Asian Journal of Pharmaceutical and Clinical Research
Vol. 4, Issue 3, 2011 ISSN - 0974-2441

Research Article

TOXICITY STUDIES OF *EURYCOMA LONGIFOLIA* (JACK)-BASED REMEDIAL PRODUCTS

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ABSTRACT

Eurycoma longifolia Jack is a popular herb that is commonly mixed with other reputedly nontoxic herbs in a non-traditional (new) formulation. In this study, the effect of mixing herbs in a polyherbal product on the overall toxicity status of the polyherbal products was studied using three commercial polyherbal products containing mixture of *E. longifolia* with other herbs, a product which contains only *E. longifolia* and an authenticated *E. longifolia*. *Eurycoma longifolia* or the herbal products were extracted with methanol-chloroform and the extracts were challenged with human cells, Hep2. Effects of the extracts on Hep2 cells viability was analysed using 3-4,5-dimethylthiazole-2,5-diphenyl-tetrazolium bromide (MTT) assay. The extracts were also tested for mutagenicity using Ames test employing *Salmonella* TA98 and *Salmonella* TA100. Cadmium, As, Pb, Mn and Cu content of the products were analysed by flame atomic absorption spectrometry. IC_{50} of crude extract of pure *E. longifolia* and a product containing only *E. longifolia* was 22.23 µg ml^{-1} and 50.00 µg ml^{-1}, respectively. Two of the polyherbal products had IC_{50} of 15.20 µg $ml^{-1}$and 18.89 µg ml^{-1}, respectively. All extracts, except a product containing mixture of *E. longifolia* and *Cistanche deserticola,* was not mutagenic. Heavy metals were detected in very low concentrations in all products. Under the condition of this study, it can be concluded that there is a risk of increased cytotoxicity and mutagenicity of extract of *E. longifolia*-based remedies compared to the toxicity of remedies containing solely *E. longifolia*. Toxicity of newly formulated polyherbal products cannot be deduced from the information of the toxicity of each individual component of the polyherbal products.

Key words: Cytotoxicity, *Eurycoma longifolia*, heavy metals, mutagenicity, polyherbal remedies.

INTRODUCTION

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[1]. It has been used as a medicinal herb in Southeast Asia mainly to increase libido and to a lesser extent to improve general health[2]. Other traditional uses include treatment of malaria, bleeding, ulcers and hypertension[3].

E. longifolia has been mixed with other reputedly nontoxic herbs in many herbal products preparation for improving general health rather than to improve strength and power during sexual activities alone. Many of the formulations are non-traditional[4]. However, mixing of *E. longifolia* with other herbs may increase the chances of *E.longifolia* based-herbal products being contaminated with toxic materials such as heavy metals and it may change the cytotoxicity status due to combination of various plants metabolites.

Although there have been a lot of *E. longifolia* preparations have been marketed, in either single or combined preparations, there has not been much study done on toxicity and safety of *E. longifolia* or *E. longifolia*-based products in humans. Animal studies in mice have shown that dose of *E. longifolia* of 1500 to 2000 mg/kg killed 50% of the test animals. Dose of 600 mg/kg daily was associated with signs of toxicity such as increased weight of liver, kidneys, spleen, and testes, and toxicity increased to 100-fold in intraperitoneal administration as compared to oral[5].

The identification of products with chemicals or compounds capable of inducing mutations is crucial in safety assessment since mutagenic compounds can potentially induce cancer [6-7]. 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[8]. Pure *E. longifolia* extracts have been shown to be nonmutagenic at 250 µg ml^{-1} [9]. However, there were little or no report of mutagenic potential of extract of polyherbal products containing *E. longifolia*. Recent analysis of 100 products that contain *E. longifolia* in Malaysia showed that 36% and 8% of these products possessed 0.52-5.3 and 10.64-20.72 ppm of mercury[4] and lead[10] respectively and these values exceeded the limit set by the Malaysian government[11]. Mercury and lead are cytotoxic and mutagenic[12].

In this study the effect of combining *E. longifolia* with other herbs on cytotoxicity was studied using tissue culture method. Mutagenic potential of the herbal products was determined by measuring gene mutation in bacteria. Heavy metals of the products were also analysed to determine their potential as toxicants in the products.

MATERIALS AND METHODS

Herbal products

Herbal products namely EG, PB, TA, and GE were purchased form retail shops in Kuala Lumpur, Malaysia. Product EG contains solely *E. longifolia* while PB, TA and GE were mixed herbal products containing *E. longifolia* as major constituents. Table-1 shows the constituents of the products and their intended usage. All herbal products were sold in bottles bearing the scientific name of the herbal plants used in the preparation of the products and production batch number. All products were fine powder (140 mesh) packed in capsules and were at least 6 months before the expiry date. Three bottles of products from different batch numbers for each herbal product were used in the analysis.

Sample of *E. longifolia*

Eurycoma longifolia was collected by the staff of the Forest Research Institute of Malaysia (FRIM) and authenticated by the botanist of the Institute. The specimen was deposited at FRIM and the root was dried and ground to powder.

Extraction of herbal products and *E. longifolia*

Herbal samples (25 g) were extracted with 50 ml methanol/chloroform solution (1:1)[13]. The extract was filtered through filter paper (Whatman no.1) and then evaporated in rotary evaporator at 40°C to approximately 3 ml and then stored in a dark glass vial at 2-4°C. The filtrate was evaporated to dryness before the cytotoxicity assay and mutagenicity test were carried-out. The dried filtrate was weighed and dissolved in ethanol.

Cytotoxicity test

Human cell lines, Hep2, (purchased from The European Collection of Cell Culture, Salisbury, UK) were cultured as described by Betancur-Garvis *et al* [14]. The cells were grown in the 75 cm^2 cell culture dish as a monolayer in the Minimum Essential Medium with Earl's salts and glutamine (MEM, BioWhittaker BE12-611F) and added with Non-

essential amino acids (BioWhittaker Be1-114E), Penicillin-Streptomycin (100 IU/ml, Sigma P-0906), Amphotericin B (0.25 µg/ml, BioWhittaker BE17-836E) and Foetal Bovine Serum (10%, BioWhittaker). Culture dish was incubated at 37°C in an incubator (5% CO_2, 95% air) for 3 days or until the cells were 70-80% confluence. When the cell lines were approximately 80% confluence, the growth medium was removed by aspiration and the cells were rinsed with 5 ml phosphate buffer solution (PBS). The washing solution was discarded and 4 ml of trypsin (0.1%)/EDTA (0.04%) solution was added.

The cells were incubated at 37°C for 5 minutes and then the culture dish was tapped to detach the cells. Phosphate buffer solution (6 ml) was added and the cells were transferred into a centrifuge tube. The cells were centrifuged at 1000 rpm for 3 min. The supernatant was discarded and 10 ml MEM was added. The cells were counted using a Haemocytometer (Neubaur improved) and then diluted to approximately 5 x 10^5 cells/ml. Cell suspension (195 µl) was seeded onto a flat bottom 96-well tissue microtitre culture plate and incubated at 37°C for 24 h. The crude extracts were diluted in the MEM in order to achieve the required working stocks and 5 µl of the diluted extracts were added to the culture wells to give final extracts concentration of 7.82, 15.63, 31.25, 62.5 and 250 µg ml^{-1}.

Each concentration was done in quadruplicate and untreated cells served as negative control. All culture plates were incubated in 5% CO_2 at 37°C for 48 h[15]. Once the incubation was completed, the cytotoxicity of the herbal extracts was evaluated by using MTT[14]. The supernatants were removed from all wells and 25 µl of 3-4,5-dimethylthiazole-2,5-diphenyl-tetrazolium bromide (MTT) in PBS (2 mg ml^{-1}) was added to each well and the plate was incubated in 5% CO_2 at 37°C for 2 hours. Then, 125 µl Dimethyl-sulfoxide (DMSO) was added to dissolve any intracellular formazan crystals and agitated for 15 min on a rotary shaker. Absorbance was measured at 492 nm in an ELISA plate reader.

The absorbance reading of test materials and untreated cells (control) were corrected by subtracting the background absorbance from wells containing no cells (blank). The percentage of cytotoxicity was calculated as, [(A-B)/A] x 100, where A is the mean optical density of control wells and B is the optical density of wells with plant extracts. The IC_{50} concentration, determined as an effective dose to reduce the growth to 50% of the control value (50% inhibition of growth), was calculated by linear interpolation[16]. In this calculation, two test points that bracket 50% inhibition was determined and the two percentages and the two concentrations were inserted into the following formula:

$$IC_{50} = \left[\frac{(\text{High \%} - \text{Low \%})}{(50\ \% - \text{Low \%})} \times (\text{High concentration} - \text{Low concentration}) \right] + \text{Low concentration}$$

The IC_{50} was used to rank the potential risk of acute toxicity of herbal products extracts.

Mutagenicity test

A commercial test kit, the Muta-Chromplate, was purchased from Environmental Biodetection Products Incorporation (EBPI, Ontario, Canada). This test kit was based on the validated Ames bacterial reverse-mutation test[17] but was performed entirely in liquid culture (fluctuation test). The following chemicals were purchased from EBPI: Davis-Mingioli salt (5.5 times concentrated), D-glucose (40%, w/v), bromocresol purple (2 mg/ml), D-biotin (0.1 mg/ml), and L-histidine (0.1 mg/ml). Two sterile standard mutagens were sodium azide (NaN_3, 0.5 µg/100 µl) and 2-nitrofluorene (2-NF, 30 µg/100 µl).

Reagent mixture comprising of Davis-Mingioli salt (21.62 ml), D-glucose (4.75 ml), bromocresol purple (2.38 ml), D-biotin (1.19 ml) and L-histidine (0.06 ml) were mixed aseptically in a sterile bottle. Reagent mixture, herbal extract, sterile distilled water and standard mutagen were mixed in several bottles at the amount indicated in Table 2. Two mutant strains, *S. typhimurium* TA98 and *S. typhimurium* TA100 were provided by EBPI. The bacteria were inoculated in nutrient broth and incubated at 37°C for 18 -24 h. The culture broth (5 µl) was inoculated into the bottles and mixed thoroughly. The content of each bottle was transferred into a multichannel reagent boat and 200 µl aliquots of the mixture were dispensed into each well of a 96-well microtitration plate using a multichannel pipette. The plate was placed in an air-tight plastic bag to prevent evaporation and incubated at 37°C for 4 days.

The 'blank' plate was observed first and the rest of the plates were read only when all wells in the blank plate were coloured purple indicating the assay was not contaminated. The 'background', 'standard' and 'test' plates were scored visually and all yellow, partially yellow or turbid wells were scored as positive while purple wells were scored as negative. Numbers of all positive wells were recorded. The 'background' plate (no herbal extract or standard mutagen added) showed the level of spontaneous or background mutation of the test bacteria. The extract was considered toxic to the test strain if all wells in the test plate showed purple coloration. For a herbal extract to be mutagenic, the number of positive well had to be more than twice the number of positive well in the 'background' plate (spontaneous mutation)[18].

Heavy metal analysis

Herbal powder was ashed at 525°C overnight. After cooling, 20% HCl was added to the samples. Samples were filtered and the filtrate was diluted with deionised water. Metals in the filtrate were analysed using Flame Atomic Absorbtion Spectrometry (Perkin-Elmer) with Air-C_2H_2 flame. Cathode lamps wavelength and slit width used were as recommended by the manufacturer. The recovery efficiency of this method was carried out by spiking 1 g herbal powder with the combined standards. The spiked sample was placed in an oven at 80°C until dried followed by dry ashing and samples were prepared as described above.

RESULTS

Figure 1 shows concentration-response curves of Hep2 cell to 72 h exposure with the herbal products extracts and *E. longifolia*. Table 3 shows that IC_{50} of *E longifolia* (EL) and the product containing only *E. longifolia* (EG) was 20.87 ± 2.23 µg/ml and 50.00 ± 9.41 µg/ml, respectively. Concentrations of IC_{50} for extracts TA, PB and GE were 15.20 ± 2.43 µg/ml, 55.77 ± 3.69 µg/ml and 18.89 ± 7.18 µg/ml respectively. Table 4 shows that none of the products was mutagenic when tested on *Salmonella* strain TA98 while mixture of *E. longifolia* and *C. deserticola* (TA) was mutagenic when tested on *Salmonella* strain TA 100. Cadmium, Pb, As and Cu were not detected in product EG, TA and GE (Table 5). Cadmium, As and Cu were found to be 0.2 ppm or less in PB and *E. longifolia*. All products contain Mn of not more than 12 ppm and Pb was detected only in *E. longifolia*.

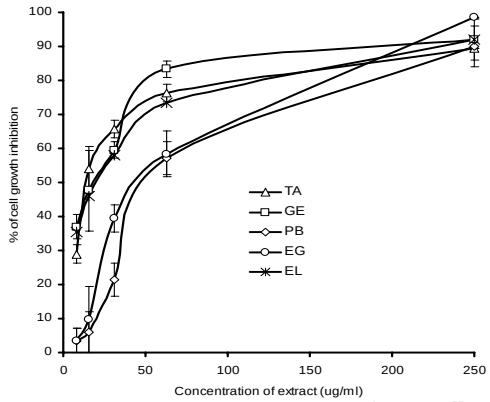

Fig. 1: Concentration-response curves of Hep2 cell to 72 h exposure with chloroform-methanol extracts of *E. longifolia* (EL), a product contining solely *E.longifolia* (EG) and three polyherbal products containing *E.longifolia* (TA, GE and PB). Each points of the curve represent the mean value of 3 samples and standard deviations.

Table 1: Herbal formulations, dosage, and intended usage of some herbal products containing *E. longifolia* purchased from retail shops in Kuala Lumpur, Malaysia.

Herbal preparation	Constituents	Plant parts used[a]	% in the formulation	Dosage	Intended use
EG	*Eurycoma longifolia*	Radix	100	600 mg, twice daily	To increase sexual stamina energy in man
PB	*Eurycoma longifolia*	Radix	40	1225 mg,	Increase passion in
	Curcumae sp	Rhizoma	10	3 times daily	women
	Honey	Whole	50		
TA	*Eurycoma longifolia*	Radix	50	1200 mg,	To increase sexual
	Cistanche deserticola	Herba	50	twice daily	stamina energy in man
GE	*Eurycoma longifolia*	n.i	30.4	600 mg, twice daily	For energy, increase
	Tacca palmate	n.i	21.4		sexual stamina and
	Zingiberis aromaticae	n.i	17.9		men's' health.
	Zingiberis officinale	n.i	14.3		
	Helminthoctachys zeylanica.	n.i	16		

[a] **Plant parts used: n.i, not indicated; Radix, the root; Rhizoma, rhizome or a creeping horizontal stem generally bearing roots on its underside; Herba, the aerial parts or the aboveground parts of plants which may include the flower, leaf, and the stem;**

Table 2: Set-up of the fluctuation assay

Treatment	Volume added (ml)				
	Mutagen Standard	Herbal extract	Reagent mixture	Deionised Water	*Salmonella* test strain
Blank	-	-	2.5	17.5	-
Background	-	-	2.5	17.5	0.005
Standard mutagen	0.1	-	2.5	17.4	0.005
Test sample	-	0.005	2.5	17.5	0.005

Table 3: Concentration of extracts of herbal products containing *E. longifolia* and extracts of *E. longifolia* that caused 50% decrease in Hep2 cell viability (IC_{50}) as calculated by linear interpolation. Sign '#' indicates significantly different from EG

E. longifolia / Polyherbal products	IC_{50} (µg ml^{-1})
EG	50.00 ± 9.41
TA	15.20 ± 2.43#
PB	55.77 ± 3.69
GE	18.89 ± 7.18#
E. longifolia (EL)	20.87 ± 2.23#

Table 4: Mutagenic activity of extract of *E. longifolia* and some polyherbal products containing *E. longifolia* in the Ames fluctuation test. All extracts were tested at 250 µg ml^{-1}

	Test on *Salmonella* TA 98		Test on *Salmonella* TA 100	
	Number of positive wells / 96 wells#	Results ($p \leq 0.05$)	Number of positive wells / 96 wells#	Results ($p \leq 0.05$)
Background	8	-	14	
2-Nitrofluorene (1.5 g/ml)	93	Mutagenic	-	-
NaN3 (0.025 g/ml)	-	-	93	Mutagenic
EC	7	Non-mutagenic	20	Non-mutagenic
PB	4	Non-mutagenic	8	Non-mutagenic
TA	4	Non-mutagenic	28	Mutagenic
GE	4	Non-mutagenic	14	Non-mutagenic
E. longifolia	8	Non-mutagenic	19	Non-mutagenic

Average value of 3 samples of different batches of products

Table 5: Heavy metals content (ppm)* of *E. longifolia* and some polyherbal products containing *E. longifolia*.

Herbal products	Cd	Pb	As	Cu	Mn
EG	ND	ND	ND	ND	.50 ± 0.01
PB	0.20 ± 0.06	ND	0.01 ± 0.00	0.20 ± 0.01	11.00 ± 0.01
TA	ND	ND	ND	ND	3.00 ± 0.01
GE	ND	ND	ND	ND	6.67 ± 0.02
E. longifolia	0.20 ± 0.06	1.67 ± 0.04	0.08 ± 0.20	0.20 ± 0.05	4.00 ± 0.07

*Average concentration ± standard deviation (n=3); ND = not detected

DISCUSSIONS

Table 3 shows that IC_{50} for the product containing only *E. longifolia* (EG) was 50.00 ± 9.41 µg/ml and this is considered to be cytotoxic. Cytotoxicity of *E. longifolia* has been reported [9,19]. However, *E. longifolia* has been consumed orally for centuries without any report of adverse effect. *Eurycoma longofolia* extract was found to be 100 times less cytotoxic if administered orally than intraperitoneal in experimental mice. Since *E. longifolia* is normally taken orally from water decoction[4], its cytotoxicity has been reduced possibly by elimination of cytotoxic substance in the liver or poorly absorbed into the blood circulation[5]. Higher IC_{50} of *E. longifolia* in product EG than pure *E. longifolia* powder suggest that *E. longifolia* used in commercial products were of different quality or were not standardised and may affect the effectiveness of the remedies.

Mixing *Curcumae* sp. with *E. longifolia* (1:4) as in PB did not result in a product with high cytotoxocity property (IC_{50} = 55.77 ± 3.69 µg/ml). However, adding *C. deserticola* to *E. longifolia* (1:1) as in TA resulted in a product with high cytotoxic status (IC_{50} = 15.20 ± 2.43 µg/ml). A crude extract with IC_{50} of < 20µg/ml is considered highly cytotoxic[20]. High cytotoxicity of the latter mixture could not be explained due to cytoxicity of *C. deserticola* as *C. diserticola* has not been reported highly cytotoxic.

Combination of *E. longifolia* with of *T. palmata, Z. aromaticae, Z. officinale* and *H. zeylanica* (reducing the proportion of *E.longifolia* in the remedy to only 30%) as in GE resulted in a high cytotoxicity product (IC_{50} = 18.89 ± 7.18 µg/ml). None of *T. palmata, Z. aromaticae, Z. officinale* and *H. zeylanica* has been considered highly cytotoxic.

Thus, higher cytotoxicity of TA and GE as compared to EG could be due to interaction between the phytochemical constituents of two or more herbal extracts in TA and GE synergistically resulting in higher cytotoxic extract. Although IC_{50} of PB is comparable to EG, concentration of *E. longifolia* in PB is 50% less indicating that there might be a positive interaction of *E. longifolia* and *Curcumae* species towards the increase of IC_{50}. These findings are not in line with the popular believed that mixture of two or more non-cytotoxic herbs should not be cytotoxic. These results support the suggestion by the World Health Organization that a combination of ingredients should be considered as 'herbal medicine of uncertain safety' and also regards this product as a new substance where safety data are then required[21].

In this study product containing *E. longifolia* alone was found to be not mutagenic using *Salmonella* strain TA98 and TA100. Extract of *E.longifolia* was reported to be non-mutagenic[9]. However, *E. longifolia* was reported to contain mutagenic and genotoxic substance alkaloids β–carboline [22-23]. This study showed that some components of *E. longifolia* that could be mutagenic are in very low concentration. However, products containing *E. longifolia* and *C. deserticola*, TA, was found to be mutagenic. There were little or no reports of mutagenicity of *C. deserticola*. These results suggested that the mutagenicity of TA could be due to the effect of herbal combination and warrant further study on the combined effect of herbal plants in mixed herbal preparation on mutagenicity.

Arsenic, Cd and Pb are toxic for human bio-systems even at low level of intake[24]. Lead may induce reduced cognitive development and may be associated with cardiovascular disease while Cd can affect kidney function, induce skeletal damage and reproductive deficiency[25]. Because of their toxicity and the fact that they are not required by the body, level of As, Cd and Pb permissible in food and herbal products has been set.

The World Health Organization prescribes limits for As, Cd and Pb for various medicinal plants of not more than 5, 0.3, and 10 ppm respectively[26]. None of the herbal products except PB contained As, Cd or Pb. In PB, Pb was not detected while Cd and As concentration were less than the permissible limits. Manganese is more prevalent in the products but presence in low concentration. Despite of containing most of the metals detected in this study, PB was not mutagenic and less cytotoxic than the rest of the products. These results suggest that there is no toxicity risk of consuming these products as far as heavy metals is concerned and heavy metals did not contribute to the cytotoxicity or mutagenicity of the products studied.

CONCLUSIONS

Under the conditions of this study, it can be concluded that some herbal products containing *E. longifolia* were highly cytotoxic. This cytotoxicity could be the result of interaction of phytochemicals in the herbal mixture. Cytotoxicity status of polyherbal products could not be deduced from the reported cytotoxicity status of each herb in the polyherbal products. Phytochemical interaction may have also contributed to mutagenicity of a product containing *E. longifolia* with a non-mutagenic herb. Heavy metals such as Lead, Cadmium and Arsenic were detected in some proucts and *E. longifolia* in concentration that did not exert mutagenic effect.

ACKNOWLEDGEMENT

The authors wish to thank the Director General of Health Malaysia and the Director of Institute for medical Research for the permission to publish this paper. This study was financially supported by the Public Service Department of Malaysia through the Federal Government Training Scholarship Program.

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