The Evaluation of Antioxidant Activity and Cytotoxicity of Leaf, Orange Fruit, and Calyx Extract of *Physalis alkekengi* on Human Lung Cancer A549 Cell Line

Babak Babakhani [a], Mahdieh Houshani [a], Sogol Motallebi Tala Tapeh [b], Seyed Afshin Hosseini Boldaji [c], Maryam Shoja Shafiee [a], Mitra Arman [d], Saeed Heidari Keshel [e,f*]

[a] Department of Biology, Tonekabon Branch, Islamic Azad University, Tonekabon, Iran; [b] Department of Chemistry, Tonekabon Branch, Islamic Azad University, Tonekabon, Iran; [c] Department of Biology; College of Sciences; Yadegar-e-Imam Khomeini (RAH) Shahre rey Branch; Islamic Azad University; Tehran; Iran; [d] Department of Biology, Payam noor University, Tehran, Iran; [e] Medical Nanotechnology Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran; [f] Department of Tissue Engineering and Applied Cell Science, School of Advanced Technologies in Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran.

Corresponding authors: Saeed Heidari Keshel, Medical Nanotechnology Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran. ***E-mail:*** saeedhey@gmail.com; ***Tel:*** +98-912 5870517

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Introduction: Lung cancer is the most common cancers, and chemotherapy treatment as one of the methods leads to the occurrence of intolerable side effect. Thus recently, natural products extracted from medicinal plants can play an important role in cancer treatment. It was aimed in this study, based on medicinal effect of the *Physalis alkekengi*, to determine the content and activity of some antioxidant compounds of leaves, orang fruits, and calyx of P. alkekengi and also the inhibitory effects of their crude extract on lung cancer A549 cell line. **Materials and Methods:** Leaf, Orange Fruit, and Calyx Extract of P. alkekengi were harvested from the region of Tonekabon city and A549 cells line was cultivated and proliferated. Then, the cells exposed to different concentrations of the leaves, fruits ,and calyx extract of P. alkekengi (31.25 to 2000 µg/ml) were incubed for 24, 48, and 72 hours. After the incubation period, the colorimetric MTT method was used to determine cytotoxicity. Also, total phenol, flavonoid, anthocyanin, and carotenoid contents were determined as well as antioxidant activity of extract using DPPH method. **Results:** The results showed that the highest total phenol, flavonoids, and anthocyanins were in leaves and the highest antioxidant activity and total carotenoids were seen in fruits. The results have also shown that the extracts of leaves, fruits, and calyxes brought the highest inhibition of cancer cell growth at the concentration level of 2000 µg/ml that were 88.55, 91.81, and 94.97% respectably. **Conclusion:** These results suggest that the leave, fruit and calyx of ethanolic extract of P. alkekengi have the most antioxidant and antimicrobial activity and cytotoxicity against A549 cell line. It seems to come with further research, and utilizes its compound in cancer treatment.

Keywords: Antioxidant Compounds; Lung Cancer; A549 cells line; Physalis Alkekengi

Introduction

Cancer is the second leading cause of death after cardiovascular diseases in most communities like Iran. Lung cancer is the most common cancer through the world and considered as an epidemic (1). Lung cancer or lung carcinoma is a malignant lung tumor characterized by uncontrolled cell growth in the lung tissues. Worldwide in 2012, lung cancer occurred in 1.8 million people and resulted in 1.6 million deaths. This makes it the most common cause of cancer related death in men and second most common in women after breast cancer (2).These cases are often caused by a combination of genetic factors and exposure to random gas, asbestos, second- hand smoke, or other forms of air pollution. Lung cancer may be seen small-cell lung carcinoma (SCLC) and non-small-cell lung carcinoma (NSCLC) (2, 3).The most common clinical manifestations are coughing (including coughing of blood), weight loss, shortness of breath, and chest pain (4). The vast majority (85%) of cases of lung cancer are due to long-term tobacco smoking. About 10-15% of cases occur in people who have never smoked (5). These cases are often caused by a combination of genetic factors and exposure to radon gas, asbestos, second-hand smoke, or other forms of air pollution. Lung cancer may be seen on chest radiographs and computed

tomography (CT) scans (4).

Conventional cancer treatments have serious side effects and the anticancer drugs used previously exhibited relatively high toxicity not only to the tumor cells, but also to the normal cells of the body part in which the cancer had developed (6). So access to high-efficacy and low toxicity drugs that specifically affect cells and being inexpensive is one of the major concerns of the medical community in the world (7). Currently, the search for novel anticancer drags is being conducted among medicinal plants. In this regard herbal medicines are more important in preventing cancers because of the low or lack of side effects. Accordingly, medical plants are a great source of hope for discovering new drags (8, 9).

Physalis alkekengi belongs to Solanaceae including 80 species in the world and two of them grow in Iran including *Physalis alkekengi* and Physalis angulate. *Physalis alkekengi* is an herbaceous, annual or perennial plant with 30-60 cm height, growing as a wild plant in Mazandaran, Gilan and Golestan. This plant extract has been used for treatment of wide range of diseases including difficult urination, kidney and bladder stone, febrile diseases, gout, general edema, and rheumatism (10, 11, 12, and 13). Therefore, its anticancer potential has been not yet fully investigated and there has been no systematic study on the anticancer effects of different parts of this plant. Thus, the main purpose of this study was to investigate the antioxidant compound and the inhibitory effects of different parts of this plant on the growth of lung cancer cells.

Materials and Methods

The plant was identified at the Herbarium of Tonekabon University. The materials used in the experiment included acetone,3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Fetal Bovine Serum (FBS), streptomycin, penicillin, 2, 2-diphenyl-1-picrylhydrazyl (DPPH), ethanol, sodium sulfate, aluminum chloride, acetate potassium, Quercetin, gallic acid, Folin–Ciocalteu, sodium carbonate, hydrochloric acid and methanol.

All chemicals and reagents used in this study were of analytical grade and were purchased from Fluka, Merck and Sigma Chemical Companies.

Plant collection and extraction

Different part of *Physalis alkekengi* including leaves, fruits and calyx were collected from Tonekabon region and samples were dried at room temperature in dark and powdered. 30 gram of each powder samples were extracted in 300 ml of ethanol and kept at room temperature for 48 hours. Then, the extracts were filtered and the solvent was evaporated on a rotary device at 40°C. The remaining was kept in refrigerator at 4°C for further tests (14).

Measurement of anthocyanin

To measure the total anthocyanin content, 0.02 g of dried plant sample was pulverized with 4 ml of hydrochloric acid containing 1% methanol in a porcelain mortar. The solution was kept in the refrigerator for 24 hours and then, centrifuged for 10 minutes at 13000 g. The supernatant was removed and absorbance of the extract was measured at 530 and 657 nm against the control (hydrochloric acid containing 1% methanol). The anthocyanin content of each extract was calculated using the following equation (15).

$$A = A_{530} - (0.25 \times A_{657})$$

Where, A is absorbance of the solution (subscripts indicate the wavelength at which the absorbance is measured).

Measurement of total phenolic content

The total phenol content was determined by the Folin–Ciocalteu method (16). A volume of 2.8 ml of distilled water, 100 µl of Folin–Ciocalteu reagents and 2 ml of sodium carbonate 2% (weight/ volume) were added to 100 µl of supernatant and incubated for 30 minutes. The absorbance of sample was measured at 720 nm compared to the control. Gallic acid was used for the preparation of calibration curve (20-200 mg L^{-1}). The data was expressed as mg gallic acid equivalents (GAE) g^{-1} DW.

Measurement of total flavonoid content

To measure the amount of total flavonoid, 1.5 ml ethanol, 100 ml aluminum chloride solution 10%, 100 µl acetate potassium 1 M and 2.8 ml distilled water were added to 500 µl of each extracts. After 40 minutes, absorbance of the mixture was measured at 415 nm compared to the control. Quercetin was used for the preparation of calibration curve (20-200 mg L^{-1}). The total flavonoid content of the extract was described as mg quercetin equivalents (QE) g^{-1} FW (17).

Measurement of carotenoid content

0.05 gram of fresh plant tissue was homogenized with 5 ml acetone in a cold porcelain mortar and ice bath. Then, 1 g

anhydrous sodium sulfate was added to the filtrated solution and was filtrated by filter paper. Solution was made to 10 ml volume by adding acetone and was centrifuged at 2600 g for 10 min. Supernatant was collected and solution absorbance was measured in 662, 645 and 470 nm in against to acetone blank. The amount of carotenoids was measured by following formulas for each extract (18).

$$C_a = 11.24\ A662 - 2.04\ A_{645}$$

$$C_b = 20.13\ A645 - 4.19\ A_{662}$$

$$C_t = 1000\ A_{470} - 1.9\ A_{Ca} - 63.14\ A_{Cb} / 214$$

In these formulas Ca, Cb, and Ct: the amount of chlorophyll a, b, and carotenoid, A_{470}: absorbance in 470 nm (for carotenoid), A_{645}: absorbance in 645 nm (for chlorophyll a), A_{662}: absorbance in 662 nm (for chlorophyll b).

Antioxidant activity assays by DPPH method

Scavenger 2, 2-diphenyl-1-picrylhydrazyl (DPPH) with slight modifications of the method described by Miliauskas *et al.,* (19) Method. Briefly, the concentrations (100–2000 µL) of extracts were prepared in ethanol. DPPH solution (0.004%) was prepared in ethanol and 2 ml of this solution was mixed with the same volume of ethanol extracts and standard ascorbic acid solution separately. The mixture was incubated for 30 minutes in the dark at room temperature and the absorbance was measured at 517 nm. The results were expressed as inhibition of free radical by DPPH in percent (%I), and calculated by using the equation:

$$\%I = [(A_{control} - A_{sample}) / A_{control}] \times 100$$

Where $A_{control}$ is the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of the sample. IC_{50}, which denotes the amount (mg) of plant extract that inhibits DPPH radicals by 50%, was calculated from the plotted graph of inhibition percentage against concentration. Ascorbic acid (AA) was used as a standard and results were expressed as ascorbic acid equivalent antioxidant activity (AEAC) using the following equation:

$$AEAC\ (mg\ AA/g\ dw) = IC_{50\ ascorbate} / IC_{50\ sample} \times 1000$$

Assessment of cell viability

A549 cell line was purchased from Pasteur Institute of Iran. This cell line was cultured in a media containing enriched RPMI 1640 with 10% Fetal Bovine Serum (FBS), streptomycin antibiotics (100µg/ml) and penicillin (100 units /ml) and kept in an incubator at 37°C, 5% CO_2 and 95% humidity. All above phases were conducted under laminar hood and in a completely sterilized environment. The culture containing cell line was transmitted to a falcon and centrifuged at 1250 rpm ×8 min in order to sub-culturing the cell line. Then 5ml of the medium culture was added to the sediment and piptage was done slowly to solve the sediment. After counting cells to determine their density, it was transmitted to a new flask and the flask was transmitted to a CO2 incubator with 95 percent humidity and 37°C temperature.

Cell viability was measured by 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. For this purpose, 104 cells were put in each well of 96-well plates. After 24 hours, concentration 31.25, 62.5, 125, 250, 500, 1000, and 2000 (µg ml-1) of *Physalis alkekengi* ethanolic extract were added to each well at 24, 48, and 72 hours. In each interval, by ELISA reader sample absorption in 540 nm wavelength. Viability percentage was calculated by the following formula (20).

$$Viability\ (\%) = sample\ OD / control\ OD \times 100$$

Statistical analysis

All measurements were conducted with three replications and data were reported as mean ± standard deviation (SD). The data were analyzed using GLM procedure by SPSS software (Ver.16) and Tukey's multiple range tests was used for mean comparisons at 5 % probability level. SPSS software was used to calculate the correlation coefficient (Pearson) between characteristics. Microsoft excel 2013 software was used for the preparation of figures.

Results

Antioxidant activity

The antioxidant activity was significant in different parts of *Physalis alkekengi.* The results indicated that antioxidant activity was low in leaf while increased in calyx and orange fruit. The highest activity was obtained in orange fruit as 1807.01±0.23 (mg g^{-1} DW) (Figure 1).

Phenolic, flavonoid, *anthocyanin,* and Carotenoid contents

The results showed that the effect of different parts of *Physalis alkekengi* on total phenol, flavonoid and ***anthocyanin*** content was different, with the highest amount of these compounds in leaf and the lowest total phenol content in fruit and calyx.

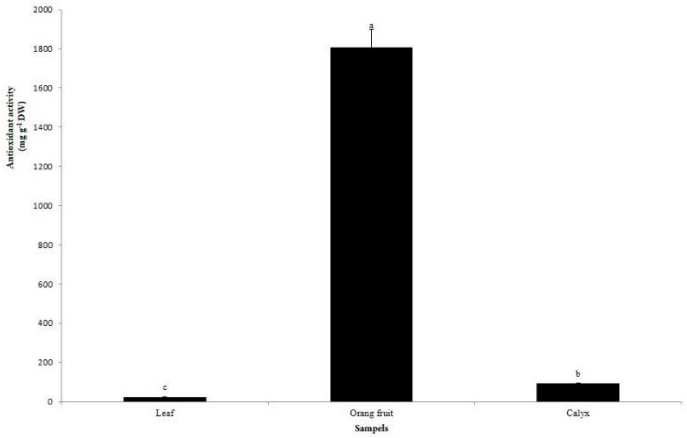

Figure 1. The effect different part of *Physalis alkekengi* on antioxidant activity. The data represent the mean of three replications and error bars indicate SD. The same letters above the bars indicate no significant differences ($p < 0.05$)

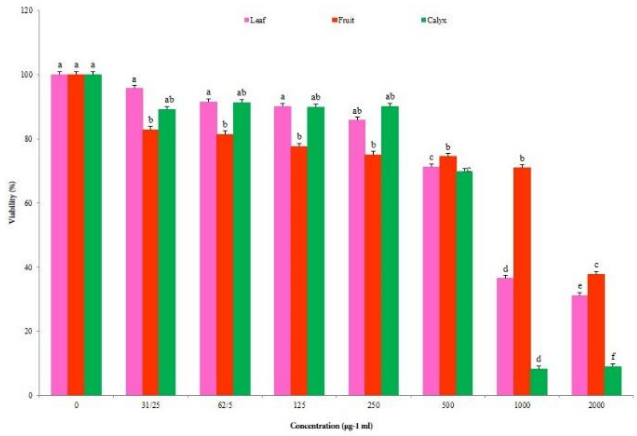

Figure 2. Effect of different concentration of leaf, orange fruit, and calyx *Physalis alkekengi* extracts on the viability of cancer cells within 24 hours

In addition, the results indicated that the carotenoid content was low in leaves, but the highest amount of that was observed in orange fruit recording 10.05±0.91 mg g^{-1} FW (Table 1).

Anticancer potential of *Physalis alkekengi* leaf, orange fruit, and calyx extracts in 24 hours

The results indicated that various concentrations of leaf, orang fruit, and calyx of *Physalis alkekengi* extracts inhibit cancer cells growth while the percentage of inhibition for each of extracts at the concentration of 2000µg^{-1}ml recorded 68.8%, 62.38%, and 91.03%, respectively. Therefore, the results indicate that inhibitory effect of calyx extract is more than that of leaf and fruit extracts (Figure 2).

Anticancer potential of *Physalis alkekengi* leaf, orange fruit, and calyx extracts in 48 hours

Generally, the results indicated that different concentration of leaf, orang fruit, and calyx of *Physalis alkekengi* extracts inhibit cancer cells growth while the highest inhibitory effect for each extract of leaf, orang fruit, and calyx obtained 83.74%, 86.77%, and 92.31% in 2000µg^{-1}ml, respectively. Therefore, the results demonstrated that inhibitory effect of calyx extract is more than that of other extracts. Further, the growth of cancer cells significantly decreased in concentrations from 250 to 2000 µg $^{-1}$ml extracts of calyx in comparing with leaf and fruit (Figure 3).

Table 1. The effect different part of *Physalis alkekengi* on non- antioxidant compound. The data represent the mean of three replications ±SD and similar upper case letters indicates no significant difference at $p < 0.05$

Samples	Total phenol (mg EGA g^{-1} DW)	Total flavonoid (mg EQ g^{-1} DW)	Total anthocyanin (mg g^{-1} DW)	Carotenoid (mg g^{-1} FW)
Leaf	31.7±2.48 ^{a}	5.31±0.13 ^{a}	22.2±0.391 ^{a}	4.71±0.041 ^{b}
Orang fruit	8.94±1.18 ^{c}	0.231±0.019 ^{c}	10.8±0.117 ^{b}	10.1±0.091 ^{a}
Calyx	18.5±1.18 ^{b}	1.98±0.111 ^{b}	5.52±0.214 ^{c}	3.21±0.059 ^{c}

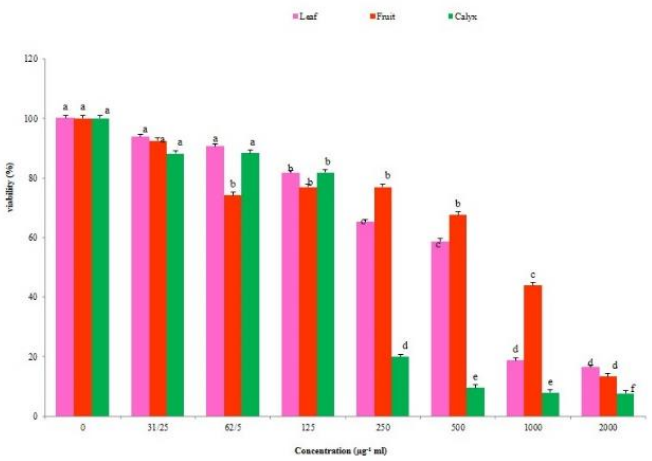

Figure 3. Effect of different concentration of leaf, orange fruit, and calyx *Physalis alkekengi* extracts on the viability of cancer cells within 48 hours.

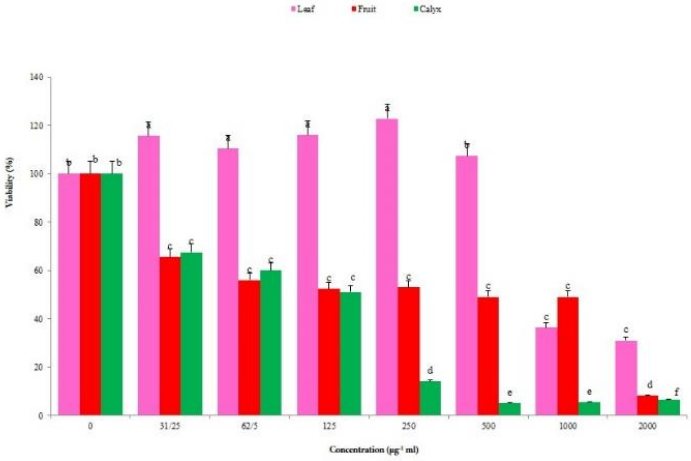

Figure 4. Effect of different concentration of leaf, orang fruit, and calyx *Physalis alkekengi* extracts on the viability of cancer cells within 72 hours.

Anticancer potential of *Physalis alkekengi* leaf, orange fruit, and calyx extracts in 72 hours

Overlay, the results indicated that different concentrations of *Physalis alkekengi* leaf, orang fruit, and calyx extracts prevent cancer cells growth while maximal inhibitory effect for each extract was observed in 2000µg^{-1}ml and the inhibition rate of calyx extract was more than that of leaf and fruit. In addition, cancer cells growth significantly decreased in concentrations from 250 to 2000 µg^{-1}ml extract of calyx in comparing with that of other extracts as the inhibition rates were 94.95%, 94.49% ,and 93.54% in concentrations of 500, 1000 ,and 2000 µg^{-1}ml, respectively. Moreover, the inhibition percentage of leaf extract in concentrations 250, 500, and 1000 µg^{-1}ml was significant comparing to fruit extract especially in 1000 µg^{-1}ml (Figure 4).

Correlations among measured factors and cancer cells growth inhibition by *Physalis alkekengi* leaf, orange fruit, and calyx extracts

The results of correlations among factors were given in Table (2 a and c). The result showing in table 2a, there is a positive and significant correlation between cancer cells growth inhibition and ***anthocyanin*** content of leaf ($p \leq 0.05$). Therefore, by increasing ***anthocyanin*** content of *Physalis alkekengi* leaves, the inhibition rate of growth of cancer cells enhanced. In addition, the results indicated that there was a correlation between leaf flavonoid and total phenolic contents and cancer cells growth inhibition, but that was not significantly. Regarding orang fruit extract, there was a positive and significant correlation between inhibition of cancer cells growth and *anthocyanin* content at the level of 1% and carotenoid content at the level of 5% (Table 2 b). Also, the results shown that there was positive correlation between total phenolic content of fruit extract and cancer cells growth inhibition, but it was not significantly. In terms of calyx extract, there also was a positively significant correlation between cancer cells growth inhibition and carotenoid content at the level of 1% (Table 2 c).

Discussion

In recent years, the use of natural compounds was focused because of their ability in inhibiting cancers, less side effects, and hopeful effectiveness. Researches were conducted to investigate the anti-cancer effects of medicinal plants and native to countries (21, 22). Medical therapy has been one of the useful methods to cure patients with cancers and different research centers in the world and in Iran try to provide effective drugs with selective impacts on cancer cells and less effect on healthy cells (22, 23). Medicinal plants are great and hopeful sources to detect new drugs. Therefore, *Physalis alkekengi* plants were collected from their natural habitats and then studied. The results of this study indicated that different concentrations of *Physalis alkekengi* ethanolic extract inhibited lung cancer A_{549} cell line and significantly decreased their growth in comparison with the control. It has also been shown that the extract of the

Physalis alkekengi in a concentration-dependent manner inhibited the growth of these cells, so that by increasing the concentration of the extract, it increased the percentage of growth inhibition, and the highest percentage was 94.97% at the concentration level of 2000µg/ml after 72 hours. Moreover, the findings indicated that *Physalis alkekengi* extracts contained antioxidant compounds like flavonoid, phenol, carotenoid, and anthocyanin and that there was a positively significant correlation among flavonoid and anthocyanin contents and the cancer cells growth inhibition; accordingly, the increases in their contents in plant enhanced cells growth inhibition. The compounds may inhibit cell cycle or activate their checkpoints to prevent DNA replication and/or activate internal and external pathways of apoptosis (24, 25).

Table 2 a. Correlation between growth inhibition of cancer cells and some non-enzymatic antioxidant compounds of the *Physalis alkekengi* leaf.

	Phenol content	Flavonoid content	Anthocyanin content	Carotenoid content	Antioxidant activity	Inhibition of cancer cell growth
Inhibition of cancer cell growth	0.310 ^{ns}	0.310 ^{ns}	0.638 *	-0.173 ^{ns}	0.186 ^{ns}	**1**
Antioxidant activity	0.255 ^{ns}	0.215 ^{ns}	0.875 **	0.445 ^{ns}	1	
Carotenoid content	0.572 ^{ns}	0.352 ^{ns}	0.538 ^{ns}	1		
Anthocyanin content	0.765 *	0.795 *	1			
Flavonoid content	0.981 **	1				
Phenol content 1						

** Correlation is significant at 0.01 levels.
* Correlation is significant at 0.05 levels.
^{ns} Correlation is not significant.

Table 2 b. Correlation between growth inhibition of cancer cells and some non-enzymatic antioxidant compounds of the *Physalis alkekengi* orange fruit.

	Phenol content	Flavonoid content	Anthocyanin content	Carotenoid content	Antioxidant activity	Inhibition of cancer cell growth
Inhibition of cancer cell growth	0.142 ^{ns}	0.310 ^{ns}	0.284 ^{ns}	0.909 **	0.986 **	**1**
Antioxidant activity	0.981 **	0.235 ^{ns}	0.812 **	0.135 ^{ns}	1	
Carotenoid content	-0.323 ^{ns}	-0.996 **	-0.689 ^{ns}	1		
Anthocyanin content	0.909 **	0.765 *	1			
Flavonoid content	0.417 ^{ns}	1				
Phenol content	1					

** Correlation is significant at 0.01 levels.
* Correlation is significant at 0.05 levels.
^{ns} Correlation is not significant.

Table 2 c. Correlation between growth inhibition of cancer cells and some non-enzymatic antioxidant t compounds of the *Physalis alkekengi* orange fruit.

	Phenol content	Flavonoid content	Anthocyanin content	Carotenoid content	Antioxidant activity	Inhibition of cancer cell growth
Inhibition of cancer cell growth	0.195 ^{ns}	0.520 ^{ns}	0.995 **	0.765 *	0.886 **	**1**
Antioxidant activity	0.322 ^{ns}	0.866 **	0.678 *	-0.813 **	1	
Carotenoid content	-0.813**	-0.995 **	0.123 ^{ns}	1		
Anthocyanin content	0.220 ^{ns}	0.477 ^{ns}	1			
Flavonoid content	0.752 *	1				
Phenol content	1					

** Correlation is significant at 0.01 levels.
* Correlation is significant at 0.05 levels.
ns Correlation is not significant.

Previous studies indicated that antioxidant compounds like phenolic acids, polyphenols, and flavonoids prevented or suppressed free radicals like hydroperoxide, superoxide, and hydrogen peroxide and also prevented oxidative processes resulting damage or mutation in genome (24, 25). Flavonoids prevented immune and cell processes related to cancer development like cell proliferation, cell differentiation, and generating new vessels (26). Other reports stated that quercetin and poly hydroxulated flavonoids inhibited cancer cell growth *in vitro* and decreased DNA generation 14% comparing to control group and prevented passing cells from G1 phase of cell cycle to S phase (27). Some researches stated that *Physalis alkekengi* contained antioxidant compounds with cytotoxic effects these compounds could induce apoptosis pathway and decrease tumor cells growth by activating autophagy-related apoptosis pathway and transcription factor BOX_2 (28, 29). Although studying about the cytotoxic effects of *Physalis alkekengi* extracts is very low, some reports indicated that plant extracts displayed different anti-cancer effects in various cancers or even on same cell lines. In most studies, there was a correlation between phenolic contents and the extracts antioxidant capacities and their effect on cancer cells. The compounds affected cancer cells through different ways; while in most studies compounds of plant extract induced apoptosis in cancer cells and then increased caspase3 gene expression. Some reports have also shown that extract compounds increased antioxidant enzymes like superoxide dismutase, catalase, and glutathione peroxidase and non-enzymatic antioxidant compounds removing free radicals and decreased new cancer cells generation (30, 31). The biochemical pathways and extract mechanisms of *Physalis alkekengi* extracts for inhibiting lung cancer cells were not investigated by this study, but this study demonstrated that *Physalis alkekengi* leaf, calyx, and fruit extracts contain high amount of antioxidant compounds and there was a positively significant correlation between antioxidant compounds and cancer cells growth inhibition. It indicated that the compounds may inhibit cancer cells growth through one of the mentioned pathways.

Conclusion

The results of this study indicated that ethanolic extract of various parts of *Physalis alkekengi* contains different antioxidant amounts. In addition, the extracts of this plant can inhibit cells growth because of their dose or concentration dependent effects on A549 cancer cell line. Therefore, future pharmacological studies can be useful in curing cancers or may be hopeful for providing an anti-cancer drug without side effects.

Conflict of Interest: 'None declared'.

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