinine are the most widely used indicator in clinical biochemistry test for monitoring renal function. Along with unchanged levels of serum urea and creatinine and kidney weights, gross and histopathological findings of kidney supported the conclusion that LLC extract treatment did not produce any renal toxicity. Serum levels of ALT, AST and ALP are commonly used as clinical biochemical markers associated with liver damage. These liver function markers did not show any significant changes between control group and LLC extract treatment groups in male or female mice.

The effect of LLC on the liver, kidneys, spleen, testis/uterus, heart and brain, was initially evaluated by comparing the absolute and relative organ weights of treated animals with control (Table 5). The group means values of absolute and relative weights of the of treated animals did not differ significantly from those of control. Further, there were no gross pathological changes to be observed on macroscopic examination of the organs.

No histopathological changes were to be observed on microscopic examination of representative cross sections of the organs (Figures 3 and 4).

CONCLUSION

Based on the results of the acute, repeated dose and sub-chronic toxicity studies described herein, it is concluded that doses of 400 mg/kg - five times the optimal hepatoprotective dose of Link Livecare™ - does not produce any toxicity, in mice. These findings support the claim of safety for Livecare™.

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