

Aqueous extract of Thai medical Herbs (Phytoplex) Inhibits Cell Proliferation and Induces Apoptosis in human cervical cancer cell line (HeLa cells)

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Abstract

Cervical cancer remains a leading cause of cancer mortality in females. Chemotherapy is important as it was a part of the main treatment for this type of cancer. However, multidrug resistance and serious side effects have been major problems in cervical cancer chemotherapy. Therefore, the search for new anticancer drugs from the native medicinal herbs, is very attractive. The combination extract of eight Thai medicinal herb recipes, namely Phytoplex, is a commercial product of The Government Pharmaceutical Organization, has anti-cancer effect on hepatocellular carcinoma cells (HepG2) *in vitro*. However, the anticancer effects and molecular mechanisms of Phytoplex on cervical cancer have not yet been studied. The aim of this study was to evaluate the inhibitory effect of Phytoplex on human cervical adenocarcinoma cells (HeLa cells) *in vitro*. HeLa cells were treated with low concentrations of Phytoplex (50, 100, 500, 1,000 µg/ml) and high concentrations of Phytoplex (2,000 and 5,000 µg/ml) compared with a positive control (0.1% mitomycin C) and a negative control (0 µg/ml of Phytoplex) for 24, 48 and 72 h. Then, cell viability was evaluated using an MTT assay. The activities of caspase-3 (apoptosis marker) and Ki-67 (proliferation marker)† were investigated using an immunofluorescence assay. At 24 h, lower concentrations of Phytoplex promoted cell viability, while higher concentrations inhibited cell viability ($P < 0.05$). In addition, the inhibitory effect of Phytoplex continuously increased from 24 to 72 h of incubation period at high concentrations of Phytoplex. Moreover, Phytoplex inhibited HeLa cell proliferation with an IC_{50} value of 1,972.43, 1,230.10 and 1,317.67 µg/ml at 24, 48 and 72 h, respectively. The activity of caspase-3 of HeLa cells treated with high concentration of Phytoplex seemed to be higher than of negative control (0 µg/ml). Activity of Ki-67 was higher in lower concentrations of Phytoplex, than that in higher concentrations of Phytoplex. Therefore, HeLa cell growth inhibition was dose- and time-dependent. The study suggested that high concentrations (2,000 and 5,000 µg/ml) of Phytoplex exerted inhibitory effect in HeLa cell growth by inducing apoptosis via activation of caspase-3.

Keywords: cervical cancer, Phytoplex, HeLa cells, herbs, viability

สารสกัดสมุนไพรไทย (ไฟโทเพล็กซ์) ด้วยน้ำ ยับยั้งการเพิ่มจำนวนของเซลล์และเหนี่ยวนำให้เซลล์ตายแบบเอพออโตซิสในเซลล์มะเร็งปากมดลูกของมนุษย์ (เซลล์ฮีลา)

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บทคัดย่อ

มะเร็งปากมดลูกเป็นสาเหตุการตายอันดับต้น ๆ ของผู้หญิง การใช้ยาเคมีบำบัดมีความสำคัญอย่างมากต่อการรักษามะเร็งชนิดนี้ แต่ปัญหาหลักในการใช้ยาเคมีบำบัดรักษามะเร็งปากมดลูกคือการดื้อต่อยาหลายชนิดและผลข้างเคียงของยาที่รุนแรง ดังนั้น การค้นหาต้านมะเร็งชนิดใหม่จากสมุนไพรพื้นบ้านจึงเป็นที่น่าสนใจอย่างมาก คำรับยาของสารสกัดสมุนไพรไทยรวม 8 ชนิด ชื่อว่าไฟโทเพล็กซ์ เป็นผลิตภัณฑ์ขององค์การเภสัชกรรม มีคุณสมบัติต่อต้านมะเร็งในมะเร็งเซลล์ตับ (เอชซีซี) ในหลอดทดลอง ในปัจจุบันยังไม่มีการศึกษาคุณสมบัติต่อต้านมะเร็งและกลไกระดับโมเลกุลของยาไฟโทเพล็กซ์ที่ใช้รักษามะเร็งปากมดลูก งานวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาประสิทธิภาพของไฟโทเพล็กซ์ในการยับยั้งการเจริญเติบโตของเซลล์มะเร็งปากมดลูก (เซลล์ฮีลา) ในหลอดทดลอง โดยนำไฟโทเพล็กซ์ความเข้มข้นต่ำ (50 100 500 และ 1,000 ไมโครกรัม/ มิลลิลิตร) และไฟโทเพล็กซ์ความเข้มข้นสูง (2,000 และ 5,000 ไมโครกรัม/ มิลลิลิตร) มาทดสอบกับเซลล์มะเร็งปากมดลูก เทียบกับกลุ่มควบคุมผลบวก (0.1% mitomycin C) และกลุ่มควบคุมผลลบ (ไฟโทเพล็กซ์ที่ความเข้มข้น 0 ไมโครกรัม/มิลลิลิตร) เป็นเวลา 24 48 และ 72 ชั่วโมง หลังจากนั้นวัดอัตราการรอดชีวิตของเซลล์โดยวิธี MTT ตรวจการแสดงออกของ caspase-3 (บ่งบอกการตายของเซลล์) และ Ki-67 (บ่งบอกการแบ่งตัวของเซลล์) โดยวิธีอิมมูโนฟลูออเรสเซนซ์ ผลการศึกษาที่ 24 ชั่วโมง พบว่าไฟโทเพล็กซ์ความเข้มข้นต่ำ มีอัตราการรอดชีวิตของเซลล์เพิ่มขึ้น ขณะที่ไฟโทเพล็กซ์ความเข้มข้นสูง ยับยั้งการเจริญเติบโตของเซลล์มะเร็ง (P < 0.05) การยับยั้งการเจริญเติบโตของเซลล์เพิ่มขึ้นอย่างต่อเนื่องจาก 24 ชั่วโมง จนถึง 72 ชั่วโมงของการเลี้ยงที่ความเข้มข้นสูง ความเข้มข้นของไฟโทเพล็กซ์ที่ทำให้เซลล์รอดชีวิตครึ่งหนึ่ง (IC₅₀) มีค่าเท่ากับ 1,972.43 1,230.10 และ 1,317.67 ไมโครกรัม/ มิลลิลิตร ที่ระยะเวลา 24 48 และ 72 ชั่วโมง ตามลำดับ การแสดงออกของ caspase-3 พบว่า เซลล์มะเร็งปากมดลูกที่ทดสอบด้วยไฟโทเพล็กซ์ความเข้มข้นสูงมีการแสดงออกของ caspase-3 สูงกว่าไฟโทเพล็กซ์ความเข้มข้นต่ำ การตรวจแสดงออกของ Ki-67 พบว่า เซลล์มะเร็งปากมดลูกที่ทดสอบด้วยไฟโทเพล็กซ์ความเข้มข้นต่ำมีการแสดงออกของ Ki-67 สูงกว่าไฟโทเพล็กซ์ความเข้มข้นสูง ดังนั้น ประสิทธิภาพของไฟโทเพล็กซ์ ในการยับยั้งการเจริญเติบโตต่อเซลล์มะเร็งปากมดลูกขึ้นกับความเข้มข้นของยาและเวลาในการทดสอบ การศึกษานี้ชี้แนะว่า ไฟโทเพล็กซ์ที่ความเข้มข้นสูง 2,000 และ 5,000 ไมโครกรัม/ มิลลิลิตร มีฤทธิ์ยับยั้งการเจริญเติบโตของเซลล์มะเร็งปากมดลูกโดยการเหนี่ยวนำให้เกิดการตายแบบเอพออโตซิสผ่านการกระตุ้น caspase-3

คำสำคัญ : เซลล์มะเร็งปากมดลูก ไฟโทเพล็กซ์ เซลล์ฮีลา สมุนไพร อัตรารอดชีวิต

Introduction

In human, cervical cancer, a Papillomavirus infection-related cancer, was the fourth most frequent cancer globally found among women during 2012, with age-standardized incidence rate (ASRs) of 14 per 100,000 (an estimated 528,000 new cases diagnosed annually). During 2012, the worldwide number of deaths from cervical cancer was 265,672 heads, accounting for 7.5% of all cancer in women (Serrano et al., 2018). Even though the incidence of cervical cancer has decreased in developed countries, this cancer is a serious public health as the second leading cause of female cancer in Thailand, with ASRs of 17.8 per 100,000 (about 8,184 new cases diagnosed annually) (Bruni et al., 2017). Chemotherapy is considered the main treatment of cervical cancer. However, it contributes to several serious side effects, including alopecia, nausea and vomiting, peripheral neuropathy and fatigue (Sun et al., 2014). Furthermore, numerous patients with recurrent cervical cancer do not show any response to the chemotherapy because of multidrug resistance (Zhu et al., 2016). Hence, the discovery and development of new chemotherapeutic drugs is urgently necessitated.

Medicinal herb is a medical system utilizing plants, their extracts, or their recipes to promote health status as well as to prevent and cure diseases for thousand years. In addition, synergistic effects of medicinal herb recipes has been reported (Che et al. 2013). In Thailand, traditional medicinal herb recipes have been incrementally used as alternative treatments for anticancer drugs due to low cost and low risk of side effects (Poonthananiwatkul et al., 2015). Besides, thai medicinal herb recipes do not

only possess anticancer activities in several human cancer cell lines, but they also improve survival rate and quality of life of cancer patients when they are used for adjuvant therapy with traditional chemotherapeutic drugs (Thisoda et al., 2013).

Phytoplex, one of the most outstanding Thai medicinal herb recipes, is a commercial product launched by the Government Pharmaceutical Organization (GPO) of Thailand, which has been ordered for curing patients with cancer by Dr. Sommai Thongprasert (traditional Thai medicine practitioner) (Akaraserenont et al., 1999). Phytoplex is a combination of eight plants: *Acanthus ebracteatus* (Ngueak-Pla-Moo, Acanthaceae), *Ammannia baccifera* (Ma-Fai-Duen-Haa, Lythraceae), *Canna indica* (Put-Ta-Rak-Sa, Cannaceae), *Clinacanthus nutans* (Pha-Ya-Yor, Acanthaceae), *Mallotus philippensis* (Tang-tuay, Euphorbiaceae), *Polygala chinensis* (Peek-Kai-Dam, Polygalaceae), *Premna herbacea* (Khao-Yen-Tai, Lamiaceae), and *Smilax corbularia* (Khao-Yen-Nuea, Smilacaceae). The previous study reported that the use of anticancer properties of a single herbal medicine could induced apoptosis in cancer cells by active ingredient in each plant of Phytoplex including β -sitosterol in *A. ebracteatus* (Awad et al., 2003), triterpenes and coumarins in *A. baccifera* (Loganayaki et al., 2012), stigmaterol and 6 beta-hydroxystigmasta-4, 22-diene-3-one in *C. indica* (Darsini et al., 2015), stigmaterol in *C. nutans* (Ng et al., 2017), rottlerin in *M. philippensis* (Gangwar et al., 2014), terpenoid in *P. chinensis* and *P. herbacea* (Yang and Dou, 2010), and quercetin in *S. corbularia* (Granado-Serrano et al., 2006). However, there was no report of anticancer properties of combine use of these plants. Moreover,

earlier studies have indicated that Phytoplex inhibited vascular endothelial cell proliferation in human umbilical vein endothelial cells (HUVECs) (Akaraserenont et al., 1999) and suppressed the growth of hepatocellular carcinoma cells (HepG2) transplanted in mice due to tumor anti-angiogenesis effect (Duansak et al., 2007). Besides, the chronic toxicity of Phytoplex demonstrated that the oral administration with Phytoplex of 0.24, 1.2, 3.6 g/kg/day for six months did not affect both health status and body weight in Wistar rats (Chivapat et al., 2010). However, the anticancer effects and actual molecular mechanisms of Phytoplex on cervical cancer have not yet been studied. Accordingly, the aim of this study was to evaluate the effect of Phytoplex on growth inhibition of human cervical adenocarcinoma cells (HeLa cells) *in vitro*, which could be developed to the adjuvant anticancer therapeutic agent of cervical cancer in human in the future.

Materials and methods

Chemicals, reagents and culture media

All chemicals and reagents were purchased from Sigma-Aldrich Co. (Saint-Louis, MO, USA) and cell culture products were purchased from GIBCO (Carlsbad, CA, USA).

Cell culture

HeLa cells (ATCC® CCL-2) were kindly provided by the Monitoring and Surveillance Center for Zoonotic Diseases in Wildlife and Exotic Animals (MoZWE), passaged every 3-4 days with trypsin/EDTA and cultured in a fibroblast medium (FM) containing Dulbecco's

modified Eagle medium (DMEM), 10% fetal bovine serum (FBS), 1% glutamax-L and 1% penicillin-streptomycin. Cells were incubated in a humidified 37°C, 5% CO₂ incubator.

Herbal extract and treatments

Phytoplex (GPO, Bangkok, Thailand), an aqueous crude extract of eight plants packed in a capsule, was extracted with phosphate buffered saline (PBS) at 100°C for 2 h. Stock solution of Phytoplex was 10 mg/ml and stored at 4°C, meanwhile further dilutions were prepared in FM. HeLa cells were treated with low concentrations of Phytoplex (50, 100, 500, 1,000 µg/ml) and high concentrations of Phytoplex (2,000 and 5,000 µg/ml). The cells treated with 0.1% mitomycin C (5 µg/ml) were used as positive control, and untreated cells (0 µg/ml of Phytoplex) were considered negative control.

Morphological observation

HeLa cells were seeded into 6-well plates with 2 x 10⁵ cells/well. After 6 h of incubation, the medium was changed with different concentrations of Phytoplex. After 24, 48 and 72 h of incubation, cell morphology was examined under an inverted microscope. Images were captured by an Axiovert 40 CFL microscope connected to AxioCam 105 color camera (Carl Zeiss, Germany).

Cell viability assay

MTT assay was applied to measure cytotoxic effect of Phytoplex on HeLa cells, as already described by Berenyi et al. (2013). Briefly, cells were applied into a 96-well plate with a density of 5 x 10³ cells/well. After 6 h

of adherence, the medium was changed with various concentrations of Phytoplex. After 24, 48 and 72 h, 20 μ l of 3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) solution at 5 mg/ml in PBS was added to each well and incubated for 3 h. Then, the media were replaced with 100 μ l of Dimethyl sulfoxide (DMSO) per well to dissolve the formation of formazan crystals by the mitochondrial dehydrogenase activity in the living cells. Each drug concentration was assayed in 8 wells and repeated at least three times. The absorbance values were measured by an ELX808 automatic microplate reader (BioTek Inc., Winooski, VT, USA) at the wavelength of 450 nm. The HeLa cell viability was calculated as a percentage from the equation shown below. The IC₅₀ value (drug concentration inhibiting cell proliferation by 50%) was determined from linear regression analysis.

The percentage of HeLa cell viability was calculated using the formula as followed:

$$\text{Viability (\%)} = \frac{(\text{Mean OD of test sample} - \text{Mean OD of the blank})}{(\text{Mean OD of the control} - \text{Mean OD of the blank})}$$

Detection of apoptosis and proliferation by immunofluorescence staining

HeLa cells were applied into a 24-well plate at a density of 2×10^4 cells/well. After 24, 48 and 72 h of treatment, they were fixed in 4% paraformaldehyde for 15 min, permeabilized with 0.25% Triton-X100 for 10 min and blocked with 1% bovine serum albumin in PBS. The fixed cells were stained with caspase-3 antibody (sc-7148, diluted 1:200; Santa Cruz Biotechnology, USA) to investigate the apoptotic activity, and stained with

Ki-67 antibody (sc-15402, diluted 1:200; Santa Cruz Biotechnology, USA) to evaluate the cell proliferation at room temperature for 1 h. Then, the samples were labeled with AlexaFluor® 594 goat anti-rabbit IgG (A11037, diluted 1:2000; Invitrogen Life Technologies, USA) at room temperature for 1 h.

The stained cells were mounted onto slide with 4', 6-diamidino-2-phenylindole (DAPI) staining (Vectashield® H-1200, Vector Laboratories, UK) and assessed under a fluorescence microscope. Images were captured using an Axioskop 40 microscope connected to AxioCam MRc camera (Carl Zeiss, Germany).

Statistical analysis

Each experiment was repeated three times. Mean data values were presented with their deviation (mean \pm SD). Statistical analyses were performed according to the SPSS statistics version 18.0 (SPSS, 2010). Analysis of variance (ANOVA) was performed by Dunnett's T3 test. A value of $P < 0.05$ was considered statistically significant.

Results

Cell morphology

Under the inverted phase contrast microscope, no typical change in morphology was observed from cells treated with Phytoplex at low concentrations (50, 100, 500 and 1,000 μ g/ml) compared with untreated cells at 24, 48 and 72 h (data not shown). The cells were well spread and appeared in regular polygonal shape with a few round cells. In Figure 1, HeLa cells treated with Phytoplex at

high concentrations (2,000 and 5,000 $\mu\text{g/ml}$) showed the decrease in cell volume and the obviously morphological changes including cellular shrinkage, rounding and vacuole formation. Moreover, cells detached from the surface and cell debris were also detected.

Cell viability

According to the MTT test, Figure 2 demonstrates that the inhibition of HeLa cell growth was drug concentration- and time-dependent. The maximum growth inhibitory effect of Phytoplex was observed at high concentrations (2,000 and 5,000 $\mu\text{g/ml}$). The percentage of viable cells treated with 2,000 $\mu\text{g/ml}$ of Phytoplex, compared to the negative control (0 $\mu\text{g/ml}$ of Phytoplex), significantly decreased from $48.3 \pm 12.92\%$ after 24 h to $16.2 \pm 3.46\%$ and $13.4 \pm 4.49\%$ by 48 and 72 h, respectively ($P\text{-value} < 0.05$). Furthermore, a strong diminishment of viable cell number was appeared by 24 h of Phytoplex treatment at 5,000 $\mu\text{g/ml}$. Moreover, the complete death of viable cells was noticed by 72 h. According to Phytoplex treatment at high concentrations (2,000 and 5,000 $\mu\text{g/ml}$) significant reduction in cell viability was shown compared to positive control (0.1% mitomycin C) at different points of treatment (24, 48 and 72 h). Furthermore, Phytoplex inhibited the proliferation of HeLa cells with an IC_{50} value of 1,972.43, 1,230.10 and 1,317.67 $\mu\text{g/ml}$ at 24, 48 and 72 h, respectively. However, the low concentrations of Phytoplex (50, 100, 500, 1,000 $\mu\text{g/ml}$) promoted cell growth compared with untreated cells at 24 h.

Cell apoptosis

Caspase-3 in HeLa cells were stained in red with caspase-3 antibody, while the nuclei were counterstained in blue with DAPI (Figure 3). The activity of caspase-3 seemed to be higher in HeLa cells treated with high concentrations of Phytoplex (2,000 and 5,000 $\mu\text{g/ml}$) and positive controls (0.1% mitomycin C) than in negative controls (0 $\mu\text{g/ml}$ of Phytoplex). The higher the concentration of Phytoplex, the more the decrease in cell number was found. Moreover, under continuous exposure to Phytoplex, an increased numbers of cells positive to caspase-3 staining was observed, indicating the progression of apoptosis across the cell population.

Cell proliferation

Ki-67, a nuclear protein, was stained in red and the nucleus was counterstained in blue with DAPI (Figure 4). Activity of Ki-67 was high in HeLa cells treated with low concentrations (50, 100, 500 and 1,000 $\mu\text{g/ml}$) of Phytoplex and negative control, whereas low activity was found in those treated with high concentrations (2,000 and 5,000 $\mu\text{g/ml}$) of Phytoplex.

Discussion

Cervical cancer has been one of the most frequent cancers causing mortality among females in Thailand and USA (National cancer institute, 2012). As cancer, an induction of cell death using cytotoxic agents such as chemotherapy was important strategies of treatment. Recently, the resistance of anticancer synthetic drug was reported worldwide (Mohammad et al., 2015). Therefore, the discoveries of new and safe drugs from native

medicinal herbs for cervical cancer treatment have become necessary. In Thailand, Phytoplex was produced as a commercial drug containing the extracts from eight Thai medicinal herbs. Currently, anti-angiogenesis effect on HUVECs and HepG2 cells was reported (Akaraserenont et al., 1999; Duansak, 2007). Thus, it is important to investigate the more benefits of this drug on other cancer cells as well as its anticancer mechanism. In this study, we demonstrated that aqueous extract of Phytoplex exhibited a dose- and time-dependant activity, having IC_{50} value of 1,972.43, 1,230.10 and 1,317.67 $\mu\text{g/ml}$ at 24, 48 and 72 h, respectively.

Apoptosis (programmed cell death) has been one of the reliable indicators for evaluating cytotoxic agents. It could be characterized by cell rounding and shrinkage, nuclear condensation and fragmentation, plasma membrane blebbing and reduced cell volume (Safarzadeh et al., 2014). Caspase-3, one of the key executioners of apoptosis, could be used to determine mechanisms of apoptosis in cellular assays (Elmore, 2007). In this study, we demonstrated that HeLa cells treated with high concentrations of Phytoplex (2,000 and 5,000 $\mu\text{g/ml}$) exhibited morphological deformation, while the proliferation was inhibited due to cell apoptosis. This was confirmed by the increase in caspase-3 activity within 24, 48 and 72 h after Phytoplex treatment via immunofluorescence assay. Therefore, we revealed that the main molecular mechanisms of Phytoplex in HeLa cells were to inhibit cell proliferation and induce apoptosis through caspase-3 activation. This finding was supported by the previous study. It revealed that β -sitosterol in *A. ebracteatus* induced cell apoptosis in human breast cancer cell line (MDA-MB-231) via

caspase-3 activation (Awad et al., 2003). In addition, this active ingredient possessed a number of anticancer effects, such as increased caspase-3 activation, decreased antiapoptotic Bcl-2 expression and increased proapoptotic Bax expression in human colon cancer cells (HT116) (Choi et al., 2003). Moreover, *A. ebracteatus* could inhibit cervical cancer growth, vascular endothelial growth factor expression and angiogenesis in a CaSki-cell transplanted in mice (Mahasiripanth et al., 2012). Besides, the triterpenes and coumarins in *A. baccifera*, that extracted by using methanol, exerted cytotoxicity against HeLa cells *in vitro*, but no cytotoxic effects were observed against NIH/3T3, a normal cell line (Loganayaki et al., 2012). The mechanism of triterpenes induced apoptosis in cancer cells via the activation of caspase-8 and -9 (Uto et al., 2013). Coumarin induced cell cycle arrest and apoptosis in HeLa cell by inducing internucleosomal fragmentation of DNA and reducing mitochondrial membrane potential. Also, coumarin increased the expression of the Bax (pro-apoptosis protein), promoted the release of cytochrome c and activated caspase 3 (Chuang et al. 2007). In addition, the former study indicated that stigmaterol and 6 beta-hydroxystigmasterol, 22-diene-3-one in *C. indica* exhibited cytotoxicity activity against P388 leukemia cells ($ED_{50} = 55.50$ and $37.50 \mu\text{g/ml}$) (Darsini et al., 2015). Moreover, stigmaterol in *C. nutans* had antiproliferative effects on HeLa cells but significant cytotoxic activities were observed against Vero, normal kidney cell line (Zakaria et al., 2017). Stigmaterol induced intracellular reactive oxygen species (ROS) generation and induced cell apoptosis through intrinsic and extrinsic caspase pathways

(Ng et al., 2017). Rottlerin in *M. philippensis* inhibited histamine-induced H1-receptor gene in HeLa cells (Gangwar et al., 2014). According to *in vivo* studies, treatments of Dalton's ascites lymphoma-mice with single herb, including *P. chinensis* and *P. herbacea*, contributed to enhance average life span and reduced tumor xenograft volume compared with the control group (Alagammal et al., 2012). Terpenoid in *P. chinensis* and *P. herbacea* drive up-regulation of Bax and down-regulation of Bcl-2 (anti-apoptotic protein), resulting in cytochrome c release, caspase activation and apoptotic cell death (Yang and Dou, 2010). Quercetin in *S. corbularia* induced apoptosis in HepG2 by the activation of caspase-3 and -9, the higher expression of pro-apoptotic Bcl-s family members (Bcl-x_s and Bax) and the lower level of anti-apoptotic (Bcl-x_L) (Granado-Serrano et al., 2006). Mitomycin C, an anticancer drug used for blocking DNA and RNA replications and stopping protein synthesis, led to an inhibition of cell mitosis (Sartorelli et al., 1994). In this study, mitomycin C was used as a positive control for apoptosis examination because it induced apoptosis in HeLa cells and human breast cancer cell line (MCF-7) via a caspase-3 (Pirnia et al., 2002; Cheng et al., 2009).

Ki-67, a marker of cancer proliferation, was associated with aggressive cancer. We demonstrated that HeLa cell proliferation was inhibited when being treated with high concentrations of Phytoplex (2,000 and 5,000 µg/ml), as confirmed by immunofluorescence assay.

The use of multiple herb extracts against cancer in complex recipes provided better efficacy than single active ingredient or herb at the equivalent dose (or concentration). This combination resulted in chemical

combination effects. The main mechanisms are hypothesized to be either potentiated or prolonged, and/or its adverse effects reduced, because of synergistic or antagonistic effects, by adding of other herbs (Kiyohara et al., 2004; Zhou et al., 2016). The former study demonstrated that the aqueous extract of *A. ebracteatus* alone had weak antiproliferative effect on HeLa cells as seen from IC₅₀ value of 6,072.50 µg/ml (Mahasiripanth et al., 2012), which was lower than that from Phytoplex in the current study (1,230.10 µg/ml at 48 h incubation period). Moreover, the aqueous extract of *M. philippensis* at 100 µg/ml alone had no antiproliferative effect to HeLa (Sharma and Varma, 2011). On the other hand, the use of single herb either the aqueous extract of *C. nutans* or the methanol extract of *A. baccifera* had antiproliferative effect against HeLa cells (IC₅₀=13 and 130 µg/ml, respectively), which was lower than that of Phytoplex (Yusmazura et al., 2017). Aqueous extract of *S. corbularia* alone possessed low cytotoxic activity against human colon adenocarcinoma (LS-174T) and human large cell lung carcinoma cell lines (COR-L23), their percentages of cell viability for 50 µg/ml concentration after 72 h incubation were 60.1 and 98.9, respectively (Itharat et al., 2004). This difference in IC₅₀ could be explained that the combined use contributed to synergistic or antagonistic effects of anticancer (Pal et al., 2003), the type of solvents for herbal extraction and the sensitivity of various kinds of cancer cell lines to anticancer herbs (Sharma and Varma, 2011). Here, we demonstrated that Phytoplex has chemical combination effects, synergistic or antagonistic effects, against HeLa cells.

Further investigations for the mechanisms of Phytoplex

on HeLa cells are needed including an alteration in cell cycle, an immunomodulatory effect and an activation of autophagic activity. Moreover, studies in animal model

should be conducted to assure the efficacy and safety so that Phytoplex would be the adjuvant anticancer therapeutic agent of cervical cancer in human in the future.

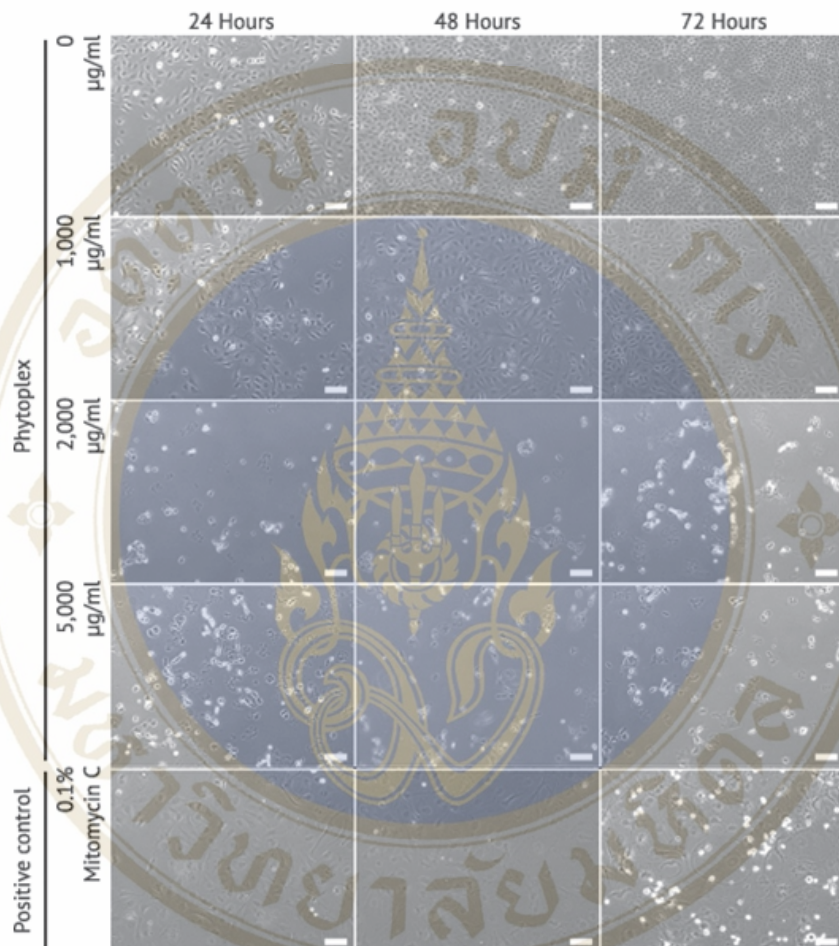


Figure 1. The morphology of HeLa cells treated with different concentrations of Phytoplex (1,000, 2,000 and 5,000 µg/ml) after 24, 48 and 72 h, compared to the negative control (0 µg/ml of Phytoplex) and the positive control (0.1% Mitomycin C). Magnification: 10x.

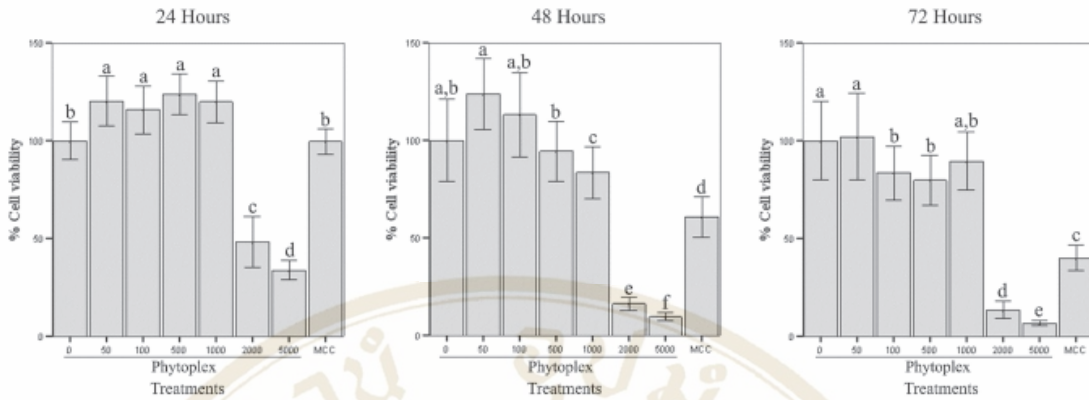


Figure 2. The percentage of cell viability after being treated with different concentrations of Phytoplex (1,000, 2,000 and 5,000 µg/ml) after 24, 48 and 72 h, compared to the negative control (0 µg/ml of Phytoplex) and the positive control (0.1% Mitomycin C). Different letters demonstrates statistical significance (P-value < 0.05, Dunnett's T3 test).

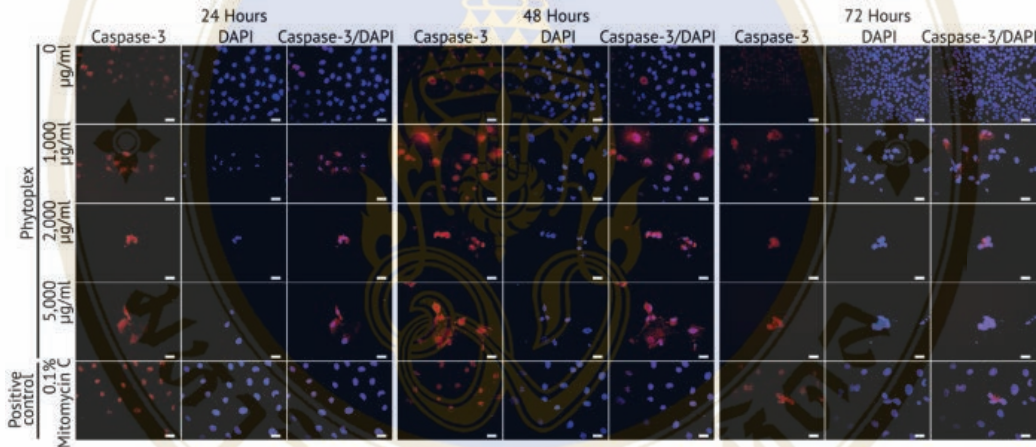


Figure 3. HeLa cells processed for immunofluorescence assay of caspase-3 antibody (red) and counterstained with DAPI (blue). Magnification: 40x.

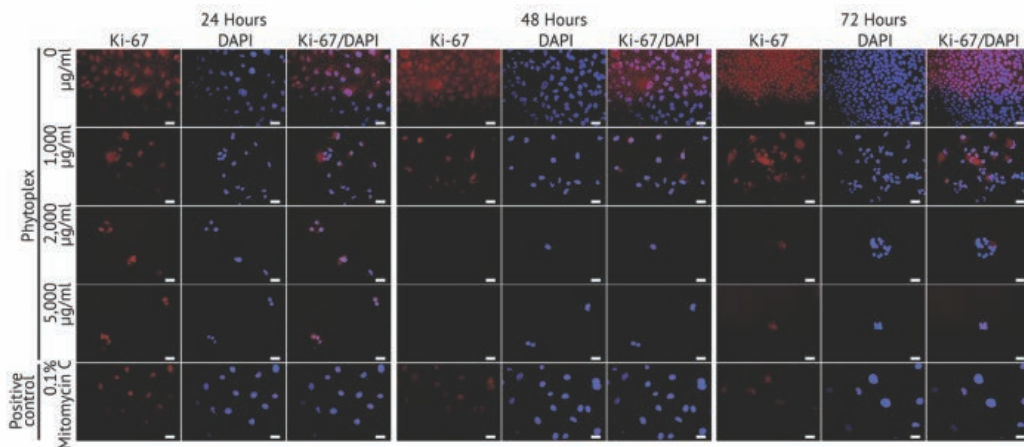


Figure 4. HeLa cells processed for immunofluorescence assay of Ki-67 antibody (red) and counterstained with DAPI (blue). Magnification: 40x.

Conclusion

Our study revealed anticancer properties of Phytoplex against HeLa cells. Phytoplex exhibits numerous anti-cancer effects including deformation of morphology, inhibition of cell growth, suppression of Ki-67 and induction of apoptosis via activation of caspase-3. Moreover, only high concentrations (2,000 and 5,000 µg/ml) of Phytoplex exerted growth inhibitory effect in HeLa cells. Hence, HeLa cell growth inhibition was a dose- and time-dependent manner.

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