Vietnam Journal of Biotechnology **20**(1): 81-87, 2022

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

Pham Thi Hai Yen[1], Nguyen Thi Nga[2], Trieu Ha Phuong[2], Nguyen Thi Cuc[2], Do Thi Phuong[2], Hoang Le Tuan Anh[3], Do Thi Thao[1,2,✉]

[1]*Graduate University of Science and Technology, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet Road, Cau Giay District, Hanoi, Vietnam*
[2]*Institute of Biotechnology, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet Road, Cau Giay District, Hanoi, Vietnam*
[3]*Center for Research and Technology Transfer, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet Road, Cau Giay District, Hanoi, Vietnam*

✉To whom correspondence should be addressed. E-mail: thaodo74@yahoo.com

Received: 11.8.2021
Accepted: 25.11.2021

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 IC_{50} 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 treaments 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 IC_{50} = 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 anti-cancer 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% CO_2, 37ºC, 100% humidified. 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 10^6 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 10^6 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 Novocyte 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 10^6 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 activitiy 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, stimulatiom 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 (Belloc *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.

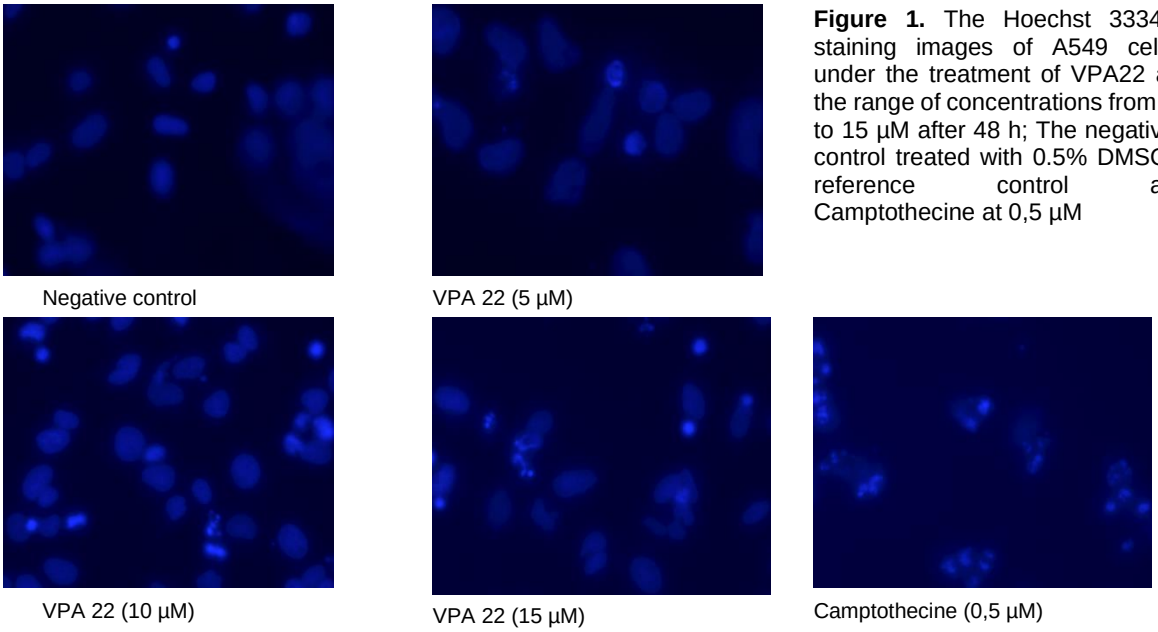


Figure 1. The Hoechst 33342 staining images of A549 cells under the treatment of VPA22 at the range of concentrations from 5 to 15 µM after 48 h; The negative control treated with 0.5% DMSO; reference control as Camptothecine at 0,5 µM

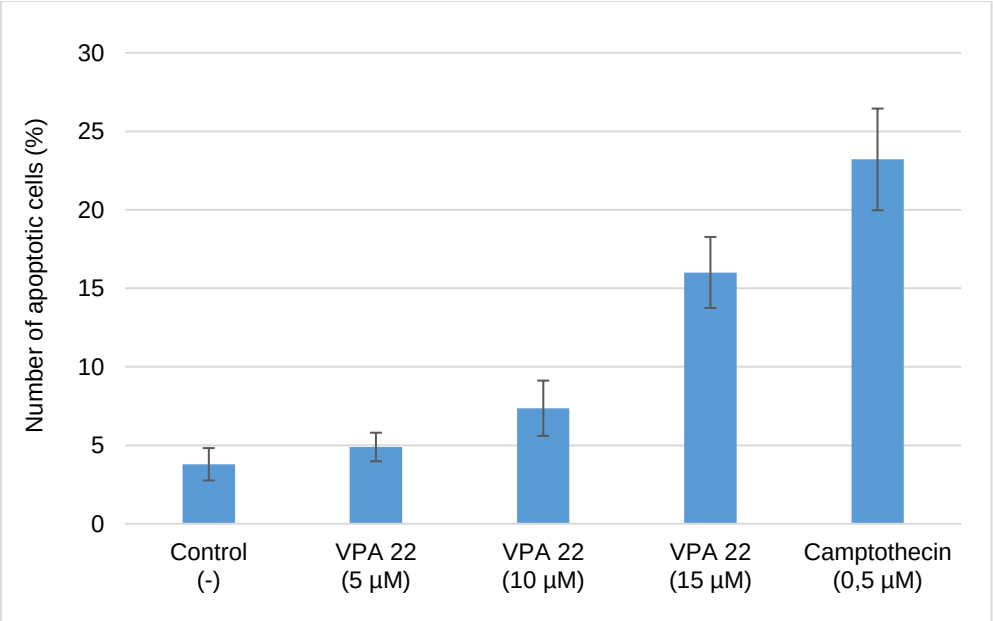


Figure 2. The percentage of apoptotic A549 lung cancer cells induced by VPA 22 on the A549 line was 4.89% (5 µM); reached 7.36%(10 µM); got 16.01% (15 µM); camptothecine reached 23.21% (0.5 µM);

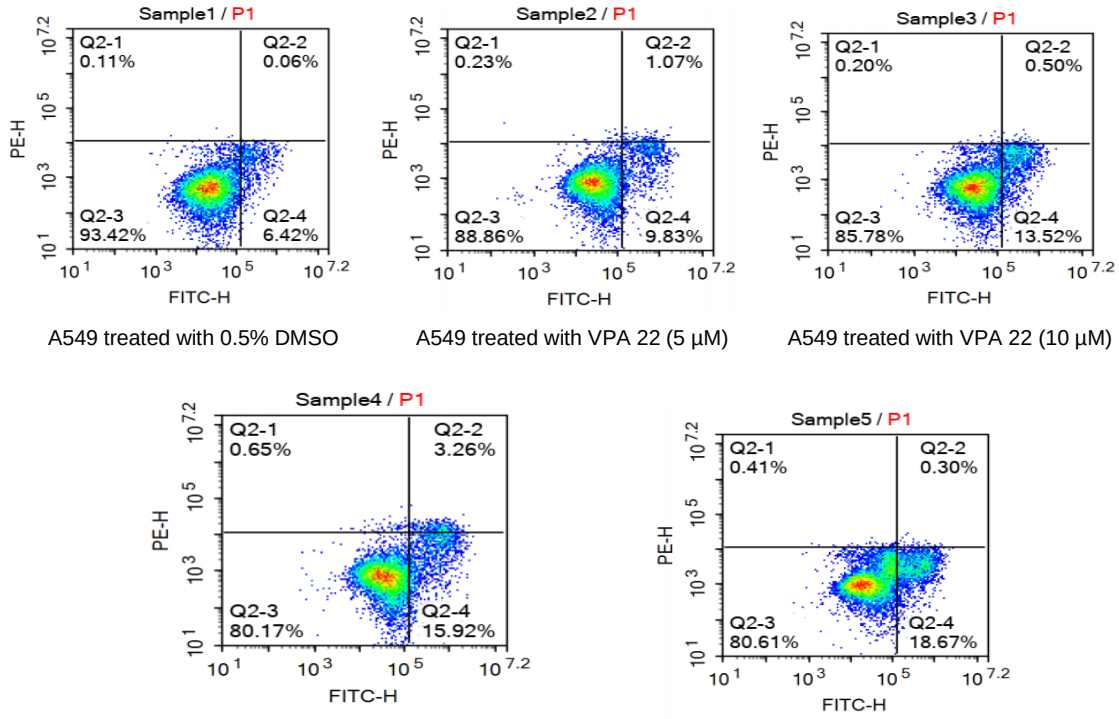
The apoptosis-inducing effect of VPA 22 on the phosphatidylserine marker

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 flulorescent labeled protein has been poplular 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.

Samples	% alive cell (Q2-3)	% early apoptotic cell (Q2-4)	% late apoptotic cell (Q2-2)	% necrotic cell (Q2-1)
Negative control	93.42	6.42	0.11	0.06
VPA 22 (5 µM)	88.86	9.83	1.07	0.23
VPA 22 (10 µM)	85.78	13.52	0.50	0.20
VPA 22 (15 µM)	80.17	15.92	3.26	0.65
Camptothecin (0,5 µM)	80.61	18.67	0.30	0.41



A549 treated with 0.5% DMSO | A549 treated with VPA 22 (5 µM) | A549 treated with VPA 22 (10 µM)

A549 treated with VPA 22 (15 µM) | A549 treated with Camptothecin (0.5 µM)

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.

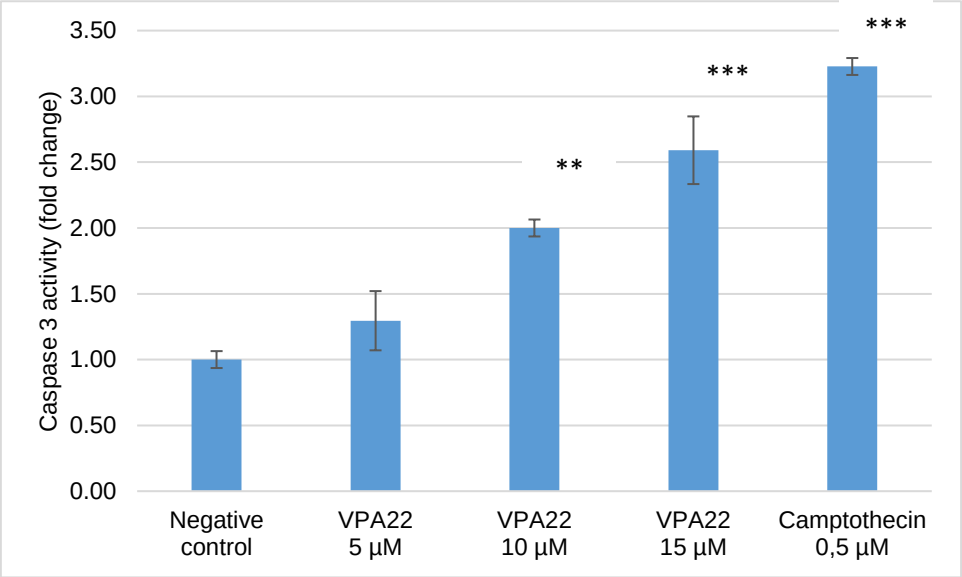


Figure 4. Propotion of caspase-3 induction under treatment of CPA 22 on A549 reached 1.3 folds (5 µM), 2.0 folds (10 µM), 2.59 folds (15 µM); camptothecine reached 3.25 lần; ***P* <0,01; ****P*<0,001

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 fragementation 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 prevously been reported.

Acknowledgments: *This research is funded by Vietnam Academy of Science and Technology (VAST) under grant number QTKR01.03/19-20.*

REFERENCES

Anh HL, Dung DT, Van Kiem P, Quang TH, Yen PT, Cuong PV, Hung TM (2018) Phytochemical constituents and cytotoxic activity of *Physalis angulata* L. growing in Vietnam. *Phytochem Lett* 27: 193–196.

Arbiser JL, Bonner MY, Gilbert LC (2017) Targeting the duality of cancer. *NPJ Precis Oncol* 1(1): 1–7.

Belloc F, Dumain P, Boisseau MR, Jalloustre C, Reiffers J, Bernard P, Lacombe F (1994) A flow cytometric method using Hoechst 33342 and propidium iodide for simultaneous cell cycle analysis and apoptosis determination in unfixed cells. *Cytometry* 17(1): 59–65.

Carneiro BA, El-Deiry WS (2020) Targeting

apoptosis in cancer therapy. *Nat Rev Clin Oncol* 17(7): 395–417.

Elmore S (2007) Apoptosis: A review of programmed cell death. *Toxicol Pathol* 35: 495–516

Iqbal J, Abbasi BA, Mahmood T, Kanwal S, Ali B, Shah SA, Khalil AT (2017) Plant-derived anticancer agents: A green anticancer approach. *Asian Pac J Trop Biomed* 7(12): 1129–1150.

Crowley LC, Marfell BJ, Scott AP, Waterhouse NJ (2016) Quantitation of Apoptosis and Necrosis by Annexin V Binding, Propidium Iodide Uptake, and Flow Cytometry. Cold Spring Harb Protoc 2016(11).

Negara KS, Suwiyoga K, Pemayun TG, Sudewi AA, Astawa NM, Arijana IG, Tunas K (2018) The role of caspase-3, apoptosis-inducing factor, and B-cell lymphoma-2 expressions in term premature rupture of membrane. *Rev Bras de Ginecol e Obstet* 40(12): 733–739.

Pfeffer CM, Singh AT (2018) Apoptosis: a target for anticancer therapy. *Int J Mol Sci* 19(2): 448.

Pozarowski P, Grabarek J, Darzynkiewicz Z (2003) Flow cytometry of apoptosis. *Curr Protoc Cytom* Unit: 18.8.

Reed JC (2000) Mechanisms of apoptosis. *Am J Pathol* 157(5) : 1415–1430.

Van Engeland M, Nieland LJ, Ramaekers FC, Schutte B, Reutelingsperger CP (1998) Annexin V-affinity assay: a review on an apoptosis detection system based on phosphatidylserine exposure. *Cytometry* 31(1): 1–9.

Van Genderen HO, Kenis H, Hofstra L, Narula J, Reutelingsperger CP (2008) Extracellular annexin A5: functions of phosphatidylserine-binding and two-dimensional crystallization. *Biochim Biophys Acta Mol Cell Res* 1783(6): 953–963.

Xu W, Jing L, Wang Q, Lin CC, Chen X, Diao J, Liu Y, Sun X (2015) Bax-PGAM5L-Drp1 complex is required for intrinsic apoptosis execution. *Oncotarget* 6(30): 30017.