

Pro-apoptotic and anti-proliferative effects of *Physalis angulata* leaf extract on retinoblastoma cells

Marsha Dechastra Chairissy, Lely Retno Wulandari, Hidayat Sujuti

Department of Ophthalmology, Faculty of Medicine, Brawijaya University, Malang 65145, Indonesia

Correspondence to: Lely Retno Wulandari. Department of Ophthalmology, Faculty of Medicine, Brawijaya University, Malang 65145, East Java, Indonesia. lelyretnowulandari@gmail.com

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Abstract

• **AIM:** To investigate the effect of *Physalis angulata* leaf extract on apoptotic and proliferation of retinoblastoma cells. Despite several previous studies evidencing the anti-cancer potential of *Physalis angulata*; however, certain study that proves its benefits in retinoblastoma cancer cells has been limited.

• **METHODS:** This study utilizes an *in-vitro* experimental study by applying Y79 human retinoblastoma cell line culture obtained from the American Type Culture Collection (ATCC; 10801 University Boulevard Manassas, VA 20110, USA). The cell was divided into 4 groups. Group I was the control group without the administration of *Physalis angulata* leaf extract. Whereas, group II, III and IV are engaged with 25, 50, and 100 µg/mL of *Physalis angulata* leaf extract respectively. After a 24h incubation, an examination with microtetrazolium (MTT) cell proliferation assay and Annexin V apoptosis detection was conducted. Statistical analysis was performed with the Tukey test.

• **RESULTS:** *Physalis angulata* leaf extract improved apoptosis and significantly reduced the number of living cells in retinoblastoma cells, along with the increase in the given dose. Based on the Tukey test, a significant difference was found in the treatment group at 50 µg/mL ($P=0.025$) and 100 µg/mL ($P=0.001$) in the measurement of apoptosis. Proliferation measurements also indicated a significant decrease in the number of living cells in the 50µg/mL treatment group ($P=0.004$), and in the 100 µg/mL treatment group ($P=0.000$). Meanwhile, a dose of 25 µg/mL indicated insignificant difference in the two measurements. Improved apoptosis and decreased number of living cells occurred at a dose of 100 µg/mL. Decreased number of living cells (in the measurement of proliferation) was due to the inhibited proliferation or improved apoptosis.

• **CONCLUSION:** *Physalis angulata* leaf extract improve apoptosis in retinoblastoma cell culture, requiring further research to inhibit proliferation.

• **KEYWORDS:** *Physalis angulata*; apoptotic; proliferation; retinoblastoma cells

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INTRODUCTION

Retinoblastoma is suspected as a primary intraocular malignancy mostly occurring in children, with the worldwide general incidents ranging for 8000 children per year. It is unbeatable fact that developed countries have survived their high life expectancies; whereas, developing and poor countries have been suffered with the finding of less than 50%^[1-3].

The pathogenesis of retinoblastoma is initiated with a disruption of cell cycle regulation leading to abnormal retinal cell growth. In retinoblastoma, mutations or inactivation of tumor suppression proteins include the retinoblastoma protein (pRB) and the transcription factor p53^[4]. Both of the pRB and p53 allow the two main pathways of tumor suppression controlling cellular responses to oncogenic stimuli such as errors in cell division, DNA damage and incompatible mitogenic signals. Each of these pathways has certain regulators and effectors to stop the cell cycle or even induce apoptosis^[5]. Inactivation of pRB prohibits cells to enter the proliferation phase resulting in uncontrolled DNA replication and imperfect replication results^[6]. A mere pRB pathway is insufficient to induce tumor formation. Dysregulation in p53 pathway will also induce anti-apoptosis and excessive cell growth^[7].

One of the weaknesses of current retinoblastoma therapy is indicated to restrict the therapeutic index range allowing toxicity in normal tissue. In addition, a high level of recurrence causes a large number of treatment failures. At present, several concerned researches have been devoted by developing anti-tumor agents (one of which is pRB) to target the mechanisms which can suppress tumor development. The two main

strategies that have been developed with the target of RB1 gene as cancer therapy are exploiting the RB1 gene mutation and reactivating tumor suppressor functions from RB1^[8-10].

Physalis angulata is a plant, evidenced to have photochemical attributes addressing the anti-cancer potential^[11]. One of the anticancer active substances successfully isolated from *Physalis angulata* leaf extract is physalin, which is a derivative of flavonoid glycosides. In addition, other ingredients were found, such as luteolin, carotenoids, and withanolides. In addition to anticancer effect, *Physalis angulata* leaf extract is also believed to have antiparasitic, anti-inflammatory, antimicrobial, anti-inflammatory, and immunosuppressive potential^[12].

Several concerned studies have proven the anticancer potential of *Physalis angulate*^[13-22]. However, there has not been any study that proves its benefits in retinoblastoma cancer cells.

MATERIALS AND METHODS

The design of this study involved an *in vitro* experimental in retinoblastoma cell line culture which was engaged with *Physalis angulata* leaf extract. The study was conducted at the Biomedical Laboratory of Faculty of Medicine in Brawijaya University, on October 2018.

The sample of this study was retinoblastoma cell line culture obtained from the American Type Culture Collection (ATCC; 10801 University Boulevard Manassas, VA 20110, USA). The cell culture was divided into four groups, including the control group (without exposure), treatment group 1 (with exposure to 25 µg/mL of *Physalis angulata* leaf extract), treatment group 2 (with exposure to 50 µg/mL of *Physalis angulata* leaf extract), and treatment group 3 (with exposure to 100 µg/mL of *Physalis angulata* leaf extract).

Inclusion criteria involved cell culture with characteristics of retinoblastoma cell line Y79 (ATCC® HTB18™) marked with multicellular cluster or grape-like cluster. Whereas, exclusion criteria involved cell culture without characteristic of retinoblastoma cell, ingrown cell culture during incubation period or cell culture with contamination from chemical material, micro-organism, or fungi experiencing an error during treatment.

Physalis angulata leaf extract is derived from extraction with absolute ethanol solvent by utilizing soxhlet extractor device. The liquid extract is diluted to acquire concentration with dose of 25 µg/mL, 50 µg/mL, and 100 µg/mL.

Proliferation and apoptosis of retinoblastoma cell involve the growth of retinoblastoma cell counted by microtetrazolium (MTT) cell proliferation assay method and by Flowcytometri Annexin V apoptosis detection kit (to reveal the percentage of living cell).

Research Procedure

The process of retinoblastoma cell culture Retinoblastoma

cell tissue was washed 3 times with sterile phosphate-buffered saline (PBS) in laminar flow. The tissue was chopped in a serum-free media to get a size of 1×1 mm². The network suspension (centrifuge) was rotated at a speed of 1200 rpm for 7min. The supernatant was removed, while the pellet was re-suspended and rotated again. The pellet was re-suspended with culture media and was pipetted. The tissue was inserted into the culture plate and was incubated at 37°C, 95% with air humidity level of 5% CO₂ for 24h.

The extraction of *Physalis angulata* leaf Totally 100 g of dry leaf powder (simplisia) from *Physalis angulata* leaves was obtained from the Center of Medicinal Plants in Materia Medika, at Batu Municipality, Malang. Dry sample powder was soaked with ethanol until the volume reached 900 mL. It was shaken and left overnight until settled (maceration process). The supernatant was filtered to obtain the filtrate. The separation between ethanol solvent and extract was performed by applying a rotary evaporator with a temperature of 70°C-80°C to achieve oily solids from *Physalis angulata* leaves. Further dilution was carried out by using DMSO of <0.05% (v/v) for the purpose of pharmacological studies.

MTT proliferation assay procedure Various *Physalis angulata* extract concentrations were added to 96 well-plates and were incubated in a incubator with 5% CO₂ at 37°C for 24h. After the incubation ends, the medium and *Physalis angulata* extract were removed and were added to each well-plates with 10 µL reagen MTT (TACS MTT Cell Proliferation assay®4890-25 Cat-K) with a concentration of 0.5 mg/mL in dark condition.

Incubation was performed at 37°C for 4h until formazan was formed. Observation of cell conditions was carried out with an inverted microscope. Formazan was eluted from the cell with 150 µL of DMSO. The reading of the results was conducted by ELISA microplate reader at 550 nm absorbance. Retinoblastoma cell proliferation was expressed in the form of percentage with the following formula:

$$100\% \text{ Retinoblastoma Cell} = \frac{(\text{Absorbance experiment} - \text{Absorbance blank})}{(\text{Absorbance control} - \text{Absorbance blank})} \times 100\%$$

Measurement of Apoptosis using Annexin V Various *Physalis angulata* extract concentrations were added to 24 well-plates and were incubated in incubator with 5% CO₂ at 37°C for 24h. After the cell was removed from the incubator, the medium and *Physalis angulata* extract were aspirated. Further, Dulbecco's phosphate-buffered saline (D-PBS), ethylenediaminetetraacetic acid (EDTA) and trypsin were added in each well-plates followed by incubation for 2min in CO₂ incubator. Afterwards, all aspirates were placed on Eppendorf Multipette® plus (Germany) and were centrifuged at 2500 rpm for 5min. Cells in each Eppendorf were washed

Table 1 Mean of observation results of apoptosis and proliferation in retinoblastoma cell culture in the control group and treatment groups

Parameters	Group	n	Mean±SD	Min.	Max.	95%CI	
						Lower bound	Upper bound
Apoptosis	K	6	0.86±0.18	0.63	1.15	0.67	1.05
	P1	6	1.06±0.31	0.74	1.57	0.73	1.39
	P2	6	1.33±0.17	1.09	1.52	1.15	1.51
	P3	6	1.54±0.34	1.04	2.03	1.19	1.89
Proliferation	K	6	89.65±1.18	88.22	91.35	88.41	90.88
	P1	6	87.84±1.01	86.41	88.88	86.76	88.90
	P2	6	86.77±1.75	84.39	89.33	84.94	88.61
	P3	6	84.80±1.01	82.95	86.05	83.74	85.86

with cold PBS. Cells were centrifuged at a speed of 1500 rpm at 4°C. Annexin V, Streptavidin and propidium iodide (PI; Annexin V Biotin Apoptosis Detection Kit e-Bioscience cat. No.BMS500BT/300) were added to the cells and were incubated for 10min at 4°C in the dark. The last step was performed by adding a 300 µL staining buffer in each well-plates. Analysis was conducted by using flowcytometry.

Statistical Analysis The normality test for each group was conducted with the Kolmogorov-Smirnov test and the homogeneity test of the data was performed with the Levene test. To determine apoptosis and retinoblastoma cell proliferation between each treatment, analysis with one way ANOVA was utilized. To compare data from each group, the Tukey test was applied, indicating significant result if $P < 0.05$.

RESULTS

Primary Outcome From the results of statistical analysis, it was revealed that there was an effect of *Physalis angulata* leaf extract on retinoblastoma cell apoptosis in decreasing the number of living cells in the treatment groups with doses of 25, 50, and 100 µg/mL. The higher the dose, the higher the effect in increasing apoptosis and inhibiting the number of living cells in retinoblastoma cells (Table 1).

Physalis angulata leaf extract increasing the retinoblastoma cell apoptosis, which was conducted on the one-way ANOVA test, indicated that the significance value for apoptosis was 0.001 (< 0.05). In addition, the Tukey test identified the treatment groups marking differences in the treatment dose of 50 µg/mL ($P = 0.025$), and 100 µg/mL ($P = 0.01$); whereas, a dose of 25 µg/mL indicated insignificant difference ($P = 0.555$; Figure 1).

Physalis angulata leaf extract fighting the retinoblastoma cell proliferation, which was conducted on the one-way ANOVA test, indicated that the significance value for proliferation was 0.000 (< 0.05). In addition, the Tukey test identified the treatment groups marking differences in the treatment dose of 50 µg/mL ($P = 0.004$), and 100 µg/mL ($P = 0.00$); whereas, a dose of 25 µg/mL indicated insignificant difference ($P = 0.097$; Figure 2).

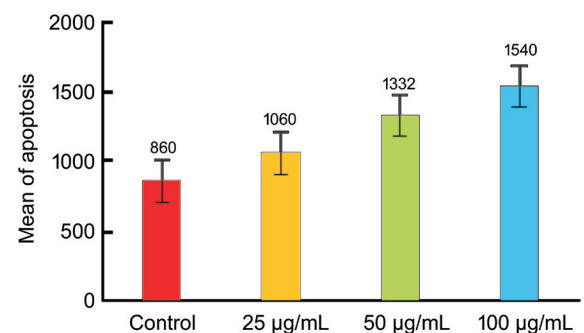


Figure 1 Apoptosis of retinoblastoma cell given *Physalis angulata* leaf extract.

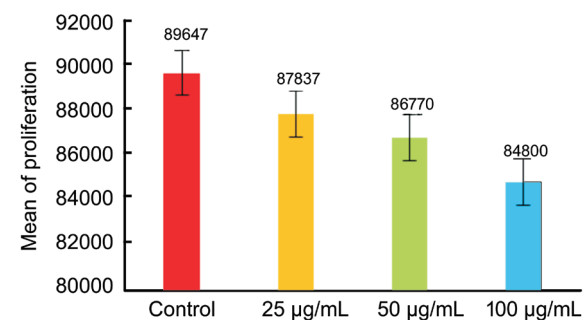


Figure 2 Proliferation of retinoblastoma cell given *Physalis angulata* leaf extract.

Secondary Outcome This research hypothesis states that: *Physalis angulata* leaf extract is likely to have pro-apoptotic effect compared to its anti-proliferative effect due to the two-fold apoptotic rate. Meanwhile, there was no decreased of living cell rate when cell proliferation was measured.

DISCUSSION

Several previous studies have proven the anticancer potential of *Physalis angulata*^[13-22]. However, certain study dealing with benefits in retinoblastoma cancer cells remains limited. In this research, the researchers promote the novelty by studying the effect of *Physalis angulata* leaf extract on apoptosis and proliferation of retinoblastoma cell. In addition, the major mechanism of cancer damage is unbalanced condition between apoptosis and proliferation.

Apoptosis is a mechanism of programmed cell death while cell

proliferation is a process which cells multiply by growing and splitting themselves into two. In tumor, there is an excessive proliferation process without the balance of apoptotic process.

The researchers performed apoptosis calculations by using Annexin V, as a quantitative method of calculating apoptosis, based on the phenomenon of phosphatidylserine released during apoptosis and the ability of Annexin V to bind to high affinity phosphatidylserine. In dead cells, the inner layer is extrinsically bound to Annexin V due to the loosing integrity of the plasma membrane, causing Annexin V to also bind the necrotic cells. In addition, to distinguish between dead cells and apoptotic cells, PI is added to cell suspension. Hence, dead cells and apoptotic cells are distinguished by double labeling of Annexin V and PI and analyzed with flow cytometry^[8,23].

From the data analysis in this study, normal data distribution was obtained and the variance homogeneity test of the data with the Lavene test was found to be significant marking a homogeneous data variant. Since the variant of the data is normal and homogeneous, the Tukey test was applied to determine the different effects of *Physalis angulata* leaf extract on retinoblastoma culture cell among the treatment groups. Analysis of the data with the Tukey test indicated a significant difference ($P < 0.05$), such as in the control group and the treatment groups with a dose of 50 $\mu\text{g/mL}$ ($P = 0.025$) and a dose of 100 $\mu\text{g/mL}$ ($P = 0.01$). Meanwhile, there was no significant difference ($P = 0.555$) between the control group and the treatment group with a dose of 25 $\mu\text{g/mL}$. Thus, it is concluded that the administration of *Physalis angulata* leaf extract can increase apoptosis in retinoblastoma cells. From the results of the analysis, the dose of 100 $\mu\text{g/mL}$ affects the greatest increase in apoptosis compared to the other doses.

This is in line with the research of Handayani^[13] which states that the ethanol extract of *Physalis angulata* can inhibit the growth of breast cancer cells by reducing the expression of c-Myc protein, increasing the wild p53 protein and Apaf-1 protein, decreasing the number of mitosis, and increasing the number of apoptosis. This is in line with the research conducted by Ooi *et al*^[14] which asserts that *Physalis angulata* extract can induce apoptosis in T-47D human breast cancer cells through c-myc-, p53-, and caspase-3-dependent pathways. Many methods in calculating cell proliferation, such as the method of direct counting with tryphan blue and the MTT assay method. Direct counting is a simple method for assessing the integrity of cell membranes with less sensitive ability. The basis of enzymatic testing in MTT assay is performed to measure the ability of living cells based on mitochondrial activity from cell culture. In this study, living cells are measured with the MTT assay method due to several advantages, such as: relatively faster, sensitive, and more accurate when compared to direct counting method. Beside

the two methods above, cell proliferation may be measured by examining one or more specific markers within a cell such as Brdu, Ki-67 or PCNA.

From the data analysis in this study, normal data distribution was obtained and variance homogeneity test with the Levene test was found to be significant marking a homogeneous data variant. Because the variant of the data is normal and homogeneous, the Tukey test was applied to determine the different effects of *Physalis angulata* leaf extract on retinoblastoma culture cell among the treatment groups. Analysis of the data using the Tukey test indicated a significant difference ($P < 0.05$), such as in the control group and in the treatment groups with a dose of 50 $\mu\text{g/mL}$ ($P = 0.004$) and a dose of 100 $\mu\text{g/mL}$ ($P = 0.00$). Meanwhile, there was no significant difference ($P = 0.097$) between the control group and the treatment group with a dose of 25 $\mu\text{g/mL}$. It is concluded that the administration of *Physalis angulata* leaf extract potentially reduced the number of living cells from retinoblastoma cells. From the results of the analysis, the dose of 100 $\mu\text{g/mL}$ reduced the biggest number of living cells compared to the other doses. The decrease in the number of living cells is due to inhibited cell proliferation, which is in accordance with several other studies stating that *Physalis angulata* extract can reduce cell proliferation^[15-16]. However in this study, there is a possibility of the decreasing number of living cells due to the increasing number of apoptosis. The research by Darma *et al*^[15], reported that *Physalis angulata* has cytotoxic activity in uterine cancer cells through modulation of expression of p53 which causes cessation of cell proliferation. In a study conducted by Fitria *et al*^[16], it was stated that *Physalis angulata* plays a role in regulation of proliferation, cell cycle and apoptosis of MDA-MB 231 breast cancer cell. Physalin and withanolides contained in *Physalis angulata* extract are suspected to provide the anti-cancer mechanism. Based on research, physalin and withanolides have anti-inflammatory, anti-cancer and anti-oxidant effects. There are 6 types of Physalin in *Physalis angulata* extract, such as physalin A, B, D, F, J and N. The highest content of physalin inhibits the flowers and leaves. Rengifo-Salgado and Vargas-Arana^[12] have found several new Withanolides, namely physagulin A, B, C and D which were obtained from methanol extract of *Physalis angulata* fresh leaves and stems.

Another study conducted by Boonsombat *et al*^[17] mentioned that physalins B, D and F isolated from *Physalis angulata* indicated strong cytotoxicity against various tumor cell lines, including KB (nasopharyngeal Ca), A431 (epithelial carcinoma), HCT-8 (Duodenum Ca), PC-3 (prostate Ca), and ZR751 (breast Ca), with half maximal effective concentration (EC_{50}) < 4 mg/mL. In a study conducted by Hsu *et al*^[18], physalin B isolated from *Physalis angulata* had selective

cytotoxicity for melanoma cells. Physalin B can induce melanoma cell apoptosis through NOXA, caspase-3, and mitochondria-mediated pathways. Wu *et al*^[19] reported that physalin F induces cell apoptosis *via* the mitochondrial pathway mediated by reactive oxygen species (ROS), and it suppresses NF- κ B activation in renal cancer cells. Damu *et al*^[20] on his study mentioned that biological evaluation of withangulatin B-H (1-7), and new minor physalin, such as physalin W, together with 14 identified compounds (withaphysanolidide, withaphysalin A, dihydrowithanolide E, physaprun A, physanolide A, and physalins B, D, F, G, I, J, T, U, and V) isolated from *Physalis angulata* plants, provide extensive cytotoxic activity against human cancer cells. In addition, angulatin B, physalins D and F indicate strong cytotoxic activity against various human cancer cell lines with EC₅₀ values ranging from 0.2-1.6 mg/mL. Another study mentioned that withanolides physangulidines A, B and C isolated from *Physalis angulata* presented significant antiproliferative activity against DU145 in prostate cancer cells^[21]. The study of Reyes-Reyes *et al*^[22] postulated that physangulidine A, one of the types of withanolide isolated from *Physalis angulata*, could disrupt the cell cycle and increase apoptosis in prostate cancer cells. This content is thought to be the anti-cancer mechanisms of *Physalis angulata* with its effect in increasing apoptosis and decreasing proliferation.

Another suspected mechanism on the effect of *Physalis angulata* extract as pro-apoptosis and anti-proliferation agent is the content of *Physalis angulata* extract which has an influence on cyclooxygenase. In a study conducted by Kalsum *et al*^[24], *Physalis angulata* extract had a large effect on decreasing the levels of cyclooxygenase (COX)-1, COX-2 and PLA-2. In a study by Sutrisna *et al*^[25], it was found that *Physalis angulata* extract could reduce COX-2 activity in breast cancer cells (MCF-7).

However, the studies about COX-2 expression in retinoblastoma provide the controversy. In a study conducted by Wang *et al*^[26] about the COX-2 expression in the normal human eye and its expression pattern in the selected eye tumor including retinoblastoma, it was found that there was weak to medium expression of COX-2. That result is different with study by Suoza Filho *et al*^[27], that found the overexpressed of COX-2 in both differentiated and undifferentiated human retinoblastoma. In addition to, from the study it was found the proliferation of retinoblastoma cells to be inhibited by a COX-2 inhibitor. Wang *et al*^[26] on his study mentioned that focusing exophytic retinoblastoma in the outer layers of the retina, a weak to moderate COX-2 staining was demonstrated in the tumor cells. Interestingly, the photoreceptor inner segment, which is a part of the outer layers of the retina, has been shown a strong constitutive expression of COX-2, although COX-2 was not

expressed in the outer nuclear layer in the normal human retina. Elevated tumor COX-2 is associated with increased angiogenesis, tumor invasion and promotion of tumor cell resistance to apoptosis. By inhibiting COX-2, it can increase p53 activity, which in turn promotes apoptosis, cell cycle arrest or senescence in damaged cells, and inhibits proliferation^[28]. Thus, it is possible that the effect of *Physalis angulata* administration through the COX pathway also plays a role in reducing proliferative and promote apoptosis in this study.

From the results of this study, the researchers assume that *Physalis angulata* leaf extract is more likely to have pro apoptotic effect than anti-proliferation. Therefore, *Physalis angulata* leaf extract is more suitable as an adjuvant chemoradiation therapy which is anti-proliferation in its future use. However, accurate data remains limited due to the need of further research with better cell quality and a longer incubation period (48 or 72h). In addition to obtain better proliferation data, it is recommended to synchronize cells for 48h before proliferation measurements are carried out. The result from this study is expected to be the basis for further research to discover the potency of *Physalis angulata* as an anticancer agent of retinoblastoma.

The limitation of this study lies on the very small amount of cell density, due to slower growth of retinoblastoma cells. There are several possible reasons such as: 1) the utilized cell was in the fourth passage (not optimal) due to genetic instability and phenotypic shift during the previous culture process; 2) there is an error during the storage process or in the previous treatments, in liquid nitrogen, 3) the number of cells which were frozen is insufficient during previous breeding. Thus, when the thawing process was carried out, the number of cells obtained is insufficient despite the 2wk process (the half-life for the retinoblastoma cell is 40h); 4) the lack of the dose range used in this study; therefore, further research with a larger replication group and more cell density and a longer incubation period (48 or 72h) is required to obtain better results and to find out the effective doses as well as the toxic doses of *Physalis angulata* leaf extract. Besides, the chemical structure of *Physalis angulata* leaf was not specifically separated due to the lack of available facilities, time and expensive cost. In the future, future research is advised to separate the chemical structure of the object and its effect on apoptosis and proliferation in normal cells.

Further research is also suggested to perform reexamination with better condition and better cell quality. Further research on the effect of *Physalis angulata* leaf extract on proliferation using Ki-67, BrdU, or other more sensitive and more specific measurement methods is also recommended to conduct 48-hour synchronization before proliferation measurement to get more accurate proliferation rate.

In addition, further research is advised to explore the active substances in *Physalis angulata* leaf extract separately (by using periodic titration to isolate the active substances) to find out the direct effect on retinoblastoma cell culture. Finally, further research to determine the effective dose and the toxic dose and the effect of *Physalis angulata* leaf extract on normal cell culture is strongly advisable.

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