



Original Article

## Apoptotic induction activity of *Dactyloctenium aegyptium* (L.) P.B. and *Eleusine indica* (L.) Gaerth. extracts on human lung and cervical cancer cell lines

Pintusorn Hansakul<sup>1</sup>, Chatri Ngamkitidechakul<sup>2</sup>, Kornkanok Ingkaninan<sup>3</sup>, Seewaboon Sireeratawong<sup>1</sup>, and Watcharin Panunto<sup>1</sup>

<sup>1</sup> Department of Preclinical Science, Faculty of Medicine, Thammasat University, Rangsit Campus, Khlong Luang, Pathum Thani, 12120 Thailand.

<sup>2</sup> Department of Biochemistry, Faculty of Medicine, Chiang Mai University, Muang, Chiang Mai, 50200 Thailand.

<sup>3</sup> Department of Pharmaceutical Chemistry and Pharmacognosy, Faculty of Pharmaceutical Sciences, Naresuan University, Muang, Phitsanulok, 65000 Thailand.

Received 8 April 2008; Accepted 18 May 2009

### Abstract

*Dactyloctenium aegyptium* (L.) P.B. (Yaa paak khwaai) and *Eleusine indica* (L.) Gaerth. (Yaa teen-ka) have long been used in traditional Thai medicine because of their diuretic, anti-inflammatory, and antipyretic effects. The present study examined the antiproliferative and cytotoxic effects of the hexane and butanolic extracts of these two grass species. All the grass extracts exhibited selective growth inhibition effect on human lung cancer (A549) and cervical cancer (HeLa) cells relative to normal human lung MRC-5 fibroblasts with IC<sub>50</sub> values in a range of 202 to 845 mg/ml. Apparently, HeLa cells were more sensitive to the extracts than A549 cells. Moreover, all the extracts induced lethality in both cancer cell lines at concentrations close to 1,000 mg/ml, indicating their selective cytotoxicity effects. ELISA assay showed that only the hexane extract of *D. aegyptium* (L.) P.B. and *E. indica* (L.) Gaerth. significantly increased the apoptotic level in extract-treated A549 cells. However, DNA ladder assay detected classic DNA ladder patterns, a characteristic feature of apoptosis, in both cancer cell lines treated with all the extracts in a dose- and time-dependent manner. Taken together, these results indicate that the cytotoxic activity of the grass extracts against lung and cervical cancer cells is mediated through the induction of apoptosis.

**Keywords:** *Dactyloctenium aegyptium* (L.) P.B., *Eleusine indica* (L.) Gaerth., antiproliferative, apoptosis

### 1. Introduction

Cancer is still a serious health problem and has a major social and economic impact worldwide. Despite recent advances in diagnosis, prevention, and therapy, cancer still

affects quality of life in patients due to some limitations of these current medical practices. Consequently, more and more people resort to alternative medicine, which is defined as health care practices used instead of standard ones. Herbal medicine, one type of the alternative medicine, is based on the use of plants or plant extracts to treat diseases and promote health and has been offered especially for cancer treatment over the last century. This alternative treatment is more widely accepted at the present time. Therefore, medici-

\*Corresponding author.

Email address: [hansakul@yahoo.com](mailto:hansakul@yahoo.com)

nal plants have become important and reliable sources for anticancer agents and worldwide efforts are ongoing to find new plants with biological activity (Newman *et al.*, 2002; Newman *et al.*, 2003; Schwartzmann *et al.*, 2002).

Thailand, located in the tropical region, is naturally rich in a large natural resource of medicinal plants and herbs used for treatment in many diseases over centuries. *Dactyloctenium aegyptium* (L.) P.B. (Thai name-Yaa paak khwaai) and *Eleusine indica* (L.) Gaerth. (Thai name-Yaa teen-ka) are Thai herbal medicines that have long been prescribed to relieve dysuria, fever, inflammatory, jaundice, and centipede & scorpion poison by being soaked in alcohol (Boonyaprapashara & Chokchaichareonporn, 2000). These two grass species belong to the Poaceae family, several members of which have been shown to possess anticancer activities such as, *Imperata cylindrica* (Linn.) Raeusch (Thai name-Yaa khaa) (Ding *et al.*, 2006), *Bambusa arundinacea* Willd. (wild bamboo) (Kim *et al.*, 2003; Ren *et al.*, 2004), *Coxi lachryma* (adlay seed) (Chang *et al.*, 2003), *Lolium multiflorum* Lam. (ryegrass) (Komiya *et al.*, 1999; Kyohkon *et al.*, 2001) (Verschoyle *et al.*, 2007).

In the present study, we examined the antiproliferative and apoptosis-inducing activities of the two grass species on human lung and cervical cancer cells versus normal lung fibroblasts, which has not been previously investigated. This could lead to the discovery of some newer anticancer agents with selective cytotoxic effects on cancer cells.

## 2. Materials and Methods

### 2.1 Plant material

The whole plants of *Dactyloctenium aegyptium* (L.) P.B. (Yaa paak khwaai) and *Eleusine indica* (L.) Gaerth. (Yaa teen-ka) were collected from Phitsanulok, Thailand, in April 2005. The voucher specimens (Totium 008 and 009, respectively) were kept and identified by the Faculty of Pharmaceutical Sciences, Naresuan University, Phitsanulok.

### 2.2 Preparation of plant extracts

The collected plant materials were washed, sliced, and completely dried in a hot air oven at 60°C. The dried materials were ground and macerated in 95% ethanol for three days and filtered. The marc was remacerated in 95% ethanol for another three days and filtered. The two sets of the filtrate were pooled and evaporated to give crude extract, which was dissolved in mixed solvents of methanol and water (9:1). The dissolved crude extract was re-extracted with an equal volume of hexane, dichloromethane, and butanol in succession at least three to four times for each solvent. The extracts obtained from each solvent were combined and concentrated to dryness under reduced pressure. The percentage yields obtained were, in the order of hexane, dichloromethane, and butanol, as follows: *D. aegyptium* (L.) P.B. (Yaa paak khwaai) (10.76, 7.01, 22.19) and *E. indica* (L.) Gaerth. (Yaa teen-ka)

(11.01, 7.02, 63.14). These extracts were dissolved in DMSO to make a stock solution and sterilized by filtration (pore size as 0.45 µm) before testing.

### 2.3 Cell culture

Two cancer cell lines were used in this study: A549 (human non-small cell lung cancer cell line) and HeLa (human cervical cancer cell line). A549 was kindly provided by Dr. Chantragan Phiphobmongkol (Chulabhorn Research Institute). All cancer cell lines were cultured at 37°C and 5% CO<sub>2</sub> in RPMI-1640 supplemented with 10% FBS and 1% antibiotic-antimycotics. The normal human fibroblast cell line MRC-5 was cultured in MEM supplemented with 10% FBS, 1 mM nonessential amino acid, 1% sodium pyruvate, and 1% antibiotic-antimycotics.

### 2.4 Antiproliferation assay

HeLa, A549, and MRC-5 cells were seeded at 1x10<sup>4</sup> cells/well in 96-well microtiter plates and incubated overnight for cell attachment. A stock of each grass extract was prepared at 10 mg/ml and further diluted in medium to produce 7 concentrations of 31.25, 62.5, 125, 250, 500, 1000, 2000 µg/ml, each of which was added to cells in triplicate wells. The plates were then incubated for 72 h at 37°C and 5% CO<sub>2</sub>. At the end of exposure time, the medium was removed and the cells were fixed by layering 100 µl of ice-cold trichloroacetic acid (10% v/v). Cell number was estimated indirectly by staining total cellular protein content of each well with sulforhodamine B (Skehan *et al.*, 1990). Absorbance was determined at 492 nm. The percentage of cell survival was calculated based on the following formula.

$$\% \text{ cell survival} = \frac{(T-T_0)}{(C-T_0)} \times 100, \quad T \geq T_0$$

$$\text{or} \quad \frac{(T-T_0)}{T_0} \times 100, \quad T < T_0$$

T = average O.D. of treated group (cells treated with plant extract for 72 h.)

C = average O.D. of control growth group (cells treated with media only for 72 h.)

T<sub>0</sub> = average O.D. of control time group (cells at the time of adding the extract "zero hour plate")

Based on the formula, the percentage of cell survival can be greater than zero, zero, or less than zero. The IC<sub>50</sub> values were calculated using GraphPad Prism software obtained by plotting the percentage of cell survival versus the concentrations and using cubic spline interpolation and were reported as mean ± standard error of mean (SEM). The IC<sub>50</sub> of plant extracts against cancer cells were compared with IC<sub>50</sub> of normal cells and statistical differences were evaluated using one-way ANOVA. Differences were considered significant at p<0.05.

## 2.5 Cell death detection by enzyme-linked immunosorbent assay (ELISA)

Apoptosis was assayed using Cell Death Detection ELISA<sup>PLUS</sup> Assay (Roche, Indianapolis, IN). It is a photometric enzyme-linked immunosorbent assay (ELISA) that quantitatively measures cytoplasmic histones associated DNA fragments (mono and oligonucleosomes), which are produced as a result of apoptosis in cells. A549 and HeLa cells ( $1 \times 10^4$  cells per well) were incubated in the culture medium only (negative control) or in the culture medium with each of the extracts in the range of 250-800  $\mu\text{g/ml}$  for 48 h. Cell lysates were placed into the streptavidin coated microplate for analysis according to the manufacturer's instructions. The apoptotic level was expressed as an enrichment factor of nucleosomes in cytoplasm and was calculated using the following formula.

Enrichment factor =

$$\frac{\text{absorbance of sample (treated)}}{\text{absorbance of the corresponding negative control}}$$

Results were expressed as the mean values of the enrichment factor  $\pm$  SD. Statistical evaluation was performed by one-way ANOVA. The results were considered significant at  $p < 0.05$ .

## 2.6 Analysis of DNA fragmentation

$1 \times 10^6$  A549 and HeLa Cells were incubated overnight and treated with each of the plant extracts at 0.5, 1.0, and 2.0 mg/ml for 24, 48, and 72 h in 6-well flat bottom plates. If the number of cells is less than  $5 \times 10^5$ , DNA will not be detectable by photography of ethidium bromide stained gel. However, there should be no more than  $5 \times 10^6$  cells to avoid getting too much amount of insoluble DNA. Cells were collected and washed twice in PBS. DNA was extracted, precipitated by addition of isopropanol, recovered by centrifugation, washed in 70% ethanol, dried, and resuspended in

hydration buffer (10 mM Tris.Cl, pH 8.5) according to the manufacturer's instructions (FlexiGene DNA Kits, Qiagen). The collected DNA (10 mg) was electrophoresed on a 2% agarose gel, stained with ethidium bromide, and visualized under UV light.

## 3. Results and Discussion

### 3.1 Antiproliferative and cytotoxic effects of the grass extracts on cancer cells

To evaluate the effects of the hexane and butanolic extracts of *D. aegyptium* (L.) P.B. and *E. indica* (L.) Gaerth. on cell proliferation, we first determined the anti-proliferative effects of the plant extracts on non-small cell lung cancer A549 cells, cervical cancer HeLa cells, and normal human lung MRC-5 fibroblasts. These cells were exposed to various doses of each plant extract for 72 h and cell viability was determined by SRB assay. The cell survival curves in Figure 1 showed that the hexane and butanolic extracts isolated from the two grass species had dose-dependent inhibitory effects on the proliferation of A549, HeLa, and MRC-5 cells after 72 h of incubation. Moreover, the survival curves revealed that all the extracts produced 50% lethality (% cell survival = -50) in A549 and HeLa cancer cells at high concentrations (1,000-2,000  $\mu\text{g/ml}$ ) except for the hexane extract of *E. indica* (L.) Gaerth. against A549 cells.

The mean  $\text{IC}_{50}$  value of all grass extracts against A549 and HeLa cancer cells was in the range of 202 to 843  $\mu\text{g/ml}$  (Table 1). The hexane extract of *D. aegyptium* (L.) P.B. showed the highest activity against A549 and HeLa cancer cells with  $\text{IC}_{50}$  of 202 and 407  $\mu\text{g/ml}$ , respectively compared to all other extracts. In addition, all four extracts were more active against HeLa cells than against A549 cells treated with the corresponding extracts. Indeed, all  $\text{IC}_{50}$  values of the extracts on HeLa cells were significantly lower than corresponding  $\text{IC}_{50}$  values on A549 cells (Table 1). On the contrary, the grass extracts had relatively low toxicity to normal human lung MRC-5 fibroblasts with inhibitory concentration  $\text{IC}_{50}$  values higher than 1000  $\mu\text{g/ml}$  except the

Table 1.  $\text{IC}_{50}$  values of extracts of *D. aegyptium* (L.) P.B. and *E. indica* (L.) Gaerth. on A549 and HeLa cancer cells versus MRC-5 fibroblasts

Plant	Extract	$\text{IC}_{50}$ ( $\mu\text{g/ml}$ )		
		MRC-5	A549	HeLa
<i>Dactyloctenium aegyptium</i> (L.) P.B.	hexane	648.1 $\pm$ 58.0	406.7 $\pm$ 14.9* <sup>†</sup>	201.8 $\pm$ 23.3*
	butanol	1258.7 $\pm$ 41.9	842.9 $\pm$ 51.4* <sup>†</sup>	613.2 $\pm$ 17.9*
<i>Eleusine indica</i> (L.) Gaerth.	hexane	1211.1 $\pm$ 88.2	688.9 $\pm$ 60.1* <sup>††</sup>	466.3 $\pm$ 24.6*
	butanol	1603.2 $\pm$ 36.0	753.7 $\pm$ 56.6* <sup>†</sup>	398.5 $\pm$ 24.9*

Each cell line was treated with the hexane and butanol extracts of *D. aegyptium* (L.) P.B. and *E. indica* (L.) Gaerth.  $\text{IC}_{50}$  values are presented as mean  $\pm$  SEM (n=3-5). \* Significantly different from MRC-5 at  $p < 0.01$ , <sup>†</sup> Significantly different from HeLa at  $p < 0.01$ , <sup>††</sup> Significantly different from HeLa at  $p < 0.05$

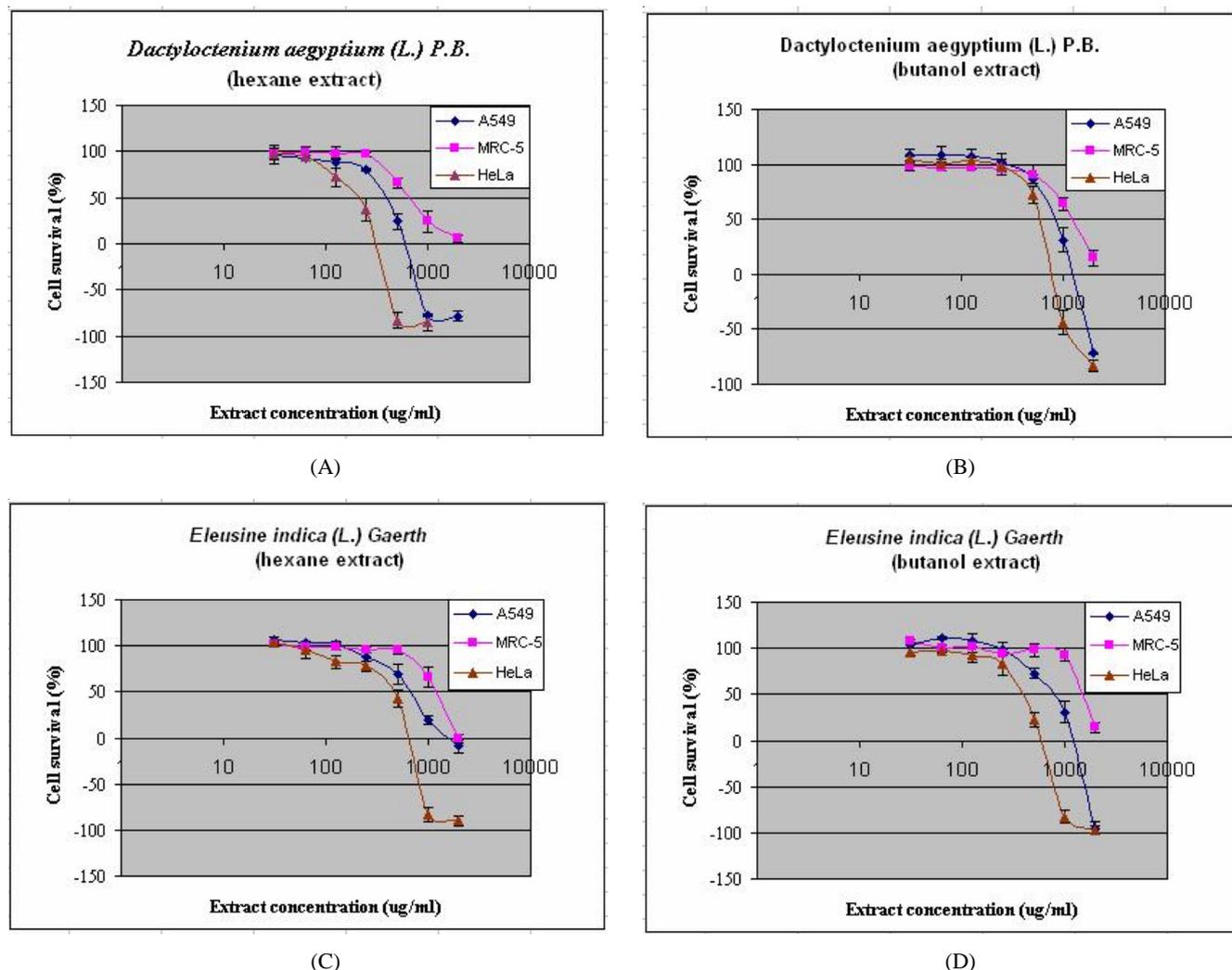


Figure 1. Effects of the hexane and butanolic extracts of *D. aegyptium* (L.) P.B. (Yaa paak khwaai) (A, B) and *E. indica* (L.) Gaerth. (Yaa teen-ka) (C, D) on cell survival (%) of A549, HeLa, and MRC-5 cells. Cells were seeded into 96-well multiplates. After incubation overnight for cell attachment, cells were incubated with the culture medium containing various concentrations of the grass extracts for 72 h. Cell survival (%) was determined by SRB assay. Each point represents the mean  $\pm$  SEM (n = 3-5).

hexane extract of *D. aegyptium* (L.) P.B. (648 µg/ml) (Table 1). These values are significantly different from those observed in both cancer cell lines, indicating that the plant extracts exhibited selective growth inhibition effects on cancer cells. Furthermore, the cell survival curves displayed a net loss of A549 and HeLa cancer cells, which was represented as negative values of the percentage of cell survival at high concentrations close to 1,000 µg/ml (Figure 1) Taken together, these data indicate that the plant extracts exerted antiproliferation (inhibition of cell proliferation) and cytotoxicity (induction of cell death) on cancer cells.

### 3.2 Apoptotic effect of the grass extracts on cancer cells

We next examined whether the cytotoxicity of the plant extracts is related to the induction of apoptosis using an ELISA-based apoptosis assay. The apoptotic level was

calculated by the relative absorbance of treated versus untreated cells and expressed as an enrichment factor. As shown in Figure 2, the hexane extract of *D. aegyptium* (L.) P.B. and *E. indica* (L.) Gaerth. significantly increased the level of apoptosis in extract-treated A549 cells by 5.8 and 2.7 fold, respectively, as compared to untreated A549 cells. In addition, the butanolic extract of *D. aegyptium* (L.) P.B. and *E. indica* (L.) Gaerth slightly induced a 1.6- and 1.7-fold increase of cell apoptosis in extract-treated A549 cells, respectively, over the control level. These results revealed that the hexane extract of these two grass species exhibited higher apoptosis levels in A549 cells than the butanol extract. This could be due to the presence of a higher concentration of the bio-active compound(s) in these hexane extracts that could effectively induce apoptosis. These results correlated with the higher antiproliferative effect of the hexane extracts as shown by their lower  $IC_{50}$  values compared to those of the

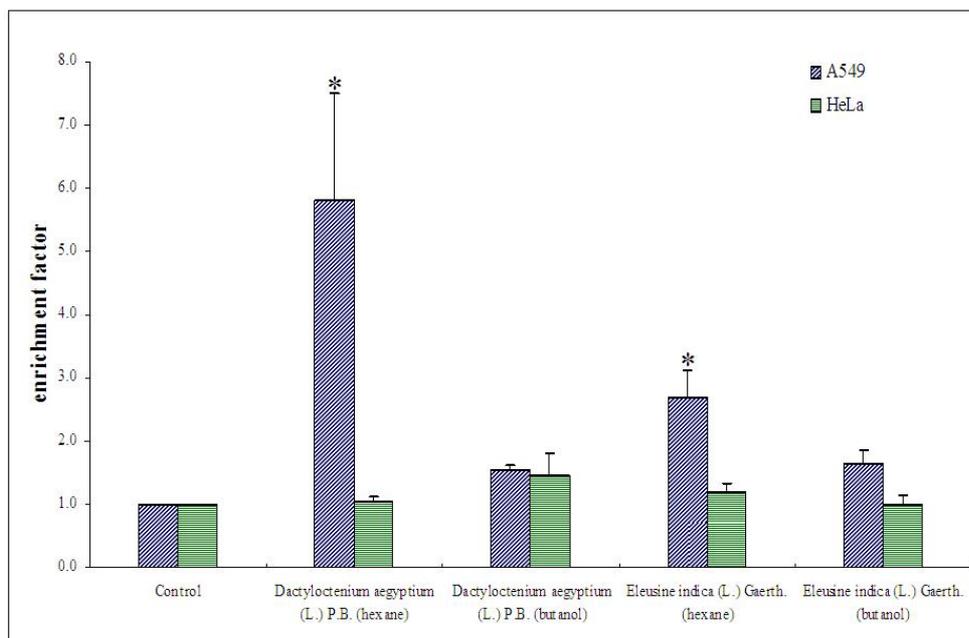


Figure 2. Effects of the hexane (A549/HeLa:250  $\mu\text{g/ml}$ ) and butanolic extracts (A549:800  $\mu\text{g/ml}$ , HeLa:500  $\mu\text{g/ml}$ ) of *D. aegyptium* (L.) P.B. (Yaa paak khwaai), the hexane and butanolic extracts (A549:500  $\mu\text{g/ml}$ , HeLa:300  $\mu\text{g/ml}$ ) of *E. indica* (L.) Gaerth. (Yaa teen-ka) on apoptosis in A549 and HeLa tumor cells. Values are presented as the mean values of the enrichment factor  $\pm$  SD. \* Significantly different from control at  $p < 0.05$ .

butanol extracts in the cell proliferation assay

On the contrary, the enrichment factors of HeLa cells treated with all the extracts were not significantly different from that of the negative control. Despite negative results, it cannot be concluded that there was no increase in apoptosis in these treated HeLa cells because it was noticed that the absorbance of untreated HeLa cells was obviously high, which thus resulted in substantially decreased relative-apoptotic values of HeLa cells. Apparently, the absorbance of untreated HeLa cells was approximately 20 times higher than that of untreated A549 cells. This too high absorbance of negative control HeLa cells could be due to a lot of dead cells or too many cells used, which could be solved with appropriately optimized culturing conditions or cell concentration. Therefore, higher induction of apoptotic level for HeLa cells might be obtained under appropriate conditions.

### 3.3 Induced DNA fragmentation of cancer cells by the grass extracts

Although the ELISA-based apoptosis assay only detected slightly increased levels of apoptosis in HeLa cells (Figure 2), the DNA ladder assay clearly showed classic DNA ladders in both cancer cell lines treated with all the plant extracts (Figure 3 and Figure 4). These DNA ladders are one of the classical features of apoptosis, which are the cleavage of the genomic DNA into oligonucleosomal DNA fragments of multiples of about 180 bp. In this assay, A549 and HeLa cells were treated with the grass extracts at 0.5, 1.0, and 2.0 mg/ml for 24, 48, and 72 h. A dose of 0.5 mg/ml was used to

induce inhibition of cell proliferation in A549 and HeLa cells because it is close to mean  $\text{IC}_{50}$  value of most extracts treated cancer cell lines (Table 1). The two doses, 1.0 and 2.0 mg/ml, were used to induce induction of cell death based on the cell survival curves (Figure 1). Nevertheless, these concentrations are only estimated values for DNA laddering analysis since apoptosis may depend on other factors such as kinetics of cell death and the amount of affected cells in the total population. Both cancer cell lines were also treated with the anticancer drug paclitaxel (Taxol) and their DNA extracts were electrophoresed in parallel as positive controls. The classic 180-bp nucleosomal DNA ladder was observed in a dose- and time-dependent manner. However, Figure 3 and Figure 4 show only the classic ladders induced by the minimum effective dose of each grass extract at the minimum incubation time for A549 and HeLa cells, respectively.

In summary, our data provide experimental evidence for the first time that the hexane and butanolic extracts of *D. aegyptium* (L.) P.B. and *E. indica* (L.) Gaerth. showed selective antiproliferative and cytotoxic effects on human lung and cervical cancer cells, but exhibited only moderate to low levels. Furthermore, these extracts exerted their cytotoxic effects to eliminate cancerous cells via specific apoptotic cell death.

### Acknowledgements

This work was supported by the National Research Council of Thailand.

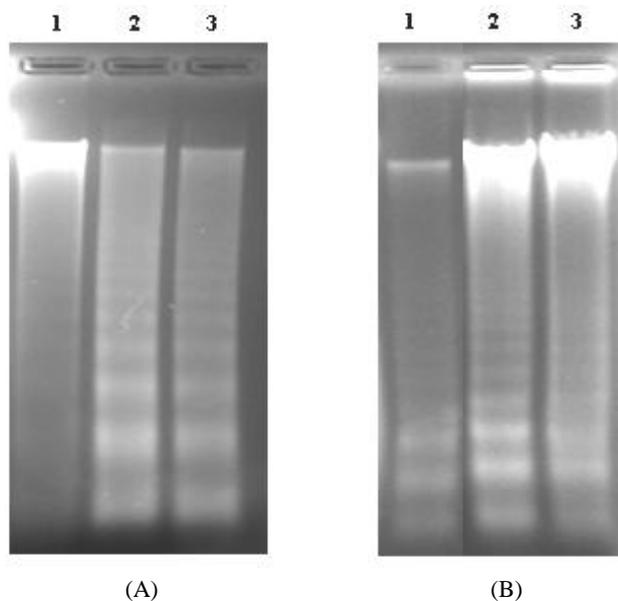


Figure 3. DNA ladder of A549 cells treated with the grass extracts. (A) Cells were treated with the culture medium only (lane 1), the hexane extract of *D. aegyptium* (L.) P.B. (Yaa paak khwaai) at 2 mg/ml for 24 h (lane 2), and the hexane extract of *E. indica* (L.) Gaerth. (Yaa teen-ka) at 2 mg/ml for 24 h. (lane 3) (B) Cells were treated with the butanolic extract of *D. aegyptium* (L.) P.B. at 2 mg/ml for 48 h (lane 1), the butanolic extract of *E. indica* (L.) Gaerth. at 2 mg/ml for 72 h (lane 2), and 12  $\mu$ M Taxol for 24 h (lane 3). DNA was isolated, subjected to agarose gel electrophoresis, stained with ethidium bromide, and visualized under UV light.

## References

- Bunyapraphatshara, N. and Chokchaichareonporn, O. 2000: Samunprai maipeunban. Vol. 4. Bangkok: Prachachon Ltd., 627-633.
- Chang, H. C., Huang, Y. C. and Hung, W. C. 2003. Antiproliferative and chemopreventive effects of adlay seed on lung cancer in vitro and in vivo. *Journal of Agricultural and Food Chemistry*. 51, 3656-60.
- Ding, G., Song, Y. C., Chen, J. R., Xu, C., Ge, H. M., Wang, X. T. and Tan, R. X. 2006. Chaetoglobosin U, a cytochalasan alkaloid from endophytic *Chaetomium globosum* IFB-E019. *Journal of Natural Products*. 69, 302-4.
- Kim, K. K., Kawano, Y. and Yamazaki, Y. 2003. A novel porphyrin photosensitizer from bamboo leaves that induces apoptosis in cancer cell lines. *Anticancer Research*. 23, 2355-61.
- Komiya, T., Kyohkon, M., Ohwaki, S., Eto, J., Katsuzaki, H., Imai, K., Kataoka, T., Yoshioka, K., Ishii, Y. and Hibasami, H. 1999. Phytol induces programmed cell death in human lymphoid leukemia Molt 4B cells. *International Journal of Molecular Medicine*. 4, 377-80.

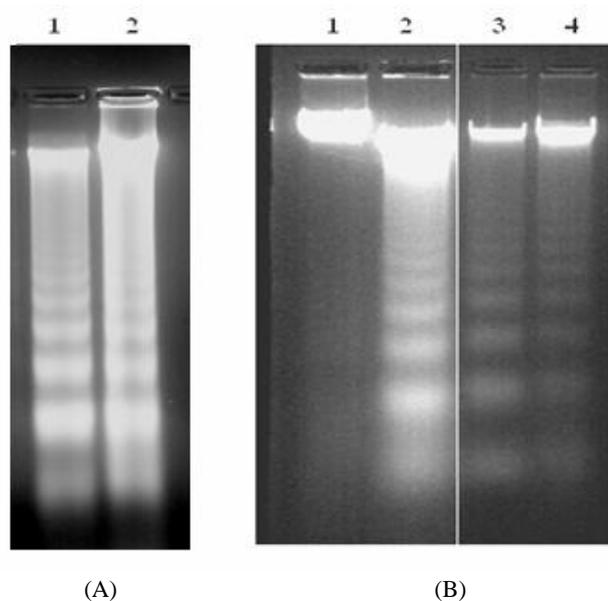


Figure 4. DNA ladder of HeLa cells treated with the grass extracts. (A) Cells were treated with the hexane extract of *D. aegyptium* (L.) P.B. (Yaa paak khwaai) at 1 mg/ml for 24 h (lane 1), and the hexane extract of *E. indica* (L.) Gaerth. (Yaa teen-ka) at 1 mg/ml for 48 h (lane 2) (B) Cells were treated with the culture medium only (lane 1), 100 nM Taxol for 48 h (lane 2), the butanolic extract of *D. aegyptium* (L.) P.B. at 2 mg/ml for 48 h (lane 3), and the butanolic extract of *E. indica* (L.) Gaerth. at 2 mg/ml for 48 h (lane 4). DNA was isolated, subjected to agarose gel electrophoresis, stained with ethidium bromide, and visualized under UV light.

- Kyohkon, M., Komiya, T., Ohwaki, S., Katsuzaki, H., Imai, K., Nishide, R., Ikeda, H. and Hibasami, H. 2001. Sodium 1-monolinolenin isolated from Italian ryegrass (*Lolium multiflorum* Lam) induces apoptosis in human lymphoid leukemia Molt 4B cells. *International Journal of Molecular Medicine*. 7, 265-8.
- Newman, D. J., Cragg, G. M., Holbeck, S. and Sausville, E. A. 2002. Natural products and derivatives as leads to cell cycle pathway targets in cancer chemotherapy. *Current Cancer Drug Targets*. 2, 279-308.
- Newman, D. J., Cragg, G. M. and Snader, K. M. 2003. Natural products as sources of new drugs over the period 1981-2002. *Journal of Natural Products*. 66, 1022-37.
- Ren, M., Reilly, R. T. and Sacchi, N. 2004. Sasa health exerts a protective effect on Her2/NeuN mammary tumorigenesis. *Anticancer Research*. 24, 2879-84.
- Schwartzmann, G., Ratain, M. J., Cragg, G. M., Wong, J. E., Saijo, N., Parkinson, D. R., Fujiwara, Y., Pazdur, R., Newman, D. J., Dagher, R. and Di Leone, L. 2002. Anticancer drug discovery and development throughout the world. *Journal of Clinical Oncology*. 20, 47S-59S.
- Skehan, P., Storeng, R., Scudiero, D., Monks, A., McMahon, J., Vistica, D., Warren, J. T., Bokesch, H., Kenney, S.

and Boyd, M. R. 1990. New colorimetric cytotoxicity assay for anticancer-drug screening. *Journal of National Cancer Institute*. 82, 1107-12.

Verschoyle, R. D., Greaves, P., Cai, H., Edwards, R. E., Steward, W. P. and Gescher, A. J. 2007. Evaluation of the cancer chemopreventive efficacy of rice bran in genetic mouse models of breast, prostate and intestinal carcinogenesis. *British Journal of Cancer*. 96, 248-54.