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

YEE K MING¹, NORAIASYAH BT ZULKAWI¹, VANDANA K CHOU DHARY², YOGENDRA K CHOU DHARY²

Biotrops Malaysia¹, Division of Product Development Berhad, Lot 21 Jalan U1/19, Section U1, Hicom-Glenmarie Industrial Park, 0150 Shah Alam Selangor, Malaysia, Ethix Pharma², Division Of Toxicology And Clinical Affairs, G-111, Landmark Business Centre, Old Bus Stand, Karbala Road, Bilaspur-495001, Chhattisgarh, India.

Email: yogendrakumar.choudhary@gmail.com

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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 micronucleated polychromatic (immature) erythrocytes.

Results: The results were expressed as 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 micronucleated 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 [5]. 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 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 LSD50 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 bone marrow of animals, usually rodents. The conducted
study involves measurement of micronucleated polychromatic
(mature) 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 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

**MATERIALS AND METHODS**

*Plant Material and Extraction Preparation*

The extract was obtained from a commercial batch of PHYSTA® from
Phytes Biotech 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
anhydrous 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 (MgCl₂), potassium chloride (KCl), sodium phosphate salts
were purchased from Ajax Finechem Pty Ltd, Australia. Bacto Agar
was purchased from Becto 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 (1 µg /plate) were employed as positive controls.
Sodium azide and mitomycin C were dissolved in deionised 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).
Ethanol, 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-FTC and CD61-RPE (labeled with of
Fluorescein-isothiocyanate and Phycocerythrin) were purchased from
eBioscience (San Diego, CA, USA) and AbD 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 (polysulphone; 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
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, 1000mg/kg and
500mg/kg of *Eurycoma longifolia* aqueous extract respectively.

**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 (9 mixture) according to
OECD 471 (The OECD guideline for testing of chemicals in a Bacterial
Reverse Mutation Test) at a GLP complaint facility (Toltox,
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 50µl standard mutagens (positive control) or
denonized water (negative control), and 500µl of growth buffer (without (59) or 500µl of 9 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 deionised water
while DMSO was used to dissolve the remaining positive control
mutagens.
mice 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-isothiocyanate (FITC) and Phycocerythrin (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°C) methanol and stored at -80°C. 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 500mg/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 100mg/kg and 250mg/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 non 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].**

<table>
<thead>
<tr>
<th>Dose (µg/plate)</th>
<th>TA 98</th>
<th>TA100</th>
<th>TA102</th>
<th>TA 1535</th>
<th>TA1537</th>
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<tbody>
<tr>
<td></td>
<td>-S9</td>
<td>+S9</td>
<td>-S9</td>
<td>+S9</td>
<td>-S9</td>
</tr>
<tr>
<td>0.005</td>
<td>157±11</td>
<td>151±7</td>
<td>152±16</td>
<td>155±13</td>
<td>143±12</td>
</tr>
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<td>0.01</td>
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<td>155±13</td>
<td>155±16</td>
<td>140±17</td>
<td>142±16</td>
</tr>
<tr>
<td>0.03</td>
<td>148±17</td>
<td>139±15</td>
<td>486±60</td>
<td>525±39</td>
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<tr>
<td>0.3</td>
<td>143±4</td>
<td>152±13</td>
<td>483±30</td>
<td>531±32</td>
<td>17±2</td>
</tr>
<tr>
<td>1.0</td>
<td>141±12</td>
<td>513±27</td>
<td>467±50</td>
<td>26±4</td>
<td>7±3</td>
</tr>
<tr>
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<td>467±50</td>
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<td>0+</td>
<td>16±16</td>
<td>519±48</td>
<td>45±32</td>
<td>20±6</td>
<td>15±5</td>
</tr>
<tr>
<td>PC</td>
<td>420±25</td>
<td>220±14</td>
<td>437±28</td>
<td>22±2</td>
<td>10±5</td>
</tr>
</tbody>
</table>

Values are mean±S.D 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): 100µl 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, NANA (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, NANA (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 i.p) 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.
Results from the Salmonella/microsome assay showed that, based on in vitro Salmonella/microsome assay and in the mouse peripheral blood cell micronucleus assay, Eurycoma longifolia aqueous extract was undertaken to evaluate the genotoxicity of a standardized extract 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 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-AAF 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 bw 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/bw 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.

**CONCLUSION**

The authors declare that there are no conflicts of interest.

**ACKNOWLEDGEMENTS**

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