Original Article

Anti-Angiogenic and Anti-Proliferative Effects of Physalis Alkekengi Hydroalcholic Extract on Breast Cancer in Mice

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Received: 13 Sep 2020 Accepted: 16 Nov 2020

Abstract

Background & Objective: The Physalis alkekengi is a herbaceous plant belonging to Solanaceae family. According to research, this plant can help inhibit cancer cell growth.
Materials & Methods: In this study, the extract of Physalis alkekengi was used to suppress angiogenesis in Balb/c mice. First, the MC4L2 cells were injected in the mice breastbone and the tumor began to grow in this area, then the mice were killed and the tumor removed from the mice and inserted in the test mice by surgical technique. Then, the mice were gavaged for 3 weeks at 50mg/kg and 100mg/kg daily Physalis alkekengi extract, and then the mice were killed and their tumor tissue removed, and tumor growth, apoptotic cell tissue and expression of VEGF gene were studied.
Results: The results of this study showed that the hydroalcholic extract of Physalis alkekengi had a significant effect on tumor growth reduction in experimental groups 2 (50mg/kg body weight) and 3 (100 mg/kg) compared to the control group. Also, the results of the apoptotic cell in tissue showed that the extract of Physalis alkekengi significantly induced apoptotic and necrotic cells in experimental groups in comparison with the control group. The results of RT- PCR showed that the expression of VEGF gene in the experimental groups (50 mg/kg body weight) was significantly ($P < 0.001$) less than that of the control group.
Conclusion: In the end, it was concluded that the extract of the Physalis alkekengi has anti-tumor properties and can be considered as an anti-cancer drug in future clinical studies.

Keywords: Anti-angiogenic, Breast Cancer, Physalis Alkekengi

Introduction

Breast cancer is the most widespread cancer among women. In less developed countries, it is the first cause of cancer death in women, and in more developed regions it is the second (1, 2). Thus, a great threat to the health system and imposed substantial cost on patients and society. Among the various types of cancer, breast cancer, which accounts for 23% of all cancers in women, is the most common cancer and the most lethal malignancy among women, and one of the most important causes of the health concern in women (3). According to the latest statistics from the Cancer Research Center in Iran, about 8500 new cases of breast cancer are reported annually in the country. Also, 1400 people die of breast cancer; there are currently around 40,000 people living with this disease in the country (4, 5). In the past few decades, there have been a number of advances in prompt diagnosis and treatment of breast cancer. But the high prevalence of relapse and progression after conventional therapies point to a need for new therapeutic strategies for definitive treatment and reduce complications of breast cancer(6, 7).

The term angiogenesis is referred to the vascularization from preexisting blood vessels. This phenomenon is the requirement for tumor growth and malignancy. If the high amount of vascularization is achieved, tumor growth and metastatic behavior are occurred (8).

For the induction of non-differentiated stromal cells to the site of tumor growth, growth factors

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and other molecules are released to occur the metastatic behavior. By providing tumor cells with the necessary signals, these cells, also known as Mesenchymal Stem Cells (MSCs), enable tumor growth. MSCs by means of chemokine secretion are shown to have vital role in breast cancer progression and metastasis (9). Therefore, impeding tumor growth through anti-angiogenic is of research interest.

Physalis Alkekengi is a herbaceous, perennial plant. This plant has a wide phytochemical profile including Steroids, Flavonoids, Alkaloids, and Terpenoid (10). Various investigations have shown that P. Alkekengi has anti-inflammatory, anti-bacterial (11), the inhibitory of tumor proliferation (12) properties. Physalin A in a dose-dependent manner inhibited the growth of H292, H460, H358, H1975 and A549 cells from lung cancer with IC50 values ranging from 5 to 30 μM (13). Physalin B was specifically active against human A375 and human A2058 melanoma cells (IC50 <4.6 μg/mg) and had low cytotoxicity against H9c2, T/G HA-VSMC and CCD-966SK (14). Further studies showed that Physalin B at a dose of 3 μg/mg induced apoptosis in a mechanism similar to that of Physalin A by activating Noxa, Caspase 3, and Bax in A375 cells(15). Physalin A showed an effect on apoptosis in HT1080 cells by increasing caspases 3 and 8 expression(16).

In this research, the anti-tumor mechanism of Physalis Alkekengi Hydroalcoholic Extract (PAHE) (PA), was evaluated especially on the pathway of angiogenesis as one of the important pathways in the dispersion of cancer cells. In this regard, the expression of VEGF gene as the main agent of angiogenesis was assayed in Balb/c mice model that MC4L2 cells were injected into the breastbone of these mice.

Materials & Methods

Extract preparation

Physalis alkekengi fruits were collected from Golestan province in northern Iran in the summer of 2017. A sample was sent to Herbarium of Pharmacy Research Center of Tehran University of Medical Sciences and was verified. Before implementing extraction procedure, the fruits were dried and powdered. Dried Chinese lantern fruits were extracted with 15mg ethanol 70% and 1% acetic acid and filtered. By means of rotary evaporator, the solvent was evaporated in vacuum conditions and the hydroalcholic extract were put in -70°C freezer until use.

Experimental groups

Four groups (control and groups I-III) of male Wistar rats [With similar conditions: gender (male) and age (three months old)] with body weight ranging from 200-250 g were chosen randomly and without any tension, from the Laboratory Animal Center of Medical Branch, Islamic Azad University. The animals had free access to food and water, maintained on a 12 h light/12 h dark cycle in a temperature-controlled environment (22°C). The studies were performed in accordance with guidelines established by the university research center (Department of Biology, School of Basic Sciences, Science and Research Branch, Islamic Azad University, Tehran, Iran) and all animals received human care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institute of Health (NIH publication 86-23 revised 1985). Usage of animals was in accordance with the ethics committee of the Science and Research Branch, Islamic Azad University, Tehran, Iran. Ethics approval was made for this research.

The administration of Chinese lantern extracts to ER^+ breast carcinoma BALB/c mice

The ER^+ breast carcinoma BALB/c mice were divided in four groups:

Control group: Seven ER^+ breast carcinoma BALB/c mice without any treatment.

The group I: Seven ER^+ breast carcinoma BALB/c mice were treated with 10 mg PAHE (per kg body weight in 1mL) daily and continued for 21- days.

The Group II: Seven ER^+ breast carcinoma BALB/c mice were treated daily with 50 mg PAHE (per kg body weight in 1mL) daily and continued for 21- days.

The Group III: Seven ER^+ breast carcinoma BALB/c mice were treated daily with 100 mg PAHE (per kg body weight in 1mL) daily and continued for 21- days.

Sampling from tumor tissue

For this purpose, animals were killed by breaking cervical vertebra. Then, a part of the tumor tissue of the animal after isolation was placed in a sterile medium for histological examination in 10% formalin, and the other part was transferred to sterilized cryovial for

subsequent molecular investigations and was frozen in liquid nitrogen.

Reverse transcriptase polymerase chain reaction

RNeasy Mini plus Kit (Qiagen, Valencia, CA, USA) was used to extract RNA from tumor tissue following the manufacturer's protocols.

After the extraction of RNA, its quality and quantity were investigated using UV spectrophotometric techniques or using the Nanodrop device (2000 Thermoscientific Inc. USA) and gel electrophoresis.

The synthesis of cDNA was done using 0.2 µg /µl Random Hezamer, 0.5µg/µl Oligo dT and 100U MMULV Enzyme, 10 µl RNA. Samples were placed at 65°C for 5 minutes. Then it was immediately put into ice. The tubes were placed at 42°C for 1 hour.

RT-PCR was performed using specific primers for VEGF and β-actin (as internal control) mRNAs (table 1).

The following PCR cycling conditions were used to amplify VEFG and β- actine genes: an initial denaturation step at 95°C, then 45 amplification cycles composed of denaturation at 95°C for 15 s, annealing at 60°C for 30 s and an extension at 72°C for 30s.

Results

Tumor growth

Based on the results, it was observed that using PAHE can limit the growth trend and dimensions of steroid receptor breast tumors at the end of the study significantly. The results of examination and comparison of cancer cells showed that the growth trend of the tumor between the control and group I (10 mg/kg body weight), seems that 10 mg/kg body weight of PAHE significantly limited tumor growth in day 16 after starting treatment ($P \leq 0.05$). Comparison of tumor growth trend between control and groups II and III (50 and 100 mg/kg body weight, respectively) indicated that these doses of the extract significantly decreased tumor growth from day 8 to 12 after starting treatment ($P \leq 0.01$) . Shown in chart 1.

Table 1. The sequences of primers used in this research

Primers	Sequences
VEFG (F)	5'-AACGATGAAGCCCTGGAGTG-3'
VEFG (R)	5'-AACAAGGCTCACAGTGAACG-3'
β- actine (F)	5- CGGTTCCGATGCCCTGAGGCTCTT-3
β- actine (R)	5- CGTCACACTTCATGATGGAATTGA-3

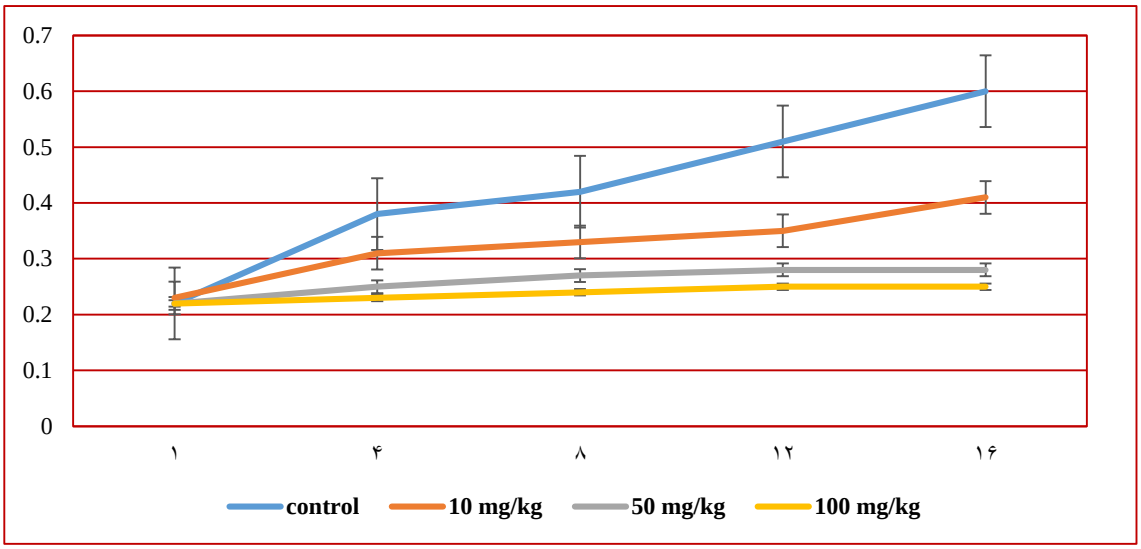

Chart 1. The tumor growth trend on days 1, 4, 8, 12, and 16 in the control and treatment groups (* p <0.05, ** p <0.01, *** p <0.001) (X±SE)

Histopathological evaluation

Histopathologic study of apoptotic and necrotic cells was performed by H & E staining ((Haemotoxylin and Eosin) by independent pathobiologist. As shown in chart 2 (A and B), the use of PAHE after 16 days at 50 mg/kg body weight significantly increased the apoptotic cells, while at a dose of 100 mg/kg induced necrosis in these tumors.

Analysis of RNA quality

The results of the investigation of the extracted RNA quality by spectrophotometer showed that in the ratio of 260/280 OD, all extracted samples were about 1.92 to 1.97, indicating good quality of RNA extraction.

Also, investigation of RNA molecule extracted using gel electrophoresis method showed that the purification of the RNA molecule has a good quality (figure 1).

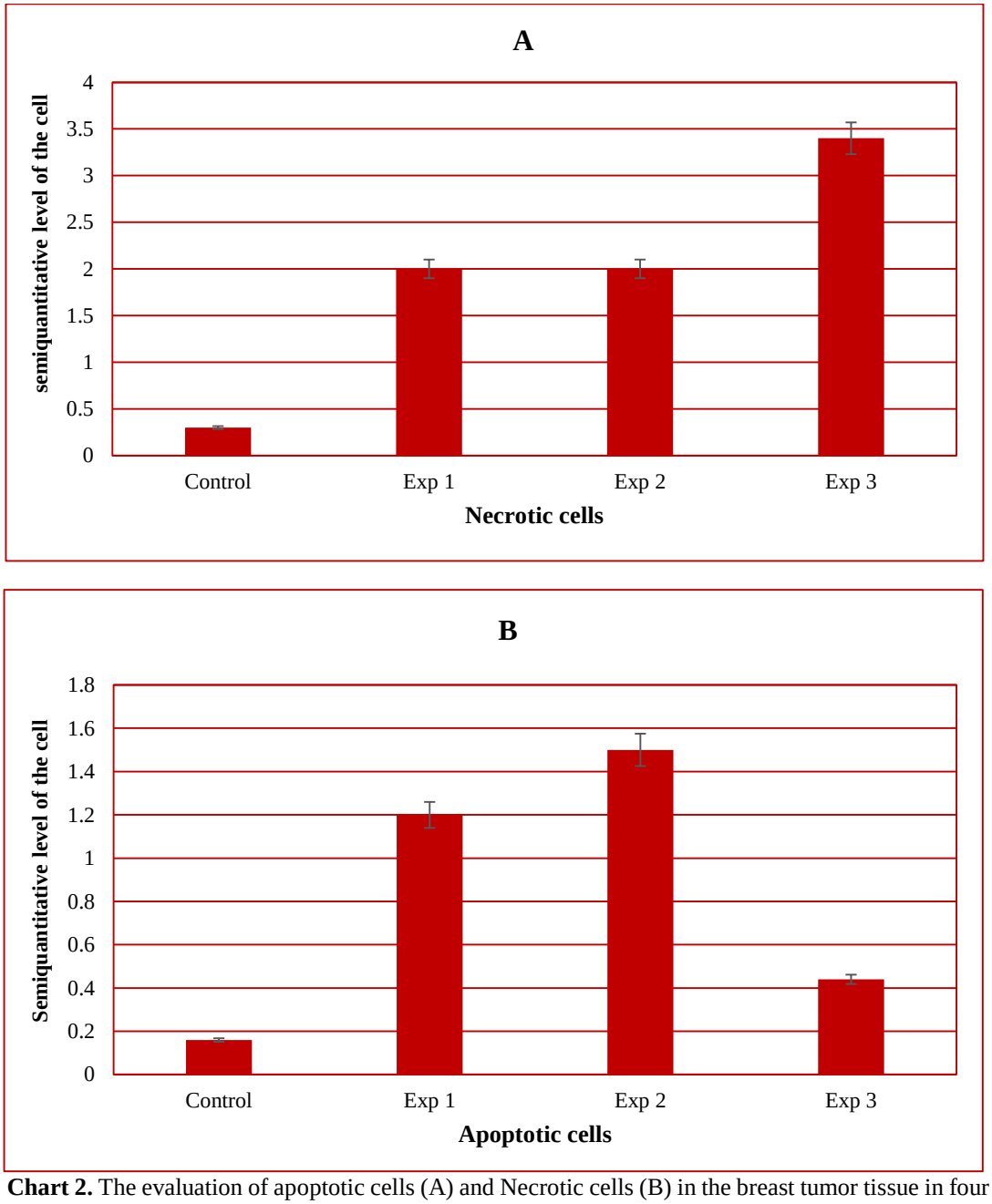

Chart 2. The evaluation of apoptotic cells (A) and Necrotic cells (B) in the breast tumor tissue in four studied groups; control and treated with hydroalcholic extract with doses of 10 mg/kg, 50 mg/kg and 100 mg/kg (* $p<0.05$).

Analysis of cDNA synthesis

The amplification of the target genes was performed in 35 cycles. After the completion of the amplification reaction, 5 µl of the sample was taken on a 1% agarose gel to evaluate the quality of the genetic fragments. Expected bands for VEGF and B-actin genes are shown in figure 2.

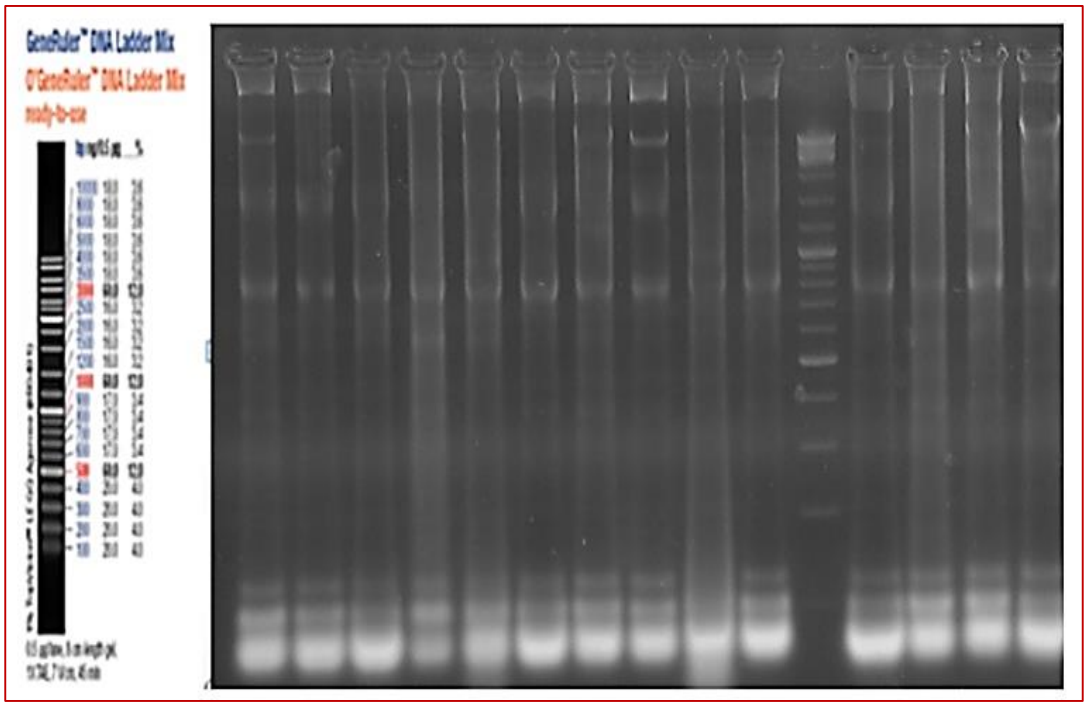

Figure 1. Extraction of RNA from tumor tissue on 1% agarose gel.

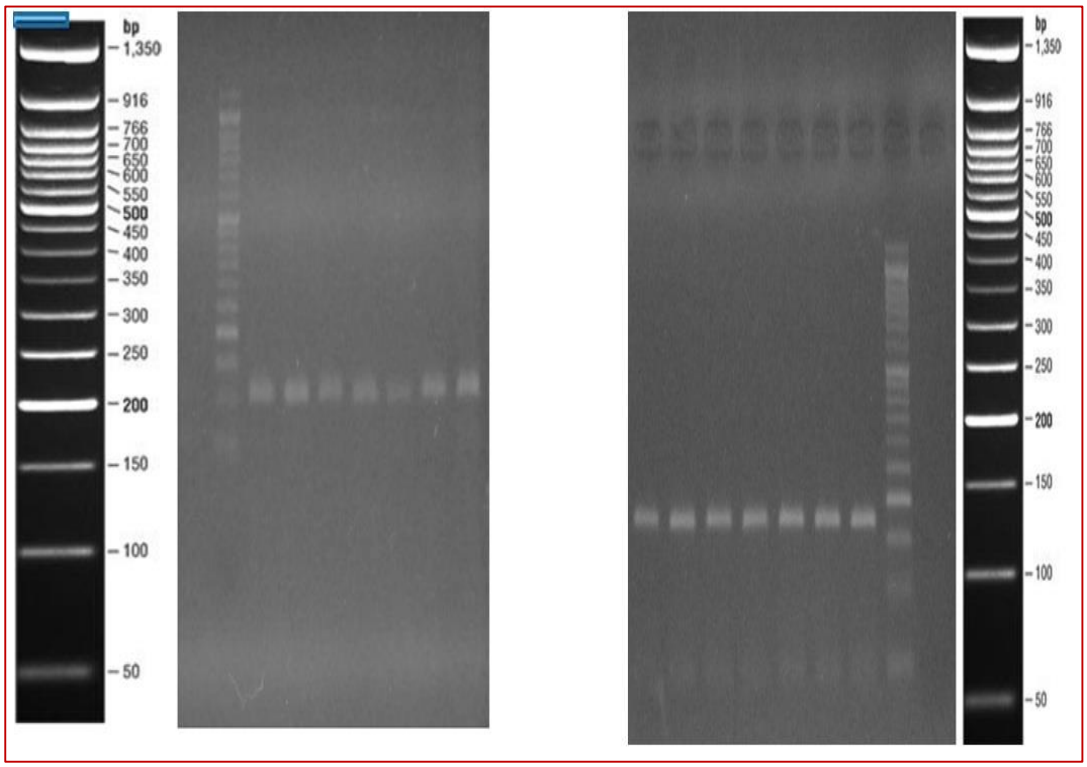

Figure 2. PCR product of VEGF gene (right) and internal control (left) cDNAs

Analysis of the melting curve of VEGF and β-Actin genes

The results of the analysis of the melting curve of the control and the target genes have been shown (figure 3). The same melting curve indicates the absence of a non-specific product. Both diagrams are consistent as shown in the figure 3, and therefore there is no by-product in the RT-PCR reaction.

The evaluation of VEGF gene expression using RT- PCR

After performing RT-PCR, the data were calculated in the corresponding formulas and the $2^{-\Delta\Delta Ct}$ values were evaluated using SPSS version 23 and Tukey and One-Way ANOVA tests and $P < 0.05$ was considered as significant level. According to the statistical analysis, the level of VEGF gene expression (chart 3) decreased compared with the internal reference gene (β-Actin) ($P < 0.001$). Also, the amount of gene expression in the 50 mg/kg body weight group was significantly reduced compared with the control group ($P < 0.001$).

Discussion

In the present study, the effect of PAHE on the expression of VEGF (Vascular

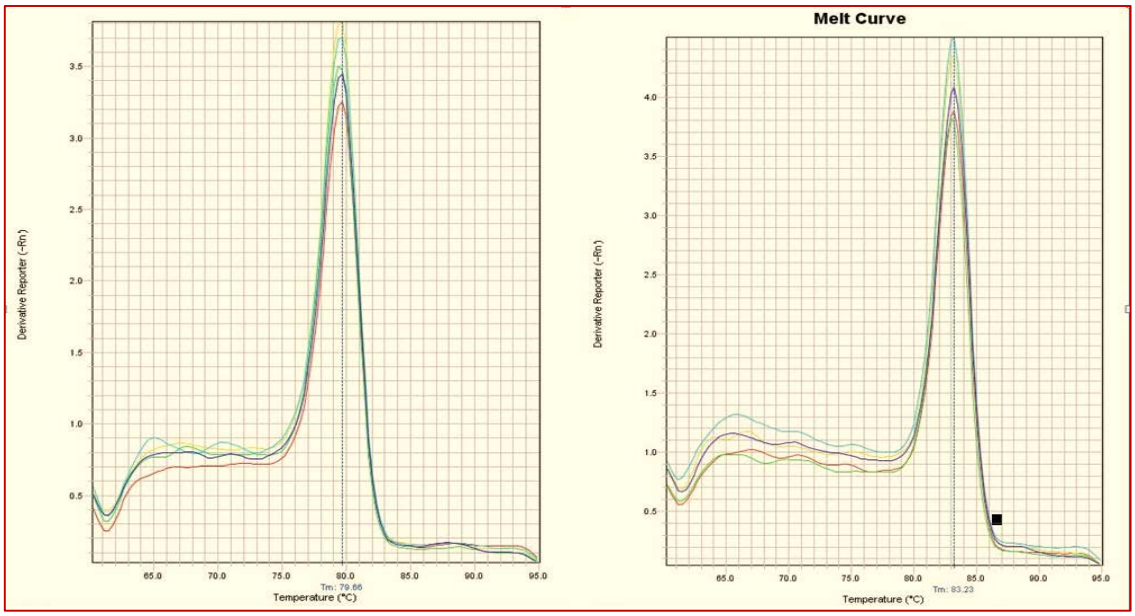

Figure 3. Melting curve of VEGF genes

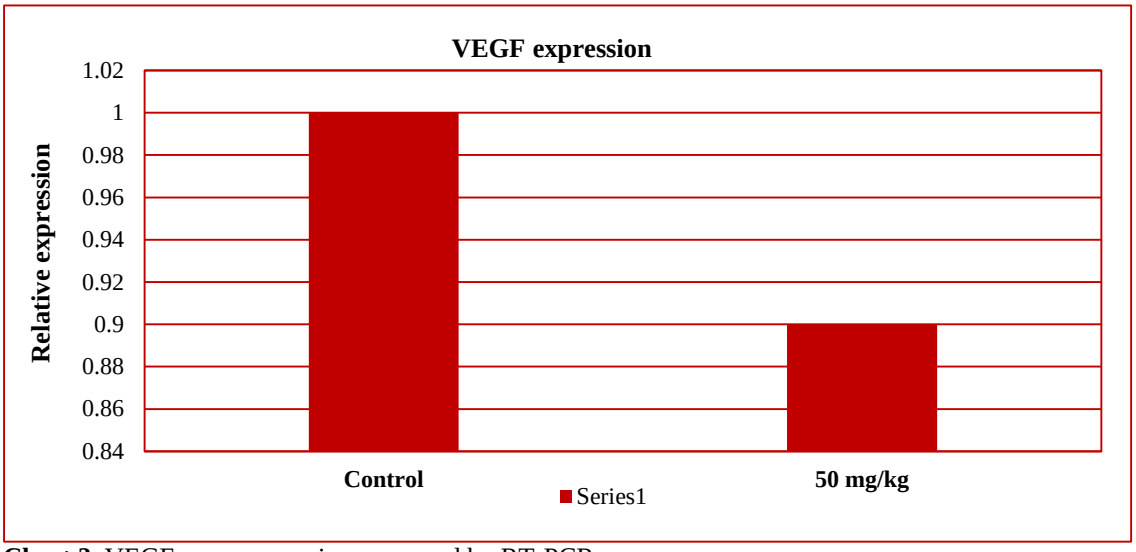

Chart 3. VEGF gene expression measured by RT-PCR

endothelial growth factor) gene, apoptosis and necrosis of cancer cell in mice with breast cancer were studied. The results showed that concentrations of 50 and 100 mg/kg body weight of Physalis alkekengi had a significant effect on decreasing the expression of VEGF gene and increasing apoptosis and necrosis of tumor tissue cells.

Various angiogenic factors are need for the tumor cells to establish angiogenesis. In breast cancer angiogenesis, the secretion of VEGF is apparent (17). Amongst the multiple angiogenic factors, the VEGF signaling pathway functions as the key regulator of tumor neovascularization (18). Our results showed that the PAHE decrease the VEFG gene expression and hence reduced angiogenesis and potential metastasis. To the best of our knowledge, this is the first study that shows the potential anti-angiogenesis effect of PAHE based on VEGF mRNA expression, tumor cells apoptosis and necrosis assays in mice.

The anti-cancer effects of other species of Physalis spp. were shown. In the study conducted by Chiang et al. (1992), it was found that physalisin B and physalin F isolated from Physalis angulata had an inhibitory effect on human leukemia cells(19). In a study, it was found that physalin B and D isolated from Physalis angulate had anti-tumor effects both in vitro and in vivo. However, the molecular mechanism of cancer cells death has not been studied in that study. Our results showed that the tumor cell death responsible for compounds in Physalis Alkekengi imposed their effects by reducing angiogenesis factors such as VEGF gene. In another study done by Damu et al, the extract of Physalis angulate showed cytotoxic effects on tumor cells (20). In this study, the biological evaluation of these compounds against the panel of human cancer cells is indicative of extensive cytotoxic activity. In another study conducted by HangZhang et al., (2009), it was suggested that a new physalin in the extract of the Physalis alkekengi has been isolated that has cytotoxic effects. The novel physalin was 5α-hydroxy-25,27-dihydro-4,7-didehydro-7-deoxyneophysalin A, which has cytotoxic effects on PC-3 and LNCaP cell lines (21). The results of current study are in line with aforementioned studies and the anti-angiogenesis effects of Physalis alkekengi are verified.

Conclusion

In general, it can be concluded that the PAHE has anti-cancerous and tumor inhibitory properties in a variety of cancers, including breast cancer, and it reduces tumor angiogenesis and increases apoptosis and necrosis of cancer cells.

According to the results of the current study, it seems that the effects of PAHE under clinical conditions and on human specimens can be of great importance, therefore, it is recommended for future studies.

Acknowledgments

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(VOUCHER NUMBER: PMP_1648).

Conflicts of Interest

The authors declare no conflict of interest.

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