

Antiproliferative effects of three roots Thai herbal recipe on human cancer cell lines

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Abstract

A very famous Thai herbal recipe in northeastern Thailand is named “sam rak,” which means “three roots.” These ethanolic roots extract were. The three roots Thai herbal recipe is composed of *Eurycoma longifolia*, *Dipterocarpus obtusifolius* and *Tamilnadia uliginosa* in a ratio of approximately 7:4:3. Ten human cancer cell lines were used in this investigation. These comprised breast (MCF-7, SKBR3, MDA-MB435), lung (A549), colon (HCT116), cervical (HeLa), leukemic (NB4 and K562), and liver (Hep3B and C3A) cancer cell lines. Human dermal fibroblasts were used as control group. ED₅₀ values of this herbal recipe ethanolic extract were determined using MTT assay. The results showed that five cancer cell lines, HCT116, HeLa, SKBR3, NB4, and K562, were significantly inhibited with ED₅₀ values less than 100 µg/mL. These five cancer cell lines were further evaluated to determine their mechanisms of action using cell cycle analysis. The results demonstrated that most of these cancer cell lines showed high percentages of apoptosis, except HCT116 which showed significant S phase arrest. Thus, this study showed that “sam rak” Thai herbal recipe has potential to be developed as a traditional anticancer recipe that could be available to people all over the country.

Keyword: Thai herbal recipe, antiproliferative effect, *Dipterocarpus obtusifolius*, *Eurycoma longifolia*, *Tamilnadia uliginos*

1. INTRODUCTION

In Nong-bua-lum-pu province, in northeastern Thailand, there is a very well-known Thai herbal recipe named “sam rak.” This means “three roots” in the Thai language. This treatment has been widely used to treat various kinds of cancers such as liver, lung, breast, colon, and cervical cancers for several decades. Most Thai cancer patients in this province use “sam rak.” The recipe is composed of roots from 3 different plants, namely, *Eurycoma longifolia*, *Dipterocarpus obtusifolius* and *Tamilnadia uliginosa* mixed in a ratio of approximately 7:4:3. The roots are ground into powder using whetstones and then dissolved with one liter of water. This blend is designed to be consumed all in one day, every day. The preliminary survey

of this three root herbal recipe by directly interviewing cancer patients demonstrated very good outcomes with regression of malignant diseases. However, scientific information both *in vitro* and *in vivo* studies has never been demonstrated.

Eurycoma longifolia, in the family of Simaroubaceae, is commonly used as an herbal medicinal plant in the traditional medicine zone of Southeast Asia, including Indonesia, Malaysia, Vietnam, Laos, and Thailand. It has been prescribed as an antimalarial, aphrodisiac, antidiabetic, antimicrobial, and antipyretic medicine. It is included in the mixture of various herbal remedies. The part usually employed by traditional doctors is the root. Based on previous reports, the bioactive compounds isolated from

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this plant are eurycolactone, eurycomaoside, eurycomalactone, eurycomanone, and 9-methoxycanthin-6-one. Interestingly, *Eurycoma longifolia* root extract has high anticancer activity against various kinds of cancer cell lines such as K562 (chronic myeloid leukemia), Caov-3 (ovarian cancer), DE-145 (prostate cancer), KB (epidermoid carcinoma), RD (rhabdosarcoma), and MCF-7 (breast cancer).^{1,2} Among the extracts from its root, the aqueous fraction showed the least antiproliferative activity when compared with methanol, chloroform, and butanol fractions with IC₅₀ over 100 µg/mL.²

Dipterocarpus obtusifolius, in the family of Dipterocarpaceae can grow to be ten to fifteen meters tall. It is distributed in Laos, Vietnam, Cambodia, and Thailand. In traditional medicine, this plant has been prescribed to relieve abdominal discomfort. Based on previous reports, the components of this plant contain sesquiterpenes, triterpenes, flavonoids, and resveratrol oligomers which exhibit various characteristics such as anticancer, antibacterial, and antioxidant properties. The compounds isolated from the stems of this plant exert antiproliferative effects against several cancer cell lines, such as HepG2 (hepatocellular carcinoma), SK-OV-3 (ovarian cancer), A549 (lung cancer), and MCF-7 cells (breast cancer).³ However, the antiproliferative effect, especially from the root of this plant, has not yet been reported.

Tamilnadia uliginosa, in the family of Rubiaceae, is a native plant growing in tropical countries such as India, Bangladesh, Sri Lanka, and Thailand. In traditional medicine, it has been used to treat various diseases and conditions such as aphrodisiac, diabetes mellitus, diarrhea and dysentery. Moreover, its raw fruit can be ingested as a vegetable that is usually eaten with Thai chili paste.⁴ The pharmacological property of the root extract has been recently reported to have antiepileptic activity.⁵ However, the root water extract of this plant has never been investigated to determine its effect on cancer cell growth inhibition.

In our experiments, this special Thai herbal recipe was investigated for the antiproliferative effect on several human cancer cell

lines such as breast (MCF-7, SKBR3, MDA-MB435), lung (A549), colon (HCT116), cervical (HeLa), leukemic (NB4 and K562), and liver (Hep3B and C3A). Human dermal fibroblasts were used as control group in this study. The cell cycle was analyzed to determine the mechanism of action in those cell lines that showed strong inhibition in response to this special Thai herbal recipe.

MATERIALS AND METHODS

Plant collection and extraction process

The roots of *Eurycoma longifolia*, *Dipterocarpus obtusifolius* and *Tamilnadia uliginosa* were collected from Nong-bua-lum-pu province in northeastern Thailand. All of the roots were identified by comparing them with data and specimens at the Forest Herbarium (BKF nos. 141423, 143892 and 23913, respectively), Department of National Parks, Wildlife and Plant Conservation, Bangkok, Thailand.

Water extract

According to the traditional preparation method, the water extract was obtained. The root was rubbed with water on the knife sharpener. One liter of water was used for rubbing 10 grams of root. The root suspension was collected and filtered. The filtrate was dried on the water bath. The amount of the root that produced one gram extract or the drug-extract ratio (DER) was recorded. DER of *Eurycoma longifolia*, *Dipterocarpus obtusifolius* and *Tamilnadia uliginosa* were 34:1, 18:1 and 38:1, respectively. Sample preparation for TLC : 200 mg of the dry extract was sonicated with ethanol for 10 min and used for thin-layer chromatography.

Ethanol extract

Ten grams of three roots were ground and macerated in 50 mL of 95% ethanol for one day. The root was repeatedly macerated and then filtered. The solvent was removed under reduced pressure to obtain the dry extract. Two hundred milligrams of dry extract was sonicated with 2 mL of 95% ethanol for 10 minutes and used for thin-layer chromatography.

The ethanolic extract was investigated for antiproliferative activity. Thin-layer chromatography on normal and reversed phases was performed.

Cell culture condition

Ten human cancer cell lines used in this experiment were breast, lung, cervical, liver, and colon cancer cell lines. Three human breast cancer cell lines, MCF-7, MDA-MB435, and SKBR3, were cultured in DMEM high glucose (Gibco™ Thermo Fisher Scientific, USA) supplemented with 10% fetal bovine serum (Gibco™ Thermo Fisher Scientific, USA), and 1% penicillin plus streptomycin (Pen-strep) (Gibco™ Thermo Fisher Scientific, USA). SKBR3 is a breast cancer cell line with over-expression of HER-2/*neu*, whereas the MCF-7 expresses both estrogen and progesterone receptors on the cell surface. MDA-MB435 shows neither estrogen nor progesterone expression on the cell surface.⁶ All three of these breast cancer cell lines were kindly provided by Dr. Pornchai O-charoenrat (Department of Surgery), Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok, Thailand.

A549 is a human lung adenocarcinoma cancer cell line which was cultured in DMEM high glucose with 10% FBS plus 1% penicillin and streptomycin. NB4 is an acute promyelocytic leukemic cell line, whereas K562 is a chronic myeloid leukemic cell line. Both cell lines were cultured in the same medium as A549. These two leukemic cell lines were kindly provided by Ms. Setsuko Miyanishi, Tenri Institute of Medical Research, Japan.⁷

HCT116 is a colon cancer cell line and HeLa is a cervical cancer cell line which was cultured in DMEM high glucose plus 10% FBS and 1% Pen-strep. Hep3B and C3A are hepatocellular carcinoma cell lines with hepatitis B surface antigens, positive and negative, respectively. Both Hep3B and C3A were cultured in MEM supplemented (Gibco™ Thermo Fisher Scientific, USA) with 10% FBS, 1% Pen-strep, 1% non-essential amino acid (Gibco™ Thermo Fisher Scientific, USA), and 1% 100 mm sodium pyruvate (Gibco™ Thermo Fisher Scientific, USA).

The control group, human dermal fibroblast, was cultured in DMEM high glucose supplemented with 10% FBS plus 1% Pen-strep, and used to represent the normal cell line. A549, HCT116, HeLa, Hep3B, C3A, and human dermal fibroblast were purchased from ATCC (American Type Culture Collection, USA).

All cell lines were incubated at 37°C with 5% CO₂, and 60-80% humidity incubator. Cells were sub-cultured every 3-4 days when reaching 80-100% confluency using trypsin and phosphate buffer saline (Gibco™ Thermo Fisher Scientific, USA).

MTT assay

The ethanolic herbal extract was diluted with 100% DMSO to make a 50 mg/mL stock solution. The final concentration of the extract was performed from the stock solution by diluting with RPMI in the presence of 10% FCS and filtering with 0.2 mm filtered membrane.

Cell proliferation assay was performed to evaluate the growth inhibition of cancer cell lines. The number of cells used to seed in each well of 96 well plates depends on the growth curve of each cell line after 48 hours. The human dermal fibroblast cells were seeded at 1x10⁴ cells per well in 96 well plates, whereas the breast, liver, colon, lung, and cervical cancer cells were seeded at 3x10⁴ cells per well. NB4 and K562 were seeded at 5x10⁴ cells in each well. After 24 hours in the incubator, cells were treated with the herbal extract at various concentrations of 1, 10, 100, and 500 µg/mL, in the final concentration of 0.1% DMSO. These treated cells were then incubated for an additional 48 hours. Then, fifty microliters of MTT (Sigma) [(3, 4, 5-dimethylthiazol2-yl)-2-5-diphenyltetrazolium bromide] at 1 mg/mL in PBS were added in each well. Cells treated with doxorubicin were used as positive control. All treated cells were incubated at 37°C with 5% CO₂ for 4 hours with MTT in darkness before processing to be analyzed using ELISA (Biotek Laboratories®, USA). The NB4 and K562 floating cells were incubated with MTT reagent overnight before processing with ELISA. The assay was done in triplicate with three independent

experiments. Cell viability was calculated using the following formula:⁸

$$\text{Cell viability (\%)} = 100 \times (\text{sample O.D.} / \text{control O.D.})$$

Cell cycle analysis

Five cell lines with ED_{50} less than 100 $\mu\text{g/mL}$ were processed to determine cell cycle distribution, namely, HCT116, HeLa, SKBR3, K562, and NB4 cell lines. For cell cycle study, SKBR3, K526, and NB4 cell lines were seeded at 1×10^6 cells per well in 6 well plates, whereas HCT116 and HeLa were seeded at 7×10^5 cells per well. Cells were incubated for 24 hours before adding the three root herbal extract at dosages of $0.5 \times ED_{50}$ and $1 \times ED_{50}$. After 48 hours, cells were harvested to evaluate the cell cycle determination using CycleTEST™ PLUS DNA Reagent Kit (BD, Biosciences, USA) following the manufacturer's protocol, on ice. Then, the stained

cells were analyzed using flow cytometry with CellQuest software (BD, USA) within 1 hour of finishing all the processes. The experiment was performed in duplicate.

Statistics

Data were expressed as mean \pm standard deviation. The R-square equation was used to calculate the ED_{50} value.

RESULTS

Thin-layer chromatography (TLC)

TLC on normal and reversed phases was performed as shown in Figure 1A and 1B. Fig 1 A showed that the ethanol extracts contained the band with the same R_f 's value as G1a (a mixture of sitosteryl and stigmasteryl- β -D glucopyranoses). The upper bands were non-polar substances such as aglycones (phytosterols,

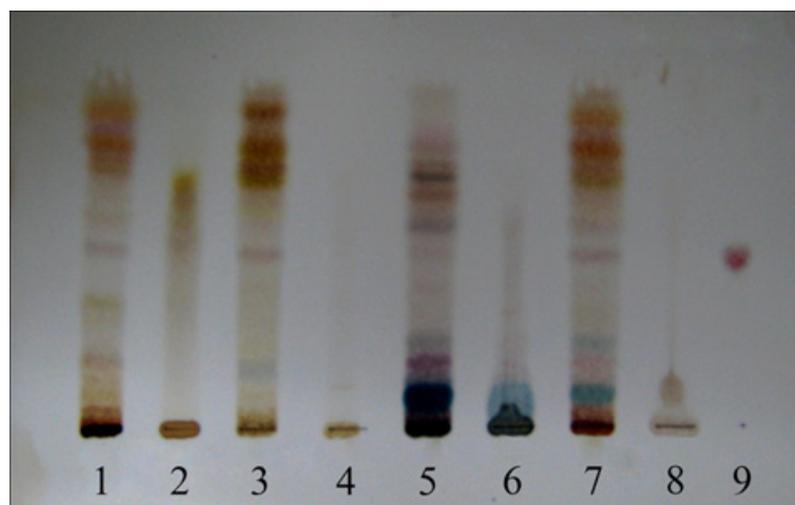


Figure 1. Thin-layer chromatogram of ethanolic and water extracts of three root herbal recipe on normal phase.

Solvent system: dichloromethane: methanol, 85: 15

Absorbent: silica gel GF₂₅₄ aluminium sheet (normal phase)

Detection: sprayed with 10% ethanolic sulfuric acid and heated on the hot plate until the colors develop

- | | |
|--|--|
| 1 = ethanolic extract <i>Dipterocarpus obtusifolius</i> , 20 μl | 6 = water extract <i>Tamilnadia uliginosa</i> , 20 μl |
| 2 = water extract <i>Dipterocarpus obtusifolius</i> , 20 μl | 7 = ethanolic extract 3 root herbal recipe, 20 μl |
| 3 = ethanolic extract <i>Eurycoma longifolia</i> , 20 μl | 8 = water extract 3 root herbal recipe, 20 μl |
| 4 = water extract <i>Eurycoma longifolia</i> , 20 μl | 9 = 0.1% G1a in solvent system, 2-3 μl |
| 5 = ethanolic extract <i>Tamilnadia uliginosa</i> , 20 μl | |

*a mixture of sitosteryl-and stigmasteryl- β -D glucopyranoses, 1: 1

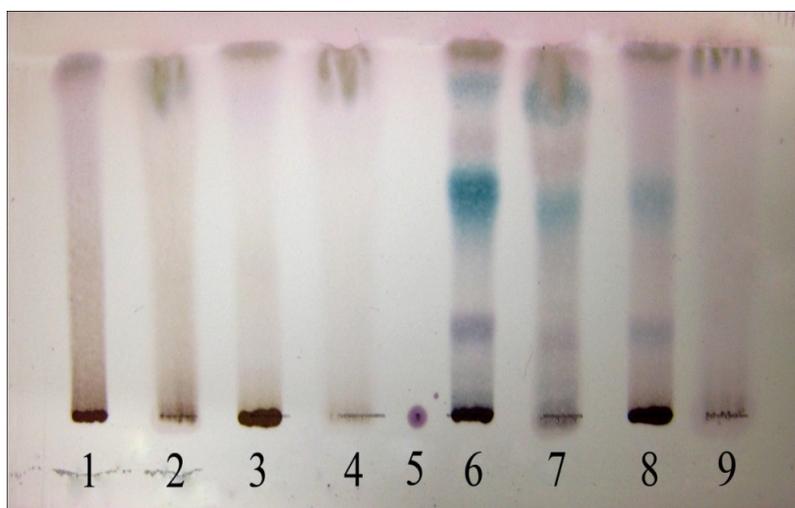


Figure 1B. Thin-layer chromatogram of ethanolic and water extract of three root herbal recipe on reversed phase.

Solvent system: methanol: water, 3: 7

Absorbent: silica gel RP-18 F254S glass plate (reversed phase)

Detection: sprayed with 10% ethanolic sulfuric acid and heated on the hot plate until the colors develop

1 = ethanolic extract *Dipterocarpus obtusifolius*, 20 μ l 6 = water extract *Tamilnadia uliginosa*, 20 μ l

2 = water extract *Dipterocarpus obtusifolius*, 20 μ l 7 = ethanolic extract 3 root herbal recipe, 20 μ l

3 = ethanolic extract *Eurycoma longifolia*, 20 μ l 8 = water extract 3 root herbal recipe, 20 μ l

4 = water extract *Eurycoma longifolia*, 20 μ l 9 = 0.1% G1 α in solvent system, 2-3 μ l

5 = ethanolic extract *Tamilnadia uliginosa*, 20 μ l

triterpenoids, lactones) and the lower bands were more polar substances such as polyphenols, sugars, etc. The TLC fingerprint was used to identify the extract and as a quality control.

Growth inhibition of herbal extract

The 50% growth inhibition (ED_{50}) of the three root herbal extract on 10 human cancer cell lines was demonstrated as shown in Figure 2. Of the 10 cancer cell lines, only five cell lines exhibited significant ED_{50} values less than 100 μ g/mL. These cell lines were HCT116, HeLa, SKBR3, K562, and NB4. The ED_{50} values of HCT116, HeLa, SKBR3, K562, and NB4 were 70, 80, 85, 75, and 70 μ g/mL, respectively. However, the growth inhibition on A549, MCF-7, and MDA-MB435 was greater than 100 μ g/mL but less than 250 μ g/mL. Meanwhile, the ED_{50} values of Hep3B and C3A were over 500 μ g/mL. Of note, this three root herbal recipe also inhibited growth

of normal fibroblasts with the ED_{50} value at approximately 250 μ g/mL.

Cell cycle analysis of five cell lines

Five cancer cell lines which exerted significant ED_{50} values less than 100 μ g/mL were evaluated to identify the mechanism of action using cell cycle determination. At ED_{50} dosage, the HCT116 colon cancer cell line showed significant S phase arrest from 8.05 to 52.81%, when compared to the control cells. HeLa cervical cancer cell line exhibited high apoptosis from 1.43 to 40.35%, at ED_{50} dose level. Surprisingly, SKBR-3 and K562 showed a mild degree of apoptosis from 3.24 to 4.89%, and 6.95 to 10.10%, respectively. Finally, NB4 demonstrated significant apoptosis from 6.80 to 69.71%, when compared to the control group. Data are shown in Table 1 and flow cytometry dot plot histograms of these five cancer cell lines were demonstrated in Figure 3.

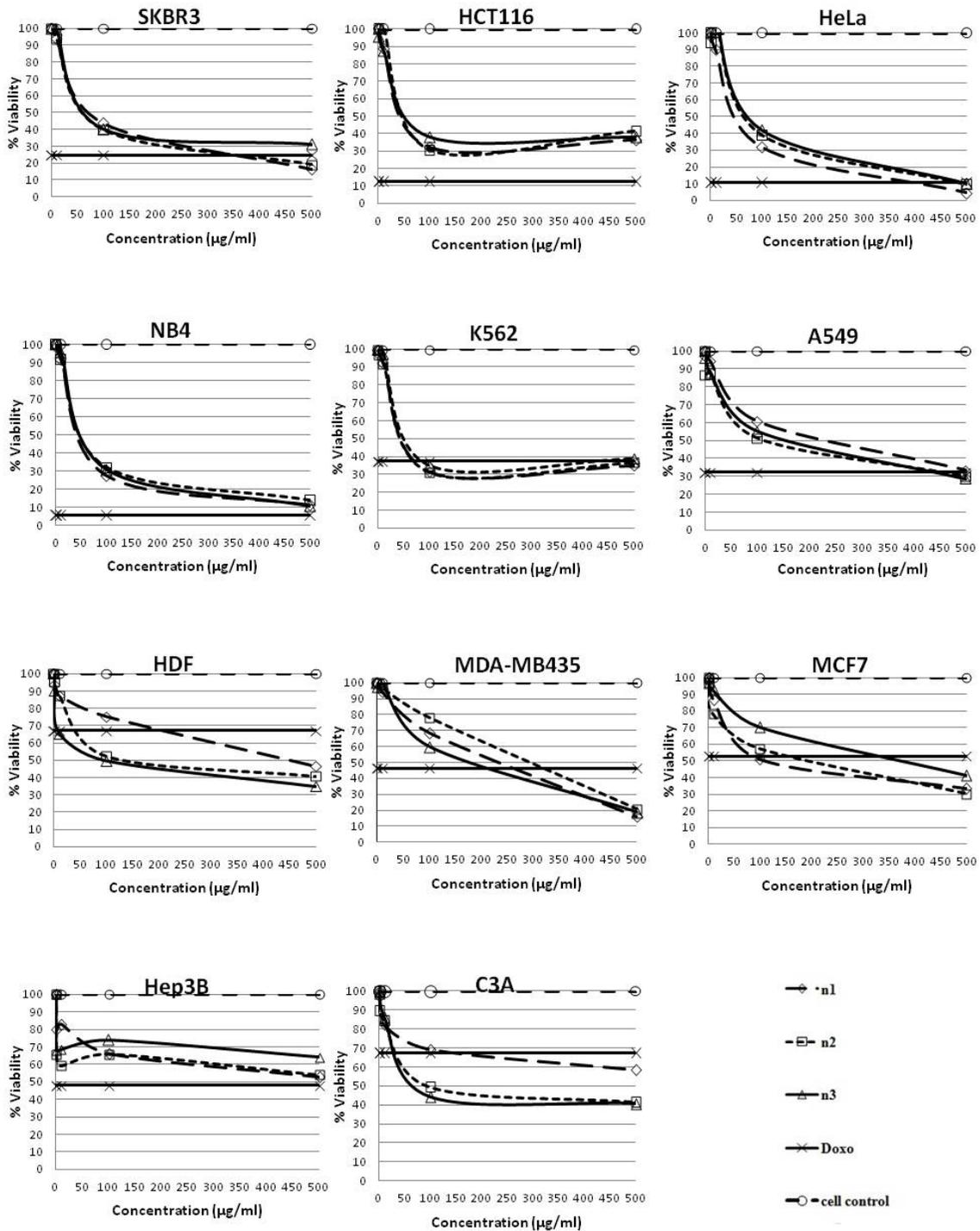


Figure 2. Graph patterns of ED50 values of ten human cancer cell lines including human dermal fibroblast cells treated with three root herbal recipe. Cells were treated with the herbal ethanolic extract at various concentrations for 48 hours before determining the antiproliferative activity using MTT assay. Only 5 cancer cell lines, specifically, HCT116, HeLa, SKBR3, K562, and NB4, showed significant inhibition by the herbal recipe. The graphs refer to the following specific types of cancer cell lines: (A) A549 = human lung adenocarcinoma cancer cell line; (B) MDA-MB435 = human breast cancer cell line; (C) MCF7 = human breast cancer cell line; (D) SKBR3 = human breast cancer cell line; (E) HCT116 = human colon cancer cell line; (F) HeLa = human cervical cancer cell line; (G) NB4 = acute promyelocytic leukemic cell line; (H) K562 = chronic myeloid leukemic cell line; (I) Hep3B = hepatocellular carcinoma cell line; (J) C3A = hepatocellular carcinoma cell line; and (K) HDF = human dermal fibroblast.

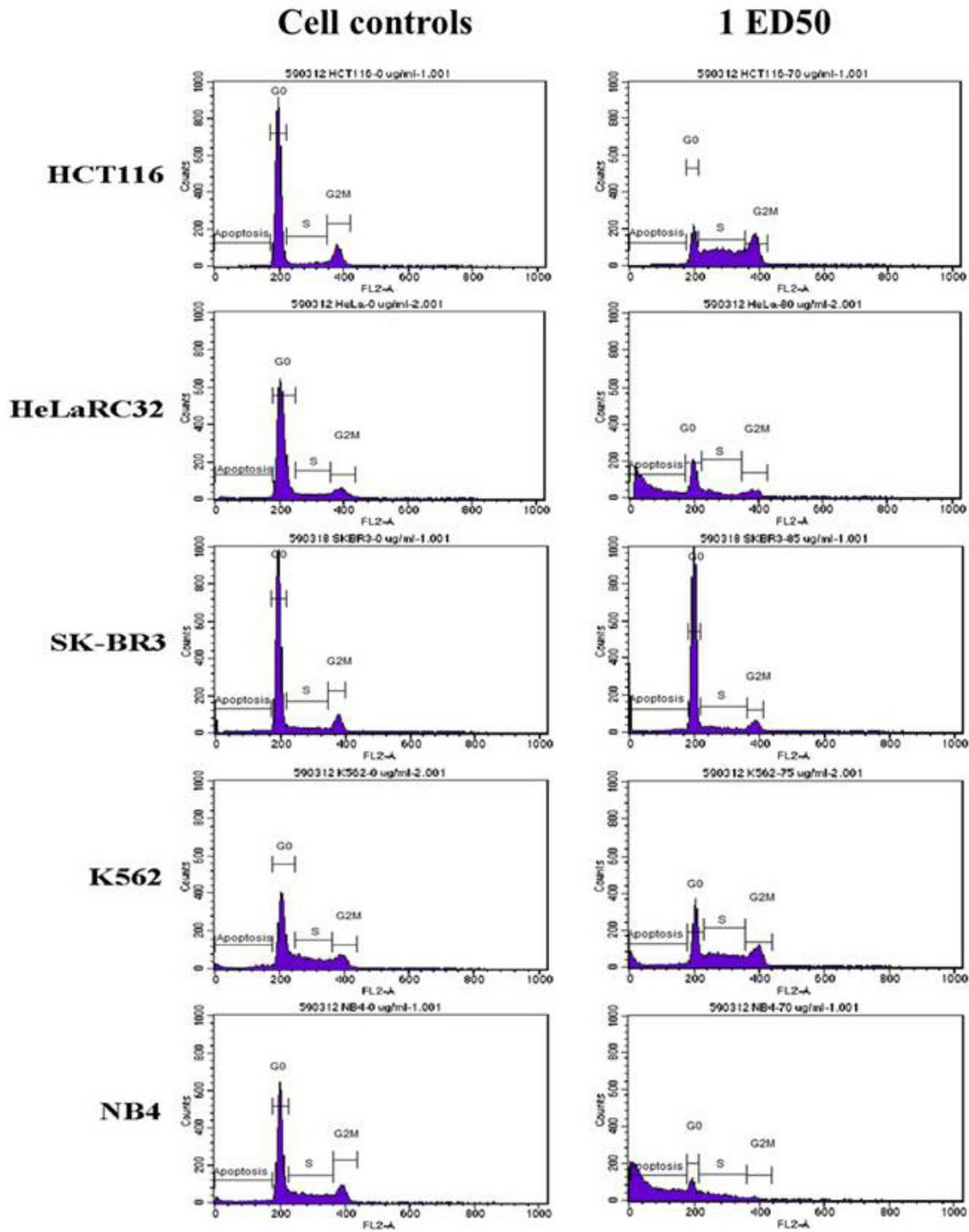


Figure 3. The histogram of cell cycle of five cell lines was showed. The inhibition of S phase was found in HCT116. HeLa and NB4 showed the highly apoptotic phase.

Table 1. Cell cycle analysis of five cancer cell lines. Analysis using flow cytometry was performed after treating the cancer cells for 48 h with 2 dosages of the 3 root-herbal extract, 0.5xED₅₀ and 1xED₅₀.

Type of cells	Herb Concentration ($\mu\text{g/mL}$)	% DNA in cell cycle phase			
		Apoptosis	G0	S	G2M
HCT116	0	0.58	75.82	8.05	14.57
	35	0.67	40.41	36.21	21.04
	70	1.32	18.19	52.89	27.27
HeLaRC32	0	1.43	77.10	10.29	10.70
	40	2.74	67.81	13.42	14.14
	80	40.35	30.80	17.18	11.22
SK-BR3	0	3.24	70.98	12.11	11.62
	42.5	3.97	72.40	11.55	10.60
	85	4.89	72.32	13.09	8.00
K562	0	6.95	50.24	27.25	14.91
	37.5	8.99	46.16	27.31	16.71
	75	10.10	31.15	37.12	21.01
NB4	0	6.80	52.24	26.55	14.18
	35	44.92	29.72	17.38	8.15
	70	69.71	12.18	15.86	2.32

DISCUSSION

A Thai herbal recipe that has been used for many decades to treat various kinds of cancers has been investigated in this study. Based on our results, the potent ED₅₀ values less than 100 $\mu\text{g/mL}$ were demonstrated in HCT116, HeLa, SKBR3, NB4, and K562 cancer cell lines. These five cell lines were further processed for cell cycle determination to search for the inhibitory mechanism of action.

Among the three breast cancer cell lines in this study, SKBR3, MCF-7 and MDA-MB435, only SKBR3 demonstrated over-expression of HER-2/*neu*. Meanwhile, MCF-7 expressed both estrogen and progesterone receptors on the cell surface, but MDA-MB435 showed neither estrogen nor progesterone expression on the cell surface. Our data demonstrated that the herbal recipe could inhibit growth of SKBR3 at an ED₅₀ level at 85 $\mu\text{g/mL}$, whereas the ED₅₀ values of both MCF-7 and MDA-MB435 were over 100 $\mu\text{g/mL}$. Thus, this herbal recipe can prominently inhibit growth of SKBR3 which

has HER-2/*neu* over-expression. We hypothesize that this specific type of receptor on the cell surface might help in modulating herb or drug sensitivity so that the drug molecules can effectively transfer through the cell membrane and actively inhibit cell proliferation.⁹ This may be the reason for the increased inhibitory effect of this herbal recipe on HER-2/*neu* over-expression breast cancer. However, the cell cycle study of SKBR3 demonstrated higher percentages of apoptosis when compared with the control, but without much significance. This means that the herbal recipe can strongly inhibit growth of SKBR3, but apparently cannot cause the death of cells to occur.

For HCT116 colon cancer cells, the herbal recipe could effectively inhibit cancer cell growth with the ED₅₀ value at 70 $\mu\text{g/mL}$. The cell cycle analysis showed that HCT116 was inhibited at the S phase of the cell cycle where inhibition of DNA duplication occurred.¹⁰ Hence, the cells could not proceed through the rest of the cell cycle phases at that moment

due to the decrease of cyclin A and cyclin B1.¹¹ This caused significant growth inhibition in this cell line.

HeLa and NB4 showed highly significant percentages of apoptosis when compared with the control group. Undoubtedly, the mechanism of action of the herbal recipe in these two cancer cell lines was induction of apoptosis. The potential pathways of the apoptosis induced by the three root herbal recipe might be from the activation of death receptor by the drug molecules externally. Otherwise, the mitochondrial pathway activation induced by p53 as an intrinsic pathway could have occurred following by the activation of caspase cascade pathway including specific apoptotic proteins.^{12,13}

The herbal recipe inhibited growth of K562, the chronic myeloid leukemic cell line, by induction of apoptosis, but at a lower percentage when compared to those of HeLa and NB4. This phenomenon was also observed in SKBR3 with potent ED₅₀. This means that the treated cells might proliferate again because the induction of apoptosis was not very high. Thus, the treated cells might recover from the drug inhibiting process unless the drug is administered continuously, and not only at one point in time. Prolonged and continuous drug administration may enhance cell death to occur.¹³

For hepatocellular carcinoma cell lines (Hep3B and C3A), the ED₅₀ values were over 500 µg/mL. It would be necessary to use a much higher dosage to inhibit growth of these two liver cancer cell lines.

Among the three plants in the herbal recipe, *Tamilnadia uliginosa* was the only one that Thai traditional doctors described in terms of adjusting the main four elements (earth, water, air, fire) in patients' bodies. The other two herbs, *Dipterocarpus obtusifolius* and *Eurycoma longifolia*, were used for inhibiting cancer cell growth in this recipe. These two herbal plants were previously discussed for their anticancer activities on several human cancer cell lines. Therefore, it is highly possible that the anti-proliferative activities of the three root herbal recipe demonstrated in our study is most likely due to the anticancer properties of *Dipterocarpus obtusifolius* and *Eurycoma longifolia*, which were similar to previous reports.

In conclusion, our experiment demonstrated that the three root herbal recipe ethanolic extract had potential antiproliferative activity, especially with 5 human cancer cell lines, specifically, HCT116, HeLa, SKBR3, NB4, and K562. The most common mechanism exhibited by these 5 cell lines following this special herbal treatment was the induction of apoptosis. Only HCT116, the colon cancer cell line, showed a different mechanism with prominent S phase arrest during cell cycle progression. Thus, this Thai herbal recipe has potential to be developed as a traditional anticancer recipe that could be available to people all over the country.

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