

# The Therapeutic Efficacy of *Physalis Alkekengi* Hydro alcoholic Extract on Estrogen Receptor-Positive Breast Cancer Mice Model in an Autophagy Manner

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## ABSTRACT

**Objective:** *Physalis Alkekengi* has several biological activities. Our aim was to assess the anti-cancer effect of hydro alcoholic extract of *Physalis Alkekengi* on the estrogen receptor-positive breast cancer in mice model.

**Methods:** Twenty-eight ER+ breast cancer BALB/c mice (four groups each including seven members) were enrolled. The *P. Alkekengi* hydro alcoholic extract (10, 50 and 100 mg/kg) was administered for two weeks against EGFR2 cancerous cells. The tumor size, histopathological features, and mRNA expression amount of ATG5 Autophagy-specific gene were investigated.

**Results:** At the two higher doses (50 and 100 mg/kg), the *P. Alkekengi* hydro alcoholic extract inhibited the breast cancer growth. Consequently, there was a significant histopathological change in the breast cancer among the groups treated with *P. Alkekengi* compared to the control group ( $p=0.0189$ ). Additionally, the *P. Alkekengi* hydro alcoholic extract significantly enhanced the mRNA expression level of the ATG5 at 50 mg/kg. **Conclusions:** The results of this study observed that the *P. Alkekengi* hydro alcoholic extract exerted a promising anti cancer effect against estrogen- positive breast cancer through induction of the Autophagy pathway though needing more exact verification.

**Keywords:** *Physalis Alkekengi*, ER+ breast cancer, BALB/c mice, Autophagy

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## INTRODUCTION

Breast cancer (BC) is among leading malignancies among women all over the globe, being second cause of mortality rate due to cancer types. Owing to an uncontrolled growth of mammary cells following mutations in the genes regulating the development of cells, this neoplasia develops. However, the exact genetic and histopathological effects behind the mechanisms leading to the BC growth are unknown. Noticeably, most of them are estrogens-dependent diseases increasing the morbidity and mortality rates [1-3]. Several anti-BC therapeutic strategies including radiotherapy, mastectomy, chemotherapy, monoclonal antibodies, endocrine therapies and herbal medicine have been employed until today without a confirmed and established approach [4-10]. Targeting of cancerous cells is a promising strategy with this regard. Considering this promotion of Autophagy in BC cells can be an effective strategy [11,12]. Those autophagy mediator drugs such as phosphoinositide 3-kinase (PI3K) class III inhibitors including wortmannin/LY29002, 3MA and Vacuolar type H<sup>+</sup> - ATPase (V-ATPase) inhibitors such as bafilomycin A1 and concanamycin A leave side effects or stimulate resistance by carcinogenic cells [13,14]. Herbal Medicines (HMs) have been consumed as organic and non-toxic compounds for various ailments, such as cancer types. Nevertheless, the effects of various doses of these HMs or compounds are unknown need to be determined [15-17]. The *Physalis Alkekengi* (*P. Alkekengi*) has been consumed for its antioxidant activity and various therapeutic effects. In addition, at laboratory experiments, *P. Alkekengi* has improved the immune system, enzymes activities, and sexual and reproductive hormones [18-20]. Our subjective was assessment of the *Physalis Alkekengi* hydro alcoholic

extract efficacy against the estrogen receptor-positive BC in Mice Model.

## Materials and methods

### Cell Culture

The EGFR2 mouse BC cell line was obtained from genetic research center. EGFR2 was cultured in DMEM/F-12 with HEPES buffer (15 mM), streptomycin (100 µg/ml), l-glutamine, penicillin (100 µg/ml), Medroxy Progesterone Acetate (10 nM) (Sigma Chemicals, Ontario, Canada) and 10% FBS (Gibco BRL, Life Technologies). The cells were grown at 37°C in a humidified atmosphere with 5% CO<sub>2</sub> [21,22].

### Animals

Twenty- eight inbred female BALB/c mice (weighting 8-10gr) were purchased and their health was confirmed during 24hr keeping, with free access to food and water *ad libitum*. Mice keeping and experiments were performed following the relevant national and international guidelines of Weather all report.

### Tumorigenicity

EGFR2 cells were treated with 0.025% trypsin (rinsed with PBS and enzymatically neutralized using 10% FBS) then centrifuged at 1200 rpm for 3-5 min and resuspended in 10-fold excess culture medium. Eventually, a number of  $1 \times 10^6$  cells suspended in 0.1 mL serum-free medium was injected in the animal's right inguinal flank [22].

### Preparation of Hydro alcoholic Extract

Aerial parts of *P. Alkekengi* were purchased from mountains of Central Iran. A voucher specimen was taken as a reference and control at the Herbarium of Medicinal Plants Research Center, Tehran University of Medical Sciences, Iran. One-hundred grams of the sample being

entirely powdered dried was extracted by 500 mL of 1:1 ratio of 70% methanol and water in a percolator apparatus for 72 hr. Following extraction, the solvent was separated using filtration and then evaporated in a rotary evaporator at 40 °C. The dried extract weighed 40 gm indicating a 37% yield.

#### Experimental Animals

Among the 28 ER+ BC BALB/c mice models four (each including six members) groups were considered for 21 days. They included A: positive control group or extract untreated group; B: ER+ BC mice receiving 10 mg/kg of the hydro alcoholic extract in 1 mL water; C: ER+ BC group which received 50 mg/kg of the hydro alcoholic extract in 1 mL water; and D: untreated ER+ group which received 100 mg/kg of the hydro alcoholic extract in 1 mL water. Animals' health was daily and regularly monitored. The size of cancer was measured by a digital Vernier caliper (Mitutoyo, Japan) on a weekly basis, and reported as cm<sup>3</sup> using the following formula [23,24].

$V = 1/6\pi LWD$ , where L = length, W = width and D = depth.

#### Hematoxylin and Eosin Staining

The mice were euthanized 24 h after the last extract administration to prevent the *P. Alkekengi* hydro alcoholic extract reaction.

Finally, animals were euthanized with cervical dislocation. Tumor tissues were set, transferred and embedded in paraffin in 10% formaldehyde. The paraffin blocks were sectioned (3 µm) and stained with hematoxylin and eosin (H&E). The sections were detected by delayed histopathologist [25-26].

#### Expression analysis of ATG5 gene

Quantitative expression of the ATG5 gene was evaluated by real-time polymerase chain reaction (PCR) (RT-qPCR) technique using specific primers, table (1) [27]. Notably, the RNA was extracted from the mice with a TRIzol® reagent (Life Technologies) kit according to the manufacturer's guidelines, and DNase I digestion (Thermo Fisher Scientific, Waltham, MA, USA) treatment was used to remove DNA contaminating. The concentration and quality of RNA were measured using the UV absorbance at

260 nm/280 nm (A260/280 ratio) and checked by gel electrophoresis. Furthermore, using Prime Script™ RT reagent kit (Fermentas, Germany), cDNA was synthesized from the extracted RNA samples. The RT-qPCR was implemented in triplicate using the SYBR® Premix Ex Taq™ II (Takara) for 30 cycles with the following conditions: 94°C for 30 s, followed by repeating cycles at 94°C for 5 s and 60°C for 30 s. The relative expressions of the ATG5 gene was measured using the method of cycle threshold (CT) ( $2^{-\Delta\Delta CT}$ ) with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the internal control or housekeeping gene to normalize the data.

**Table 1.** list and sequence of the used ATG5 and GAPDH primers

Primers	Sequence
ATG 5-F	5'-TTTGCATCACCTCTGCTTTC-3'
ATG 5-R	5'-TAGGCCAAAGGTTTCAGCTT- 3'
GAPDH-F	5- GAA GGT GAA GGT CGG AGT CA-3
GAPDH-R	5- TTG AGG TCA ATG AAG GGG TC-3

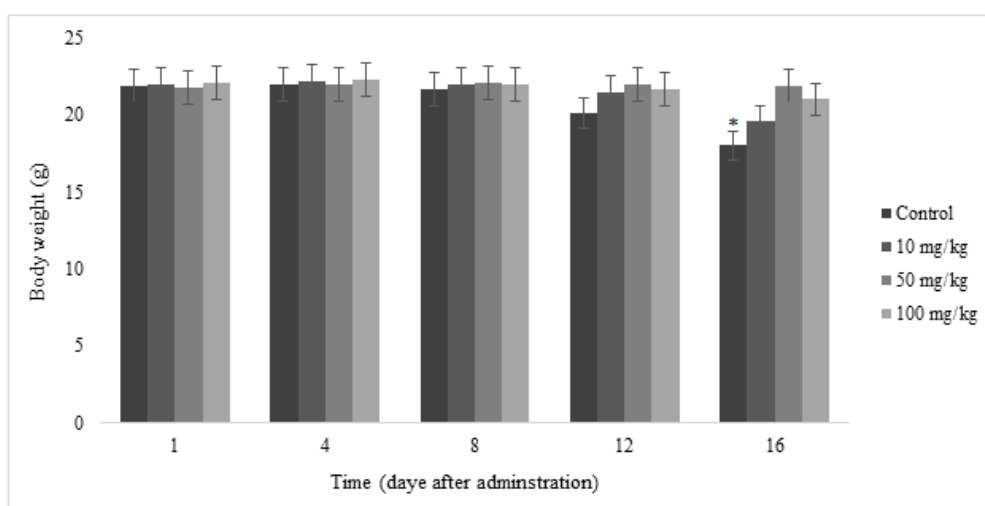
#### Statistical Analysis

Statistical analysis of data was done using SPSS version 20(New York, USA) and analyzed using Chi-Square and ANOVA tests. The results were statistically significant where the p value was < 0.05.

#### Results

##### Animal Weight

The animal weight changes resulted in treatment with the *P. Alkekengi* hydro alcoholic. The results have strongly defended the beneficial effects of *P. Alkekengi* extraction animal health, figure (1). In this regard, no significant changes in mice weight were observed in the *P. Alkekengi* extract groups. In contrast, the average weight of the control was significantly reduced ( $P < 0.05$ ) compared to the treatment groups 16 days after beginning the study.



**Figure 1.** The body weight of mice following the exposure to *P. Alkekengi* extract

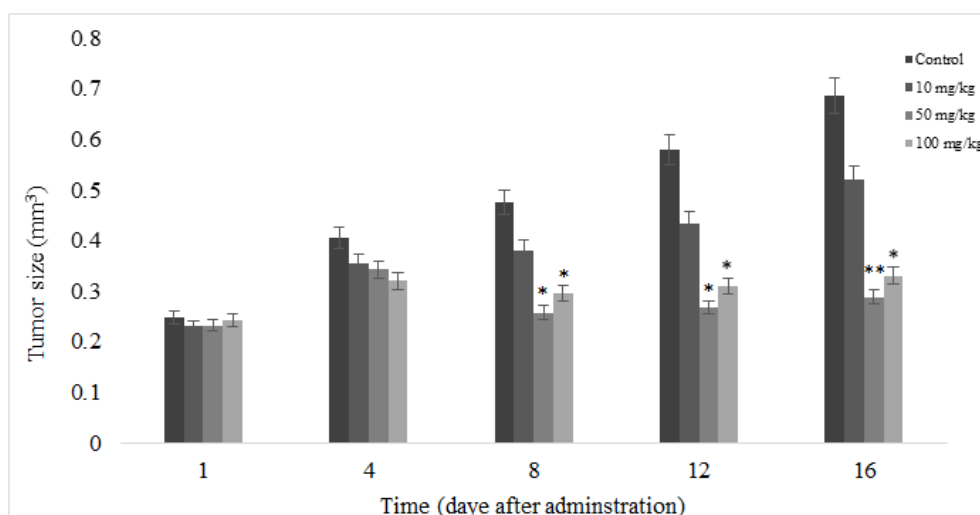
#### Analysis of tumor size

The effects of *P. Alkekengi* hydro alcoholic extract on the animal's tumor progression and development was shown. In the treatment groups, the tumor size among the

different doses of *P. Alkekengi* lose to 0.6 mm was in the greatest dimension with dosage of 10mg/kg. The smallest detectable malignant lesion of size was close to 0.3 mm in diameter for dosage of 50mg/kg. Furthermore, the

untreated mice control group was larger than 0.7mm in histological size. Therefore, in the groups of drug administration, the average tumor size was significantly

less than the control group. Statistically significant differences were found between the compared groups with regard to tumor size, figure (2).



**Figure 2.** The cancer size of mice exposed to various concentrations of extract

### Histopathological Index

The highest diagnostic accuracy of the histopathological lesions of tumor was achieved using an independent pathologist for each subject. Histopathological, most (n = 28) cancer in the groups were composed with a high dosage of *P. Alkekengi*, in which neoplastic cells within tissue lesions often had increased mitotic rates (were frequently in mitosis), greater nuclear pleomorphism or abundant polygonal and or highly pleiomorphic cells, prominent nucleoli, hyper chromatic nuclei and loss of

tubular morphology compared with normal control tissues.

Additionally, in some cases, cells were significantly arranged with necrosis and hemorrhage or congestion patterns of varying degrees, and in some areas, these cells ranged from multinucleated giant cells with higher lymphocytes, plasma cells (lymphoplasmacytic infiltration), and rare Neutrophils (accumulations of mixed inflammatory cells). Taken together, at (50 mg/kg) and (100mg/kg), histopathological alterations were observed in the breasts (table2).

**Table 2.** Macroscopic and histological indices in breast cancer tissue following treatment with H & E staining

Indices \ Groups	Control	10 mg/kg	50 mg/kg	100 mg/kg
Hyper chromatic nucleus	0	+1	+1	+2**
Mitotic Figures	0	0	+1	+2**
Hyperemia and bleeding	+1	+2	+3	+3
Tumor-inflammatory cells infiltration	0	+1	+2	+3*
Pleomorphism of tumor cells	0	+1	+2	+3*
Apoptotic cells	0	+1	+1	0
Necrotic cells	0	+2	+2	+3*
Polynucleotide cells	0	0	+1	+2**

Severe (3+); moderate (2+); mild (1+) between normal and moderate, and none (0) as normal

Significant\* than control group, Significant\*\* than control group

### Analysis of Gene Expression by Real-Time PCR

Based on the assessments, a significant up-regulation in the *ATG5* gene, as one of the main Autophagy genes, was observed (1.82-fold change) in the ER+ breast tumor tissues of mice following treatment with the *P. Alkekengi* extract compared to that of control group ( $P < 0.05$ ).

### Discussion

Nowadays, cancer therapy, especially breast cancer, has become one of the principal challenges of treatment that

the entire population needs. Although multiple hypotheses have been proposed for its association, but yet there is still no explicit and clear concept. The present study compared the performance of histopathology investigation in evaluating breast cancer lesions on various dosages of *P. Alkekengi*. The result proposes the potential and important role that histopathology technique plays in the types of groups undergoing treatment with *P. Alkekengi*. Also, these findings revealed that the diagnostic performance between four groups

(10,50,100 mg/kg and control) was significantly different. To the best of our knowledge, the effects of *P. Alkekengi* hydro alcoholic extract on breast cancer have not been published in mice so far. In recent studies, alternative species of the family Solanaceae showed that the dichloromethane extract of *Physalis pubescence* L. has the properties of cancer-prevention due to the natural compounds extracted from *P. pubescence* L. that possess anti-cancer activities [28,29]. Moreover, most medicinal herbs have antioxidant activity due to the anticancer property of phenolic compounds [30]. In addition, in the present study, *P. Alkekengi* as an anticancer compound could elevate the levels of *ATG5* gene expression with a significant difference ( $p < 0.05$ ) in the more effective dosage of *P. Alkekengi* (50 mg/kg) when compared to that of control group. Based on this result, we were able to show the potential of the *P. Alkekengi* extract to promote the Autophagy mechanisms into malignant ER+ breast tumor cells. On the other hand, the findings showed that *P. Alkekengi* concentrations ranging from 50 and 100 mg/kg, remarkably inhibited the proliferation of cancer cells with infiltrating inflammatory cells and tumor cell necrosis etc. in a dose-dependent manner. These histopathological observations were made during drug administration in high doses toward low dosages. Therefore, our data indicated a potential role for *P. Alkekengi* in breast carcinoma prevention. In parallel, Ding et al., have reported the anti-tumor activity of *P. pubescence* L., in prostate cancer cells of microscopic images [28]. Thus, the findings in this study are consistent with the effect of *P. pubescence* L. on tumor cells. In addition, numerous studies of the therapeutic effects of *P. Alkekengi*, due to its antioxidant properties or activity on different diseases have been noted such as diabetes mellitus, atherosclerosis, cancer, cardiovascular diseases, infection and toxicities [31-36]. Furthermore, Torabzadeh et al. suggested that the effect of an aqueous extract of *P. alkekengi* on the cancer cytotoxicity of the U937 cell line was positive and in another study by Li et al., it was revealed that the anticancer cytotoxic activity of this plant on tumor cells with the human cell line HeLa and Hepatoma cell lines SMMC-7721 and HL-60 is verified [37,38]. Also, several studies on various cancers clearly by Lahijani et al., Nicholson & Ricke and Beiraghdar et al., have shown that the administration of *P. alkekengi* extract remarkably decreased the tumoral level which may associate with the reduction in prostate [39-41]. In summary, our data showed that the effects of *P. Alkekengi* were attributed to its natural compounds which damage the tumor tissue. Afterwards, this study may provide evidence for the potential application of *P. Alkekengi* in the treatment of breast carcinoma and this plant may provide better treatment results by targeting some cellular death cascades including the Autophagy mechanism. Further research is warranted to identify these anti-cancer ingredients of *P. Alkekengi* with overdose and validate its utilization in animal studies.

#### Funding

This study was supported by the authors themselves.

#### Authors' contribution

All the authors have participated in the study equally.

#### Conflict of interest

None

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