

Full Length Research Paper

***In vitro* antineoplastic effect of *Ficus hispida* L. plant against breast cancer cell lines**

B. Pratumvinit¹, T. Srisapoomi¹, P. Worawattananon¹, N. Opartkiattikul¹, W. Jiratchariyakul² and T. Kummalue^{1*}

¹Department of Clinical Pathology, Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand.

²Department of Pharmacognosy, Faculty of Pharmacy, Mahidol University, Bangkok 10400, Thailand.

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Stems of *Ficus hispida* L. have long been prescribed as one of the constituents in various Thai traditional remedies for cancer therapy. In the present study, crude ethanol extract and its sequential fractions from *F. hispida* L.: water, methanol: water, methanol and ethyl acetate fraction were tested *in vitro* against SKBR3, MDA-MB435, MCF7 and T47D human breast cancer cell lines. The results have shown that the methanol extract exhibited antineoplastic activity against T47D cells. The cytotoxic activity was further examined by MTT assay with more dilution, colony forming assay and cell cycle analysis. The IC₅₀ of this extract against T47D cell was 110.3 ± 9.63 µg/mL by MTT assay and colony forming assay confirmed the cell growth inhibition in a dose-dependent manner. Cell cycle analysis demonstrated a rising of apoptotic cell population in herbal treated cells. Therefore, *F. hispida* L. used in traditional medicine may provide some benefits in the treatment of breast cancer.

Key words: *Ficus hispida* L., T47D, SKBR3, MCF7, MDA-MB435, MTT, colony count, cell cycle, antineoplastic.

INTRODUCTION

Epithelial malignancies of the breast are the most common cause of cancer in women (excluding skin cancer), accounting for about one-third of all cancer in women (Lippman, 2006). Although the incidence of breast cancer in Thailand is lower than that in developed countries (Vatanasapt et al., 2002), breast cancer still represents the third leading cancer in women after cervical cancer and lung cancer with an estimated age standardized incidence rate of 20.7 per 100,000 female (Srivatanakul, 2008; Vatanasapt et al., 2002). Treatments in cancer usually require a multimodality approach including medical, surgical and radiological treatments to reduce mortality and improve palliation in women where cure is not a possibility. Hortobagyi (1998) and Shenkier et al. (2004)

Current medical treatments in breast cancer compose of chemotherapy, hormonal treatment and targeted therapy which, however are sophisticated, expensive and not widely available. Therefore, a search for novel anti-cancer agents from natural products may provide an alternative and cost-effective treatment modality (Steva-

novice et al., 2006).

Thailand has various kinds of medicinal plants which have been used to treat many diseases for a long time. Among these potential Thai plants, *Ficus hispida* L. is one of ingredients in Thai traditional medical formulations, which has been widely prescribed for cancer therapy.

F. hispida L. (Locally known as *Ma Dau Plong*), is a traditional Thai plant from family Moraceae. It is a shrub or moderate sized tree and grows well in damp and in shady places. It is widely distributed throughout India, Sri Lanka, Myanmar, southern regions of the China, New Guinea and Queensland in Australia. Almost all parts of this plant can be used in traditional medicine for the treatment of various ailments, for example, an anti-diarrheal, emetic, astringent, hepatoprotective, antitussive, antipyretic, anti-inflammatory, depurative, vulnerary, hemostatic, antiulcer agent as well as in the treatment of anemia (Mandal and Ashok Kumar, 2002; Peraza-Sanchez et al., 2002). Additionally, their stems have been utilized for the treatment of malignancies in Thai traditional medicinal formulations, but there is no scientific data to support their pharmacological activities. Therefore, we performed this study to determine the activity of *F. hispida* L. against human

*Corresponding author. E-mail: sitkm@mahidol.ac.th. Tel./Fax: (662) 418-1367.

breast cancer cell lines.

MATERIALS AND METHODS

Plant identification

F. hispida L. was collected from Ratchaburi province in the eastern part of Thailand and was identified by Miss Leena Phuphatthana-pong, a taxonomist at the Forest Herbarium (BKF), Royal forest department, Ministry of Agriculture and cooperatives, Bangkok, Thailand. A voucher specimen No.139132 was deposited at the same place.

Plant material and extraction procedures

Dried stems of *F. hispida* L. were purchased from a Thai Herbal Pharmacy (Chao-Krom-Po). They were cut into small fragments and ground into powder. Their powder was macerated three times with 4 L of 95% ethanol for seven days. Crude extract was acquired by concentrating the extract under reduced pressure.

Diaion® HP-20 chromatography

Crude extract was then processed as following to attain four fractions (Kummalue, 2005). First, the ethanol extract was chromatographed on Diaion® HP-20 column (Mitsubishi Chemical Corp., Japan) and equilibrated with water (100 ml). The ethanol extract was dissolved in distilled water (100 mL) and sonicated for 15 min in an ultrasonic bath to get the water soluble fraction. The suspension was centrifuged at 20,000 rpm for 30 min. The supernatant was applied onto the Diaion® HP-20 column and the eluent was collected (fraction 1). The precipitate was dissolved in methanol water (1:1/100) and methanol (150 ml) to acquire fraction 2 and methanol fraction (fraction 3) respectively. Each supernatant was repeatedly performed as described above. The precipitate was dissolved in ethyl acetate (fraction 4). The chemical composition of each fraction was monitored on thin layer chromatography.

Preparation of the plant extracts

The water fraction (fraction 1) of *F. hispida* L. was dissolved in water while other fractions were dissolved in dimethyl sulfoxide (DMSO, Sigma, St. Louis, USA) before performing experiments. The final concentrations of the tested compound were prepared by diluting the stock with the culture medium (Kummalue, 2005).

Human cell lines

Four human breast cancer cell lines used in the present study were SKBR3, MDA-MB435, MCF7, and T47D which were kindly provided by Dr. Pornchai O-charoenrat (Mahidol University, Bangkok). All cancer cell lines were invasive ductal carcinoma and originally derived from pleural effusion, but their receptor status were different. SKBR3 breast cancer cell line is estrogen receptor-negative (ER-) /progesterone receptor-negative (PR-) and has over-expression of HER2/neu receptor. Conversely, MDA-MB435 breast cancer cell line is ER-/PR- and do not express HER2/neu receptor while T47D and MCF7 cell lines are ER+/PR+ and negative HER2/neu receptor (Lacroix and Leclercq, 2004). All cell lines were maintained in Dulbecco's modified eagle's medium (DMEM) (Gibco, Thailand) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Gibco, Thailand), glutamine (Gibco, Thailand) and 1% penicillin-streptomycin (Gibco, Thailand). Cells were maintained at 37°C in a

humidified atmosphere of air/CO₂ (19:1) and subcultured with 0.05% trypsin-EDTA (Gibco, Thailand).

F. hispida L. treatment of cells

0.025 of *F. hispida* L. was dissolved in DMSO 250 µL first and then further dissolved in 250 µL of media to get a concentration of 50,000 µg/ml as a stock solution. The stock was diluted to the required concentration before use with growth media. The cells were exposed to *F. hispida* L. at different concentration (0 - 200 µg/mL) for 48 h.

Cell in media containing equivalent amount of DMSO served as negative control for crude extract and fraction 2, 3, and 4 while sterile water served as negative control for fraction 1.

Cell viability assay

The viability of the cells was assessed by MTT (3, 4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma, USA) assay which is based on the reduction of MTT by the mitochondrial dehydrogenase of intact cells to a purple formazan product (Denizot and Lang, 1986; Mosmann, 1983).

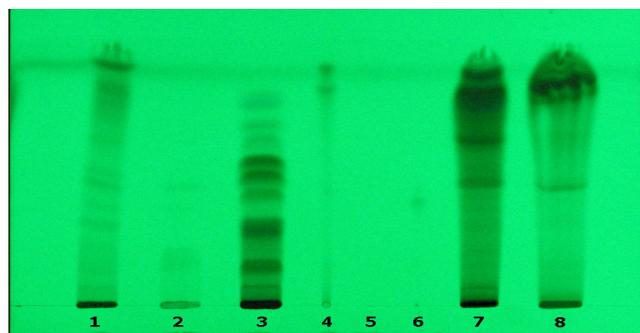
1 x 10⁴ cells per well were plated in a 96-well plate (Costar, Corning, NY). After 24 h incubation, cells were treated with each herbal extract with various concentrations (1, 10, 50, 100 µg/ml) for 48 h. Then, MTT 50 µl (1 mg/ml in PBS) was added in each well and cells were incubated for 4 h. The supernatant in each well was removed carefully and 100 µl of DMSO were added. Amount of formazan was determined by measuring the absorbance at 595 nm using an ELISA plate reader (EL808 ultra microplate reader, Biotek laboratories, USA). Data were calculated as percentage viability using the following formula: Cell death (%) = (control OD - sample OD)/control OD x 100; Cell viability (%) = 100 - (% cell death). Cell viability assay was performed in duplication with three independent experiments.

Colony-forming assay

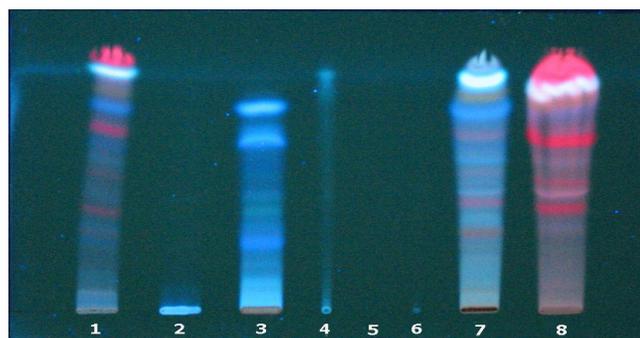
Breast cancer cell lines were plated on 12-well plate (Costar, Corning, NY) at a density of 7 x 10² cells per well and treated with *F. hispida* L. at the concentration of 50, 100 and 150 µg/ml. Media was changed after 48 h of incubation, colonies were further observed until 7 days. Colonies were then stained with 1 g of crystal violet in 50% methanol for 30 min and counted by using ChemiDoc XRS (Biorad).

Cell cycle analysis

Analysis of the cell cycle in control and treated cancer cells were determined. The DNA of cells was stained with propidium iodide, and the proportion of cells in different phases of the cell cycle was recorded. Briefly, cancer cells (1x10⁶ cells per well) were incubated in 100 mm x 20 mm plate (Corning, NY). After 24 h incubation, cells were treated with each herbal extract at the concentration of 50, 100, 200 µg/mL for 48 h. Cells in plates were trypsinized and collected, washed with PBS, fixed with 1 mL ice-cold 70% ethanol for 30 min at -20°C. Then, cells were washed with PBS and resuspended in 500 µl (10 µg/ml) of Ribonuclease A (RNase) (Sigma, Germany) and incubated for 30 min at 37°C. After the incubation, 500 µl (50 µg/mL) of propidium iodide staining solution (PI) (Sigma, Germany) was added in the dark and the samples were analyzed by FACScaribur using CellQuest software.



Under UV 254



Under UV 366

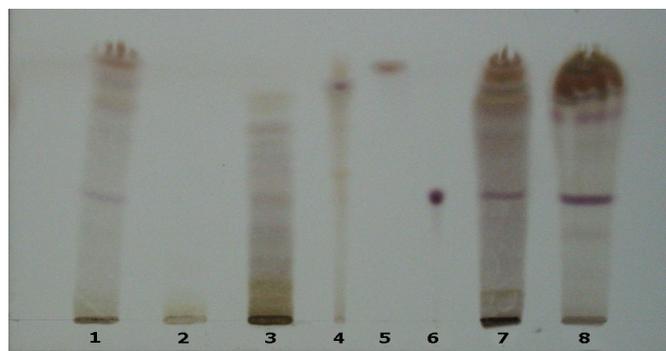
Spray with H₂SO₄

Figure 1. Thin-layer chromatogram of *F. hispida* L. ethanol (crude) extract and fractions from Diaion® HP-20. TLC chromatogram of *Ficus hispida* L. extract

Adsorbent: silica gel GF₂₅₄ Alufolien, Merck; Solvent system: Ethyl acetate: Acetic acid: Formic acid: Water (100:11:11:2); Detection: Under UV 254, UV 366 spray with 1% FeCl₃ and 10 % sulphuric acid in ethanol, heat at oven 110°C, 1- 2 min.; 1 = ethanol extract; 2 = Water fraction; 3 = Water:methanol fraction; 4 = β-sitosterol; 5 = α-amyrin; 6 = G1a; 7 = Methanol fraction; 8 = Ethyl acetate fraction.

Statistical analysis

The results were presented as mean ± standard error. The values of IC₅₀ were calculated by using regression analysis. Data were analyzed using Student t-test and analysis of variance (ANOVA).

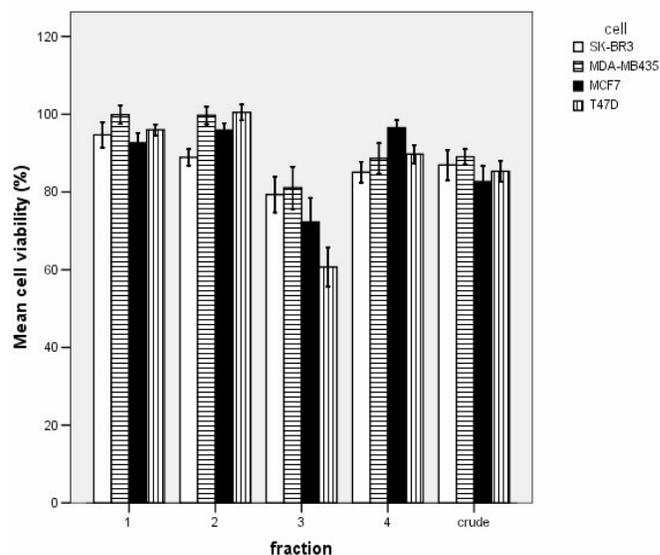


Figure 2. Cytotoxic effects of *F. hispida* L. extracts on the growth of SK-BR3, MDA-MB435, MCF7 and T47D breast cancer cell lines after 48 h of treatment. The results were expressed as a mean of the cell viability after treatment with all four concentration of each herbal fraction ± standard error of the mean. Crude = ethanol extract, fraction 1 = water extract, fraction 2 = water: methanol extract, fraction 3 = methanol extract, fraction 4 = ethyl acetate extract

The level of significance was set at $p < 0.05$.

RESULTS

Yield of extracts from plant materials

F. hispida L. was extracted as described in extraction procedure. The thin layer chromatography of herbal extract was demonstrated in figure 1.

Antineoplastic effect of *F. hispida* L. on human breast cancer cell lines by colorimetric assay (MTT based)

The antineoplastic effect of *F. hispida* L. on various breast cancer cell lines was determined. Cells were treated with each herbal extract at the concentration of 1, 10, 50, 100 µg/ml for 48 h. Cells incubated with sterile water were used as a control for fraction 1, whereas cells incubated with 0.5% DMSO were used as a control for crude extract, fraction 2,3 and 4. Methanol fraction (fraction 3) had a superior cytotoxic effect against all four breast cancer cells compared to the other four fractions (Figure 2). For SKBR3 cells, fraction 3 of this herb decreased cell viability by $20.67 \pm 4.59\%$ while fraction 1 decreased cell viability by $5.32 \pm 3.29\%$ ($p < 0.05$).

In MDA-MB435 cells, fraction 3 inhibited the cell growth by $18.93 \pm 5.45\%$ but fraction 1 and 2 inhibited the cell growth by $0.13 \pm 2.4\%$ ($p < 0.01$) and $0.3 \pm 2.27\%$ ($p < 0.01$) respectively. In addition, fraction 3 decreased MCF7 cell survival by $27.73 \pm 6.2\%$, however; fraction 1, 2, 4 decreased cell survival by $7.35 \pm 2.55\%$ ($p < 0.01$),

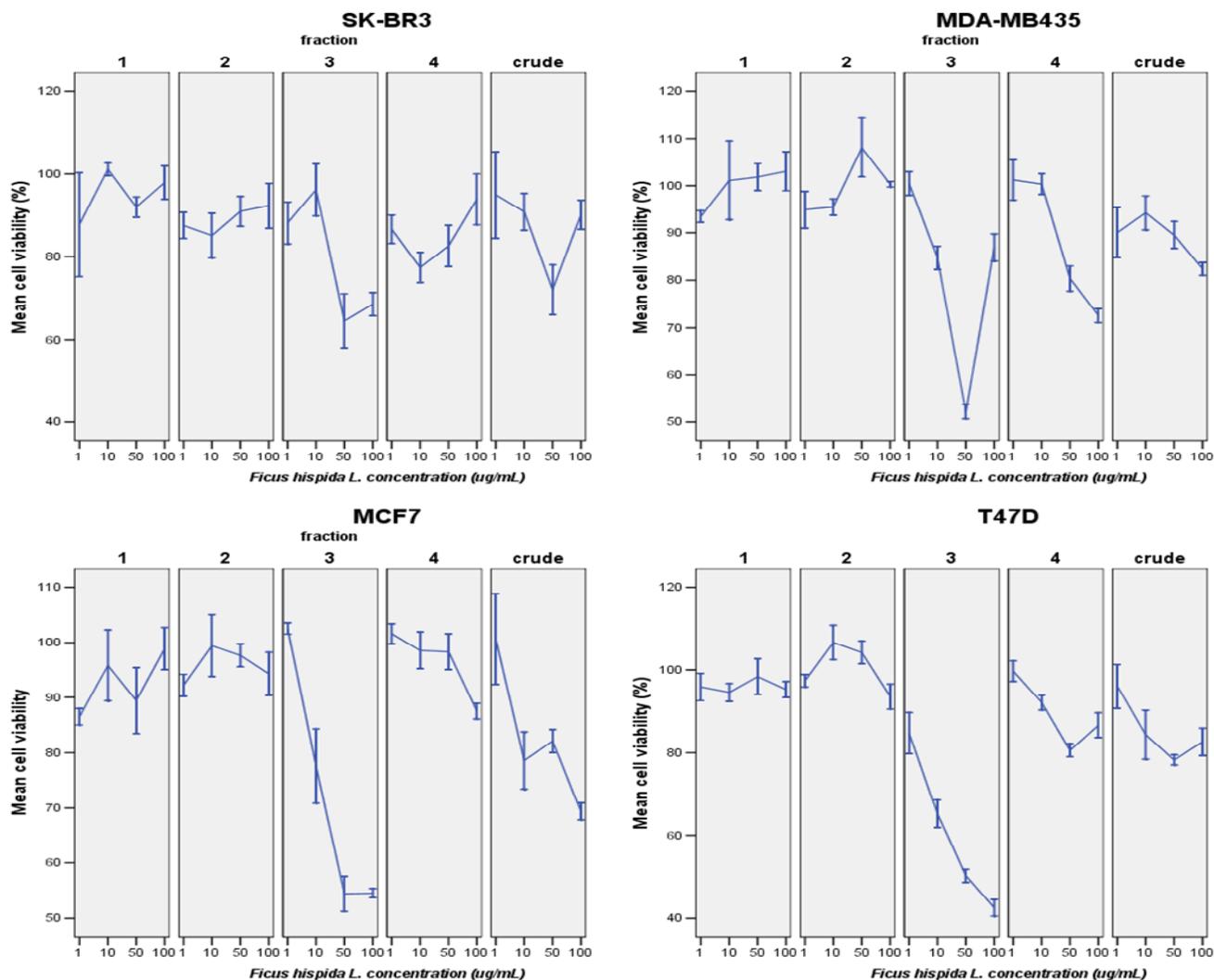


Figure 3. Cytotoxicity of *F. hispida* L. on four breast cancer cell lines, Cells were seeded at 1×10^4 cells/well in 96 well plate for 48 h before adding *F. hispida* L. at concentrations of 1, 10, 50, 100 µg/ml. Crude = ethanol extract, fraction 1 = water extract, fraction 2 = water:methanol extract, fraction 3 = methanol extract, fraction 4 = ethyl acetate extract.

$4.10 \pm 1.8\%$ ($p < 0.001$) and $3.5 \pm 1.96\%$ ($p < 0.001$) respectively. For T47D cells, fraction 3 of *F. hispida* L. decreased cell viability by $39 \pm 5\%$ but crude extract, fraction 1, 4 decreased cell viability by $14.69 \pm 2.72\%$ ($p < 0.001$), $4.02 \pm 1.39\%$ ($p < 0.001$), $10.27 \pm 2.34\%$ ($p < 0.001$) respectively. Cell viability was not decreased in T47D cells after treatment with fraction 2 of this herb. After 48 h treatment, the 50% inhibition of cell viability was observed only in T47D cancer cell line after the treatment with *F. hispida* L. methanol fraction ($r^2 = 0.81$) (Figure 3). To explore the inhibitory effect of *F. hispida* L. methanol fraction on T47D cancer cell line, the methanol fraction of the herb was diluted at the concentration of 30, 40, 50, 60, 70, 80 µg/ml and treated on T47D cells with three independent experiments and the IC₅₀ value was 110.3 ± 9.63 µg/ml after 48 h treatment (Figure 4).

Antineoplastic effect of *F. hispida* L. methanol fraction on T47D human breast cancer cell lines by colony forming assay

The antineoplastic effect of *Ficus hispida* L. (methanol fraction) on T47D breast cancer cell line by MTT assay was confirmed by using colony count. In the clonogenic assay, *F. hispida* L. (methanol fraction) at the concentration of 50 µg/mL suppressed cell colony growth by 2.71% ($p < 0.001$) when compared with control (0.5% DMSO) as demonstrated in Figure 5.

Cell cycle analysis

The cell cycle was analyzed in T47D cell treated with *F. hispida* L. methanol fraction for 48 h at the concentration

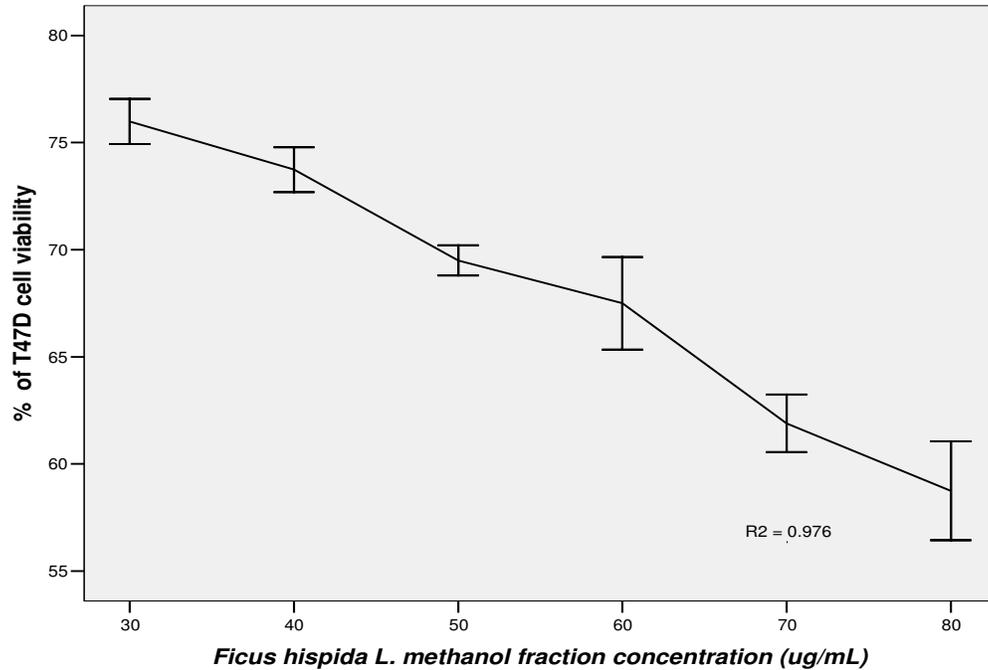


Figure 4. Percentage of cell viability of T47D cell after treatment with *F. hispida* L. methanol fraction at the concentration of 30, 40, 50, 60, 70 $\mu\text{g/ml}$ determined by MTT (3, 4, 5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide) assay. Values are expressed as a mean \pm standard error of independent three experiments.

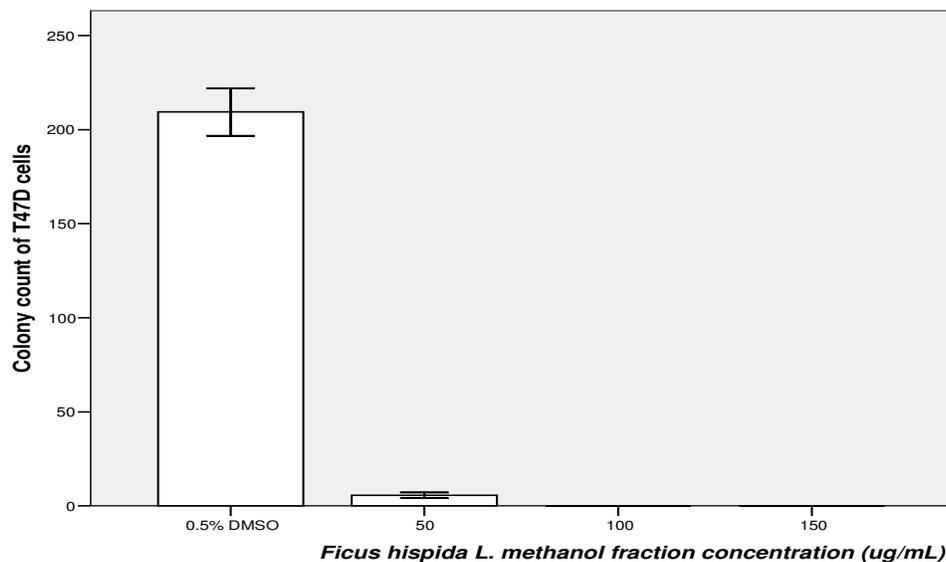


Figure 5. Cytotoxic effects of *F. hispida* L. methanol fraction against T47D cell by colony forming assay at the concentration of 50, 100, 150 $\mu\text{g/ml}$ compared with 0.5% DMSO. Values are expressed as a mean \pm standard error of triplicate experiments.

of 50, 100, 200 $\mu\text{g/ml}$. The percentage of cells in each phase of cell cycle was compared with 0.5% DMSO and determined by flow cytometry. Quantitation of apoptosis evaluated by the percentage of cells in the sub-G1 phase

revealed $8.945 \pm 2.115\%$ apoptotic population in the 0.5% DMSO-treated T47D cell which was used as negative control. The apoptotic cell population increased in a dose-dependent manner in the T47D cell treated with

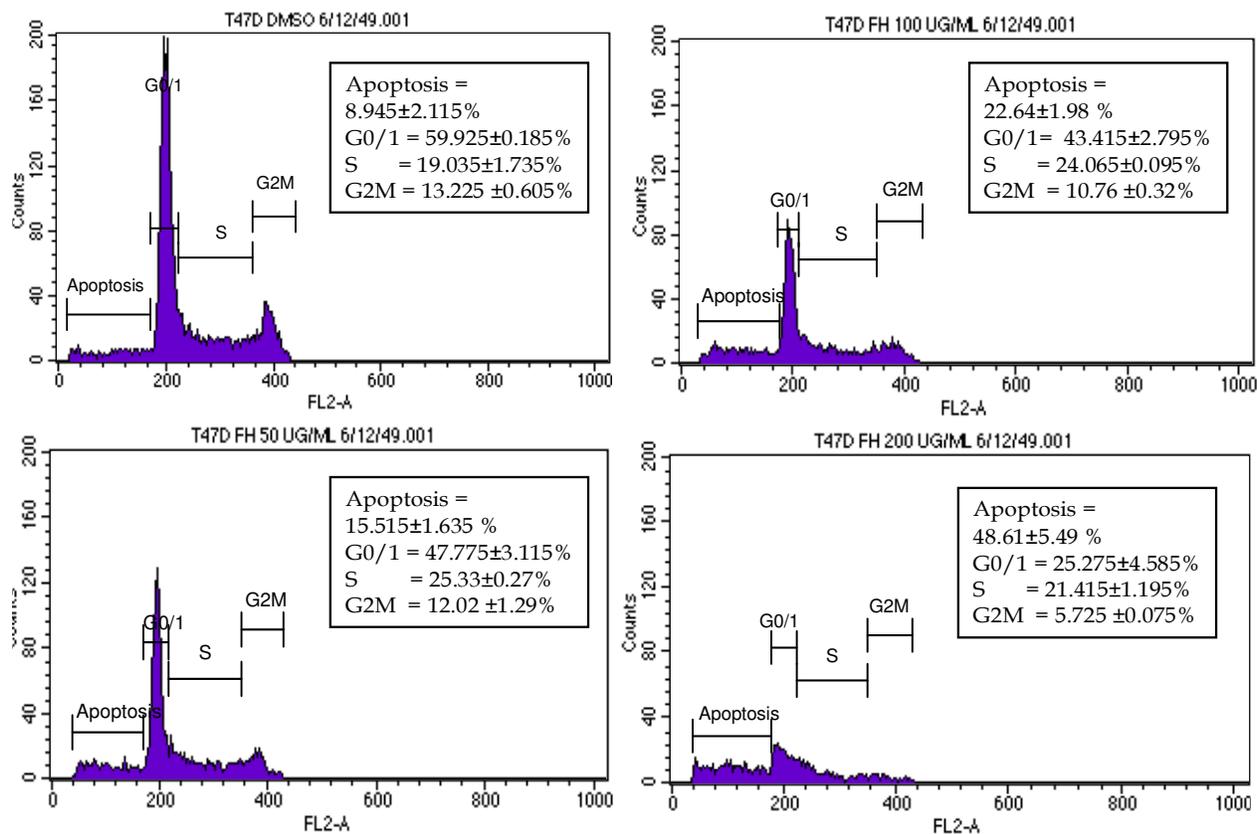


Figure 6. Cell cycle analysis of T47D cancer cells using flow cytometry after 48-h treatment with 0.5% DMSO (negative control) and *F. hispida* L. methanol fraction at the concentration of 50, 100, 200 µg/ml, respectively. The cells were fixed and stained with PI, and the DNA content was analyzed by FACScalibur flow cytometry. Values are means ± standard error of two independent experiments.

the herbal extract The apoptotic cell population were $15.515 \pm 1.635\%$, $22.64 \pm 1.98\%$ and $48.61 \pm 5.49\%$ ($p < 0.001$) in T47D cells treated with *F. hispida* L. fraction 3 at the concentration of 50, 100 and 200 µg/ml respectively as shown in Figure 6. Data from this study suggested that the antineoplastic effect of *F. hispida* L. methanol fraction on T47D cells was mediated by induction of cell apoptosis.

DISCUSSION

The Stem of *F. hispida* L. is one of the ingredients used as constituent in Thai traditional anticancer remedies. O-methyltylophorinidine, a constituent of *F. hispida* leaves was shown to be highly cytotoxic for the lung (Lu1, ED₅₀ = 0.018 µg/ml), colon (Col2, ED₅₀ = 0.02 µg/ml), nasopharynx (KB, ED₅₀ = 0.02 µg/ml) and prostate (LNCaP, ED₅₀ = 0.018 µg/ml) cancer cell lines. In addition, CHCl₃ extract from mixture of leaves and twigs of *F. hispida* L. was shown to be highly cytotoxic for the cell lines in which it was evaluated (LU1, ED₅₀ = 0.5 µg/ml; Col2, ED₅₀ = 0.1 µg/ml and LNCaP, ED₅₀ = 0.03

µg/ml) (Peraza-Sanchez et al., 2002). Our study was performed to demonstrate the cytotoxic effect of *F. hispida* L. stems against four breast cancer cell lines. The results illustrated that methanol fraction of *F. hispida* L. had stronger antineoplastic activity than other fractions in breast cancer cell lines especially in T47D cells. Our study did not support traditional use of water extracts of *F. hispida* L. The possible mechanism of selectivity of this plant extract against T47D cell would be some cytotoxic effects of this extract mediated by estrogen and/or progesterone receptors of T47D cells.

After 48 h incubation, we observed that *F. hispida* L. methanol fraction inhibited the growth of T47D cell line from both MTT assay and colony forming assay in a dose-dependent manner. With MTT colorimetric assay, the IC₅₀ of *F. hispida* L. methanol fraction against T47D cell line was 110.3 ± 9.63 µg/ml. However, in colony forming assay, *F. hispida* L. methanol fraction at a low concentration of 50 µg/ml can inhibit reproduction of T47D cells more than 50% when compared with 0.5% DMSO. The colony forming assay, thus, seemed to have IC₅₀ of less than 50 µg/ml. The discrepancy of MTT assay and clonogenic assay may come from different

methods and different dilutions. MTT assay is a functional assay which measures the metabolic components that are necessary for cell growth. This assay is sensitive and can be analyzed semiautomatically (Park et al., 2000). In contrast, clonogenic assay is a reproductive assay which determine growth rate which depend on cellular reproductive potential.

Other natural products such as *Saururus cernuus*, *Psychotria klugii* Standl, have been shown to inhibit breast cancer cell growth including T47D cell by inhibiting the activation of hypoxia-inducible factor-1 (HIF-1). (Hossain et al., 2005; Hodges et al., 2004; Zhou et al., 2005) Curcumin (Verma et al., 1998) and genistein (natural flavone compound found in soy) have also been reported to have cytotoxic effects on T47D cancer cell lines. From cell cycle analysis, *F. hispida* L. methanol fraction used in the present study increased apoptotic cell population of T47D cancer cell. The apoptotic population in cell cycle analysis attained approximately 50% at the concentration of 200 µg/ml which was different from IC50 in MTT assay and clonogenic assay. The IC50 from MTT assay which is 110.3 ± 9.63 µg/ml illustrated the inhibition of cell growth at 50% but the dose of herbal extract from cell cycle analysis should be higher in order to cause 50% apoptosis. Moreover, apoptotic cell population in cycle analysis analyzed the state of individual cells in contrast to the clonogenic assay which analyzed the colonies and required at least 50 cells to detect the reproductive capacity of the each cell (Franken et al., 2006). However, the exact mechanism by which *F. hispida* L. induces apoptosis of breast tumor cells are still not known and remains to be determined in future investigations.

F. hispida L. methanol fraction exerted an inhibitory effect on the growth of T47D breast cancer cell lines in a dose-dependent manner. In addition, our results demonstrated that apoptosis may play some role in *F. hispida* L. induced antineoplastic activities in T47D. However, further studies at molecular level are required to identify the active components and specific mechanism that could induce growth inhibition from this herbal extract.

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