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Original Article

EVALUATION OF THE GENOTOXICITY OF *EURYCOMA LONGIFOLIA* AQUEOUS EXTRACT (PHYSTA®) USING *IN VITRO* AMES TEST AND *IN VIVO* MAMMALIAN MICRONUCLEUS TEST

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ABSTRACT

Objective: In this study the genotoxic potential of *Eurycoma longifolia* aqueous extract (PHYSTA®) was investigated by the *in vitro* Ames test (Salmonella/microsome mutation assay) and the *in vivo* mouse peripheral blood cell micronucleus test. *Eurycoma longifolia* also known as Tongkat Ali in Malaysia, is traditionally used in South East Asia to treat fever, intestinal worms, mouth ulcers, headache, erectile dysfunction and many other general pains. It is traditionally used as a health tonic and anti-stress remedy. Recent studies of *Eurycoma longifolia* pharmacological profile have revealed antioxidant properties and other potentially useful biological activities thereby lending some scientific support to its use in folk medicine. Evaluation of the genotoxic potential is one of the most important nonclinical safety studies required for registration and approval for marketing of pharmaceutical products.

Methods: The Salmonella/microsome mutation assay (TA 98, TA 100, TA 102, TA 1535 and TA 1537; plate incorporation method) was performed in the presence or in the absence of extrinsic metabolic activation (S9 mixture). In the mouse micronucleus assay, *Eurycoma longifolia* aqueous extract was administered intraperitoneally (100, 250 and 500 mg/kg body weight; single dose) to male and female NMRI mice (N= 5 per dose per sex) and peripheral blood was analyzed after 44 and 68 h after treatment using a flow cytometer to determine micro nucleated polychromatic (immature) erythrocytes.

Results: The results were expressed as the relative proportion of polychromatic erythrocytes among total erythrocytes (relative PCE). Tested at doses up to 5 mg/plate, the *Eurycoma longifolia* aqueous extract (PHYSTA®) was not toxic to Salmonella tester strains and did not increase the number of revertant colonies over the background incidence. In the mouse peripheral blood cell micronucleus assay, the extract did not alter the relative PCE, nor did it increase the incidence of micro nucleated polychromatic erythrocytes.

Conclusion: Based on the aforementioned findings, it is concluded that the *Eurycoma longifolia* aqueous extract (PHYSTA®) has no mutagenic potential and considered to be non-genotoxic with respect to clastogenicity.

Keywords: *Eurycoma longifolia, Ames test, Micronucleus assay, Genotoxicity.*

INTRODUCTION

The use of medicinal plants has substantially increased in the last decades and a World Health Organization survey indicated that 70–80% of the world population still relies on herbal-based traditional medicine for their primary healthcare [1]. In Malaysia where more than 15,000 flowering plant species grow, over 3000 species have been identified as possible medicinal plants and current Malaysian market for herbal and natural products has been estimated to be worth USD 1.4 billion [2].

Eurycoma longifolia (Simaroubaceae), locally known as "Tongkat Ali", is a small evergreen shrub tree commonly found in the tropical forests of South East Asia (Indonesia, Thailand, Malaysia and the Philippines). It is a dioecious plant, with male and female flowers produced in large panicles, on different trees. The pinnate leaves, 20–40 cm long with ovate–lanceolate leaflets, are spirally arranged. Across Southeast Asia, *Eurycoma longifolia* root is used as a traditional remedy for treating malaria, cancer, anxiety, ulcers, fatigue, infertility and impotence [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]. Malaysian traditional medicine is known to have utilized at least 1300 different plants, with Eurycoma longifolia root holding a prominent place in the local culture.

Eurycoma longifolia root 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 [5]. Human clinical observation with placebo control was conducted for *Eurycoma longifolia* aqueous extract showing the extract to be non toxic even at a high dose of 600 mg to liver function, renal function, hematological profile, lipid profile, body electrolytes and body immune system, as well as the cancer markers, specifically Prostate Specific Antigen and the various hormones in the body [6]. Animal studies in mice have shown that LD50 of *Eurycoma longifolia* aqueous extract was more than 3000 mg/kg [7]. Acute, subacute and subchronic studies in rat have shown that 1000 mg/kg of *Eurycoma longifolia* water extract has no observed adverse event and toxicity [8].

Notwithstanding the potential usefulness of herbal drugs, numerous reports of adverse effects and fatalities have highlighted that traditional herbal medicines also need to be evaluated regarding their safety. It is of note that some bioactive compounds present in plants have been reported to interfere with drug kinetics and to produce adverse effects related or unrelated to their pharmacological actions, such as allergic reactions, mutagenic and carcinogenic effects, and several other toxic effects [9]. Evaluation of the genotoxic potential is one of the most important nonclinical safety studies required for registration and approval for marketing of pharmaceutical products. Furthermore, studies on the genotoxicity of medicinal plants used by the population are needed to identifying those which pose mutagenic and carcinogenic risks.

The *in vitro* Ames test, which was conducted using strains of *Salmonella typhimurium*, is a widely used bacterial assay for the identification of chemicals that can produce gene mutations, and it shows a high predictive value with rodent carcinogenicity tests [10]. The *in vivo* mammalian micronucleus test is used for the detection of damage induced by the test item to the chromosomes or the mitotic

apparatus of erythroblasts by analysis of erythrocytes as sampled in the bone marrow of animals, usually rodents. The conducted study involves measurement of micronucleated polychromatic (immature) erythrocytes in peripheral blood, which is equally accepted since the spleen of mice is unable to remove micronucleated erythrocytes.

The present studies were undertaken to evaluate the genotoxicity of a standardized *Eurycoma longifolia* aqueous extract (PHYSTA®) in the Ames test and in the mouse *in vivo* micronucleus test. The study was conducted to comply with OECD Principles of Good Laboratory Practice and OECD Guidelines for Testing of Chemicals, Section 4, No. 471 "Bacterial Reverse Mutation Test" and OECD Guidelines for Testing of Chemicals, Section 4, No. 474 "Mammalian Erythrocyte Micronucleus Test", adopted July 21, 1997.

MATERIALS AND METHODS

Plant material and extract preparation

The extract was obtained from a commercial batch of PHYSTA® from Phytes Biotek Sdn Bhd, Malaysia. The standardized aqueous 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) 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; 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.

Genotoxicity assay

Salmonella typhimurium/Microsome assay

Chemicals

Dimethylsulfoxide (DMSO), nicotine-adenine dinucleotide phosphate (NADP) and glucose-6-phosphate (G6P) were purchased from Sigma Aldrich Chemical Co® (St. Louis, MO, USA). Magnesium chloride ($MgCl_2$), potassium chloride (KCl), sodium phosphate salts were purchased from Ajax Finechem Pty Ltd, Australia. Bacto Agar was purchased from Bacto Laboratories Pty Ltd, Australia

Positive control mutagens

Sodium azide (SA), Acridine mutagen ICR191, Mitomycin-C, 2-nitrofluorene (2-NF), 2-aminofluorene (2-AF) and 2-aminoanthracene (2-AA) were all purchased from Sigma Aldrich Chemical Co® (St. Louis, MO, USA).

Bacterial strain

Salmonella typhimurium strains TA 98, TA100, TA 102, TA 1535 and TA 1537; were sourced/origin from Moltox (Molecular Toxicology Inc, USA).

Metabolic activation system (S9 mixture)

Lyophilized rat liver S9 fraction induced by Aroclor 1254 was purchased from Celsis, *In vitro* Technologies.

Mutagenicity assay method

The *Salmonella typhimurium*/microsome assay was performed by the standard plate incorporation method with and without addition of an extrinsic metabolic activation system (S9 mixture) according to OECD 471 (The OECD guideline for testing of chemicals in a Bacterial Reverse Mutation Test) at a GLP complaint facility (TetraQ, Australia; GLP No. 15153). Basically, 100 µl of an overnight grown culture (containing approximately 1–2×109 bacteria per ml) was added into culture tubes which contained overlay agar, 100 µl of *Eurycoma longifolia* aqueous extract (0.005, 0.01, 0.03, 0.05, 0.3, 1, 3 and 5 mg/plate) or 50 µl standard mutagens (positive control) or deionised water (negative control), and 500 µl of sodium phosphate buffer (without S9) or 500 µl of S9 mixture. After 48 hours of incubation at 37 0C, all plates were checked for the presence of the background lawn and compared to the negative control group plates. Numbers of revertant bacterial colonies were counted and compared with those in negative and positive control plates. Every experiment was carried out in triplicate. Sodium azide (4 µg/plate), Acridine mutagen ICR191 (2 µg/plate), Mitomycin-C (0.5 µg/plate), 2-nitrofluorene (2 µg/plate), 2-aminofluorene (µg/plate) and 2-aminoanthracene (1 µg/plate) were employed as positive controls. Sodium azide and mitomycin C were dissolved in de ionised water while DMSO was used to dissolve the remaining positive control mutagens.

Mouse erythrocyte micronucleus assay

Chemicals

Li-heparin tubes were obtained from Sarstedt Germany (41.1503.005). Methanol, Propidium iodide (PI), Cyclophosphamide (CPA), Hank's balanced salt solution was purchased from Sigma Aldrich Chemical Co® (St. Louis, MO, USA). Sodium chloride was purchased from Delta Select, Munchen Germany. Specific antibodies against anti-mouse CD71-FITC and CD61-RPE (labeled with of Fluroscein-isothiocynate and Phycoerythrin) were purchased from eBioscience (San Diego, CA, USA) and AbDl Serotec (Kidlington, UK) respectively.

Experimental animals

Specific-pathogen-free male and female NMRI mice (n = 50), aged 6–12 weeks were used in this study. The animals were obtained from Charles River, 97633 Sulzfeld, Germany. The research was conducted in accordance with the Principle and Guide to Ethical Use of Laboratory Animals, MOH and OECD TG 474 Guidelines for Mammalian Erythrocyte Micronucleus Test (OECD, 1997) at a GLP complaint facility (BSL Bioservice Scientific Laboratories GmbH). The animals were housed in IVC cage (poly sulphone; Type II L; five mice of identical sex per cage) bedded with Altromin saw fiber. The animals were provided with commercially available rodent feed (Altromin 1324 maintenance diet for rats and mice) and water ad libitum. The animals were maintained under controlled environmental conditions of 12 hours light/dark period, 22±3 °C room temperature and humidity at 55±10 %.

Treatment

Intraperitoneal route was selected to maximize the exposure in an absence of oral bioavailability data of *Eurycoma longifolia* aqueous extract. In a pre-experiment study, three male animals received single intraperitoneal dose of 2000 mg/kg, 1000 mg/kg and 500 mg/kg of *Eurycoma longifolia* aqueous extract respectively. Animals with dose of 2000 mg/kg and 1000 mg/kg intraperitoneally showed severe toxicity. Animal with 500 mg/kg intraperitoneally showed clear tolerable toxicity, hence 500 mg/kg was chosen as the maximum tolerable dose in the study. The animals were randomly assigned to 7 groups (5 animal of each sex per group) and tail tagged. Three treatment groups received the single dose of *Eurycoma longifolia* aqueous extract (500, 250 and 100 mg/kg b. w) by intraperitoneal route. A fourth group (positive control) was treated with a single intraperitoneal injection of CPA 40 mg/kg b.w. 24 hours prior to euthanasia and fifth group served as negative control. All mice were weighed on a daily basis and were also observed for any clinical sign of toxicity after treatment. Sampling of peripheral blood was carried out on animals after 44 and 68 hours after treatment. The sixth and seventh groups were the additional group to evaluate plasma content of Eurycomanone (to ensure exposure and bio-availability of *Eurycoma longifolia* aqueous extract). The sixth group received intraperitoneally single dose of 500 mg/kg *Eurycoma longifolia* extract and seventh group was the negative control. The groups were sacrificed after 2 hours of administration.

Blood preparation for the flow cytometry and micronucleus scoring

Blood was obtained from the tail vein after its incision and collected in the Li-heparin tubes. Blood cells were fixed immediately in ultra cold (-80 0C) methanol and stored at-80 0C. After 16 hours of fixation, it is washed with Hank's balanced salt solution, centrifuged at 600 x g for 5 minutes and supernatant discarded. The cell populations were discriminated using specific antibodies against anti-mouse CD71-FTC (expressed at surface of immature erythrocytes) and hamster anti-mouse CD61-RPE (expressed at surface of platelets) and DNA content of micronuclei was

determined by the use of DNA specific stain (PI). Evaluation of all samples was performed using a flow cytometer (FACScan, BD Biosciences). Antibodies were labeled with Fluorescein-isothiocynate (FITC) and Phycoerythrin (PE) and measured for fluorescent intensity. Ten thousand (10,000) immature erythrocytes per animal were scored for incidence of micronucleated polychromatic (immature) erythrocyte. The results were expressed as relative proportion of polychromatic erythrocytes among total erythrocytes (relative PCE) by calculating ratio polychromatic erythrocytes (PCE) to normochromatic erythrocytes (NCE).

Blood preparation for plasma Eurycomanone evaluation

Blood was obtained by cardiac punctual and collected in the Li-heparin tubes. Blood cells were fixed immediately in ultracold (-80 0C) methanol and stored at-80 0C. The plasma was then quantified for Eurycomanone using Liquid Chromatography Mass Spectrum method (table 2, fig. 5).

Statistical analysis

Statistical comparisons were made by non-parametric tests (Kruskal–Wallis followed by the Mann–Whitney test). In any case a difference was considered as statistically significant when P ≤0.05.

RESULTS AND DISCUSSION

Salmonella typhimurium/microsome assay

Since *Eurycoma longifolia* aqueous extract had shown no toxicity towards tester strains, a dose as high as 5 mg per plate was fixed as the upper limit of the dose range tested. *Eurycoma longifolia* aqueous extract, tested in doses up to 5 mg per plate, did not increase the number of histidine revertant colonies over the negative control values (table 1). Results therefore indicated that *Eurycoma longifolia* aqueous extract was not mutagenic in the Salmonella/microsome assay.

Mouse erythrocyte micronucleus assay

The animal treated with single dose intraperitoneal 500 mg/kg of *Eurycoma longifolia* aqueous extract showed toxic effect such as reduction of spontaneous activity, constricted abdomen, piloerection and half eyelid closure; and single dose intraperitoneal 100 mg/kg and 250 mg/kg showed no toxicity effects. The relative PCE remained unaltered in the treated groups, a finding that indicated target cell exposure and non toxic effect of *Eurycoma longifolia* aqueous extracts to the peripheral blood erythrocytes (fig. 1 & 2). The proportion of polychromatic erythrocytes with micronuclei (MNPCEs) noted in treated groups was reduced none significantly at 44 hours and 68 hours from the background incidence recorded in the vehicle-control group (fig. 3 & 4). The positive control drug (CPA; 40 mg/kg body weight i. p.), however, markedly increased the frequency of MNPCEs over the background incidence thereby confirming that the assay was sensitive to detect genotoxic substances. Eurycomanone (marker for *Eurycoma longifolia* aqueous extract) was found in the blood of the treated animal and not in the negative control proved the bio-availability of the *Eurycoma longifolia* aqueous extract by intraperitoneal route.

Table 1: Mutagenicity testing of *Eurycoma longifolia* aqueous extract in the salmonella/microsome assay [TA 98, TA100, TA102, TA 1535 and TA 1537 tester strains]

Dose (µg/plate)	TA 98		TA100		TA102		TA 1535		TA 1537	
	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
0.005			157±11	151±7						
0.01			152±16	155±13						
0.03			154±16	140±17						
0.05	34±3	23±5	148±17	139±15	486±60	525±39	21±4	12±2	13±5	6±2
0.3	28±2	22±3	143±4	152±13	483±30	531±32	17±2	11±2	9±3	7±5
1.0	27±8	22±2	149±15	143±12	513±27	467±50	26±4	7±3	8±1	5±3
3.0	32±2	25±7	145±12	137±13	559±34	524±21	25±8	10±4	10±2	8±4
5.0	37±7	22±3	146±15	129±10	501±27	568±74	22±2	10±1	10±2	7±2
0	41±8	23±3	146±15	162±16	519±48	454±32	20±6	15±5	7±2	4±1
0+	25±7	19±8	123±7	120±9	409±14	437±28	22±2	10±5	9±2	7±2
PC	452±5	693±41	1964±92	240±25	2203±141	2511±231	2207±203	140±14	1023±231	65±4

Values are mean±SD of 3 plates. With (+S9) and (-S9) without addition of liver post-mitochondrial fraction (S9) from rats pretreated with aroclor 1254. Doses 0–negative control (solvent): 100 µl H2O; 0+–negative for positive control: DMSO or H2O; PC, positive control: for TA98/-S9, 2-NF (2µg/plate); TA98/+S9, 2-AA (1 µg/plate); TA100/-S9, NANa3 (4 µg/plate); TA100/+S9, 2-AA (0.5 µg/plate); TA102/-S9, MMC (0.5 µg/plate); TA102/+S9, 2-AA (4 µg/plate); TA1535/-S9, NANa3 (4 µg/plate); TA1535/+S9, 2-AA (2 µg/plate); TA1537/-S9, ICR191 (2 µg/plate); TA1537/+S9, 2-AA (2 µg/plate).

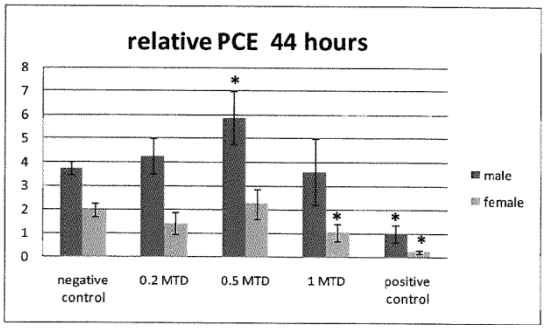

Fig. 1: Effects of *Eurycoma longifolia* aqueous extract (100, 250 & 500 mg/kg b. w i. p) and of a positive control drug (CPA 40 mg/kg b. w ip) on Relative PCE at 44 h. Data are presented as mean and SD and comparisons were made by the Kruskal–Wallis and the Mann–Whitney U-tests. Differences (P<0.05) are indicated by an asterisk (*). N= 5 male+5 female mice per dose group

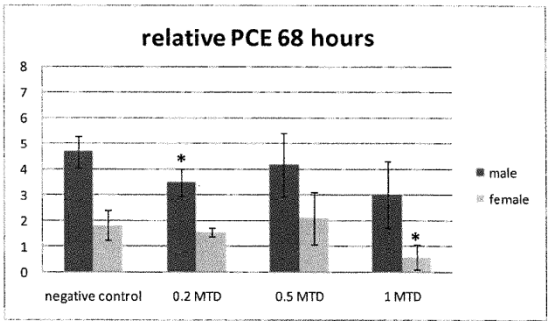

Fig. 2: Effects of *Eurycoma longifolia* aqueous extract (100, 250 & 500 mg/kg b. w i. p) and of a positive control drug (CPA 40 mg/kg b. w i. p) on Relative PCE at 68 h. Data are presented as mean and SD and comparisons were made by the Kruskal–Wallis and the Mann–Whitney U-tests. Differences (P<0.05) are indicated by an asterisk (*). N= 5 male+5 female mice per dose group

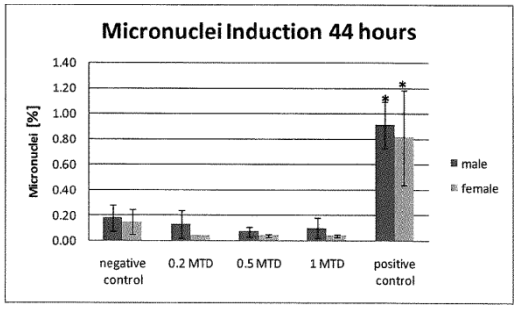

Fig. 3: Effects of *Eurycoma longifolia* aqueous extract (100, 250 & 500 mg/kg b. w i. p) and of a positive control drug (CPA 40 mg/kg b. w i. p) on the incidence (%) of MNPCE at 44 h. Data are presented as mean and SD and comparisons were made by the Kruskal–Wallis and the Mann–Whitney U-tests. Differences (P<0.05) are indicated by an asterisk (*). N= 5 male+5 female mice per dose group

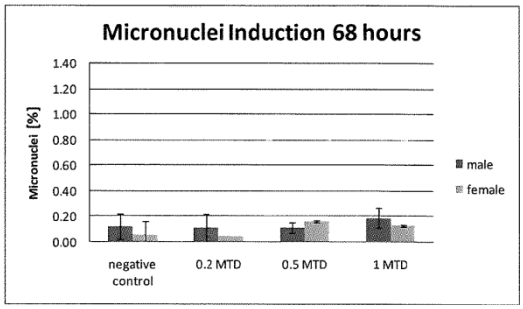

Fig. 4: Effects of *Eurycoma longifolia* aqueous extract (100, 250 & 500 mg/kg b. w i. p) and of a positive control drug (CPA 40 mg/kg b. w i. p) on the incidence (%) of MNPCE at 68 h. Data are presented as mean and SD and comparisons were made by the Kruskal–Wallis and the Mann–Whitney U-tests. Differences (P<0.05) are indicated by an asterisk (*). N= 5 male+5 female mice per dose group

Table 2: Eurycomanone concentration in blood sample measured by LCMS

Group		Concentration of Eurycomanone (ng/ml)
500 mg/kg i. p. of	Male	105.4
Eurycoma longifolia aqueous extract		134.2
		119.8
		139.1
		59.3
	Female	122.8
		64.8
		67.7
		133.6
		131.9
Negative control	Male	Not detected
		Not detected
		Not detected
		Not detected
		Not detected
	Female	Not detected
		Not detected
		Not detected
		Not detected
		Not detected

LOQ is 200ng/ml, LOD is 50ng/ml

CONCLUSION

The identification of products with chemicals or compounds capable of inducing mutations is crucial in safety assessment since mutagenic compounds can potentially induce cancer [11, 12]. Gene mutations can be measured in bacteria, where they cause a change in the growth requirements. The Ames test, which is conducted using Salmonella typhimurium, is a widely used bacterial assay for the identification of chemicals that can produce gene mutations, and it shows a high predictive value with rodent carcinogenicity tests. In some isolated studies, pure *Eurycoma longifolia* extracts have been shown to be non-mutagenic at 250 µg/ml [13]. The present studies were undertaken to evaluate the genotoxicity of a standardized *Eurycoma longifolia* aqueous extract, namely Physta™ in the Salmonella/microsome assay and in the mouse peripheral blood cell micronucleus assay.

Results from the Salmonella/microsome assay showed that, tested up to a very high dose (5 mg of dry extract per plate), *Eurycoma longifolia* aqueous extract did not produce any increase of the number of histidine revertant colonies over the negative (solvent) control values obtained for tester strains TA 98, TA 100, TA 102, TA 1535 and TA 1537; either in the presence or in the absence of extrinsic metabolic activation (Aroclor 1254-induced rat liver S9). Since the standard mutagens used in this study (SA, ICR191, MMC, 2-NF, 2-AF and 2 AA) induced a clear positive response, the foregoing results indicated that the *Eurycoma longifolia* aqueous extract was not mutagenic in the assay.

The clastogenicity of *Eurycoma longifolia* aqueous extract was evaluated in NMRI mice. Previous studies had indicated that the systemic toxicity of *Eurycoma longifolia* aqueous extracts given by the oral route to rodents is very low. In Wistar rats, an acute oral toxicity study found no mortality and no overt toxicity up to the highest dose of *Eurycoma longifolia* extract tested (>2000 mg/kg b.w. po) while a 28 & 90-day repeated dose study found no deaths and no other adverse effects on rats treated orally with doses of *Eurycoma longifolia* aqueous extract up to 1000 mg/kg b. w/day [8]. Considering the lack of overt toxicity in the aforementioned rodent studies and in a preliminary experiment with NMRI mice, 500 mg/kg of b. w was set as the upper limit of the dose range tested in this study. The intraperitoneal administration of *Eurycoma longifolia* aqueous extract to male and female mice did not cause any alteration of the relative PCE thereby indicating that it was not clastogenic or aneugenic. While a single non-clastogenic dose of the positive control drug (CPA) markedly enhanced the occurrence of micronuclei, single treatment with doses of *Eurycoma longifolia* aqueous extract ranging from 100 up to 500 mg/kg/b. w once did not induce any increase of micronucleated PCE over the background frequency recorded in the vehicle-control group. The results of the *in vivo* assay were thus consistent with the outcome of the *in vitro* mutagenicity test. Both assays strongly suggest that consumption of

Eurycoma longifolia aqueous extracts does not pose genotoxic hazards.

This study indicated that *Eurycoma longifolia* aqueous extract was not mutagenic in the *in vitro* Salmonella/microsome assay, or clastogenic in the *in vivo* mouse peripheral blood cell micronucleus test. Based on these results it is concluded that mammalian toxicity of the standardized *Eurycoma longifolia* aqueous extracts (PHYSTA®) is low and their use pose no genotoxic risks to individuals.

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CONFLICT OF INTERESTS

The authors declare that there are no conflicts of interest

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