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Comparison of Cytotoxic Activities between *in Vitro* and Field Grown Plants of *Typhonium flagelliforme* (Lodd.) Blume

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An *in vitro* cytotoxicity screening of the *Typhonium flagelliforme* extracts indicated high cytotoxicity effect on human lung carcinoma NCI-H23 cells and human mammary gland carcinoma T-47D cells, but the extracts were not active on human liver carcinoma HepG2 cells. NCI-H23 cells were more susceptible to *T. flagelliforme* extracts than T-47D cells. ED_{50} values of the hexane fractions of the mature plant and the *in vitro* plantlet of *T. flagelliforme* on NCI-H23 cells were less than 2 μg/mL. Extract from the mature plant was relatively more cytotoxic than the one from *in vitro* plantlet except for the hexane fraction. The chloroform and butanol fraction of the mature plant had higher cytotoxicity effect than the fraction from *in vitro* plantlet on NCI-H23 cells. All the 3 fractions (hexane, chloroform, and butanol) of the mature plant exhibited higher cytotoxicity effects on human mammary gland carcinoma T-47D cells than the 3 fractions of *in vitro* plantlet. However, the human liver carcinoma cells were resistant to *T. flagelliforme* extracts except for higher concentration of hexane fractions of both the mature and the *in vitro* plants and the chloroform fraction of the mature plant. Micropropagated plantlets of *T. flagelliforme* could hence be used as herbal materials for the treatment of human lung and breast cancers.

Keywords: carcinoma cells, cytotoxicity, ED_{50} values, *in vitro* plantlets, *Typhonium flagelliforme*

Typhonium flagelliforme (Lodd) Blume (Araceae), commonly known as rodent tuber in Malaysia, Singapore and China, has long been used in the preparation of traditional medicine in India, China and South East Asian countries. This plant propagates by forming small tubers from the subterranean stolons (Hay, 1993). In China, the leaves of the *Typhonium divaricatum,* possibly the same plant as *T. flagelliforme* which was misidentified previously, were prepared as tincture to be taken in small amounts for the treatment of internal injuries. The tubers were used for the treatment of pulmonary disorders (Perry and Metzger, 1980). For the past few years in Malaysia, *T. flagelliforme* has been taken orally, either as juice extracts or as dry powder, in combination with other herbal extracts for combating breast, lung, colon and liver cancers (Teo and Chng, 1996). Crude extracts of *T. flagelliforme* were found to reduce ^{3}H-thymidine incorporation in a murine lymphoid cell line (Neoh, 1992). Itokawa and Takaya (1993) discovered that the extracts of *T. flagelliforme* fresh roots, rhizomes, stems and leaves had cytotoxic activity against P388 cells. *T. flagelliforme* extracts were also tested on rats and had similar hepatocarcinogenesis effect as glycy-rrhizin, a type of chemotherapy drug for curing liver diseases (Thilakavathy et al., 1999). High demand for this plant in cancer treatment coupled with its slow propagation rate and restricted distribution in only damp and shady areas caused the declining of *T. flagelliforme* population in its natural habitat. Therefore, there is an urgent need of an alternative propagation method to conserve the plant and sustain the supply of raw plant materials that are in great demand for the cancer treatments. This problem has made us to embark on a search for an efficient micropropagation technique for mass propagation of *T. flagelliforme*. A micropropagation technique was successfully established and 420 plantlets could be produced from each bud explant in 12 months using the *in vitro* multiple shoots regeneration method (Su et al., 2000). The *in vitro* plantlets can be massively produced using a 5-L fermentor (Koh and Chan, 2003). The *in vitro* *T. flagelliforme* plantlets could thus be used to provide raw material for the production of potential anticancer compounds or the production of field planting materials. The objective of this study was to examine and to compare the cytotoxicity effect of the *T. flagelliforme* extracts derived from the *in vitro* plantlets and the matured mother plants on three types of human carcinoma cell lines namely T-47D (mammary gland

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Abbreviations: IBA, indole-3-butyric acid; MS, Murashige and Skoog; BA, N^{6}-benzyladenine

carcinoma), NCI-H23 (lung carcinoma), and HepG2 (liver carcinoma).

MATERIALS AND METHODS

Plant Material

Four weeks old *in vitro* plantlets and 6 months old mature *T. flagelliforme* were used. The *in vitro* plantlets (Fig. 1) were mass produced in 5-L fermentors using a modified Murashige and Skoog (MS) (1962) liquid medium supplemented with 1.33 μmol L^{-1} N^6-benzyladenine, 2.46 μmol L^{-1} indole-3-butyric acid and 4% sucrose according to Koh and Chan (2003). The cultures were placed under cool-white fluorescent lamps providing continuous lighting with 32 μE m^{-2} s^{-1} light intensity and temperature of 25 ± 2°C. After 4 weeks, the *in vitro* plantlets were removed from the fermentors and were washed under running water to remove traces of liquid medium. The 6 months old mature plants were collected from the herbal garden of School of Biological Sciences, Universiti Sains Malaysia, Penang, Malaysia and washed thoroughly with tap water and then with distilled water. The *in vitro* plantlets and the mother plants were sun dried until constant weight was obtained before extraction.

Preparation of Extracts

The dried plant materials were macerated using an electric blender. The powdered matured plant materials were soaked in methanol at room temperature in darkness for 24 h. The methanol extract was filtered with Whatman filter paper no.1 and the plants were further soaked twice in methanol. The filtered methanol extracts were combined and evaporated to dryness under reduced pressure. The concentrated methanol extract was then suspended in 150 mL water for 15 min. The suspension was partitioned with 100 mL n-hexane in a separation funnel. Hexane partition was repeated for two more times with 75 mL water each. After removing the hexane fraction, the aqueous layer was partitioned again with chloroform then with n-butanol. All 3 fractions were evaporated to dryness. The dried *in vitro T. flagelliforme* plantlets were treated equally.

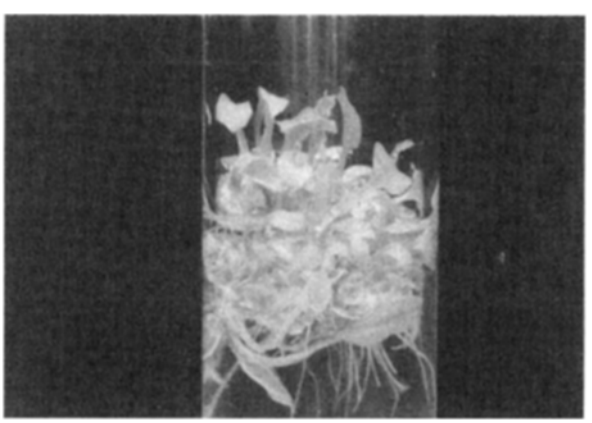

Figure 1. Normal and healthy 4 week old *T. flagelliforme* plantlets produced in aeration tubes.

Cultures of Human Cancer Cell Lines

Three different human tumour cell lines were used: T-47D (mammary gland carcinoma), NCI-H23 (lung carcinoma) and HepG2 (liver carcinoma). All cell lines were purchased from American Type Culture Collection. T-47D and NCI-H23 cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, and EBSS medium supplemented with 10% fetal bovine serum was used to maintain HEPG2 cells. All cell lines were cultured at 37°C in an atmosphere of 5% CO_2 in air (100% humidity).

In Vitro Cytotoxicity Assay

Cells at the exponential growth phase were harvested, centrifuged at 1000*g* for 5 min and resuspended in complete medium at 0.5×10^5 or 1.0×10^5 cells mL^{-1}. A volume of 100 μL of the cells was added to each well of a flat bottom 96-well plate. After 24 h incubation in a 5% CO_2 humidified incubator at 37°C, 10 μL of the test agent was added in triplicates to give final concentrations of 200, 100, 75, 50, 25, 10 and 1 μg mL^{-1} medium. The test agents used were hexane, chloroform and butanol extracts of the mature plants and the *in vitro* plantlets of *T. flagelliforme*. The concentration of DMSO used to dissolve the extract was adjusted to 1% and similar concentration of solvent was used in control wells.

After incubation for 72 h, cell survival was determined by methylene blue staining as described by Yamazaki et al. (1986). In this method, glutaraldehyde was added to each well to a final concentration of 2.5% (v/v) and the surviving cells were fixed for 15 min. After removing the dead cells by washing twice with 0.15 M sodium chloride, the fixed cells were stained with 0.1 mL 0.05% (w/v) methylene blue for 15 min. After washing off the excess dye with 0.15 M sodium chloride twice, 0.2 mL of 0.33 M hydrochlo-

ric acid was added to each well and the plate was rotated on a shaker until the dye was well blended before absorbance was read at 660 nm with a multi-well plate reader (Vmax Kinetic, USA). Wells with complete medium, test agent and methylene blue but without cells were used as blanks. The percentage of inhibition was obtained using the following equation:

$$\% \text{ inhibition} = \frac{[\text{absorbance of control cell - absorbance of stimulated cells}] - \text{absorbance of blank}}{\text{absorbance of control cell-absorbance of blank}} \times 100$$

Effective concentration (EC_{50}) was defined as the concentration required to reduce cell growth by 50%, and *T. flagelliforme* plant extracts with EC_{50} value of less than 20 µg/ml were considered as active extracts (Geran et al., 1972). EC_{50} values of the plant extracts were determined by computerized Prism® program (GraphPad Software, USA). The cytotoxicity effect of *T. flagelliforme* plant extracts on the three different tumor cell lines were analyzed using ANOVA, means were compared using Student's *t*-test, and Tukeys multiple range test was used at $P = 0.05$ for comparison of inhibition between different concentrations.

RESULTS AND DISCUSSION

Hexane fraction of *in vitro T. flagelliforme* plant at 10 µg/ml exhibited 95% inhibition on NCI-H23 (lung carcinoma) cells whereas 81% inhibition was exhibited at the same concentration from the hexane fraction of the mature plant (Fig. 2a). This indicated that hexane fraction of the *in vitro* plantlets was significantly ($p = 0.05$) more cytotoxic than the hexane fraction of the mature plants on NCI-H23 cells. This was further confirmed by the EC_{50} value of the hexane fraction of the *in vitro* plantlets (1.5 µg mL^{-1}) that is lower than the EC_{50} value of the hexane fraction from the mature plants (1.8 µg mL^{-1}) (Table 1). By contrast, chloroform fraction from the *in vitro* plantlets exhibited 75% inhibition at 25 µg mL^{-1} as compared to 83% inhibition from the chloroform fraction of the mature plant at 10 µg mL^{-1} (Fig. 2b). This indicated that chloroform fraction of the mature plants had higher cytotoxicity effect than the fraction from *in vitro* plantlets. This was also confirmed by the relatively lower EC_{50} value of chloroform fraction from the mother plants (5.2 µg mL^{-1}) as compared to the *in vitro* plantlets (14.6 µg mL^{-1}) (Table 1). Butanol fraction of both the *in vitro* and the mother plants also

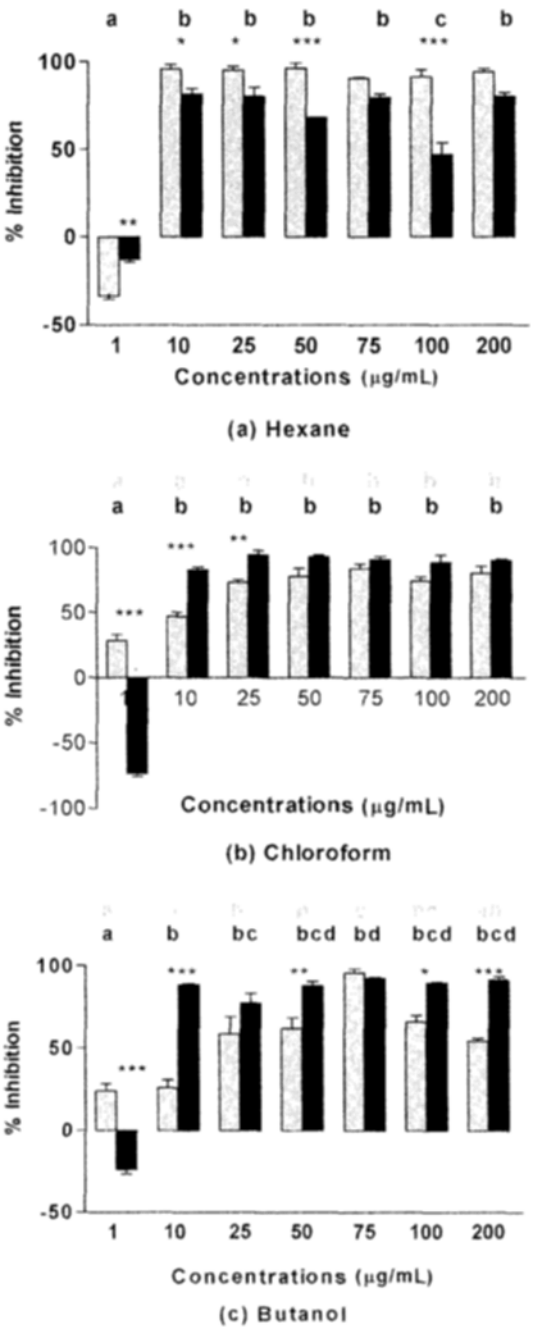

Figure 2. Cytotoxicity effect of three different fractions of *T. flagelliforme* extracts on NCI-H23 cells at different concentration. Each value represents the mean ± s.e. Asterisks indicate significant difference between the *in vitro* (▒) and the mature plant (■) fractions at respective concentrations of column below (ANOVA followed by Student's *t*-test where * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). Same letters on top of each column represent no significant difference in the multiple concentration comparison within each plant source (ANOVA, Tukey's multiple range test, $P = 0.05$). Top row of letters represent the *in vitro* fractions and second row of letters represent the mature plant fractions.

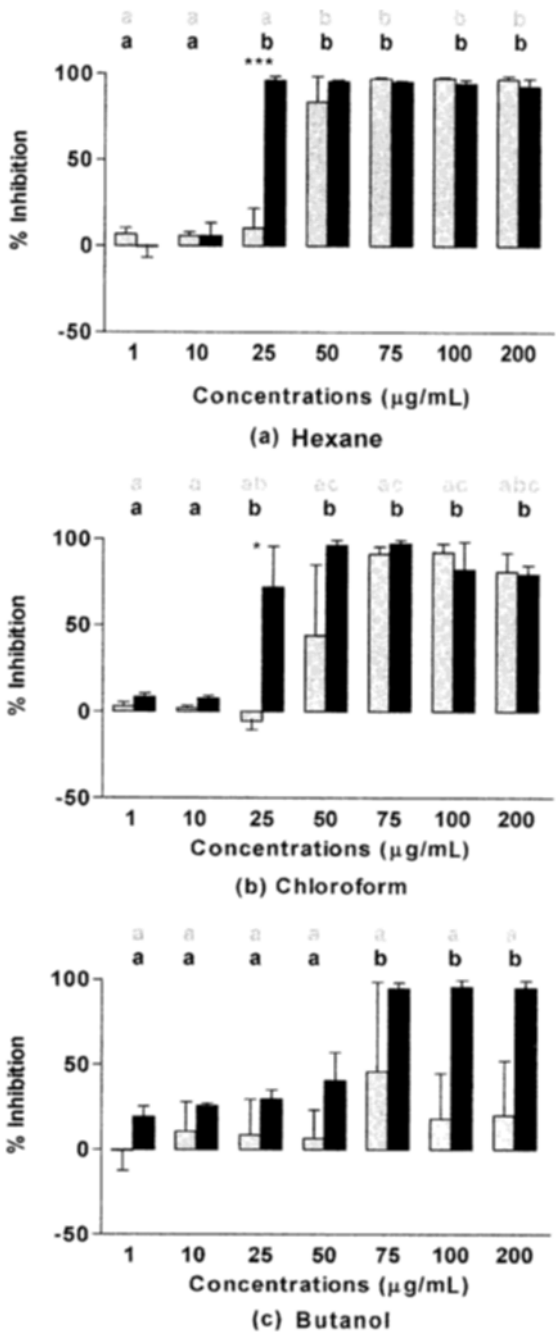

Figure 3. Cytotoxicity effect of three different fractions of *T. flagelliforme* on T-47D cells at different concentrations. Each value represents the mean ± s.e. Asterisks indicate significant difference between the *in vitro* plantlet ( ) and the mature plant (■) fractions at respective concentrations (ANOVA followed by Student's *t*-test where $*P < 0.05$, $**P < 0.01$, $***P < 0.001$). Same letters on top of each column represent no significant difference in the multiple concentration comparison within each plant source (ANOVA, Tukey's multiple range test, $P = 0.05$). Top row of letters represent the *in vitro* fractions and second row of letters represent the mature plant fractions.

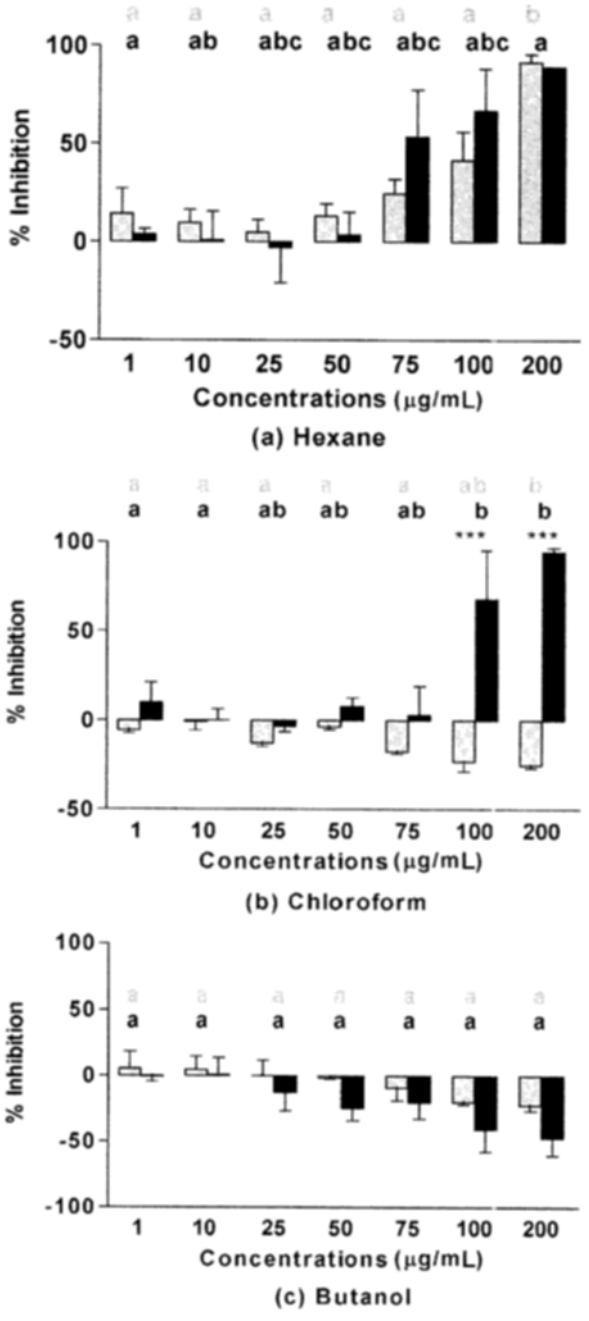

Figure 4. Cytotoxicity effect of three different fractions of *T. flagelliforme* on HepG2 cells at different concentrations. Each value represents the mean ± s.e. Asterisks indicate significant difference between the *in vitro* plantlet ( ) and the mature plant (■) fractions at respective concentrations (ANOVA followed by Student's t-test where $*P < 0.05$, $**P < 0.01$, $***P < 0.001$). Same letters on top of each column represent no significant difference in the multiple concentration comparison within each plant source (ANOVA, Tukey's multiple range test, $P = 0.05$). Top row of letters represent the *in vitro* plant fractions and second row of letters represent the mature plant fractions.

Table 1. EC_{50} values (μg/mL) of hexane, chloroform, and butanol fractions of *T. flagelliforme* extracts on different human tumour cell lines.

Tumour Cell Lines	Extract Fractions	EC_{50} values (μg/mL)	
		In vitro plantlets	Mature plants
NCI-H23	Hexane	1.5	1.8
(Lung)	Chloroform	14.6	5.2
	Butanol	18.7	1.2
T-47D	Hexane	39.1	12.2
(Breast)	Chloroform	50.0	23.0
	Butanol	>100	54.6
Hep G2	Hexane	>100	>100
(Liver)	Chloroform	>100	>100
	Butanol	NA	NA

NA, non active

showed similar pattern where higher inhibition (88%) was exhibited at a lower concentration (10 μg mL^{-1}) from the mother plants (Fig. 2c). EC_{50} value of butanol fraction from the mother plants (1.2 μg mL^{-1}) was also lower than that of the *in vitro* plantlets with an EC_{50} value of 31.6 μg mL^{-1} (Table 1). This suggested that the extracts of both the mature plants and the *in vitro* plantlets of *T. flagelliforme* had high cytotoxicity effect on NCI-H23 (lung carcinoma) cell line. Only the hexane fraction from the *in vitro* plant extracts was relatively more cytotoxic than the mother plants.

For breast tumor cells (T-47D), hexane fraction from the *in vitro* plantlets exhibited 83% growth inhibition at 50 μg mL^{-1} but hexane fraction of the mature plants exhibited higher growth inhibition (96%) at a lower concentration (25 μg mL^{-1}) (Fig. 3a). This indicated that hexane fraction of the mature plants was more cytotoxic than hexane fraction of the *in vitro* plantlets. This was also confirmed by the lower EC_{50} value of hexane fraction of the mature plants (12.2 μg mL^{-1}) as compared to the *in vitro* plantlets (39.1 μg mL^{-1}) (Table 1). Chloroform fraction of the *in vitro* plantlets showed 44% growth inhibition on T-47D cells at 50 μg mL^{-1} and 93% growth inhibition at 75 μg mL^{-1} (Fig. 3b). Chloroform fraction of the mature plants achieved 72% growth inhibition from a lower concentration (25 μg mL^{-1}) and this rate was mostly maintained to relatively higher concentrations (50–200 μg mL^{-1}). This showed that the mature plants had higher cytotoxicity effect against T-47D cell than the *in vitro* plantlets. This was further confirmed by EC_{50} value of the chloroform fraction of the mature plants (23.0 μg mL^{-1}) that was lower than the *in vitro* plantlets (50.0 μg mL^{-1}) (Table 1). Butanol fraction of the *in vitro* plantlets showed no significant difference in growth inhibition in all the concentrations used. For the butanol fraction, the highest growth inhibition was only 46% at 75 μg mL^{-1} (Fig. 3c). Whereas for the mature plants, the butanol fraction exhibited 95% growth inhibition at 75 μg mL^{-1} and the EC_{50} is 54.6 μg mL^{-1}. Generally, cytotoxicity effect of *T. flagelliforme* extracts on T-47D cells was moderate with EC_{50} value of more than 20 μg mL^{-1}, except for hexane fraction of the mature plants which had lower EC_{50} value of 12.2 μg mL^{-1}. All fractions of the mature plants exhibited higher cytotoxicity effect on T-47D cells compared to the *in vitro* plantlets.

For human liver carcinoma (HepG2) cells, *T. flagelliforme* extracts showed low cytotoxicity effects where most of the fractions exhibited EC_{50} values of over 100 μg mL^{-1} (Table 1). Hexane fraction of the *in vitro* plantlets produced relatively low inhibitory effect (5-20%) at lower concentrations (1 to 50 μg mL^{-1}), and the rate increased to 45% and 92% at 100 μg mL^{-1} and 200 μg mL^{-1}, respectively. However, hexane fraction of the mature plants achieved almost 50% growth inhibition at 75 μg mL^{-1}, and the rate increased to 70% and 90% at 100 μg mL^{-1} and 200 μg mL^{-1}, respectively (Fig. 4a). The EC_{50} value was more than 100 μg mL^{-1} for the *in vitro* plantlets and 77.0 μg mL^{-1} for the mature plants. Chloroform fraction of the *in vitro* plantlets did not show any cytotoxicity effect on HepG2 cells. On the contrary, it was observed to promote growth of the HepG2 cells at all the concentrations tested (Fig. 4b). For the chloroform fraction of the mature plants, growth inhibition was achieved only at high concentrations (100 and 200 μg mL^{-1}). Butanol fraction of both the *in vitro* and the mature plants also showed no cytotoxicity effect on HepG2 cells but induced growth of HepG2 cells at high concentrations (50–200 μg mL^{-1}) (Fig. 4c). This result indicated that HepG2 cells were resistant to *T. flagelliforme* extracts except for high concentration hexane fractions of both plant sources and chloroform frac-

tion of the mature plants. Chloroform fraction of the *in vitro* plantlets and butanol fraction of both plant sources promoted growth of HepG2 cells. This result suggested that the extracts may contain growth induction compounds that were active on HepG2 cells but not on T-47D and NCI-H23 cells.

From these cytotoxicity assays, it was indicated that human lung carcinoma NCI-H23 cells were most susceptible to *T. flagelliforme* extracts followed by human mammary carcinoma T-47D cell line. However, there was no significant cytotoxicity effect from all the extracts of both the *in vitro* plantlets and the mature plants on human liver carcinoma HepG2 cells. All EC_{50} values of all three different fractions of the *in vitro* plantlets and the mature plants tested on NCI-H23 were lower than 20 $\mu g\ mL^{-1}$. Thus these extracts are potentially active against human lung carcinoma.

The lower EC_{50} value exhibited by the mature plants indicated that the mature plants contained more cytotoxic compounds than the *in vitro* plantlets. This result was supported by HPLC test carried out by Dennis (2001) that showed extracts of the mature *T. flagelliforme* plants exhibited additional minor peaks in addition to the common major peak with the *in vitro* plantlets. These minor peaks could reflect the presence of additional growth inhibitory compounds in the mature plants. The *in vitro* plantlets were only 4 weeks old while the mature plants were 6 months old field-grown plants, and during the field growth, additional cytotoxic compounds became synthesized in the plants.

Ong (1996) reported that *T. flagelliforme* contains haemolitic saponine that also presents in ginseng and had antiviral property (Hostettmann and Marston, 1995). Leaf of the plant was found to contain alkaloids, well known anti-cancer components (Goh et al., 1993). Choo et al. (2001) identified the chemical constituents of the hexane fraction of *T. flagelliforme* that included methyl esters of hexadecanoic acid, octadecanoic acid, 9-octadecenoic acid, and 9,12-octadecadienoic acid. In addition, several common aliphatics were also identified as dodecane, tridecane, tetradecane, pentadecane, hexadecane, heptadecane, octadecane, nonadecane, and eicosane. Interestingly, it was found that none of the identified compounds has been known to have cytotoxic activity (Choo et al., 2001). This fact suggests that the cytotoxic effects exerted by the chloroform and hexane extracts on both NCI-H23 and T-47D cells might have resulted from synergistic interactions amongst the compounds present in the extracts. It has been well reported that the synergistic effects amongst a broad range of structurally diverse compounds might contribute to the overall pharmacological activity of a plant extract (Hamburger and Hostettmann, 1991; Tan, 2003).

Neoh (1992) reported that the hexane extract from the leaves of the mature plant exhibited anti-proliferative effect on the lymphoid cell lines. However, Choo et al. (2001) showed that the chloroform fraction exhibited higher cytotoxic activity than the hexane fraction on P388 murine leukaemia cells. Our studies indicated that the hexane fraction exhibited significantly higher growth inhibition on NCI-H23 and T-47D cells followed by the chloroform and the butanol fractions. The reason for the different levels of cytotoxic activity of the extracts on different cancer cell lines is still unclear. However, it is tempting to speculate and hypothesize that different cancer cell lines may have varying degree of susceptibility to individual plant extracts due to the presence and absence of important bioactive compounds in that particular extract based on the type of solvent used for the extraction process of the extracts. It has been well reported that different bioactive compounds produced different levels of cytotoxic effect against different cancer cell lines (Colman de Saizarbitoria et al., 1997; Li et al., 1998; Ho and Chen, 2003).

This study showed that *T. flagelliforme* contained anti-cancer properties that were effective especially on human lung carcinoma NCI-H23 cells. This suggested its potential use in treating lung cancer. Overall, extracts of the *in vitro* plantlets exhibited lower cytotoxicity on NCI-H23 and T-47D cells compared to the mature plant extracts. However, *in vitro* system of producing plants could provide a weather and disease independent, continuous and homogenous supply of plant material. Furthermore, such material could be more easily manipulated than the whole plants to increase potential yields of known compounds or used to generate undiscovered compounds.

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