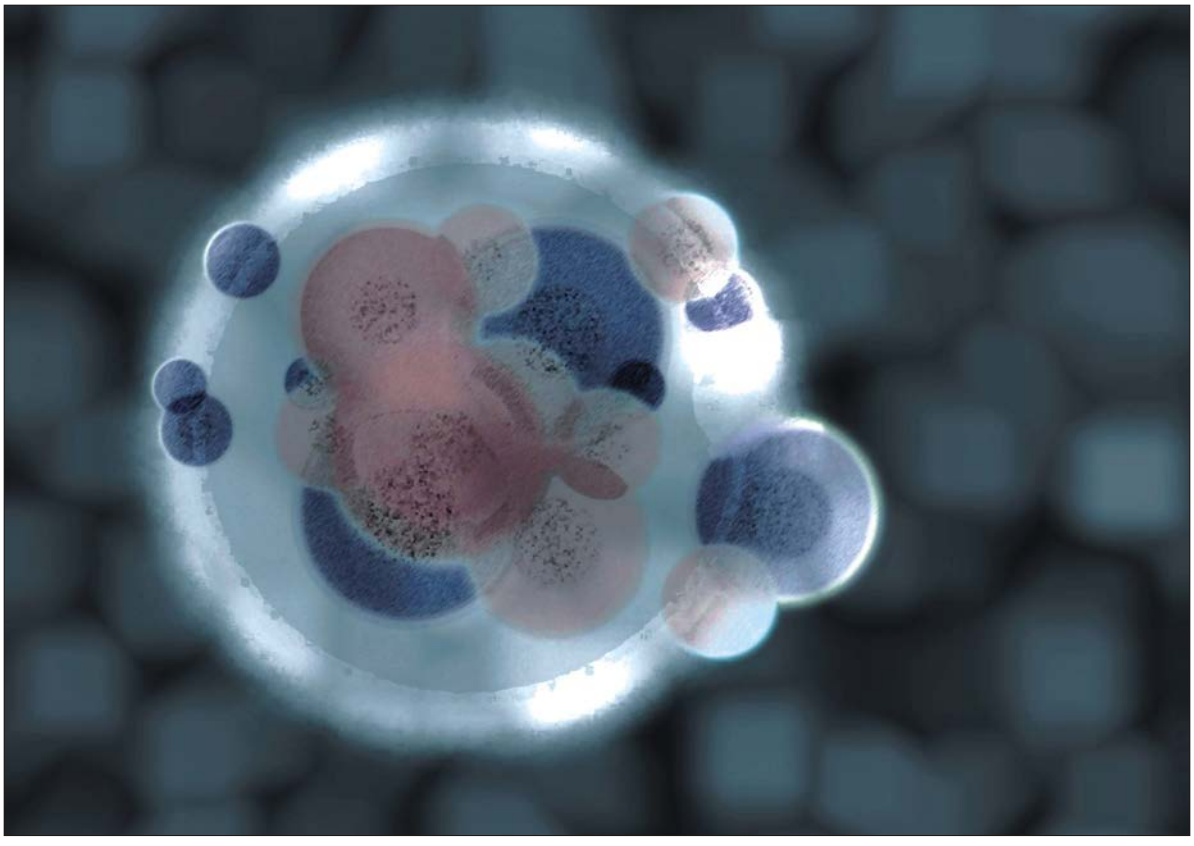

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Research Article
Antiproliferation of Fibrosarcoma Cell Line (L929) by Hua-Khao-Yen-Tai Thai Medicinal Plant (*Dioscorea membranacea* Pierre)

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Abstract

Background: Extracts of many Thai medicinal plants affected anti-proliferation of cancer cell line. This study was to determine cytotoxic activity and apoptotic gene expression of fibrosarcoma cell line (L929) treated with Thai medicinal plant, *Dioscorea membranacea* Pierre. **Materials and Methods:** The ethanolic crude extracts of Hua-Khao-Yen-Tai (*Dioscorea membranacea* Pierre) were tested for anti-proliferative activity, apoptotic gene expression and protein expression of L929 by using MTT assays, RT-PCR and Western blot analysis. **Results:** The MTT assays showed 50% of viability at 15 μg mL^{-1} after treatment for 24 h and 25 μg mL^{-1} at 48 h of L929. The L929 cells showed character of apoptosis: Cell shrinkage, membrane blebbing and nuclear condensation when treated with Hua-Khao-Yen-Tai ethanolic extracts. The expression level of BAX gene increased more than control but Bcl-2 decreased lower than control. The results of Western blot analysis showed up-regulation of BAX protein and down-regulation of Bcl-2 protein when compared with control cells at 24 and 48 h. **Conclusion:** Results indicated that the ethanolic crude extracts of Hua-Khao-Yen-Tai induced apoptosis and anti-proliferation on fibrosarcoma cell line. This could be beneficial to the further development of cancer treatments in the future.

Key words: Thai medicinal plant, *Dioscorea membranacea,* fibrosarcoma cell line L929, cancer, antiproliferation, apoptosis, BAX, Bcl-2

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Cancer is the uncontrolled growth of abnormal cells in the body. Cancerous cell are also called malignant cells. The cancer may also spread to more distant part of body through the lymphocytic system or bloodstream. There are serveral causes of cancer such as tobacco, diet, obesity, radiation, stress, virus and genetic[1]. Hepatoma cancer is the one of the most common cancers in the world found approximately 95% in Thailand[2]. The cause of cancer are many viruses and chemical agents such as hepatitis B and C virus, alcohol, alfatoxin and polymer polyvinyl chloride (PVC). The treatment of cancer is liver transplantation, chemotherapy and radiation. Furthermore, several countries used traditional plants to cure cancer. For example, Itharat *et al.*[3] used Thai medicinal plants: *Dioscorea membranacea* Pierre ex Prain and Burkill, *Dioscorea brimanica* Prain and Burkill (Dioscoreaceae) and *Siphonodon celastrineus* Griff Celastraceae) treated human large cell lung carcinoma cell line COR-L32, the human breast adenocarcinoma cell line MCF-7 and human colon adenocarcinoma cell line LS-174T. The result of this experiment exhibited highly cytotoxic activity to cancer cell. In Moongkarndi *et al.*[4] evaluated ethanolic crude extracts of nine Thai medicinal plant against Human breast cancer cell line. The result showed IC_{50} of these plant against cancer cells. Moreover, Saetung *et al.*[5] showed specific activity against lung cancer cell lines and less cytotoxic activity compared to normal cell by using ethanolic plant extracts (*Dioscorea membranacea* and *Curcuma zedoaria*). Triphala (TPL), an Indian ayurvedic formulation, induced loss in viability and inhibited the clonogeric growth of breast cancer[6,7] MCF-7. The chamomile extracts caused minimal growth inhibition in normal cell, whereas decreased cell viability in human cancer. Jaengsuwan and Itharat[8] reported that ethanolic crude extracts of Hua-Khao-Yen-Tai, Thai medicinal plant (*Dioscorea membranacea* Pierre) could induce apoptosis and anti-proliferation on hepatoma HepG2 cell lines. Hua-Khao-Yen-Tai is an alternative way for cancer treatment and effects of its extract on other types of cancer cell lines have not been determined. The aim of this study is to determine cytotoxic activity and apoptotic gene expression of fibrosarcoma cell line (L929) treated with Thai medicinal plant, *Dioscorea membranacea* Pierre.

MATERIALS AND METHODS

Preparation of plant extracts: Hua-Khao-Yen-Tai (*Dioscorea membranacea* Pierre) were washed and dried. Twenty grams of Hua-Khao-Yen-Tai were added with 200 mL of 50% alcohol. After that N_2 gas were sprayed on the top of materials and shaken at 150 rpm for 72 h. The ethanolic crude extracts were filtered and concentrated to dryness under reduced pressure. The ethanolic extracts were dissolved in DMSO and sterilized by filtration.

***In vitro* assays for cytotoxic activity:** The L929 were cultured in DMEM medium supplement with 10% FBS for 24 h in 96 well plate. After that, L929 were washed with medium for 3 times and then added serum-free medium. Following this step L929 were treated with the Hua-Khao-Yen-Tai extracts for various dose and incubated at 37°C for 24 and 48 h. After incubation, treated cells were washed with PBS for twice time, added MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] and incubated for 3 h, after that removed the MTT and added DMSO. Plates were shaken in the dark condition for 30 min. The absorbance was recorded on a microplate reader at 490 and 570 nm. The effect of Hua-Khao-Yen-Tai on growth inhibition was assessed as percentage of cell viability and IC_{50} (Inhibiting concentration).

RNA extraction and RT-PCR: The L929 were treated with Hua-Khao-Yen-Tai at 15 μg mL^{-1} at 24 and 25 μg mL^{-1} at 48 h. After incubation, attached and detached cells were collected by centrifugation at 3000 rpm. Pellet cells were extracted with TriZol and cDNA was synthesized with oligod T21 mer. The cDNA was amplified for apoptotic gene: BAX, Bcl-2 and GAPDH (internal control) by PCR. The PCR product were analyzed by 1.5% agarose gel electrophoresis and stained with ethidium bromide solution.

Western blot analysis: The L929 were treated with 15 μg mL^{-1} for 24 and 25 μg mL^{-1} at 48 h of Hua-Khao-Yen-Tai ethanolic crude extracts. After that, attached and detached cells were collected by centrifugation. Protein lysis buffer was added into pellet-cell for protein extraction. Pellet was vortexed and centrifuged to collect supernatant measured protein concentration. Fifty microgram of protein was separated on 10% SDS-PAGE and transferred to nitrocellulose membrane. This membrane was blocked with 5% skim milk for 1 h and incubated with primary anti-mouse monoclonal antibody: BAX, Bcl-2 and β-actin (internal control) for 3 h. Following this step, membrane was washed and incubated with HRP-conjugated anti-mouse secondary antibody for 1 h. The reaction were detected with ECL on hyperfilm.

RESULTS

Cytotoxicity activity: Figure 1 showed viability of L929 treated with Hua-Khao-Yen-Tai crude extracts for dose-dependence after 24 and 48 h treatment. The results

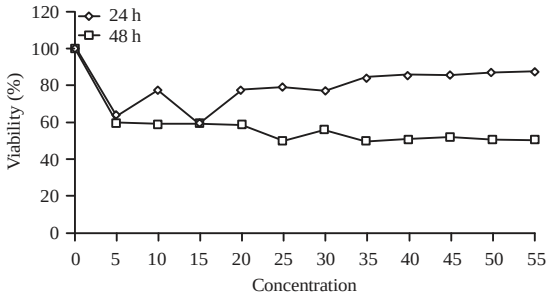

Fig. 1: IC_{50} of L929 for dose-dependent at 24 and 48 h

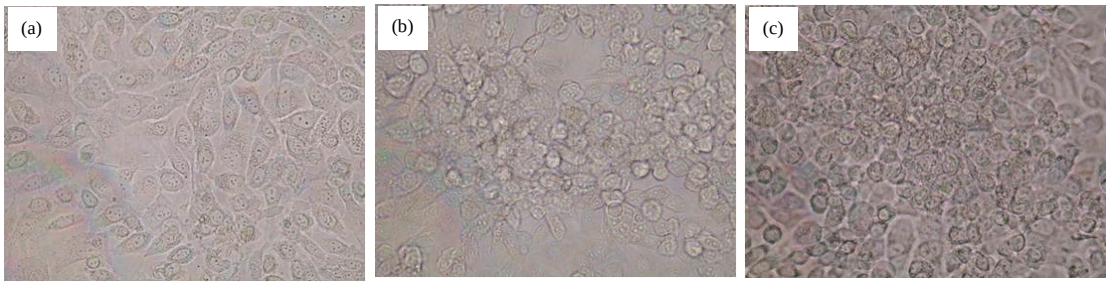

Fig. 2(a-c): Hua-Khao-Yen-Tai ethanolic crude extracts induced L929 to apoptosis, (a) L929 cells without treatment, (b) L292 cells were treated with 15 μg mL^{-1} of extracts at 24 h and (c) L929 cells were treated with 25 μg mL^{-1} of extracts at 48 h

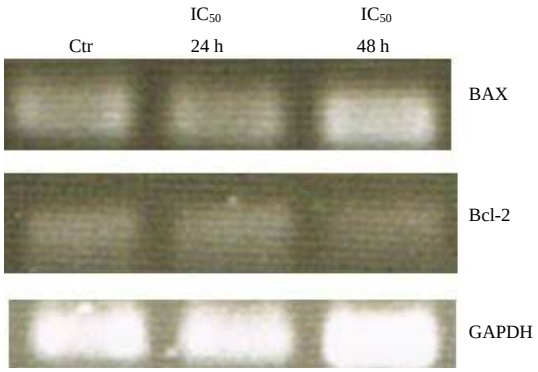

Fig. 3: *Dioscorea membranacea* Pierre crude extracts induced apoptotic gene expression in L292, Ctr: Control (untreated), IC_{50} 24 h: L292 cells was exposed at IC_{50} (15 μg mL^{-1}) for 24 h and IC_{50} 48 h: L292 cells was exposed at IC_{50} (25 μg mL^{-1}) for 48 h

showed IC_{50} at 15 μg mL^{-1} at 24 h and 25 μg mL^{-1} at 48 h of Hua-Khao-Yen-Tai ethanolic crude extracts. Consequently, the ethanolic crude extracts from Hua-Khao-Yen-Tai at 15 μg mL^{-1} induced 50% of cell death for L929 at 24 h whereas induced 50% of cell death at 48 h with 25 μg mL^{-1}.

Morphological alterations: Control cells exhibited morphological of L929 in the absence of Hua-Khao-Yen-Tai extracts (Fig. 2a). After treatment at IC_{50}: 15 μg mL^{-1} at 24 and at IC_{50}: 25 μg mL^{-1} at 48 h, L292 showed apoptotic cell characters; nuclear condensation, membrane blebbing and cell shrinkage and occurred some necrosis. As for at 48 h, L292 had apoptotic cell characters more than 24 h (Fig. 2b, c).

Apoptosis gene expression: The L292 were treated with Hua-Khao-Yen-Tai ethanolic crude extracts at 24 and 48 h (IC_{50}: 15 and 25 μg mL^{-1}, respectively). The apoptotic gene expression was determined by RT-PCR method. The result in Fig. 3 showed the expression of BAX gene increased more than control and the expression level of BAX gene depended on time while the expression level of Bcl-2 inverted with BAX gene. Furthermore, the expression level of Bcl-2 at 48 h decreased more than the others. The expression level of BAX gene and Bcl-2 gene were compared to GAPDH being internal control of gene expression.

Protein expression: Apoptotic gene expression was confirmed by Western blot analysis. After treatment of L929, the result in Fig. 4 showed up-regulation of BAX protein and down-regulation of Bcl-2 protein at 24 and 48 h compare with control cells, all of apoptotic protein level was equated to the b-actin being internal control protein.

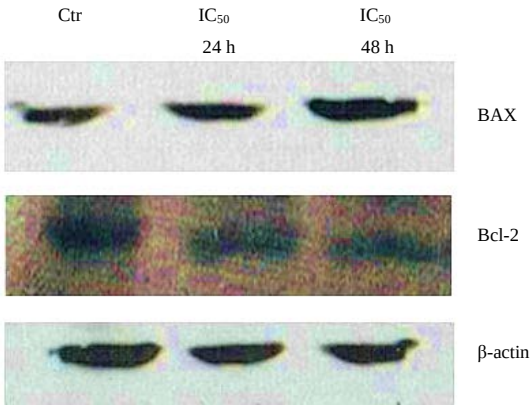

Fig. 4: *Dioscorea membranacea* Pierre (Hua-Khao-Yen-Tai) crude extracts induce pro-apoptotic protein in L929, Ctr: Control (untreated), IC_{50} 24 h: L929 cells was exposed at IC_{50} (15 μg mL^{-1}) for 24 h and IC_{50} 48 h: L292 cells was exposed at IC_{50} (25 μg mL^{-1}) for 48 h

DISCUSSION

Hua-Khao-Yen-Tai is a Thai medicinal plant. It mostly has been used in Thai traditional medicine. Five species of Hua-Khao-Yen-Tai in Thailand included *Dioscorea birmanica*, *Similax corburalia*, *Similax glabra*, *Pygmaeo premnaherbacea* and *Dioscorea membranacea* having been extensively used for treatment of cancer. This experiment showed that the IC_{50} of ethanolic crude extracts opposite to L929 at 15 μg mL^{-1} at 24 and at 25 μg mL^{-1} at 48 h. This data correlated with the results of Itharat *et al.*[3]. Jaengsuwan and Itharat[8] reported the *Dioscorea membranacea* rhizome ethanolic crude extracts was active against lung, colon, breast and liver cancer cells (IC_{50} at exposure time 72 h: 6.2, 16.7, 12.0 and 51 μg mL^{-1}, respectively). Sung *et al.*[9] reported that *Salvia miltiorrhiza* root extracts obtained using 100% ethanol and 100% acetone as solvents exhibited more potent effects compared with extracts obtained using 70 and 30% aqueous ethanol and the active components of *S. miltiorrhiza* ethanol extracts, known as tanshinones exhibit a high cytotoxic potential.

Treatment of L929 with Hua-Khao-Yen-Tai ethanolic crude extracts at 15 μg mL^{-1} and at 25 mg mL^{-1} showed decrease 50% of proliferation and induced L929 cells into apoptotic program at 24 and 48 h, respectively. The molecular mechanism of apoptosis promoted apoptotic genes and reduced anti-apoptotic genes. The RT-PCR showed high level of BAX gene and low level of Bcl-2 gene at 24 and 48 h. Furthermore, the apoptotic protein (BAX) up-regulated while anti-apoptotic protein (Bcl-2) down-regulated at 24 and 48 h. This result correlated with many researches which treatment cancers with traditional plants and induced cancer cell to apoptosis. For example, Saekoo *et al.*[10] reported that the Dioscorealide B was a bioactive compound, isolated from *Dicoscorea membranacea*. Dioscorealide B induced human breast cancer cell line MCF-7 into apoptosis via the activation of caspase-9 and -7. Their results showed the mRNA level of p53 and BAX level increased whereas Bcl-2 decreased after treatment. Jaiaree *et al.*[11] used ethanolic crude extracts of *Dioscorea brimanica* Prair and Burkill (DBE). The result showed cytotoxic activity against lung cancer, A549 and COR-L23 (IC_{50}: 7.45 and 8.17 μg mL^{-1}, respectively). Recently, *Panax ginseng* fresh leaves exhibited anticancer activity in lung cancer cell line (A549) and the regulation of EGFR/p38 MAPK/p53 pathway might be the possible mechanism of its anti-activity[12].

The result obtained in this study indicated that Hua-Khao-Yen-Tai ethanolic crude extracts induced to cytotoxicity in L929 through apoptosis pathway. It is interesting in traditional plants for cancer patient treatment. However, there are plentiful researches about Thai and Chinese traditional plant reported that these extracts induced several type of cancer cells line to apoptosis but do not effects to normal cells[5,6,8-10,13-16]. In China, Sa *et al.*[17] determined *Similax glabra* Roxb (SGR) especially root to anti-proliferation affect in human hepatoma cell line; HepG2 and Hep3B. They showed that SGR extracts inhibited HepG2 and Hep3B cell growth by causing cell cycle arrest of either S phase or S/G2 transition and induced to apoptosis. Mohammadi *et al.*[18] extracted *Dysosma versipellis* which is traditional Chinese medicine herbs. These extracts inhibited the growth of human carcinoma cells, PC3 and Bcap-3. Chen *et al.*[19] also showed apoptosis in PC3 carcinoma cells treated with dichloromethanolic extract of *Urtica dioica*. Huang *et al.*[14] studied wagonin, one of flavonoid compounds isolated from Chinese herbal plants *Scutellaria baicalensis* Georgi, induced G1 arrested of MCF-7 cells, decreased the Bcl-2 but increased of BAX and p53. The recently study revealed the correlations between the flavonoids of *Rhamnus davurica* with their anti-proliferative activities against human cancer cells of HT-29 and SGC-7901 *in vitro* exhibited distinct inhibitory effects with IC_{50} values[20] at 24.96±0.74 and 89.53±4.11 μg mL^{-1}. Furthermore, in Sri Lanka, Ediriweera *et al.*[21] used decoction *Nigella sativa* seeds, *Hemides musindicus* root and *Similax glabra* rhizome to treat human hepatoma HepG2 cell line. After 24 h exposure to the decoction, HepG2 were in the late stage of apoptosis and/or necrosis. Consequently, the traditional plant is another ways to treat cancer and extracts is antioxidant to protect cells from stress. Ediriweera *et al.*[21] also showed potential

anticancer activity of the bark of *Mangifera zeylanica*, an endemic plant in Sri Lanka that has been traditionally used for cancer therapy through induction of apoptosis occurred in the breast and ovarian cancer cell lines.

CONCLUSION AND FUTURE RECOMMENDATIONS

The ethanolic extracts from Hua-Khao-Yen-Tai inhibited L929 proliferation. For L929 morphology, these cells showed apoptotic character after treatment with the extracts moreover, increasing the level of BAX gene and protein whereas decreasing the level of Bcl-2 gene and protein. The study indicates that *Dioscorea membranacea* Pierre (Hua-Khao-Yen-Tai) ethanolic crude extracts induced cytotoxicity in L929 cells through apoptosis pathway. This could be beneficial to the further development of cancer treatments in the future.

Dioscorea membranacea Pierre (Hua-Khao-Yen-Tai) should be propagated by tissue culture technique because plants could be cultivated in order to examine the quantity of essential element. In the future, the best breeding of *Dioscorea membranacea* Pierre could be identified to produce for commercial purposes.

SIGNIFICANCE STATEMENT

Dioscorea membranacea Pierre (Hua-Khao-Yen-Tai) ethanolic crude extracts induced cytotoxicity in fibrosarcoma cell line (L929) through apoptosis pathway.

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