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Cytotoxic effect of crude *Vitex glabrata* fruit extracts on human cancer cell lines

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ABSTRACT

Vitex glabrata (VG) or Khai-noa fruit extracts were prepared from three methods; squeezing extract (SE), maceration extract (ME), and ethanol extraction (EE) to evaluate their effects *in vitro*. By the (1,1-diphenyl-2-picrylhydrazyl) scavenging assay, all extracts revealed very weak antioxidant. Results of (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, a tetrazole) assay showed the increase in cell viability of SE at 100 and 500 μg/ml on TK6 cells after 24 and 48 h treatments ($P < 0.01$) without affecting Caco-2 cells. Conversely, a dose- and time-dependent cytotoxicity of all extracts revealed at 1000-2000 μg/ml on both cells ($P < 0.01$). SE showed cytotoxicity on PC-3 cells and anticancer activity when confirmed by clonogenic assay in a dose-dependent manner. SE also showed cytotoxicity on HT-29 cells after 24 h treatment but the cells could recurrent in the clonogenic assay. No effect was observed on MCF-7 cells after SE alone, SE co-treated with MMC treatment, and in the clonogenic assay. Moreover, HT-29 cells revealed a dose-dependent decrease in viability when treated with SE co-treated with MMC by 24 h. Hence, SE can selectively promote cell viability on TK6 and anticancer activity on PC-3. These results suggest that the different potential of SE depends on different cell lines.

Keywords: *Vitex glabrata*, fruit extract, cytotoxicity, cell proliferation, anticancer

INTRODUCTION

Vitex glabrata R. Br. (Verbenaceae, VG), commonly known as Khai-Nao, is a medicinal plant that can be found in some regions of Thailand. The ovoid to obovoid fresh succulent drupe fruits are edible when turn purple-black.[1] It has been used and believed to improve cognition, enhance memory,[2] bone maintaining, and promote the improvement of various symptoms and diseases.[3]

The phenolic compounds/flavonoids and their derivatives, containing in fruits have a wide range of biological activities including antioxidant and anticancer activities.[4-7] In general, the natural antioxidants have a capability of inactivation of reactive radical species or free radicals, which are associated with oxidative damage, causing many diseases such as inflammatory diseases, cardiovascular diseases, neurological diseases, cancer, and aging-associated diseases.[4] In Thailand, VG ripe fresh fruits collected from Kanchanaburi[8] and Ratchasima[9] contain the total phenolic compounds in the range of 335.33–408.93 mg GAE/L, respectively, depending on the extraction methods and determining using Folin–Ciocalteu assay.[8,9] The crude water extract of *Vitex agnus-castus* (VAC) fruits showed stronger antioxidant activity than those of the other extracts[5,10] and casticin, a flavonoids derivative, isolated from VAC fruits possessed a marked antioxidant activity compared with the control ascorbic acid.[6] The *Vitex* fruits extracts also showed an anticancer activity. The crude ethanolic extract of VAC fruits exhibited anticancer activity by inducing cell apoptosis and cell cycle inhibition on ovarian cancer (SKOV-3), gastric singlet ring carcinoma (KATO-III), colon carcinoma (COLO-201), and small cell lung carcinoma (Lu-134-A-H).[7] Furthermore, the ethanolic extract of *Vitis rotundifolia* fruits decreased cell growth of human colorectal cancer cells, HCT116 and SW480, by down-regulating the protein and mRNA levels of cyclin D1 and CDK4 which played

a role in cell cycle mechanisms.[11] Casticin isolated from some *Vitex* fruits induced apoptosis on many human cancer cell lines such as prostate cancer (PC-3), colon cancer (HT-29) and Caco-2, and breast cancer (MCF-7) through different molecular mechanisms.[12-14]

For VG, the methanolic extract from leaves showed an inhibitory effect on estrogen-dependent breast cancer, MCF-7 and T47D, cell proliferation.[15] Although few studies have reported that VG ripe fruit contains the phenolic compounds, there is no available information elsewhere both *in vitro* and *in vivo* to show the antioxidant activity or cytotoxic effect on both normal and cancer cell lines. However, antioxidants have been reported to cause mutagenesis and carcinogenesis in humans such as epigallocatechin gallate (EGCG), a most abundant flavonoid in green tea. EGCG is a strong antioxidant that promotes significant death by inducing DNA double-strand breaks and apoptosis in human lung and skin normal cell lines.[16] Therefore, in this study, we evaluate the antioxidant activity of three different VG fruit crude extracts by (1,1-diphenyl-2-picrylhydrazyl) (DPPH) radical scavenging assay. These extracts contain a mixture of phenolic compounds that may toxic to normal cells. Hence, we investigated the cytotoxic effect of the extracts on a normal cell using a normal human lymphoblast (TK6) and on human epithelial colorectal adenocarcinoma (Caco-2) cell lines. Although the Caco-2 is colon cancer cells, their morphology and function resemble the enterocytes lining the small intestine when they differentiate under the culture conditions.[17,18] The Caco-2 cell is wildly used as an intestinal *in vitro* model in toxicology studies to evaluate the cytotoxic effect of the oral administration chemicals and also used in pharmacology studies to evaluate the ability of chemicals to cross the intestinal barrier, as well as to study their transport mechanisms which correlate well with the human *in vivo* absorption.[17-19] The extracts and their concentrations which showed no toxic to TK6 and Caco-2 cells were chosen to carry out the cytotoxic/anticancer activity on three cancer cell lines; human colorectal cancer (HT-29), human breast cancer (MCF-7), and human prostate cancer (PC-3). Crude VG fruit extracts with potentially useful biological activities will be chosen for further detailed investigation through these *in vitro* screening methods.

MATERIALS AND METHODS

Fruits Extraction

Hand-picked VG fresh fruit from Rapee Sagarik Orchid Garden, Kasetsart University, Bangkhen was identified by Assoc. Prof. Saranya Watcharothai, Ph.D., Department of Botany, Faculty of Science, Kasetsart University, and the voucher specimen numbered BKs 01506 (Collector: S. Intarasam No.01) was deposited at the Bangkok herbarium, Bangkok, Thailand.

To obtain the extracts, the seedless cleaned fruits were extracted using three different methods. (1) squeezing extract (SE); the chapped flesh fruits were shaken in distilled water in the ratio of 1:1 (w/v) and squashed. (2) Maceration extract (ME); seedless fruits were dried at 45°C in a hot air oven. The ground fruit powder was soaked in distilled water at the ratio of 1:2 (w/v) for 24 h. To gain SE and ME, the juice of (1) and (2) was filtered with a pile of sterile gauze followed by Whatman filter paper (Whatman, Buckinghamshire, UK) to remove any debris. The filtrates were dried using lyophilization technique and stored at −80°C until used. (3) Ethanolic extraction (EE); the 500 g of fruit powder was extracted with 70% ethanol by the soxhlet apparatus. To gain the EE, the solvent was eliminated under low-pressure condition by a rotary evaporator. The percentage of each yield extract was calculated based on dry weight as the following;

$$\%\text{yield} = (\text{Actual mass}/\text{Predicted mass}) \times 100$$

When: Actual mass = Weight of the extract after lyophilization

Predicted mass = Weight of the VG fleshy fruits

Before carrying out the experiments, SE and ME were freshly dissolved in distilled water and the EE was dissolved in dimethyl sulfoxide (DMSO; Fisher Scientific, UK). Various concentrations of each extract were prepared from the stock solution as indicated by dissolving the extracts in the complete culture medium. The solution was filtrated using Whatman sterile endotoxin-free syringe filter (Whatman, Buckinghamshire, UK) before being used.

Antioxidant Activity Evaluation by DPPH Radical Scavenging Assay

The DPPH assay was modified from Shekhar and Anju (2014).[20] DPPH is a free radical which can accept an electron or hydrogen radical from the donors. The purple DPPH in methanol (Macron, USA) can be bleached to yellow when it accepts the hydrogen atom or electron from the antioxidants. Firstly, 100 μM solution of DPPH (Sigma, USA) was dissolved in methanol. Then, 10 μg/ml of each extract at the concentrations of 100, 500, 1000, 1500, and 2000 μg/ml were added to 190 μl of DPPH in a 96 well plate. The absorbance was measured at wavelength 515 nm (OD_{515}) by a microplate reader after 30 min of incubation at room temperature. Antioxidant activity was determined by calculating the percentage of reproduction of DPPH absorbance. The DPPH scavenging percentage was calculated as the following equation:

$$\%\ \text{DPPH radical scavenging} = ([OD_{515}\text{of control}] - [OD_{515}\ \text{of sample}]/OD_{515}\ \text{of control}) \times 100$$

Vitamin C (ascorbic acid, Sigma Aldrich, USA) was used as a positive antioxidant control at concentrations of 1, 2, 4, 8, and 16 μg/ml. The DPPH scavenging activity of the sample extracts and vitamin C was presented as half-maximum inhibition concentration (EC_{50}) calculated from % DPPH scavenging. The lowest value of EC_{50} means the highest antioxidant activity. The experiment was done in triplicate for standard and each extract.

Cell Culture and Maintaining

MCF-7 and PC-3 cell lines were a generous gift from Prof. Eric W.-F. Lam, Ph.D., Imperial College London. HT-29 cell line was a kind gift from Asst. Prof. Nattanan T-Thienprasert, Ph.D., Department of Biochemistry, Faculty of Science, Kasetsart University. The TK6 and Caco-2 were kindly given by Miss Prapaipat Klungsupya, Ph.D., Pharmaceuticals, and Natural Products Department, Thailand Institute of Scientific and

Technological Research (TISTR), Techno Polis, Pratum Thani, Thailand.

TK6 cells were cultured as a cell suspension in RPMI 1640 medium (Gibco-Invitrogen, CA). HT-29, Caco-2, MCF-7, and PC-3 cells were cultured in DMEM. Cells were cultured in specific medium supplemented with 10% fetal bovine serum (FBS, Gibco-Invitrogen, CA) and incubated at 37°C with 5% CO_2 in a humidified atmosphere.

Cytotoxicity Experiments of VG Fruit Extracts by (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, a tetrazole) Assay

MTT assay was wildly used to identify the cell viability and proliferation activity. MTT, a yellow solution when dissolved in PBS, is reduced to insoluble purple formazan crystal only by active mitochondrial reductase enzymes in live cells. This formazan crystal can be dissolved in DMSO (Fisher Scientific, UK) and measured the absorbance at 570 nm. MTT was freshly prepared by adding 500 μg/ml in PBS before use in the experiments. The MTT assay was done by following the method of Scudiero *et al*. (1988)[21] with some modification.

The cytotoxic effect of the three crude extracts was preliminarily screened on TK6 and Caco-2 cells to gain the appropriate activity of the extracts and the concentrations. Each cell line at a density of 1×10^4 cells/well was cultured at 37°C with 5% CO_2 for 24 h before treating with SE, ME or EE at final concentrations of 100, 500, 1000, 1500, and 2000 μg/ml. The control assay for the treatments of water extracts was set up using complete medium without any SE or ME. For the treatment of ethanolic extract, cells cultured in complete medium containing 0.3% DMSO without EE served as a control. This amount of DMSO was an equivalent amount to the treatment cells. The cells were further incubated at 37°C with 5% CO_2 for 24 and 48 h. After incubation, cells were washed twice with PBS, resuspended in fresh complete medium and 20 μl of MTT solution was added to each well. The plates were incubated at 37°C with 5% CO_2 for 2 h to allow the formazan crystal to form. To harvest, the supernatant was removed and 150 μl of DMSO was added. The absorbance was measured using a microplate reader. The absorbance of the controls represents 100% viability. The percentage of cell viability was then calculated. The 50% inhibition concentration (IC_{50}) values were calculated by the linear and logarithmic correlation equation.

SE was selected from its marked biological activity to conduct the cytotoxic activity on cancer cell lines. MCF-7, PC-3, and HT-29 cells at a density of 1×10^5 cells/well were seeded and cultured to allow cell attachment overnight before treating with SE at final concentrations of 0, 0.1, 1, 10, 50, and 100 μg/ml. The cells were further incubated at 37°C with 5% CO_2 for 24 h. The cell viability was determined using MTT assay. The percentage of cell viability and IC_{50} values were calculated as mention above. Each experiment and concentration was tested in four replications and the experiment was done in triplicate.

Activity of SE in Mitomycin C (MMC)-Induced Apoptosis Co-treatment

MMC (MMC; Merck, Singapore), a chemotherapy drug, was used as an apoptosis-inducing agent in cancer cells.[22] The cell density, culturing and maintaining procedures of MCF-7, PC-3, and HT-29 cells were the same as 2.4. However, the cells were treated with various SE concentrations combined with MMC for 24 h. The 15, 20, and 25 μg/ml of MMC was used to co-treat with SE for MCF-7, PC-3, and HT-29 cells, respectively. MTT assay was used to evaluate the percentage of cell viability. Each concentration was tested in four replications and the experiment was done in triplicate.

Cell death observation was done using the nucleic acid binding dye, acridine orange (AO), and propidium iodide (PI). AO can permeate both live and dead cells that emit green fluorescence. PI can enter only dead cells with poor membrane integrity and necrotic nucleated cells and generate red fluorescence. The widely used AO/PI double staining method was performed based on the protocol of Wang *et al*. (2005).[23] By the time of treatment, representative PC-3 cells were trypsinized (trypsin (Gibco BRL, USA; EDTA (Vivantis, USA) and stopped the reaction by adding the complete medium. The trypsinized cells were washed twice with PBS, then stained with an aliquot of 1 μl of 0.3 mg/ml AO/PI. After incubated the cells at room temperature for 10 min, the stained cell suspension (10 μl) was placed on a clean glass slide and covered with a coverslip. The cells were immediately observed under a fluorescence microscope (Olympus, Japan). Cells were photographed, viable, apoptotic, and necrotic cells were observed.

Activity of SE on Long-Term Cell Survival by Clonogenic Assay

The clonogenic assay was modified from Khongkow *et al*. (2016).[24] After the MCF-7, PC-3, and HT-29 cells were separately seeded into 6-well plates at a concentration of 500 cells/well and incubated at 37°C with 5% CO_2 for 24 h, cells were treated with the indicated SE concentrations and further incubated for 72 h before changing the medium. Then, the cells were maintained in a complete medium without SE for 11 days before harvesting. The medium was changed every 3 days. To harvest after removing the medium, cells were washed twice with PBS. At room temperature, cell colonies were fixed with 4% paraformaldehyde (Schartau Chemi S.A., Spain) for 15 min and were stained with 0.5% crystal violet solution (Merck, Germany) for 1 h. The excess dye was removed by immersing the plates in running tap water. To quantify, the dye was dissolved from the air-dried colonies using 33% acetic acid (Macron, USA), and the absorbance was measured at 592 nm using a microplate reader. In this study, the colorimetric analysis was used to determine cell survival instead of colony counting for calculating the plating efficiency. It is because the nature of some cancer cells forms small colonies that difficult to count make this method improper to apply in this assay.

Statistical Analysis

All data were reported as the mean ± standard error (SE) of triplicated measurement. All analyses were performed using One-way ANOVA by Duncan multiple Range Test.

RESULTS AND DISCUSSION

Yield of VG Fruit Crude Extracts

The extracts from different extraction methods showed distinct features. The EE was viscous dark-brown color with a 2.285% yield. The ME, after lyophilization was light-brown crystal with a 3.202% yield, and the SE was a fluffy brown crystal with a 3.856% yield.

Antioxidant Activity Evaluation by DPPH Radical Scavenging Assay

The percentage of DPPH radical scavenging of SE, ME, EE, and ascorbic acid significantly increased ($P < 0.05$) in a dose-dependent manner [Figure 1]. The EC_{50} values were 369.25 (y = 48.656x–74.908, R^2 = 0.9924), 585.46 (y = 44.434x–72.96, R^2 = 0.966), 627.30 (y = 52.392x–96.566, R^2 = 0.9879), and 6.74 (y = 5.3135x+ 14.187, R^2 = 0.8834) µg/ml for ME, SE, EE, and the positive antioxidant ascorbic acid, respectively. EC_{50} means the concentration of sample extract that can scavenge 50% DPPH, a free radical. According to Blois (1958),[25] the sample, which had an EC_{50} >150 µg/ml, was a weak antioxidant. It meant that ME was a very weak antioxidant and the antioxidant activity was reduced in the SE and EE, respectively. It can be concluded that the antioxidant potential of ascorbic acid was approximate 54-, 86-, and 93-fold potency of ME, SE, and EE, respectively. It may due to the mixture of many compounds in these crude extracts.

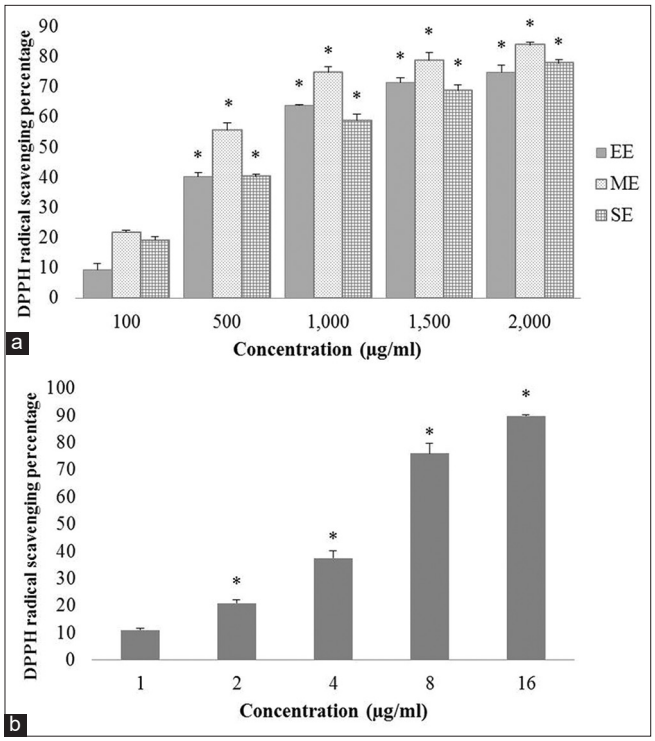

Figure 1: (1,1-Diphenyl-2-picrylhydrazyl) (DPPH) radical scavenging activity. (a) The percentages of DPPH scavenging of EE, ME, and SE show a dose-dependent increase. (b) The percentages of DPPH scavenging of positive antioxidant ascorbic acid also show a dose-dependent increase. The results represent the mean ± SE. *indicates significant difference at $P < 0.05$ SE = squeezing extract, ME = maceration extract, EE = ethanolic extract

This study in 100 µg/ml of the extracts, the compounds, which can act as antioxidants, have not achieved its effective concentration yet. Therefore, the antioxidant compounds consisting of each extract can scavenge DPPH at the lowest percentage, and % DPPH scavenging is increased at higher concentrations which are due to the increase in the effective concentration of antioxidant as shown in Figure 1. Moreover, it may due to the species of *Vitex* and the procedures of extraction. Rani and Sharma (2013)[26] concluded that the methanol extract and diterpenoid (ferruginol) isolated from *V. otundifolia* fruits exhibited antioxidant activity. The flavonoids and diterpenoids isolated from ethyl acetate extract and the caffeic and chlorogenic acids isolated from the mixture of *o*-phosphoric acid from VAC fruit were also included. For VG, the pharmacological component other than the total phenolic compounds[8] is in the processes of our study.

Cytotoxicity Experiments of VG Fruit Extracts by MTT Assay

After 24 h of treatment on TK6 cells, at 100, 500 and 1000 µg/ml, SE significantly ($P < 0.01$) exhibited the respective increase in % cell viability at 148.12 ± 3.62, 140.35 ± 2.68, and 111.05 ± 1.72, whereas at 1500 and 2000 µg/ml, it significantly ($P < 0.01$) exhibited the respective decrease in % cell viability at 72.83 ± 0.95 and 50.07 ± 0.59 when compared with the untreated control. By 48 h of treatment, only 100 and 500 µg/ml of SE significantly ($P < 0.01$) exhibited the respective increase in % cell viability at 138.59 ± 9.80 and 125.22 ± 3.75, whereas the decrease in cell viability was found at 1000–2000 µg/ml when compared with the untreated control. No significant of cell viability was found on TK6 cells treated with ME and EE, conversely, the decrease in cell viability was obviously seen on cells treated with ME and EE at a respective concentration of 1000–2000 and 500–2000 µg/ml at 24 and 48 h periods when compared with the untreated controls [Figure 2a and b]. Both the increase and decrease in cell viability showed as a dose- and time-dependent manner. The cytotoxicity of the extracts was evaluated using IC_{50} values by following the criteria of Ballantyne (1999).[27] The IC_{50} values at 24 and 48 h after treatment of SE, ME, and EE (at 24 h) were potentially nontoxic because they showed IC_{50} >1000 µg/ml. However, EE was potentially harmful at 48 h period because it showed 100 <IC_{50} <1000 µg/ml. Therefore, SE at the concentration of ≤100 µg/ml was used in the other experiments. The increase in cell viability of SE at a low concentration on TK6 cells correlated with the very recent study on casticin isolated from *Vitex trifolia* that could promote T- and B- lymphocyte proliferation in leukemia mice.[28]

However, when the concentrations of SE were increased, the consequent decreased in cell viability was observed. It might be from some compounds that reached the effective concentration of toxicity that caused to reduce cell viability.

No significant decrease in cell viability was found on Caco-2 cells treated with SE and ME at 100–1000 µg/ml at 24 h, but the decrease was found at the higher concentrations ($P < 0.01$) when compared with the untreated controls. At 48 h after treatment, SE still showed no significant decrease in cell viability at 100–500 µg/ml, and the obvious cell decrease revealed at higher concentrations ($P < 0.01$) when compared

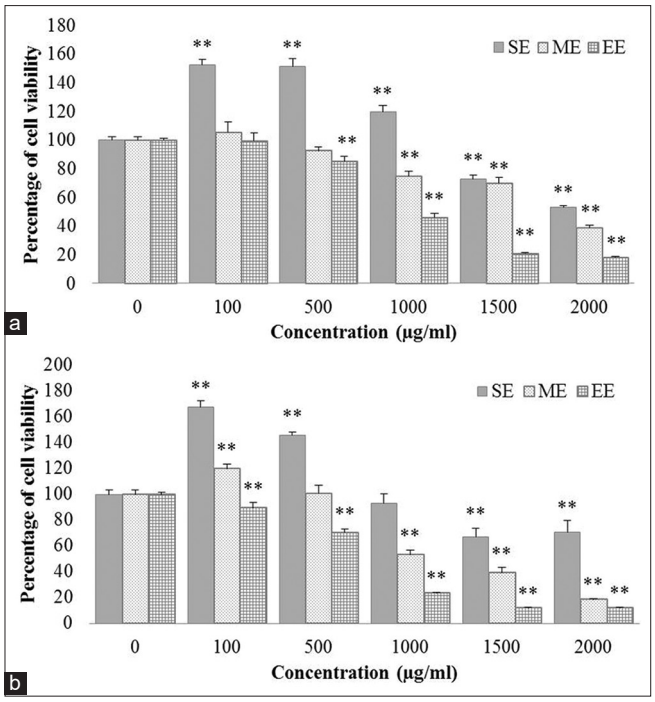

Figure 2: The effect of SE, ME, and EE on the viability of TK6 cell (a) after 24 h and (b) 48 h of treatment by MTT assay. Both the increase and decrease in cell populations showed in a dose- and time-dependent manner when compared with each untreated control. The results represent the mean ± SE. ** indicates significant difference at $P < 0.01$ SE = Squeezing extract, ME = Maceration extract, EE = Ethanolic extract

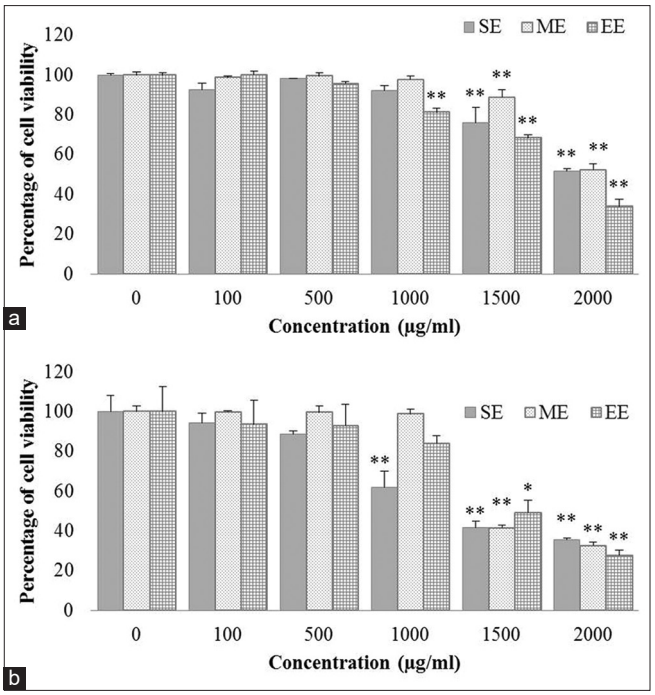

Figure 3: The effect of three *Vitex glabrata* fruit extracts on the viability of Caco-2 cell after 24 h (a) and 48 h (b) treatment by MTT assay. The results represent the mean ± SE. * indicates significant difference at $P < 0.05$ when compared with each untreated control, ** indicates significant difference at $P < 0.01$ when compared with each untreated control SE = Squeezing extract, ME = Maceration extract, EE = Ethanolic extract

with the untreated controls. No significant decrease in cell viability induced by ME at 100–1000 µg/ml was found, but the cell decrease was found at the higher concentrations ($P < 0.01$) when compared with the untreated controls. EE showed a decrease in cell viability at 1000–2000 µg/ml at 24 h and 1500–2000 µg/ml at 48 h periods ($P < 0.01$), but it showed no significant decrease in cell viability at lower concentrations when compared with the untreated controls. The decrease in cell viability was exhibited in a dose- and time-dependent manner [Figure 3]. Nevertheless, SE, ME, and EE were potentially non-toxic to Caco-2 cells because their IC_{50} values were >1000 µg/ml.[27,29]

Differentiate Caco-2 cells resemble the human intestinal epithelium, which was generally used in toxicology study to evaluate the cytotoxic effect of the oral administration compounds.[19] After treatment at 100–1000 µg/ml (24 h) and 100–500 µg/ml (48 h), no significant decrease in Caco-2 viability was found. Whereas, the decreased in cell viability was showed at the higher concentrations resemble the treatment on TK6 cells. Hence, SE at the concentrations of ≤100 µg/ml was chosen to investigate the cytotoxic activity on three different cancer cell lines, PC-3, HT-29, and MCF-7. Because at this concentration SE exhibited an increase in normal cell viability and no toxic to Caco-2 cells both at 24 and 48 h after treatment.

Activity of SE on cancer cell lines

Results of MTT assay exhibited the non-dose-dependent decrease in cell viability of PC-3 (79.61 ± 5.07–72.78 ± 4.89%, $P < 0.01$) followed by HT-29 (90.07 ± 0.72–84.11 ± 3.98%, $P < 0.05$) with respective concentrations, except at 100 µg/ml HT-29 showed $P < 0.01$ significance when compared with their SE untreated controls [Figure 4]. To determine SE-induced apoptosis, PC-3 cells were used to examine the morphological changes after 24 h of SE treatment at 100 µg/ml and analyzed by AO/PI double staining. The SE-treated PC-3 cells exhibited cell shrinkage, chromatin condensation and nuclear fragmentation [Figure 5b and c]. Most of the cells expressed early apoptosis with apoptotic bodies when compared with the SE untreated control [Figure 5a]. No effect was found on MCF-7 cells [Figure 4]. The respective IC_{50} values of SE on PC-3 and HT-29 cells were 241.35 ($y = -0.1393x + 83.62$, $R^2 = 0.2978$) and 473.26 ($y = -0.0881x + 91.694$, $R^2 = 0.3793$) mg/ml, respectively, which were potentially toxic according to the criteria of Ballantyne (1999).[27,29]

The previous study on crude VAC fruit extract was revealed the significant arrested PC-3 cells in the G0/G1 phase and S phase accumulation leading to a dose-dependent increase in apoptosis.[30] The VAC fruit extract also reduced the prostate cancer growth rate and the enzymes involved in prostate cancer induction such as 5-α reductase, Na^+/K^+ ATPase, prostate-specific antigen, and cyclooxygenase-2 in the prostate cancer-induced rats.[30] Casticin isolated from VAC fruits could inhibit the growth of prostate cancer cell lines, PC-3, BHP-1, and LNCaP by arresting cancer cells in the G2/M phase from the disruption of mitotic spindles and also through caspase pathway.[31,32]

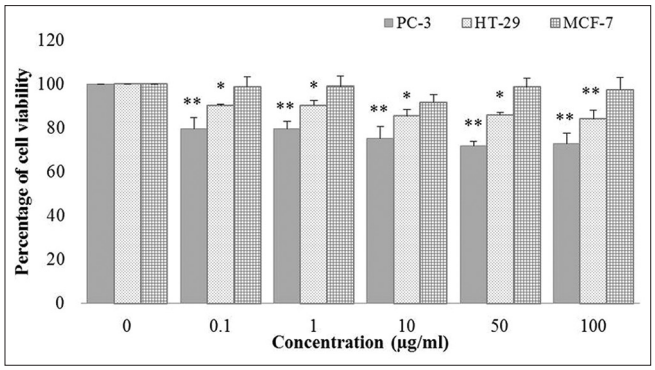

Figure 4: The effect of SE on the viability of PC-3, HT-29 and MCF-7 after 24 h of treatment by MTT assay. SE shows the decrease in viability of PC-3 followed by HT-29 cells and without any effect on MCF-7 cells. The results represent the mean ± SE.* indicates significant difference at $P < 0.05$ when compared with each SE untreated control, ** indicates significant difference at $P < 0.01$ when compared with each SE untreated control

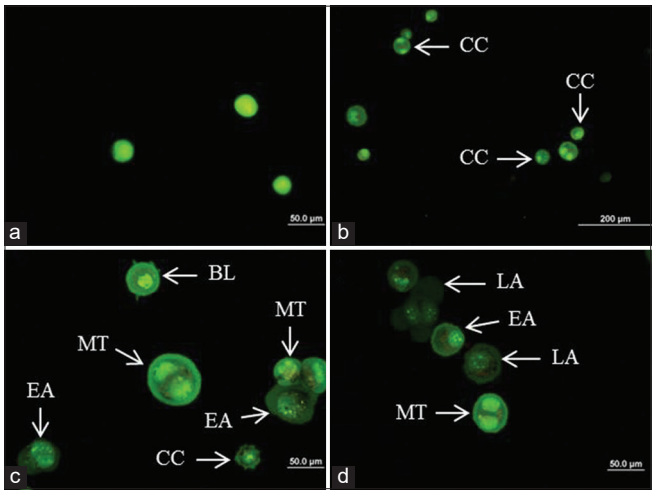

Figure 5: Fluorescent microscopic image of PC-3 apoptotic cells after 24 h treatment by AO/PI double staining. (a) untreated control (normal) cells (b-c) cells treated with SE 100 µg/ml (d) cells treated with MMC 20 µg/ml. CC = Chromatin condensation; BL=Blebbing; EA=Early apoptosis; MT=Mitotic division; LA=Late apoptosis

The induction of PC-3 cell apoptosis in this study may involve with the compounds in SE that influence on estrogen receptor (ER) and/or epidermal growth factor (EGF) leading to apoptosis. ER to which estrogen binds and induces cellular responses, is a nuclear protein complex that appears in the cytoplasm. The ER has 2 main classes, ERα and ERβ.[33] PC-3 cell was reported to have a higher ERβ level than ERα[33] but Mishra *et al*. (2015)[34] demonstrated that there was no expression of *ERα* in PC-3 cells. The ERβ was thought to function as prostate cancer growth inhibitor, which reduces prostate cancer by an apoptosis pathway,[35,36] and the VAC extract was reported to bind specifically to ERβ subtype using ligand-binding assay.[32] Whereas, the alcoholic extract of VAC fruit could decrease prostate cancer in the rat.[30] However, the mechanisms of ERβ in growth inhibition of prostate cancer remains unclear.[37] EGF is a ligand that plays a role in PC-3 cell survival and proliferation. When EGF was inhibited, PC-3 cells showed apoptotic cell death. It was found that quercetin, a plant flavonoid found in fruits and vegetables, induced PC-3 cell apoptosis by down-regulating EGF that induced Bcl-2 expression and upregulating the Bax protein levels with the increase in caspase-3 activity.[38]

HT-29 cells also present the ERβ protein but in low level and ERβ reduction is associated with the advanced stages of colon cancer or disappears in the dedifferentiated H-29 cells compared with the normal colon tissues.[37,39,40] Hence, he decrease in viability of HT-29 cells can expect in few different pathways of apoptosis. The flavonoids containing in the ethanolic extract of VAC fruits reduced the viability of HT-29, and also COLO 201 and COLO 320 human colon cancer cell lines by causing the DNA fragmentation.[41] Moreover, the anthocyanins were reported to induce apoptosis. Anthocyanins, the water-soluble pigments, are the largest subgroups of flavonoids that give red, blue, and purple color in fruits, flower, and vegetables.[42] The VG ripe fruits have deep purple in color, so the anthocyanins are included in the phenolic compounds. Anthocyanins extracted from wild berry fruits of *Lonicera edulis* was reported to inhibit HT-29 cell growth through an apoptosis after 24 h of exposure in a dose-dependent manner.[43] Moreover, delphinidine, a major anthocyanin, was reported to arrest PC-3 cell growth in G2/M phase and the apoptosis was mediated *in vitro* by the activation of caspase pathways in a dose-dependent manner. The significant decrease in p53 and Bcl-2 but increase in Bax protein levels was found. In an *in vivo* nude mice PC-3 xenograft model, this chemical showed the significant decrease in tumor dimension.[44]

Ohayama *et al*., (2003)[7] have reported previously that the alcoholic crude extracts of VAC fruits showed cytotoxic effect on MCF-7 cells by necrosis induction. The extract from *V. pseudo negundo* fruits had cytotoxic effect on MCF-7 cells. Furthermore, the casticin isolated from *V. trifolia* fruits induced apoptosis in MCF-7 cells by FOXO3a activation and deactivation of FoxM1.[14] VAC extract was reported to bind specifically to ERβ subtype using ligand-binding assay,[32] unfortunately, MCF-7 cells appeared ERα.[34] In this present study, SE did not affect the MCF-7 cells. SE is a crude water extract, so that the mixture of constituent influences on different cell strains and exhibits different responses depending on the effective concentrations. From this finding, we consider as a promise to continue our experiments in many aspects.

Activity of SE in MMC-induced apoptosis co-treatment

Nowadays, a trend to take herb extracts with standard treatment for cancer patients is increasing. Single herb extracts or in the form of food supplements are used to improve their patients' health and decrease the side effects of anticancer chemotherapy. This experiment aimed to investigate the interaction between SE and MMC in terms of cytotoxicity induction on cancer cells. Results of MTT assay showed that SE did not interact with MMC because the viability of PC-3 and MCF-7 cells was very slightly changed when compared with the controls of each cell line received MMC alone. Co-treatment of SE with MMC on HT-29 cells resulted in a significant decrease in dose-dependent cell viability, especially at 100 µg/ml (41.52 ± 1.45%, $P < 0.01$) when compared with its control received MMC alone [Figure 6].

Among all the cancer cells treated, HT-29 cells exhibited the most pronounced cytotoxic effect on the enhancement of MMC-induced apoptosis. The percentage of cell death was 30.85, which was higher than that of HT-29 treated with 100 µg/ml SE alone (15.90) in the 3.3.2 previous experiment. However, the enhanced MMC-induced cytotoxicity by SE in HT-29 cells did not investigate cell death characteristics. Thus, the cell death pathway by SE may be cell line dependent. It seems to be SE showed more effect of cytotoxicity when MMC was included. It remains to be determined which compound in SE is the active trigger for cytotoxicity in HT-29 cells and in which pathways when co-treated with MMC.

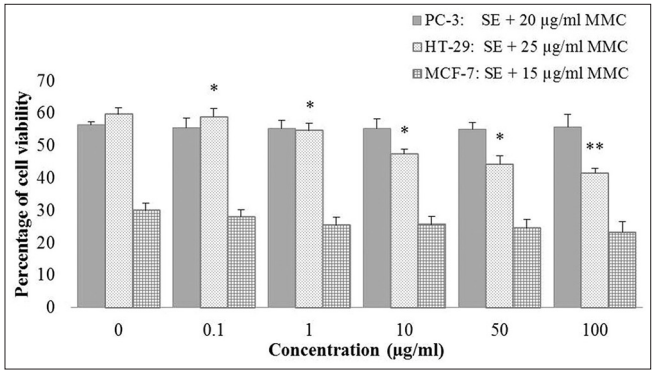

Figure 6: The co-treatment effects of SE and MMC-induced apoptosis on PC-3, HT-29 and MCF-7 cell lines after 24 h of treatment by MTT assay. Only HT-29 cells show a dose-dependent decrease in cell viability. The different MMC concentrations are the IC_{50} values obtaining from pre-treatment on each cell line. The results represent the mean ± SE. * indicates significant difference at $P < 0.05$ when compared with each control received MMC alone, ** indicates significant difference at $P < 0.01$ when compares with each control received MMC alone

Activity of SE on Long Term Cell Survival by Clonogenic Assay

The modified clonogenic assay was performed to confirm the potential of SE to suppress the growth of PC-3, HT-29, and MCF-7 cell lines. Cell survival based on the capacity of a single cell to grow into a colony. As shown in Figure 7, SE extensively reduced the colonization only of PC-3 cells. To avoid a human error of counting the clonogenic survival by plating efficiency assay of a plate that plenty of small colonies are formed, the colorimetric analysis of cell survival was performed. In accordance with the clonogenic plates, it was found that SE

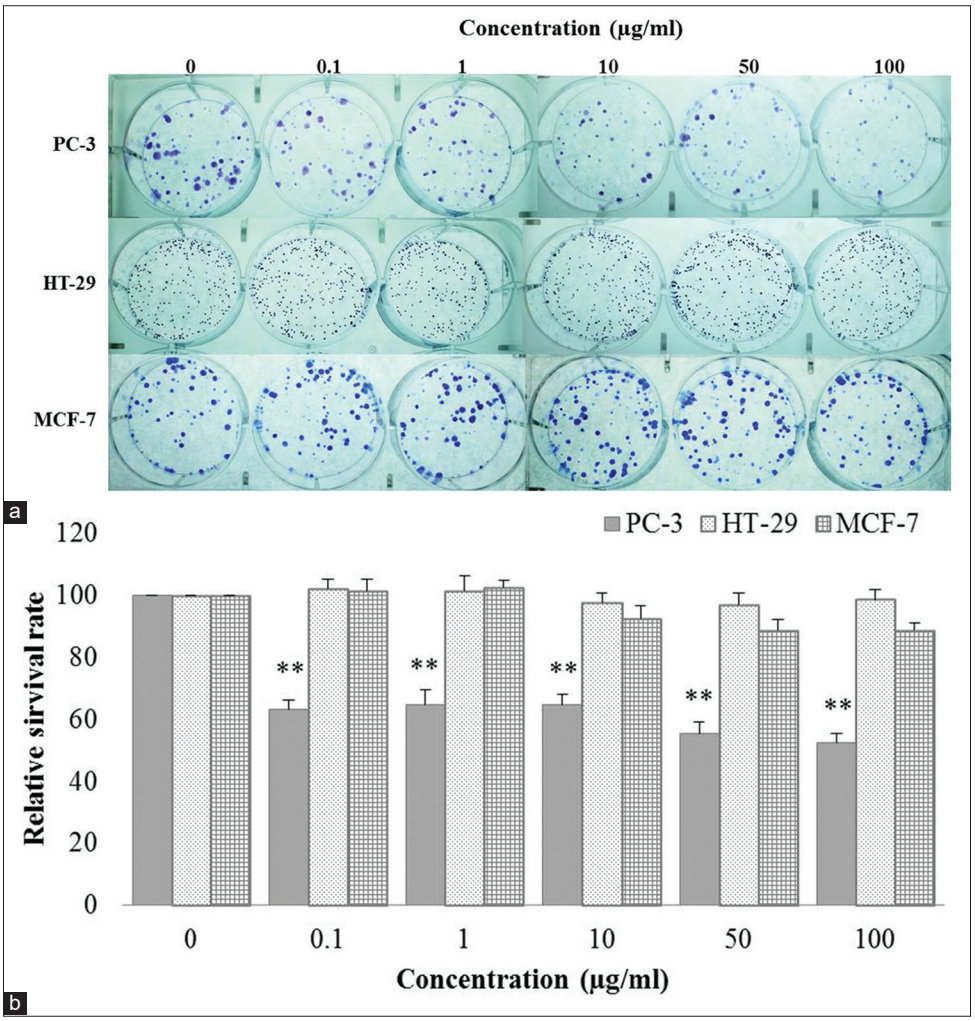

Figure 7: Long-term cell survival effect of squeezing extract on PC-3, HT-29, and MCF-7 cells by (a) clonogenic assay. PC-3 cells show the reduction of cell survival rate at all extract concentrations when compares with the SE untreated control. (b) Relative survival rates represent in the mean ± SE. ** indicates a significant difference at $P < 0.01$

successfully affected the self-renewing capacity only of PC-3 in a dose-dependent manner, especially at 100 μg/ml the relative survival rate was 52.24 ± 3.03% ($P < 0.01$)) when compared with the untreated controls [Figure 7]. No significant decrease in survival rate was found on MCF-7 cells in a dose-dependent manner, and SE showed no effect on HT-29 cells in long-term experiment when compared with their SE untreated controls [Figure 7]. These results suggested that the SE has anticancer activity against PC-3 cells. The clonogenic assay has been used to detect cells that have retained their capacity to produce a large number of self-renewing progeny after radiation and chemotherapy treatments. It also correlates tumorigenicity analysis *in vivo* and predicts the clinical response toward several agents in cancer patients.[45] Hence, the results in the clonogenic assay confirm the SE property in decreasing the viability of PC-3 cells and suggest that SE has selective potential anticancer activity on PC-3 cells, limiting PC-3 cell growth after treatment.

Taking all results together, SE is an interesting crude extract that has cytotoxicity on specific cancer cell lines, PC-3 and HT-29, and promotes viability of non-cancer TK6 cell line at lower concentrations after 24 and 48 h treatment. Since SE shows different properties on different cell types, identification of the active compound is necessary. Similar to casticin isolated from *Vitex* fruits, inhibited the growth of specific human cancer cell lines such as PC-3, HT-29, Caco-2, and MCF-7 through different molecular mechanisms.[11,15,17] However, casticin isolated from ethanolic extract of VAC fruits appeared no cytotoxicity on peripheral blood mononuclear cells isolated from healthy volunteers when treated with concentrations showing significant cytotoxicity.[46] Moreover, knowing the active compound or even the isolated of the active compound helps to control the amount of the compound from the variation among extraction batch in the further experiments. Anyhow, the isolated compounds from VG fruits may be developed to the anticancer drug against the specific cancer cell types as casticin.[46]

CONCLUSION

The results suggest that SE, ME, and EE were very weak antioxidant. SE, the SE extract of VG fruits, is reported for the 1st time to have highly significant potential in normal lymphoblast TK6 cell growth at 100–500 μg/ml over the control SE untreated group, without affecting the colon cancer Caco-2 cells, after 24 and 48 h treatment. Conversely, at 1000–2000 μg/ml, SE showed a dose- and time-dependent toxic to both TK6 and Caco-2 cells. Among cancer cell lines, SE revealed the highly significant toxic to prostate cancer PC-3 cell, which was confirmed by the clonogenic assay that SE had an anticancer activity selectively on PC-3 cells in a dose-dependent manner. SE showed a significant toxic to colon cancer HT-29 after 24 h treatment but HT-29 cells could recurrent and form colonies, which confirmed by the clonogenic assay. The viability of HT-29 cells was decreased more in the SE co-treated with MMC treatment than that of SE alone. SE showed no toxic to MCF-7 cells, which was confirmed by clonogenic assay. These findings suggest that SE has the potential to promote TK6 cell viability and has the anticancer activity selectively on PC-3 cells. It seems that the capacity of SE depends on the different cell lines.

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