

Full Length Research Paper

Apoptotic inducibility of *Sapindus rarak* water extract on A549 human lung cancer cell line

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Sapindus rarak DC., Thai herbal medicine named “Ma-Gum-Dee-Kwao” in Sapindaceae family, has been widely prescribed in treatment of skin diseases and also combination with several herbal plants to treat various diseases including malignancies. Bio-based assay of water extract from *S. rarak* on two human breast cancer cell lines, T47D and MCF7; two human lung cancer cell lines, A549 and SK-LU1; and one colon cancer cell line, Caco-2 was investigated. We observed a significant growth inhibition only in A549 cell line with ED₅₀ value 84.194 ± 2.597 µg/ml. Cell cycle analysis and apoptotic study were performed in A549 human lung cancer cell line and found significant increase of apoptotic cells without significant changes in cell cycle phase. Mechanism of apoptotic induction was demonstrated using Western blotting and indicated that the caspase pathway was involved in herb signal pathway. In summary, the extract-induced apoptosis of A549 cells was primarily mediated through caspase dependent pathway especially caspase 3 and 9 which suggested that *S. rarak* water extract might be a potential anticancer agent against human lung cancer cell line. This research might also be a hypothesis generating study in the future.

Key words: *Sapindus rarak*, apoptosis, A549, caspase, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT).

INTRODUCTION

Cancer is one of the most serious clinical problems worldwide and affects not only developed countries but also developing countries as well. In Thailand, lung cancer is the second most common cause of cancer, accounting for 25.9 per 100,000 in males, whereas breast cancer is the second most common cause of cancer in females, accounting for 17.2 per 100,000 (Sriplung, 2004). Though, current treatment modalities are improving in therapeutic efficacy and quality of life, both malignancies, especially in advanced stages, remain with poor prognosis due to only a modest increase in survival rate. Drug resistance and toxicity are also found to be the major problems in chemotherapeutic treatment. Therefore, novel anticancer agents are still being sought

for more effective therapies to treat advanced stages of these diseases. For centuries, many chemotherapeutic agents have been developed from natural products including higher plants, for example, paclitaxel, which is a microtubule disrupting agent, targets primarily tubulin derived from *Taxus brevifolia* (Schiff et al., 1979). Vincristine and vinblastine, chemotherapies used to treat various hematologic malignancies such as lymphoma, were isolated from *Catharanthus roseus* Linn. G.Donn (Shoeb, 2006). In Thailand, *Sapindus rarak* DC., named in Thai “Ma-Gum-Dee-Kwao”, is a herbal plant in a Sapindaceae family. It is commonly found in several areas of Thailand and its seed has long been prescribed for treating skin diseases. Additionally, it has been widely used in combination with other herbal plants to treat several malignancies such as lung and breast cancer (Sintusarn, 1992). However, there is no scientific report concerning the potential anticancer activity of this plant, especially the effect of *S. rarak* on human lung and

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breast cancer cells *in vitro*.

In this study, the antiproliferative effect of *S. rarax* water extract on two human lung cancers, two human breast cancer cell lines, and one colon cancer cell line was investigated. Furthermore, A549 human lung cancer cell line was used to assess the mechanism of possible herb-induced anticancer effect, including cell cycle distribution and apoptosis. Moreover, this report also demonstrated the expression of caspase activity to elucidate the apoptotic pathway of this herbal plant.

MATERIALS AND METHODS

Plant extraction process

The fruit pulps of *S. rarax* were gifted from Thai Herbal Drug Store "Chao-Krom-Pao". They were ground and then extracted with distilled water (ratio 1:30) on a hot plate for 120 min. The aqueous solution was filtered and evaporation to dryness by water bath.

Plant extract preparation

The herbal extract was dissolved in water and sterile filtered through 0.22 μm . For all experiments, the sterile herbal water extract was diluted with culture medium to the final concentrations of 1 to 500 $\mu\text{g/ml}$.

Cell lines and culture

Two human lung cancer cell lines, A549 and SK-LU1, and two human breast cancer cell lines, T47D and MCF7, including one colon cancer cell line, Caco-2, were used in this investigation. A549 and SK-LU1 were purchased from American Type Culture Collection (ATCC, Rockville, MD) whereas two human breast cancer cell lines were kindly provided by Dr. Pornchai O-charoenrat, Department of Surgery, Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand. A colon cancer cell line was kindly provided by Ministry of Public Health, Thailand. A549 is a cisplatin resistance adenocarcinoma lung cancer whereas SK-LU1 is a cisplatin sensitive adenocarcinoma lung cancer cell lines. T47D and MCF-7 are breast cancer cell lines that are ER receptor-positive and HER2/*neu*-negative (Lacroix and Leclercq, 2004). All cell lines except SK-LU1 were maintained in DMEM media supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin-streptomycin in a humidified atmosphere with 5% CO₂. SK-LU1 was maintained in MEM medium supplemented with 10% heat-inactivated fetal bovine serum, 1% sodium pyruvate, non-essential amino acid and 1% penicillin-streptomycin in a humidified atmosphere with 5% CO₂.

Antiproliferative assay

Antiproliferative assay of control and treated cells were evaluated by using the MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl-tetrazolium bromide) assay in triplicate and three independent experiments (Mueller et al., 2004; Skehan et al., 1990). Human cancer cells at 1×10^4 cells per well were seeded in a 96-well plate and incubated for 24 h before treating with the herbal extract. Cells were treated with various final concentrations of *S. rarax* water extract at 1, 10, 50, 100, 150 and 500 $\mu\text{g/ml}$ for 48 h. Fifty microlitres of MTT solution (1 mg/ml of PBS) were added to each using an ELISA reader at a wavelength of 595 nm. Effective dose

inhibiting 50% of cells was calculated as ED₅₀ and formula of cell growth inhibition was as followed (Hsu et al., 2004a; Kummalue et al., 2007).

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

Cell cycle analysis

To determine the effects of herbal extract on cell growth, the cell cycle analysis was performed. A549 human cancer cells were plated at a density of 1×10^6 cells per 100 mm culture dish for 24 h before treating the cells with the herbal extract diluted with fresh medium at various dosages to the final concentrations of 110, 165, and 220 $\mu\text{g/ml}$. After an hour of incubation, the cell cycle analysis was performed using the CycleTEST™PLUS DNA Reagent Kit (BD, Biosciences, USA). Briefly, cells were collected, incubated and washed by the solution A, B and C as described in the protocol. All samples were analyzed within 3 h by FACScalibur using CellQuest software.

Apoptotic assay

To determine the induction of apoptosis by the herbal extract, 1×10^6 cells were seeded in 100 mm culture dish and incubated for 24 h. The herbal extract in fresh medium was added in each plate at various dosages to the final concentrations of 82.5, 110 and 137.5 $\mu\text{g/ml}$ for early detection of apoptosis. After half an hour incubation, cells were harvested and labeled with Annexin V-PI following the protocol of Annexin V-FITC Apoptosis Detection kit (BD, Bioscience, USA) before analysis by flow cytometry within 1 h.

Western blot analysis

To determine the activation of caspase activity in herb treated cells, the Western blot was performed. Cells at 1×10^6 cells were seeded in 100 mm culture dish and incubated for 24 h. Cells were treated with herbal extract to the final concentrations of 82.5, 110 and 137.5 $\mu\text{g/ml}$. After half an hour incubation, cells were collected and suspended in lysis buffer containing 10% glycerol, 0.063 M Tris base, 3% SDS, pH 6.8 (Lin et al., 2006; Yang et al., 2009). All protein concentrations were determined by using a Bio-Rad Protein assay (BIO-RAD laboratories, USA) (Hsu et al., 2004b). For Western blot analysis, equal amount of proteins at 50 μg was separated in 15% SDS-polyacrylamide gel electrophoresis for caspase 9 and 12% gel for caspase 3, then transferred to polyvinylidene fluoride membrane (PVDF). The membrane was blocked with 5% non fat dry milk following the manufacturer's protocol (Santa Cruz Biotechnology, USA) and incubated for 1 h at room temperature on rocking platform. The blot was incubated with primary antibodies 1:200 against caspase 9, and procaspase 3 (Santa Cruz Biotechnology, USA) for an hour at room temperature and finally incubated with an appropriate horseradish peroxidase conjugated secondary antibody 1:1000. The reaction bands were visualized using a SuperSignal West Femto Maximum Sensitivity Substrate (Pierce Biotechnology, USA). β -actin was used as internal control. Results were visualized by using the Chemidoc™XRS (BIO-RAD Laboratories, USA).

Statistical analysis

Data were presented as means \pm standard deviation. All experiments were performed in triplicate with three independent experiments. P value < 0.05 was considered as significant difference.

Table 1. ED₅₀ values of 5 human cancer cell lines treated with *Sapindus rarak* water extract at the concentrations of 1, 10, 50, 100, 150 and 500 µg/ml.

Human cell lines	ED ₅₀ ± SD (µg/ml)
T47D	277.600 ± 37.72
MCF-7	258.213 ± 19.922
Caco-2	109.242 ± 13.172
SKLU1	106.372 ± 18.865
A549	84.194 ± 2.597

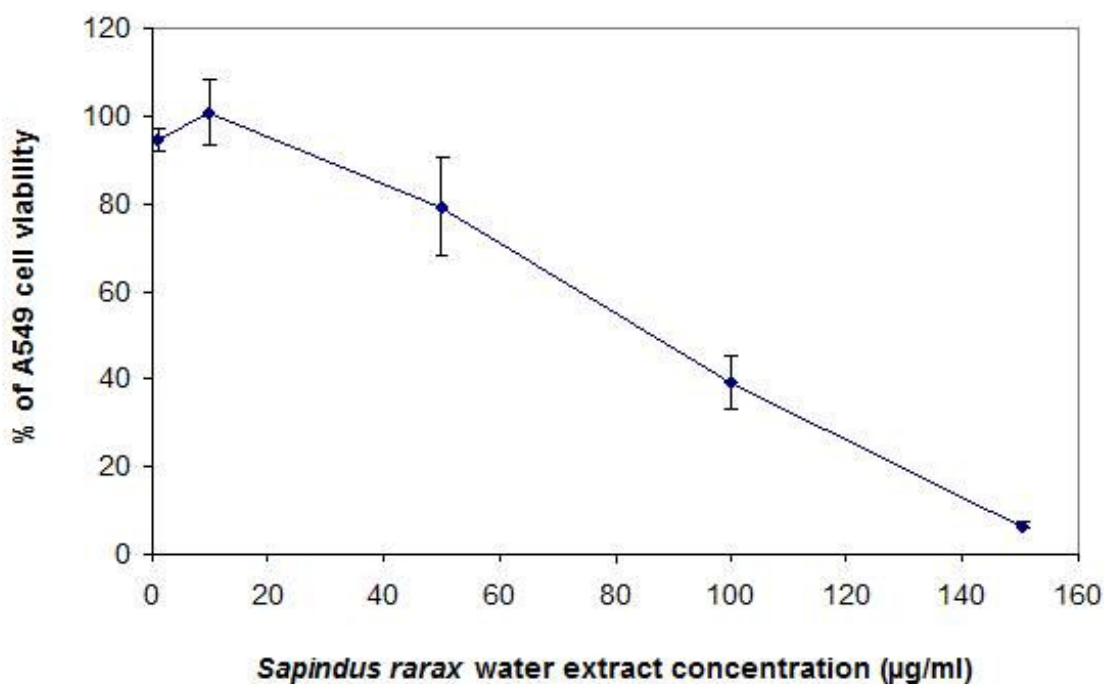


Figure 1. Percentage of cell viability of A549 cells after treatment with *Sapindus rarak* water extract at the concentrations of 1, 10, 50, 100, 150 µg/ml determined by MTT [3- (4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Values are expressed as a mean ± standard error of three independent experiments.

RESULTS

Effect of *S. rarak* water extract on human cancer cells growth

The ED₅₀ values of *S. rarak* water extract on five human cancer cell lines were shown in Table 1. Interestingly, the most prominent growth inhibition was detected in A549 lung cancer cell line with ED₅₀ value 84.194 ± 2.597 µg/ml in a dose-dependent manner as demonstrated in Figure 1. For SK-LU1 and Caco-2 cell lines, the growth inhibition was at a modest level, with ED₅₀ values of 106.372 ± 18.865 and 109.242 ± 13.172 µg/ml, respectively. However, no growth inhibition effect was detected in the other two human breast cancer cell lines,

that is, MCF7 and T47D. Therefore, only the A549 human lung cancer cell line was further used to characterize the effect and growth inhibitory mechanism of *S. rarak* water extract.

Effect of *S. rarak* on cell cycle distribution

The cell cycle distribution of herbal treated A549 cancer cells was shown in Figure 2. After treating cells with the herbal extract for one hour, the number of A549 cells in cell cycle phase did not significantly change when compared with the control. However, the number of treated cells undergoing apoptosis at one hour was significantly increased from 0.72 ± 0.06 to 18.10 ± 4.96%

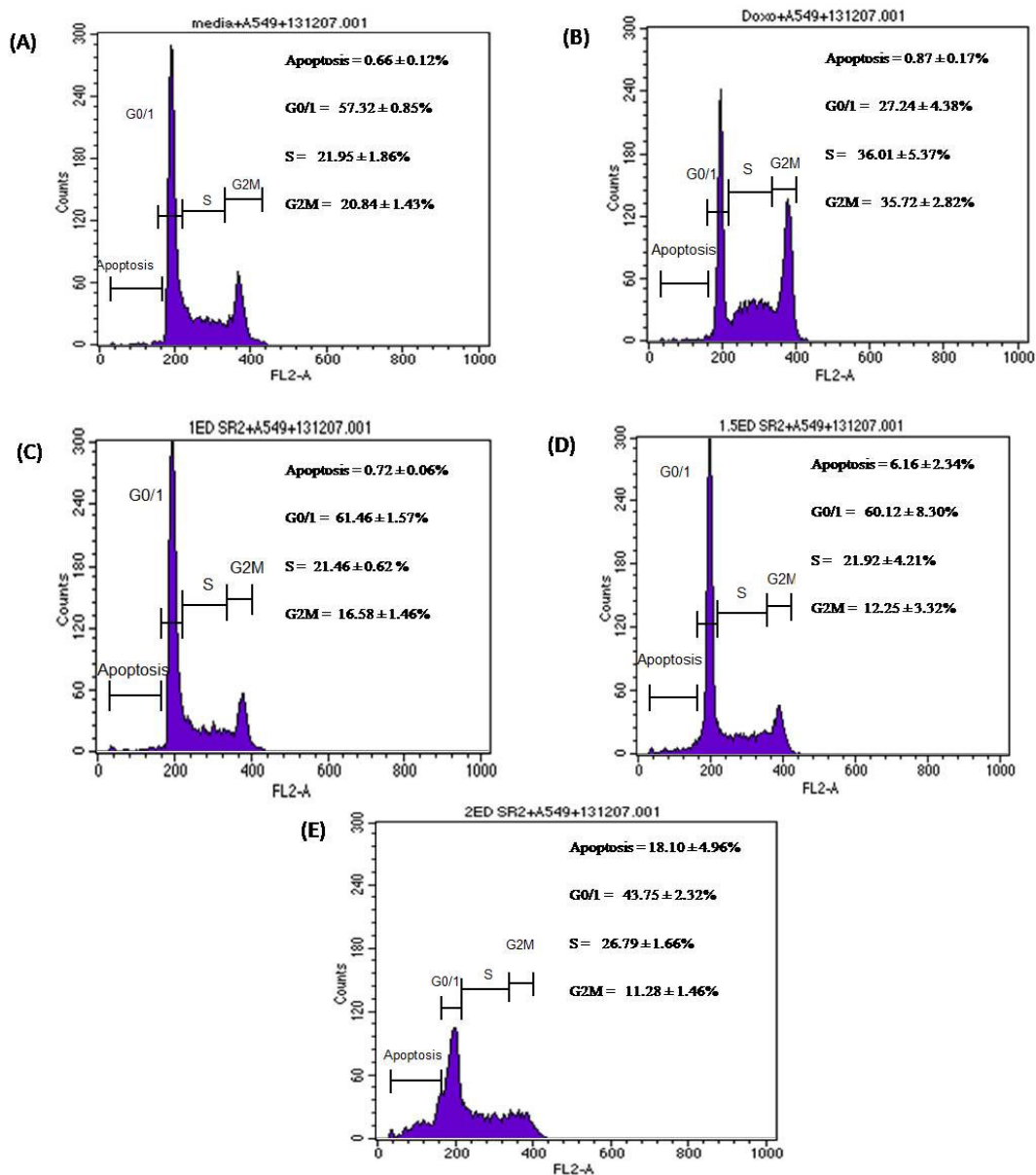


Figure 2. Cell cycle analysis of herbal treated A549 cells using flow cytometry. (A) Negative control; (B),(C),(D),(E) A549 cells treated with 1 µg/ml Doxorubicin and *Sapindus rarak* water extract at the concentrations of 110, 165, 220 µg/ml, respectively, for 1 hr. The percentage of G0/1, S, G2M and apoptotic phases was analyzed by FACScalibur flow cytometry using CellQuest Software. Values are means ± standard error of three independent experiments.

in a dose-dependent manner with significant p value < 0.05 at 165 and 220 µg/ml which indicated that growth inhibition of treated cells might be operating through the apoptotic process.

Induction of apoptosis by water extract of *S. rarak*

The effect of *S. rarak* water extract on induction of apoptosis of A549 cells was demonstrated in Figure 3.

Herb-treated cells at concentration 137.5 µg/ml for half an hour showed marked induction of apoptosis, with early apoptosis of 17.05 ± 6.58% and late apoptosis of 59.13 ± 3.04% whereas control cells showed early apoptosis of 6.15 ± 0.70% and late apoptosis of 12.19 ± 3.19%. Therefore, the number of cells undergoing apoptosis increased significantly about 3 to 5 fold with p value < 0.05. Additionally, cells when treated with herbal extract at various concentrations exhibited apoptosis in a dose dependent manner.

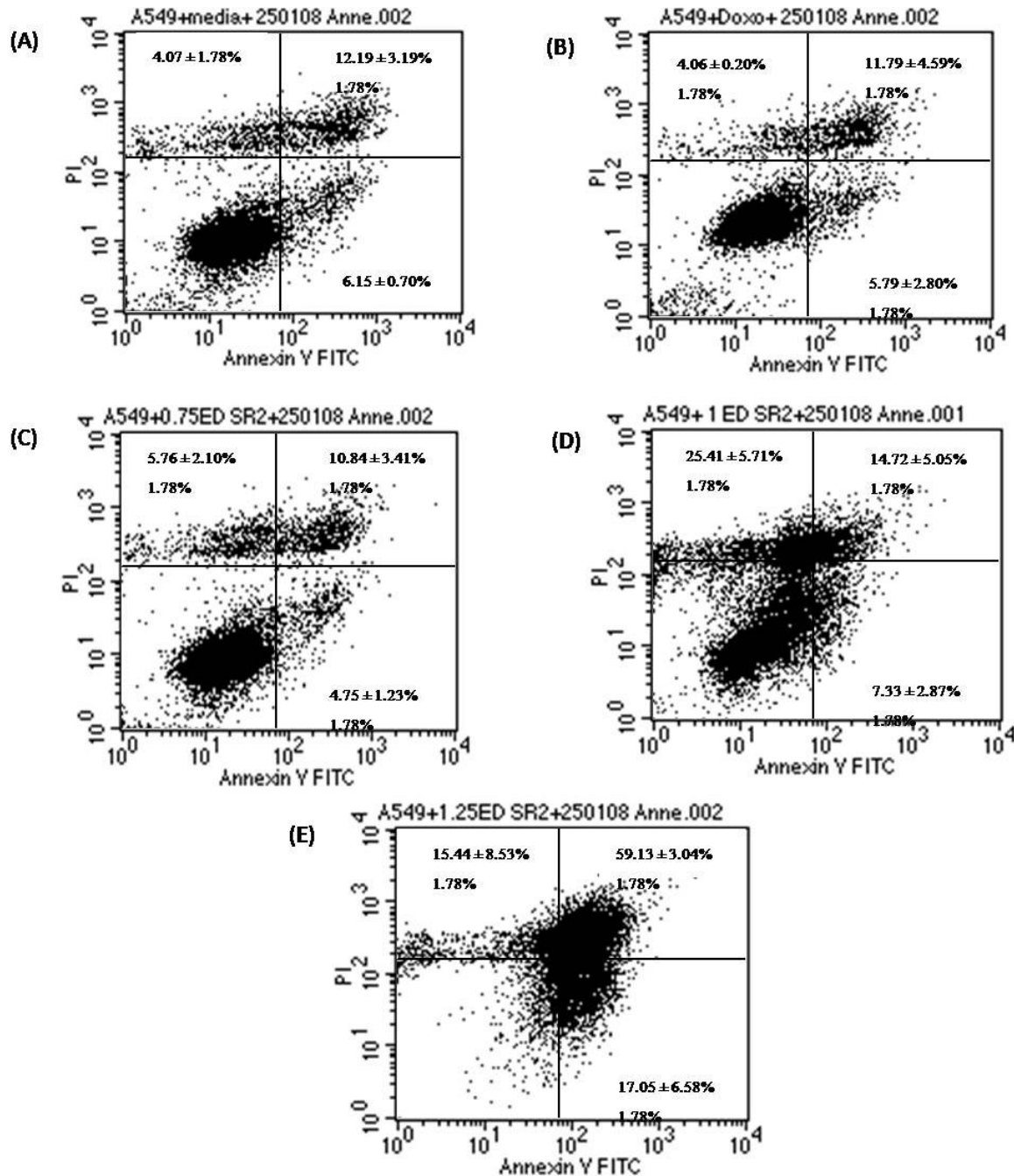


Figure 3. Annexin V binding and propidium iodide uptake in A549 cells induced by *Sapindus rarak* water extract. (A) Negative control; (B), (C), (D), (E) A549 cells treated with 1 μ g/ml Doxorubicin and *Sapindus rarak* water extract at the concentrations of 82.5, 110, 137.5 μ g/ml, respectively, for 0.5 hr. The dual parameter fluorescent dot plots showed the viable cells populations in the lower left quadrant (Annexin V⁻ PI⁻), the cells at the early apoptosis are in the lower right quadrant (Annexin V⁺ PI⁻), and the ones at the late apoptosis are in the upper right quadrant (Annexin V⁺ PI⁺). Values are means \pm standard error of three independent experiments.

Effect of *S. rarak* water extract on caspase activity

The herbal extract induced apoptosis of A549 cells by activation of caspase activities was shown in Figure 4. The extract significantly increased caspase 9 level, with

product size 46 KDa, and marked detection was observed at 110 μ g/ml with bands density from 31064 to 71623 INT/mm² (p value < 0.05). However, at concentration of 137.5 μ g/ml, the herb extract reversely decreased the level of caspase 9 in A549 treated cells

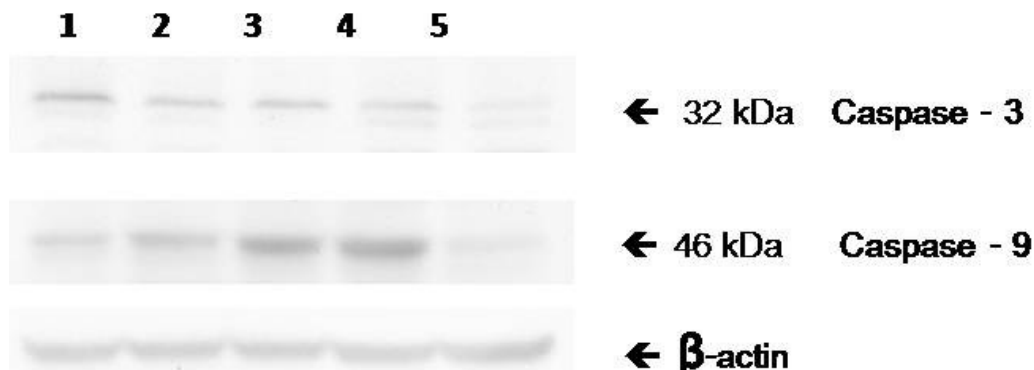


Figure 4. Caspase activation in A549 cells after treatment with *Sapindus rarax* water extract determined by Western blot. Negative control (lane 1); A549 cells treated with 1 µg/ml Doxorubicin (lane 2) and *Sapindus rarax* water extract at the concentrations of 82.5, 110, 137.5 µg/ml (lane 3, 4, 5), respectively, for 0.5 hr. Expression of β-actin was used as an internal control.

with density of bands from 31064 to 22736 INT/mm². The level of procaspase 3, with product size 32 KDa, in herb treated cells was significantly decreased after half an hour incubation at concentration 137.5 µg/ml with band density from 52630 to 14366 INT/mm² (p value < 0.05). In addition, the activation of procaspase 3 increased progressively in a dose dependent manner.

DISCUSSION

Thai traditional doctors used water extract of *S. rarax* to treat several diseases, including malignancies. However, there is still no scientific data on the therapeutic activity of this herbal plant in cancer. Therefore, this is the first report of this plant on cancer which showed that *S. rarax* water extract has the antiproliferative activity on A549 human lung cancer cell line. The antiproliferative effect of this herbal extract showed the highest response on A549 cell line when compared with other cell lines used in this study in dose dependent manner. The reason might be because different cell lines have different surface receptors and various sensitivity. Therefore, each plant will have different effect on different cell types. Based on the antiproliferative effect, and cell cycle analysis in A549 cells was performed and we found that herbal treated A549 cells showed no significant change in any cell cycle phase. The underlying mechanism of the extract to inhibit cell growth was further investigated and demonstrated that the growth inhibition of A549 cells was induced by apoptosis which is one of the most common anticancer effects, a pathway previously reported with some other plant extracts (Cheng et al., 2004; Eom et al., 2008; Ma et al., 2008; Sa et al., 2008; Zhao et al., 2008). Apoptosis is an important mechanism that balances cell division and cell death for controlling the tissue kinetics (Hengartner, 2000; Kerr et al., 1972). It is regulated by several effector proteins including caspase enzymes, such as caspase 3

and 9 (Earnshaw et al., 1999; Nicholson, 1999; Thornberry and Lazebnik, 1998; Thornberry et al., 1997). In order to determine the pathway of apoptosis, the activities of caspase 9 and procaspase-3 were studied using Western blotting.

The treatment of A549 cells with herbal extract resulted in significant increase in the expression of caspase 9. Interestingly, the decreased expression in caspase 9 in herbal treated cells at high concentration of 137.5 µg/ml was also exhibited, which might result from the decrease in the number of viable cells. Activated caspase 3 is the key executioner of apoptosis, and the decrease in procaspase 3 was demonstrated in Western blot. Based on this study, the activation of both caspase enzymes were in a dose dependent manner. From previous reports, apoptosis is controlled by two distinct pathways and one of them is the death receptor mediated pathway. This pathway is initiated by the interaction of the ligand with its receptor resulting in the activation of caspase 3, including caspase 8 (Ashkenazi and Dixit, 1999). The other pathway, the mitochondrial mediated pathway, involves in releasing cytochrome c from mitochondria which ultimately activates caspase 9, which in turn activates caspase 3 (Green and Reed, 1998). In the present study, the apoptotic pathway of this herbal plant was caspase dependent pathway since the activation of both caspase 3 and 9 was also detected.

S. rarax methanolic extract has been investigated and reported to have the inhibitory effect on pancreatic lipase activity and further investigation of this fraction revealed several constituents with saponins (Asao et al., 2009). Saponins are triterpene or steroid glycosides that are distributed in more abundance in some plants such as *S. rarax*. Based on several reports, saponins from other plants have already been studied and demonstrated to have activities involving in chemopreventive effect in terms of cytotoxic activity and inhibitory effect including induction of apoptosis (Tundis et al., 2009; Xiao et al.,

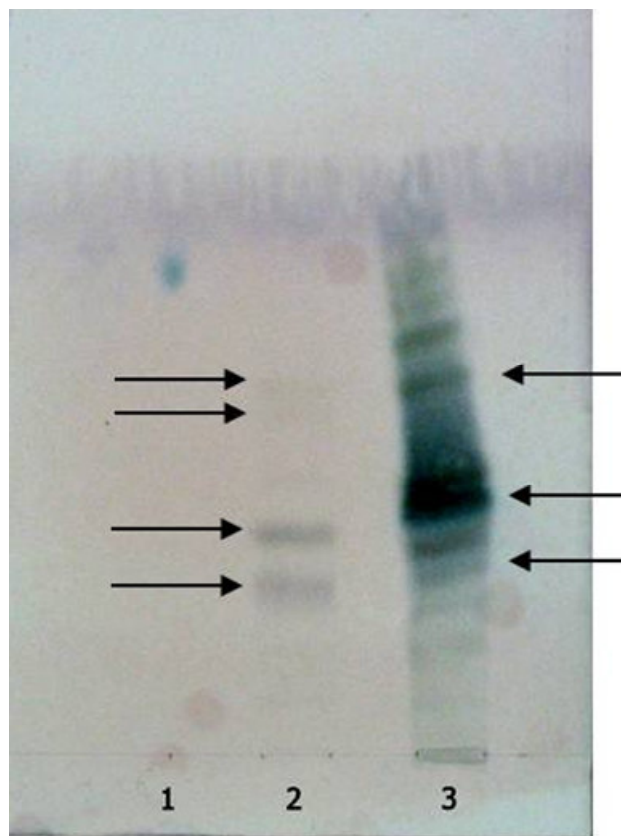


Figure 5. Thin layer chromatograms of *Sapindus rarak* water extract which showed the saponin fraction compared with the control in lane 2. Adsorbent : silica gel 60 GF₂₅₄, Alufolien, Merck; Solvent system: butanol: ethanol: water: acetic acid (10.8:3.6:2.7:0.2); Detection: spray with 10% aq. H₂SO₄, and heat in the oven at 110 °C for 1-2 minute.; 1= Phytosteryl glucoside (0.01 mg/mL, 1 µL); 2= Saponins from Tea seed (60%, 10 mg/mL, 4 µL); 3= Water extract of *Sapindus rarak* (10 mg/mL, 4 µL).

2009). As shown in Figure 5, thin layer chromatograms of *S. rarak* water extract showed dense bands which might be the biological active constituent at the same level with the saponin fraction control in lane 2. Therefore, the activities of the water extract should result from the saponin component in this herbal plant.

In conclusion, *S. rarak* water extract inhibited cell proliferation or induction of cell death in A549 human lung cancer cell line by the induction of an apoptotic process which was confirmed by Annexin V-FITC. The experiments showed that the *S. rarak* induced apoptosis in A549 cells by mediating through the activation of the caspase pathway which showed enhanced expression of caspase enzymes 9 and procaspase 3 in Western blot. This research might be a hypothesis generating studies. Further evidences are required to determine whether this special herbal water extract, which has long been used in Thai traditional medicine, could be developed as a novel anticancer agent for the treatment of human lung cancer.

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