DETERMINATION OF APOPTOTIC INDUCTIVE ACTIVITIES OF PHYSAGULIN P FROM PHYSALIS ANGULATA PLANT IN VIETNAM

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SUMMARY

Currently, multimode treatment is current therapy for cancer that causes various unwanted side effects and costliness for patients. Therefore, finding new natural ingredients derived from plants of which are less toxic and more targeted, are considered to be urgent for development of potential effective drugs to treat cancer due to safety and economic concerns. Physagulin P (VPA22) was known as a novel structural compound, isolated from the species Physalis angulata of the genus Physalis in Vietnam. This active substance has demonstrated cytotoxic effect on lung cancer cell line (A549) with the IC₅₀ value = 13.47±2.73 µM. In our study, VPA 22 was investigated for apoptotic inducing activity on A549 cells by using Hoechst staining, flowcytometry analysis and caspase-3 colorimetric assay. VPA 22 treatment changed the morphology of A549 tending to apoptotic cells whose percentage ranged from 10.09% to 19.18% at the concentration of 5 µM and 15 µM, respectively. In addition, the active ingredient at concentration of 5 µM to 15 µM also significantly activated caspase-3 in turn from 1.30 folds to 2.59 folds. These results together indicated that VPA 22 activated the caspase pathway to trigger out apoptosis on A549 cells and subsequently resulted in inhibition of cancer cell proliferation.

Keywords: A549, apoptosis, caspase-3, Hoechst, Physalis angulata, physagulin P

INTRODUCTION

Cancer was the second lead to the death worldwide, just behind the cardiovascular diseases. According to the published record by Global Cancer Data (GLOBOCAN) in 2020, there were 19.3 million of new cancer patients and 10.0 million cancer deaths reported. In Vietnam, the number of new cancerous cases rapidly increase. According to the statistics of International Agency for Research on Cancer (IARC), there were more than 16 million new cancer patients, nearly 123,000 deaths and more than 350,000 people living with cancer.

However, cancer treatments were currently followed a single mechanism and induced a number of serious side effects. The cost for cancer treatment also increases every year. Therefore, the investigation for novel drugs originated from plants that give environmental friendliness and safe, effective, low-cost treatments was focused (Iqbal et al., 2017).

Active ingredient named physagulin P
(VPA22) was the compound which was the first time isolated from Physalis angulata, from the plant belonging to the Physalis genus in Vietnam. This compound demonstrated the antiproliferative activity on A549, Hela and PANC-1 cancer cell lines. The cytotoxicity of Physagulin P on A549 lung cancer cell line was higher than that on other cell lines with IC50 = 13.47 ± 2.73 μM (Anh et al., 2018). However, this compound has not been reported in detail information about its anticancer mechanism underneath. Apoptotic stimulation was popular known as a pathway for cancer cell proliferative suppression. In cancer, apoptosis was often inhibited by several pathways including over expression of carcinogenic proteins and/or inhibition of pro-apoptotic proteins (Carneiro, El-Deiry, 2020). These changes might induce extrinsic resistance to anticancer therapies that are currently and ordinarily used as chemotherapeutic intervention (Pfeffer, Singh, 2018). Therefore, the promising novel anticancer agents are plant-derived compounds that exhibit antitumor activity through the activation of the apoptosis pathway. Thus, in this study, we report some potential apoptotic inducing activities of the compound physagulin P.

MATERIALS AND METHODS

Materials

A549 cancer cell line was purchased from the American Type Culture Collection (ATCC, USA). Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS), trypsin – EDTA 0.05%, antibiotic (Anti-anti) from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA), Hoechst 33342 (Aldrich, St. Louis, MO, USA). eBioscience™ Annexin V-FITC Apoptosis Detection kit (Invitrogen, Carlsbad, CA, USA). Caspase-3 assay kit (Biovision, Milpitas, CA, USA)

Cell culture method

Cancer cell line (A549, ATCC, USA) was cultured with DMEM supplemented with 10% FBS, 1% antibiotic and incubated at 5% CO₂, 37°C, 100% humidification. Sub-culture was carried out every 3 days.

Staining cells with Hoechst 33342

Cells were placed into a 24-well culture plate at 1 x 10⁶ cells/mL and cultured overnight before treating with the studied sample at different concentrations. After 48 h of incubation, cells were stained with Hoechst 33342 at 5 μg/mL for 30 min. The cells were then observed under fluorescence microscopy at Excitation/Emission (nm) wavelength of 350/461 nm to identify the apoptotics. Apoptotic cells displayed a brighter nucleus (due to condensed chromatin) or a nucleus that has fragmented into small pieces. The percentage of apoptotic cells was determined from each 200 cells observed under the microscope.

Detection of apoptosis using AnnexinV-FITC flow cytometry analysis

Annexin V-FITC Apoptosis Detection Kit has been used by following the instruction of the kit manufacturer to indentify the apoptosis cells. A549 cells (1 x 10⁶ cells/mL) after treated with sampled was harvested with trypsin and washed with PBS. Then, resuspension of cell pellets in 100 μL binding buffer adding with 5 μL of annexin V-FITC was carried out. After 15 minutes, the stained cells was washed with binding buffer before adding 5 μL of propidium iodide (PI) in 200 μL binding buffer. A total of 10,000 cells from each sample were analyzed using the Novocyt flowcytometry system (ACEA Bioscience Inc) and NovoExpress software to determine the percentage of apoptotic cells.

Caspase-3 inducible assay

Caspase-3 activity was performed using Biovision's Caspase-3 colorimetric kit according to the manufacturer's instructions. A549 cells, at the seeding concentration as 1 x 10⁶ cells/mL, after being incubated with the sample at various concentrations for 48 h were lysed with 50 μL of cell lysis buffer for 10 min to obtain total protein. Then, the reaction consisted of 50 μL of lysed protein solution (containing 100 μg of protein), 50 μL of DTT (10 mM) and 5 μL of DEVD-pNA (200 μM) in a 96-well plate incubated at 37 °C.
for 2 hours. The OD value was determined through ELISA system (BioTek ExL800) at 405 nm. The capacity of the samples to stimulate caspase-3 activity was determined in comparison with the negative control (without sample incubation).

**Statistical analysis**

Data were expressed as means ± standard deviation of the mean (SD). Statistical differences were analyzed by one-way analysis of variance (one-way ANOVA). A value of $P<0.05$ was a statistically significant difference.

**RESULTS AND DISCUSSION**

**Study on the apoptotic inductive activity of physagulin P by staining the cellular nucleus with Hoechst 33342**

The properties of cancer cells include maintenance of proliferative signals, suppression of growth inhibitory signals, avoidance of apoptosis, unlimited proliferation potential, as well as stimulation of vessel formation and trigger invasion and metastasis (Arbiser et al., 2017; Xu et al., 2015). Thus, stimulation of programmed cell death on cancer cells was known as one of promising anticancer patterns. Apoptosis was a process of programmed cell death that occurs in multicellular bioorganisms. Biochemical events lead to characteristic changes in the morphology of the apoptotic cell and caused to the death of that cell. Apoptosis is characterized by changes in cell morphology, chromatin condensation, DNA cleavage, and nuclear fragmentation. These typical morphological features of apoptotic cells could be observed through staining using Hoechst 33342 (Bello et al., 1994).

According to Anh et al. (2018), active ingredient as physagulin P (VPA22) was capable of causing cytotoxicity of A549 with IC$_{50}$ value of 13.47 µM. Therefore, in this study, lung cancer cells (A549) were incubated with VPA22 at concentrations of 5 µM, 10 µM and 15 µM to determine the ability to induce apoptosis through Hoechst 33342 staining. The results showed that VPA22 had capability of inducing nuclear concentration or fragmentation at the studied concentrations (Fig. 1). However, the percentage of cells exhibiting apoptosis depended on the compound concentration (Fig. 2). At a concentration of 15 µM, percentage of apoptotic cells reached up to 16.01%, which gradually decreased on cells incubated with lower concentrations of VPA22.

**Figure 1.** The Hoechst 33342 staining images of A549 cells under the treatment of VPA22 at the range of concentrations from 5 to 15 µM after 48 h; The negative control treated with 0.5% DMSO; reference control as Camptothecine at 0.5 µM.
Figure 2. The percentage of apoptotic A549 lung cancer cells induced by VPA 22 on the A549 line was 4.89% (5 µM); reached 7.36% (10 µM); got 16.01% (15 µM); camptothecine reached 23.21% (0.5 µM);

The apoptosis-inducing effect of VPA 22 on the phosphatidylserine marker

Beside of the change in cell nucleus morphology, apoptosis also caused a series of other alternation including asymmetric distribution of phospholipids of the plasma membrane leading to re-arrange of the position of phosphatidylserine (PS) from the inside to the outside of the plasma membrane (Van Engeland et al., 1998). In addition, PS had a high affinity for binding to annexin V, thus this fluorescent labeled protein has been popular used as a molecular agent to identify PS-expressing apoptotic cells in vitro as well as distinguish apoptotic and/or necrotic cells double stained with PI (Lisa et al., 2016; Elmore, 2016). The usage of fluorescent probed annexin V and PI could identify cell apoptosis through microscopy or flow cytometry. In particular, flow cytometry could identify thousands of cells in a single experiment that easily classified populations of cells with similar characteristics (van Genderen et al., 2008). Specifically, the cell population stained with annexin V-FITC and PI was divided into 4 sub-groups analyzed by flow cytometry such as normal cell population that was unstained with annexin V-FITC and PI, the early apoptosis cells stained with annexin V-FITC only, the late-apoptotic cell group exhibited both annexin V-FITC and PI and the necrotic cell death cluster presented PI (Pozarowski et al., 2003).

The result from this study indicated that the percentage of apoptosis cell was increased gradually with rising of studied concentration and higher than that of the negative control (Table 1 and Fig. 3). The percentage of apoptosis cells after being treated with active ingredient VPA 22 increased from 10.09% at the concentration of 5 µM to 19.18% at the concentration of 15 µM, while this percent was 6.53% in the negative control. Besides, VPA 22 have not recorded to cause necrosis at the studied concentrations due to the percentage of necrotic cells was in the range of 0.20 - 0.65%. These results demonstrated that VPA 22 induced apoptosis, especially in the early stages of apoptosis on A549 cells.
Table 1. Proportion of apoptosis cells.

<table>
<thead>
<tr>
<th>Samples</th>
<th>% alive cell (Q2-3)</th>
<th>% early apoptotic cell (Q2-4)</th>
<th>% late apoptotic cell (Q2-2)</th>
<th>% necrotic cell (Q2-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>93.42</td>
<td>6.42</td>
<td>0.11</td>
<td>0.06</td>
</tr>
<tr>
<td>VPA 22 (5 µM)</td>
<td>88.86</td>
<td>9.83</td>
<td>1.07</td>
<td>0.23</td>
</tr>
<tr>
<td>VPA 22 (10 µM)</td>
<td>85.78</td>
<td>13.52</td>
<td>0.50</td>
<td>0.20</td>
</tr>
<tr>
<td>VPA 22 (15 µM)</td>
<td>80.17</td>
<td>15.92</td>
<td>3.26</td>
<td>0.65</td>
</tr>
<tr>
<td>Camptothecin (0.5 µM)</td>
<td>80.61</td>
<td>18.67</td>
<td>0.30</td>
<td>0.41</td>
</tr>
</tbody>
</table>

Figure 3. Impact of VPA 22 on A549 cell apoptosis through Kit Annexin V/ FITC. Samples were analyzed using a cytometric system. The x-axis represents the FITC-Annexin V staining level, the y-axis represents the PI staining level in Log.

VPA 22 apoptotic inductive activity through caspase-3 indicator

During apoptosis, proteases called caspase protein induce morphological and metabolic changes in apoptotic cells. There were 2 pathways of apoptosis that were the extracellular pathway and the intracellular pathway, but both were activated through caspase-3 protein (Reed, 2000). In apoptotic cells, caspase-3 activated the DNA endonucleases, which disrupted the chromosomal DNA inside the cell nucleus, causing chromatin condensation and DNA fragmentation. Caspase-3 also altered the cytoskeleton and degraded cells into apoptotic stage (Negara et al., 2018). Thus, caspase-3 was considered to be an important enzyme of the apoptosis pathway. Active substance VPA 22 was demonstrated strongly induced caspase-3 expression at the studied concentrations.
compared with negative control (Fig. 4). Especially, VPA 22 had the ability to stimulate caspase 3 up to 2.00 and 2.59 folds at concentrations of 10 µM (P < 0.01) and 15 µM (P < 0.001), respectively. However, caspase 3 level of cell treated with VPA 22 at the concentration of 5 µM decreased to 1.30 folds, which was not statistically significant difference comparing with that of the negative control. Thus, these results indicated that the natural compound VPA 22 activated the caspase pathway to trigger apoptosis in A549 lung cancer cells.

**Figure 4.** Propotion of caspase-3 induction under treatment of CPA 22 on A549 reached 1.3 folds (5 µM), 2.0 folds (10 µM), 2.59 folds (15 µM); camptothecine reached 3.25 lăn; **P <0.01; ***P<0.001

CONCLUSIONS

Our results showed that physagulin P (VPA 22) activated apoptosis of A549 cells and subsequently leading to inhibition of cellular proliferation. The compound presented apoptotic inductive effects by increasing number of cells with DNA condensation and fragmentation up to 19.18% at the 15 µM treatment. The compound also exhibited its significantly induced activity on apoptotic caspase 3 at both concentrations which were 10 µM and 15 µM after 48 h of treatment. By using flow cytometry analysis, VPA 22 did confirm its typical effects with 19.18% of early and late apoptosis induction. The results from this study on physagulin P apoptotic induced effects have not previously been reported.

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REFERENCES


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