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Original Article

REPRODUCTIVE AND DEVELOPMENTAL TOXICITY OF *EURYCOMA LONGIFOLIA* AQUEOUS EXTRACT (PHYSTA®) IN WISTAR RATS

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ABSTRACT

Objective: Reproductive and developmental toxicities of *Eurycoma longifolia* aqueous extract (PHYSTA®) in male and female wistar rats were examined concerning its effect on male and female reproductive performance such as gonadal function, mating behavior, conception, development of the conceptus and parturition.

Methods: *Eurycoma longifolia* aqueous extract at a dose of 250, 500 and 1000 mg/kg b. w. was administered daily by oral gavage during 14 day premating and 14 days mating period in both male and female, during gestation period and up to post natal day 3 in females. Animals were observed for clinical signs, morbidity, mortality, body weight development, food consumption and pub observation (number of live pups/litter, live birth index, implant sites, fetal resorption etc.). Male and females were sacrificed on day 29 and post natal day (PND) 4 respectively; then subjected to necropsy.

Results: The result indicated non-significant predominant clinical signs, no mortality, and no toxicological relevance effect on body weight. In addition increased food consumption with no treatment related effect on litters, precoital interval and duration of gestation was observed. Pre and post natal data (Corpora lutea, implant sites, live pups, pre and post percent pre-implantation loss) remain unaffected. All pregnancies resulted in normal birth and delivery index remain unaffected. Survival of pups from PND 0 to PND 4 remained unaffected along with no treatment related gross external findings. No gross pathology findings and no statistically significant absolute and relative organ weight was observed. The histopathological evaluation does not revealed any lesions considered to be treatment related.

Conclusion: In conclusion, the repeated dose administration of *Eurycoma longifolia* aqueous extract (PHYSTA®) to male Wistar rats for 28-29 days and female Wistar rats up to 54 days at dosages of 250, 500 and 1000 mg/kg b.w. revealed no major toxicological findings. As per the present investigation the non-observed-adverse event level (NOAEL) for *Eurycoma longifolia* aqueous extract (PHYSTA®) is believed to be 1000 mg/kg body weight for reproductive and developmental toxicity.

Keywords: Reproductive toxicity, *Eurycoma longifolia*; Developmental toxicity, PHYSTA.

INTRODUCTION

Historically, herbal medicine has been an important component of healthcare all over the world. With the advances in medical and biological sciences that resulted in the introduction of promising synthetic orthodox therapies for many conditions, the use of herbal medicine declined in the 20th century. Lately, however, there has been a resurgence of interest in the use of phytomedicinal products in the treatment of diseases [1, 2]. The World Health Organization (WHO) has documented the rapidly growing interest and economic importance of Traditional Medicine in health systems all over the world.

Eurycoma longifolia (Jack) is a small tree that grows along the hilly slopes of the rainforests of Southeast Asia, including Indonesia, Malaysia, Thailand, Laos, Cambodia and Vietnam [3]. It has been used as a medicinal herb in Southeast Asia mainly to increase libido and to a lesser extent to improve general health [4]. Other traditional uses include treatment of malaria, bleeding, ulcers and hypertension [5].

Eurycoma longifolia is reputed as an aphrodisiac and remedy for decreased male libido. Study showed *Eurycoma longifolia* aqueous extract demonstrated significant improvements in libido, sexual performance, satisfaction, and physical functioning with well tolerated daily dose of 300 mg in man [6]. Animal studies in mice have shown that LD50 of *E. longifolia* water was more than 3000 mg/kg [7]. Acute, subacute and subchronic studies in rat have shown that 1000 mg/kg of *E. longifolia* water extract has no-observed-adverse event and toxicity [8]. Although there have been a lot of *E. longifolia* preparations have been marketed and few safety and human studies have been conducted, there has not been study done on toxicity and safety of *E. longifolia* or *E. longifolia*–based products concerning reproductive and development.

To expand our understanding of *E. longifolia* aqueous extract toxicity on reproductive outcomes and to evaluate further our interpolation of toxicities in humans systematically, specific life stages not covered by other types of toxicity studies were evaluated. The effects that may occur as a result of pre and postnatal chemical exposure were evaluated. Given the importance of *E. longifolia* aqueous extracts to reproductive outcomes, the possibility for systemic toxicity in pregnant and development, and the likelihood for effects on reproductive organs for males and females, we further evaluate these potential risks. Other parameters such as gonadal function are considered including the oestrous cycle, epididymal sperm maturation, mating behavior, conception, pregnancy, parturition, and lactation. The study was therefore designed to elucidate the possible effects of *E. longifolia* aqueous extract (PHYSTA®) on male, female fertility and embryo fetal development in Wistar rats. The study was conducted to comply with OECD Principles of Good Laboratory Practice and OECD Guidelines for Testing of Chemicals, Section 4 No 421 "Reproductive/Development Toxicity Screening Test".

MATERIALS AND METHODS

Preparation of extract

The extract was obtained from a commercial batch of standardized *E. longifolia* aqueous extract (PHYSTA®) from Phytes Biotek Sdn Bhd, Malaysia. The standardized extract was prepared by a water extraction of *E. longifolia* roots using the patented high pressure water extraction technology (Patent no. US 7,132,117 B2),

comprising the steps of a) subjecting the dried root to hot water extraction by percolation; b) filtering; c) followed by concentration by condensation; d) freeze drying without any carrier such as maltodextrin or lactose; and e) size reduction obtaining the dry extract powder. The dry extract powder was standardized for content of (1)>22% of protein; (2)>35% of Glycosaponin and (3) 0.8–1.5% Eurycomanone.

Animals

Young healthy male and nulliparous, non-pregnant female Wistar rats; 10 per treatment group, was purchased from Charles River, 97633 Sulzfeld, Germany. They were acclimated for 5 days, weighed, given a unique identification number and were randomly assigned to one of the four different dosages (0, 250, 500, and 1000 mg/kg b.w. of *E. longifolia* extract) groups.

They were housed in an automated climate control facility where the temperature was maintained at 22±3 °C, with a relative humidity of 55±10% and 12/12 h light–dark cycle. The animal use and care guidelines established by the Art 9.2, No. 7 of the German Act on Animal Welfare were strictly followed. Rats were housed in IVC cages III II, polysulfone cages on altromin saw fibre bedding (Lot No: 150610).

Experimental design

During the premating period 1 male and 1 female was housed per cage. After 14 days of administration of *E. longifolia* aqueous extract for both male and female, animals were paired (mating). During the mating period, 1 male and 1 female was matched from each treatment group and cohabited for a maximum of 14 days. The subsequent morning and the next morning there onwards the vaginal smear of female was checked to confirm the evidence of mating. Rats were separated after positive identification of a vaginal plug and/or sperm, after which a designation of gestational day [GD] 0 was made. Females with unsuccessful mating were allowed to mate with other male of the same group. Females with no evidence of copulation up to 14 day mating period were sacrificed 26 days after the last day of the mating period.

Males and females were sacrificed on day 29 and post natal day (PND) 4 respectively then subjected to necropsy. Randomly, body weights of males were taken weekly during entire study and at termination, whereas females were weighed weekly during pre mating period, on GD 0, GD 7, GD 14, GD 20 and on PND 0 (within 24 hours of parturition) and PND 4 along with pups. Food consumption was measured weekly until the rats gave natural birth but not during the mating period.

Dosing, feeding and animal care

The test item was formulated in sterile water. The dose formulation was prepared on each day of administration by dissolving/ dispersing required amount of extract for each dose concentration in sterile water. The dose formulation was verified for the nominal concentration in study week 1 (first week of pre-mating period), week 3 (first week of mating), week 5 (gestation) and week 7 (gestation/lactation) and checked for homogeneity in study week 1 and 5 with coefficient of variation within 20%. Four groups comprised of 10 adults males and 10 non-pregnant nulliparous female Wistar rats were dose daily by oral gavage with 0, 250, 500 and 1000 mg/kg b.w. per day of *E. longifolia* aqueous extract at dose volume of 10 ml/kg b.w. For each animal the individual dosing volume was calculated on the basis of the most recently measured body weight. Sterile water was used as vehicle and served as control group. Control animals were handled identically as treated groups. The highest doses of 1000 mg/kg b.w. were selected based on information of the highest dose from repeated dose toxicity studies. The item formulation was prepared freshly and administered daily during 14 days premating and 14 days mating period in both male and female, during the gestation period and up to post natal day 3 in females. Males were dosed for 28 days. Females were dosed throughout the study, 14 days of premating, mating, 14 days of gestation and 4 days lactation before sacrificed. Males were dosed daily for 28 days including 14 days of premating and then sacrificed. Additionally, all animals were fed an ad libitum supply of maintenance diet for rats Altromin 1324 and tap water, sulphur acidified to a pH of approximately 2.8.

Clinical observations

Animals were observed 1-2 times daily for general clinical signs.

Litter observations

Each litter was examined as soon as possible after delivery of the dam to establish the number and sex of pups, stillbirths, live births, runts and the presence of gross abnormalities. Live pups were counted, sexed and litters weighed within 24 hours of parturition, PND 0 and PND 4.

Pathology, Histopathology and organ weights

The sacrificed rats were examined macroscopically for any abnormalities and gross pathological changes. The numbers of implantation sites and corpora lutea were examined grossly in all pregnant females. The testes and epididymides of all males, and ovaries, uterus with the cervix of all females were weighed along with liver, kidneys, adrenals, thymus, spleen, brain, and heart. The following tissues from control and high dose group (1000 mg/kg b.w.) were examined for full histological evaluation: thymus, liver, kidney, adrenal, spleen, uterus with cervix, ovaries, vagina, testes, epididymides, accessory sex organs (prostate, seminal vesicles with coagulating glands) and all organs showing macroscopic lesions of all adult animals. All gross lesions were examined microscopically. These examinations were not extended to other dosage groups because no treatment related changes observed in the high dose group compared to control group.

Dead pups and pups were killed at post-partum day 4 and were carefully examined for gross external abnormalities.

Statistical analysis

All data of each group were expressed as mean±SEM (n = 10). Statistical analyses were performed with 'Graph Pad Prism (Version V)' software and were carried out using one way ANOVA followed by Dunnet multiple comparison test to reveal any differences between control and test groups. Statistical significance was set at $p<0.05$.

RESULTS AND DISCUSSION

Mortality and clinical observation

No mortality was observed during the entire period of the study. Predominant clinical signs were observed in the high and medium dose group. The animals were piloerection, aggressive behavior, moving the beddings, salivation and nasal discharge.

Body weight

In males, statistically significant decrease in body change was observed only between treatment day 28 and terminal sacrifice (day 29) in the high dose group of 1000 mg/kg when compared to controls. As there was no effect on overall body weight change and due to lack of dose dependency and minimal differences of the body weight change, this effect on body weight change was considered to be unrelated to the *E. longifolia* aqueous extract. In females, although significant body weight change was observed in the high dose group of 1000 mg/kg during premating day 1-7, no significant body weight change was observed 7-14 days compared to control. During gestation period, statistically significant body weight changes were observed in the low dose group of 250 mg/kg b. w. However, due to lack of consistency and no dose dependency, no toxicological relevance could be attributed to the finding.

Litter data

Statistical analysis of litter weight data revealed no treatment related effect on group mean litter weight, total litter weight, male litter weight and female litter weight when compared with controls. No treatment related effect was observed on the total number of pup born, number of males, number of females, sex ratio, live pups, still birth and runt. No treatment related gross external findings were observed in pups from any of the treated group during the lactation period.

Table 1: Body weight change (g)–males

		Group (n = 10 each group)			
	Day of treatment	**0 mg/kg**	**250 mg/kg**	**500 mg/kg**	**1000 mg/kg**
Pre mating	1-7	12.70±6.45	17.20±5.88	13.10±4.33	11.60±8.04
	7-14	22.60±6.00	22.90±7.82	15.20±7.58	18.20±7.63
Mating/Post Mating	14-21	6.50±3.54	7.70±5.36	5.50±5.80	10.10±4.93
	21-28	7.40±4.25	10.40±4.01	10.40±5.02	11.50±6.31
	28-29	3.60±2.41	1.30±3.09	1.50±3.37	-0.30*±2.95
	1-29	52.80±16.67	60.26±14.20	45.70±15.43	51.10±18.84

*significant (p<0.05) as determined with the individual data

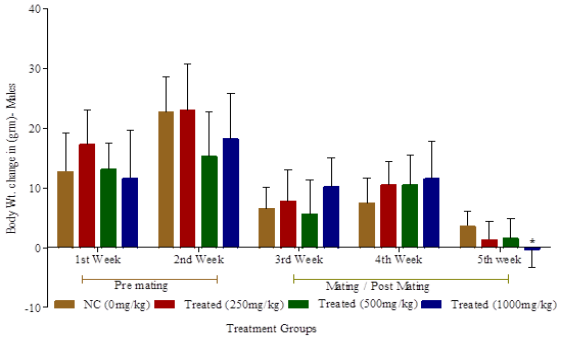

Fig. 1: Body weight of males supplemented with *E. longifolia* aqueous extract for the entire period of the study

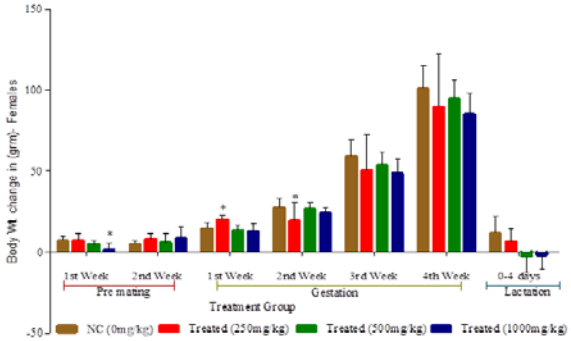

Fig. 2: Body weight of females supplemented with *E. longifolia* aqueous extract for the entire period of the study

Table 2: Body weight change (g)–females

		Group (n = 10 each group)			
Day of treatment		**0 mg/kg**	**250 mg/kg**	**500 mg/kg**	**1000 mg/kg**
Pre mating	1-7	7.10±2.81	7.30±4.30	4.90±2.42	1.70*±3.74
	7-14	4.80±2.35	8.20±3.39	6.00±5.75	8.60±6.79
Gestation	0-7	14.70±3.50	20.00*±2.93	13.50±3.37	12.63±5.07
	7-14	27.40±5.87	19.25*±11.29	26.80±3.79	24.25±3.06
	14-20	59.20±10.45	50.25±22.10	54.10±7.56	48.63±8.50
	0-20	101.30±13.89	89.50±33.20	94.40±11.74	85.50±12.35
Lactation	0-4	11.70±10.53	6.38±7.87	-2.80±9.67	-2.13±8.79

*significant (p<0.05) as determined with the individual data

Table 3: Precoital Interval and duration of gestation (days)

Parameter	**0 mg/kg**	**250 mg/kg**	**500 mg/kg**	**1000 mg/kg**
Precoital Interval	5.50±5.083	1.78*±0.972	2.20*±1.229	2.10*±1.449
	n = 9	n = 9	n = 10	n = 10
Duration of Gestation	21.60±0.516	21.50±0.535	21.60±0.516	21.75±0.463
	n = 10	n = 8	n = 10	n = 8

*significant (p<0.05) as determined with the individual data

Table 4: Reproductive performance of pairs during a 21-day breeding phase of rats exposed to *E. longifolia* aqueous extract

Reproductive parameters	***E. longifolia* aqueous extract dosages (mg/kg b.w.)**			
	0.0 n–10:10[a]	**250 n–10:10**	**500 n–10:10**	**1000 n–10:10**
Dams at term	10	8	10	8
Precoital interval	5.50±5.083	1.78*±0.972	2.20*±1.229	2.10*±1.449
Duration of Gestation	21.60±0.516	21.50±0.535	21.60±0.516	21.75±0.463
Corpora lutea	14.00±1.76	14.75±2.43	14.70±2.41	14.00±4.34
Implantation sites	12.60±1.58	12.25±2.76	12.00±2.75	12.63±1.85
Pups per litter	12.30±1.42	10.38±4.81	11.10±2.38	11.88±2.17
Live pups per litter on PND0	12.20±1.40	10.38±4.81	11.00±2.31	11.00±3.21
Live pups per litter on PND4	12.20±1.40	10.25±5.09	10.80±2.35	10.75±2.82
% Pre implantation loss	9.15±11.93	16.71±15314	16.43±22.15	6.79±11.90
% Pre implantation loss	2.20±3.54	15.75±31.29	6.77±13.34	10.55±19.36
% Mortality/cannibalism (PND1-4)	0.77±2.43	12.50±35.36	2.10±4.77	4.74±9.35
Viability index 9 (%)	99.23±2.43	87.50±35.36	97.90±4.77	95.94±7.78
Copulation index (%)	100	90	100	100
Fertility index (%)	100	80	100	80
Delivery index (%)	100	100	100	100

Data represent mean±SD and were analyzed by one way ANOVA followed by Dunnett's multiple comparison test, to identify differences between treatments, [a]Starting number of breeding pairs, * Significant (p<0.05) *E. longifoloia* aqueous extract differences, between treatment groups and controls. One male and one female from each treatment group were cohabited for 14–21 days and then separated.

Reproductive performance

No treatment related effect was observed on the duration of gestation when compared with controls.

Statistically significant decrease in precoital interval was observed in all treated groups when compared with controls showing positive effects that could be attributed to *E. longifolia* aqueous extract.

Pathology

Gross pathological observation of males, females at scheduled necropsy revealed no treatment related findings.

The histopathological evaluation of males and females reproductive organs did not reveal any histopathological lesions considered to be to *E. longifolia* aqueous extract related.

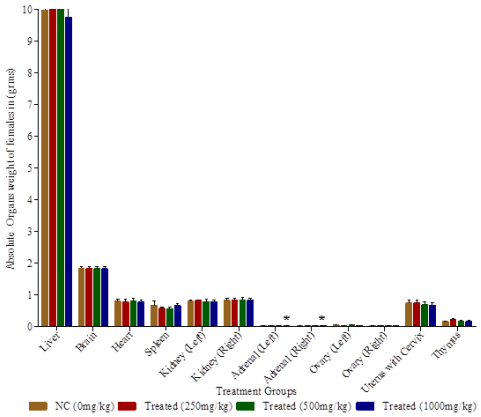

Fig. 3: Absolute organs weight of females supplemented with *E. longifolia* aqueous extract for the entire period of the study

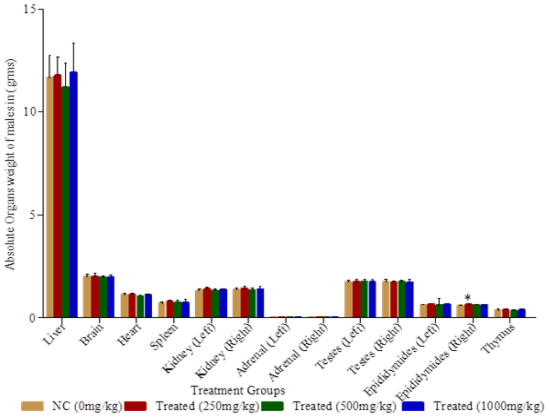

Fig. 4: Absolute organs weight of males supplemented with *E. longifolia* aqueous extract for the entire period of the study

Table 5: Absolute organs weight of females supplemented with *E. longifolia* aqueous extract for the entire period of the study

Parameters	*E. longifolia* aqueous extract dosages (mg/kg b.w.)			
	0.0 n–10[a]	250 n–10	500 n–10	1000 n–10
Liver	9.97±0.91	9.98±1.45	10.03±0.98	9.75±1.18
Brain	1.84±0.06	1.82±0.09	1.82±0.08	1.82±0.08
Heart	0.79±0.08	0.77±0.10	0.79±0.10	0.78±0.05
Spleen	0.67±0.15	0.57±0.05	0.56±0.07	0.67±0.07
Kidney (Left)	0.79±0.05	0.82±0.03	0.78±0.08	0.78±0.06
Kidney (Right)	0.82±0.08	0.84±0.05	0.84±0.08	0.83±0.07
Adrenal (Left)	0.03±0.005	0.03±0.003	0.03±0.003	0.03*±0.004
Adrenal (Right)	0.03±0.006	0.03±0.004	0.03±0.004	0.03*±0.002
Ovary (Left)	0.05±0.006	0.04±0.009	0.05±0.009	0.04±0.01
Ovary (Right)	0.04±0.009	0.04±0.005	0.04±0.006	0.04±0.006
Uterus with Cervix	0.74±0.11	0.75±0.08	0.68±0.11	0.67±0.09
Thymus	0.16±0.03	0.21±0.04	0.17±0.03	0.17±0.04

Data represent mean±SD and were analyzed by one way ANOVA followed by Dunnett's multiple comparison test, as determined with individual data, [a]The number of females per treatment group,* Significant ($p<0.05$) *E. longifolia* aqueous extract as determined with the individual data

Table 6: Absolute organs weight of males supplemented with *E. longifolia* aqueous extract for the entire period of the study

Parameters	*E. longifolia* aqueous extract dosages (mg/kg b.w.)			
	0.0 n–10[a]	250 n–10	500 n–10	1000 n–10
Liver	11.66±1.12	11.77±0.90	11.19±1.18	11.90±1.46
Brain	2.01±0.12	1.99±0.17	1.98±0.08	1.97±0.12
Heart	1.12±0.09	1.13±0.08	1.05±0.09	1.11±0.07
Spleen	0.70±0.08	0.79±0.08	0.75±0.11	0.74±0.15
Kidney (Left)	1.31±0.11	1.40±0.09	1.31±0.12	1.36±0.07
Kidney (Right)	1.36±0.11	1.42±0.10	1.33±0.12	1.38±0.14
Adrenal (Left)	0.03±0.004	0.03±0.004	0.03±0.002	0.03±0.004
Adrenal (Right)	0.03±0.008	0.03±0.003	0.02±0.003	0.03±0.003
Testes (Left)	1.73±0.12	1.74±0.13	1.77±0.12	1.74±0.12
Testes (Right)	1.73±0.14	1.72±0.08	1.75±0.10	1.72±0.14
Epididymides (Left)	0.62±0.05	0.67±0.05	0.62±0.34	0.64±0.05
Epididymides (Right)	0.59±0.04	0.65*±0.04	0.61±0.03	0.61±0.03
Thymus	0.36±0.08	0.39±0.04	0.34±0.05	0.38±0.08

Data represent mean±SD and were analyzed by one way ANOVA followed by Dunnett's multiple comparison test, as determined with individual data, [a]The number of males per treatment group, * Significant ($p<0.05$) *E. longifolia* aqueous extract as determined with the individual data

Table 7: Organ weight ratios of males supplemented with *E. longifolia* aqueous extract for the entire period of the study

Parameters	*E. longifolia* aqueous extract dosages (mg/kg b.w.)			
	0.0 n–10[a]	250 n–10	500 n–10	1000 n–10
Terminal body weight	368.10±22.87	374.60±19.13	361.30±15.67	367.60±25.27
Organ to body weight ratios				
Liver	3.16±0.19	3.14±0.18	3.09±0.20	3.23±0.22
Brain	0.55±0.04	0.53±0.04	0.54±0.02	0.53±0.04
Heart	0.30±0.02	0.30±0.01	0.29±0.02	0.30±0.01
Spleen	0.19±0.01	0.21±0.02	0.20±0.02	0.20±0.03
Kidney (Left)	0.35±0.02	0.37±0.02	0.36±0.02	0.37±0.02
Kidney (Right)	0.37±0.03	0.38±0.02	0.36±0.02	0.37±0.03
Adrenal (Left)	0.008±0.001	0.008±0.001	0.008±0.001	0.008±0.001
Adrenal (Right)	0.009±0.001	0.008±0.001	0.007±0.000	0.008±0.000
Testes (Left)	0.47±0.03	0.46±0.02	0.45±0.10	0.47±0.05
Testes (Right)	0.47±0.04	0.46±0.02	0.45±0.10	0.47±0.05
Epididymides (Left)	0.17±0.01	0.18±0.01	0.17±0.01	0.17±0.02
Epididymides (Right)	0.16±0.01	0.17±0.01	0.17±0.01	0.16±0.01
Thymus	0.09±0.02	0.10±0.01	0.09±0.01	0.10±0.02

Data represent mean±SD and were analyzed by one way ANOVA followed by Dunnett's multiple comparison test, to identify differences between treatments, [a]The number of males per treatment group.

Table 8: Organ weight ratios of females supplemented with *E. longifolia* aqueous extract for the entire period of the study

Parameters	*E. longifolia* aqueous extract dosages (mg/kg b.w.)			
	0.0 n–10[a]	250 n–10	500 n–10	1000 n–10
Terminal body weight	250.50±16.54	246.63±16.61	245.30±15.64	235.13±16.53
Organ to body weight ratios				
Liver	3.98±0.30	3.88±0.49	4.09±0.35	4.14±0.38
Brain	0.73±0.05	0.71±0.11	0.74±0.05	0.77±0.05
Heart	0.31±0.01	0.30±0.04	0.32±0.04	0.33±0.02
Spleen	0.26±0.05	0.22±0.04	0.23±0.03	0.28±0.03
Kidney (Left)	0.31±0.02	0.32±0.04	0.32±0.02	0.33±0.03
Kidney (Right)	0.33±0.03	0.33±0.05	0.34±0.04	0.35±0.02
Adrenal (Left)	0.01±0.002	0.01±0.002	0.01±0.001	0.01±0.002
Adrenal (Right)	0.01±0.002	0.01±0.002	0.01±0.001	0.01±0.001
Ovary (Left)	0.02±0.002	0.02±0.003	0.02±0.004	0.02±0.005
Ovary (Right)	0.02±0.003	0.02±0.003	0.02±0.002	0.02±0.002
Uterus with Cervix	0.29±0.03	0.30±0.06	0.28±0.06	0.29±0.05
Thymus	0.06±0.01	0.08±0.10	0.06±0.01	0.07±0.01

Data represent mean±SD and were analyzed by one way ANOVA followed by Dunnett's multiple comparison test, to identify differences between treatments, [a] The number of females per treatment group.

CONCLUSION

The study has shown that standardized *E. longifolia* aqueous extract (PHYSTA®) revealed no toxicological findings at the dosages of 250, 500 and 1000 mg/kg b.w. Based on the data generated from the study, the No-Observed-Adverse Effect Level [NOAEL] is 1000 mg/kg b.w. for reproduction/developmental toxicity screening in males and females Wistar rats.

The study provided initial information on possible effects on reproductive and development of *E. longifolia* aqueous extract, and also as dose range information for more extensive reproductive and developmental toxicity study [9]. Further reproductive and developmental study should be considered to extend the toxicity information of *E. longifolia* aqueous extract such as two-generation reproductive and development toxicity study. Teratology should also be considered in combination with neurotoxicity and immunology toxicity study.

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CONFLICT OF INTERESTS

The authors declare that there are no conflicts of interest.

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