



Cell viability enhancing and cellular protection activity of *Vitex glabrata* R.Br. crude fruits extract on TK6 and L929 cell lines

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ABSTRACT

Studying the plant-based traditional medicine is a starting point for the advancement of modern studies. *Vitex glabrata* (VG) or Khai-Noa is a medicinal plant in Thailand, which fruits have been used by Thai ancients to improve cognition and enhance memory. This study aimed to investigate the cytotoxic effects and the protective activity against hydrogen peroxide (H₂O₂)-induced cytotoxicity and mitomycin C (MMC)-induced apoptosis of water crude extract of VG fruit (VGCE) on human lymphoblast (TK6), and mouse fibroblast (L929) by MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay. Here, the water crude extract of VG fruit was used, which resemble the native usage. The results showed that the VGCE was not toxic to both cell lines and significantly ($P < 0.01$) promoted the increase in cell viability, especially at 1 µg/ml VGCE. The extract significantly ($P < 0.01$) exhibited dose-dependent protection on TK6 cells against H₂O₂ after 24 and 48 h treatments. The protection of TK6 and L929 cells from MMC induced apoptosis was significantly found at 1 µg/ml and 0.1 µg/ml VGCE ($P < 0.01$), respectively. These findings reveal the capacity of VGCE to promote cell viability and protect the cells from H₂O₂- and MMC-induced cell death.

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

INTRODUCTION

Recently, medicinal plants have become famous as their biological activities could prevent many diseases. Recently, the use of plant extracts to treat and/or prevent many degenerative diseases has been increased, including inflammatory diseases, cardiovascular diseases, neurological diseases, aging-associated diseases, and cancer. Several reports indicated that the phenolic compounds such as flavonoids, phenolic acid, tannins, and diterpenes from fruits have antioxidant and/or anti-genotoxic activities by scavenging free radicals from the cells and protecting the cells from oxidative damage-inducing DNA fragmentation.^[1-3] Antioxidants are the compounds that have the ability to neutralize free radicals, thus preventing free radicals from mediating oxidative damage in cells.^[4] Oxidative damage from free radicals associated with many degenerative diseases.^[5] Almost researches in fruits of *Vitex* species

revealed the potencies of phenolic compound/flavonoids, which were the main active compounds in antioxidant activity and anticancer.^[1] The crude water extract of *V. agnus-castus* (VAC) fruits showed strong antioxidant^[4,6] and anticancer activities by inducing cell apoptosis and cell cycle inhibition on ovarian cancer (SKOV-3), gastric singlet ring carcinoma (KATO-III), small cell lung carcinoma (Lu-134-A-H),^[7] and colon carcinoma (COLO-201).^[7,8] The growth of non-cancer cells, peripheral blood mononuclear cells (PBMNC) isolated from healthy volunteers and mixed lymphocytes (T- and B-lymphocytes excised from mice spleens) including mouse fibroblast (L929) was inhibited by the extracts of VAC fruits and *V. rotundifolia* isolate, respectively,^[9,10] and VAC fruit extract showed no cytotoxic effect on non-cancer fibroblast cell lines, HCF, and HE-21.^[7] Interestingly, these results suggest that the cytotoxicity of *Vitex* fruit extracts vary among different cell types, which may propose cytotoxicity in specific cancer cells and did not affect the specific non-cancer cells.^[11,12]

When the reactive oxygen species (ROS) such as superoxide ($O_2^{\cdot-}$), hydroxyl radical ($\cdot OH$), peroxy radical ($ROO^{\cdot}$), as well as non-radical species, hydrogen peroxide (H_2O_2) exceeds the antioxidant mechanism levels, it leads to oxidative damage. Hence, the protective mechanisms from plant extracts limit the risk of biomolecules, cells, and tissue damage, leading to degenerative diseases. An antioxidant mechanism of cells to eliminate ROS and H_2O_2 is an endogenous detoxifying defense, which occurs to preserve optimal cellular function.^[13,14] H_2O_2 , a weak oxidizing agent, can across the cell membrane rapidly and inactivate few enzymes directly by oxidation. H_2O_2 can react with Fe^{2+} , and/or Cu^{2+} ions to form hydroxyl radical, which induces cytotoxicity.^[14] *V. negundo* (VN) extracts have been reported to have antioxidant activity against H_2O_2 . The crude ethanolic extract^[13] and the bicyclic [2.2.1] hept-5-ene-2-carbonitrile^[15] from VN leaves and the crude methanolic extract from VN bark demonstrated H_2O_2 scavenging activity in a dose-dependent manner.^[14]

The presence of genotoxic compounds in the environment, food, and active ingredients in drugs can affect the DNA and, therefore, causes several diseases including cancer.^[5] Mitomycin C (MMC) is a genotoxicant used in the treatment of various cancers as the third-line chemotherapeutic agent. MMC forms covalent DNA intra- and inter-strand crosslinks causing DNA double-stranded breakage that prevents DNA replication and transcription leading to the induction of apoptosis.^[16] MMC has been widely used for genotoxicity studies as a positive mutagen agent. It acts as a reference agent for genotoxic study as mentioned by OECD, 1977.^[17] MMC has been reported in several studies that it generates ROS from the metabolizing mechanism in the cells and creating different types of DNA adducts.^[18,19] Moreover, MMC revealed the p53 gene alteration in the expression, which induces the apoptosis pathways of the cells.^[19]

VG (Verbenaceae), Thai name: Khai-Nao and Common name: Black currant tree; Smooth Chastetree, is an indigenous herbaceous plant that distributes in Bangladesh, Cambodia, India, Indonesia, Laos, Malaysia, Myanmar, Papua New Guinea, Singapore, Thailand, Vietnam, and in North America.^[20] The use of VG as folk medicine is limited in Indian and Thai medicine. Thai ancients have used the fruits on believing to improve cognition and enhance memory including the treatment of diabetes, bone maintaining, and neurotonic. It is also used for the treatment of fever, diarrhea, and as a tonic.^[21]

Resemble the other *Vitex* fruits, the ripe VG fresh fruits collected from Kanchanaburi^[22] and Nakhon Ratchasima,^[23] Thailand, also contained the total phenolic compounds.^[22,23] The dichloromethane and ethyl acetate extracts of VG wild fruits collected from Nakhon Ratchasima, Thailand, also presented a H_2O_2 scavenging activity.^[23] To date, there have been no available academic reports on the activities of this VG fruit extract and its relevant roles in medicine. Therefore, the objectives of the current study were to investigate the cytotoxic effect and the protective activity against H_2O_2 and MMC of VGCE on non-cancer cell lines, the human lymphoblast (TK6) and L929. The experiments were conducted on TK6 and L929 cell lines since the VGCE may toxic to normal cells as VAC fruit extract that showed a selective cytotoxicity against cell types.^[7,11,12] Investigating, the protective activity

of VGCE on cell lines was based on the total phenolic compound containing^[22,23] that presented a H_2O_2 scavenging activity.^[23] The knowledge obtained from the study of plant-based traditional medicine serves as a starting point for the advancement of the insight studies.

MATERIALS AND METHODS

Plants Material

VG tree, 10–25 m high with tri-foliolate leaves, was at the Rapee Sagarik Orchid Garden, Kasetsart University, Bangkok, Thailand. The fresh drupes were ovoid with 2–2.5 cm long that turns purple-black when they were ripen. The botanical identification was made by Assoc. Prof. Saranya Watcharothai, Ph.D., Department of Botany, Faculty of Science, Kasetsart University. The fruit specimen was deposited at the Bangkok herbarium, Bangkok, Thailand, with voucher specimens numbered as BKs 01506 (Collector: S. Intarasam No.01).

Preparation of VG Fruit Crude Extract

Thai ancients used VG fruits as crude material for treating various disease conditions,^[21] so the crude extract was prepared by chapping the ripe VG fruits after being washed with distilled water. The chapped fruits were quickly shaken with distilled water (1:1 w/v), then squashed the flesh and, respectively, filtrated the suspension with a pile of sterile gauze and Whatman NO.1 filter paper (Whatman, UK). The suspension was lyophilized using a freeze dryer (Scanvac, Labogene, Denmark). The % yield of the extract was calculated based on dry weight as follows:

$$\% \text{ yield} = [\text{Actual mass} / \text{Predicted mass}] \times 100$$

When: Actual mass = Weight of the extract after lyophilization

Predicted mass = Weight of the fresh VG fruits

Before the treatments, concentrated stock solutions of VG fresh fruit crude extract (VGCE) were freshly prepared by dissolving the VGCE crystal in distilled water and, respectively, filtered with 0.45 and 0.2 μm sterile endotoxin-free syringe filter (Whatman, UK). The stock solution was diluted to the indicated concentrations using the cell culture complete medium.

Thin-Layer Chromatography (TLC) Analysis of the Extract

The method was modified from Sabine (1996).^[24] The VGCE was analyzed by TLC on pre-coated silica gel 60 GF₂₅₄ plate (Merck, USA) using butanol:acetic acid:water (4:1:1 v/v) as a solvent system. The completely dried plates were detected under UV 254 and 366 nm to classify the groups of phytochemical constitute, including flavonoids, terpenoids, and steroids. To detect flavonoids, the plate was sprayed with natural products-polyethylene glycol reagent (NP/PEG) (Sigma-Aldrich, USA). The green fluorescence was produced under UV 366 nm. To detect terpenoids and steroids, the plate was sprayed with anisaldehyde sulfuric acid reagent (Sigma-Aldrich, USA) and heated at 100°C for 5 min. The purple spot

was observed under UV 366 nm. The distance of each spot was calculated for the relative front (Rf) value to defined the ratio of the distance moved by the solute as the following equation:

$$\text{Relative front (Rf)} = \frac{\text{Distance from baseline traveled by solute}}{\text{Distance from baseline traveled by solvent(solvent front)}}$$

Cell Culture and Maintaining

Two normal cell lines, human lymphoblast (TK6) and mouse fibroblast (L929) cells were kindly given from Miss Prapaipat Klungsupya, Ph.D., Pharmaceuticals, and Natural Products Department, Thailand Institute of Scientific and Technological Research (TISTR), Techno Polis, Pratum Thani, Thailand.

The TK6 cell line was cultured in RPMI 1640 medium and L929 cell line was cultured in DMEM. 10% FBS was added as a supplement and incubated at 37°C with 5% CO₂. All cell culture chemicals were purchased from Gibco-Invitrogen, Carlsbad, CA.

Cytotoxicity of VGCE by MTT Assay

TK6 and L929 cells were separately seeded at a respective density of 2×10^4 and 2×10^3 cells/well into a sterile 96-well plate and incubated at 37°C for 24 h. Then, cells were treated with final VGCE concentrations at 0, 0.1, 1, 10, 50, and 100 µg/ml and further incubated for 24 h. After treatment, RPMI 1640 was removed by centrifugation at 1200 rpm for 10 min, while the DMEM was removed by pipetting out and the cells were washed twice with phosphate-buffered saline (PBS). The 100 µl fresh complete medium was added to each well followed by 10 µl of 5 mg/ml MTT solution and further incubated under the protection from any light at 37°C with 5% CO₂ for 2 h to allow the reduction of MTT by mitochondrial enzyme and formed the formazan crystal.^[25] To measure the absorbance, formazan crystal was dissolved in 150 µl DMSO (Fisher Scientific, UK) and the solution was measured at 570 nm using a microplate reader (Biotek, Powerwave XS, Belgium). The absorbance of the untreated control represented 100% cell viability. Every experiment was done in triplicate. The percentage of cell viability was calculated from the following equation:

$$\% \text{ cell viability} = \left[\frac{\text{Absorbance of Treatment} - \text{Absorbance of Blank}}{\text{Absorbance of Control} - \text{Absorbance of Blank}} \right] \times 100$$

Protective activity of VGCE Against H₂O₂-induced Cytotoxicity

TK6 cells were seeded at a density of 2×10^4 cells/well in 96 well plates and incubated at 37°C with 5% CO₂ for 24 h. Then, the cells were treated with VGCE at 0, 1, 10, 50, 100, and 200 µg/ml and further incubated for 24 and 48 h. To harvest, the cells were centrifuged, washed twice with PBS and resuspended in cold complete medium. To prepare H₂O₂ solution, the H₂O₂ stock solution was freshly diluted in cell

culture medium and rapidly added to the wells at the final concentration of 50 µM H₂O₂, and then incubated at 4°C for 5 min.^[26,27] The cells were washed twice with cold PBS and resuspended in fresh complete medium before cell viability examination by MTT assay. The experiment was done in triplicate.

Cell Viability Experiment of VGCE Against MMC-induced cytotoxicity

TK6 and L929 cells were separately seeded in the sterile 96-well plates at respective densities of 2×10^4 and 2×10^3 cells/well and incubated at 37°C for 24 h. Then, cells were treated with various final VGCE concentrations at 0, 0.1, 1, 10, 50, and 100 µg/ml combination with mitomycin C (MMC; Merck, Singapore) at the IC₅₀ values of each cell line as followed 10 µg/ml for TK6 and 15 µg/ml for L929 cells (data not shown). The treated cells were further incubated for 24 h. After that, the cell viability was examined by MTT assay. Every experiment was done in triplicate.

Apoptosis Analysis by Hoechst Dye Staining

TK-6 cell line was a representative cell for apoptosis analysis by Hoechst dye staining assay modified from Zou et al. (2008).^[28] The cells were treated following the same condition as the VGCE against MMC-induced cytotoxicity assay. Then, 18 h before reaching the incubation time, the treated cells were added 15 µl of cytochalasin B (Sigma, St Louis) to inhibit cytokinesis. After completion of the incubation time at 24 h, cells were washed twice with PBS and then 5 µl of 0.5 mg/ml Hoechst 33342 (Thermo Fisher Scientific, USA) staining solution diluted with PBS was added and further incubated at 37°C with 5% CO₂ for 10 min. To harvest, the cells were washed with PBS, the cold Canoy's fixative (3 ml methanol, Macron, USA and 1 ml glacial acetic acid, Qrec, New Zealand) was gently added and mixed with the cell pellets. The fixation process was done twice to fully preserve the cells. After that, cell pellets were washed with PBS. The stained TK6 cells were observed under the fluorescence microscope (Olympus, Japan) equipped with BX51-DP 70 controller program in picture capture. A total of 700 cells of each concentration was differentiated the morphological changes and the observation was done in triplicate.

Long-Term Cell Survival by Clonogenic Assay

The clonogenic assay was modified from Khongkow et al. (2016).^[29] To avoid over population, a density of 20 cells/well of L929 cells was seeded in the 6-well plates at 37°C for 24 h. On the next day, cells were treated with VGCE at 0, 0.1, 1, 10, 50, and 100 µg/ml and further incubated for 3 days following the recommendation of Riddell et al. (1986).^[30] After that, fresh complete medium free of VGCE was replaced and the cells were allowed to grow for another 11 days. The complete medium was changed every 3 day. After 11 days of culture, the PBS washed cells were fixed with 4% paraformaldehyde (Schartau Chemi S.A., Spain) for 15 min and then were stained with 0.5% crystal violet solution for 1 h at room temperature. The excess dye was gently removed by immersing the plates

in running tap water. After air dry, to quantify the colonies, the stained dye was dissolved using 1 ml of 33% acetic acid and shaken for 1 h at room temperature. The absorbance of 100 µl of dye solution was measured at 592 nm using a microplate reader.^[29] The experiment was done in triplicate. The percentage of relative survival rate was calculated from the following equation:

$$\% \text{ Relative survival rate} = \left[\frac{\text{Intensity of Treated sample}}{\text{Intensity of Control}} \right] \times 100$$

Statistical Analysis

All data were reported as mean $\pm$ standard error (SE) of the triplicated measurement. The statistical significance was determined using one-way analysis of variance (ANOVA) with Duncan's multiple-range *post hoc* test.

RESULTS AND DISCUSSIONS

Phytochemical Analysis of VGCE by TLC

In this study, TLC profile was performed to investigate VGCE nature by analyzing TLC chromatogram. Here, VGCE showed chromatographic fingerprints with dark quenching and fluorescent chromatographic bands under the detection of UV 254 and 366 nm, respectively [Figure 1], suggesting the presence of chromatophores. After spraying with anisaldehyde sulfuric acid reagent [Figure 1e], a purple fluorescent band appeared under UV 366 nm at Rf 0.21, which suggested the presence of terpenoids and steroids. A blue-green fluorescent band at Rf 0.42 appeared after NP/PEG reagent spraying, which suggested the presence of phenolics/flavonoids.

The fruit extracts of many *Vitex* species mainly composed of flavonoids and terpenoids. Six flavonoids^[31] and casticin^[32] isolated from *V. trifolia* fruit extracts showed cell cycle inhibition for mammalian cancer cells growth. Diterpenoids

and flavonoids,^[33] including total phenolic compounds and total flavonoids,^[4,34] were found in VAC fruit extracts, which showed antioxidant and anticancer activities. Moreover, the findings from this current study correlated with the findings of the total phenolic compounds from VG fruits collected from Kanjanaburi,^[22] and Nakhon Ratchasima,^[23] Thailand, that composed of total phenolic compounds.

Cytotoxicity of VGCE by MTT Assay

The VGCE gave a yield of 3.0154 % (w/w) with the fluffy brown crystal. Results of MTT assay exhibited an increase in cell viability of TK6 and L929 cells at all concentrations compared with the untreated control. Figure 2 significantly showed the highest cell viability exhibited at 1 µg/ml, in which L929 cells showed $142.89 \pm 4.20\%$, followed by $133.71 \pm 6.50\%$ of the TK6 cells ($P < 0.01$).

Determination of cell viability or cytotoxic activity generally used to evaluate the activity of the extracts to reveal their biological activities. The results from this study were similar to the property of ethanolic crude extract of VAC fruits at 5 µg/ml that showed the greatest influence on HE-21 human embryo fibroblast proliferation.^[7,35] The capacity of VGCE to promote cell viability might be regarding flavonoids containing in the water extract that could easily be released by water.^[22,36] Recently, it was found that casticin a polymethoxyflavone isolated from *V. trifolia* could promote T-cell and B-cell proliferation in leukemia mice.^[37]

Protection Property of VGCE Against H₂O₂-induced Cytotoxicity

The protection activity of VGCE in protecting TK6 cells from H₂O₂-induced cytotoxicity by generating ROS, causing oxidative stress was investigated. In Figure 3, comparing the groups received H₂O₂ alone with the control groups (0 µg/ml of VGCE) at 24 and 48 h after treatments, the result showed that H₂O₂ was toxic to the cells in a time-dependent manner. Figure 3 also showed the increase in cell viability of TK6 at all VGCE concentrations when compared with the groups received H₂O₂ alone after 24 and 48 h treatment. The highest % cell viability was still at 1 µg/ml with respective 80.78 ± 1.69 and $75.64 \pm 1.13\%$ after 24 and 48 h of VGCE treatments, significantly ($P < 0.01$). From the capacity of H₂O₂ that could enter the cell membrane rapidly and induce cytotoxicity,^[14] without the help of VGCE, TK6 cell viability of the groups received H₂O₂ alone decreased at both periods of the treatment. Thus, VGCE exhibited the protection property that could help protect the TK6 cells from H₂O₂ toxicity. Besides, in this present study, we also investigated the repair property of VGCE against H₂O₂-induced cytotoxicity by treating TK6 cells with 50 µM H₂O₂ for 5 min before VGCE treatment. The results of MTT assay exhibited that VGCE did not recruit the TK6 cell viability when compared with the untreated controls and the groups treated with H₂O₂ alone at 24 and 48 h after treatment (data not shown). From these results, in consequence, VGCE possesses a dose- and time-dependent protection activity against H₂O₂-induced cytotoxicity.

Several extracts derived from the *Vitex* species, which compose of flavonoids, have the antioxidant activity that helps

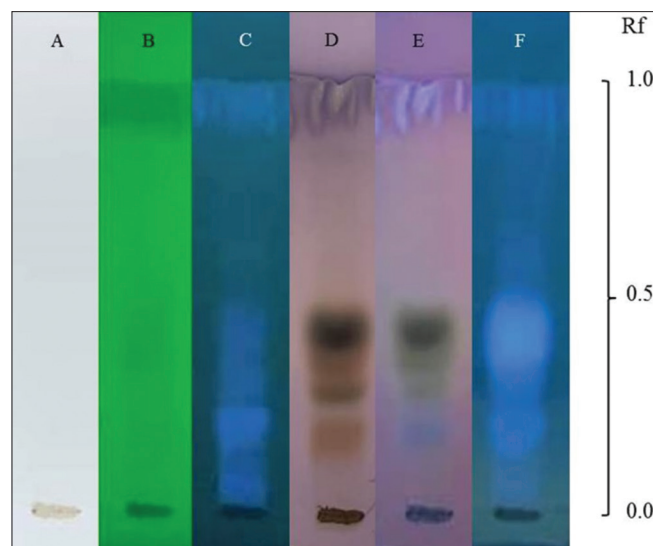


Figure 1: TLC fingerprints of SE; stationary phase: silica gel 60 GF₂₅₄, solvent system:butanol:acetic acid:water (4:1:1 v/v/v). (A) white light (B) UV 254 nm (C) UV 366 nm (D) white light after spraying with anisaldehyde sulfuric acid reagent (E) UV 366 nm after spraying with anisaldehyde sulfuric acid reagent (F) UV 366 nm after spraying with NP/PEG reagent

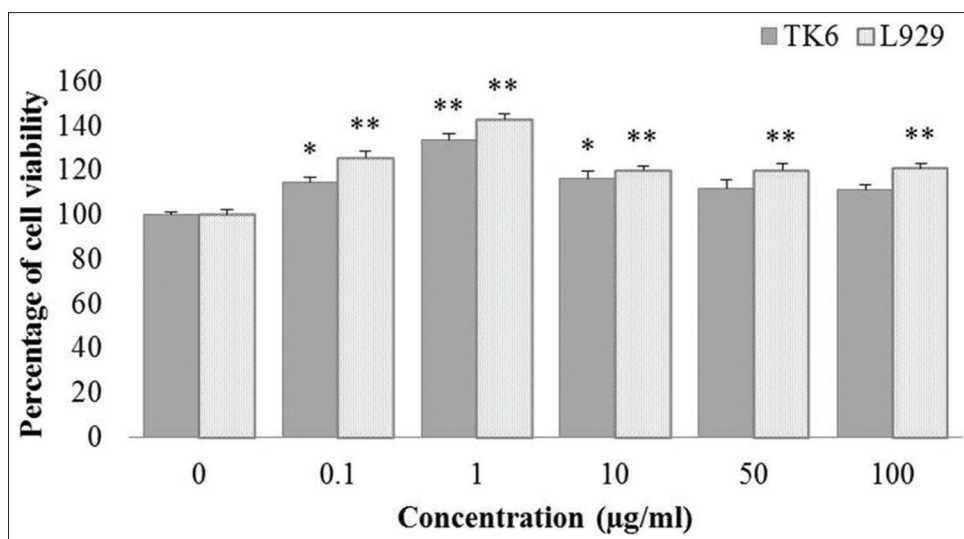


Figure 2: Cell viability of VGCE on TK6 and L929 cells after 24 h treatment by MTT assay. The extract significantly shows dose-dependent cell viability compared with the untreated controls (0 µg/ml VGCE). The results represent the mean $\pm$ SE. *Indicates significant difference at $P < 0.05$, **Indicates significant difference at $P < 0.01$

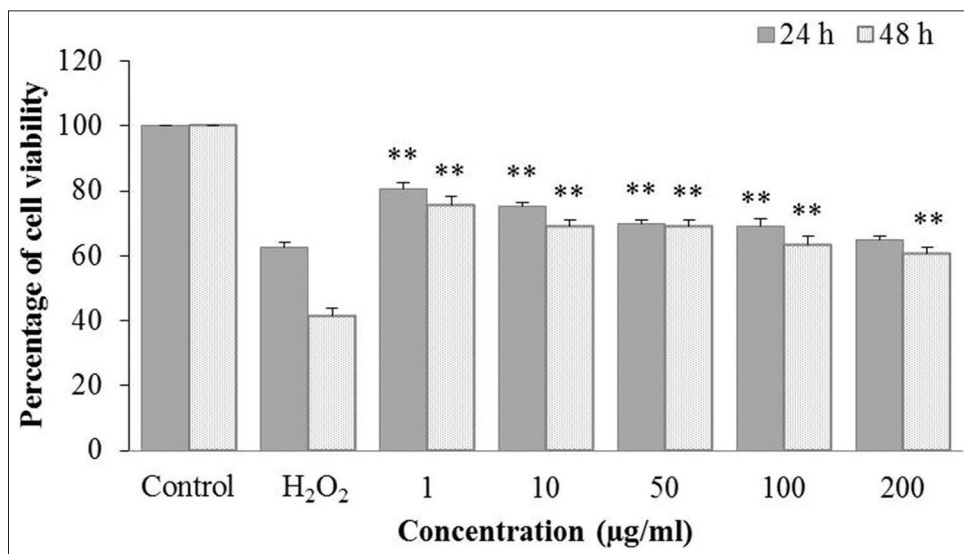


Figure 3: The protective effect of VGCE on 50 µM H₂O₂-induced TK6 cytotoxicity after 24 and 48 h of treatment by MTT assay. Cells of the control groups (0 µg/ml VGCE) are normalized and expressed as 100% viability. At 1 µg/ml of VGCE, cell viability significantly increases ($P < 0.01$) around 20 and 35% when compares with the cells received H₂O₂ alone at 24 and 48 h after treatment, respectively. VGCE reveals a time-dependent increase in TK6 cell viability when compares with the cells received H₂O₂ alone. The results represent the mean $\pm$ SE. **Indicates a significant difference at $P < 0.01$

to prevent oxidative stress.^[38] Diterpenoids and flavonoids isolated from VAC fruit exhibited antioxidant activity on a standard rat brain homogenate.^[39] Negundoside isolated from *V. negundo* showed the hepatoprotection activity against CCl₄-induced toxicity and oxidative stress.^[40] Besides, the crude methanolic extract of *V. doniana* fruit showed the H₂O₂ scavenging activity in the hepatotoxicity induced by acetaminophen.^[40] Islaih *et al.* (2005)^[41] concluded the target genes of TK6 cells involving in the *p53*, *TNF*, *ERK*, or *JNK* pathways after H₂O₂-induced cytotoxicity. These target genes induced cell cycle regulation, DNA repair and apoptosis, cell survival, and cell proliferation. The protective activity of VGCE against H₂O₂ resembled the dichloromethane and ethyl acetate

extracts of VG wild fruits collected from Nakhon Ratchasima, Thailand, that presented the H₂O₂ scavenging activity.^[23]

Cell Viability Experiment of VGCE Against MMC-induced Cytotoxicity

The current study was to evaluate the protective activity of VGCE against MMC-induced apoptosis using the MTT assay. The TK6 and L929 cells were cotreated with various VGCE concentrations after pre-culture at 37°C with 5% CO₂ for 24 h. In Figure 4, TK6 and L929 cell lines received MMC alone exhibited the decrease in cell viability 52.08 ± 4.41 and $56.15 \pm 3.01\%$, respectively, when compared with the

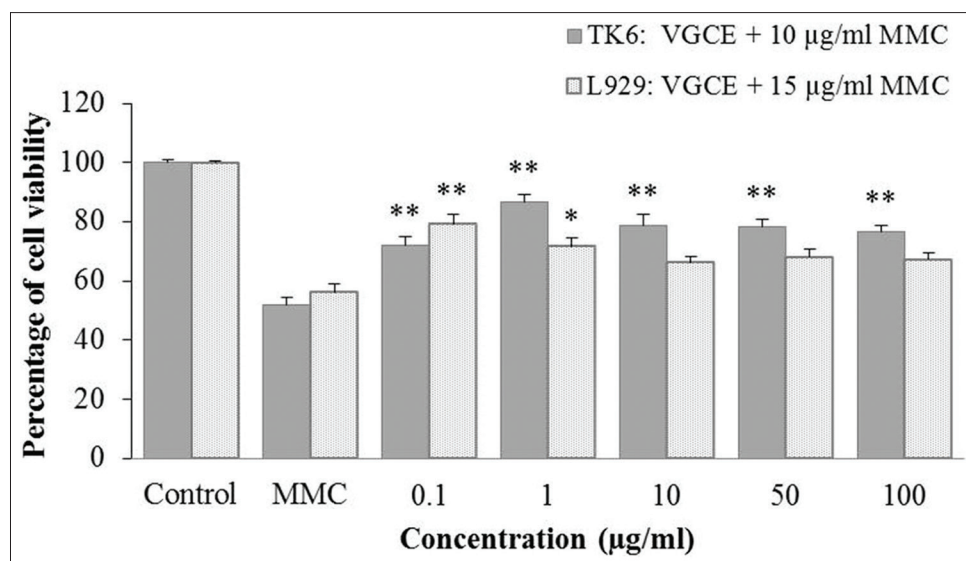


Figure 4: The percentage of cell viability of TK6 and L929 cell lines cotreatment with various concentrations of VGCE and the respective 10 and 15 µg/ml MMC by MTT assay at 24 h after treatment. Cells of the control groups (0 µg/ml VGCE) are normalized and expressed as 100% viability. Cell survival of TK6 cotreated between VGCE and MMC significantly increased at all concentrations of VGCE when compares with the cells received MMC alone. The highest cell viability at 1 µg/ml of VGCE combines with MMC, TK6 cell viability significantly increases ($P < 0.01$) around 34% when compares with the cells received MMC alone. For L929 cells, at 0.1 µg/ml followed by 1 µg/ml of VGCE combination with MMC, reveal the significant cell survival around 23 and 10%, respectively, when compares with the cells received MMC alone. The results represent the mean $\pm$ SE. * Indicates significant difference at $P < 0.05$ and ** indicates a significant difference at $P < 0.01$ when compares with the group received MMC alone

untreated control groups (without neither VGCE nor MMC). All VGCE concentrations exhibited a dose-dependent increase in cell viability against MMC-induced apoptosis. For TK6 cells, the highest viability revealed at 1 µg/ml ($85.64 \pm 2.52\%$), whereas for L929 cells revealed at 0.1 µg/ml ($79.25 \pm 6.16\%$) with a significant difference at $P < 0.01$. The results of MTT assay revealed the protective activity of VGCE against MMC-induced DNA damage leading to apoptosis.

Reduction of MMC-induced Apoptotic Rate in VGCE Treated TK6 cells

To evaluate the property of VGCE in reduce the apoptotic rate in the cells treated with MMC genotoxicity agent, apoptosis analysis was performed in MMC/VGCE cotreated TK6 cells using Hoechst 33342 staining. The cotreatment of VGCE with 10 µg/ml MMC revealed the significantly decreasing ($P < 0.01$) in percentages of apoptotic cells when compared with cells treated with MMC alone [Figure 5]. The lowest percentage of cell apoptosis was at 1 µg/ml VGCE cotreated with MMC. The result obtained from this apoptosis analysis could emphasize the activity of VGCE in protection activity against MMC-induced apoptosis.

MMC has been reported in several studies that it generates ROS from metabolizing mechanism in the cells^[18,19] and causes the alteration in p53 gene expression, which induces the apoptosis pathways of the cells.^[19] TK6 cells exposed to MMC expressed four DNA repair genes, two cell cycle regulation genes, four genes involved in apoptosis, and two genes important in the antioxidant defense. The downregulation of three cell cycle-regulate genes (*Cyclin A2*, *Cyclin B1*, and *CDC20*) was included. The TK6 cells collected at the 24 h after MMC exposure showed a strong G2/M

arrest.^[41] In this current study, it found that all concentrations of VGCE could decrease the % cell apoptosis, especially at 1 µg/ml. On the other hand, VGCE could increase the cell viability of both TK6 and L929 cell lines. Hence, the VGCE showed the protective activity against MMC-induced apoptosis possibly through the ROS elimination. MMC also showed the decrease in cell viability of L929. As well as, the normal human dermal fibroblast exposed to 40 µg/ml MMC showed the cell proliferation inhibition after 4 h of induction leading to apoptosis with the marked increase in mRNA production of basic fibroblast growth factor (*bFGF*).^[42] The bFGF is a small polypeptide that controls cell proliferation, cell survival, differentiation, and migration in almost every cell, especially fibroblasts.^[43]

In the current study, the controls TK6 and L929 cells received MMC alone may arrest in the G2/M phase for the natural defense, or preparing for apoptosis. For the increase in cell viability of the treated TK6 and L929 cells, there might be another mechanism behind the G2/M arrest that VGCE may induce cell viability in the presence or absence of p53 initiation.^[16] For L929, EGF receptors and bFGF are important in fibroblast growth induction. Hence, VGCE is expected to help promote lymphocyte viability to enhance the immune system in chemotherapeutic patients similar to the previous report that the mushroom compounds could stimulate the immune system, and rejuvenating the immune system after radiotherapy and chemotherapy in cancer treatment.^[44]

Long-Term Cell Survival by Clonogenic Assay

This cytotoxicity experiment was conducted on L929 cells to ensure the long term exposure of various VGCE concentrations

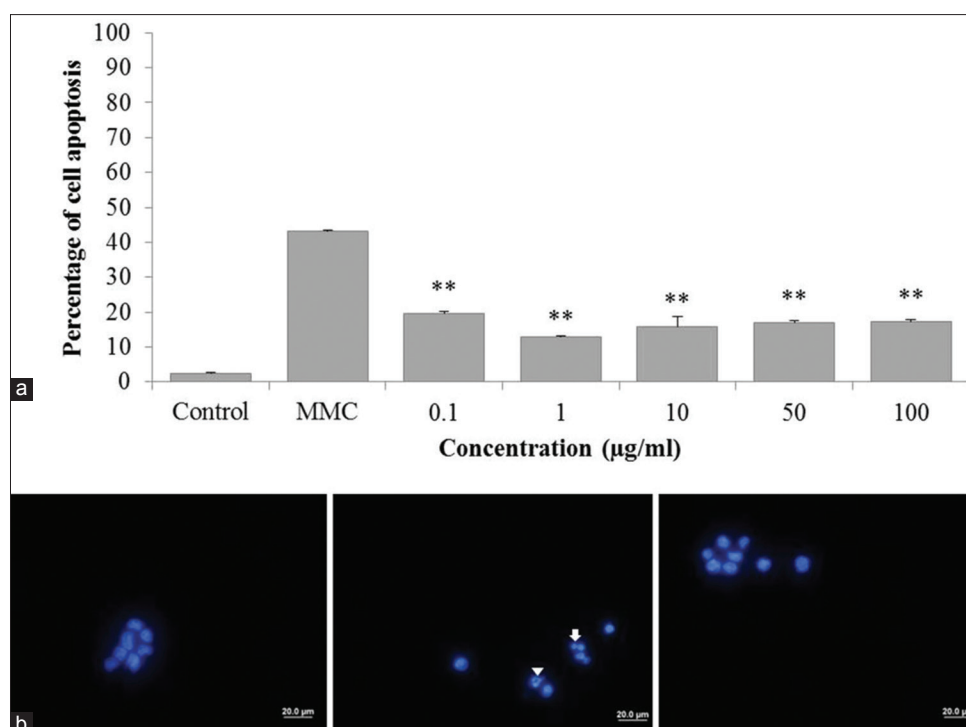


Figure 5: Apoptosis analysis of TK6 cell line cotreatment with various concentrations of VGCE and 10 µg/ml MMC showing the decrease in apoptosis cell death at all concentrations of VGCE. (a) All concentrations of VGCE cotreated with MMC exhibited the significant decrease in % cell apoptosis compared with the cells received MMC alone. The results represent the mean $\pm$ SE. **Indicates a significant difference at $P < 0.01$ when compares with the group received MMC alone. (b) Fluorescent microscope images of TK6 cells stained with Hoechst 33342 after 24 h of VGCE cotreatment with MMC (10 µg/ml). Left panel: Control cells without VGCE and MMC show typical nuclear morphology Middle panel: MMC (10 µg/ml) treatment showing cell shrinkage with chromatin condensation, nuclear fragmentation (arrowhead), and a binucleated cell (arrow). Right panel: VGCE (1 µg/ml) and MMC (10 µg/ml) cotreatment showing cells with normal nuclear morphology

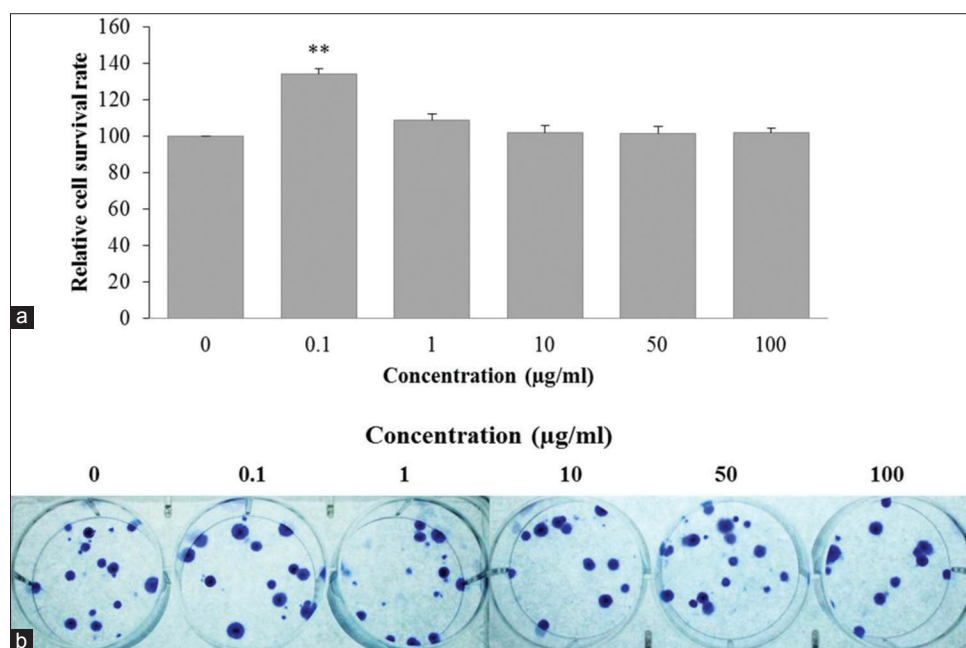


Figure 6: Clonogenic assay of L929 cells treated with VGCE. (a) VGCE at very low concentration (0.1 µg/ml) reveals the significant increase in cell viability compared with those of the other concentrations and also the untreated (0 µg/ml VGCE) control. The results represent the mean $\pm$ SE. **Indicates a significant difference at $P < 0.01$. (b) Clonogenic images of the L929 cell line showing colonies formed after 11 days after VGCE treatment.

that were not toxic *in vitro*. One of the important factors that reveal the health of cells is the capability to renew several numbers of new cells after exposure to any reagents. In the case of the cell was affected, new progeny could not reproduce. This information revealed that clonogenic assay may imply long-term cytotoxicity.^[45] The results of clonogenic assay in Figure 6a exhibited the nontoxic effect of long-term VGCE exposure on L929 cells at all concentrations. The highest rate of L929 cell survival ($133.22 \pm 8.51\%$) still revealed at 0.1 µg/ml of VGCE, significantly ($p < 0.01$), while the others were higher than that of the unexposed cell control. Besides, L929 cells still exhibited their specific characteristic in colony formation [Figure 6b].

The increase in viability of L929 cells may be from the increase in the number of epidermal growth factor (EGF) receptors and/or bFGF induced by VGCE at an appropriate concentration of 0.1 µg/ml. The EGF receptors and bFGF were mentioned above, involving fibroblast proliferation.^[11,42] Therefore, the long-term effect of VGCE should be continued on normal human fibroblasts for the expression of EGF receptor and/or bFGF and also their mechanisms of cell proliferation. It might benefit the healing and repair processes of non-cancer people that are the main functions of the fibroblasts.^[46,47]

CONCLUSIONS

The findings from this study pointed out that VGCE at very low concentrations significantly ($P < 0.01$) promoted the cell viability of non-cancer TK6 and L929 cell lines. VGCE revealed the protective activity against ROS induction by H_2O_2 on the TK6 cell line at 24 and 48 h after VGCE treatments. The extract also revealed the protective activity against MMC-induced apoptosis, since the % cell apoptosis decreased in the cotreatment of VGCE and MMC when compared with the cells treated with MMC alone. The findings from this study provide a rationale for further exploration of the mechanisms in these aspects.

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