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ORIGINAL RESEARCH

The Autophagy-Inducing Mechanisms of Vitexin, Cinobufacini, and *Physalis alkekengi* Hydroalcoholic Extract against Breast Cancer in vitro and in vivo

Esraa Ghazy[1] · **Hassanien Sagban Taghi**[2]

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Abstract

Objectives Owing to inefficiency of chemotherapy towards cancer treatment, formulation and application of herbal drug compounds will open new avenues with this regard. In this study, the anticancer effects of itexin, cinobufacini, and *Physalis alkekengi* (*P. alkekengi*) were assessed.

Methods Herein, synergistic effects of vitexin, cinobufacini, and *P. alkekengi* hydroalcoholic extract were assessed against estrogen-receptor (EGFR2)-positive breast cancer mouse model. Sixty ER + breast cancer BALB/c mice (six groups each including ten members) were included. The anticancer effects of *P. alkekengi* hydroalcoholic extract, vitexin, and cinobufacini were administered against EGFR2 cancerous cells for 14 days. The tumor size, cytotoxic effects, and expression of *Beclin-1*, *LC3-II*, and *ATG5* autophagy-related genes were investigated using RT-qPCR technique. The data was analyzed using chi-square, ANOVA, and multinomial logistic regression tests.

Key Findings The 50% lethal dose (LD50) of *P. alkekengi* and vitexin against the breast cancer cells included 12 mg/kg, respectively, while cinobufacini LD50 was 24 mg/kg but had no toxicity against CRL7242 breast normal cells. Furthermore, 24 mg/kg of the *P. alkekengi*, vitexin, and cinobufacini significantly increased the *ATG5*, *Beclin-1*, and *LC3-II* gene expression.

Conclusion Considering anticancer effects of *P. alkekengi*, vitexin, and cinobufacini against breast cancer through induction of the autophagy pathway, the compound formulations can be applied as anticancer therapies.

Keywords *Physalis alkekengi* · Vitexin · ER + breast cancer · Cinobufacini · Autophagy

Introduction

Breast cancer (BC) is the second leading cause of deaths related to cancers among over 170 countries as per world health organization (WHO) reports [1]. The disease has several subtypes such as estrogen- and progesterone-receptor breast cancer. Most cases include estrogen-receptors expression type with higher morbidity and death rates [2–5].

Although some anti-BC treatment methods such as radiology, mastectomy, endocrine therapies, chemical drugs, and specific antibodies have been used considering each type, no verified method exists without side effects [6–13]. Some pivotal mechanisms which inhibit cancer cells multiplication include autophagy and apoptosis. The induction of autophagy within the BC cells has been performed using chemotherapy [14–17]. However, side effects or induction of resistance have been conferred using phosphoinositide 3-kinase (PI3K) class III inhibitors [18] and vacuolar-type H + –ATPase (V-ATPase) inhibitors [18–20].

The application of herbal medicines (HMs) as anticancerous agents has been conducted leaving various efficacies. The identification and dose determination of their bioactive fractions for anticancer aims are a critical stage with this regard [21–24]. Some bioactive compounds with anticancer effects have been introduced such as that 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, enzyme activities, and sexual and

✉ Esraa Ghazy
management.editores@gmail.com

[1] Department of Pharmacy, Al-Rasheed University College, Baghdad, Iraq

[2] College of Pharmacy, Al-Bayan University, Baghdad, Iraq

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reproductive hormones [25–27]. Our subjective was assessment of the *Physalis alkekengi* hydroalcoholic extract efficacy against the estrogen-receptor-positive BC in mice model.

Materials and Methods

Cell Culture

Herein, EGFR2 BC cell line of mouse was purchased from Pasteur Institute. This cell line was cultured into the DMEM/F-12 with HEPES buffer (15 mM), penicillin (100 µg/ml), L-glutamine, streptomycin (100 µg/ml), 10% FBS (Gibco BRL, Life Technologies), and medroxyprogesterone acetate (10 nM) (Sigma Chemicals, Ontario, Canada). The cells were incubated in a humidified atmosphere at 37 °C containing 5% CO2 [28, 29].

Laboratory Animals

Forty healthy inbred female BALB/c mice (weighting 6–8 g) were obtained and kept for 24 h before experiment, supplemented with food and water ad libitum. Mice housing conditions and experiments were implemented as per the relevant international guidelines of the Weatherall report.

Tumorigenicity

EGFR2 cells were treated with 0.025% trypsin (rinsed with PBS and enzymatically neutralized using 10% FBS) and finally centrifuged in 1200 rpm for 3–5 min and resuspended in tenfold excess culture medium. Then, a number of 1×10^6 cells suspended in 0.1 mL serum-free medium was inoculated into the animal's right inguinal flank [29].

Preparation of Extract and Compounds

Aerial parts of *P. alkekengi* were prepared from the central country's mountains. After drying, 100 g of the sample was dried and powdered and subjected for extraction by 70% methanol (500 mL) and H2O with 1:1 ratio in a percolator apparatus for 72 h. After the completion of extraction, the extract (dried = 40 g, 73%) was separated from solvent using filtration, and the solvent was evaporated in a rotary evaporator at 40 °C. Moreover, the vitexin and cinobufacini fractions were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Experimental Animals

Sixty ER + BC BALB/c mice models six (10 members in each) groups were selected for study which last during 21 days. The animal housing was performed following ethical aspects using complete nutrients and water under ambient conditions and enough light monitored daily in regular. Group A included untreated group (negative control) receiving normal saline; group B received 10 mg/kg *P. alkekengi* hydroalcoholic extract in 1 mL water; groups C and D received vitexin and cinobufacini extracts, respectively; group E received a combination of *P. alkekengi* and cinobufacini; and group F received a combination of *P. alkekengi* and vitexin with equal amounts.

The size of each tumor (cm^3) was measured using a digital vernier caliper (Mitutoyo, Japan) for each week and was documented using below formula:

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

Cell Cytotoxicity Assessment

The cytotoxic effects of *P. alkekengi*, vitexin, and cinobufacini compounds were assessed against CRL7242 breast normal cell line for 24 h. Various concentrations of each ranging 6–24 mg/mL were inoculated into cell culture flask, and the cellular shapes were observed under light microscopy.

Gene Expression Analyses

The *Beclin-1*, *LC3-II*, and *ATG5* gene expression levels were measured using the quantitative real-time polymerase chain reaction (RT-qPCR) technique and primers exhibited in Table 1. The total RNA was isolated from each cancerous tissue using a TRIzol® method. The UV absorbance of

Table 1 Primers used in this study

Primers	Sequence: 5′–3′	Annealing (°C)	Reference
ATG 5-F	F: 5′-TTTGCATCACCTCTGCTTTC-3′ R: 5′-TAGGCCAAAGGTTTCAGCTT-3′	58	[36]
GAPDH	F: 5-GAA GGT GAA GGT CGG AGT CA-3 R: 5-TTG AGG TCA ATG AAG GGG TC-3	60	
LC3-II	F: GAGAAGCAGCTTCCTGTTCTGG R: GTGTCCGTTCACCAACAGGAAG	60	
Beclin-1	F: GGCTGAGAGACTGGATCAGG R: CTGCGTCTGGGCATAACG	60	

Table 2 The effects of various concentrations of herbal compounds against the tumor size

Groups/dose (mg/kg)	1.5	3	6	12	24	*p* Value
P. alkekengi	0.6 mm	0.6 mm	0.4 mm	0.3 mm	0.3 mm	0.001
Vitexin	0.5 mm	0.5 mm	0.3 mm	0.2 mm	0.2 mm	<0.001
Cinobufacini	0.6 mm	0.6 mm	0.4 mm	0.2 mm	0.2 mm	<0.001

260/280 nm (A260/280 ratio) was observed for RNA quality. Furthermore, using PrimeScript™ 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, South Korea) for 30 cycles using the following conditions: 94 °C for 30 s, followed by 30 cycles at 94 °C (5 s), annealing temperature (Table 1) (30 s), 72 °C (30 s), and final extension (5 min). The relative expression of each gene was evaluated using the $2^{-\Delta\Delta CT}$ method which used the threshold cycle (CT). Notably, the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the housekeeping or internal control gene.

Statistical Analysis

Statistics of our study was implemented employing the SPSS (New York, USA) version 20. For comparisons of mean expression levels and anticancer effects of compounds, the chi-square and ANOVA tests were applied. For each test, the p value < 0.05 was given a significant finding.

Results

Mice Weight

Following the treatment of mice with compounds for 14 days, their weight was undergone with no significant changes. Regarding *P. alkekengi* extract (group B), mice weight before and after treatment by 1.5–24 mg/kg concentrations included 6.7 ± 3.4 and 6.8 ± 1.2 g, respectively ($p = 0.719$, paired t-test analysis). In addition, groups vitexin (C) and cinobufacini (D) (1.5–24 mg/kg concentrations) mean weight before exposure included 6.8 ± 2.5 and 7.1 ± 1.1, respectively, and after the treatment were 6.9 ± 0.4 ($p = 0.811$) and 7.2 ± 3.3 ($p = 0.699$), respectively.

The Tumor Size Measurement

Physalis alkekengi, vitexin, and cinobufacini exerted anticancerous effects against mice tumors size in vivo (Table 2). In all groups, with treatment by > 6 mg/kg of herbal compound, a significant reduction of tumor size was observed.

Cell Cytotoxicity

The cell cytotoxicity concentrations of (50%) of *P. alkekengi*, vitexin, and cinobufacini included > 24 mg/mL. Indeed, no cell cytotoxicity was observed when cell line was exposed to these compounds.

Gene Expression

The exposure of breast cancer cells to various concentrations of *P. alkekengi*, cinobufacini, and vitexin resulted in alteration of *ATG5*, *Beclin*, and *LC3-II* autophagy-associated genes. The mean threshold cycle value and expression levels of these genes exposed to 24 mg/mL of each herbal compound have been depicted in Table 3. Accordingly, *P. alkekengi* hydroalcoholic extract and vitexin at concentrations 12 and 24 mg/kg increased the expression of *ATG*, Beclin-1, and *LC3-II* genes significantly compared to negative control. Additionally, the cinobufacini compound increased the expression of *ATG* and *Beclin-1* genes at 24 mg/mL. However, lower concentrations of each agent exerted no expression change in these genes.

Discussion

In this study, following the treatment of mice with *P. alkekengi*, vitexin, and cinobufacini for 14 days, their weight was undergone no significant changes. This result highlights that these herbal drug compounds had no dangerous effect on mice growth. In addition, *P. alkekengi*, vitexin,

Table 3 The expression levels (CT/fold change) of autophagy-associated genes exposed to 24 mg/mL of herbal drug compounds

Compound	*ATG* (CT/fold)	*Beclin-1* (CT/fold)	*LC3-II* (CT/fold)	*GAPDH* (CT)
P. alkekengi	21/7.4	22/6.7	23/5.6	25
Vitexin	23/6.2	22/7.1	22/6.8	24.8
Cinobufacini	24/4.7	25/2.3	29/0.8	25.1
Control	31/1	32/1	31/1	26
p Value	<0.001	<0.001	<0.001	0.983

and cinobufacini exerted anticancerous effects against mice tumors size in vivo. In all groups, treatment by > 6 mg/kg of herbal compound caused a significant reduction in the tumor size. There is no similar study assessing the efficacy of these drugs. A previous study exhibited that *P. alkekengi* had anticancer effects [30]. Other studies have exhibited the anticancer effects of vitexin through inducing the apoptosis in vitro [31, 32]. Another member of the *Physalis* family, *P. pubescens L.* has exerted anticancer effects [33, 34]. Noticeably, compounds with antioxidant traits play a role in cancer prevention and treatment [35]. Because of nontoxicity of herbal drug compounds at doses of anticancer effects, they are efficient for preparation of formulations as alternative agents. We observed that the cell cytotoxicity concentrations of (50%) of *P. alkekengi*, vitexin, and cinobufacini included > 24 mg/mL. Indeed, no cell cytotoxicity was observed when normal lung cell line was exposed to these compounds. Furthermore, induction of anticancer drug resistance by herbal drugs is scarce; however, we did not assess the induction of resistance. It is notable that use of combination therapies has inhibited the development of resistance.

Studies have demonstrated that *LC3* and *Beclin* gene expression decreased during lung cancer [36]. Therefore, agents which increase the expression of these genes can induce the autophagy in cancer cells are promising in this regard. In our study, the exposure of breast cancer cells to various concentrations of *P. alkekengi*, cinobufacini, and vitexin resulted in alteration of *ATG5*, *Beclin*, and *LC3-II* autophagy-associated genes in a dose-dependent manner. *Physalis alkekengi* hydroalcoholic extract and vitexin at concentrations 12 and 24 mg/kg increased the expression of *ATG*, Beclin-1, and *LC3-II* genes significantly compared to negative control. Additionally, the cinobufacini compound increased the expression of *ATG* and *Beclin-1* genes at 24 mg/mL. However, lower concentrations of each agent exerted no expression change against the genes. In several studies, the vitexin inhibited multidrug resistance by HCT-116^{DR} cells and caused increase in the expression of both apoptosis- (BID and Bax) and autophagy-associated (ATG5, Beclin-1, and LC3-II) genes in vitro and in vivo [31, 32, 37].

Conclusion

In this study, *P. alkekengi*, vitexin, and cinobufacini herbal drug compounds had no harmful effects against mice and no cytotoxic effects against breast normal cells. *Physalis alkekengi* hydroalcoholic extract and vitexin high concentrations exerted anticancer effects through a significant increase in the expression of *ATG*, Beclin-1, and *LC3-II* genes compared to control. Additionally, the cinobufacini compound increased the expression of *ATG* and *Beclin-1* genes at the highest concentration. However, lower concentrations of each agent conferred no expression change in the autophagy-related genes.

Author Contribution All the authors have participated in the study equally.

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Declarations

Conflict of Interest The authors declare no competing interests.

References

1. Waks AG, Winer EP. Breast cancer treatment: a review. JAMA. 2019;321(3):288–300.
2. Purushothaman A, et al. Antiproliferative and apoptotic effects of Shemamruthaa, a herbal preparation, in 7, 12-dimethylbenz (a) anthracene-induced breast cancer rats. J Evid Based Complementary Altern Med. 2015;20(4):259–68.
3. Hedenfalk IA, et al. Gene expression in inherited breast cancer. 2002.
4. Angeline Kirubha S, et al. Evaluation of mammary cancer in 7, 12-Dimethylbenz (a) anthracene-induced Wister rats by asymmetrical temperature distribution analysis using thermography: a comparison with serum CEA levels and histopathology. Biomed Res Int. 2012;2012.
5. Ghafouri-Fard S, et al. An update on the role of long non-coding RNAs in the pathogenesis of breast cancer. Pathol Res Pract. 2021:153373.
6. Richardson MA. Research of complementary/alternative medicine therapies in oncology: promising but challenging. J Clin Oncol. 1999;17(11):38–43.
7. Indap M, et al. Anticancer activity of phenolic antioxidants against breast cancer cells and a spontaneous mammary tumor. Indian J Pharm Sci. 2006;68(4).
8. Maganha EG, et al. Pharmacological evidences for the extracts and secondary metabolites from plants of the genus Hibiscus. Food Chem. 2010;118(1):1–10.
9. Dias MC, et al. Effects of Ginkgo biloba on chemically-induced mammary tumors in rats receiving tamoxifen. BMC Complement Altern Med. 2013;13(1):93.
10. Cuzick J. Breast cancer prevention in the developing world. Breast Cancer Res. 2010;12(Suppl 4):S9.
11. Jemal A, et al. Global cancer statistics. CA Cancer J Clin. 2011;61(2):69–90.
12. Bouchardy C, et al. Recent increase of breast cancer incidence among women under the age of forty. Br J Cancer. 2007;96(11):1743.
13. Ghafouri-Fard S, et al. Function of circular RNAs in the pathogenesis of colorectal cancer. Biom Pharmacother. 2021;140:111721.
14. Kondo Y, Kondo S. Autophagy and cancer therapy. Autophagy. 2006;2(2):85–90.
15. Akar U, et al. Silencing of Bcl-2 expression by small interfering RNA induces autophagic cell death in MCF-7 breast cancer cells. Autophagy. 2008;4(5):669–79.
16. Wei T, Xiaojun X, Peilong C. Magnoflorine improves sensitivity to doxorubicin (DOX) of breast cancer cells via inducing apoptosis and autophagy through AKT/mTOR and p38 signaling pathways. Biomed Pharmacother. 2020;121:109139.

17. Zhao Q, et al. Pristimerin induces apoptosis and autophagy via activation of ROS/ASK1/JNK pathway in human breast cancer in vitro and in vivo. Cell Death Discov. 2019;5(1):1–13.
18. Ghayad SE, Cohen PA. Inhibitors of the PI3K/Akt/mTOR pathway: new hope for breast cancer patients. Recent Pat Anticancer Drug Discov. 2010;5(1):29–57.
19. Hendrix A, et al. Vacuolar H+ ATPase expression and activity is required for Rab27B-dependent invasive growth and metastasis of breast cancer. Int J Cancer. 2013;133(4):843–54.
20. Ghafouri-Fard S, et al. MicroRNAs as important contributors in the pathogenesis of colorectal cancer. Biomed Pharmacother. 2021;140:111759.
21. Hoshyar R, et al. Antitumor activity of aqueous extract of Ziziphus jujube fruit in breast cancer: an in vitro and in vivo study. Asian Pacific J Reprod. 2015;4(2):116–22.
22. Rafieian-Kopaei M. Medicinal plants and the human needs. J HerbMed Pharmacol. 2012;1.
23. Bahmani M, et al. Nephrotoxicity and hepatotoxicity evaluation of Crocus sativus stigmas in neonates of nursing mice. J Nephropathol. 2014;3(2):81.
24. Ghafouri-Fard S, et al. Emerging role of circular RNAs in breast cancer. Pathol Res Pract. 2021:153496.
25. Keshtkaran R, Vessal M. Effect of the hydroalcoholic extract of winter cherry fruits (*Physalis alkekengi*) on serum lipid profile and paraoxonase activity of healthy male rats. Zahedan J Res Med Sci. 2017;19(5).
26. Naseri MKG, Mohammadian M, Pharm ZGN. Antispasmodic effect of *Physalis alkekengi* fruit extract on rat uterus. Int J Reprod Biomed. 2008;6(5):193–0.
27. Nasimi M, Heydari NM, Shiravi A. Effects of (*Physalis alkekengi*) fruit's alcoholic extract on development of placentas in pregnant wistar rats. 2009.
28. Isanejad A, et al. MicroRNA-206, let-7a and microRNA-21 pathways involved in the anti-angiogenesis effects of the interval exercise training and hormone therapy in breast cancer. Life Sci. 2016;151:30–40.
29. Khori V, et al. Effects of exercise training together with tamoxifen in reducing mammary tumor burden in mice: possible underlying pathway of miR-21. Eur J Pharmacol. 2015;765:179–87.
30. Esmailpoor A, et al. *Physalis alkekengi* hydroalcoholic extract enhances the apoptosis in mouse model of breast cancer cells. Gene Rep. 2019;15:100366.
31. Zhou Y, et al. Vitexins, nature-derived lignan compounds, induce apoptosis and suppress tumor growth. Clin Cancer Res. 2009;15(16):5161–9.
32. Liu X, et al. Vitexin induces apoptosis through mitochondrial pathway and PI3K/Akt/mTOR signaling in human non-small cell lung cancer A549 cells. Biol Res. 2019;52(1):7.
33. Ding W, et al. Physapubescin B exhibits potent activity against human prostate cancer in vitro and in vivo. J Agric Food Chem. 2015;63(43):9504–12.
34. Yu Y, et al. Withaferin A targets heat shock protein 90 in pancreatic cancer cells. Biochem Pharmacol. 2010;79(4):542–51.
35. Asadi-Samani M, et al. A systematic review of Iran's medicinal plants with anticancer effects. J Evid Based Complementary Altern Med. 2016;21(2):143–53.
36. Jiang Z-F, et al. Decreased expression of Beclin-1 and LC3 in human lung cancer. Mol Biol Rep. 2012;39(1):259–67.
37. Bhardwaj M, et al. Vitexin induces apoptosis by suppressing autophagy in multi-drug resistant colorectal cancer cells. Oncotarget. 2018;9(3):3278.

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