Academic Sciences International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 4, Issue 3, 2012

Research Article

ACUTE, SUB ACUTE AND SUBCHRONIC 90-DAYS TOXICITY OF EURYCOMA LONGIFOLIA AQUEOUS EXTRACT (PHYSTA) IN WISTAR RATS

YOGENDRA K CHOUDHARY[1*], PRAVEEN BOMMU[1], YEE K MING[2], NORAISYAH B ZULKAWI[2]

Ethix Pharma[1*], Division of Toxicology and Clinical Affairs, Level I, Off Campus CIIMER, Karbala Road, Bilaspur, Chhattisgarh, 495001, India, Biotropics Malaysia Berhad[2], Division of Product Development, Level 52, Menara TM, Jalan Pantai Baharu, 50672, Kuala Lumpur, Malaysia. Email:yogendrakumar.choudhary@gmail.com

Received: 11 Feb 2012, Revised and Accepted: 22 Mar 2012

ABSTRACT

The acute, subacute and subchronic 90-days toxicity of the *Eurycoma longifolia* aqueous extract was studied extensively in wistar rats. In the acute toxicity test, oral administration of 2000 mg/kg of the *Eurycoma longifolia* aqueous extract produced neither mortality nor changes in behavior or any other physiological activities. In subacute toxicity study, no mortality or toxic signs were observed when the three doses of 250, 500 and 1000 mg/kg of *Eurycoma longifolia* aqueous extract were administered orally for a period of 28 days. There were no significant differences in the body and organ weights between controls and treated animals of both sexes. However a slight change was observed in feed consumption in female rats which was unrelated to the treatment. In the blood chemistry analysis, no significant changes occurred, including glucose, creatinine, urea, aspartate transaminase (AST), potassium, sodium, total billirrubin, total cholesterol, total protein, albumin, clotting time (CT) of both sexes except slight increase in alanine transaminase (ALT) levels in female rats, which was further investigated as unrelated to treatment. Hematological analysis showed no differences in any of the parameters examined (WBC count, platelet and hemoglobin estimation) in either the control or treated group of both sexes. Pathologically, neither gross abnormalities nor histopathological changes were observed. Subchronic 90-days toxicity was studied by daily oral dose (ten females, ten males) of 250, 500 and 1000 mg/kg for 90 days. No critical incidence of mortality and clinical abnormalities were reported. Ophthalmological, sensory and motor activity examination did not reveal any ocular and neurotoxic effects. The body weight gain and food consumption was not affected and was found to be comparable between control and treated animals of both sexes. In addition, the rats were analyzed for final body and organ weights, necropsy, and hematological, blood chemical, urinalysis and histopathological parameters. Hematological analysis and clinical blood chemistry was comparable to the control group. The data on urinalysis indicated no adverse effect due to the treatment. No gross or histopathology findings were observed in the treatment groups. NOAEL for the *Eurycoma longifolia* aqueous extract was found to be more than 1000 mg/kg orally (p.o), under the conditions of this investigation.

Keywords: *Eurycoma longifolia*, Toxicity, Acute toxicity, Sub-acute toxicity, 90-days toxicity.

INTRODUCTION

Eurycoma longifolia or commonly know as Tongkat Ali, is Malaysia's wonder plant for male vitality and general energy. The most potential activity of the plant is found in the roots. It's name can also be refered as "Ali's staff or walking stick" in reference to its effects on male sexuality. *Eurycoma longifolia* has traditionally been consumed to increase overall energy, enhance sexual potency, strengthen erection, boost the metabolism and improve fertility[1,2]. *Eurycoma longifolia* is a proven testosterone booster[2,4]. This herb is particularly renowned for its ability to increase testosterone levels significantly. Since testosterone is primarily responsible for the growth and development of male reproductive organs and normal testosterone level maintains energy level, mood, fertility and desire. *Eurycoma longifolia* acts by positively affecting hormonal balance which naturally stimulates the body to produce more of the free testosterone. This testosterone supplementation in turn increases muscle strength and muscle mass, which are important for physical function and athletic performance[3-5]. Across Southeast Asia, Tongkat Ali (*Eurycomanone longifolia*) root is used as a traditional remedy for treating malaria, cancer, anxiety, ulcers, fatigue, infertility and impotence [6-9]. Malaysian traditional medicine is known to have utilized at least 1300 different plants, with Tongkat Ali root holding a prominent place in local culture. The Malaysian government, through its Forest Research Institute of Malaysia (FRIM) program, entered into the partnership program with the Massachusetts Institute of Technology (MIT) reported the effects of patented Tongkat Ali Root Extract[10], branded as Physta. Physta is standardized on Eurycomanone, proteins, carbohydrates and glycosaponins.

The present study was designed to determine the acute, subacute and subchronic 90-days oral toxicity of the *Eurycoma longifolia* aqueous extract (Physta) in Wistar rats.

MATERIALS AND METHODS

Preparation of the extract

The extract was obtained from a commercial batch of PHYSTA from Phytes Biotek Sdn Bhd, Malaysia. The standardized extract was prepared by a water extraction of *Eurycoma longifolia* roots using the patented high pressure water extraction technology (Patent no. US 7,132,117 B2), concentrated by condensation and filtered at 1-4 micron followed by freeze drying without maltodextrin or lactose. The extract was further standardized for its bioactive content of (1) >22% of Eurypeptides; (2) >35% of Glycosaponin and (3) >1% Eurycomanone.

Acute toxicity

The acute toxicity of the *Eurycoma longifolia* aqueous extract was evaluated in wistar rat as per the OECD guideline[11, 12].

In sighting study, a single female rat (overnight fasted) received *Eurycoma longifolia* aqueous extract starting at 300 mg/kg orally by gavage. The animal was observed for toxic symptoms continuously for 48 hrs. As no toxic sign observed till 48 hrs, another female rat recieved highest dose of 2000 mg/kg of *Eurycoma longifolia* aqueous extract, orally by gavage and observed for 48 hrs. As there was no clinical sign even at highest dose, the main study was carried out in female wistar rats (four female rats, weight: 199 -207.5 g, age 8-12 weeks) received *Eurycoma longifolia* extract at 2000 mg/kg orally by gavage. The animals were observed for toxic symptoms continuously for the first 4 h after dosing. The number of survivors was noted after 24 h and these animals were then maintained for further 13 days with observations made daily. Finally the animals were necropsied at the end of day 14.

Subacute toxicity

The sub acute toxicity of the *Eurycoma longifolia* aqueous extract was evaluated in wistar rat as per the OECD guideline[13].

Experimental animals

Sixty Wistar rats (weight: 160–237 g; age: 6–8 weeks old) were randomly assigned into six groups (n = 10), five females and five males in each group. Groups of five rats were housed together in polypropylene cages (males separated from females) with 12 h

light/dark cycle in a temperature and humidity controlled environment. Treatments were administered orally by gavages once a day for 28 days. The first and second groups of animals, serving as control and control reversal, received distilled water alone; the third, fourth, fifth and sixth group received the *Eurycoma longifolia* aqueous extract at doses of 250, 500, 1000 and 1000 mg/kg, respectively; the sixth group of animals, served as high dose reversal. All animals were supplied with Rat pellet feed (Amrut brand, Pranav Agro Industries Ltd., Sangil) and RO water *ad libitum* during the testing periods.

All rats were weighted and observed daily for physiological, hematological, biochemical and behavioral changes upto 28 days. Animals belonging to second and sixth group were additionally observed for another 14 days during the post observation period. Gross pathology for group first, third, fourth, fifth and second, sixth was performed on day 28 and 42 respectively.

Observation

Clinical signs were observed at least once a day through the 28 days of dosing. Body weight, food intake and weight gain were measured once a week. Ophthalmological examination of each animal was carried out using ophthalmoscope prior to the first treatment and sacrifice. Sensory and motor activity was observed during the 4th week of dosing for the first, third, fourth, fifth groups and 2nd week of post treatment observation period for second and sixth group respectively. Motor activity was measured by using animal activity meter (Columbus, U.S.A).

Blood analysis

On day 28th all surviving animals were fasted overnight, and anesthetized afterwards for blood collection from the orbital sinus. Blood samples were collected into three tubes: (1) 3.2% buffered sodium citrate tubes; (2) heparinized centrifuge tubes; (3) dry non-heparinized centrifuge tubes. A blood analysis (hematology, coagulation and chemistry) was carried out by using HUMACOUNT (M/s Humane). The blood in the sodium citrate tubes was used for Clotting time (CT). The heparinized blood was used for a hematological study which included red blood cell count (RBC), hemoglobin concentration (Hb), hematocrit (HCT), White blood cells (WBC), platelets (PLT), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and mean corpuscular volume (MCV).

The non-heparinized blood was allowed to coagulate before being centrifuged and the serum separated. The serum was assayed for Total protein, albumin, glucose, aspartate transaminase (AST), alanine transaminase (ALT), urea creatinine, cholesterol, total billirrubin, sodium and potassium. The clinical biochemistry was performed using RT-1904C Chemistry Analyser (M/s. Rayto).

Tissue analysis

Immediately after collecting the blood samples, tissue were collected and preserved using 10% neutral buffered formalin solution. The organs such as Brain, Heart, Thymus, Spleen, Liver, Kidney, Adrenals, Testes, and Epididymides were removed and weighted immediately for subsequent analysis. Spinal cord, intestines, stomach, thyroid, trachea, seminal vesicles, ovary, lymph nodes, bone marrow, lungs, prostate, uterus, urinary bladder and the sciatic nerve were also extracted.

Tissues from the control group and the group treated with the high dose were subjected to dehydration process, embedded in paraffin, sectioned at 3 to 5 micron and followed by hematoxillin-eosin staining. The pathological observations of all tissues were performed on gross and microscopic bases. Histological plates of the preserved tissues were encrypted for analysis by a pathologist.

Subchronic 90-days toxicity

The subchronic 90-days toxicity of the *Eurycoma longifolia* aqueous extract was evaluated in wistar rat as per the OECD guideline[14].

Experimental animals

One twenty Wistar rats (weight: Male, 165–200 g and Female, 141-184 g; age: 7–8 weeks old) were randomly assigned into six groups (*n* = 20), ten females and ten males in each group. Groups of three rats were housed together in polypropylene cages (males separated from females) with 12 h light/dark cycle in a temperature and humidity controlled environment. Treatments were administered orally by gavages once a day for 90 days. The first and second groups of animals, serving as control and control reversal, received analytical grade water (10 mg/kg); the third, fourth, fifth and sixth group received the *Eurycoma longifolia* aqueous extract at doses of 250, 500, 1000 and 1000 mg/kg, respectively; the sixth group of animals, served as high dose reversal. All animals were supplied with extruded rat pellet feed ('Provimi', M/s Provimi Animal Nutrition Pvt. Ltd., Bangalore) and potable water passed through Aqua guard with U.V. irradiation, *ad libitum* during the testing periods.

All the rats were examined daily for toxicity, morbidity and mortality. They were subjected for detailed clinical examination before initiation of the study and weekly thereafter. Opthalmoscopic examination was conducted on all rats before initiation and at termination of treatment. Additionally in the thirteen week of treatment the animals were examined for sensory, grip strength and motor activity. Body weight and food consumption were recorded weekly. Biochemistry, hematology and urinalysis were performed at the termination of the treatment and at end of recovery period. All animals sacrificed terminally were subjected to detailed necropsy. All organ weight was recorded. Tissues from control and high dose level group were subjected to histopathological evaluation.

Observation

Mortality and clinical signs were observed at least once a day through the 90 days of dosing and also during the recovery period. Detailed clinical examination (change in gait, posture, response to handling, clonic or tonic movements, stereotypes and bizarre behavior) was performed before initiation of the treatment, weekly thereafter during treatment and recovery. Ophthalmological examination of each animal was carried out using ophthalmoscope (Heine-mini 2000) prior to the first treatment and termination of treatment period. In thirteen week of treatment (day 86/87), all rats were examined for functional observation battery. Body weight was recorded at day 0, weekly thereafter and at necropsy (day 91). Weights of reversal groups were recorded weekly during post-treatment and at necropsy (day 119). Feed consumption was recorded weekly.

Blood analysis

On day 91st and 119th all surviving animals was fasted overnight, and anesthetized afterwards for blood collection from the retro-orbital plexus. Blood samples were collected in tubes containing Heparin (for clinical chemistry) and EDTA (for haematology). Blood smears were also made on glass slides. A blood analysis (hematology and clinical chemistry) was carried out by using 'Coulter A$^{c.}$ T diff' Hematology Analyser, Synchron CX-5 Pro Fully Automatic Random Access Analyser and commercially available diagnostic kits (Beckman Coulter, Inc, Miami, Florida, USA). The clotting time was determined manually. The heparinized blood was used for a hematological study which included hemoglobin concentration (Hb), packed cell volume (PVC), red blood cell count (RBC), White blood cells (WBC), platelets (PLT), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular volume (MCV), differential WBC count [Neutrophils (N), Lymphocytes (L), Eosinophils (E), Monocytes (M)]

The non-heparinized blood was allowed to coagulate before being centrifuged and the serum separated. The serum was assayed for Total protein, albumin, globulin, alkaline phosphatase, aspartate aminotransferase (AST), alanine aminotransferase (ALT), glucose, urea nitrogen, urea, creatinine, total billirrubin, calcium, phosphorus, total cholesterol, triglycerides, sodium and potassium.

Urine samples

On day 88th and end of reversal period (day 117), the rats were placed in specially designed urine collection cages during 2–4 h; 3–5 ml of urine was collected for immediate urinalysis. Qualitative analysis was performed using Multistix® SG Multiple Reagent

Diagnostic strips (Bayer Diagnostic India Ltd., Baroda, India) and microscopic examination was performed for presence of epithelial cells, leucocytes, erythrocytes, casts, granular casts, crystals, other abnormal constituents and triple phosphate crystals.

Tissue analysis

At termination of treatment period (day 91) and at the end of reversal period (day 119), rats were anesthetically sacrificed and tissue were collected and preserved using 10% neutral buffered formalin solution. Testes and eyes were collected in modified Davidson's fixative. The organs such as Kidney, liver, adrenals, testes, epididymides, uterus, thymus, spleen, brain, ovaries, and heart were removed and weighted immediately for subsequent analysis. Gross lesions, Spinal cord, eye, thyroid, parathyroid, adrenals, pancreas, trachea, lungs, aorta, oesophagus, prostate, intestines, stomach, skin, seminal vesicles, ovary, lymph nodes, bone marrow, mammary glands, uterus, urinary bladder, skeletal muscle and the sciatic nerve were also extracted.

Tissues from the control group and the group treated with the high dose were subjected to dehydration process, embedded in paraffin, sectioned at 3 to 5 micron and followed by hematoxillin-eosin staining. The pathological observations of all tissues were performed on gross and microscopic bases. Histological plates of the preserved tissues were encrypted for analysis by a pathologist. Histopathological examination was not extended to lower groups and recovery group animals as no treatment related changes were observed in high dose group animals.

Statistical analysis

Statistical evaluation was performed using Graph Pad Prism Ver.5.0, for Windows XP. All results are presented as Mean ±S.E.M. Data were analyzed using Student's't'- test. Data were analyzed using one-way analysis of variance (ANOVA-Snedecor and Cochran, 1980) and, when appropriate, by a Dunnett's pair wise comparison (Scheffe, 1953). Results were considered significant at $p < 0.05$.

RESULTS

Acute toxicity

No death was recorded in the 14 days of observation period in the male and female animals given 2000 mg /kg of the *Eurycoma longifolia* aqueous extract orally. The animals did not show any changes in the general appearance during the observation period (Table 1).

Table 1: Toxic signs assessment of rats in acute toxicity of the aqueous extract from *Eurycoma longifolia* (2000 mg/kg)

Animal no.	Days																	
	0																	
	30 min	1 hr	2 hr	4 hr	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Sighting Study																		
1	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
2	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Main Study																		
3	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
4	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
5	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
6	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N

N – Normal

Sub acute toxicity

General signs

No deaths or significant changes in general behavior or other physiological activities were observed at any point in the present study.

Body weight, food, and water intake

No significant changes were observed in body weight gain. A significant decrease in feed intake was found in some female groups in first week but no body weight decline was seen and males of the same groups were unaffected. Sensory, motor and grip strength were reported normal and no abnormalities seen in the ophthalmological examination.

Hematological and plasma biochemical data

The hematological analysis (Table 2), showed no significant changes of RBC, Hb, Ht, WBC, and platelets in the male and female treatment group compared to the control group.

Table 2: Hematological values of rats in subacute toxicity of the aqueous extract from *Eurycoma longifolia*

	Male						Female					
	Control		*Eurycoma longifolia* aqueous extract [mg/kg]				Control		*Eurycoma longifolia* aqueous extract [mg/kg]			
	G1	G2*	250	500	1000	1000**	G1	G2*	250	500	1000	1000**
WBC[a]	13.88 ±5.50	12.42 ±1.41	13.00 ±2.68	11.83 ±4.58	14.46 ±4.02	11.32 ±2.82	14.44 ±2.37	13.20 ±6.09	12.22 ±2.13	11.30 ±3.15	15.19 ±4.39	10.95 ±4.50
RBC[b]	7.90 ±1.29	7.74 ±0.39	7.64 ±1.48	7.51 ±0.77	7.83 ±1.24	7.67 ±1.05	6.81 ±0.20	7.56 ±0.59	7.47 ±0.88	7.18 ±0.98	7.27 ±0.76	8.08 ±0.52
Hb[c]	11.12 ±1.75	10.92 ±1.05	11.30 ±1.30	11.06 ±0.82	11.22 ±1.49	11.06 ±0.52	11.90 ±0.69	10.84 ±0.38	12.02 ±0.58	11.36 ±0.84	12.44 ±0.60	11.10 ±1.01
HCT[d]	37.91 ±5.45	39.43 ±1.74	38.55 ±6.49	39.54 ±5.70	38.43 ±7.83	36.52 ±5.01	34.50 ±2.85	39.26 ±3.03	38.31 ±4.96	33.81 ±3.65	35.92 ±4.28	41.17 ±2.09
MCV[e]	48.18 ±3.14	51.00 ±1.38	50.70 ±3.01	52.64 ±4.93	48.78 ±2.78	47.80 ±4.97	50.62 ±2.79	51.96 ±2.32	51.28 ±2.06	47.56 ±5.83	49.44 ±3.58	51.00 ±2.13
MCH[f]	14.25 ±2.55	14.15 ±1.55	15.08 ±2.27	14.80 ±1.17	14.77 ±3.77	14.59 ±1.48	17.49 ±1.02	14.40 ±1.14	16.29 ±2.25	16.02 ±1.98	17.22 ±1.49	13.79 ±1.70
MCHC[g]	29.45 ±3.91	27.76 ±3.20	29.77 ±4.44	28.42 ±4.58	30.61 ±9.18	30.65 ±3.38	34.68 ±3.64	27.72 ±2.01	31.78 ±4.28	34.02 ±5.53	34.89 ±2.85	27.08 ±3.49
PLT[h]	900.20 ±136.04	794.20 ±136.04	870.00 ±165.27	807.20 ±172.20	922.40 ±163.09	784.00 ±170.70	962.20 ±138.97	772.60 ±231.42	788.00 ±171.45	864.40 ±147.89	789.80 ±130.7	700.40 ±84.98
G[i]	21.52 ±3.29	20.08 ±5.62	22.36 ±4.93	17.14 ±2.76	19.82 ±4.05	21.52 ±2.18	22.40 ±3.24	19.48 ±5.32	21.44 ±6.06	18.26 ±4.78	20.64 ±2.58	22.20 ±3.85
L[j]	73.40 ±3.37	75.38 ±5.15	72.92 ±3.04	77.12 ±3.67	75.00 ±3.88	73.54 ±1.68	72.52 ±1.56	75.66 ±4.63	73.28 ±5.39	75.84 ±5.38	74.94 ±2.45	73.74 ±2.33
M[k]	5.08 ±0.94	4.54 ±0.94	4.72 ±2.31	5.74 ±2.41	5.18 ±1.80	4.94 ±0.66	5.08 ±1.94	4.86 ±1.35	5.28 ±1.21	5.90 ±0.85	4.42 ±2.11	4.06 ±2.01
CT[l]	94.20 ±5.54	96.00 ±7.42	93.60 ±10.78	98.00 ±11.16	101.60 ±12.66	97.00 ±10.56	100.40 ±12.30	91.00 ±11.40	102.40 ±7.92	95.00 ±16.66	98.00 ±11.58	102.20 ±7.79

Values are expressed as mean ±SEM for *n* = 10 rats per group, [a] White blood cell (×103 mm3), [b] Red blood cell (×106 mm3), [c] Hemoglobin concentration (g/dl), [d] Hematocrit (%), [e] Mean corpuscular value (FL), [f] Mean corpuscular hemoglobin (pg), [g] Mean corpuscular hemoglobin concentration (g/dl), [h] Platelet Count (×103 mm−3), [i] Granulocyte (%), [j] Lymphocyte (%), [k] Monocytes (%), [l] Clotting time, * Control reversal, ** High dose reversal.

The biochemical analysis (Table 4), showed no significant differences in any of the parameters examined in either the control or treated group of the male and female rats. A significant increase in ALT was seen in one male group, however it was absent in females of the same group. Hence the changes are taken as incidental and not caused by the test item.

Tissue analysis

There were no significant differences between the control and treated groups in the organ weights of male and female rats (Table 6). Pathological examinations of the tissues on a gross basis indicated, Hydrometra of uterus in one female rat (common incidental occurrence in this species). No alterations were seen in the microscopic examination of the internal organs; the cellular appearances were unremarkable in both groups and sexes.

Table 4: Blood chemistry values of rats in subacute toxicity of the aqueous extract from *Eurycoma longifolia*

	Male						**Female**					
	Control		***Eurycoma longifolia* aqueous extract [mg/kg]**				**Control**		***Eurycoma longifolia* aqueous extract [mg/kg]**			
	G1	**G2***	**250**	**500**	**1000**	**1000****	**G1**	**G2***	**250**	**500**	**1000**	**1000****
TP[a]	6.70 ±0.15	6.69 ±0.16	6.77 ±0.19	6.68 ±0.14	6.65 ±0.17	6.67 ±0.12	6.86 ±0.14	6.68 ±0.07	6.67 ±0.15	6.70 ±0.17	6.74 ±0.21	6.64 ±0.28
ALB[b]	3.41 ±0.42	3.24 ±0.65	3.54 ±0.58	3.69 ±0.35	3.20 ±0.37	3.54 ±0.35	3.24 ±0.30	3.15 ±0.25	3.34 ±0.32	3.20 ±0.28	3.32 ±0.21	3.21 ±0.36
GLU[c]	109.07 ±28.28	130.45 ±27.48	97.05 ±32.01	73.78 ±34.17	102.68 ±47.59	116.19 ±19.49	108.17 ±20.43	98.07 ±17.26	95.64 ±39.89	116.21 ±18.20	110.62 ±24.39	104.66 ±18.12
AST[d]	73.24 ±6.30	75.36 ±11.87	76.13 ±11.79	80.99 ±6.11	78.94 ±6.00	82.84 ±10.13	79.02 ±15.34	65.41 ±5.34	79.74 ±11.77	82.77 ±11.94	75.11 ±11.45	74.33 ±7.52
ALT[e]	26.92 ±3.18	27.20 ±3.21	31.26 ±2.13[s]	28.02 ±2.73	27.64 ±1.89	27.83 ±3.16	25.30 ±6.45	27.34 ±2.02	25.16 ±3.79	25.32 ±2.25	28.58 ±1.62	26.83 ±3.30
UREA[f]	40.28 ±1.90	38.60 ±3.07	41.10 ±3.22	42.50 ±2.87	40.22 ±1.12	38.26 ±1.52	43.04 ±2.47	38.02 ±2.29	40.50 ±2.70	41.96 ±4.81	41.02 ±1.92	38.66 ±3.22
CRT[g]	0.47 ±0.15	0.46 ±0.18	0.57 ±0.22	0.41 ±0.08	0.57 ±0.06	0.48 ±0.26	0.51 ±0.13	0.50 ±0.18	0.52 ±0.06	0.47 ±0.18	0.51 ±0.17	0.54 ±0.16
CHL[h]	105.18 ±19.18	95.29 ±17.24	88.60 ±16.23	101.68 ±9.35	88.15 ±10.14	89.00 ±24.80	88.53 ±19.3	103.90 ±15.24	96.90 ±22.55	101.26 ±16.02	99.91 ±9.72	86.46 ±20.99
TBN[i]	0.48 ±0.08	0.38 ±0.11	0.52 ±0.08	0.44 ±0.06	0.47 ±0.06	0.53 ±0.13	0.59 ±0.19	0.55 ±0.18	0.52 ±0.09	0.59 ±0.11	0.47 ±0.17	0.39 ±0.05
NA[j]	143.25 ±5.18	147.89 ±2.46	146.82 ±7.33	146.74 ±4.86	145.75 ±11.65	148.38 ±6.21	147.74 ±3.88	146.06 ±7.16	147.16 ±2.03	149.55 ±2.60	146.39 ±6.07	143.30 ±4.55
K[k]	6.69 ±1.94	6.17 ±1.47	6.80 ±1.17	6.35 ±0.91	6.51 ±0.62	6.04 ±2.01	5.51 ±1.61	6.02 ±1.63	6.76 ±1.37	6.09 ±1.03	6.06 ±1.34	6.41 ±1.58

Values are expressed as mean ±SEM for *n* = 10 rats per group. [a] Total Protein, [b] Albumin, [c] Glucose, [d] Aspartate aminotransferase, [e] Alanine aminotransferase, [f] Urea, [g] Creatinine, [h] Cholesterol, [I] Total billirubin, [j] Sodium, [k] Potassium, * Control reversal, ** High dose reversal, S-: Significantly lower over the control group (p < 0-05).

Table 6: Relative organ weight of rats in subacute toxicity of the aqueous extract from *Eurycoma longifolia*

	Male						**Female**					
	Control		***Eurycoma longifolia* aqueous extract [mg/kg]**				**Control**		***Eurycoma longifolia* aqueous extract [mg/kg]**			
	G1	**G2***	**250**	**500**	**1000**	**1000****	**G1**	**G2***	**250**	**500**	**1000**	**1000****
ADRENALS	0.0182 ±0.0012	0.0185 ±0.0009	0.0178 ±0009	0.0178 ±0.0009	0.0181 ±0.0007	0.0188 ±0.0012	0.0301 ±0.0020	0.0316 ±0.0024	0.0305 ±0.0016	0.0298 ±0.0015	0.0301 ±0.007	0.0294 ±0.0025
EPIDIDYMES	0.3725 ±0.0148	0.3682 ±0.0178	0.3720 ±0.0213	0.3718 ±0.0161	0.3769 ±0.0134	0.3805 ±0.0154						
THYMUS	0.1256 ±0.0095	0.1255 ±0.0169	0.1209 ±0.0050	0.1258 ±0.0166	0.1152 ±0.0140	0.1337 ±0.0090	0.1238 ±0.0139	0.1266 ±0.0177	0.1319 ±0.0085	0.1326 ±0.0147	0.1414 ±0.0101	0.1319 ±0.0090
SPLEEN	0.3986 ±0.0073	0.4035 ±0.0092	0.3939 ±0.0093	0.4014 ±0.0167	0.4077 ±0.0150	0.4005 ±0.0065	0.3997 ±0.0246	0.4042 ±0.0113	0.4061 ±0.0149	0.4080 ±0.0079	0.4036 ±0.0158	0.4136 ±0.0230
HEART	0.3875 ±0.0063	0.3925 ±0.0117	0.3933 ±0.0104	0.3815 ±0.0177	0.3890 ±0.0186	0.3978 ±0.0143	0.4011 ±0.0258	0.3989 ±0.0190	0.3930 ±0.0331	0.3965 ±0.0269	0.3848 ±0.0264	0.3835 ±0.0228
BRAIN	0.8531 ±0.0096	0.8511 ±0.0164	0.8621 ±0.0091	0.8521 ±0.0088	0.8506 ±0.0037	0.8606 ±0.0090	0.8533 ±0.0144	0.8411 ±0.0139	0.8639 ±0.0232	0.8629 ±0.0110	0.8608 ±0.0137	0.8540 ±0.0385
TESTES	1.0232 ±0.0115	1.0224 ±0.0123	1.0257 ±0.0157	1.0247 ±0.0095	1.0233 ±0.0056	1.0183 ±0.0120						
KIDNEYS	0.8360 ±0.0169	0.8402 ±0.0147	0.8269 ±0.0063	0.8229 ±0.0072	0.8175 ±0.0109	0.8356 ±0.0095	0.8376 ±0.0218	0.8426 ±0.0246	0.8265 ±0.0263	0.8171 ±0.0223	0.8342 ±0.0160	0.8184 ±0.0151
LIVER	3.1786 ±0.0162	3.1710 ±0.0099	3.1703 ±0.0163	3.1678 ±0.0104	3.1695 ±0.0061	3.1785 ±0.0100	3.1655 ±0.0252	3.1610 ±0.0145	3.1794 ±0.0184	3.1825 ±0.0135	3.1568 ±0.0203	3.1794 ±0.0309

Values are expressed as mean ±SEM for *n* = 10 rats per group.

Subchronic 90-days toxicity

General signs

No treatment related mortality or significant changes in general behavior or other physiological activities were observed at any point in the present study. One male rat (Animal ID Re8701) at 1000 mg/kg dose group died on day 73 of the study, which was further investigated as incidental and not treatment-related.

Body weight, food, and water intake

No significant changes were observed in body weight gain and feed intake. Sensory, motor and grip strength were reported normal and no abnormalities seen in the ophthalmological examination.

Hematological and plasma biochemical data

The hematological analysis (Table 3), showed no significant changes of RBC, Hb, WBC, PVC, clotting time and platelets in the male and female treatment group compared to the control group. Although eosinophil values were found statistically significant, it has no biological significance as the values have a big variance. However this decreased eosinophil may be monitored in future non-clinical and clinical studies.

The biochemical analysis (Table 5), showed no significant differences in any of the parameters examined (Total protein, albumin, globulin, alkaline phosphatase, aspartate aminotransferase (AST), alanine aminotransferase (ALT), glucose, urea nitrogen, urea, creatinine, total billirrubin, calcium, phosphorus, total cholesterol, triglycerides, sodium and potassium) in either the control or treated group of the male and female rats.

Urinalysis

The urinalysis in the control and treated groups of both male and female rats did not indicate any abnormalities.

Table 3: Hematological values of rats in subchronic 90-days toxicity of the aqueous extract from *Eurycoma longifolia*

	Male						Female					
	Control		*Eurycoma longifolia* aqueous extract [mg/kg]				Control		*Eurycoma longifolia* aqueous extract [mg/kg]			
	G1	G2*	250	500	1000	1000**	G1	G2*	250	500	1000	1000**
	13.99	14.22	14.57	14.30	14.14	14.39	13.93	14.12	14.23	14.15	14.07	13.87
Hb[a]	±0.74	±0.28	±0.37	±0.48	±0.48	±0.74	±0.48	±0.40	±0.55	±0.63	±0.41	±0.59
PCV[b]	37.67	37.94	39.26	38.70	38.39	38.43	37.45	37.28	38.22	37.81	37.70	36.80
	±1.95	±0.83	±1.08	±1.59	±1.07	±1.65	±1.42	±1.03	±1.52	±1.82	±1.15	±1.53
RBC[c]	7.76	8.12	8.29	8.14	8.12	7.94	7.54	7.50	7.73	7.67	7.60	7.44
	±0.56	±0.26	±0.27	±0.43	±0.37	±0.27	±0.29	±0.23	±0.37	±0.33	±0.27	±0.33
MCH[d]	18.06	17.53	17.59	17.60	17.44	18.12	18.48	18.83	18.43	18.45	18.52	18.66
	±0.93	±0.39	±0.46	±0.59	±0.53	±0.52	±0.71	±0.37	±0.56	±0.35	±0.41	±0.67
MCV[e]	48.64	46.77	47.39	47.60	47.34	48.40	49.66	49.71	49.49	49.28	49.63	49.51
	±2.54	±1.16	±1.26	±1.00	±1.27	±0.96	±1.18	±0.85	±1.17	±1.02	±0.95	±1.60
MCHC[f]	37.14	37.48	37.12	36.96	36.83	37.43	37.22	37.88	37.24	37.43	37.32	37.69
	±0.44	±0.37	±0.71	±0.59	±0.47	±0.37	±1.17	±0.47	±0.47	±0.48	±0.43	±0.37
WBC[g]	5.05	7.35	6.44	6.82	7.29	7.71	4.68	6.51	4.70	4.86	4.71	5.62
	±0.68	±1.34	±1.13	±1.44	±1.06	±1.21	±0.97	±1.63	±0.98	±1.57	±0.90	±0.63
N[h]	19.80	18.90	21.10	19.30	24.10	15.00	14.60	16.20	17.80	15.20	15.60	18.90
	±7.79	±5.70	±7.49	±8.46	±9.15	±4.74	±5.72	±4.21	±4.78	±9.34	±5.82	±4.91
L[i]	78.60	80.10	76.90	78.90	75.00	84.56	84.30	83.10	81.40	84.50	84.20	80.30
	±7.95	±5.55	±6.52	±8.24	±8.91	±5.05	±5.81	±4.01	±5.13	±9.49	±5.83	±5.12
E[j]	1.10	1.00	1.40	1.70	0.70	0.33	0.90	0.50	0.40	0.30	0.20	0.80
	±1.52	±0.94	±1.35	±1.42	±0.82	±0.71	±0,57	±0.53	±0.52	±0.48[S-]	±0.63[S-]	±0.79
M[k]	0.50	0.00	0.60	0.10	0.20	0.11	0.20	0.20	0.40	0.00	0.00	0.00
	±0.53	±0.00	±0.84	±0.32	±0.42	±0.33	±0.42	±0.42	±0.70	±0.00	±0.00	±0.00
PLT[l]	793.70	780.90	800.10	829.70	876.80	779.56	785.80	857.00	801.30	839.10	761.00	798.40
	±88.19	±84.40	±57.90	±61.08	±89.03	±86.64	±123.44	±98.36	±108.74	±71.83	±166.53	±47.46
CT[m]	164.50	134.00	157.20	162.90	159.10	126.33	176.40	124.10	178.50	156.80	149.70	113.40
	±19.70	±22.85	±16.25	±24.61	±24.38	±11.94	±22.47	±19.38	±22.47	±26.33	±23.05[S-]	±13.73

Values are expressed as mean ±SEM for *n* = 10 rats per group. [a] Hemoglobin concentration (g/dl), [b] Packed cell volume, [c] Red blood cell (×106 mm−3), [d] Mean corpuscular hemoglobin (pg), [e] Mean corpuscular value (FL), [f] Mean corpuscular hemoglobin concentration (g/dl), [g] White blood cell (×103 mm–3), [h] Neutrophils (%), [i] Lymphocyte (%), [j] Eosinophils (%), [k] Monocytes (%), [l] Platelet Count (×103 mm-3), [m] Clotting time, * Control reversal, ** High dose reversal, S- : Significantly lower over the control group. (p < 0-05).

Table 5: Blood chemistry values of rats in subchronic 90-days toxicity of the aqueous extract from *Eurycoma longifolia*

	Male						Female					
	Control		*Eurycoma longifolia* aqueous extract [mg/kg]				Control		*Eurycoma longifolia* aqueous extract [mg/kg]			
	G1	G2*	250	500	1000	1000**	G1	G2*	250	500	1000	1000**
TP[a]	7.02	7.28	7.28	7.46	7.16	7.37	7.62	7.76	7.49	8.70	9.31	7.45
	±0.32	±0.55	±0.42	±0.42	±0.35	±0.27	±0.40	±0.44	±0.44	±0.59[S+]	±0.58[S+]	±0.46
ALB[b]	1.69	1.64	1.79	1.97	1.79	1.62	2.11	1.74	2.07	2.54	2.74	1.91
	±0.14	±0.13	±0.13	±0.09[S+]	±0.18	±0.13	±0.22	±0.16	±0.18	±0.27[S+]	±0.26[S+]	±0.09
GLB[c]	5.33	5.64	5.49	5.49	5.37	5.74	5.51	6.02	5.42	6.16	6.57	5.54
	±0.31	±0.54	±0.33	±0.39	±0.30	±0.27	±0.36	±0.40	±0.36	±0.46[S+]	±0.49[S+]	±0.46
ALT[d]	34.90	44.20	33.70	38.10	32.00	34.11	26.70	28.30	32.50	39.70	37.30	28.00
	±10.72	±11.00	±8.38	±12.22	±6.55	±6.68	±5.81	±3.77	±11.07	±15.18[S+]	±5.72[S+]	±4.40
AST[e]	91.20	156.80	85.40	108.20	93.80	117.67	88.20	87.70	120.40	110.20	115.00	81.20
	±18.21	±43.12	±13.89	±16.23[S+]	±11.93	±33.51	±18.73	±21.09	±63.23	±18.62	±19.17	±18.15

ALP[f]	78.70	80.50	82.80	97.40	78.00	82.44	39.90	48.10	39.40	35.40	35.70	37.80
	±20.47	±19.70	±15.24	±35.24	±13.34	±17.10	±16.84	±14.07	±10.93	±8.10	±5.72	±20.10
GLU[g]	120.30	110.50	101.60	115.70	99.80	106.89	89.00	99.20	100.10	94.70	101.40	89.90
	±25.47	±18.39	±13.21	±34.35	±9.13	±20.76	±9.17	±10.10	±38.20	±14.17	±12.86	±9.31[S-]
UN[h]	20.40	20.70	21.80	22.20	20.00	19.22	22.60	21.60	23.50	23.80	25.50	26.00
	±4.72	±3.40	±3.08	±3.43	±3.50	±2.22	±1.58	±3.24	±3.10	±3.99	±5.62	±3.56[S+]
UREA[i]	43.66	44.30	46.65	47.51	42.80	41.14	48.36	46.22	50.29	50.93	54.57	55.64
	±10.10	±7.28	±6.60	±7.33	±7.48	±4.76	±3.38	±6.93	±6.63	±8.55	±12.03	±7.62
CRE[j]	0.52	0.46	0.52	0.60	0.52	0.45	0.55	0.49	0.60	0.53	0.48	0.48
	±0.06	±0.07	±0.009	±0.08[S+]	±0.07	±0.05	±0.09	±0.05	±0.09	±0.05	±0.05	±0.07
CHL[k]	51.40	58.40	54.70	50.20	53.80	55.89	39.90	36.80	39.70	47.50	47.90	41.70
	±10.75	±5.68	±8.99	±7.64	±8.18	±10.83	±11.30	±6.61	±8.65	±10.88	±11.03	±7.09
TRI[l]	60.40	104.40	66.90	74.20	86.20	84.00	40.70	60.10	44.30	52.00	62.10	81.20
	±28.22	±34.55	±34.86	±32.45	±28.04	±39.88	±18.84	±23.72	±18.55	±17.49	±20.86	±29.42
BIT[m]	0.20	0.16	0.16	0.21	0.20	0.16	0.23	0.19	0.29	0.30	0.35	0.19
	±0.07	±0.08	±0.08	±0.07	±0.07	±0.07	±0.07	±0.11	±0.19	±0.13	±0.13	±0.80
NA[n]	157.64	147.30	153.24	157.84	152.27	148.67	146.51	147.04	148.95	145.87	146.42	147.45
	±3.74	±1.99	±1.95[S-]	±2.50	±2.41[S-]	±2.27	±5.19	±1.38	±2.42	±1.81	±0.87	±0.80
K[o]	5.88	5.37	5.20	5.35	5.25	5.08	5.71	4.42	6.55	6.21	6.66	4.55
	±0.83	±0.48	±0.38[S-]	±0.57	±0.42[S-]	±0.28	±1.49	±0.39	±1.20	±0.44	±0.69	±0.50
CA[p]	10.95	10.51	11.31	11.72	11.40	10.66	11.14	10.55	11.11	12.68	13.17	10.60
	±0.57	±0.27	±0.51	±0.36[S+]	±0.20[S+]	±0.21	±0.42	±0.27	±0.27	±0.59[S+]	±0.51[S+]	±0.21
P[q]	7.45	6.24	7.16	8.99	8.21	6.06	6.76	5.23	6.69	8.63	9.59	4.75
	±1.33	±0.57	±1.08	±1.85[S+]	±0.93	±0.65	±2.06	±0.62	±1.37	±1.83	±1.98[S+]	±0.69

Values are expressed as mean ±SEM for *n* = 10 rats per group. [a] Total Protein (g/dl), [b] Albumin (g/dl), [c] Globulin (g/dl), [d] Alanine aminotransferase (IU/L), [e] Aspartate aminotransferase (IU/L), [f] Alkaline phosphatase (IU/L), [g] Glucose (mg/dl), [h] Urea Nitrogen (mg/dl), [i] Urea (mg/dl), [j] Creatinine (mg/dl), [k] Cholesterol (mg/dl), [l] Triglyceride (mg/dl), [m] Total billirubin (mg/dl), [n] Sodium (mMol/L), [o] Potassium (mMol/L), [p] Calcium (mMol/L), [q] Phosphorus (mMol/L), * Control reversal, ** High dose reversal. S-/S+: Significantly lower/higher over the control group (p < 0-05).

Tissue analysis

There were no significant differences between the control and treated groups in the organ weights of male and female rats (Table 7). Pathological examinations of the tissues on a gross basis indicated no abnormalities. No treatment related alterations were seen in the microscopic examination of the internal organs. Some incidental and spontaneous lesions were observed, with less frequency and not dose dependent.

Table 7: Relative organ weight of rats in chronic toxicity of the aqueous extract from *Eurycoma longifolia*

	Male						**Female**					
	Control		***Eurycoma longifolia* aqueous extract [mg/kg]**				**Control**		***Eurycoma longifolia* aqueous extract [mg/kg]**			
	G1	**G2***	**250**	**500**	**1000**	**1000****	**G1**	**G2***	**250**	**500**	**1000**	**1000****
ADRENALS	0.017	0.013	0.015	0.016	0.015	0.014	0.032	0.027	0.032	0.033	0.032	0.029
	±0.002	±0.002	±0.002	±0.001	±0.003	±0.001	±0.004	±0.003	±0.005	±0.005	±0.004	±0.004
TESTES	0.89	0.90	0.939	0.90	1.02	0.94						
	±0.23	±0.09	±0.048	±0.04	±0.09	±0.09						
KIDNEYS	0.72	0.69	0.706	0.75	0.80	0.72	0.74	0.74	0.73	0.75	0.73	0.75
	±0.06	±0.05	±0.049	±0.05	±0.09[S+]	±0.06	±0.06	±0.06	±0.06	±0.05	±0.06	±0.09
LIVER	3.00	2.68	2.714	2.87	2.97	2.71	3.08	2.84	3.03	2.90	2.91	2.95
	±0.22	±0.28	±0.264	±0.23	±0.28	±0.23	±0.39	±0.24	±0.39	±0.12	±0.17	±0.20
BRAIN	0.53	0.54	0.533	0.54	0.58	0.57	0.82	0.84	0.87	0.83	0.84	0.83
	±0.06	±0.04	±0.036	±0.03	±0.04	±0.06	±0.04	±0.07	±0.07	±0.05	±0.03	±0.07
THYMUS	0.08	0.08	0.096	0.10	0.11	0.09	0.14	0.14	0.13	0.14	0.13	0.15
	±0.03	±0.01	±0.014	±0.02	±0.01[S+]	±0.02	±0.02	±0.03	±0.03	±0.03	±0.02	±0.05
HEART	0.30	0.29	0.292	0.29	0.32	0.30	0.34	0.35	0.34	0.33	0.36	0.35
	±0.02	±0.02	±0.028	±0.02	±0.03	±0.02	±0.02	±0.03	±0.02	±0.01	±0.02[S+]	±0.04
SPLEEN	0.19	0.18	0.185	0.18	0.19	0.18	0.22	0.23	0.20	0.21	0.21	0.23
	±0.04	±0.03	±0.021	±0.02	±0.02	±0.02	±0.03	±0.03	±0.02	±0.03	±0.04	±0.04
EPIDIDYMIDES	0.34	0.35	0.369	0.35	0.40	0.38						
	±0.07	±0.03	±0.025	±0.03	±0.03	±0.03						
OVARIES							0.063	0.037	0.062	0.056	0.055	0.037
							±0.010	±0.006	±0.012	±0.009	±0.005	±0.006
UTERUS							0.41	0.38	0.38	0.37	0.35	0.31
							±0.25	±0.14	±0.17	±0.14	±0.08	±0.06

Values are expressed as mean ±SEM for *n* = 10 rats per group. S+: Significantly higher over control group (p<0.05)

DISCUSSION AND CONCLUSION

The acute toxicity study does not show any toxic symptoms, changes in behavior or mortality at 2000 mg/kg doses. On the basis of above observations, the acute oral LD50 of *Eurycoma longifolia* aqueous extract was determined as > 2000 mg/kg body weight and the test item was classified according to the Globally Harmonized System (GHS) Category 5 or Unclassified.

The subacute and subchronic 90-days toxicity guideline applied considers a full study with three dose levels when at least 1000 mg/kg day does not show toxicity effects, as was observed in the

acute toxicity experiment. The objective was to evaluate toxicity profile, arising from repeated oral admistration of *Eurycoma longifolia* aqueous extract to rats for a period of 28 days and 90 days. Also, to find out the reversibility of any observed toxic effects or withdrawal Syndrome. The high dose of *Eurycoma longifolia* aqueous extract (1000 mg/kg day) in the subacute and subchronic 90-days study was applied because human exposure indicates the use of a high dose level in accord with the subacute and subchronic 90-days guideline. A lower dose of 250 mg/kg day was used to determine dose related toxic effects. In a subacute toxicity study, it appeared that the *Eurycoma longifolia* aqueous extract at the doses used did not produce any marked changes in both male and female rats, as evidenced by the absence of toxic symptoms, no changes in water/food ingestion, or weight gain. All animals survived until the scheduled euthanasia and no gross pathological alteration was found in the internal organs. Organ weight revealed that *Eurycoma longifolia* aqueous extract, at the doses used, did not produce organ swelling, atrophy or hypertrophy. Moreover, the microscopic evaluation did not find any abnormalities in the 1000 mg/kg *Eurycoma longifolia* aqueous extract group compared to the control group. Microscopic evaluation in the 250 mg/kg group was not performed in accordance with the subacute and subchronic 90-days guideline that considers unnecessary the microscopic examination of organs in the low dose group when no histopathological abnormalities are found in the high dose group. The comparable biochemical results in the control group and *Eurycoma longifolia* aqueous extract treated groups were consistent with the morphological analysis.

In summary the *Eurycoma longifolia* aqueous extract was found to be nontoxic when oral acute, subacute and subchronic 90-days toxicities in rats were performed. Mutagenicity and carcinogenicity studies are necessary to further support the safe use of this plant product. Based on the above investigation, the NOAEL of *Eurycoma longifolia* aqueous extract for Wistar rats can be considered as greater than 1000 mg/kg body weight under the condition of this investigation.

ACKNOWLEDGEMENT

This project was possible, thanks to the scientific support of Intox Pvt. Ltd. India (GLP Certification No. GLP/C-024)

REFERENCES

1. Gimlette JD, Thompson JW. A Dictionary of Malayan Medicine. Kuala Lumpur: Oxford University Press; 1997.
2. Chan KL, Choo CY, Morita H, Itokawa H. High performance liquid chromatography in phytomedical analysis of *Eurycoma longifolia*. Planta Med 1998; 64:741-45.
3. Perry LM, Metzger J. Medicinal plants of East and Southeast Asia: attributed properties anduses. USA: Massachusetts Institute of Technology Press; 1980:389.
4. Ismail Z, Ismail N, Lassa J. Malaysian herbal monograph. Kuala Lumpur: Malaysian Monograph Committee; 1999.
5. Jagananth JB, Ng LT. Herbs the green pharmacy of Malaysia. Kuala Lumpur: Vinpress Sdn Bhd : Malaysian Agricultural Research and Development Institute (MARDI); 2000:45-6.
6. Burkill IH. A dictionary of the economic products of the Malay Peninsula, Kuala Lumpur: Government of Malaysia and Singapore; 1966.
7. Kardono LBS, Angerhofer CK, Tsauri S, Padmawinata K, Pezzuto JM, Kinghorn AD. Cytotoxic and antimalarial constituents of the roots of Eurycoma longifolia. J Nat Prod 1991; 54:1360-67.
8. Chan KL, Lee SP, Yuen KH. Antipyretic activity of Quassinoids from Eurycoma longifolia Jack. In: Ghazally I, Murtedza M, Laily D, eds, Chemical prospecting in Malayan forest. Selangor: Pelanduk Publications; 1995:219-2.
9. Zakaria Y, Rahmat A, Pihie AHL, Abdullah NR, Houghton PJ. Eurycomanone induce apoptosis in HepG2 cells via up regulation of p53. Cancer Cell International Journal 2009; 9.
10. Sambandan T G et al. Bioactive fraction of *Eurycoma* Longifolia. US Patent- 7132117; 2006. EU Patent-13123491; 2007. Malaysian Patent MY -134867; 2007.
11. The Organization for Economic Co-operation and Development (OECD). Principles of GLP; Environment Monograph; 1998:45.
12. The Organization for Economic Co-operation and Development (OECD). The OECD Guidelines for Testing of Chemicals: 423 Acute Toxic Class Method OECD Sec. 4; 2001.
13. The Organization for Economic Co-operation and Development (OECD). The OECD Guidelines for Testing of Chemicals: 407 Repeated Dose Oral Toxicity –Rodent: 28 Day Study; 1995.
14. The Organization for Economic Co-operation and Development (OECD). The OECD Guidelines for Testing of Chemicals: 408 Repeated Dose 90-Day Oral Toxicity Study in Rodents: OECD, Sec. 4; 1998.