

EVALUATION OF THE EFFECT OF AMYGDALIN ON *survivin* GENE EXPRESSION IN MCF-7 BREAST CANCER CELL LINE

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Shahrokhiyan I., A. Doosti, H. Sazegar (2018): *Evaluation of the effect of amygdalin on survivin gene expression in MCF-7 breast cancer cell line.*- Genetika, Vol 50, No.2, 647-657.

The effects of amygdalin as an herbal substance on prevention of cancer cells proliferation it has been shown. Also, *survivin* gene is one of the important genes on inhibition of apoptosis and controlling of the cell cycle in cancer cell lines. For this purpose, the present study was undertaken for the first time to evaluate the effects of amygdalin on *survivin* gene expression in MCF-7 and HDF. The MCF-7 (Breast cancer cells) and normal HDF were treated by different doses of amygdalin (0.5 to 10 mg/ml). Then, the MTT assay was done on each cell groups on 24, 48, and 72 h after treatment. In each period time the cells were detached and used for RNA extraction and cDNA synthesis. Finally, the *survivin* gene expression in each cell groups was evaluated by quantitative real-time PCR (q-RT-PCR) analysis. The MTT assay was showed that 5 mg/ml of amygdalin concentration at 48 hours after treatment it was suitable for evaluation of this compound on MCF-7 and HDF cell lines. The survey results of q-RT-PCR were showed in both amygdalin-treated MCF-7 (MCF-7+AMG) compare to the treated HDF (HDF-AMG as an control group) the expression level of *survivin* gene were decreased but this reduction only in MCF-7+AMG group was statistically significant ($p < 0.05$). Our findings indicated that amygdalin in particular; decrease the *survivin* mRNA in MCF-7 breast cancer cells compare to normal healthy cells and leads to apoptosis and the death of cancer cells. It is suggested that in future study it must be better the effects of amygdalin supplemented with surfactant investigate against other

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cancer cells and evaluate the more molecular markers to specify the anti-cancer activity potential of this herbal compound.

Key words: Breast cancer, Gene expression, HDF, MCF-7, Survivin

INTRODUCTION

Breast cancer is the second cause of death in women and the probability of this cancer during their lifetime is 1 to 8 (13%) (FALLAHZADEH *et al.*, 2014; ARAGON *et al.*, 2015). The risk for breast cancer increases with age and depending to family history, genetic mutations, environmental agents, high-fat diet, late pregnancy (above age 30), nulliparity, early age of menarche, and late age of menopause (ASIF *et al.*, 2014; ANJUM *et al.*, 2017).

So far, the exact cause of breast cancer has not been identified and there are various genetic and environmental factors involved in its development. The genetic mutations in some genes like *BRCA1*, *BRCA2*, *TP53*, *CDH1*, *MRE11A*, *NBN*, *PALB2*, *PTEN*, *RAD50*, *RECQL*, *RINT1*, *CHEK2*, *ATM*, and *Survivin* increase the chances of developing of breast cancer and testing for an abnormality in these genes promotes early diagnosis of breast cancer (DOOSTI *et al.*, 2011; STEVENS *et al.*, 2013; OLLIER *et al.*, 2015).

Survivin is a member of the inhibitor of apoptosis (IAP) protein family which plays an important role in regulating of the cell cycle and inhibits caspases and blocks cell death (JAISWAL *et al.*, 2015; SHAMSABADI *et al.*, 2016). This protein is also known as BIRC5, EPR-1, and API4 which has only one BIR domain and does not have a CARD (Caspase Activation Recruitment Domain) domain. This gene is an important molecular biomarker in cancer and highly expressed in most cancer cell lines like Michigan Cancer Foundation-7 (MCF-7) and its application in tumour diagnostic, prognostic and as well as for anti-cancer therapies it has been shown (JAISWAL *et al.*, 2015). The existence of a BIR domain in this gene gives it the intrinsic property of caspases and leads to inhibition of apoptosis induced by Bax and Fas (SAH *et al.*, 2006; BARRETT *et al.*, 2011; JAISWAL *et al.*, 2015). This gene has 3 introns, four major exons including E1, E2, E3, and E4 and as well as four hidden exons 2B, 2a, 3B, and 3a (OUHTIT *et al.*, 2007; PENNATI *et al.*, 2007). *Survivin* spans 14.7 kb at the telomeric end of chromosome 17 (on the 25q band) and encodes an intracellular protein with 142 amino acids and 16.5 KDa molecular weight (ALTIERI, 2003; JAISWAL *et al.*, 2015).

Amygdalin or B-17 with the brand name of Laetril is classified as a cyanogenic glycoside that found in many herbs, but mostly there is in apricot seeds, pear core, cherry corn, bitter almonds, apples, peaches and plums. This chemical compound was first isolated in 1830 and used as an anticancer agent in Russia in early 1845 and in the 1920s in the United States. The molecular form of amygdalin is C₂₀H₂₇NO₁₁, with a molecular weight of 457.72 (SOKKAR *et al.*, 2013; AGHADAVOD, 2016). Amygdalin can be decomposed into HCN and benzaldehyde, which is an anti-tumor composition and benzaldehyde can induce analgesic action. Therefore, this compound can be used to improve cancer and reduce the pain. The anti-tumor effects of the amygdalin are one of the important topics that have been of great interest in recent years, and its anti-cancer functions can be decomposed carcinogens in the body, blocking the supply of tumor cells and inhibiting the growth of cancer cells. This substance has been particularly considered for prostate, lung, colon, and rectum cancers (SONG and XU, 2014; BLAHETA *et al.*, 2016; QADIR and FATIMA, 2017).

Due to the important role of survivin in the development, tumorigenicity, and bioavailability of tumor cells, the present study was aimed to investigate the effects of amygdalin

as an anti-cancer agent on expression level of *survivin* gene in MCF-7 (breast cancer cell line) and normal HDF (as a control cell line).

MATERIALS AND METHODS

Cell lines culture

Human breast cancer cell line (Michigan cancer foundation or MCF-7) and human dermal fibroblast (HDF) were obtained from Cellular and Molecular Research Center (Shahrekord University of Medical Sciences, Shahrekord, Iran). MCF-7 and HDF cells were cultured in two flasks (JET BIOFIL, Guangzhou, China) containing RPMI1640 and Dulbecco's modified Eagle's medium (DMEM) media (both Gibco, Grand Island, NY, USA), respectively that supplemented by 10% fetal bovine serum (FBS) with 10 % (v/v) fetal bovine serum-FBS, and 1% streptomycin (100 µg/ml) and penicillin (100 U/ml) (CMG, Iran) penicillin/streptomycin antibiotics (all reagents from Gibco, NY, USA). Both cultures were incubated in a 5% CO₂ atmosphere at 37°C to reach the cell confluence to 70-80%. For cell passage the media of each cell line were discarded and each flask were washed twice with phosphate-buffered saline (PBS) and dissociated with 0.25% Trypsin-EDTA solution at 37°C for 5 min. The enzyme activity was neutralized by DMEM and RPMI1640 containing 10% FBS and were centrifuged at 1200 rpm for 5 min.

MTT assay of Amygdalin

For MTT analysis equal volumes of MCF-7 and HDF suspensions were mixed by trypan blue (0.3% in PBS) and incubated for 5 min and then were counted by Neubauer haemocytometer chamber under microscope. A total of 6,000 cells were seeded into each well of 96-well plates that contain 500 µl of DMEM with 10% FBS per each well and incubated in a CO₂ incubator at 37°C for 24 h. Then, amygdalin was added to the cells of each well at concentrations of 0.5 to 10 mg/ml (10 different concentrations) as triplet and the plate was then placed in the CO₂ incubator at 37°C. In this stage, control group (culturing of MCF-7 in media without amygdalin) and for each amygdalin concentration HDFs (as a normal cells) were used. After 24, 48 and 72 hours treatment with amygdalin, the media of the cells were discarded, and each well was washed slowly by 100 µl of PBS. A 5 mg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dye (MTT) powder (Roche Diagnostics, Penzberg, Germany) was prepared in PBS and stored under dark condition. 100 µl of this MTT solution was added to each well in dark condition and incubated in a CO₂ incubator at 37°C for 4 hours. Finally, 100 µl Dimethyl sulfoxide (DMSO) was added to each well and was shaken on a flat surface to mix well. DMSO cause to dissolve of formosan crystals. After 30 minutes of incubation, the plate was placed on a shaker for 5 minutes and then the optical absorption was read by the ELISA reader (Awareness STAT FAX 2100 microplate reader) on a wavelength of 490 to 620 nm.

RNA extraction and cDNA synthesis

Total RNA was isolated from each treated MCF-7 and HDF cells with amygdalin at 24, 48 and 72 hours after amygdalin addition and control group (cells not exposed to amygdalin) using TRIzol LS Reagent purchased from Life Technologies (cat. no. 10296-010; US Patent no. 5,346,994) according to the manufacturer's protocol. Each extracted RNA sample was measured by Thermo Scientific™ NanoDrop 2000 (Wilmington, DE, USA) at a wavelength of 260 to 280 nm according to the method described by SAMBROOK and RUSSELL, 2001. For investigation

of gene expression each RNA sample (0.2 µg) was used to synthesize template cDNA using the PrimeScript RT reagent kit (TaKaRa BIO, Shiga, Japan) using random hexamer alone or combined with specific primers according to manufacturer's instruction.

Reverse transcriptase PCR (RT-PCR)

The specific oligonucleotide primers for amplification of *survivin* and *GAPDH* (as a housekeeping gene) genes were obtained from SHEN *et al.*, 2008 study. The sequences of each primer were analyzed by basic local alignment search tool (BLAST) in GenBank data (Table 1). The RT-PCR reaction mixture was performed in a total volume of 25 µL final volume contained 2.5 mM MgCl₂, 200 µM dNTP, 1 U *Taq* polymerase (all Cinna Gene, Iran) and 1 µg of template cDNA and diffusion water. A negative control contained all reagents without cDNA together all samples were placed in an Eppendorf AG 22331 Hamburg thermal cycler (Eppendorf, Hamburg, Germany). The PCR temperature conditions began with an initial denaturation at 94°C for 5 minutes; followed by 35 PCR cycles 1 min initial denaturation at 94°C, 30 seconds annealing at 60°C, 1 min extension at 72°C and a final extension at 72°C for 5 min.

Table 1. The sequence of primers used for RT-PCR reaction

Gene	Primers name	Sequence	Product length (bp)
<i>survivin</i>	Surv-F	5'-AGAACTGGCCCTTCTTGGAGG-3'	170
	Surv-R	5'-CTTTTATGTTCCCTCTATGGGGTC-3'	
<i>GAPDH</i>	GAPDH-F	5'-GAAGGTGAAGGTCCGAGTC-3'	226
	GAPDH-R	5'-GAAGATGGTGATGGGATTC-3'	

Aliquots of 10 µl of RT-PCR products were applied to the gel 1% agarose gel electrophoresis at 80 V constant voltages for 30 min. The TBE buffer (10.8 g of Tris-base 89 mM, 5.5 g of boric acid 2 mM, EDTA (pH=8.0) 4 mL of 0.5 M EDTA (pH= 8.0) that were combined in sufficient H₂O and were stirred to dissolve) was used as an electrophoresis buffer. GeneRuler

A 100 bp DNA Ladder Plus (Thermo Fisher Scientific, San Jose, California, USA) was used as a molecular weight DNA to determine the amplification of *GAPDH* cDNA sample for confirmation of cDNA synthesis. The agarose gel electrophoresis of amplified was stained with ethidium bromide and was visualized under UV light and photographed by UVIdoc gel documentation system (Uvitec, UK).

Quantitative real-time PCR (q-RT-PCR) analysis

The expression level of *survivin* gene in treated and non-treated MCF-7 and HDF cells by amygdalin compare to *GAPDH* gene (reference gene) were measured by q-RT-PCR. The q-RT-PCR and fluorescent melting curve analysis was done on the Corbet Rotor-Gene 6000 (Corbett, Australia). The q-PCR reaction was done in a final volume of 25 µL and 50 ng of diluted cDNA (2.5 µL) was mixed with 12.5 µL of FastStart Universal SYBR Green Master Rox (Roche Applied Science, Indianapolis, IN, USA), 2 µM of each primer (1 µl), and 9 µL of

infusion water. The thermal cycling program was an initial denaturation at 94°C for 2 min, followed by 45 cycles of denaturation for 20 s at 94°C, 15 s of annealing at 60°C, and 15 s of extension at 72°C. The melting-curve assays were evaluated by machine from 55°C to 95°C at a transition rate of 0.1°C/s to determine the primer dimers or non-specific product formation. The ΔCT values were calculated as the difference between the cycles of threshold (Ct) ranges using the $2^{-\Delta\Delta\text{Ct}}$ relative quantitative method.

Statistical analysis

Each experiment was repeated at least three times. The relative expression ratio was calculated by the Rest 2009 software. All statistical analyses were performed using the Social Sciences software (SPSS, Inc., Chicago, IL, USA) version 22 followed by independent T-test to investigate the significance of the data. Graphs were made using GraphPad Prism version 5.01 (2003, San Diego, CA) software. P -value < 0.05 was considered to be statistically significant.

RESULTS

Cell lines preparation

Both MCF-7 and HDF cells were cultured in RPMI 1640-enriched with 10% FBS and also 1% of the combination of penicillin and streptomycin antibiotics. Cells were stored in a CO₂-incubator at 37°C. The media of each cell line were replaced every three days and after cell density reached to 80% were passaged into other flasks (Figure 1).

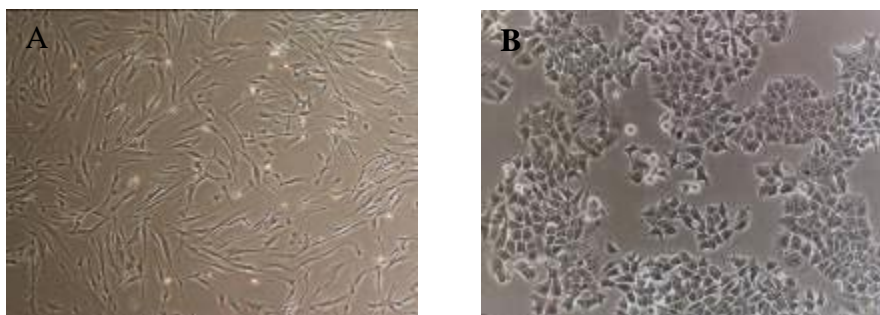


Figure 1. A) HDFs and B) MCF-7s cultured in media in day 3

MTT assay results

The MTT assay were performed in 24, 48, and 72 hours on treated cell line with amygdalin for investigation the viability of each MCF-7 and HDF (as a control) to determine the minimum concentration of this compound in their media. The findings were showed in both MCF-7 and HDF the 5 mg/ml of amygdalin was the best concentration for treatment. In 24 hours after treatment of cell lines by amygdalin the effects of this material was low and after 72 hours its efficiency was decreased. Therefore, 48 hours after the treatment was an appropriate time for investigation of the effect of amygdalin on cellular stability in future tests (Figure 2).

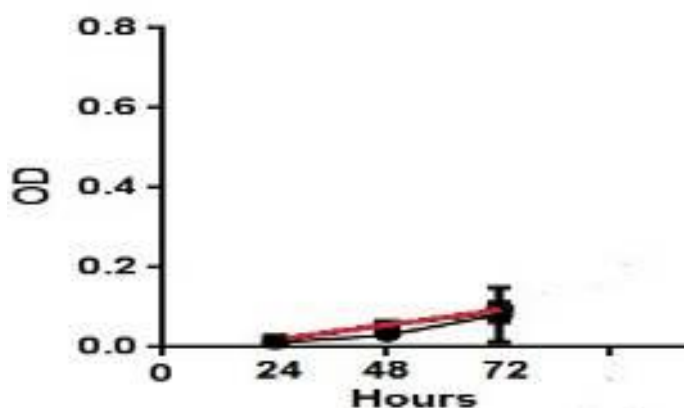


Figure 2. The comparison of the survival rate of treated MCF-7 (black) and HDF (red) with amygdalin. 48 hours was the best time of treatment in terms of the sustainability and survival in both cell lines

Verification of cDNA synthesis

The RT-PCR reaction for reference gene (*GAPDH*) was performed to ensure that cDNA production was accurate for all specimens. The investigation of the products of RT-PCR reactions for *GAPDH* gene on 1% agarose gel electrophoresis was revealed 226 bp fragments and were confirmed the authenticity of cDNAs synthesized (Figure 3).



Figure 3. The accuracy of cDNA synthesis by RT-PCR technique on a 1% agarose gel electrophoresis (Lane M is 100 bp molecular weight marker (Thermo Fisher Scientific, San Jose, California, USA), lanes 1 to 6: amplified cDNA for *GAPDH* gene (226 bp) in both MCF-7 and HDF, and lane 7: negative control (without template cDNA))

Gene expression analysis

Analysis of relative *survivin* gene expression data compare to *GAPDH* gene using q-real-time RT-PCR in treated cancer cell line (MCF-7) and control group (HDF) by amygdalin were showed that the expression levels of the *survivin* gene in treated MCF-7 (MCF-7+AMG) compare to the treated HDF (control group) was reduced statistically significant (p -value <0.05). Also, the *survivin* gene expression was decreased in treated HDFs by amygdalin (HDF+AMG) compare to the normal HDFs but this reduction was not statistically significant (p -value >0.05) (Figure 4).

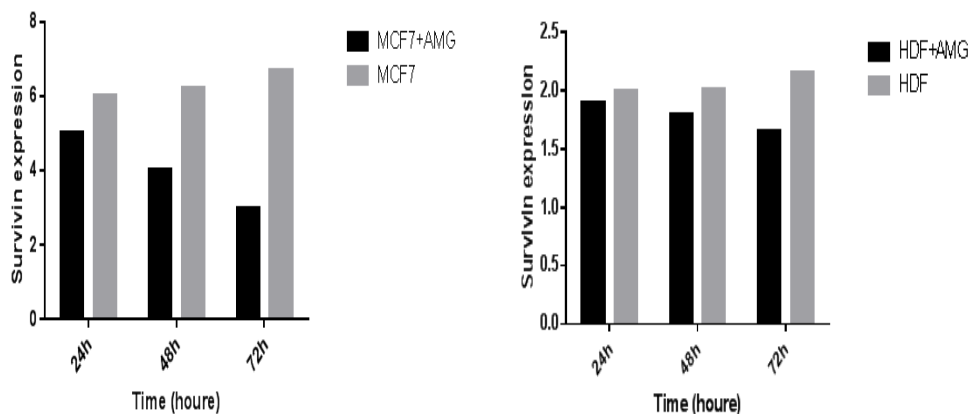


Figure 4. The compression of *survivin* gene expression in treated and non-treated MCF-7 and HDF with amygdalin and decreasing in *survivin* gene expression in treated both cells by this compound was observed but this reduction in exposed MCF-7s was significant

DISCUSSION

Survivin gene by encoding an apoptotic inhibitor protein plays an important role in regulating cell cycle and its expression has increased in most cancers and it is associated with resistance to chemotherapy, increased relapse of the disease, and lead to death of the patient (KHAN *et al.*, 2015; FINLAY *et al.*, 2017). In recent years, the anticancer and anti-tumor effect of amygdalin in different cancers has been produced. Amygdalin into the cancer cells is broken down and its cyanide released is and its cyanide can inhibit tumor proliferation and suppress the cancerous cells (QIAN *et al.*, 2015; LEE *et al.*, 2016).

The present study was performed to investigate the effects of amygdalin on *survivin* gene expression in breast cancer cell line (MCF-7) and healthy HDFs using molecular technique. The MCF-7 (breast cancer cell line) and HDF (normal cells as a control) were treated by amygdalin and MTT assay were performed in 24, 48, and 72 hours after the treatment and it was found that 48 hours after treatment by amygdalin at a 5 mg/ml concentration was suitable for treatment of cells and molecular investigation. The results of q-real-time RT-PCR on expression level of *survivin* gene were showed that the expression levels of this gene in treated MCF-7 (MCF-7+AMG) compare to the treated HDF (HDF-AMG) was reduced statistically significant ($p <0.05$). Though in treated HDFs by amygdalin (HDF+AMG) compare to the normal HDFs the

survivin gene expression level was decreased but this reduction of expression was not statistically significant.

WANG *et al.*, 2013 investigated the effect of up-regulating of *survivin* on the expression of breast cancer-resistant protein (BCRP) during dilution and sequestration of P53 in the expression of NF-KB in MCF-7/5FU cancer cells. In their study the protein expression (BCRP) was demonstrated by expressing *survivin* in the up-regulation drug delivery system and these findings determined by comparing the MCF-7/5FU with the MCF-7 cell lines.

In another study performed by Makarević and colleagues investigated the effect of amygdalin in bladder cancer. According to their findings amygdalin stops the growth and reproduction of the bladder cells. Other researchers confirmed the symptoms of amygdalin on inducing of apoptosis. The rapid activation of caspas-3 during the down-regulation of the BCL2 protein and the up-regulation of BAX proteins in the presence of 1 mg/ml of amygdalin was determined in the DU145 and LNCaP prostate cell line. Cellular apoptosis was detected in vaginal cancer (HELA) and in prolapsed leukemia (MAKAREVIĆ *et al.*, 2014). While in our study, after MTT assay 5 mg/ml of amygdalin was suitable for controlling and inhibition of *survivin* mRNA expression in MCF-7 cancer cell and HDF (normal cell). In recent study the effect of amygdalin on cell growth and telomerase activity in human cancer and MRC-5 fibroblast cell lines investigated and it was found that above 10 mg/ml concentration of amygdalin inhibited the growth rate of cancer cells. Also, their research indicated that telomerase activity significantly down-regulated in amygdalin-treated cancer cell lines (A-549, MDA-MB-231, MCF-7, and U87-MG) with the decreased expression of telomerase reverse transcriptase (TERT) and telomerase RNA component (TERC) compared with control cancer cells (MOON *et al.*, 2015). Their findings confirmed the results of the present study on effects of amygdalin on inhibition of MCF-7 growth and leading to the mortality and prevention of the spreading of these cancer cell lines after treating by this herbal compound. But in our study only the effects of amygdalin on *survivin* mRNA expression in MCF-7 compare to HDF was evaluated and the inhibitory effects of this compound on the *survivin* protein in cancer cells was shown.

Survivin is very commonly express in cancer cells and its presence in healthy cells is so low. This protein inhibits apoptosis by controlling caspase-3 in mitochondria and inhibits cell proliferation and angiogenic promoters. As a result, Survivin is introduced as a multi-functional protein and reducing the expression of *survivin* is closely related to tumor death and drug inhibition. In fact, many studies have shown that patients with high survival mRNA expressions have a short life (WANG *et al.*, 2016; GUO *et al.*, 2016; HU *et al.*, 2017). Therefore, amygdalin that used in this study via decreasing of *survivin* mRNA expressions leads to apoptosis and cell death in cancer cell line like MCF-7, and this naturally occurring chemical compound is useful for prevention and inhibition of the spread of cancer.

CONCLUSIONS

In conclusion, after 48 hours exposing of MCF-7 with amygdalin this compound by decreasing the expression level of *survivin* mRNA in this cancer cell lines leads to induction of apoptosis and ultimately cell death. Although in amygdain-treated HDFs the *survivin* gene expression was decreased but this reduction range in breast cancer cell line (MCF-7) was more and significant and indicated that amygdalin induce apoptosis in MCF-7 breast cancer cells via the impact on *survivin* gene expression and could lead to death of these cancer cells. It is

suggested that the effect of amygdalin supplemented with surfactant on cancer cells investigated and more markers to confirm the anti-cancer effect of this chemical compound evaluated.

ACKNOWLEDGEMENTS

The present article was based on the MSc thesis and the authors would like to express their gratitude to the Research Deputy of Islamic Azad University of Shahrekord Branch and all staffs in the Biotechnology Research Center of mentioned university for their cooperation.

Received, December 31th, 2017

Accepted June 18th, 2018

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**OCENA EFEKTA AMIGDALINA NA EKSPRESIJU GENA *survivin* KOD MCF-7
ĆELIJA KARCINOMA DOJKE**Iman SHAHROKHIYAN¹, Abbas DOOSTI^{2*}, Hossein SAZEGAR¹¹ Departman za biologiju, Fakultet za osnovna istraživanja, Shahrekord ogranak, Islamski Azad Univerzitet, Shahrekord, Iran² Biotehnološki istraživački centar, Shahrekord ogranak, Islamski Azad Univerzitet, Shahrekord, Iran**Izvod**

U radu su prikazani efekti amigdalina kao biljne supstance na sprečavanje proliferacije ćelija karcinoma. *Survivin* gen je jedan od važnijih gena za inhibiciju apoptoze i kontrolisanje ćelijskog ciklusa u ćelijama karcinoma. U tu svrhu, ova studija je prvi put izvedena da bi se procenili efekti amigdalina na ekspresiju gena *survivin* u MCF-7 i HDF. MCF-7 (ćelije karcinoma dojke) i normalni HDF tretirani su različitim dozama amigdalina (0.5 do 10 mg / ml). Zatim je MTT test izvršen na svakoj grupi ćelija na 24, 48 i 72 h posle tretmana. U svakom periodu ćelije su odvojene i korišćene za ekstrakciju RNK i sintezu cDNK. Konačno, ekspresija *survivin* gena u svakoj ćelijskoj grupi procenjena je pomoću kvantitativne PCR (q-RT-PCR) analize. MTT test je pokazao da je 5 mg/ml koncentracije amigdalina 48 sati nakon tretmana pogodno za procenu efekta ovog jedinjenja na ćelije MCF-7 i HDF. Prikazani su rezultati q-RT-PCR u tretmanu amigdalinom MCF-7 (MCF-7 + AMG) i u tretiranim HDF (HDF-AMG kao kontrolnom grupom). Nivo ekspresije *survivin* gena se smanjio, ali ova redukcija je samo u MCF-7 + AMG grupi bila statistički značajna ($p < 0.05$). Naši rezultati su pokazali da je amigdalin smanjio *survivin* mRNA u MCF-7 ćelijama karcinoma dojke upoređujući se sa normalnim zdravim ćelijama i doveo do apoptoze i smrti ćelija karcinoma. Predloženo je da u budućoj studiji efekat amigdalina bude dopunjen istraživanjem površinski aktivne supstance protiv drugih ćelija raka i da se primeni više molekularnih markera kako bi se odredio potencijal za aktivaciju anti-karcinom efekta ovog biljnog jedinjenja.

Primljeno 31.I.2017.

Odobreno 18. VI. 2018.