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Mutagenic and cytotoxic properties of three herbal plants from Southeast Asia

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Abstract. Three popular medicinal plants regarded as improving human sexual function in some parts of Southeast Asia were analysed for their mutagenic properties using modified Ames test (fluctuation test). Extract of one of the plants, *Tacca integrifolia* Ker-Gawl., was found to be mutagenic using *Salmonella typhimurium* strains TA98 and TA100. Extract of *T. integrifolia*, *Eurycoma longifolia* Jack and *Helmintostachys zeylanica* (L.) Hook were cytotoxic to human cell lines, Hep2 and HFL1, with IC_{50} ranging from 11 µg/ml to 55 µg/ml. Extract of *E. longifolia* was the most cytotoxic with IC_{50} of 11 µg/ml and 13 µg/ml on Hep2 and HFL1 cell lines respectively. Combined extract of *T. integrifolia* and *H. zeylanica* was more cytotoxic than single extract on both Hep2 and HFL1 cell lines while combined extract of *E. longifolia* and *H. zeylanica* was more cytotoxic than single extract on Hep2 cell lines. Under the conditions of this study it can be concluded that *T. integrifolia* is mutagenic and the combined extracts of the medicinal plants was highly cytotoxic.

INTRODUCTION

Despite the increasing availability of effective conventional medical treatments, plant-derived and herbal remedies continue to provide a popular alternative for men and women seeking to improve their sex life (Adimoelja, 2000; Rowland & Tai, 2003). In Southeast Asia, aphrodisiac activity has been described for the root of *Eurycoma longifolia*, *Helminthostachys zeylanica* and *Tacca integrifolia.*

Eurycoma longifolia Jack, is a tall, slender shrub-tree, commonly found along the hilly jungle slopes of Malaysia, Burma, Indochina, Thailand, Indonesia and Philippines. The roots are used as traditional treatments for dysentery, fever, persistent fever, malaria, syphilis, smallpox and sexual insufficiency (impotency) (Hadiah, 1996; Kuo *et al.*, 2003; Kuo *et al.*, 2004).

Helminthostachys zeylanica (L.) Hook is a terrestrial, herbaceous, fern-like plant of southeastern Asia and Australia. The genus has clusters of sporangia on stems of fertile, spike-like fronds. The rhizome of this annual plant is short, creeping, underground, and stout. They can bear either a solitary frond or several fronds. Leaves are lanceolate with the margins entire or irregularly serrate. The rhizome or the roots of this plant possess an array of medicinal properties. It is used as an antipyretic and antiphlogistic agent (Huang *et al.*, 2003), as an aperients, anodyne, to treat malaria, syphilis, jaundice and impotency (Suja *et al.*, 2004). Dagar & Dagar (1987) also reported that it possessed antiviral properties.

Tacca integrifolia Ker-Gawl. is a tropical herb with a core distribution in Southeast Asia (Zhang *et al.*, 2006). *Tacca integrifolia* is a plant with a cylindric rhizome. It has large leaf blades on a petiole up to 15 inches long. Flower scapes on well established plants can grow to 3 ft. tall. The flowers are a purple colour. It has been used for

treatment of gastric ulcer, enteritis, hepatitis, controlling blood pressure and improving sexual function (Kitjaroennirut *et al.*, 2005).

In spite of the common usage of the plants and plant extracts in Southeast Asian countries such as Malaysia, Indonesia and Thailand there is very little information on the safety of these products. Consumers and the public at large would benefit from understanding of herbs, their purported or beneficial effects and their potential side effects. The identification of chemicals or compounds capable of inducing mutations is crucial in safety assessment since mutagenic compounds can potentially induce cancer (Hecht, 1999; Sugimura, 2000). 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 (Lewis *et al.*, 1993).

Eurycoma longifolia and *H. zeylanica* have been reported to be cytotoxic (Jiwajinda *et al.*, 2002; Huang *et al.*, 2003; Nurhanan *et al.*, 2005) but there appears to be no report on the cytotoxicity of *T. integrifolia*. Cytotoxicity is defined as the adverse effects resulting from interference with structures and/or processes essential for cell survival, proliferation, and/or function. These effects may involve the integrity of membrane, cellular metabolism, the synthesis and degradation or release of cellular constituents or products, ion regulation, and cell division (Seibert *et al.*, 1996). Tissue culture techniques using cultured human cells have been developed and cytotoxicity test using human cell culture have shown good correlation with data obtained using animal study (Barile *et al.*, 1995; Barile & Cardona, 1998).

It has been a practice by some herbal drug producers to mix two or more herbs with perceived aphrodisiac activity into one remedy (Mohd Fuat *et al.*, 2006). Azas *et al.* (2002) reported that herbal combination increased the cytotoxic effects. Mohd Fuat *et al.* (2006) found that 40% of some popular herbal mixtures retailed in Malaysia were cytotoxic to human cell lines Hep2 and HFL1. Thus, the present study aimed to evaluate the mutagenicity of three popular herbal plants in South-east Asia and the cytotoxic effects of the individual plants and combination of plants on human cell lines.

MATERIALS AND METHODS

Medicinal plants

Helminthostachys zeylanica, *E. longifolia*, and *T. integrifolia* were collected by the staff of the Forest Research Institute of Malaysia (FRIM) and authen-ticated by the botanist of the Institute. The specimen was deposited at FRIM where the rhizome of *H. zeylanica*, *T. integrifolia* and the root of *E. longifolia* were dried and ground to powder.

Herbal extracts

Herbal powder (10 g) was extracted with 75 ml methanol/chloroform solution (1:1) (Tyagi *et al.*, 1996; Bidla *et al.*, 2004; Choi *et al.*, 2004; Krishnan *et al.*, 2004). The slurry was stirred with a magnetic stirrer for 24 h at room temperature (22 ± 2°C) and then filtered through a Whatman No. 1 filter paper (Prozeski *et al.*, 2001). The filtrate was dried by rotary vacuum evaporation at 50ºC. The dried extract was reconstituted in dimethyl-sulfoxide (DMSO) to 10 000 µg/ml (stock solution). For cytotoxicity test, single and combined herbal extracts were tested. To prepare combined herbal extracts, equal volume of stock solution of *H. zeylanica* extract and either *E. longifolia* or *T. integrifolia*, was mixed.

Mutagenicity test

Muta-Chromplate kit

A commercial test kit, the Muta-Chromplate, was used to evaluate the mutagenicity of the herbal extracts. The kit was purchased from Environmental Biodetection Products Incorporation (EBPI, Ontario, Canada). This test kit was based on the validated Ames bacterial reverse-mutation test (Ames *et al.*, 1975) but was performed entirely in liquid culture (fluctuation test).

Test bacterial strains
Two mutant strains, *S. typhimurium* TA98 and *S. typhimurium* TA100 were provided by EBPI. The bacteria were maintained on nutrient agar at 3º ± 1ºC. The bacteria were inoculated in nutrient broth and incubated at 37ºC for 18 - 24 h prior to the test.

Chemicals
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) for *S. typhimurium* TA100 and 2-nitrofluorene (2-NF, 30 µg/100 µl) for *S. typhimurium* TA98. All chemicals were kept at 3 ± 1ºC until used.

For test with metabolic activation, S9 metabolic activation components comprising of a mixture of $MgCl_2$ (0.4 M) and KCl (1.65 M), glucose-6-phospahte (1.0 M), nicotine amide di-nucleotide phosphate (NADP, 0.1 M), phosphate buffer (pH7.4) and rat liver extract were used. Standard mutagen for test using metabolic activation was 2-amino-anthracene (2AA) (10µg/100 µl). All chemicals were kept frozen until used.

Preparation of reagent mixture
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.

Preparation of S9 activation mixture
The S9 components were prepared by mixing $MgCl_2$ and KCl, (0.4 ml), glucose-6-phosphate (0.09 ml), nicotine amide di-nucleotide phosphate (0.81 ml), phosphate buffer (9.98 ml), sterile distilled water (6.72 ml) and rat liver extract (2 ml) in a sterile bottle.

Mutagenicity assay
Reagent mixture, herbal extract, sterile distilled water, standard mutagen and S9 activation mixture were mixed in several bottles at the amount indicated in Table 1 and were inoculated with an overnight culture broth of *S. typhimurium* test strains. The content of each bottle was dispensed into each well of a 96-well microtitration plate and the plate was incubated at 37ºC for 4 days.

Interpretation of results and statistical analysis
Yellow or turbid wells were scored as positive while purple wells were scored as negative. 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 significantly higher than the number of positive well in the 'background' plate (spontaneous mutation)

Table 1. Set-up of the fluctuation assay with and without S9 activation

Treatment	Volume added (ml)					
	Standard (NaN_3, 2-NF, or 2AA)	Herbal extract	Reagent mixture	S9 mix	Deionised Water	Test strain
Blank	–	–	2.5	–	17.5	-
Background I	–	–	2.5	–	17.5	0.005
Background II	–	–	2.5	2.0	15.5	0.005
Standard mutagen	0.1	–	2.5	–[a]	17.4	0.005
Test sample 1	–	0.005	2.5	–	17.5	0.005
Test sample 1	–	0.005	2.5	2.0	15.5	0.005

[a], S9 mix (2.0 ml) was added when 2AA was used.

and this was determined statistically as described by Gilbert (1980).

Cytotoxicity

Maintenance of cell lines

Two human cell lines, Hep2 and HFL1 were cultured in culture dish containing minimum essential medium with Earl's Salt (MEM) and Ham's F12 medium respectively. Both culture media were supplemented with non-essential amino acids and foetal bovine serum (Whelan & Ryan, 2003). The culture dishes were incubated at 37ºC in a 5% CO_2 incubator for 72 h.

Treatment of cell with herbal extract

The treatment of the cell lines with the crude herbal extracts was carried out as described by Badisa *et al.* (2004). When the cell growth was confluence, the cells were harvested and then suspended in the appropriate growth medium (MEM or Ham's F12). Approximately 5 x 10^4 cells were seeded onto wells of a flat bottom 96-well microtitre plate and the plate was incubated at 37ºC in a 5% CO_2 incubator for 24 h.

Serial dilution of the herbal stock extracts was done in the appropriate growth medium and each dilution was added in quadruplicate to the culture wells. All culture plates were incubated in 5% CO_2 incubator at 37ºC for 72 h.

Cytotoxicity test of herbal extracts.

Determination of cytotoxicity of the herbal extracts was carried out using the 3-4,5-dimethylthiazole-2,5-diphenyl-tetrazolium bromide (MTT) method described by Betancur-Gavis *et al.* (1999).

Estimation of IC_{50}

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 (Becton Dickinson, 2002). 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:

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

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

RESULTS

Table 2 and Table 3 show the mutagenic activity of *E. longifolia*, *H. zeylanica* and *T. integrifolia*. *Tacca integrifolia* was found to be mutagenic using both *Salmonella* strains TA 98 (without S9 activation) and TA 100 (required S9 activation). Both *E. longifolia* and *H. zeylanica* did not show

Table 2. Mutagenic activity of herbal plant extracts using *S. typhimurium* TA98

Herbal plants	Without S9		With S9	
	Number of positive wells / total number of wells	Results	Number of positive wells / total number of wells	Results
Background	9/96		8/96	
Standards	93/96	+	95/96	+
H. zeylanica	0/96	t	3/96	–
T. integrifolia	30/96	+	11/96	–
E. longifolia	8/96	–	9/96	–

+, significant increase in the number of positive wells compared to the related control ($p \leq 0.05$); – , no significant effect observed; t, toxic for TA98 strain

Table 3. Mutagenic activity of herbal plant extracts using *S. typhimurium* TA100

Herbal plants	Without S9		With S9	
	Number of positive wells / total number of wells	Results	Number of positive wells / total number of wells	Results
Background	16/96		18/96	
Standards	94/96	+	96/96	+
H. zeylanica	4/96	–	16/96	–
T. integrifolia	16/96	–	28/96	+
E. longifolia	17/96	–	17/96	–

+, significant increase in the number of positive wells compared to the related control ($p \leq 0.05$); –, no significant effect observed.

mutagenic properties under the present experimental condition.

Extracts of *E. longifolia*, *H. zeylanica* and *T. integrifolia* were cytotoxic to both Hep2 and HFL1 cell lines (Figures 1- 4). Table 4 shows that IC_{50} of the plant extracts was between 55 µg/ml and 11 µg/ml. Extract of *E. longifolia* was the most cytotoxic with IC_{50} values of 11 µg/ml and 13 µg/ml on Hep2 and HFL1 cell lines respectively followed by *T. integrifolia* (IC_{50} values of 27 µg/ml and 15 µg/ml on Hep2 and HFL1 cell lines respectively) and *H. zeylanica* (IC_{50} values of 55 µg/ml and 50 µg/ml on Hep2 and HFL1 cell lines respectively).

Combined extract of *H. zeylanica* and *T. integrifolia* was more cytotoxic than extract of either plant alone on both Hep2 and HFL1 cell lines (Figure 1 and Figure 2). Table 4 shows that combination of *H. zeylanica* and *T. integrifolia* had lower IC_{50} (15 µg/ml and 10 µg/ml on Hep2 and HFL1 respectively) than either *H. zeylanica* or *T. integrifolia*. Combined extract of *H. zeylanica* and *E. longifolia* was more cytotoxic than extract of either plant alone on Hep2 cell lines except when tested at 10 µg/ml (Figure 3). Using HFL1 cell lines, cytotoxicity of combination of *H. zeylanica* and *E. longifolia* was higher than cytotoxicity of *H. zeylanica* extract alone but was comparable to cytotoxicity of *E. longifolia* extract at higher concentration (39 µg/ml and 78 µg/ml) (Figure 4).

DISCUSSION

Extract of *T. integrifolia* was mutagenic using both *S. typhimurium* strains TA 98 and TA100 indicating that it contained a base-pair and frame-shift mutagens. This could be the first report of mutagenicity of this plant. Results showed that *H. zeylanica* and *E. longifolia* were not mutagenic at 250 µg/ml. *Eurycoma longifolia* may be mutagenic as it contained alkaloids β-carboline. Alkaloids β-carboline was reported to be mutagenic and genotoxic as well (Picada *et al.*, 1997; Boeira *et al.*, 2002). This study has shown that some active components of *E. longifolia* that could be mutagenic were present but in very low concentration.

Eurycoma longifolia was the most cytotoxic extract which inhibited 50% of Hep2 and HFL1 cell growth at 11µg/ml and 13 µg/ml respectively. Many phytochemicals have been isolated from *E. longifolia* extract and found to be highly cytotoxic. Amongst the cytotoxic components of *E. longifolia* were canthin-6-one alkaloids, β-carboline alkoloids, and some quassinoids. Canthin-6-one isolated from this plant was cytotoxic to many cancer cells and β-carboline alkoloids inhibited growth of human lung cancer (A-549) and human breast cancer (MCF-7) cell lines (Kardono *et al.*, 1991; Kou *et al.*, 2003). Some quassinoids from this plant was reported to be cytotoxic to multiple human cancer cell lines (Jiwajinda

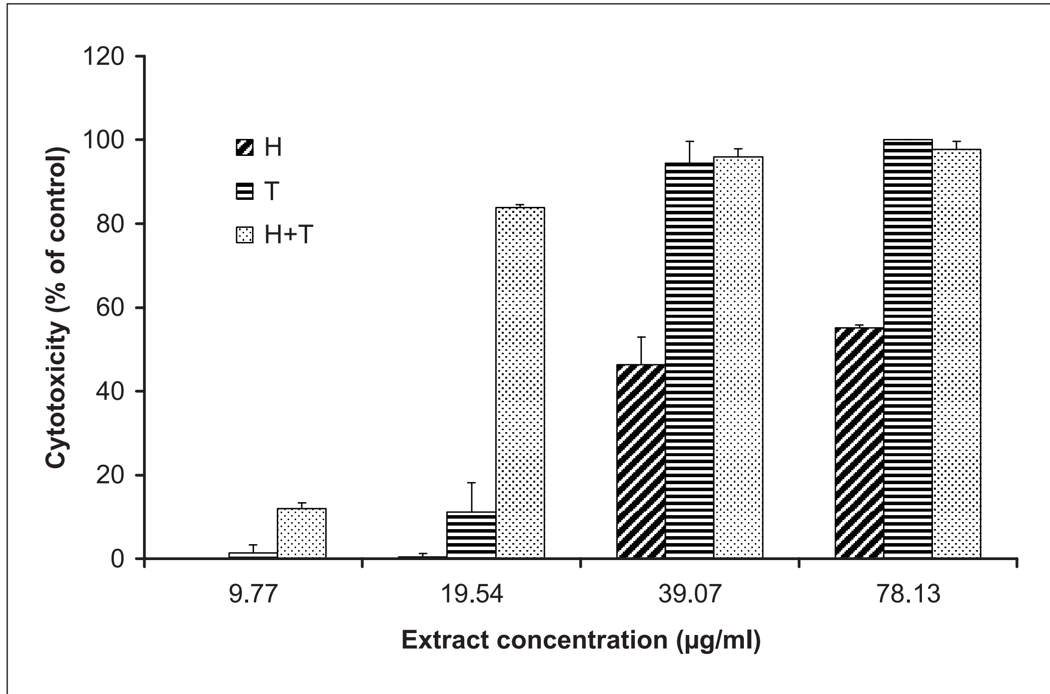

Figure 1: Cytotoxicity of *H. zeylanica* (H), *T. integrifolia* (T) and combination of *H. zeylanica* and *T. integrifolia* (H+T) on Hep2 cell lines. Error bars are standard deviations of 4 determinations.

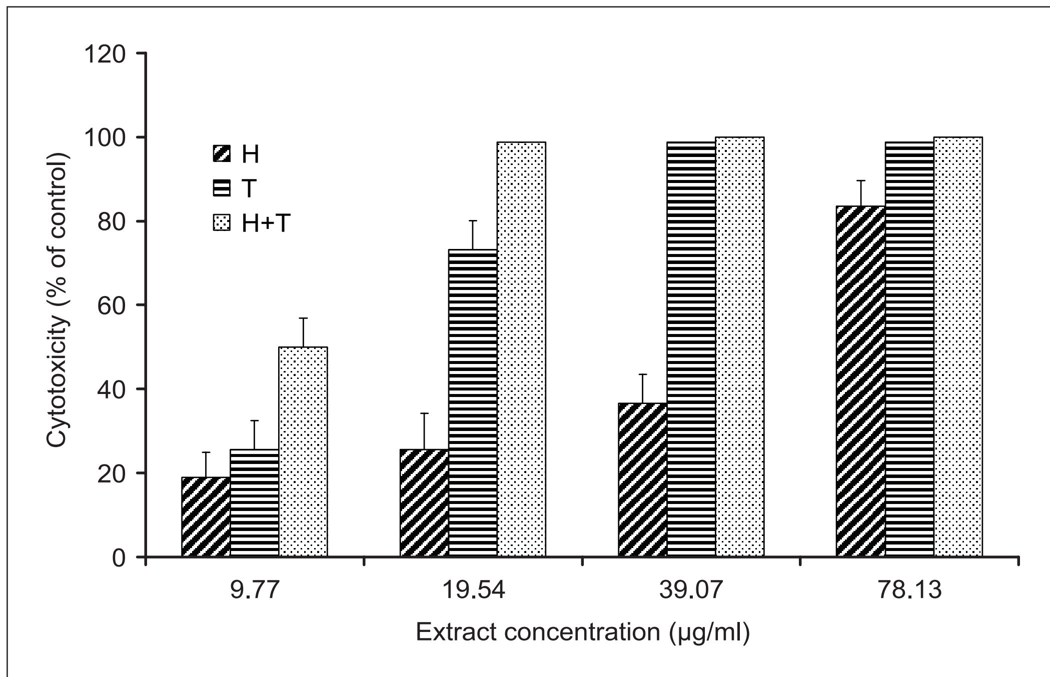

Figure 2. Cytotoxicity of *H. zeylanica* (H), *T. integrifolia* (T) and combination of *H. zeylanica* and *T. integrifolia* (H+T) on HFL1 cell lines. Error bars are standard deviations of 4 determinations.

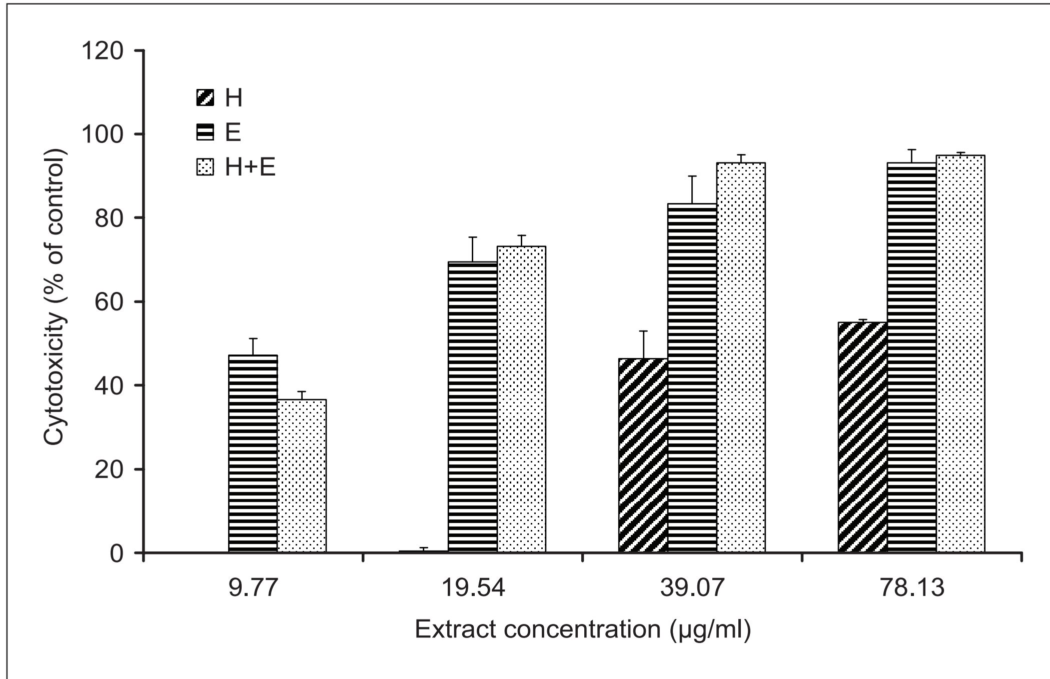

Figure 3. Cytotoxicity of *E. longifolia* (E), *H. zeylanica* and combination of *E. longifolia* and *H. zeylanica* (H+E) on Hep2 cell lines. Error bars are standard deviations of 4 determinations.

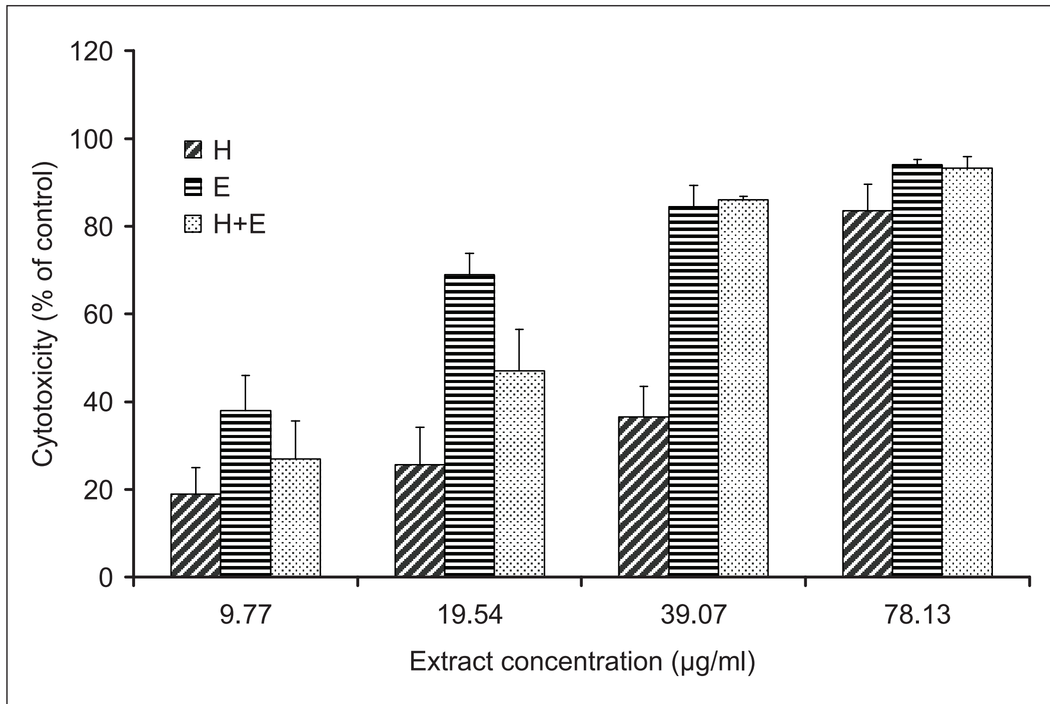

Figure 4. Cytotoxicity of *E. longifolia* (E), *H. zeylanica* (H) and combination of *E. longifolia* and *H. zeylanica* (H+E) on HFL1 cell lines. Error bars are standard deviations of 4 determinations.

Table 4. Values of IC_{50} of plants and plant combination on Hep2 and HFL1 cell lines. Values were calculated from the mean of two replicates.

Herbs / herbal combination (1:1)	IC_{50} (µg/ml)	
	Hep2	HFL1
H. zeylanica (H)	55.4	50.2
T. integrifolia (T)	26.6	14.8
E. longifolia (E)	11.0	12.9
H. zeylanica + *T. integrifolia* (H + T)	15.0	9.9
H. zeylanica + *E. longifolia* (H + E)	13.4	23.1

et al., 2002). Satyavivad *et al.* (1998) reported that toxicity of *E. longifolia* decreased by approximately 100-fold if taken orally indicating that the digestive tract filtered out many toxic compounds. High cytotoxicity of *E. longifolia* as found in this study suggests that *E. longifolia* should only be consumed orally.

Tacca integrifolia was found to be highly cytotoxic with IC_{50} of 15 µg/ml and 27 µg/ml on HFL1 and Hep2 cell lines respectively. Amongst the chemical constituents of *T. integrifolia* as reported by Tripathi & Tiwari (1981) only betulinic acid was reported to be cytotoxic to a specific cell especially melanoma cell lines and neuroblastoma cells (Fulda *et al.*, 1999) but did not affect normal cells (Zuco *et al.*, 2002). *Tacca integrifolia* was found to be toxic to normal cells (HFL1) which suggest that constituent other than betulinic acid may have been involved.

Helminthostachys zeylanica extract was less cytotoxic than *E. longifolia* and *T. integrifolia*. In fact, it could be considered as weak cytotoxic according to the criteria of Wall *et al.* (1987), Suffness & Pezzuto (1990) and Zee-Cheng (1997). Studies on effect of ethanol extract of *H. zeylanica* on mice showed that the extract was toxic at high dose (Suja *et al.*, 2004). This finding was in agreement with the present result that the IC_{50} was slightly higher than the IC_{50} of substance classified as highly cytotoxic. Suja *et al.* (2004) pointed out that *H. zeylanica* was useful as traditional medicine because it has been consumed at the dose lower than the toxicity level.

Combining two or more herbal plants which possess similar property of improving sexual functions has been a practice by some herbal drug manufacturers (Bent *et al.*, 2003; Mohd Fuat *et al.*, 2006). Increased toxicity when herbs were combined was reported by Azas *et al.* (2002) who found that various combinations of extracts from 4 different plants were strongly synergistic against malaria parasites. Combination of α-zingiberene, β-sesquiphellandrene, bisabolene and curcumene, the constituents of *Zingiber officinale*, was 66 times more effective as an antiulcer than the activity of any of the individual constituent acting alone (Beckstrom-stenberg & Duke, 1994). In the current study, combination of *H. zeylanica* with *T. integrifolia* was more cytototoxic than either plant alone on both Hep2 and HFL1 cells. This is an indication of a synergistic effect of constituents of *H. zeylanica* and *T. integrifolia* towards the overall cytotoxicity. Combination of *H. zeylanica* with *E. longifolia* also resulted in higher cytotoxicity to Hep2 cells than the cytotoxicity of either plant alone at the concentrations tested despite the fact that the IC_{50} of the combined extract was comparable to the extract of *E. longifolia* alone. It can be concluded that there is a higher toxicity risk of consuming combination of *H. zeylanica* with either *T. integrifolia* or *E. longifolia* and products which use these plants combinations should be avoided. These results also showed that cytotoxicity of a combination of medicinal plants could not be deduced from the information of cytotoxicity of each plant in the combination and herbal products with new plant combinations should be tested for cytotoxicity before it could be released into the public market.

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