

STUDIES ON AMYGDALIN

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Article Received on 21/02/2018

Article Revised on 14/03/2018

Article Accepted on 04/04/2019

ABSTRACT

Amygdalin is considered one of the nitrilosides, natural cyanide containing substances abundant in the plants seeds of the prunasin family that have been used to treat cancers and also as pain reliever. Particularly, D-amydalin (D-mandelonitrile-b-D-gentiobioside) is known to exhibit selective killing effect on cancer cells. Apoptosis, programmed cell death, is an important mechanism in cancer treatment. In the present study, we extract amygdalin from sweet apricot kernels, cashew seeds, and bitter almond seeds by ethanol extraction at 37°C, then determination and quantitation the amygdalin concentration in these extractions by application of high performance liquid chromatography HPLC procedure. The highest extract containing amygdalin of the three plants investigated whether this extract induces apoptotic cell death in human breast cancer cell MCF-7 in comparing with other concentrations of pure amygdalin which purchased from Sigma Aldrich. In the present results, we found that the bitter almond extraction containing the highest concentration of the three plants extractions. MCF-7 cells treated with amygdalin exhibited several morphological characteristics of apoptosis. Here we have shown that the treatment with amygdalin decreased expression of BCL-2, an anti-apoptotic protein, and increased caspase-3 enzyme activity in MCF-7 breast cancer cells. The present study reveals that amygdalin may offer a valuable option for the treatment of breast cancers.

INTRODUCTION

Cancer is currently the second leading cause of death around the world until now.^[1] Chemotherapy or radiotherapy, surgery are the best method to relieve patient pain. However, chemotherapy lead to adverse side effects in patients.^[2] Breast cancer has been considered as one of most commonly diagnosed types of cancer among women.^[3]

Chemoprevention by use of natural materials that have the potential to delay, prevent or reverse the development of breast cancer is an excellent option. The search for new chemo-preventive and anticancer agents that are more effective and less toxic has great interest in phytochemicals.^[4] Many natural chemo preventive compounds are shown to decrease the risk of cancer through induction of apoptosis, programmed cell death.^[9]

Mitochondrial mediated apoptosis is stimulated by multiple molecular events including reduce in B-cell lymphoma 2 (BCL-2), upregulation in BCL-2-associated X protein (Bax), and activation of caspase3.^[6] Mounting evidence has supported that amygdalin stimulate apoptotic cell death of various cancer cells such as promyelocytic leukemia, prostate cancer, cervical and liver cancer cells.^[7]

Amygdalin (D-mandelonitrile -β-D gentiobioside; (C₂₀H₂₇NO₁₁) (Figure 1) vitamin B17; previously called laetrile), one of the many nitrilosides, has been used for cancer treatment and pain relief.^[8] Old Chinese medicine used amygdalin as a useful component and it has since been used as a cough auxiliary medicine and cancer therapy.^[9]

Amygdalin, which belongs to the aromatic cyanogenic glycoside group, is cosmopolitan in distribution in the rosaceous plant seeds like *Prunus persica* (peach), *Prunus armeniaca* (apricot) and *Prunus amygdalus amara* (bitter almond).^[10]

Amygdalin has been shown to be an anti-tussive, anti-asthmatic, anti-atherogenic, inhibition and or prevention of fibrosis, anti-inflammatory, anti-ulcer and anti-tumor activities.^[7]

Amygdalin was isolated by two French chemists, Robiquet and Boutron and in 1837, it was named emulsion by Liebig and Wöhler "A chronology of significant historical developments in the biological sciences".^[8] Extraction of amygdalin from food plants is a crucial field of any analytical procedure as its potential for rapid degradation. An efficient extraction process

would cause complete detection of amygdalin without any losses or degradation.^[11]

Laetrile is a cyanogenic agent, which hydrolyze to form cyanide on interacting with an enzyme β -glucuronidase, which is a member of the glycosidase family of enzymes that catalyze the breakdown of complex carbohydrates. Hydrogen cyanide is reported to be the main agent in Laetrile which is lead to killing of cancer cells, and when the drug hydrolyzes in the presence of β -glucuronidase, hydrogen cyanide is formed. However, according to Dorr RT, the concentration of β -glucuronidase in malignant cells is no more in abundance than in normal healthy body cells.^[12]

$C_{20}H_{27}NO_{11}=457.4$

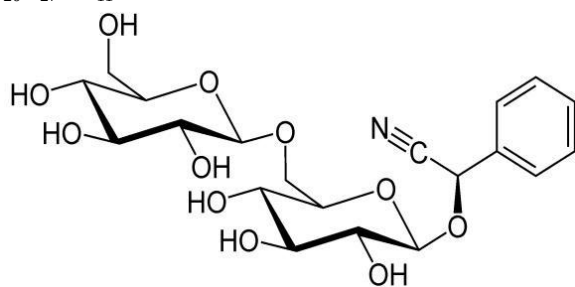


Fig. 1: Chemical structure of amygdaline (CAS—29883–15–6).

Another suggested enzyme to play a role is Rhodanese, which is responsible for converting cyanide into thiocyanate to render it harmless within the human body. It has been postulated that this enzyme is exclusive to non-cancer cells, thereby stop the non-cancer cells from cyanide toxicity, and the toxic effects of Laetrile on cancer cells are due to an imbalance in these two enzymes.^[13]

Many of the reports on efficiency of amygdalin extraction were based on the use of water or methanol. Quantification was mostly carried out in almond, apricot and Chinese herbal medicines which have amygdalin as a major ingredient. We have optimized the extraction of amygdalin which extracted by ethanol method and used a modified RP-HPLC method to measure the amount of amygdalin in sweet apricot kernel, bitter almond and cashew seeds.^[14]

Chemo-protective effect of amygdalin depends mainly on stopping of several cellular signal transduction pathways which have role in growth, differentiation, and malignant transformation, or activation of several apoptotic enzymes as Caspase-8 that lead to cleavage of BID, leading to upregulation of Bax that lead to mitochondrial Cytochrome c release and induced Caspase-3 activation with inhibition of BCL-2 gene expression.^[15]

According to the above mentioned, this study was designed aiming to extract amygdalin by ethanolic extraction at 37°C followed by identification and

quantitation by HPLC method.^[14] and investigate the chemo preventive effect of amygdalin against MCF-7 cell line and determine the gene expression level of caspase-3 and BCL-2 genes.^[16,17]

MATERIAL AND METHODS

Sample materials and reagents

Three types of plant material used in this work sweet apricot kernels, Cashew seeds and Bitter Almond seeds found in Rosaceae family were used^[9] and collected on October 2018 as: Cashew nut purchased from state of Tanil nadu in India. Apricot purchased from Pakistan state Pinjab. Bitter almond purchased from Governor of Irbid, Jordan. Solvents for high performance liquid chromatography (HPLC) analysis were purchased from J.T. Baker (Phillipsburg, NUSA). Working standard of amygdalin 98% was purchased from Sigma Aldrich Egypt. The MCF-7 cell line was purchased from Vacsera, AL Giza, Egypt. DMEM, penicillin, streptomycin, trypsin and PBS purchased from LONZA, fetal bovine serum(FBS) from Sigma Aldrich Egypt. All other chemicals used in this study were obtained from El Nasr Pharmaceutical Chemicals.

Sampling and Extraction Procedure

5 g from each type of three seeds were ground in a blender, and 2 g was weighed into a conical flask. Ethanol (50 ml) was added, and extractions were carried out in shaking water bath (37°C).The extracts were filtered and ethanol was completely evaporated from the filtrate with a rotary evaporator. Diethyl ether (10 ml) was added to the dried sample and the mixture was vortexed (1 min) at room temperature (20 ° ± 2°C) to precipitate amygdalin. The diethyl ether was allowed to evaporate.^[10]

HPLC analysis

In this study, amygdaline in the three plants seeds were extracted and isolated from their seeds matrix using reflux procedure and subsequently identified and determined by high performance liquid chromatography An HP 1100 chromatographic system consisting of a quaternary pump, degasser, diode array detector, and HP ChemStation Data system was used. (Millipore Milli Q). Separation was achieved on.^[18,19] a Agilent C₁₈ column (250 × 4.6-mm i.d., 0.45 µm). and the samples were analyzed at a UV wavelength of 215 nm. The mobile phase consisted of methanol–water (15:85 for 30 min) and the flow rate was 0.8 mL/min. The column temperature was 30°C. The injected volume of samples was 20 µL, by loop. The amygdalin contents were determined using an amygdalin standard curve.^[20,21]

Calibration curve and detection limit

Amygdalin was dissolved in methanol and five different standard solutions containing 0.2, 0.4, 0.6, 0.8, and 1 mg/ml of it were obtained. The calibration curve was constructed according to the peak area and the concentration of amygdalin. Then the standard solution of the lowest concentration was diluted gradually and

injected into the instrument to determine the detection limit when the signal-to-noise ratio is 10 (Table 1,2 & Figure 2).

Table 1: Parameters of calibration curve.

Con. (mg/ml)	peak area
0.1	2963.7876
0.2	5918.6616
0.4	11945.4755
0.6	17800.5531
0.8	23722.1786
1	29712.1568

Table 2: Analytical statistics data for the determination of amygdaline.

Correlation coefficient (r)	0.999993762
Slope	29692.18521
intercept	2.839843836
standard	10356.92127
LOD(μ g/ml)	0.91472
LO(μ g/ml)	3.27

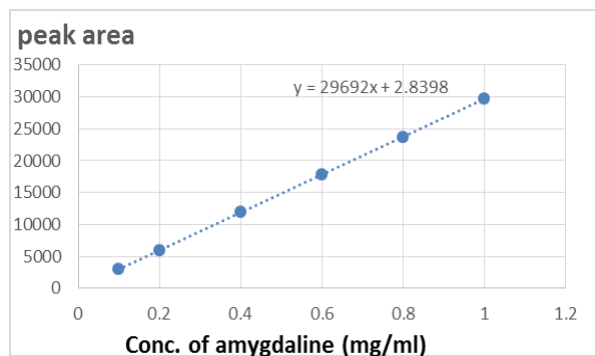


Fig. 2: Calibration curve for amygdaline by HPLC.

Quantitative Analysis of Amygdaline in Sample

Under the optimized conditions, a series of working solutions of amygdaline are injected via the injection valve of the HPLC instrument to construct a calibration graph. This is done by comparing their peak areas with the concentration standard solutions of Amygdaline from which the amount of amygdaline in each of the selected sample are determined by linear regression. The optimized conditions are summarized as in (Table 3).

Cell-line preparation

The cell line was cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 0.1 mg/ml streptomycin in a humidified incubator containing 95% air and 5% CO₂ at 37°C. MCF-7 cells (5x10⁵ cells / ml).

Table 3: Optimized chromatographic conditions for the separation and determination of amygdaline.

Parameter	Condition
Column type:	a Agilent C18 column
Dimensions:	(250 × 4.6-mm i.d., 5 μ m).
Pump:	Quaternary pump
Detector:	UV 215nm
Injection volume:	20 μ l
Column temperature:	30°C
Mobile phase:	The mobile phase consisted of methanol–water (15:85 for 30 min) and the flow rate was 0.8 mL/min.

were seeded on six well culture plate containing 2 ml DMEM after 48 hr incubation the medium was removed and fresh medium containing various concentrations of amygdalin ranged between 0.625 to 20 mg/ml were added & one well seeded without drug as untreated control and incubated for 48 hr at 37 °C in 5% CO₂ incubator until further analysis.

Cytotoxic effect on human cell line MCF-7 using MTT assay

This cytotoxic activity test was conducted and determined by Bioassay-Cell Culture Laboratory, in Nile Center for experimental Researches (NCER) Mansoura, using Vybrant® MTT Cell Proliferation Assay Kit (V-13154). The MTT assay is a colorimetric assay depend on reduction of yellow MTT (3- (4,5- dimethylthiazol-2-yl) -2,5- diphenyl tetrazolium bromide) to purple formazan.^[16] Briefly, 7000 cells/well were treated with

various concentrations of amygdalin ranged between 0.625 to 20 mg/ml. After 48 hr incubation 10 μ L of MTT was added to each well and incubated at 37°C for 4hr. The formazan crystals that formed were dissolved by adding 100 μ l/well of 10% Sodium dodecyl sulphate HCL. The absorbance was read at 570 nm. The percentage of change in viability was calculated according to the formula: (Reading of extract / Reading of negative control) -1) x 100.

Molecular determinations

Total RNA was extracted from human MCF-7 cell using RNeasy Mini Kit purchased from Qiagen, Catalog numbers: 74104 and 74106. and using the manufacture instructions. The Synthesis of cDNA was occurred by occurred by using the Thermo Scientific™ RevertAid™ First Strand cDNA Synthesis Kit purchased from Thermo Scientific, code #K1622. Real time PCR

amplification was performed using Maxima SYBR Green qPCR Master Mix (2X) kit purchased from Thermo scientific, catalog #K0251, Mitupatum *et al.*^[17], to detect the gene expression of BCL-2 and Caspase-3. The amount of target gene expression levels were quantified using the formula of $2^{-\Delta\Delta Ct}$ Livak & Schmittgen.^[22] The primer sequences of the desired genes, was designed according to (Table 4). As bitter almond seeds contain the highest concentration of amygdalin of three plants we compare the effect of 7.5 mg/ml (LC₅₀) concentration of bitter almond seed extract to 7.5 mg/ml of pure amygdalin on the apoptotic effect on MCF-7 by activation of caspase-3 and decrease of BCL-2.

Table 4: Primers sequences used in preparations.

Gene	Primers	Ann.Tem. ^o C
BCL-2	5-TTGTGGCCTTCTTTGAGTTCGGTG-3	51.9
	5--GGTGCCGGTTCAGGTAAGTCA-3	
Caspase-3	5'-AGAGGGGATCGTTGTAGAAGTC-3'	51.9
	5'-ACAGTCCAGTTCTGTAAACCACG-3'	
GAPDH	5- AAGGTGAAGGTCGGAGTCAAC-3	51.9
	5- GGGGTCATTGATGGCAACAATA-3	

RESULTS

Amygdalin analysis by HPLC

It was necessary to establish a suitable HPLC method to determine amygdalin concentration in the different seeds extraction. Agilent C₁₈ column and methanol–water as mobile phase were used. It was found that when the ratio of methanol and water was 15:85, amygdalin could be completely separated with the other ingredients. But if the ratio of methanol was a little large, amygdalin would not be separated well. If the ratio of methanol was small, it would lead to long analytical time. Because amygdalin was the only analyte, other ingredients had to be eluted as quickly as possible to cut down on analytical time when the peak of amygdalin was finished. For this reason, a gradient elution method methanol–water is 15:85 for 30 min was also attempted, and a good chromatographic separation was obtained within 50 min.

The calibration curve between peak area (A) and concentration of amygdalin showed excellent linearity ($r^2 = 0.99999$), and the limit of detection (LOD) was 0.91472 ug/ml and limit of quantitation (LOQ) for amygdaline was 3.2 ug/ml.

From the calibration curve and the equation can determine the concentration of amygdalin in the extraction of sweet apricot kernels, bitter almond and cashew seeds (**Figure 3-6**).

The content of amygdaline in plant samples was 1.7 mg/g in sweet apricot kernels, 4.9 mg/g in cashew seeds and 38.6 mg/g in bitter almond seeds respectively (**Table 5, Figure 7**).

Statistical analysis

Data analyzed by SPSS version 24. Results expressed as mean+ SD, $P < 0.05$ considered significant. Two-way ANOVA was run to test effect of dose of amygdaline and type of cell on concentration of light (Viability of cells). Duncan's multiple range test was used to differentiate between significant means. Pearson correlation test applied to test association between BCL-2 dose and caspase-3 dose (**IBM Corp. Released 2016. IBM SPSS Statistics for Windows, Version 24.0. Armonk, NY: IBM Corp.**).

Cytotoxic effect of Amygdalin on MCF-7 cell line

The MTT assay was performed to assess the rate of proliferation of MCF-7 cells after treatment with varying concentrations of pure amygdalin. We examined the in vitro effects of different concentrations of amygdalin ranged between 0.625 to 20 mg/ml on the viability of MCF-7 cells after 48 hr using the MTT assay. The result showed that amygdalin has a concentration inhibitory effect on MCF-7 cells. At the concentration of 3.75 mg/ml, 25% concentration viability was detected and 7.5 mg/ml, 50% viability was detected during the 48hr treatment, whereas maximum cytotoxicity LC₉₀ was observed at a concentration of 15 mg/ml (**Table 6**).

Effect of Amygdalin on the level of gene expression of BCL-2 and Caspase-3 by using real time PCR

The results of gene expression showed that, the extracted amygdalin from bitter almond seeds has nearly equal effect to pure amygdalin with the same concentration 7.5 mg/ml (LC₅₀) (**Figure 8**). There were significant increase in the level of gene expression Caspase-3 in MCF-7 by increasing the concentrations of amygdalin with significant decrease in the expression level of BCL-2 gene in amygdalin cells by increasing the concentration of amygdalin (**Figure 9**).

Table 5: The concentration of amygdaline in plant samples.

Sample	peak area	con. mg/g
Sweet apricot kernels	5201.91113	1.7509909
Cashew seeds	14757.2	4.9691061
Bitter almond seeds	114775	38.6539909

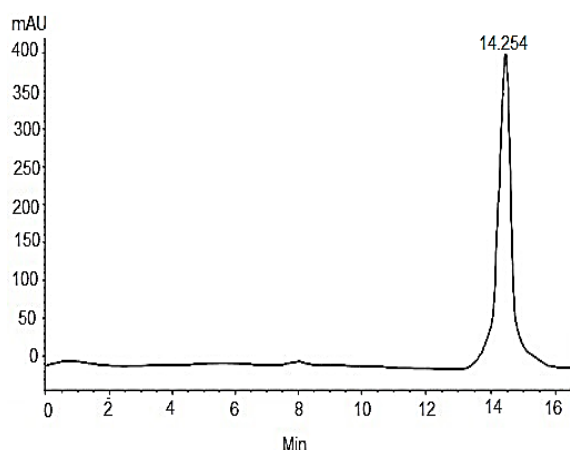
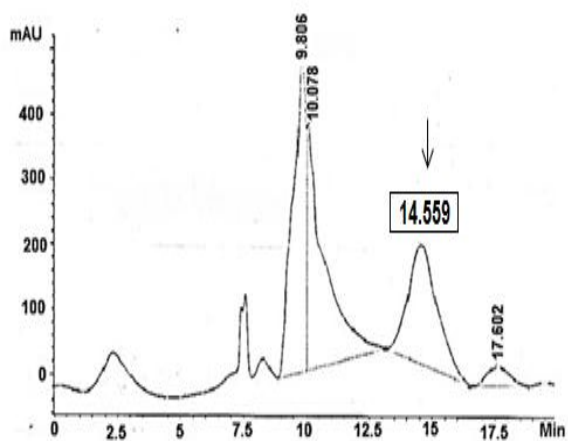
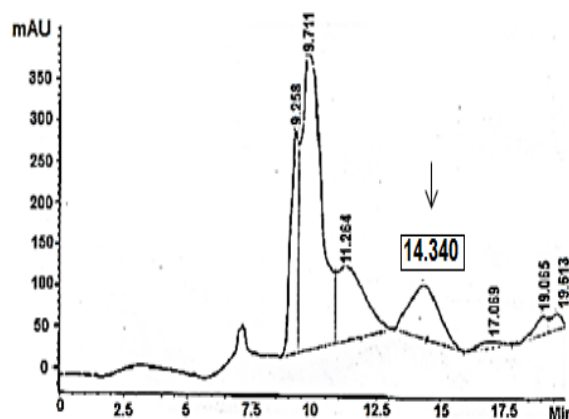
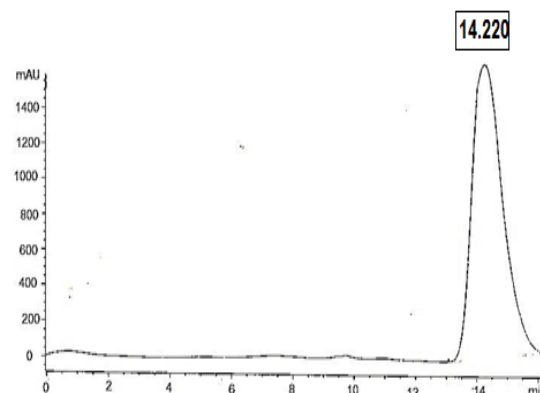
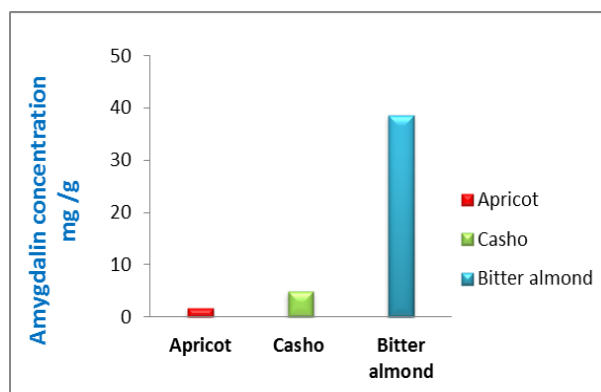
Table 6: LC₂₅, LC₅₀ & LC₉₀ of Amygdalin on MCF-7 by using MTT assay.

Sample Code	LC ₂₅ (mg/ml)	LC ₅₀ (mg/ml)	LC ₉₀ (mg/ml)
Amygdalin treated MCF-7 cells	3.75	7.5	15
Negative Control	0	0	0

LC₂₅: Lethal concentration of the sample which causes the death of 25% of cells in 48 hr.

LC₅₀: Lethal concentration of the sample which causes the death of 50% of cells in 48 hr.

LC₉₀: Lethal concentration of the sample which causes the death of 90% of cells in 48 hr.

**Fig. 3: HPLC chromatograms of amygdalin standard (amygdalin STD).****Fig. 4: The chromatographic curve comparing the height of the peak (mAu) with the retention time (Min) by HPLC. The retention time of chromatographic curve by HPLC for cashew seeds extract is 14.559 minutes.****Fig. 5: The chromatographic curve comparing the height of the peak (mAu) with the retention time (Min) by HPLC for apricot kernels, the retention time is 14.340 minutes. From the equation ($Y = X29692 + 2.8398$) we calculate the concentration of amygdalin in each gram extract. Y=Peak area & X=Concentration of amygdalin in the sample.****Fig. 6: The chromatographic curve comparing the height of the peak (mAu) with the retention time (min) by HPLC for bitter almond seeds extract. The retention time is 14.220 minute.****Fig. 7: The bitter almond seeds is the highest one containing amygdalin then cashew seeds then sweet apricot kernels.**

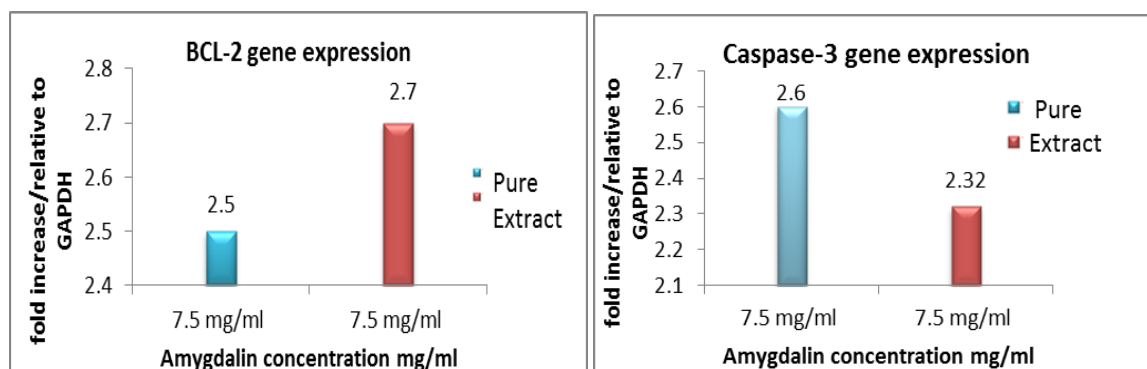


Fig. 8: Illustrate comparison between concentration 7.5 mg/ml of extract bitter almond seed against concentration of 7.5 mg/ml of pure amygdalin in gene expression of BCL-₂ and caspase-3.

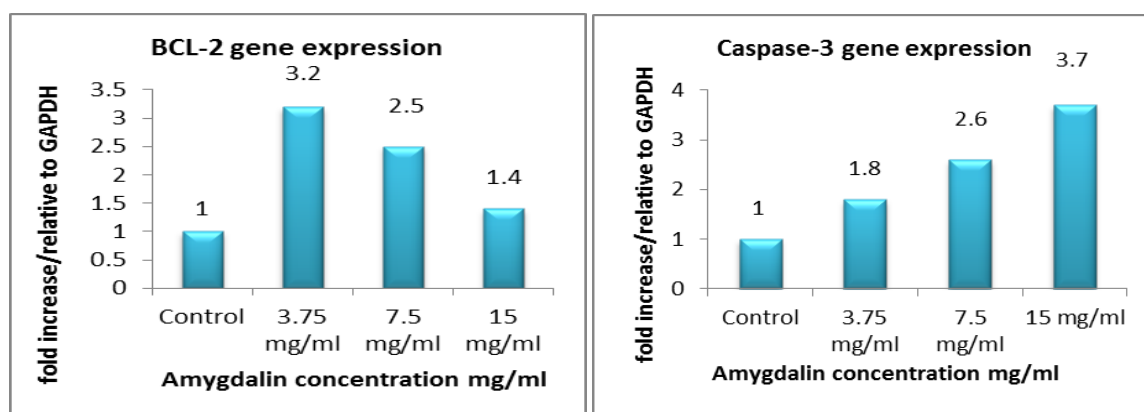


Fig 9: Analysis of PCR product of BCL-₂ and Caspase-3 gene in MCF-7 cell line by using real time PCR.

Expression of Caspase-3 gene was significantly elevated while BCL-₂ gene expression was significantly reduced after treatment with different concentrations of pure amygdalin. Fold change increase as compared to control sample in caspase-3 while in BCL-₂ decrease as compared to control sample. Data representing as Mean $\pm$ SE %. P-values >0.01.

DISCUSSION

In the present study we report the extraction and HPLC determination of amygdalin in seeds of Cashew nut, sweet apricot and Bitter almond after extraction according to (Figure 2-7). The aim of this study was to establish an optimal method for the extraction of amygdalin from the seeds of this species.

Naryal *et al.*^[11], revealed that the geographical elevation had no marked influence on kernel amygdalin content. Similarly, seed and kernel physical characters have no significant effect on amygdalin content in apricot kernel. High variability within genotypes suggested that genotype played a significant role on amygdalin content in apricot kernel. Low amygdalin content in genotypes with white seed coat phenotype confirmed our earlier findings that white seed coat phenotypic marker is associated with sweet kernel. Therefore, white seed coat phenotype can be taken as a marker for low amygdalin content in future studies.

Another study showed the precision of the HPLC method was determined by repeated analysis ($n = 5$) of amygdalin extracts obtained using treatment M1. A maximum coefficient of variation (% CV max) of 0.0949, as well as detection (LD) and quantification (LQ) limits of 0.0505 and 0.0548 mg/g, respectively,

were determined; thus indicating a high reliability of the method.^[23]

Viorica-Mirela *et al.*^[24], appreciated that the amygdalin concentration in samples which contained kernel from apricots and plums is between 3-24 mg/kg, and a high value is found in apricots. In oil samples there is no amygdalin, and this aspect have positive implications over the quality and use of oils in foods industry by using HPLC method.

Bolarinwa *et al.*^[10], HPLC method gave a good separation chromatogram within 15 min with an excellent linearity (correlation $R^2 = 0.9998$) between the peak area and the concentration of amygdalin. Amygdalin in extracts of fruit kernels was also clearly separated. The LOD was 0.1 μ g/ml and the LOQ was 0.3 μ g/ml. The LOD of amygdalin obtained in this study was lower than that obtained from a micellar- electrokinetic chromatography method (2 μ g/ml).^[25]

HPLC determination of amygdalin from fruit kernels and food products requires the establishment of a good mobile phase with the right dilution ratio. Methanol is a good mobile phase for amygdalin separation by HPLC.^[10] Having considered this factor, methanol and water were used at a ratio of 25:75, (v:v) in an isocratic

elution method. Methanol and water in a ratio of 15:85, (v:v) in a gradient elution method was reported to completely separate amygdalin from apricot and *Prunus tomentosa* thunb within 50 min.^[10]

In another study amygdalin detection was achieved by UV detection in an isocratic elution with an excellent linearity between the peak area and the concentration of amygdalin. The detection limit of RP-HPLC used for the analysis is 0.1 g/ml. The amygdalin peak was completely separated from other materials without any pre-treatment. The recovery of amygdalin was greater than 98%.^[26]

Kwon *et al.*^[27], concluded that the mobile phase combination of a 10 mM sodium phosphate buffer (pH 2.3) containing 13.5% acetonitrile in reversed phase HPLC was effective in separating and analyzing the D-amygdalin and neoamygdalin. The emulsin, a hydrolyzing enzyme, was completely inactivated at above 90°C. In addition, in order to suppress the epimerization of D-amygdalin, the extraction time was reduced to less than 8 min. By their extraction method, the extraction efficiency of D-amygdalin in ma-whang-tang was greatly improved. This study would be useful for Chinese herbal prescriptions containing *Armeniaca Semen*.

Another study revealed that a simple extraction and HPLC method for identifying and quantifying of amygdaline in Iraqi plant seeds had been established with uncomplicated optimization procedure. The analytical figures of merit such as linearity, detection limit, accuracy, and precision obtained by HPLC technique as validation method proved that it was reproducible and selective for the analysis of amygdaline in plant seeds. Among the types of seeds studied, bitter kernel had higher amygdaline content than the sweet one and *Citrullus colocynthis* kernel.^[14]

In the present study, we showed that amygdalin inhibits proliferation of breast cancer cells according to (Table 6). We further showed that amygdalin induces apoptosis in MCF-7 cell. Given that breast cancer is considered to be one of the most frequent malignancies in women^[28], our results may provide useful information on developing the anti-cancer strategy.

MTT assay can accurately determine the count of live cells and is indispensable for the assessment of cytotoxicity relevant to screening of anticancer drugs⁽²⁹⁾. By using the assay in the present study, we found that amygdalin reduced the viability of MCF-7 cells in a dose-dependent manner. The effect of amygdalin on cell viability appeared to be cell type dependent as the viability of the amygdalin-treated FL cells did not show significant changes in comparison with non-amygdalin-treated FL cells. A similar phenomenon was demonstrated by observation of cultured cells under a microscope: The numbers of amygdalin-treated HeLa

cells were markedly less than those of non-amygdalin-treated control HeLa cells, whereas the numbers of FL cells were not affected by amygdalin treatment.^[30]

Arshi *et al.*^[31], revealed the cytotoxicity effect of amygdalin was evaluated on four human cancer cells i.e. A549, MCF-7, AGS and HDF. All cells were treated with different concentration of amygdalin for incubation time of 24, 48 and 72 hr. MTT was used to measure cell viability. In agreement with Arshi study, we have shown this inhibitory effect of amygdalin in MCF-7 cell. This suppression effect could be a justification of how amygdalin arrest or diminish tumor proliferation.

Makarević *et al.*^[32], investigation demonstrates that amygdalin treatment significantly reduces the growth rate of both castration-sensitive (LNCaP) and castration-resistant (PC3) cell lines but not DU-145 without toxic effects. These findings agree with reports showing growth-inhibiting, anti-proliferative and pro-apoptotic effects of amygdalin on promyelocytic leukemia, colon cancer, cervical cancer^[30] and bladder cancer cells TCCSUP.^[33]

Lee and Moon^[34], investigated the cytotoxic effects of amygdalin on human breast carcinoma cells. They performed MTT assay upon treatment with various concentrations of amygdalin for 24 hr. Amygdalin inhibited proliferation of MCF-7, MDA-MB-231 and Hs578T cells in a dose-dependent manner.

In the present study, we investigated the expression of the effect of amygdalin through the regulation of BCL-2 and caspase-3 expression in MCF-7 human breast carcinoma cell according to (Figure 8,9).

Members of the BCL-2 family of proteins are characterized by their ability to form a complex combination of heterodimers with Bax and homodimers with itself. When Bax is overexpressed in cells, apoptotic death in response to a death signal is accelerated: this founding has resulted in its designation as a death agonist. When BCL-2 is overexpressed, it heterodimerizes with Bax, and cell death is suppressed. Caspases, a family of cysteine proteases, are integral parts of the apoptotic pathway. Caspases have been identified as effectors of the apoptotic process. Caspase-3, in particular, has many cellular targets and produces typical morphologic features of apoptosis.^[35] The present results have also revealed increasing of active caspase-3 expression and up-regulation of caspase-3 activity in the cells exposed to amygdalin and decrease in BCL-2 expression.

Chen *et al.*^[30], investigated that amygdalin induce an increase caspase-3 & Bax and decrease in BCL-2. They examined whether caspase-3 and BCL-2 protein could be induced in MCF-7 cells treated with amygdalin as determined by using western blotting. After 24 hr of exposure to amygdalin at concentrations of 1.25, 2.5, 5,

10, and 20 mg/ml, Caspase-3 & Bax genes in HeLa cells were activated by amygdalin in a dose-dependent manner. Increased concentrations of amygdalin at the amounts, resulted in corresponding increases of caspase-3 & Bax activity and reduction in BCL-2.

A study on the effect of amygdalin on the expression of caspase-3 and BCL-2 proteins was investigated. They demonstrated that amygdalin induces apoptotic cell death by caspase-3 activation through the down-regulation of anti-apoptotic BCL-2 protein and the up-regulation of pro-apoptotic Bax protein in DU145 and LNCaP prostate cancer cells. Based on these results, amygdalin showed considerable promise in the treatment of prostate cancers.^[32]

Many experiment results supported that, amygdalin has antitumor activity. Amygdalin and other cyanogenic sugar, are also considered to be a potential alternative antitumor drug.^[36] Mechanism of amygdalin. Kwon *et al.*^[37], confirmed that amygdalin can induce apoptosis in human promyelocytic leukemia (HL-60) cells.

Park *et al.*^[38], have shown that amygdalin inhibited the proliferation of human colon cancer SNU-C4 cell, and the mechanism is the inhibition of expression of cell cycle related genes. Milazzo *et al.*^[9], identified that amygdalin can induce apoptosis in prostate cancer DU145 and LNCaP cells by regulating the expression of Bax and of BCL-2.

In conclusions, recent studies have reported that various natural products induce the apoptosis of cancer cells, and inhibit metastasis and tumor cell growth, suggesting the use and benefit of these natural compounds as of novel medical treatment of human cancer. In vitro researches have revealed that amygdalin, which is popularly used among patients, is an anticancer substance with pro-apoptotic features in terms of human cancer cells. Our study obviously indicated that amygdalin changes the expression level of some critical genes in breast human cancer cells. Based on these results, amygdalin might be employed as a therapeutic anticancer drug.

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