Antitumor impact of amygdalin on adaptive immune response in BALB/c mice with breast cancer

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Amygdalin is a potential therapeutically target in cancer. Here, we evaluated the therapeutic effect of amygdalin in the mice model of breast cancer. We assessed the percentage of CD4, CD8 T lymphocyte, intracellular IFN-γ, and Granzyme B in spleen cells of tumorized mice treated with 50 and 150 mg/kg of amygdalin (AG50 and AG150), and determined the expression of caspase 3 and p53, tumor size, and survival rate of Balb/c mice in tumor tissue after amygdalin administration. No significant difference was observed in the frequency of CD4+ and CD8+ T cells in the three study groups. However, a significantly increased level of granzyme B in CD8+ T cells, as well as a significant decrease in the level of IL-10 in CD4+ T cells was detected in the AG50 group compared to the AG150. There was no significant difference in the expression of caspase 3 and P53 between the two groups. A significant change was seen in tumor size and survival rate of AG50 and AG150 groups compared to the controls. Our findings indicate that the antitumor effect of amygdalin in vivo was probably due to stimulating the effective immune response, and not the apoptotic genes induction.

Keywords: Apoptosis, Granzyme B, Tumor growth

Cancer is an inflammatory disease in which a variety of innate and adaptive immune cells are involved in infiltrating the human body⁴. During malignancy conditions, immune T cells can generate acute inflammations through secreting cytokines that could play an effective role in the process of cancer treatment⁵. Immune-based cancer therapies that have the potential to induce tumor-specific acute inflammatory responses can ultimately lead to tumor rejection. Breast cancer is one of the most common cancer diagnosed in women in 2022. Lung cancer, Colorectal cancers and breast cancer account for the 51% of all new diagnoses and just breast cancer account for 31% of all new diagnoses⁶. Natural herbal compounds such as berberine have revealed to have anticancer effects, particularly in breast cancer, through induction of apoptosis⁷. It is also suggested that amygdalin has various impacts such as immune-modulatory, anti-inflammatory, anti-bacterial and antioxidative roles⁸.

Amygdalin has cytotoxic functions on the cell cycle through modulating immune function and apoptosis induction⁹. In vitro studies revealed that amygdalin could significantly inhibit the proliferation of different cell lines as well as their DNA content due to promoting cell arrest in the G0/G1 phase⁹. It is suggested that amygdalin downregulated cdk1 and cyclin B and restrains the growth of renal cell carcinoma ex vivo⁹. It was found that amygdalin has the potential competence to stimulate the intrinsic apoptotic pathway in HeLa cells through a change in the regulation of apoptotic related genes such as caspase 3, Bax, and bcl2⁹. However, to our knowledge, there was no report on the cellular and molecular impacts of amygdalin on an animal model with breast cancer induced by 4T1 cells that need to be clarified.

Hence, in the present study, we have made an attempt to evaluate the antitumor effect of amygdalin on protective immune response and apoptotic gene expression profile in breast cancer BALB/c mice induced by 4T1 cells. The outcome of this study may represent a natural and affordable anticancer drug known as amygdalin, which can be used as complementary and alternative medicine.
Materials and Methods

Animal

Twenty-four female inbred BALB/c mice from 6-8 weeks old, 25-30 g were purchased from the Pasteur Institute of Iran (Tehran, Iran) for the current experiment. They were randomly divided into three groups (N=8 in each group) for treatment after tumor induction with an individual protocol. We kept the mice in plastic cages and gave access to food and water ad libitum with a 12 h light/dark cycle throughout the study. The room temperature and humidity were kept at 23±1°C and 55±10%, respectively. This study was approved by the ethical committee of the Yazd University of Medical Science with an ethical code: IR.SSU.SPH.REC.1399.206.

Cell line

A mouse mammary tumor (4T1) was provided by the Pasteur Institute, Tehran, Iran. The cells were seeded at 37°C in a humidified atmosphere with 5% CO2. 4T1 cells were cultured in RPMI 1640 medium supplemented with penicillin (100 units), 10% fetal bovine serum (FBS), and 100 μg/mL of streptomycin. The culture medium was changed every 2-3 days until 90% of the cell confluence. Then, the cells were used for tumor induction in mice.

Tumorigenesis and Treatment

All mice were kept for five days due to the environmental adaptation before starting the injections. After adaptation, the animals were inoculated on their left flanks with 4T1 cells (5×10^5 in 100 μL phosphate-buffered saline (PBS)) using a 26G hypodermic needle. Regular checks were performed to assess tumor growth for seven days until the palpable tumor has been detected. Then, the BALB/c mice were injected intraperitoneally with 50 and 150 mg/kg of amygdalin (AG50 and AG150, respectively) as experimental groups. The control group of mice (N=8) was treated with PBS. All mice were kept for five days due to the injections. After the 1st, 7th, 8th and 14th day of AG administration. On euthanizing three mice per group, their spleens were removed by smoothly homogenizing spleens with the top of a 5-mL syringe as a plunger, we could collect single cells by cell strainer under sterile conditions. Red blood cells from the cell suspension were removed by adding distilled water and 10X PBS (9 mL×1 mL). After that, we centrifuged the cell suspension at 500 g for 5 min at room temperature, and splenocytes excited in the cell pellets were resuspended in RPMI-1640 medium supplemented with 10% FBS.

Flow cytometry analysis

Splenocytes (10^6 cells/mL) in a medium containing 2 μL/mL brefeldin A (eBioscience) were treated at 37°C for 6 h. As positive controls, we used groups treated with 2 μL/mL PMA/ionomycin with Brefeldin A (Biolegend, USA) at 37°C for 6 hours. Then the activated splenocytes were washed and stained with anti-mouse CD4-FITC (Santa Cruz Biotechnology, Santa Cruz, CA), anti-mouse CD8a-FITC (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-mouse CD3-APC (Biolegend, USA) for 25 min at 4°C in separated tubes. Then the cells were stained with anti-IFN-γ-PE, anti-Granzyme B-PE/Cy7, and anti-IL-10-PE/Cy7 (Biolegend, USA) for 30 min in a dark place at room temperature after we washed the activated splenocytes with staining buffer, fixed, and permeabilized with fixation/permeabilization buffer (Biolegend, USA).

Gene expression

For investigating the apoptotic-related genes, the tumor tissues were removed from the sacrificed mice in all groups and applied for total RNA extraction by using an easy cDNA Synthesis kit (Parstous Biotechnology, Iran) according to its instructions. For evaluating the purity and concentration of extracted RNAs, we used a spectrophotometer with the absorbance of the A260/A280 ratio and 260 nm, respectively (PhotoBiometer, Eppendorf, Germany). All complementary DNA (cDNA) was synthesized from 1 μg RNA by utilizing Revert Aid First Strand cDNA Synthesis Kit (Parstous biotechnology, Iran). The cDNA product was kept at −20°C until use. The primer sequences were summarized in Table 1.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primer sequence (5'-3')</th>
<th>PCR product</th>
<th>Accession numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td>P53</td>
<td>F-GTATTTCACCCCTCAAGATCC</td>
<td>84 bp</td>
<td>NM_011640.3</td>
</tr>
<tr>
<td>Caspase 3</td>
<td>F-CTCGCTCTTGAGGATGTTG</td>
<td>201 bp</td>
<td>NM_00128404</td>
</tr>
<tr>
<td>GAPDH</td>
<td>F-CACGTCACACCAGAACGAGCTG</td>
<td>147 bp</td>
<td>NM_00128972</td>
</tr>
</tbody>
</table>

[p53, Tumor protein P53; F, Forward; R, Reverse; and GAPDH, Glyceraldehyde 3-phosphate dehydrogenase]
To determine the mRNA levels of caspase 3 and p53 in both case and control groups, a quantitative real-time polymerase chain reaction (qRT-PCR) method was performed. Gapdh was considered a reference gene for the normalization of two target gene expressions. Master Mix Green with high ROX™ (Amplicon) was utilized for PCR reaction using the StepOne system (Applied Biosystems, CA, USA). Each PCR run was performed in a final volume of 20 µL containing cDNA (2 µL), forward primer (1 µL), reverse primer (1 µL), master mix (10 µL), and 6 µL nuclease-free water. All run methods consisted of one cycle of holding stage (10 min at 95°C), followed by 40 cycles of amplification stage at 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. A melting curve stage was run after the cycling stage in the range of 60-95°C to verify the specificity of the amplicons. The relative expression level of each gene was analyzed by the $2^{-\Delta\Delta Ct}$ method.

**Statistical analysis**

Statistical analyses were performed with IBM SPSS 22 Statistics (IBM SPSS, NY, USA). All values were shown as mean ± SD. The normal distribution was assessed by Kolmogorov-Smirnov and Shapiro-Wilk tests. Statistical significance was assessed by using independent t-tests or one-way ANOVA plus Tukey HSD was employed. A log-rank (Mantel-cox) test was used to compare the survival rate of the mice. $P<0.05$ was considered a significant value.

**Results**

**Effect of amygdalin on tumor growth**

There was no significant difference in the tumor size between AG50 and AG150 (Fig. 1). The survival rates of mice were significantly prolonged in AG50 and AG150 groups compared to the controls. There was also no significant difference between AG50 and AG150 concerning the survival rates of mice (Fig. 2). In this study, the data showed a declined level of tumor size in both AG50 and AG150 groups rather than in the controls (Fig. 3). No remarkable tumor growth inhibition and prolonged survival time were seen in...
both groups. Table 2 describes the effects of amygdalin on TTE (Time to reach the endpoint), MST (Median survival time), TGD (Tumor growth delay), and ILS (Increase life span) rates compared to the controls.

Table 2 — Therapeutic effects of amygdalin in tumorized mice model between study groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>TTE (Mean days ± SD)</th>
<th>MST (Days)</th>
<th>TGD (%)</th>
<th>ILS (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AG50</td>
<td>65 ± 4.08</td>
<td>65</td>
<td>27.70</td>
<td>44.44</td>
</tr>
<tr>
<td>AG150</td>
<td>63.3 ± 4.71</td>
<td>60</td>
<td>24.42</td>
<td>33.33</td>
</tr>
<tr>
<td>Control</td>
<td>50.9 ± 8.34</td>
<td>45</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

[N: Number of mice in each group; MST: Median survival time; TTE: Time to reach the endpoint; TGD: Tumor growth delay; ILS: Increase life span; AG50 and AG150, mice treated with 50 and 150 mg/kg of amygdalin, respectively; and Control: mice treated with phosphate buffer saline (PBS)]

Effect of amygdalin on apoptotic genes

The highest mRNA levels of *p53* belonged to the control group, whereas a nonsignificant lower level of mRNA was seen in the AG50 and AG150 in comparison to the controls (Table 3). The expression of mRNA of *caspase 3* was higher in the AG150 compared to the control group, while in the AG50 the mRNA level of caspase 3 was lower than in the control group. In both groups, the mRNA level of caspase 3 was not significant (Table 3).

Table 3 — Comparison of mRNA level of apoptotic genes between the study groups

<table>
<thead>
<tr>
<th>Genes</th>
<th>AG50</th>
<th>AG150</th>
<th>Control</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>P53</td>
<td>0.39±0.23</td>
<td>0.39±0.19</td>
<td>1.45±0.72</td>
<td>0.23bc</td>
</tr>
<tr>
<td>Caspase 3</td>
<td>1.50±0.87</td>
<td>3.76±2.94</td>
<td>1.95±0.95</td>
<td>0.74bc</td>
</tr>
</tbody>
</table>

[The data are presented as Mean ± SEM according to the Mann-Whitney test. *p53*: Tumor protein P53. *Caspase 3*: Cysteine aspartic acid protease 3. AG50 and AG150, mice treated with 50 and 150 mg/kg of amygdalin, respectively; and Control: mice treated with phosphate buffer saline (PBS). *P* <0.05]

Effect of amygdalin on immune response

Regarding the intracellular assessment of cytokines by using the flow cytometry technique, our results demonstrated no significant change in the level of IFN-γ, IL-10 in CD4+ and CD8+ T cells between the three groups and % CD4+ and CD8+ T cells (Fig. 4). Moreover, the data showed a significant increase in the concentrations of IL-10 on CD4+ T cells in the AG50 group compared to the AG150. In contrast, there was a significantly decreased level of Granzyme B secretion in AG150 compared to the AG50 (Fig. 5).

![Fig. 4 — Intracellular production of IFN-γ of (A) CD4+ T cells; and (B) CD8+ T cells in spleens of mice treated with different dosages of amygdalin. There was no significant difference between the different groups. The data are presented as Mean ± SD according to the one-way ANOVA test. AG50 and AG150, mice treated with 50 and 150 mg/kg of amygdalin, respectively; and Control: mice treated with phosphate buffer saline as controls; and IFN-γ, Interferon-gamma](image)

![Fig. 5 — Intracellular production of IL-10 of (A) CD4+ T cells; and (B) Granzyme B of CD8+ T cells in spleens of mice treated with different dosages of amygdalin. There were significantly lower in contrast with higher production of IL-10 and Granzyme B in treated mice with AG50 than those with AG150. (P=0.0134 P=0.0246 IL10 in CD4+, respectively). The data are presented as Mean ± SD according to the one-way ANOVA test. AG50 and AG150, mice treated with 50 and 150 mg/kg of amygdalin, respectively; PBS, mice treated with phosphate buffer saline as controls; and IL10, interleukin 10](image)
Discussion

For the first time, it was Schrader and co-workers who discovered amygdalin in 1803 as recalled by Xing & Yang\textsuperscript{10}. Robiquet et al.\textsuperscript{11} introduced the main source of amygdalin isolated from bitter almonds. Park et al.\textsuperscript{12}, by analyzing the cDNA microarray results of human colon cancer cell line treated by amygdalin dose-dependently and time-dependently, found a down-regulation of cell cycle-related genes: ATP-binding cassette, exonuclease 1 (EXO1), sub-family F and topoisomerase I (TOP1). The aforementioned data indicated the antitumor effects of amygdalin. One study showed that amygdalin enhanced the immune function in peripheral blood mononuclear cells stimulated by PHA (phytohemagglutinin), promoted the secretion of IL-2 and IFN-\(\gamma\), and prevented the secretion of TGF-\(\beta\)\textsuperscript{13}. In another research, amygdalin is considered a promoter for proliferation of T lymphocytes in the range of 100-400 mg/L (0.22–0.87 mmol/L) with optimum effect at the 200 mg/L\textsuperscript{14}. In our study, the production of IL-10 in CD4+ T cells in the AG\textsubscript{50} group was significantly lower than those in the AG\textsubscript{150} group (Fig. 5A), while we detected the reverse results in the production of Granzyme B in CD8+ T cells in AG\textsubscript{50} compared to AG\textsubscript{150} group (Fig. 5B). IL-10 and granzyme B, as anti-inflammatory and inflammatory cytokines, have beneficial effects on tumor therapy. These results were in line with Baroni et al study that shows amygdalin can increase the level of IFN-\(\gamma\) and IL-2 which secrete by T-lymphocytes in human peripheral blood\textsuperscript{13}. These data surprisingly revealed that amygdalin with the dosage of 50 mg/kg has a better effect than amygdalin with a dosage of 150 mg/kg in terms of immune response; whereas no difference has been detected in the percentage of T cells between the two groups.

In this study, we tried to find the effects of different dosages of amygdalin on the expression of caspase 3 and \(P53\) in tumor tissue\textsuperscript{15} obtained from a tumorized mouse model. Different studies showed the role of amygdalin in the cell apoptosis and cell cycle regulation of breast and prostate cancer cell lines\textsuperscript{7,16}. Lee & Moon\textsuperscript{16} treated a triple-negative breast cancer cell line (Hs578T) with amygdalin at a dosage of 52.9 mg/mL. They demonstrated that amygdalin could increase the protein expression of Bax and Caspase3, while it caused the downregulation of Bcl-2 as an antiapoptotic protein. Makarević et al. reported that different concentrations of amygdalin could inhibit the cell proliferation of prostate cancer cell lines (LNCaP, DU-145, and PC3) by enhancing the cell arrest in the G2/M and S phases against an increase in G0/G1 stage\textsuperscript{7}. Arshi and colleagues reported amygdalin could downregulate anti-apoptotic genes (Survivin, XIAP) time-dependently in different types of human cancer cells (A549, MCF7, AGS)\textsuperscript{17}. Moradipoodeh et al.\textsuperscript{18} showed that amygdalin could induce apoptotic death in a dose-dependent manner in the SK-BR-3 cell line by differentially changing the protein expression of Bax and Bcl-2. Studies on other cancers such as cervical, bladder, and prostate cancer cells treated with amygdalin showed that amygdalin inhibited cell growth and promotes apoptosis in these cell lines\textsuperscript{9,19,20}. However, our data demonstrated no significant difference in the mRNA level of target proapoptotic genes between the study groups, but its effects were detectable. These controversial results maybe because of the dosage or route of injection as well as the effects of other genes or proteins. Based on our data, we concluded that the inhibition of growth induced by amygdalin was not due to apoptosis, in agreement with Juengel et al.\textsuperscript{8}.

In terms of evaluating the beneficial effect of amygdalin on tumor size as well as survival rates of mice with breast cancer like other anticancer compounds such as melatonin\textsuperscript{25}, the data of current research indicated a significant decrease in tumor size in AG\textsubscript{50} and AG\textsubscript{150} groups compared to the controls (Fig. 1). This result was in line with other research\textsuperscript{21,22}. Abboud et al.\textsuperscript{21} showed that amygdalin with the dosage of 4, 8, 16, 32 and 65 mmol/L could inhibit the growth of breast cancer cell lines (T47D and MCF-7). The inhibitory effect of AG showed a direct relationship with the amygdalin concentration in \textit{in vitro} studies. However, no significant difference was seen in the tumor size and survival rates between AG\textsubscript{50} and AG\textsubscript{150} group in our research while the results obtained from each dose showed a decrease in tumor size and increased survival compared to the control group. Due to the toxicity of AG, a dose of 150 may have toxic effects on other tissues besides the tumor treatment that did not investigated in this study. It seems that low doses could be sufficient for a therapeutic effect on the tumor, and increasing the drug concentration has no positive consequence on tumor volume and mice survival rate, even it could increase toxicity on other tissues. To our knowledge, in contrast with \textit{in vitro} experiments, there were few types of research\textsuperscript{23} that investigated the antitumor
effect of oral dose of AG (175 mg/kg) in vivo, in contrast to our study design using intraperitoneal AG in vivo administration (50 and 150 mg/kg). The reason is that the Intraperitoneal route is the most common way of drug administration due to some advantages such as a quite long absorption from the repository site with less cytotoxicity. It was also shown by Lian et al. that intravenously injection of 50 mg/kg amygdalin in a xenograft model of nude mice with colorectal cancer could significantly reduce a tumor weight and size in agreement with ours. It indicated the positive and dose-independent effects of amygdalin in the treatment of breast cancer in mice.

The design of the current study was based on the in vivo experiment on BALB/c mice induced by breast cancer. The low cost of amygdalin could be considered one of its benefits. It seems that amygdalin would be a promising candidate for chemo-preventive agents for complementary and alternative medicine. Future studies will be needed to use amygdalin as a pretreatment drug. It is also needed to use different types of breast cancer cell lines for tumor induction in mice and it is also needed to investigate other immune parameters such as cytokines in future studies. This aspect will help us to understand its functional mechanism for affecting the tumor cells.

Conclusion
Generally in cancers, the effect of various intervention take pace in two ways: direct effect on the tumor; and indirect effect on the immune system. The results of this study show indirect effect of the proposed intervention. It is indicated that this intervention has some beneficial effects on the adoptive immune system of breast cancer BABL/c mice induced by 4T1 cells. With respect to the direct effect of our intervention, there is no direct significant effect of our intervention on the P53 gene, tumor suppressive protein, and Caspase-3, one of the key protease in the apoptotic pathway. The present study revealed that amygdalin stimulated the mouse's immune response against tumor cells and improved their survival rate and life span. It also indicated the therapeutic potential of this compound for the treatment of breast cancer.

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Conflicts of interest
Authors declare no competing interests.

References


