Original Article

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A Thai Herbal Recipe Induces Apoptosis in T47D Human Breast Cancer Cell Line

T. Kummalue[1], Y. U-pratya[2], U. Lueangamornnara[2] and W. Jiratchariyakul[3]*

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

[2] Department of Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand

[3] Department of Pharmacognosy, Faculty of Pharmacy, Mahidol University, Bangkok 10400, Thailand

Abstract

One of the most common Thai herbal recipe used by cancer patients was investigated for antiproliferative activity on human breast and lung cancer cell lines: T47D and A549, respectively. This herbal recipe was composed of five medicinal plants, i.e., *Gelonium multiflorum*, *Rhinacanthus nasutus, Acanthus ebracteatus, Smilax corbularia,* and *Smilax glabra,* in the equal proportion. The herbal recipe was boiled in 3 liters of distilled water until water was approximately 1 liter left. Then, the evaporating and drying processes were performed by using desicccators to yield the herbal extract. The ED_{50} values of T47D and A549 were evaluated using MTT method at 48 hours. The antiproliferative effect on T47D was demonstrated with ED_{50} value at 6,066 μg/ml, whereas no ED_{50} value was found with A549. The apoptotic study was done and found that at dosage of 3,000 μg/ml, the apoptosis of T47D was about 50%. Therefore, this Thai herbal recipe showed the promising antiproliferative activity on human breast cancer cell line, T47D, with the mechanism of inducing apoptosis in this cell line.

Keyword: T47D, *Gelonium multiflorum*, *Rhinacanthus nasutus, Acanthus ebracteatus, Smilax corbularia, Smilax glabra*

INTRODUCTION

Thailand is full of medicinal plants that have long been prescribed in Thai traditional medicine for over centuries. Usually, Thai herbal recipes, which are composed of several plants in one recipe, are used to treat various diseases including infections and malignancies. In fact, most herbal recipes have never been investigated, though many patients still use them without notifying the effects and potency in the scientific views. Based on our previous data, some Thai herbal recipes treating malignancies have no antiproliferative effect on several human cancer cell lines[1,2].

Several Thai medicinal plants have their own specific pharmacological effects on different kinds of cancers. In this study, one Thai herbal recipe composed of *Gelonium multiflorum*, *Rhinacanthus nasutus, Acanthus ebracteatus, Smilax corbularia,* and *Smilax glabra* in the equal proportion was investigated. This herbal recipe has long been prescribed by Thai traditional doctors for treating malignancies, such as breast cancer and lung cancer. However, *in vitro* investigation of this herbal recipe has never been performed. Therefore, this prompted us to investigate the antiproliferative activity on human breast cancer cell lines: T47D, and human lung cancer cell line: A549. The ED_{50} values of both cell lines were evaluated using MTT assay. The mechanism of antiproliferative activity on human cancer cells was also determined. Our investigation find that this Thai herbal recipe has the anticancer effect especially on T47D breast cancer cell line by inducing apoptosis.

***Corresponding author:** Department of Clinical Pathology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand E-mail address: tanawan.kum@mahidol.ac.th Tel: 662-419-6587-8 Fax: 662-418-1367

MATERIALS AND METHODS

Preparation of the extract

Thai herbal recipe was composed of stems and leaves of *Gelonium multiflorum*, *Rhinacanthus nasutus, Acanthus ebracteatus,* and rhizomes of *Smilax corbularia* and *Smilax glabra.* They were put together and boiled in 3 liters of distilled water until the extract was left about 1 liter. Then, the extract was evaporated at 37°C until dry powder was formed. The testing solution was used by dissolving the extract with 100% DMSO. A serial dilution with cell culture medium was performed to gain the final concentrations.

Cell culture conditions

Human breast cancer cell line: T47D was cultured in DMEM (GibThai®) supplemented with 10% fetal bovine serum (Gib Thai®) including 1% penicillin-streptomycin (GibThai®). This human breast cancer cell line was kindly provided by Dr. Pornchai O-charoenrat (Department of Surgery, Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand). T47D expresses both ER receptor and HER2/*neu* receptor[3]. A549, a cisplatin resistant adenocarcinoma human lung cancer cell line, was purchased from ATCC (American Type Culture Collection) and was cultured in the same medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin at 37°C with 5% CO_2[4].

MTT assay

Cells at $1x10^4$ cells per well were seeded in each well of 96 well plate for 24 hours before adding the herbal extract. Various final concentrations of herbal extract at 10, 100, 500, 1000, 5000, and 10,000 µg/ml were titrated and doxorubicin was used as the positive control. After 48 hours incubation, MTT 50 µl (1 mg/ml diluted with PBS) was added in each well and incubated for 4 hours before changing the medium. Then, 100% DMSO 100 µl was added in each well and rotated for 10 min at RT before measuring the O.D. at 595 nm with ELISA reader (Biotek Laboratories®, USA). Data were used to calculate the percentage of cell viability with ED_{50} by the following equation[5]:

$$\text{Cell viability (\%)} = \frac{\text{OD sample}}{\text{OD control}} \times 100$$

Apoptotic assay

Cells at $2x10^5$ cells per well were seeded in each well of 6 well plate and cultured in completed medium overnight. The next morning, cells were treated with herbal extract at the final concentrations of 3,000 and 6,000 µg/ml with doxorubicin as the positive control. After 24 and 48 hours, cells were harvested to study the percentage of apoptosis using Annexin V: PE Apoptosis Detection kit (BD Biosciences®) following the protocol and analysed by flow cytometry.

Cell cycle analysis

Each cell line at $2x10^5$ cells per well was seeded in each well of 6 well plate and cultured in completed medium overnight. The next morning, cells were treated with herbal extract at the final concentrations of 3,000 and 6,000 µg/ml. Doxorubicin was used as the positive control. After 24 and 48 h, cells were harvested to determine the cell cycle phase using CycleTest™ Plus (BD Biosciences®) following the protocol and analysed by flow cytometry.

RESULTS

ED_{50} value of Thai herbal recipe on T47D and A549 human cancer cell lines

To evaluate the potency of growth inhibition of this herbal remedy, the ED_{50} was calculated. The antiproliferative activity of this herbal recipe on T47D showed the ED_{50} value at 6,066 µg/ml as shown in Figure 1A. Apparently, the effect of herbal recipe on this cell line was shown in the concentration dependent manner. However, no ED_{50} value of A549 human lung cancer cells was detected as shown in Figure 1B.

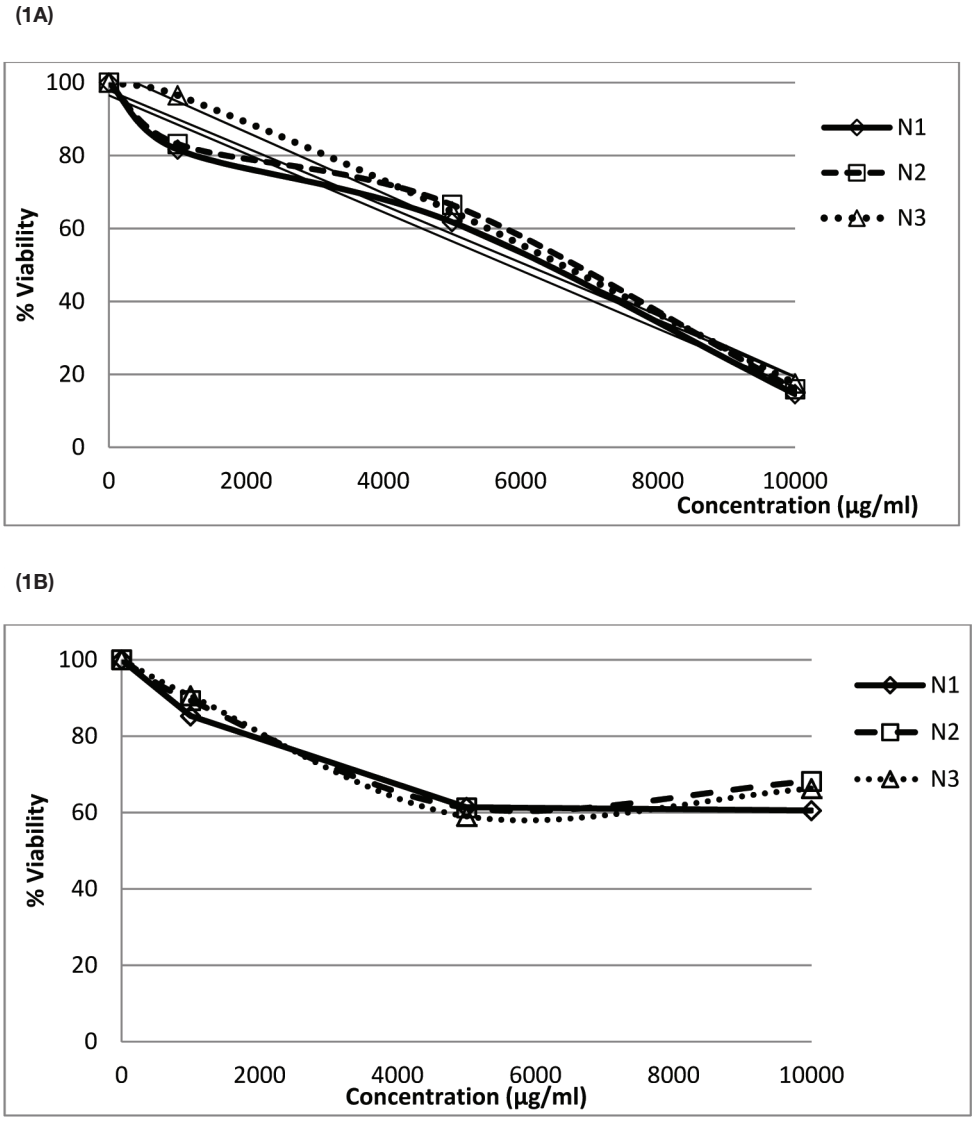

Figure 1. Showing the ED_{50} value of Thai herbal recipe with three independent experiments. (1A) The effect of herbal recipe on T47D human breast cancer cell line. The ED_{50} value of T47D is about 6,066 µg/ml, while $y = -0.0084x + 103.21$ with $R^2 = 0.9936$ (1B) The antiproliferative effect on A549 human lung cancer cell line. No ED_{50} value was detected. (N1= experiment 1, N2= experiment 2, N3= experiment 3)

Apoptosis of T47D induced by herbal extract

From the ED_{50} value, the growth of T47D breast cancer cell line could be inhibited by this herbal recipe. Therefore, we further studied the mechanism of growth inhibition especially cell cycle determination and apoptosis only in T47D with various concentrations of herbal extract at 3,000 and 6,000 µg/ml for 24 and 48 hours. We found that at 3,000 µg/ml with 48-hour incubation time, approximately 35% of cells showed early apoptosis, whereas about 50% showed late apoptosis. In contrast, the control group showed only 1% in early apoptosis phase and about 6% in late apoptosis at the same time. The apoptosis detected in this cell line showed dose dependent manner. The analyses of apoptosis of T47D cells were illustrated in Table 1.

Table 1. Analyses of T47D apoptosis from flow cytometry. The apoptosis of herb-treated T47D human breast cancer cells at 24 and 48 hours. Late apoptosis was detected at the dosage of 3,000 µg/ml with 31% and 50% at 24 and 48 hours, respectively. The dosage of 6,000 µg/ml at 48 hours could induce apoptosis at approximately 76%

T47D	Concentration	Annexin V+PI-	Annexin V+PI+	Annexin V-PI+
	0	2.4	6.12	1.23
24 hrs	3000	27.97	31.08	12.7
	6000	1.74	35.58	54.67
	0	1.47	6.75	2.18
48 hrs	3000	35.14	50.57	8.74
	6000	6.33	76.74	14.47

Cell cycle analysis of herbal recipe on T47D cell line

Determination of T47D cell cycle progression at dosage of 3,000 and 6,000 µg/ml for 24 and 48 hours was performed. At 24 hours, the high proportion of cells at sub G1 peak was detected which was the high level of apoptotic cells, as illustrated in Figure 2. Moreover, at dosage of 6,000 µg/ml for 48 hours, only apoptosis of these cells could be found (data not shown).

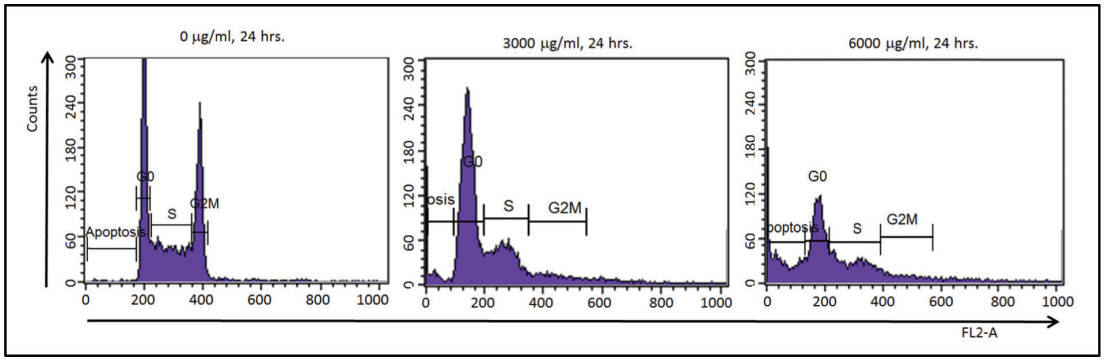

Figure 2. Cell cycle determination of herb-treated T47D cells. The effect of herbal recipe on this cell line at 24 hours showed high sub G1 peak which were apoptotic cell group. The induction of apoptosis was in dose dependent manner with the dosage of 6000 µg/ml at 24 hours showing 36% apoptosis.

DISCUSSION

Many Thai herbal recipe have been prescribed by Thai traditional doctors and lots of cancer patients in Thailand use herbal recipe to treat their own diseases without notify their own conventional doctors. Most herbal recipe have never been investigated in the scientific methods to evaluate their efficacies both *in vitro* and *in vivo*. This event will have much influence on the therapeutic response on cancer patients especially their survival rates. Therefore, the purpose of this experiment is to survey and determine the antiproliferative effect of one common herbal recipe that cancer patients used to treat themselves. This herbal recipe was gained from well known Thai herbal Pharmacy where cancer patients usually get the herbal recipe.

This Thai herbal recipe contained five medicinal plants, i.e., *G. multiflorum*,

R. nasutus, A. ebracteatus, S. corbularia, and *S. glabra.* We found that this herbal recipe had the anti-proliferative effect only on T47D breast cancer cells with the ED_{50} value at 6066 μg/ml. The influence of this herbal extract on cell growth was shown in a dose dependent manner. Moreover, this herbal recipe had higher antiproliferative effects on human cancer cells when compared to our previous studied herbal recipe[1,2]. From our investigations, we found that the prominent mechanism involving in anti-proliferative effect of this herbal recipe resulted from apoptosis. Interestingly, our results showed that this herbal recipe could induce 50% late apoptosis at the dosage of only 3000 μg/ml, whereas the ED_{50} value was at 6066 μg/ml. This discrepancy might be due to the high sensitivity of flow cytometric quantification with propidium iodide staining when compared to colorimetric assay[6].

Based on previous report, *G. multi-florum* had no effect on several human breast and lung cancer cell lines including T47D and A549[7]. Therefore, it is possible that the antiproliferative effect of this herbal recipe was not derived from this plant and would be the effect of other plants, such as *R. nasutus*, *A. ebracteatus, S. corbularia,* and *S. glabra* which had been reported to inhibit growth of several human cancer cells.

R. nasutus is belonging to the family of Acanthaceae and has long been used to treat several diseases including infections and malignancies. In this Thai herbal recipe, stems and leaves of *R. nasutus* were boiled with other plants to get the water extract. Based on a previous report, antitumor activity of root of *R. nasutus* on human cervical cell line was demonstrated[8]. Moreover, the anti-proliferative effect of the aerial parts of this plant on human oral squamous carcinoma cell line and human promyelocytic cell line was also determined[9]. Bioactive naphtho-quinone esters, such as Rhinacanthin-C, from *R. nasutus* have been found to be the potential compounds as anticancer agents.[10] Of note, the mechanism of growth inhibition from this plant is demonstrated *via* cytochrome P450CYP2A6 and CYP2A13 pathways[11.] However, the antiproliferative activity of the extract from stems and leaves of this plant on T47D breast cancer cell line has not yet been illustrated.

A. ebracteatus, plant in the family of Acanthaceae, has long been investigated for its antitumor activity during the last decade. Its growth inhibitory effect with crude extract from stems and leaves on several human cancer cell lines has been demonstrated, such as human cervical cell lines, and human hepatocellular carcinoma cell line[12]. Interestingly, the compounds found in the crude hot water extracts of this plant are galactose, 3-O-methylgalactose and arabinose[13]. However, the properties of these compounds as antitumor agents have not been shown especially on human breast cancer cells. After boiling, these carbohydrates could form the complex with some proteins and, perhaps, exhibit the anticancer effect on cancer cell line. More investigations to ascertain this concept are further needed.

Both *S. corbularia* and *S. glabra* are the common components in most Thai herbal recipe for treating cancer diseases. Several studies of these two Thai herbal plants on human cancer cell lines have been reported. Some herbal recipe containing rhizomes of *S. glabra* had growth inhibition on hepatocellular carcinoma cells.[14] Several compounds were isolated from rhizomes of *S. glabra*, such as flavonoids, engeletin, astilbin, and smilaxin[15]. It has been reported that mitochondrial apoptosis induced by *S. glabra* has the effects on the anticancer properties of this plant[16] .

CONCLUSION

This commonly used Thai herbal recipe containing five medicinal plants shows some potential antiproliferative activity on human breast cancer cell line, T47D by

inducing apoptosis. Only *G. multiflorum* has been investigated on human breast cancer T47D and found no effect on this cell line. The other four medicinal plants containing in this recipe have been reported to inhibit cancer cell growth *in vitro*, though the investigations have not been involved in T47D breast cancer cells. Several compounds derived from these four medicinal plants have also been illustrated with antiproliferative activity on some human cancer cells. Notably, more specific compounds from boiling technique might be another reason for increasing the antiproliferative activity on specific human cancer cells. In conclusion, this Thai herbal recipe used by cancer patients might have potential for therapeutic purposes and *in vivo* evaluation is needed for further investigation in the future.

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