sciendo

Original Study • DOI: 10.2478/ahem-2022-0013 • AHEM • Vol. 76 Issue 1/2022 • 132-142

AHEM

Advances in Hygiene and Experimental Medicine
Postępy Higieny i Medycyny Doświadczalnej

Effect of amygdalin on MCF-7, MDA-MB-231 and T-47D breast cancer cells in the in vitro study

Original Study

Aleksandra Zielińska*[1], Justyna Płonka-Czerw[2], Agnieszka Nowak[3], Dariusz Kuśmierz[2]

[1]Department of Medical Genetics, Faculty of Pharmaceutical Sciences in Sosnowiec, Medical University of Silesia, Sosnowiec, Poland
[2]Department of Cell Biology, Faculty of Pharmaceutical Sciences in Sosnowiec, Medical University of Silesia, ul. Jedności 8, Sosnowiec, Poland
[3]Chair and Department of Chemistry, School of Medicine with the Division of Dentistry in Zabrze, Medical University of Silesia in Katowice, Poland

Received 30 March, 2021, Accepted 12 January, 2022

Abstract

Introduction. Amygdalin is a chemical compound found in the seeds of many edible plants. Different studies using cancer cell cultures in vitro indicate its potential anti-cancer activity. Various types of cancer cells showed different responses to different doses of amygdalin. This may suggest many in vitro models of the activity of this compound. The aim of the study was to evaluate the effect of amygdalin on MCF-7, MDA-MB-231, and T-47D breast cancer cells and on HFF-1 normal dermal fibroblasts (control cell culture) in vitro. Cell proliferation, viability, and the changes in mRNA transcript levels of basic proteins (BAX, caspase 3 and BCL-2) involved in apoptosis were analyzed.
Materials and Methods. MCF-7, MDA-MB-231, T-47D, and HFF-1 cell lines were purchased from the ATCC. Amygdalin derived from apricot kernels was purchased from Sigma-Aldrich. CVDE, WST-1, and LDH assays were used to evaluate the effects of amygdalin on cell proliferation and viability. Molecular evaluation of gene transcription levels was performed using the RT-qPCR technique.
Results. Amygdalin causes a dose-dependent decrease in proliferation and metabolic activity of MCF-7, MDA-MB-231, and T-47D cells in the in vitro cultures. In all cell cultures amygdalin affects the mRNA levels of pro-apoptotic BAX and caspase 3 proteins and anti-apoptotic BCL-2 protein.
Conclusions. Amygdalin anti-cancer activity may be selective in relation to different cell types. It seems that examined breast cancer cells are more sensitive to amygdalin than normal cells.

Keywords

breast cancer • amygdalin • cell culture • T-47D • MDA-MB-231 • MCF-7 • HFF-1 • BAX • BCL-2 • caspase 3

1. Introduction

Amygdalin ($C_{20}H_{27}NO_{11}$) belongs to organic cyanogenic compounds from the group of glycosides. It occurs in large amounts in plants from the *Rosaceae* family, for example, in seeds of apricots, apples, cherries, pears, bitter almonds [1, 2]. The natural function of amygdalin, as well as other cyanogenic compounds, is to protect plants against insects and larger herbivores. The product of its decomposition is hydrogen cyanide (59 mg of HCN can be released from 1 gram of amygdalin) [1]. Unhydrolyzed amygdalin has no harmful effect. However, its decomposition products are highly toxic [3]. A common mistake, repeated in the literature and in the nomenclature of amygdalin preparations, is the interchangeable use of the names "amygdalin" and "laetrile". These names refer to two chemical compounds that differ in their structure (Figure 1). Laetrile ($C_{14}H_{15}NO_7$) is an amygdalin derivative that was synthesized by Dr. Ernst Theodore Krebs Jr. while researching less toxic form of amygdalin. In the structure of amygdalin there is a combination of two glucose molecules and mandelonitrate, while in the structure of laetrile there is only one glucose molecule [3].

Amygdalin was used as a drug in unconventional anticancer therapy for the first time in 1845 [4]. Since then, several hypotheses have been developed to explain the potential anti-cancer properties of this compound. One of the hypotheses is based on the initial reaction of amygdalin hydrolysis to its basic components: D-glucuronic acid and L-mandelonitrile [3,4]. Further decomposition of L-mandelonitrile leads to benzaldehyde and hydrocyanic acid (HCN) [5]. The production of hydrocyanic acid from amygdalin is mediated by the β-D-glucosidase enzyme, which may be 1000 to 3000 times more active in cancer cells than in normal cells [6]. This difference in enzymatic activity contributes to the accumulation of excess amounts of released

* E-mail: azielinska@sum.edu.pl

© 2022 Zielińska *et al.* This work is licensed under the Creative Commons Attribution-NonCommercial-NoDerivs 4.0 License.

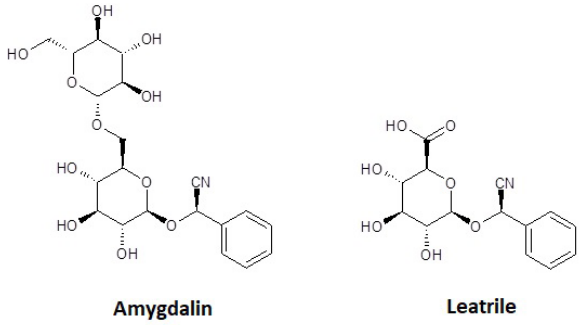


Fig. 1. Comparison of the chemical structure of amygdalin and laetrile.

hydrocyanic acid in the tumor cells. Further detoxification of hydrocyanic acid to thiocyanate requires the enzyme rhodanase, which is more active in normal tissues but has almost negligible activity in cancer cells. It is believed that the combination of high β-glucoidase activity leading to the release of cyanides with a deficiency in the activity of rhodanase, neutralizing cyanide, may be responsible for the selective mechanism of amygdalin cytotoxic action in relation to cancer cells, since it has a negligible effect on normal cells [7]. It has also been postulated that the mechanism of action of amygdalin related to the release of cyanides, cytotoxic to cancer cells, may trigger reactions that mobilize the body's immune response against pathological cells [4, 8]. Moreover, attempts were made to explain the death of cancer cells as a consequence of acidification of the cytoplasm due to destabilization of lysosomes [9]. It is assumed that not only amygdalin breakdown products, but also the unhydrolyzed starting compound itself, may have biological activity [2]. The metabolism of amygdalin largely depends on the bacterial microflora in the human intestines. Individual differences in its composition and in its enzymatic activity (depending on the diet) determine individual differences in the response to administration of amygdalin [10]. Since β-glucosidases produced by bacteria carry out the breakdown of amygdalin, taking it orally is associated with a greater risk of cyanide release and consequently poisoning [3, 11, 12]. Mammalian β-glucosidases likely hydrolyze amygdalin in a way different from that of bacterial enzymes. It is presumed that then other reaction intermediates are formed [2]. Intravenous amygdalin is mainly excreted in the urine, and hydrogen cyanide is not released [3, 11, 12]. In mice with inhibited intestinal flora, a dose of 300 mg/kg body weight administered to the stomach did not kill the rodents. However, in the control group, in which the mice had an active bacterial flora, the mortality at the same dose, administered by the same route, increased by 60% [11]. The LD_{50} for orally administered amygdalin is 880 mg/kg body weight in rats, 8 g/kg body weight in mice intraperitoneally and 25 g/kg body weight by intravenous injection. The highest dose, tolerated by rabbits and dogs, administered orally is 0.075 g/1 kg body weight, and intravenously or intramuscularly is 3 g/kg body weight. In humans the tolerated dose for intravenous administration is estimated at 0.07 g/kg body weight. Taking orally doses of 0.6-1 g daily may not cause poisoning [11]. Most likely sensitivity to amygdalin is an individual trait, as there are reports of intravenous doses of 2-9 g/kg body weight [8]. Among the recorded cases of amygdalin poisoning, severe and fatal poisoning including accidental poisoning after ingestion by children can be distinguished [2]. Studies on amygdalin as a substance with anti-cancer activity are still rare and the results are controversial. Proponents consider amygdalin to be a natural anti-cancer substance, while opponents warn that amygdalin is ineffective and has a toxic effect [13]. The studies carried out so far with the use of cell cultures of various cancer cell lines in vitro, however, confirm the ability of amygdalin to limit proliferation and induce apoptosis. Such effects of amygdalin action have been observed, among others, in the in vitro experiments on, e.g., promyelocytic leukemia cells of HL-60 line [14], bladder cancer cells of RT112, UMUC-3 and TCCSUP lines [15], breast cancer cells of Hs578T and SK-BR-3 lines [16, 17], non-small cell lung cancer line of H1299 and PA lines [18], prostate cancer cells of DU145 and LNCaP lines [8], cervical cancer cells of HeLa line [19], or colon cancer cells of the SNU-C4 line [12].

The aim of the study was to assess the effect of various doses of amygdalin on the proliferation and viability of breast cancer cells of the T-47D, MDA-MB-231, and MCF-7 lines and normal human fibroblasts of the HFF-1 line (comparative control) in in vitro cultures and to assess the effect of amygdalin at a concentration of 50 mg/ml for the expression of the genes for the pro-apoptotic proteins BAX and caspase 3 and the gene for the anti-apoptotic protein BCL-2 at the level of transcription in in vitro cultures of the above-mentioned cell lines.

2. Materials and Methods

2.1 Cell lines

All human breast cancer cells T-47D, MDA-MB-231, and MCF-7, and the human normal dermal fibroblasts HFF-1 (Table 1, Figure 2) were purchased from the American Type Culture Collection (ATCC; www.atcc.org).

2.2 Amygdalin

Amygdalin was purchased from Sigma-Aldrich (cat. No. 10050-5G); it was derived from apricot kernels and its purity was ≥ 97%.

2.3 Cell culture condition

Cell lines were cultured according to standard mammalian tissue culture protocols and sterile technique. All cells were cultured in DMEM medium (Lonza) supplemented with heat-inactivated 10% fetal bovine serum (FBS; Sigma) and 1% antibiotics mix (containing 10,000 units/ml penicillin and 10,000 units/ml streptomycin) (Lonza) at 37°C in humidified atmosphere of 5% CO_2 in air.

Table 1. Human cell lines used in the study.

Cells characteristics	Cel lines*			
	T-47D	MCF-7	MDA-MB-231	HFF-1
Morphology	Ephithelial	Ephithelial	Ephithelial	Fibroblasts
Tissue	Mammary gland/ derived from metastatic site: pleural effusion	Mammary gland/ derived from metastatic site: pleural effusion	Mammary gland/ derived from metastatic site: pleural effusion	Skin: foreskin
Disease	Ductal carcinoma/ estrogen-dependent	Adenocarcinoma/ estrogen-dependent	Adenocarcinoma/ estrogen-independent	Normal
Donor	54 years adult/female	69 years adult/female	51 years adult/female	Newborn/male

**ATCC*

2.4 Viability and proliferation assay

Effects of amygdalin on HFF-1, T-47D, MDA-MB-231, and MCF-7 cell proliferation and viability were estimated with the CVDE, WST-1, and LDH assay.

CVDE assay is used to assess the number of adherent cells: living cells in cell culture. This assay involves the spectrophotometric measurement of the amount of crystal violet bound by the DNA of living cells attached to the bottom of the culture vessel. Xenometrix reagent kit (CVDE - Crystal Violet Dye Elution Kit) was used to perform the assay. Absorbance was measured at 540 nm.

WST-1 assay is used to assess the metabolic activity of cells. This assay is based on the ability of living cells, i.e., metabolically active cells, to convert tetrazole salts to formazan. This reaction involves cellular enzymes: mitochondrial dehydrogenases. The number of cells in the culture translates into the overall enzymatic activity, and hence the specific processing intensity of tetrazole salts. The reaction is connected with a color change of the culture medium, which can be analyzed with a spectrophotometer. This assay was performed using a Roche reagent kit (Cell Proliferation Reagent WST-1). Absorbance was measured at 440 nm.

LDH assay is used to assess the number of cells dying in culture. This assay allows the analysis of the enzymatic activity of lactate dehydrogenase released into the culture medium by cells that have interrupted integrity of their cell membranes. Lactate dehydrogenase, through the reduction of NAD^+ to $NADH/H^+$ in the reaction of lactate conversion to pyruvate, reduces tetrazole salts to formazan. The effect of this reaction is connected with discoloration of the culture medium. The Roche reagent kit (Cytotoxicity Detection Kit LDH) was used to perform this assay. Absorbance was measured at 500 nm.

In each of the above assays, absorbance measurements were taken with the Biogenet ASYS UVM340 instrument.

All data are presented as a percentage of the viability or proliferation in relation to control cell culture (cell culture not treated with amygdalin). Percentages of viability and proliferation were calculated considering the controls as 100%. Each experiment was repeated three times. A p value < 0.05 was considered to be significant.

General workflow for conducting the CVDE, WST-1, and LDH assays was similar and consisted of several steps. In the first step, cells were seeded into 96-well plates (Nunc). 3,000 cells were seeded into each well of the plate after detaching the cells from the culture flask with a trypsin-EDTA solution (Gibco) and determining the number of cells with a Bürker chamber. In each well, cells were grown in 100 µl of full DMEM medium. The prepared 96-well plate was placed in a CO_2 incubator for 24 hours.

In the second step, the medium was removed from the each of the wells of the 96-well plate and replaced with a medium supplemented with amygdalin at the specified concentration (5, 10, 20, 50, and 100 mg/ml). Solutions with various concentrations of amygdalin in the medium were prepared by a series of dilutions. Each amygdalin concentration was tested in triplicate. Then, a 96-well plate with cell cultures treated with various concentrations of amygdalin was placed in a CO_2 incubator for 48 hours.

In the third step, after removing the 96-well plate from the incubator, the CVDE, WST-1 and LDH tests were performed. Each test was performed according to protocol for the reagent kit.

2.5 Preparation of cell cultures treated with amygdalin at a concentration of 50 mg/ml

After the cells had been detached from the surface of the culture flask using trypsin-EDTA solutions, the cell number was determined with a Bürker chamber and 300,000 cells were seeded into 5 cm diameter Petri dishes. The cells of each of the tested lines were seeded into two dishes. In each dish, cells were grown in 5 ml of full DMEM medium. Next, the dishes were placed in a CO_2 incubator for 24 hours. Subsequently, the medium was removed from the dishes and replaced with full DMEM medium with the addition of amygdalin at a concentration of 50 mg/ml or full DMEM medium without the addition of amygdalin in the control cultures. Then all cell cultures were placed in a CO_2 incubator for 48 hours. After incubation time, the cell culture dishes were transferred from the incubator to an inverted microscope table and the condition of each culture was visually assessed.

The observations were documented by taking microscopic photos (Figure 2). Then, after removing the medium from the cell culture, the cells were harvested by cell scraper and transferred to a 2.2 ml Eppendorf tube. Total RNA was extracted from the prepared cell pellets.

2.6 Total RNA extraction

Total RNA was isolated from cultured cells using the Zymo Research Quick-RNA MiniPrep kit according to the manufacturer's protocol. The quantitative and qualitative assessment of extracts was conducted on the basis of absorbance measurements at the wavelength of λ= 260 nm (while assuming that 1.0 OD 260 corresponds to 40 ug RNA in 1 mL of extract). Spectrophotometric measurements were made using a HP8452A spectrophotometer (Hewlett Packard®).

2.7 Evaluation of transcriptional activity of *BAX, BCL-2*, and *CASP3* using RT-qPCR

Evaluation of transcriptional activity of *BAX*, *BCL2,* and *CASP3* was performed for all individual cell culture types, treated with amygdalin at a concentration of 50 mg/ml. Reference controls were cell cultures of each individual cell not treated with amygdalin.

To evaluate the number of mRNA copies of *BAX*, *BCL2,* and *CASP3* genes in the extracts of total RNA, the RT-qPCR method was used. DNA Engine OPTICON™ fluorescence detector (MJ Research) with a Quanti Tect Sybr Green RT-PCR Kit (Qiagen) and specific complementary primers to amplify the sequence were used to run the reaction. Primer sequences were designed with use of Primer Express TM Version 1.0 computer software (ABI Prism), based on the data from MEDLINE (http://www.ncbi.nlm.nih.gov) (Table 2). A reaction mixture for each RNA extract with a special primer set was prepared in triplicate (each data point is the average of a triplicate). The RT-qPCR protocol was a 30-min RT reaction at 50°C, a 15-min PCR activation at 95°C and then 45 cycles of 15 s denaturation at 94°C, a 30 s annealing at 60C, and a 30 s PCR extension at 72°C. Finally, a melting-curve analysis was performed to confirm the RT-qPCR specificity. Negative controls with no total RNA were included in each run of the RT-qPCR.

The standard curve used in the evaluation was prepared on the basis of simultaneously amplified 18S rRNA fragments in concentrations of 100, 200, 500, 1000 and 4000 copies. 18S rRNA standards were purchased from Applied Biosystems (TaqMan® DNA Template Reagents Kit). The standard curve was used to estimate the initial copy number of RNA template used in the RT-qPCR reaction. The results are presented as the mRNA copy number of a specific gene transcript, converted to 1 µg of total RNA in a sample.

2.8 Statistical analysis

Data distribution for the trial met the requirements of normal distribution (Shapiro-Wilk test) and the homogeneity of variance (Levene test and Brown-Forsythe test). In the case of viability and proliferation assay statistically significant differences were found between the analyzed means by performing the F test in the analysis of variance. The control group was compared with four groups tested by Dunnett's test. The data considered in this way was presented in the form of charts presenting the arithmetic mean for each sample and the corresponding standard deviations.

In the case of data obtained through RT-qPCR method, the control group and one of the tested groups were compared using t-test, provided that the variances of those trials were homogenous or using t-test with separate estimation of variance (Cochran-Cox test) if they were not. The obtained data are presented in graphs in the form of the arithmetic mean and the corresponding standard deviations.

All tests were performed with the significance level of $\alpha = 0.05$ using Statistica 12.0 software.

Table 2. Nucleotide sequences of the primers used in the RT-qPCR reaction.

Primer name	Nucleotide sequence	Transcript detected	GenBank database access number
KAS3F	5'-GGCCTGCCGTGGTACAGAACTGG-3'	CASP3 mRNA	NM032991
KAS3R	5'-AGCGACTGGATGAACCAGGAGCCA-3'		
BAXF	5'-CCTGTGCACCAAGGTGCCGGAACT-3'	BAX mRNA	L22474
BAXR	5'-CCACCCTGGTCTTGGATCCAGCCC-3'		
BCL-2F	5'-TTGTGGCCTTCTTTGAGTTCGGTG-3'	BCL2 mRNA	M14745
BCL-2R	5'-GGTGCCGGTTCAGGTACATCAGTCA-3'		
18SrRNAF	5`-CAGTTATGGTTCCTTTGGTCGCTC-3'	18S rRNA	M10098
18SrRNAR	5`-GTTGATAGGGCAGACGTTCGAATG-3'		

3. Results

3.1 Microscopic evaluation of the effect of amygdalin on cultures of HFF-1 human normal dermal fibroblasts and T-47D, MDA-MB-231, and MCF-7 breast cancer cells in vitro

Microscopic images of the cell cultures showed differences in the degree of confluence of cell cultures after 48 hours of incubation between the control cultures (without the addition of amygdalin) and the cultures treated with amygdalin at a concentration of 50 mg/ml (tested cultures). In case of normal fibroblasts (HFF-1), the density of the control and test culture was comparable. On the other hand, in the case of all cultures of cancer cells, T-47D, MDA-MB-231, and MCF-7, treated with amygdalin, a clearly lower degree of confluence was observed compared to cultures without amygdalin addition (Figure 2).

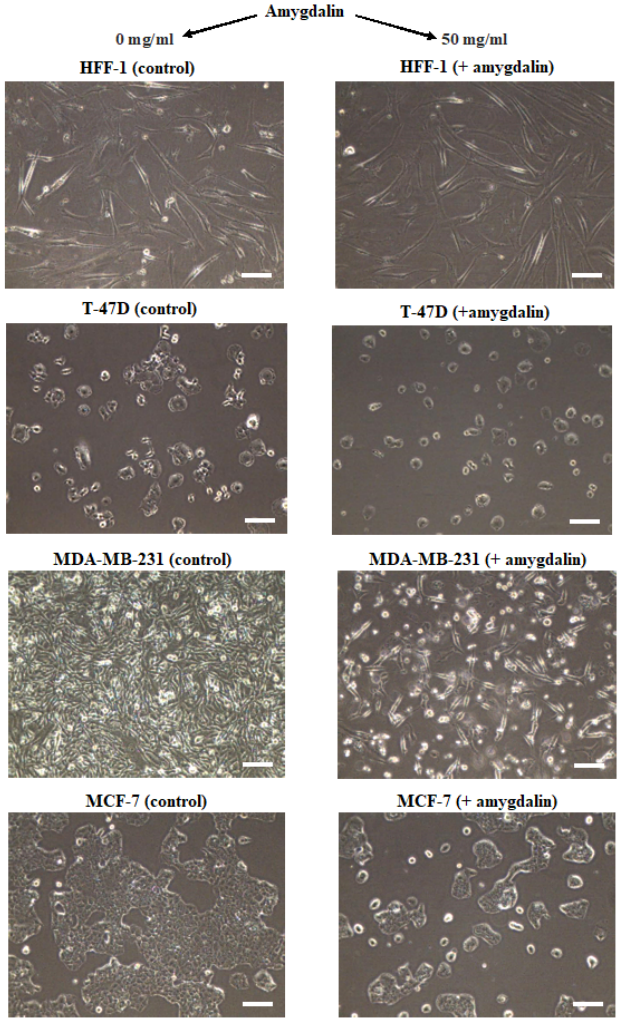


Fig. 2. Comparison of the confluence degree between the cell cultures treated with amygdalin at a concentration of 50 mg/ml and the control cultures – not treated with amygdalin. The microscopic image [100x] was collected after 48 hours of cell cultures incubation. Scale bar = 100 µm.

3.2 The effect of amygdalin on the proliferation and viability of breast cancer cells and normal dermal fibroblasts in vitro

In all cell cultures, both normal and cancer cells, a dose-dependent effect of amygdalin on cell proliferation and viability was observed. However, no amygdalin dose-dependent increase in percentage of dying cells in each of the tested cell cultures was observed (LDH assay). A difference in the effect of amygdalin on the percentage of attached and viable cells (CVDE assay) was observed between cultures of cancer cells (T-47D, MDA-MB-231, and MCF-7) and normal fibroblasts (HFF-1) (Figure 3). The higher dose of amygdalin was connected with the smaller percentage of viable cells in the cultures. In HFF-1 normal cell cultures and in T-47D and MDA-MB-231 breast cancer cell cultures, only the highest applied amygdalin dose (100 mg/ml) caused a statistically significant decrease in the percentage of viable cells in the culture ($p = 0.0114$, $p = 0.0035$, $p = 0.0010$, respectively). On the other hand, in the cancer cell cultures of the MCF-7 line a significant reduction in the percentage of viable cells was noted at the amygdalin concentration of 20, 50, and 100 mg/ml ($p = 0.0372$, $p < 0.0001$, $p < 0.0001$, respectively).

A decrease in the metabolic activity (WST-1 assay) of all cell cultures (of breast cancer cells and normal fibroblasts) was observed with an increase in amygdalin dose (Figure 3). This effect was most intense in cultures of normal human dermal fibroblasts (HFF-1 line): even the lowest dose of amygdalin (5 mg/ml) caused a significant decrease in the metabolic activity of cells ($p = 0.0181$). In the cultures of MDA-MB-231 and MCF-7 cells, significant reductions in the metabolic activity of cells (by approx. 50% and 60% respectively) were noted at the amygdalin concentration of 50 mg/ml ($p = 0.0002$, $p = 0.0173$, respectively) and of 100 mg/ml ($p < 0.0001$, $p = 0.0005$, respectively). In the culture of T-47D cells any significant reduction in metabolic activity was noted.

The effect of amygdalin on the percentage of dying cells was observed (LDH assay) in all cultures, both of cancer cells (T-47D, MDA-MB-231 and MCF-7) and normal human dermal fibroblasts (HFF-1) (Figure 3). As the dose of amygdalin increased, a decrease in the percentage of dying cells was observed in all cell cultures. In cultures of HFF-1 normal human dermal fibroblasts and T-47D and MDA-MB-231 breast cancer cells, this effect was observed at amygdalin concentrations of 20 mg/ml and higher ($p = 0.0417$, $p = 0.0002$, $p = 0.0107$). In MCF-7 cell culture, the percentage of dying cells was reduced at amygdalin concentrations of 50 mg/ml and 100 mg/ml ($p < 0.0001$, $p < 0.0001$, respectively).

3.3 Quantitative assessment of the mRNA expression levels of BAX, BCL-2, and caspase 3 proteins

In all cell cultures (HFF-1 normal fibroblasts and T-47D, MDA-MB-231, and MCF-7 cancer cells), an effect of amygdalin on the BAX, BCL-2, and caspase-3 mRNA copy number was observed, compared to control cultures that were not treated with amyg-

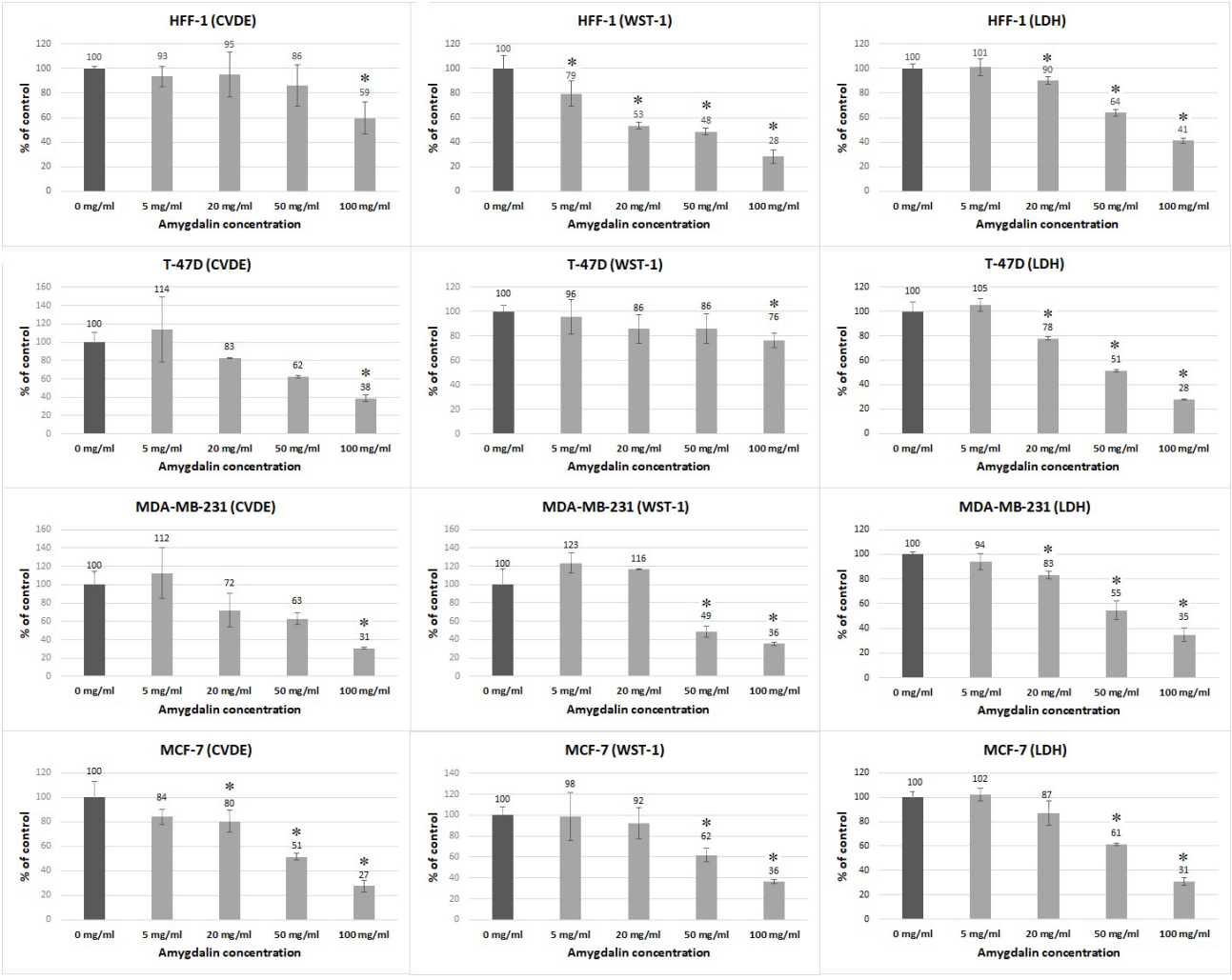


Fig. 3. The effect of different doses of amygdalin (0 – 100 mg/ml) on the percentage of attached, viable cells (CVDE test), on the metabolic activity of cells (WST-1 test) and on the percentage of dying cells (LDH test) in the HFF-1, T-47D, MDA-MB-231, and MCF-7 in vitro cultures. Statistical significance means p <0.05 (*).

dalin (Figure 4). In HFF-1, T-47D, and MDA-MB-231 cell cultures amygdalin at a concentration of 50 mg/ml caused a statistically significant increase in the BAX mRNA copy number ($p = 0.0115$, $p < 0.0001$, $p = 0.0009$ respectively) and caspase-3 mRNA copy number ($p = 0.0007$, $p = 0.0080$, $p = 0.0002$ respectively). In the HFF-1 and MDA-MB-231 cell cultures amygdalin caused a statistically significant increase in the BCL-2 mRNA copy number ($p = 0.0002$, $p = 0.0005$, respectively). In the T-47D cell culture amygdalin caused a statistically significant decrease in the BCL-2 mRNA copy number ($p = 0.0028$). However, in MCF-7 cell culture the same dose of amygdalin caused the statistically significant decrease of BAX and BCL-2 mRNA copy number ($p = 0.0130$, $p < 0.0001$ respectively) and a not statistically significant decrease of *CASP3* mRNA copy number.

In the T-47D and MCF-7 breast cancer cells treated with amygdalin at a concentration of 50 mg/ml a statistically significant increase in *BAX/BCL2* ratios was noted ($p = 0.0009$, $p = 0.0003$) (Figure 5). The opposite effect was noted in case of HFF-1 cells: amygdalin caused statistically significant decrease in *BAX/BCL2* ratio ($p = 0.0017$). No statistically significant change in *BAX/BCL2* ratio was observed in case of MDA-MB-231 cells.

4. Discussion

In our study we conducted a comparative analysis of amygdalin high dose (50 mg/ml) impact on expression on mRNA level of *BAX* and *CASP3* pro-apoptotic genes and of *BCL2* anti-apoptotic gene in cell cultures of various breast cancer cell lines (T-47D, MDA-MB-231, MCF-7) and in cultures of normal dermal fibroblasts of HFF-1 line. In addition, an analysis of the impact of amygdalin on viability, proliferation, and metabolic activity of cells of all tested cell lines in the in vitro cultures was also performed.

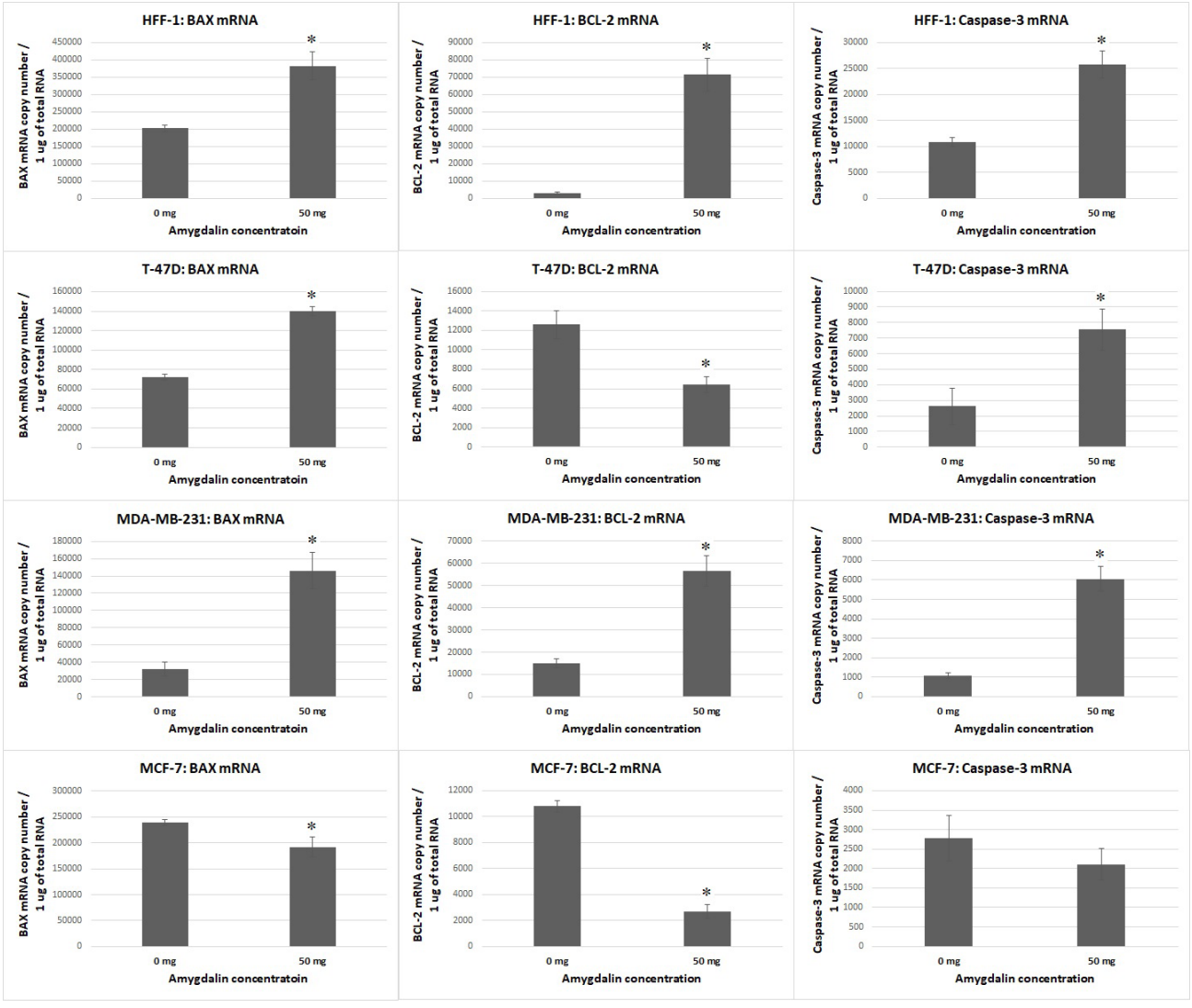


Fig. 4. The effect of amygdalin at a concentration of 50 mg/ml on the BAX, BCL2, and caspase-3 mRNA level in human breast cancer cells (T-47D, MDA-MB-231, MCF-7) and normal dermal fibroblasts in vitro compared to control (cell cultures not treated with amygdalin). Statistical significance means p <0.05 (*).

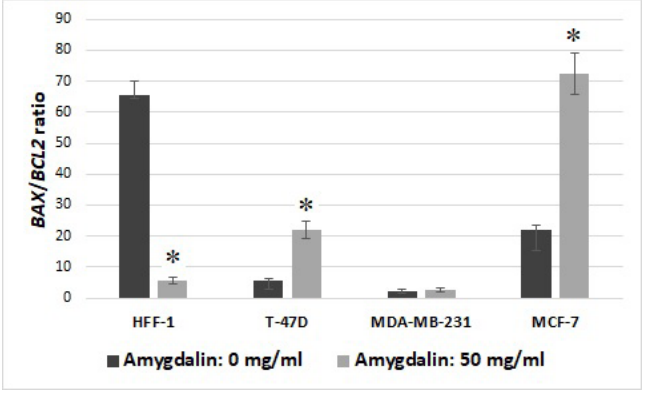


Fig. 5. The BAX/BCL2 ratio (BAX mRNA copy number/BCL-2 mRNA copy number) in culture cells treated with amygdalin (at concentration of 50 mf/ml) compared to control (culture cells not treated with amygdalin). Statistical significance means p <0.05 (*).

Studies carried out by other authors have focused on the analysis of amygdalin effect on various types of cancer cells, most often of commercially available cell lines. However, there are few studies aimed at comparative analysis of amygdalin impact on different cell lines of the same cancer type.

We selected three different established breast cancer cell lines (from ATCC) for our study. Two of them were estrogen-dependent (T-47D-derived from pleural effusion of ductal carcinoma and MCF-7-derived from pleural effusion of breast adenocarcinoma) and one (MDA-MB-231-derived from pleural effusion of breast adenocarcinoma) consisted of estrogen-independent breast cancer cells.

Our study show that amygdalin affects the proliferative and metabolic activity of T-47D, MDA-MB-231, and MCF-7 breast cancer cells and HFF-1 human normal dermal fibroblasts in vitro.

In addition, amygdalin affects the pattern of expression of BAX and caspase 3 pro-apoptotic proteins and BCL-2 anti-apoptotic protein at the transcription level in all types of in vitro cultures: breast cancer cells as well as normal cells.

The action of amygdalin was dependent on its concentration: higher doses of amygdalin caused stronger effects. The highest amygdalin concentrations, at 100 mg/ml, caused a statistically significant reduction in the viability (CVDE assay) of all cell cultures – breast cancer cells (T-47D, MDA-MB-231) as well as normal cells (HFF-1). However, cells of the MCF-7 line were most sensitive to the action of amygdalin; amygdalin doses ≥ 20 mg/ml resulted in statistically significant reduction in cell viability.

The strongest impact of amygdalin influence on metabolic activity was observed in the culture of normal HFF-1 cells. Even the lowest applied dose of amygdalin (5 mg/ml) caused a statistically significant decrease in the metabolic activity of these cells in vitro. Higher amygdalin concentrations reduced metabolic activity by approx. 50% and more. In the cultures of the MDA-MB-231 and MCF-7 breast cancer cells, only amygdalin concentrations of 50 mg/ml and higher contributed to a statistically significant decrease in cell metabolic activity (generally by more than 50%). In the culture of T-47D cancer cells only the highest dose of amygdalin caused a statistically significant decrease in metabolic activity. No effect of amygdalin on the increase in the percentage of dying cells was observed, both in cultures of cancer cells and normal cells.

However, the results of the presented study indicate a different response of individual, studied cell types to amygdalin application [3]. This may indicate a probable selective action of amygdalin on different cell types. Certainly, amygdalin inhibits proliferation and regulates the expression of one of the most important genes involved in apoptosis.

Apoptosis, programmed cell death, occurs both in pathological and physiological processes (as an element of embryonic development, cell replacement, tissue remodeling, maintenance of neuron function and mitochondrial function, and calcium utilization). Its important role is to eliminate damaged cells in a way that does not cause local inflammation. Certain cells should be directed to apoptosis: cells with irreversible DNA damage, including cancer cells, which often acquire the ability to escape the control of the mechanisms of programmed death [20]. The action of many anti-cancer drugs is based on the activation of programmed death of pathological cells by interfering with the process of nucleic acid synthesis. A cell may enter the path of programmed death also through the action of various stimuli, such as oxygen deficiency, nutrient deficiency, ionizing radiation, loss of contact with neighboring cells, or damage to telomeres. The characteristic morphological features of apoptotic cells include chromatin condensation, controlled DNA fragmentation, cell shrinkage, and cell breakdown into the so-called apoptotic bodies. Also at the molecular level, common changes involving a wide variety of molecules can be identified. Proteins from the Bcl-2 family, including anti-apoptotic (e.g., BCL-2) and pro-apoptotic (e.g., BAX, BAK, BID, BAD) proteins, play key roles in apoptosis and are located on mitochondrial and endoplasmic reticulum membranes. Caspases are another very important group of proteins involved in apoptosis [8, 21].

BCL-2 protects the cell against apoptosis, and its overexpression may promote the survival of cells with damaged genetic material. This protein is unlikely to accelerate division, but it increases cell viability by inhibiting the activity of pro-apoptotic proteins from the Bcl-2 family. This complex of co-interactions and its final effect, however, depend on other proteins from the Bcl-2 family, the proteins containing only the BH3 domain in their amino acid structure [8, 21, 22].

BAX protein is one of the factors promoting cell death. BAX undergoes conformational changes in response to stress stimuli, intra- or extracellular. As a result, a channel is formed on the outer mitochondrial membrane of the Bcl-2 protein family. It enables the cytochrome c outflow from the mitochondrial intermembrane space to the cytoplasm and it indirectly leads to effector caspases activation, leading to degradation of cell components [22, 23].

The induction of BAX protein overexpression translates into the activation of apoptosis, which is very beneficial in cancer therapy. The final fate of the cell depends on the balance between proteins from the Bcl-2 family, with opposite properties: pro- and anti-apoptotic. Determining the quantitative ratio of BAX/BCL-2 allows for a quick assessment of the intensity of apoptotic processes in cells [8, 21].

However, this model explains in a very simplified way the coexisting relationships in the cell, although usually the advantage of the amount of BAX over the amount of BCL-2 determines apoptosis activation. There are many various factors affecting the final effect of BAX and BCL-2 proteins in cells: mutual influence on the change of pro- and anti-apoptotic activity; interaction of other Bcl-2 family proteins; the occurrence of forms of the BCL-2 protein incapable of affecting the apoptotic process; preservation of the anti-apoptotic properties of BCL-2, even when the protein loses the ability to bind to the BAX protein; participation of proteins from the Bcl-2 family in processes not related to apoptosis [22].

Proteins of the Bcl-2 family play many functions, including the so-called non-canonicals that do not relate to the programmed cell death process. They are able to influence the functioning of mitochondria, and regulate autophagy and the immune response to viral infections. The relationships between proteins from the Bcl-2 family and their functions are still not fully understood [21].

In contrast to many types of cancer cells, *BCL2* gene expression in breast cancer cells is associated with a better prognosis for the patient. Patients with a moderate or high degree of *BCL2*

expression live longer without recurrence compared to patients with low levels of this gene expression. Surprisingly, BCL-2 negative tumors are usually more aggressive, estrogen-dependent and less responsive to radiotherapy and hormone therapy. The expression of the *BCL2* is associated with the presence of estrogen receptors on the cell surface. In MCF-7 breast cancer cells, the expression level of the *BCL2* increases under the influence of estradiol and decreases under the influence of P53 protein. The decreased level of BAX protein in breast cancer cells is associated with a worse prognosis and a poorer treatment response in the metastatic phase. Expression of this protein is not associated with the presence of estrogen receptors [24].

Our study confirmed the influence of amygdalin on the expression, at the transcription level, of key genes involved in the apoptotic pathway. In the culture of the T-47D cells treated with a high dose of amygdalin (50 mg/ml), the *BAX* mRNA level increased and the *BCL2* mRNA level decreased, both statistically significant compared to control (cell culture not treated with amygdalin). In the culture of the MDA-MB-231 cells treated with amygdalin, the *BAX* mRNA expression level increased significantly, while the *BCL2* mRNA expression level increased also significantly but to a much lesser extent. In the culture of MCF-7 cells, a statistically significant slight decrease in *BAX* mRNA expression and a significant decrease in *BCL2* mRNA expression level were observed. Generally, in case of all breast cancer cell cultures treated with amygdalin the changes of *BAX*/*BCL2* ratios compared to control cell cultures translated in favor of the BAX mRNA level (although in case of MDA-MB-231 the change was not statistically significant). However, different results were observed in case of HFF-1 normal fibroblasts culture. Although amygdalin influenced the statistically significant increase in both BAX and BCL2 mRNA levels in HFF-1 culture, the *BAX*/*BCL2* ratio compared to control cell culture translated statistically significant in favor of the *BCL2* mRNA (for an anti-apoptotic protein). This may suggest the activation of apoptotic processes in breast cancer cells in vitro. These data, however, are not confirmed by the LDH test, which did not show the increase of dying cells in cultures, despite increased expression of pro-apoptotic *BAX* and *CASP3* at the transcription level. Probably this may be related to the delay in starting a fully active apoptosis process.

The effect of amygdalin on the *BAX* and *BCL2* expression was also noted in other cancer cells in the in vitro cultures. The increased level of BAX protein and the decrease level of BCL-2 protein was observed in the DU145 and LNCaP prostate cancer cells treated with amygdalin in vitro. BAX overexpression induced apoptosis in prostate cancer resistant to some chemotherapeutic agents [8]. HeLa cancer cells exposed to amygdalin, also showed an increased level of BAX protein and a decreased level of BCL-2 protein [19]. Such changes were also observed in the Hs578T breast cancer cells [16].

Caspases are pro-inflammatory and pro-apoptotic factors. Active forms of these enzymes influence each other's functions and initiate a cascade of signals in the cell. Caspases belonging to the pro-apoptotic factors lead directly to apoptosis through mutual activation and controlled degradation of cell components. They are produced as a zymogens – inactive forms – which is necessary to control their activity in the cell. Activation of caspases usually involves their proteolysis followed by dimerization of the specific resulting products. Apoptotic caspases can be divided into initiating caspases (caspase 8 and 9) and effector caspases (caspase 3, 6, 7) [25]. Studies confirmed that some changes in the nucleotide sequence of the *CASP3* gene (encoding the caspase 3 protein) translate into an increased risk of different cancer types – such as neck and head cancer, lung cancer, multiple myeloma, non-Hodgkin's lymphoma, and endometrial cancer [25].

Our study showed a statistically significant increase in the caspase 3 mRNA level in T-47D, MDA-MB-231 and HFF-1 cell cultures (test: $p = 0.0080$, $p = 0.0002$, $p = 0.0007$ respectively). It is possible that the increase in level of *CASP3* transcripts may be related to the increased activity of the caspase 3 protein in the cell, and consequently it can affect the intensification of apoptosis. These results are compatible with results of studies performed on other cell lines. The increase in caspase 3 activity was observed after exposure to amygdalin in DU145 and LNCaP prostate cancer cells [8] and in HeLa cervical cancer cells [19]. However, in the Hs578T breast cancer cells amygdalin reduced the amount of procaspase 3 [16]. A similar effect was observed in our study in case of MCF-7 cell culture – amygdalin at concentration of 50 mg/ml reduced the *CASP3* mRNA level. However, it was not statistically significant.

The results of our study confirm previous reports on the properties of amygdalin. Undoubtedly, it is a chemical compound with potential anti-cancer activity. It limits the proliferation and metabolic activity of cancer cells in vitro. Moreover, it influences changes in the level of gene expression for key proteins involved in the apoptosis process. It probably influences the increase of gene expression, especially for pro-apoptotic proteins.

A study by Makarević et al. (2014) [15], which analyzed the effect of amygdalin on bladder cancer cells in vitro, showed that the number of cells entering the stage of early apoptosis slowly increased only after prolonged exposure to amygdalin (2 weeks). The short duration of action of this substance on cancer cells contributed only to the limitation of proliferative activity, which was not caused by the cytotoxic effect of amygdalin, but by a strong influence on factors regulating the cell cycle. On the other hand, Chang et al. (2006) [8], in studies of the amygdalin effect on prostate cancer cells in vitro, observed a rapid activation of caspase 3 with a decrease in amount of the BCL-2 protein and an increase in amount of the BAX protein after 24 hours of incubation. It can be concluded that probably amygdalin shows

different spectrum of impact on different types of cancer cells, and cell death may be the result of different correlations between the factors involved in the mechanisms regulating this phenomenon.

The results of our study lead to the conclusion that generally amygdalin may show selective activity in relation to various types of breast cancer. In relation to viability, proliferation, and metabolic activity, T-47D, MDA-MB-231, and MCF-7 breast cancer cells are much more sensitive to amygdalin than normal dermal fibroblasts of HFF-1 line in the in vitro condition – normal cells require higher amygdalin concentrations to show an effect similar to that seen in pathological/cancer cells at lower amygdalin concentrations.

In our study each of the tested breast cancer cell lines (T-47D, MDA-MB-231, and MCF-7) treated with high dose of amygdalin – at concentration of 50 mg/ml – showed different, individual patterns of expression of *BAX*, *BCL2*, *CASP3* genes. However, despite the individual responses with regard to changes in transcriptional activity of the studied genes, an increase in *BAX/BCL2* ratio in favor of *BAX* mRNA level was observed in all cell cultures of selected breast cancer cell lines (only in the case of MDA-MB-231cell line the change was not statistically significant). Presumbly, the effect may be related to apoptotic pathway activation. At the same time, in case of HFF-1 normal dermal fibroblast cells, a reverse effect was noted. In cell cultures of HFF-1 normal cells exposed to a high dose of amygdalin, a statistically significant decrease in the *BAX/BCL2* ratio was noted, which suggests an increase in transcriptional activity of the anti-apoptotic *BCL2* gene. This may indicate activation of protective mechanisms in response to the cytotoxic effects of amygdalin.

Amygdalin seems to be an interesting compound; to determine its anti-cancer potential, it is necessary to conduct further both in vitro and in vivo studies with use of a wide spectrum of different cell types.

Authors' Contribution

A.Z.: research concept and design, supervising the project, carrying out the experiments, acquisition of data, data analysis and interpretation, writing – original draft preparation, writing – review and editing, visualization, literature review, final proofreading and approval of the version for publication, funding acquisition; J.P.C.: research concept and design, supervising the project, carrying out the experiments, acquisition of data, data analysis and interpretation, visualization, literature review, final proofreading and approval of the version for publication; A.N.: research concept and design, carrying out the experiments, writing – original draft preparation, literature review, final proofreading and approval of the version for publication; D.K.: carrying out the experiments, acquisition of data, data analysis and interpretation, final proofreading and approval of the version for publication.

ORCID

Aleksandra Zielińska https://orcid.org/0000-0003-3394-967X
Justyna Płonka-Czerw https://orcid.org/0000-0003-0201-525X
Agnieszka Nowak https://orcid.org/0000-0002-1947-9299
Dariusz Kuśmierz https://orcid.org/0000-0002-9028-3176

Funding

Sources of support and grant numbers: Medical University of Silesia, PCN-1-056/N/0/B.

Conflict of Interest

The authors have no potential conflicts of interest to declare.

Ethics Approval

Ethics approval was not required for this study.

References

[1] Wahab MF, Breitbach ZS, Armstrong DW, Strattan R, Berthod A. Problems and pitfalls in the analysis of amygdalin and its epimer. J Agric Food Chem. 2015; 63: 8966-8973.

[2] Blaheta RA, Nelsonb K, Haferkampa A, Juengela E. Amygdalin, quackery or cure? Phytomedicine. 2016; 23: 367-376.

[3] Unproven methods of cancer management. Laetrile. CA: Cancer J Clin. 1991; 41: 187-192.

[4] Laetrile/Amygdalin: PDQ Cancer Complementary and Alternative Medicine Editorial Board, 2019. http://www.ncbi.nlm.nih.gov/pubmedhealth/PMH0032851/ (05.11.2020).

[5] Zuoqing S, Xiaohong X. Advanced research on anti-tumor effects of amygdalin. J Cancer Res Ther. 2014; 10 (Special Issue): C3-C7.

[6] Newmark J, Brady RO, Grimley PM, Gal AE, Waller SG, Thistlethwaite JR. Amygdalin (laetrile) and prunasin beta-glucosidases: Distribution in germ-free rat and in human tumor tissue. Proc Natl Acad Sci USA. 1981; 78: 6513-6516.

[7] Abboud MM, Al Awaida W, Alkhateeb HH, Abu-Ayyad AN. Antitumor action of amygdalin on human breast cancer cells by selective sensitization to oxidative stress. Nutr Cancer. 2019; 71: 483-490.

[8] Chang HK, Shin MS, Yang HY, Lee JW, Kim YS, Lee MH, Kim J, Kim KH, Kim CJ. Amygdalin induces apoptosis through regulation of Bax and Bcl-2 expressions in human DU145 and LNCaP prostate cancer cells. Biol Pharm Bull. 2006; 29: 1597-1602.

[9] Milazzo S, Lejeune S, Ernst E. Laetrile for cancer: A systematic review of the clinical evidence. Support Care Cancer. 2007; 15: 583-595.

[10] Kim YS, Kim JJ, Cho KH, Jung WS, Moon SK, Park EK, Kim DH. Biotransformation of ginsenoside Rb1, crocin, amygdalin, geniposide, puerarin, ginsenoside Re, hesperidin, poncirin, glycyrrhizin, and baicalin by

human fecal microflora and its relation to cytotoxicity against tumor cells. J Microbiol Biotechnol. 2008; 18: 1109-1114.
[11] Song Z, Xu X. Advanced research on anti-tumor effects of amygdalin. J Cancer Res Ther. 2014; 10 (Suppl 1): 3-7.
[12] Park HJ, Yoon SH, Han LS, Zheng LT, Jung KH, Uhm YK, Lee JH, Jeong JS, Joo WS, Yim SV, et al. Amygdalin inhibits genes related to cell cycle in SNU-C4 human colon cancer cells. World J Gastroenterol. 2005; 11: 5156-5161.
[13] Makarevic J, Rutz J, Juengel E, Kaulfuss S, Reiter M, Tsaur I, Bartsch G, Haferkamp A, Roman A, Blaheta RA. Amygdalin blocks bladder cancer cell growth in vitro by diminishing cyclin A and cdk2. PLoS One. 2014; 9: 105590.
[14] Kwon HY, Hong SP, Hahn DH, Kim JH. Apoptosis induction of Persicae semen extract in human promyelocytic leukemia (HL-60) cells. Arch Pharm Res. 2003; 26: 157-161.
[15] Makarević J, Rutz J, Juengel E, Kaulfuss S, Reiter M, Tsaur I, Bartsch G, Haferkamp A. Blaheta RA. Amygdalin blocks bladder cancer cell growth *in vitro* by diminishing cyclin A and cdk2. PLoS One, 2014; 9: e10559.0.
[16] Lee HM, Moon A. Amygdalin regulates apoptosis and adhesion in Hs578T triple-negative breast cancer cells. Biomol Ther. 2016; 24: 62-66.
[17] Moradipoodeh B, Jamalan M, Zeinali M, Fereidoonnezhad M, Mohammadzadeh G. In vitro and in silico anticancer activity of amygdalin on the SK-BR-3 human breast cancer cell line. Mol Biol Rep. 2019; 46: 6361-6370.
[18] Qian L, Xie B, Wang Y, Qian J. Amygdalin-mediated inhibition of non-small cell lung cancer cell invasion in vitro. Int J Clin Exp Pathol. 2015; 8: 5363-5370.
[19] Chen Y, Ma J, Wang F, Hu J, Cui A, Wei C, Yang Q, Li F. Amygdalin induces apoptosis in human cervical cancer cell line HeLa cells. Immunopharm Immunot. 2013; 35: 43-51.
[20] Elemore S. Apoptosis: A revive of programmed cell death. Toxicol Pathol. 2007; 35: 495-516.
[21] Hardwick JM, Soane L. Multiple functions of BCL-2 family proteins. Cold Spring Harb Perspect Biol. 2013; 5: a008722.
[22] Kale J, Osterlund EJ, Andrews DA. BCL-2 family proteins: changing partners in the dance towards death. Cell Death Differ. 2018; 25: 65-80.
[23] Saleem M, Asif J, Asif M, Saleem U. Amygdalin from apricot kernels induces and causec cell cycle arrest in cancer cells: An update review. Anticancer Agents Med Chem. 2018; 18: 1-6.
[24] Eguchi H, Suga K, Saji H, Toi M, Nakachi K, Hayashi SI. Different expression patterns of Bcl-2 family genes in breast cancer by estrogen receptor status with special reference to pro-apoptotic Bak gene. Cell Death Differ. 2000; 7: 439-446.
[25] McIlwain DR, Berger T, Mak TW. Caspase functions in cell death and disease. Cold Spring Harb Perspect Biol. 2015; 7: a026716.