Journal of Shahrekord University of Medical Sciences
doi:10.34172/jsums.2020.17 2020;22(3):106-112 http://j.skums.ac.ir

Original Article

Study of anticancer activity of cecropin B on 7, 12-dimethylbenz (a) anthracene-induced breast cancer

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Abstract

Background and aims: Antimicrobial peptides constitute a family of bioactive peptides that are involved in the body defense. Recently, their anti-cancer properties, especially by inducing apoptosis, have been proven in in vitro studies. Therefore, in this study, the effects of cecropin B as an antimicrobial peptide on breast cancer growth, hematological parameters, and histopathological changes in rats were evaluated.

Methods: Twenty-four female rats were randomly divided into 4 groups. The cancer group, control group, cecropin B group, and cancer group treated with cecropin B. The tumor size was measured at the beginning and the completion of the treatment period. Blood samples were collected for assessment of the hematological parameters and Bax and Bcl2 levels. Tumor tissues were removed for histopathological analysis.

Results: The tumor size had a significant increase in the cancer group and cancer group treated with cecropin at the end of the treatment. A significant decrease in mean cell volume, white blood cell count and Bcl2 level and a significant increase in hemoglobin and Bax levels were observed in the cancer group treated with cecropin B compared to cancer group. Changes in other parameters were not significant. Histopathological study showed the invasion of mitotic cells to stromal and muscular tissues of the breast in the cancer group, while focal destruction of tissue and cell death were observed in the cancer group treated with cecropin B.

Conclusion: The results showed that cecropin B has been able to reduce tumor growth and have little side effects on hematologic factors probably through apoptosis.

Keyword: Cecropin B, Apoptosis, Breast cancer, Hematological parameters

Received: 10 November 2019, Accepted: 12 December 2019, ePublished: 29 June 2020

Introduction

Cancer is a complex disease resulted from the disruption in several signaling pathways, differentiation, and cell apoptotic pathways as well as abnormal cell proliferation in the body. Radiotherapy and chemotherapy are the two most common treatments which can cause many side effects since they are non-specific ways that also damage healthy cells. In addition, drug resistance often causes treatment failure (1). Therefore, a more effective and specific treatment strategy is needed that only targets cancer cells without harming normal cells. Immunotherapy seems to be one of the first-line therapies for cancer.

Antimicrobial peptides constitute a group of bioactive peptides and a major part of the innate immune responses that are involved in the body defense. These peptides, generally 12-50 amino acids in length, are classified as either non-ribosomal or ribosomal synthesized peptides according to the production mode. The first group is mostly produced by bacteria while the second category by virtually all living things and bacteria. Amphipathic structures with a net positive charge have been identified as the main factors of antimicrobial activity. Most of these peptides cause membrane damage via binding to the anionic membrane of Gram-positive and negative bacteria. On the other hand, some of them lead to the death of cells through stopping protein synthesis in the cell wall, inhibiting the activity of some certain enzymes, and disrupting metabolic processes of DNA or RNA (2). It has been recently demonstrated that antimicrobial peptides are able to inhibit the tumor cell proliferation especially by inducing death in cancer cells through apoptosis without any effect on the proliferation of the normal cells (3). The main reason for this difference can be the existence of different compounds in the membrane structures of cancer and healthy cells. The membranes of normal cells have a neutral charge due to containing compounds such as phosphatidylethanolamine and sphingomyelin. In contrast, the negative charge of the membranes of cancer cells is associated with the presence of the anionic molecules such as phosphatidylserine.

Therefore, electrostatic reactions between the anionic compounds in the membranes of cancer cells and cationic antimicrobial peptides play a special role in killing these cells. The presence of higher numbers of microvilli on the surface of cancer cells compared to normal cells provides a wider cell surface area for binding antimicrobial peptides (3,4). Anghel et al approved anticancer effects of both peptides (cecropins A and B) on two breast cancer cell lines (M14K and MDA-MB-231) with no adverse impact on normal cells. In another study, cecropin B caused the death of these cells by creating temporary channel-like pores in the membrane of gastric carcinoma cells (5). Arasu et al showed the antitumoral and apoptotic effects of magainin 2 against colon cancer cell line 320 DM without any effect on normal cell lines (6). Kuo et al reported cell death in human osteosarcoma cell line MG63 by peptide Msp1 because of the induced apoptosis via the mitochondrial pathway in these cells (7). The induction of apoptosis by peptide FF/CAP18 in HCT116 colon cancer cells (8) and colon cancer cells (line HCT-116) (9) has been also confirmed. As the antimicrobial peptides select cancer cells purposefully, they do not cause drug resistance unlike chemotherapy drugs, and they are resistant against serum and urine proteolysis (10). Due to their biochemical structures, the antimicrobial peptides will probably serve as good treatments for cancer. Although numerous in vitro studies in the field of anti-cancer effects have been carried out, in vivo studies have rarely been conducted. Therefore, in this study, after modeling the breast cancer using carcinogen 7, 12-dimethylbenz[a] anthracene (DMBA) in rats, the effects of cecropin B as an antimicrobial peptide on the growth of resulted tumors were evaluated.

Materials and Methods

Animals

Twenty-four Wistar rats with a mean weight of 250±20 g were purchased from the animal house of Islamic Azad University of Falavarjan and housed in a room with controlled temperature (23 ± 2°C), humidity (50 ± 5%), light (12 h light/12 h dark cycle), and *ad libitum* access to food and water. The rats were randomly divided into four groups. In the control group, the rats were fed with water and food during the experiment and only for inducing injection stress in them, 0.5 ml sesame oil was injected into the nipple of their left breast. In the cancer group, at the beginning of the test, their chest hair was shaved and 50 mL of DMBA (60 mg/kg) dissolved in sesame oil was injected into the nipple of their left breast. In the cancer group treated with cecropin B, as in the previous group, breast cancer was induced in the rats. After the formation of tumors (26 days after carcinogen injection) and reaching about 10 mm in diameter, 50 µL (25 mg / kg) of cecropin B was intratumorally injected into each growing tumor daily for 3 weeks. In the cecropin B group, the rats were injected with the same volume of cecropin B as in the previous group without cancer induction (11,12).

Tumor volume measurement

Tumor volume was measured twice using a caliper with an accuracy of 0.2 mm, once in the second week when they became palpable and their approximate size reached 10 mm and then again at the completion of the treatment period. Then, tumor volumes were calculated according to the formula a × b2 × 0.5, where a represents the largest diameter and b represents the smallest (13).

Measuring hematological parameters

At the end of the test, the rats were anesthetized and the blood samples were directly taken from the heart. The blood samples were transferred into special tubes in order to evaluate the number and index of blood cells using the cell counter device (Mindray BC 6800 model).

Measuring the concentrations of Bax and Bcl2

After collecting blood samples, thoracic regions of animals were anatomized and a part of the breast tissue was removed and washed with phosphate-buffered saline (pH = 7.4). Afterwards, 1 mg of each sample was isolated and homogenized with 1 mL phosphate buffer. Then, the samples were centrifuged at 10000 rpm for 20 minutes at 4°C and the supernatant was collected. Afterward, Bax and Bcl2 concentrations were determined using ELISA kits (MyBioSource, CA, USA). Briefly, 5 different concentrations of standard solution were prepared and together with streptavidin-HRD were added to the standard wells. 50 µL of the sample supernatant, streptavidin-HRD, and testing antibody (Bax or Bcl2) were added to each test well. Only the chromogen A and B solutions, as well as stop solution were added to the blank well. The plate was incubated at 37°C for 60 minutes and then was washed carefully. At the next stage, it was added first to the plate of chromogen A and then to the chromogen B plate and incubated in darkness for 10 minutes at 37°C. Finally, the stop solution was added to every well and after 15 minutes, light absorption at a 450 nm wavelength was read and the concentrations of these two factors were calculated based on the standard curve (14).

Preparation and staining of tissue samples

A part of the breast tissue from each animal was carefully extracted and transferred to 10% formalin. Following 24-hour fixation, the tissues were transferred to a series of ethanol concentrations. Next, the samples were placed in xylene and then molten paraffin wax. 5 µm sections were made from paraffin-embedded tissues. After hematoxylin-eosin staining (15), the prepared slides were evaluated for histopathological changes by an optical microscope.

Statistical analysis

The data were expressed as mean ± SD and analyzed using SPSS software version 20.0. A paired-samples *t* test was used to measure the tumors sizes and one-way analysis of variance (ANOVA) and Tukey post hoc multiple comparison tests were used to analyze other data. The significance level was set at $P \leq 0.05$.

Results

The Results of Measuring Tumor Size

According to Figure 1, the mean volume of cancer tumors in rats of the cancer group at the end of the experiment showed a significant increase ($P \leq 0.01$) compared to the beginning of tumor formation. In cancer group treated with cecropin B, a significant increase in the volume of tumors was seen at the end of the treatment period ($P \leq 0.05$).

The results of histological observations

Histopathological changes in the breast tissue of the experimental groups can be seen in Figure 2. Figure 2A shows the breast tissue in the cecropin B group with normal adipose and muscular tissue that is similar to the images of the control group (not provided). Figure 2B clearly displays a massive invasion of the cancer cells into adipose and muscular tissues of cancer rats. Figure 2C shows a part of this tissue with active mitotic cells at a higher magnification. In Figures 2D and 2E, the focal tissue destruction and cell death in the breast tissue of the cecropin B group can be observed, respectively.

The results of the measuring Bax and Bcl2

According to Figure 3, in the cancer group and cancer group treated with cecropin B, Bax concentration significantly reduced ($P \leq 0.001$ and $P \leq 0.01$, respectively) compared to the control group, while Bcl_2 concentration significantly increased ($P \leq 0.001$ and $P \leq 0.01$, respectively). However, no significant difference in Bcl_2 and Bax levels was found

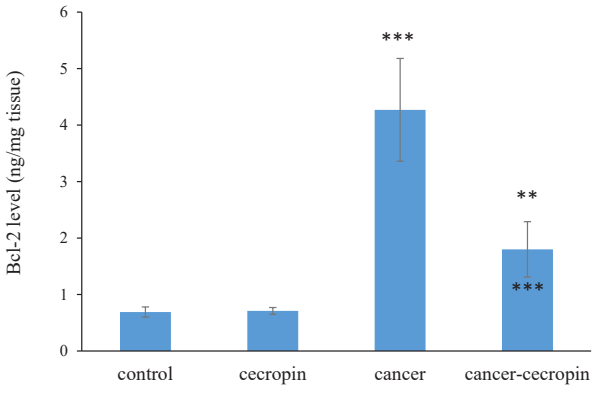

Figure 1. Comparison of the volume of the tumors at the beginning and end of the test in the cancer-cecropin group and the cancer group. Data are expressed as Mean±SD. The significance level is defined as ** $P \leq 0.01$, * $P \leq 0.05$.

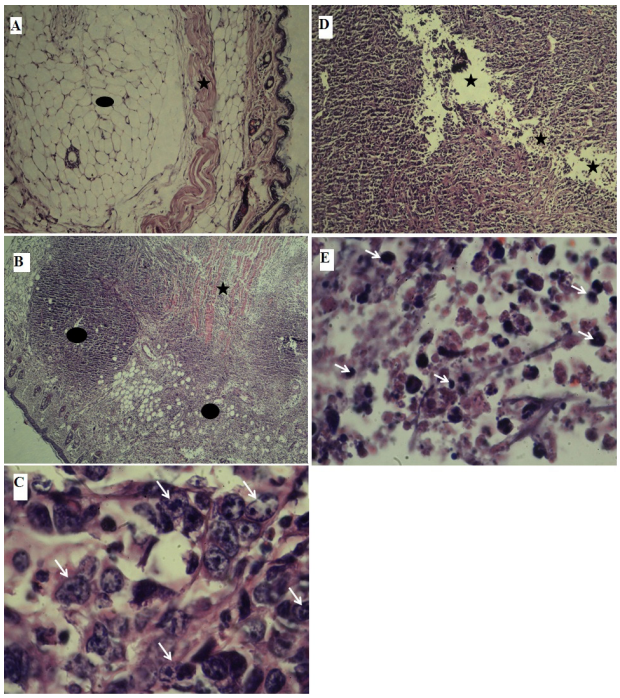

Figure 2. Hematoxylin and eosin (H&E)-stained sections of breast tissue. A: Normal adipose (oval) and muscle tissue (star) of the breast in the cecropin B group (×40). B: A massive invasion of cancer cells into adipose (oval) and muscular (star) tissues in cancer croup (×40). C: Arrows indicate active mitotic cells in cancer group (×1000). D: Stars indicate focal tissue destruction in cancer group treated with cecropin B (×40). E: Necrosis (stars) and Inflammatory cells in cancer group treated with cecropin B (×1000).

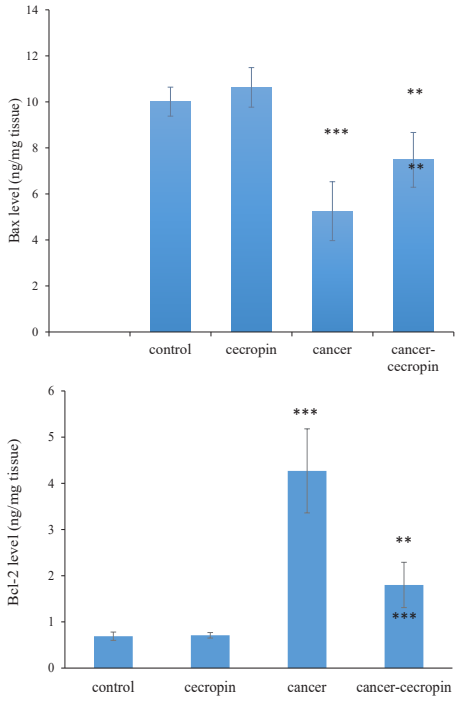

Figure 3. Evaluation of Bax and Bcl-2 levels in experimental groups. Data are expressed as Mean±SD. The significance level is defined as *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$. The stars above the error bars represent the comparison of experimental groups with the control group and the stars under the error bars indicate the comparison of the cancer-cecropin group with the cancer group.

Table 1. Evaluation of hematological parameters in experimental groups

Hematological parameters	Control	Cancer	Cancer-Cecropin B	Cecropin B
WBC (10^3 μL^{-1})	7.80±1.64	11.86±1.27***	*9.81±0.51*	8.23±0.99
RBC (10^6 μL^{-1})	7.15±0.56	4.72±1.41***	5.47±0.71*	6.71±0.67
HGB (g/dL)	13.18±1.06	9.48±1.27***	11.41±0.81*	13.11±0.63
HCT (%)	44.55±4.94	36.67±2.15**	38.10±2.29	45.46±4.03
MCV (fL)	62.38±2.49	77.11±3.99***	67.56±5.60**	67.74±5.28
MCH (pg)	18.95±0.65	20.04±0.25**	19.77±0.21*	19.13±0.63
RDW (%)	15.32±1.44	20.91±2.89***	19.46±2.17**	15.59±1.03
PLT (10^3 μL^{-1})	727.67±140.37	1016.30±69.73***	934.00±62.31**	679.50±141.93
PDW (fL)	14.67±0.08	15.00±0.21**	14.73±0.16	14.43±1.48
MPV (fL)	7.43±1.06	5.82±0.16*	6.10±0.63	7.60±1.25

Data are presented as mean ±SD. The significance level is defined as *** $P<0.001$, ** $P<0.01$, * $P<0.05$. The stars on the right side indicate the comparison of experimental groups with the control group and the stars on the left side indicate a comparison of the cancer-cecropin group with the cancer group.

between the cecropin B group and the controls. In comparison to the cancer group, increased level of Bax ($P\leq 0.01$) and decreased level of Bcl_2 ($P\leq 0.001$) were observed in cancer group treated with cecropin B.

The results of the hematological tests

The mean number of blood cells and their indices are summarized in Table 1. As can be seen, the number of platelets (PLT) in rats of both cancer group and cancer group treated with cecropin B considerably increased respectively ($P\leq 0.001$ and $P\leq 0.05$) compared with the control group. In the cancer group, the mean platelet volume (MPV) declined ($P\leq 0.05$) compared to the control group, while in the cecropin B group, the observed decrease was not significant. Platelet distribution width (PDW) did not show any significant changes compared to the control group.

In the cancer group, the mean number of red blood cells (RBCs), hemoglobin concentration (HGB), and hematocrit (HCT) decreased compared to the control group ($P\leq 0.001$, $P\leq 0.001$, and $P\leq 0.01$, respectively), while mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and red blood cell distribution width (RDW) increased ($P\leq 0.01$, $P\leq 0.001$, and $P\leq 0.001$, respectively). In cancer group treated with cecropin B, RBC and HGB reduced ($P\leq 0.05$) while HCT and MCV did not change compared to the control group. Meanwhile, MCH and RDW levels in this group increased compared to the control group ($P\leq 0.05$ and $P\leq 0.01$, respectively).

A significant increase ($P\leq 0.001$) in white blood cells (WBCs) was observed in cancer rats compared to the controls. This increase was also detected in cancer rats treated with cecropin B compared to the control group ($P\leq 0.05$). No significant differences in tested parameters were observed between the cecropin B group and the control group. Following the comparison of these parameters between the cancer group and cancer group treated with cecropin B, a significant decrease in WBC ($P\leq 0.05$) and MCV ($P\leq 0.01$) and an increase in HGB ($P\leq 0.05$) were observed, while changes in other parameters were not significant.

Discussion

Given that in several studies, it has been proved that the expression of some genes and macromolecules in DMBA-induced breast cancer in rats mimics a model similar to human breast cancer (16,17), this animal model was used in the present study. Almost 2 weeks after carcinogen injection, the tumors formed and became palpable. The mean volume of cancer tumors showed a significant increase in rats of the cancer group at the end of the experiment compared to the beginning of the formation of tumors. In cancer group treated with cecropin B, an increase in tumor size was also observed but with a slower rate compared to the cancer group. This reduced growth of cancer tumors under the influence of cecropin B treatment can be attributed to reduction of proliferation, inhibition of cell division, or induction of cell death. Additionally, cell death in the tissues of these tumors was confirmed in histopathological studies.

The increase in the number of active mitotic cells and massive invasion of them to stromal and muscular tissues of the breast in the cancer group rats confirmed the cancer induction whereas the focal tissue destruction and cell death in breast tissue was observed in the treated rats. Cell death was reported in a number of cancer cell lines including breast adenocarcinoma, human mesothelioma, and bladder cancer cell lines by cecropin A and B without harming normal cells (5,10). Xia et al induced abdominal ascites in mice by intraperitoneal injection of BGC823 cell lines and then observed reduction of tumor sizes after treating the animals with cecropin XJ (11). In another study, breast cancer was induced in mice by transplantation of MCF-7 tumor cells, and after intratumoral injection of antimicrobial peptides *Bombesin* and *Magainin* 2, a decrease in tumor size was observed (18). A similar study reported tumor size reduction following the injection of antimicrobial peptide [D]-K6L9 in prostate and breast

tumors transplanted into immune-deficient mice (12). Cecropins are AMPs which were first isolated from the hemolymph of the *Hyalophora cecropia* moth and later were found in mammals too. The mechanism of selective toxicity of cecropins to cancer cells has not been clarified yet. A group of researchers believe that cecropins directly kills the cells via electrostatic interactions with components of anionic membranes of cancer cells. On the other hand, some others think that the most important anti-cancer mechanism of these peptides is the induction of apoptosis after the increase of reactive oxygen species (ROS) in tumor cells (19). Apoptosis can be initiated through one of two fundamental pathways: (1) the death receptor pathway (by the TNF family proteins) or (2) the mitochondrial pathway (by the Bcl-2 family proteins). Since the increased expression of anti-apoptotic Bcl-2 protein and the decreased expression of pro-apoptotic Bax protein lead to the survival of cancer cells in human breast cancer and the induced breast cancer in rats (20), in the following of our research, we evaluated the possible effect of cecropin B on Bax and Bcl-2 levels in breast tissues of the experimental groups. In cancer group and cancer group treated with cecropin B, Bax concentration significantly reduced compared to the control group, while Bcl_2 concentration significantly increased. However, no significant difference in Bcl_2 and Bax levels was found in rats that received only cecropin B compared with the controls that may indicate a lack of harmful effects of this peptide on normal cells. In comparison to the cancer group, an increased level of Bax and a decreased level of Bcl_2 were observed in cancer group treated with cecropin B.

Bax concentrations in tumors of both cancer group and cancer group treated with cecropin B were lower compared to the control group, but by comparison of this factor between both cancer groups, a significant increase of it was observed in cancer group treated with cecropin B compared to the cancer group. Although the level of Bcl-2 in both aforementioned groups increased compared with that of control group; it declined significantly in cancer group treated with cecropin B compared to the cancer group. Therefore, it seems that cecropin B could induce apoptosis not entirely but partly in tumor cells. In line with these findings, Jin et al by performing the TdT-mediated dUTP nick end labeling (TUNNEL) assay could observe the induction of apoptosis in hepatocellular carcinoma cell line BEL-7402 after treatment with cecropin extracted from *Musca domestica*. In addition, they reported increased expression of caspase-3 and caspase-8 (20). Alshatwi et al evaluated the effect of antimicrobial peptide *violacein* on cell lines MCF-7 and observed increased ROS production following the increased expression of TNF-α, Bax, and caspase-3 as well as declined expression of Bcl-2 and MDM2 in cancer cells compared to the normal cells (21). In an in vivo study, antimicrobial peptaibols decreased the growth of tumors resulted from injection of hepatocarcinoma cells in mice because of the translocation of Bax to mitochondrial membrane and activation of the apoptotic pathway (22). An increased expression of Bax, caspase-3, and caspase-9 and a decreased expression of Bcl-2 at both transcription and translation levels in HepG-2 cell lines were reported after treatment with cecropin (14).

Today, a group of anti-cancer drugs were designed based on targeting Bcl-2 family proteins. These drugs would lead to activation of this complex and induction of cell death in cancer cells by binding to pro-apoptotic BH3-only proteins via directly activating the pre-apoptotic Bax/Bak complex or indirectly inhibiting anti-apoptotic Bcl-2 protein (23). In our study, cecropin A with a mechanism similar to that of these drugs may have caused induction of apoptosis. However, to confirm this result further supplementary tests are needed.

Unfortunately, one of the side effects of anticancer drugs, especially chemotherapy drugs, is hematotoxicity induction in the body. Hence, continuing this research, levels of a group of hematological parameters in experimental groups were evaluated. In rats that were treated only with cecropin B, no significant changes in these parameters were observed compared with the control group. Therefore, it can be imagined that cecropin B was toxic specifically to cancer cells and since it had no adverse effect on normal cells, it would not affect hematologic parameters. In a study by Jin et al, cecropin had no effect on the hemoglobin content, erythrocytes, leukocytes, and other blood parameters compared to the saline-treatment group (19). In cancer rats, a significant increase in WBC count was observed that can be a sign of tissue inflammation. These results are in line with the observed leukocytosis in rats after induction of breast cancer (24) and in 758 patients with non-hematological cancers, the incidence of infections after cancer metastasis in the body was noted as its secondary cause (25). PLT, MPV and PDM also showed significant changes in the rats of this group so that thrombocytosis was completely evident. A group of researchers regard thrombocytosis as a symptom of metastases. They believe that the PLT not only help cancer cells escape from the immune system but also provide the condition for attachment of these cells to vascular endothelium and their metastasis by fibrin formation (26). Thrombocytosis in patients with metastatic thyroid cancer and lung cancer was reported but not in patients with no metastasis (27).

We also observed the increase of WBC and PLT in cancer group treated with cecropin B but these changes were less severe compared to the cancer group. It seems that the cecropin B could somewhat reduce the adverse effects of cancer on the number of this type of blood cells. We suggest that cecropin B due to induction of cell death as well as its antiangiogenic and anti-bacterial properties could somewhat prevent the spread of tumor cells and the development of secondary infections. In this study,

a significant decrease in hemoglobin, hematocrit, and RBC and an increase in RDW were observed in cancer group. These changes can also be symptoms of microcytic anemia. In many infectious, inflammatory, and neoplastic diseases, anemia is very common which is called anemia of chronic disease (28). In our research, the cancer may have made rats more prone to become anemic. After treatment with cecropin B, the changes of these factors were similar to those of cancer group but with a lower intensity. Therefore, it seems that inhibitory effect of cecropin B on tumors growth blocked harmful effects of the cancer on RBC and its associated factors. However, further studies are required on the mechanism of the effect of cecropin B on hematological parameters.

Conclusion

The results of the histopathological and morphological evaluation of tumors as well as changes in the levels of two factors related to apoptosis after treatment of rats with cecropin B showed that the induction of cell death (possibly through mitochondrial apoptosis pathway) prevented somewhat the overgrowth of the tumors. Hence, pathological changes and haematological parameters reduced due to cancer.

Conflicts of Interests

None.

Ethical Approval

The permission for the use of animals in the experiments was obtained from the institutional review board of the Islamic Azad University of Flavarjan Branch (No: IR.IAU.FALA.REC.1395.002), after considering the project and its aims.

Authors Contribution

FG: Study design
MF: Study design, data collection and manuscript drafting

Funding/Support

This study was supported by the Research area of Islamic Azad University, Falavarjan Branch (Isfahan, Iran).

Acknowledgments

This article was derived from a master's degree thesis in Microbiology which was approved by the Department of Microbiology of Islamic Azad University, Falavarjan Branch in October 2015, with the code of 17230507931034. The authors are grateful to Department of Microbiology for the cooperation and supplying the experimental equipment.

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