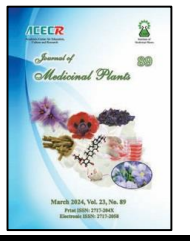
J. Med. Plants 2024; 23(89): 57-67

Institute of Medicinal Plants

Journal of Medicinal Plants

Journal homepage: www.jmp.ir



Research Article

Expression of Apoptotic genes induced by Flaxseed extract in human Prostate cancer cell line

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ARTICLE INFO

Keywords:
Prostate cancer
PC-3 cell line
Flaxseed
Apoptosis
Gene Expression

ABSTRACT

Background: Prostate cancer is known as the second common cancer among men, and is introduced as the fifth cause of death. Apoptosis known as "Programmed Cell Death", can be measured as a defense mechanism in response to cell damage, caused by diseases or exposure to toxic material, and has an important role in controlling cell population. For this, researchers are interested in studying cancer treatment for inducing apoptosis. Flaxseed, *Linum usitassimum*, has numerous benefits among other oilseeds. It contains a large amount of α-Linoleic Acid (ALA), dietary fiber, proteins, and Phytoestrogen. **Objective**: The main goal of this research was to evaluate the expression of apoptotic genes induced with flaxseed extract in human prostate cancer cells in order to find out the cell death pathway induced by flaxseed extract. **Methods:** In this study, PC-3 prostate cancer cells were cultured and the treatments were done using the flaxseed extract. The MTT test, IC50 calculations, Flowcytometry, and Real-Time PCR test for apoptotic genes were done and all the results underwent analysis. **Results**: The IC50 dose was obtained at 809 µg/ml ($P < 0.05$). Flowcytometry test was done for 600, 800, and 1000 µg/ml of extraction, and the result showed that most of the cells underwent necrosis. The Flowcytometry result was confirmed by qPCR test and showed the overexpression of TNF gene ($P < 0.05$). **Conclusion**: The results showed the overexpression of all the studied genes after treatment by flaxseed extract. The Flowcytometry result was confirmed by qPCR and showed that the TNF gene was overexpressed.

Abbreviations: AML, Acute myeloid leukemia cells; ALA, α-Linolenic Acid; DMEM, Dulbecco's modified Eagle's medium; EDTA, Ethylenediaminetetraacetic acid; IC_{50}, half-maximal inhibitory concentration.

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doi:

Received 7 April 2024; Received in revised form 31 July 2024; Accepted 6 August 2024

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[DOI: 10.61186/jmp.23.89.57]

1. Introduction

Cancer is major health problem in the world while is the second leading cause of death worldwide [1, 2]. It refers to a variety of diseases that cause the greatest global burden for both men and women [The dramatic increase in cancer rates can be attributed to genetics or to multiple risk factors such as population growth, aging, abnormal lifestyle, smoking, etc. [4], which lead to genetic and epigenetic changes and mutations that allow cells to proliferate excessively and develop cancer [5, 6]. Prostate cancer is the second common diagnosed cancer in men and the fifth risk factor of death worldwide [7, 8]. Since Iranian men are ethnically and racially different from most men in Asian countries, the incidence of prostate cancer is expected to differ among them [9].

Flaxseed, *Linum usitassimum*, from the Lineaceae family, is a very popular nutrient-rich seed due to its high content of alpha-linolenic acid (ALA), fiber, protein, and phytoestrogens [10]. ALA is an unsaturated fatty acid, which is one of the essential fatty acids with anti-inflammatory, antithrombotic, and antiarrhythmic effects [11]. Several studies have shown that the presence of about 5 % flaxseed in the daily diet inhibits the development and progression of prostate cancer in transgenic mice [12-14]. Flaxseed oil is widely recognized as a rich source of nutrients such as fiber-based lignans and high levels of ω fatty acids [11, 15]. In 2019, Buckner et al. studied the effects of treating cultured melanoma, breast cancer, and cervical cell lines with flaxseed oil to explore the mechanism of cell growth. The results showed that flaxseed oil treatment inhibited the growth of cultured malignant cells and could induce apoptosis [16]. In 2020, Tannous et al. also studied the anticancer effects of flax lignan derivatives on different acute myeloid leukemia cells. They investigated the potential anticancer effects of flax lignans on acute myeloid leukemia (AML) cells in vitro and sought to decipher the underlying molecular mechanisms. The results of this study demonstrate that ENL ligands have promising anticancer effects on AML cell lines in vitro, by promoting DNA fragmentation and the intrinsic apoptosis pathway, highlighting the health-protective benefits of flaxseed in leukemia [17]. Based on the literature, of lower proliferation rates with flaxseed supplementation is consistent with previous in-vitro study in LNCaP, DU-145 and PC-3 prostate cancer cell lines which also found inhibited cell viability with treating by flaxseed-derived lignans [13].

This research aims to study the effect of treatment with flaxseed on prostate cancer cells. We tried to investigate the expression of genes related to apoptosis induced following treatment with ethyl acetate extract of flaxseed.

2. Materials and methods

2.1. Cell culture

The human prostate cell line, PC-3 was cultured (Figure 1). It was provided through the Stem Cell Technology Research Center (Bon Yakhteh Co), cultured in standard tissue culture flasks using Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10 % fetal bovine serum (FBS), and 1 % penicillin and streptomycin [18-19]. All sapmles were maintained under standard conditions at 37 °C with 100 % humidity for culturing. Treatment with Trypsin/ethylenediaminetetraacetic acid (EDTA; 0.25 %, Gibco) was done on the cell suspensions [20-22]. For cells confluence we waited until it reached 1–2 × 105 cells/cm^2 in the cell culture procedure.

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2.2. Flaxseed extraction

The extraction was done using100 grams of grounded flaxseeds, and 300 ml of ethyl acetate in an ultrasonic bath at ambient temperature for 30 minutes. The wet extract of ethyl acetate was put under a vacuum in the rotary device, 240 bar; evaporated and a very thick oil was obtained. Then, it was dried and stored in a refrigerator and a dark environment.

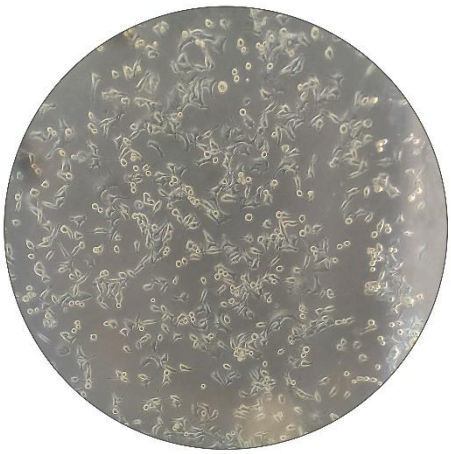

Fig. 1. Microscopic images of the PC-3 human prostate cell line (x 10)

2.3. Treatment

DMSO was used as an intermediate substance for dissolving in culture media. 50 µL of the extract was mixed with 100 µL of DMSO to reach a final volume of 150 µL (5000 µg). 90 ml of the extract was dissolved in 3 ml of the culture medium. The resulting extract was filtered. Cells were treated using different dilutions of the oil between 0.1 and 1500 µg/ml [23].

2.4. MTT

The MTT test is a colorimetric assay that uses a reduction in a yellow tetrazolium salt (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, or MTT) to assess cellular metabolic activity as a proxy for cell viability. Viable cells contain NAD (P) H-dependent oxidoreductase enzymes which reduce the MTT reagent to formazan, an insoluble crystalline product with a deep purple color. Then, formazan crystals are dissolved using the DMSO, and absorbance is measured at 500-600 nanometers using a plate reader. The darker the solution is, the greater the number of viable and metabolically active cells. In this study, 1,000-100,000 cells per well in a 96-well plate were incubated. The separated medium was removed and the cells were washed with PBS, the medium was added to reach a final concentration of 0.5 mg/mL. At the time of the MTT, DMSO was used to dissolve the formazan crystals. The solution was triturated and incubated at room temperature or 37 °C for 1 to 4 hours until the cells were lysed and the purple crystals dissolved. Finally, absorbance was measured at a wavelength of 570 nanometers[22].MTT test was done after 72 hours of cell treatment, IC50 dose was determined for the MTT test groups, and data were analyzed.

2.5. Flowcytometry

Flow cytometry was performed to study cell apoptosis. An automated, multicolor flow cytometer system (BD FACSCalibur, BD

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Biosciences, San Jose, CA, USA) was used for the assay. The flow cytometry assay was performed through several steps. First, cells were detached from the cell culture dish. Then, 2 ml of PBS was added and the solution was centrifuged at 1000 rpm for 5 min and then washed to remove the culture medium. A 500-ml cell suspension volume was then prepared using a 1x binding buffer. Annexin V-FITC (cat number: 640945, Biolegend) and PI were used for staining. Due to the overlap of FITC and PI (525 nm for FITC and 620 nm for PI, respectively), the sample was divided into 4 tubes: one unstained tube, one tube with Annexin V-FITC, one tube with IP, and one tube with IP and FITC. The first and third tubes were stored at 4 °C. For the second and fourth tubes, 5 µl of Annexin V-FITC was added and the samples were incubated for 15 min in the dark at 4 °C. After incubation, 1x binding buffer was added and the sample was centrifuged for 5 min at 1000 rpm. Next, another 500 µl of 1x binding buffer was added. For the reading step, 3 µl of PI was added for staining in the third and fourth tubes [24, 25].

2.6. Real time

RNA and DNA were extracted using TRI reagent (Sigma), according to its protocol [26]. Briefly, chloroform was added and then centrifuged to separate the three layers. The aqueous layer was separated and after washing with isopropanol (Merck), the mixture was centrifuged. The supernatant was removed, 75 % ethanol (Merck) was added, and the mixture was centrifuged. The solution was then dried in the oven and the RNA plate was re-suspended in DEPC-treated water. For DNA, the remaining two layers were mixed with 100 % ethanol and centrifuged. The supernatant was discarded and the mixture was oven dried. The DNA plate was re-suspended in DEPC water. cDNA was obtained using the Easy cDNA kit (Takara). RT-PCR was performed by ABI 7500. Agarose gel electrophoresis was performed to confirm RNA quality, and SDS gel electrophoresis was performed for real-time PCR and PCR products. Real-time analysis and normalization were performed using QGENE software, as mentioned in MIQE. CASP8, CDK4, CyclinD1, TNF, Bcl-2 and B-actin were the genes whose expression was assessed and analyzed. Primer sequences are given in table 1.

Table 1. Primer Sequences of the Genes Selected for the Experiment

Gene type	Forward	Reverse
Cyclin D1	5´- GGCGGAGGAGAACAAACAGA- 3´	5´- TGAGGCGGTAGTAGGACA-3´
CDK4	5´-GCT GCT GGA AAT GCT GAC-3´	5´- CAC TCC ATT GCT CAC TCC-3´
TNF	5´- AAG ATC ATC CAA GAT ATT GAC C-3´	5´- TGC TCC CAC TTT CTT TCC-3´
CASP8	5´- GTTGTGTGGGGTAATGACAATCT- 3´	5´-TCAAAGGTCGTGGTCAAAGCC-3´
BCL-2	5´- GATAACGGAGGCTGGGATG-3´	5´- CAGGAGAAATCAAACAGAGGC-3´
B-actin	5´- CTT CCT TCC TGG GCA TG-3´	5´-GTC TTT GCG GAT GTC CAC-3´

3. Results

3.1. MTT result

In order to obtain the IC_{50} dosage, a graph based on based on cell viability and logarithmic amount of flaxseed extract was drawn (Figure 2). The results show that after 72 hrs. of treatment with drug, 809,8 µg/ml of drug was able to kill 50 percent of cells. The absorbance of cells treated with different doses of flaxseed extract is illustrated in figure 2b. All results showed statistically significant ($P < 0.05$) differences.

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3.2. Flow cytometry results

By using MTT assay, we figured out the dose of IC50 of 809.8 µg/ml of flaxseed in which 50 % of PC3 cells were dead. To further investigate if this extract has an antiapoptotic effect or not, the annexin v test was done. As it is presented in figure 3, the flow cytometry results show that the treatment of PC3 cells with the IC_{50} dose of flaxseed oil leads to a dose-dependent increase in 12.2 % necrotic PC3 prostate cells. Figure 4 is a bar chart showing the percentages of necrosis and apoptosis for the control PC3 prostate cells, and the treated cells on day 3 (doses of 800 µM).

In the negative control sample, most of the cells are in the Q_4 region, which indicates that the cells are alive. The positive control sample contains cells treated with 10 % alcohol, which is lethal for cells. The result of flow cytometry shows that the cells have entered the stage of apoptosis, Q_2, and Q_3. In the sample of cells treated with 600 mg/ml extract, the cells have entered the Q1 phase or necrosis, and some of them show early apoptosis. In cells with800 mg/ml extraction treatment, most of the cells have entered the stage of necrosis or Q_1 with less percent in the late apoptosis, Q_2.

When cells were treated with a dose higher than IC_{50}, 1000 mg/ml, they shifted to the necrosis quadrant or Q_1, and a lower amount of cells shifted to the late apoptosis quadrant or Q_2.

3.3. qPCR results

For more investigation of whether the cell death after treatment with the 800 µg/ml of flaxseed extract after 3 days of treatment was caused by apoptosis or not, the mRNA expression of some genes which are related to apoptosis and necrosis were evaluated, such as CASP8, CDK4, CyclinD1, TNF, Bcl-2, and B-actin as the internal control, on which the changes in mRNA expression were normalized. Results of gene expression in the current survey are depicted in figure 4. The *β*-actin gene as the internal reference control had no changes in expression. The results show that the expression level of the CyclinD1 gene multiplied 5.79 times in the treated group in comparison with the control group. Subsequently, the expression of BCL2, CDK4, TNF, and CASP8 has been multiplied by 2.77, 3.27, 2.95, and 2.47 respectively.

(a) (b)

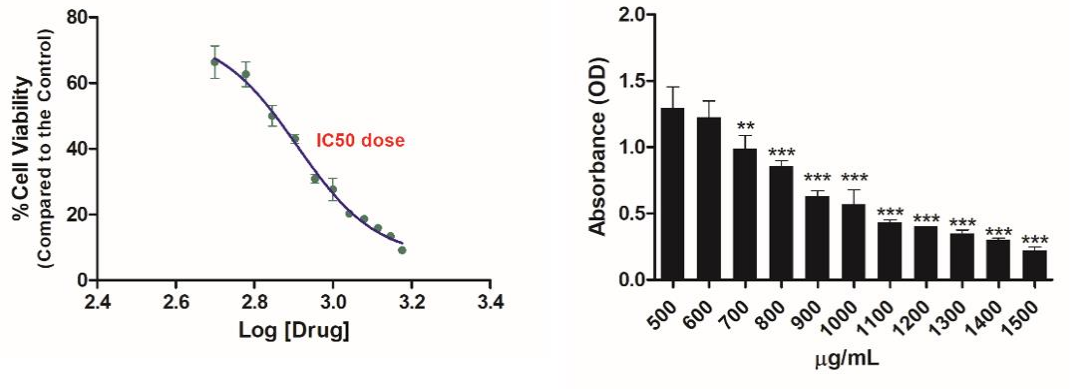

Fig. 2. Viability findings of control prostate PC-3 (a) IC_{50} at logarithmic drug dose (b) Absorbance at various doses of oil treatment

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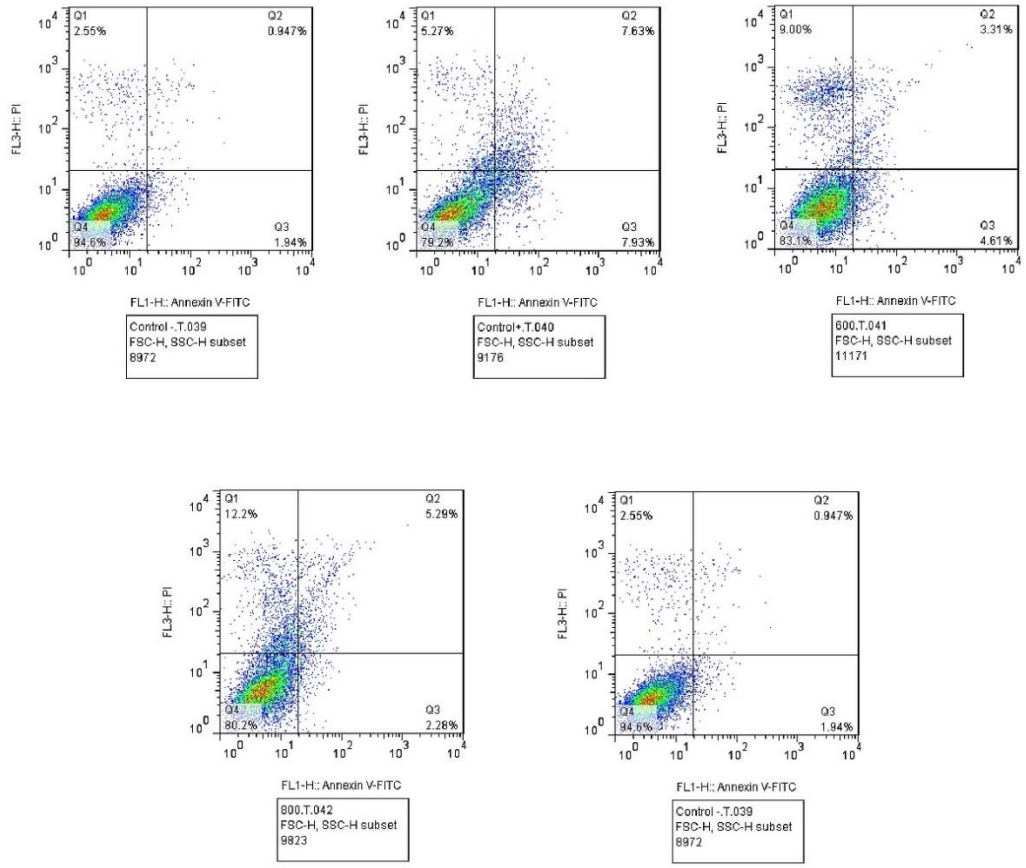

Fig. 3. Verification of apoptotic and necrotic cell death using the standard method of flow cytometry; a) untreated prostate PC-3 cells, b) treated prostate PC-3 cells on day 3 (oil doses of 800 µg/ml)

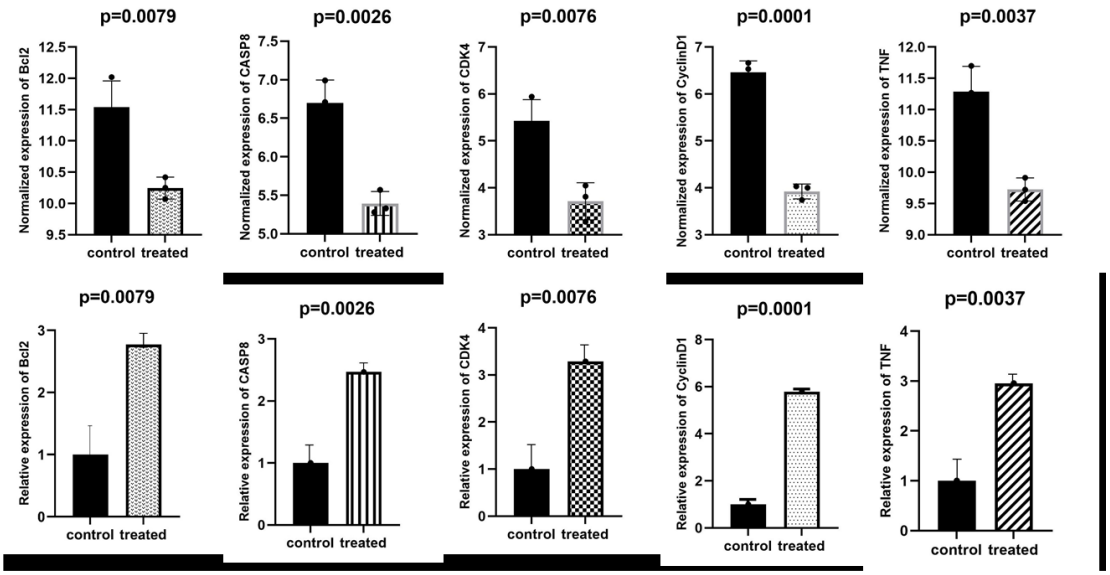

Fig. 4. mRNA expression of selected genes that are related to apoptosis and necrosis (a) normalized expression (b) relative expression

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4. Discussion

Prostate cancer is the second most common cancer among men. In the last two decades, there has been a significant increase in prostate cancer incidence, which might be due to the aging of the population, the development of diagnostic modalities, and the increase of the screening rate [27]. Flaxseed is one of the most important sources of Lignan for mammals, and preclinical studies have shown its protective properties against carcinogenic chemicals [28]. Clinical studies have also shown that the use of flaxseed and Lignan can reduce breast cancer incidence and mortality caused by this cancer [29].

Based on the flow cytometry results, we found that cell treatment with flaxseed extract oil induced cell necrosis. By increasing the extract dosage from 600 to 1000 µg/ml, the cell population has shifted towards the necrosis quadrant. In the dose of 1000 µg/ml, the cell population has shifted towards the necrosis quadrant. One of the important reasons for this change seems to bathe lethal concentration of 1000 µg/ml. At the concentration of 600 µg/ml, the percentage of necrotic cells is higher than apoptotic cells. At the concentration of 800 µg/ml, which is within the IC_{50} dose range, the population of necrotic cells is more than apoptotic cells. Hence, it can be concluded that the ethyl acetate extract of flaxseed has a key role in both the induction of apoptosis and the induction of necrosis; however, its effect in inducing necrosis seems to be higher than apoptosis. For achieving a more accurate assessment of the apoptosis and necrosis of cells treated with the flaxseed extraction, a real-time PCR test was performed, and different genes involved in necrosis, apoptosis, and cell cycle were checked.

Ethylacetate extract of flaxseed used in this research contains various substances that might induce apoptosis and necrosis, or raise the cell growth. For this reason, we tried to select the genes related to apoptosis, cell cycle growth, and progression, as well as cell necrosis. This seems to help us for better analytical understanding regarding the effect of the extract on cell death, or the growth and progression of the cell cycle.

The results of gene expression showed that the expression was maximum in Cyclin D1, and respectively in CDK4, TNF, Bcl-2, and CASP8. The activity of the CyclinD1 gene is in the regulation of the cell cycle. By regulating the activity of CDK4, CyclinD1 leads the cell to pass through the G1 phase and go to the S phase. In addition to cell cycle progression, this gene also plays a role in cell migration, DNA damage or repair, as well as chromosomal stability. It has also been found that increased expression of CyclinD1 might be related to the increase in endocrine resistance of the cancer cells. During late apoptosis, internal and external pathways of apoptosis are seen [30-31].

Furthermore, it was observed that with the treatment dose of 800 µg/ml, 5.29 % of the cells were in the late phase of apoptosis. In this stage, DNA fragmentation happens, and the CyclinD1 gene protein plays a role in DNA damage and repair. Also, CDK4 gene expression has increased by 3.28 times compared to the control sample. The activity of this gene is influenced by the activity of cyclin D1 and this cyclin plays a regulatory role in the activity of this protein. Therefore, we can consider a relationship between increased expression of cyclin D1 and CDK4.

The expression of the TNF gene in cells treated with flaxseed extract has increased about 3 times compared to the control cells. The flow cytometry data also shows that the higher the

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treatment usage of the extract, the more cells enter the necrosis phase. So, by a dose higher than IC50, the main cell population changes towards the necrosis quadrant. So, it can be concluded that the ethylacetate in flaxseed extract might cause cell necrosis, and probably be suitable for destructing the vessels involved in tumor angiogenesis.

Considering the 2.77-fold increase in Bcl-2 gene expression in the treated cells using flaxseed extract, and inhibiting cell death induced due to the TNF expression, it can be mentioned that Bcl-2 gene overexpression due to the increase in TNF gene expression prevents cell death and necrosis.

By an increase in the expression level of CASP8 (approximately 2.5 times) in the treatment group, about 8 % of cells have entered the apoptosis phase, which can confirm the result of the real-time PCR reaction.

Finally, there are some suggestions for future research in continuation. We have a limited selection for gene expression, while more genes or pathways might shed light on a deeper understanding of the actin mechanism of flaxseed extract on cancer cells. HPLC study for defining the substances of the flaxseed extract is also suggested. It is the potential to use other herbal extracts and compare them with flaxseed extract or in combination treatment for investigating their effect on cancer cells.

5. Conclusions

Treating the cells with flaxseed extract leads to an increase in the expression of the studied genes related to apoptosis, cell cycle, and necrosis. Based on the data, treating PC3 cells with ethyl acetate extract of flaxseed led to a significant increase in the expression of Cyclin D1 and CDK4 gene which are important in cell cycle progression and are anti-apoptotic genes. Meanwhile, the expression of another anti-apoptotic gene –BCL2- was increased moderately to reduce the chance of cell death.

TNF-α and CASP8 are two genes that are related to cell death. As it was seen from the flow cytometry results that the number of cells suffered from necrosis type of cell death which was induced by TNF gene expression was more than the apoptosis type of cell death which was further confirmed by the real-time PCR results.

In conclusion, the flaxseed oil extract by ethyl acetate stimulates cell cycle progression and necrosis more than apoptosis in the PC3 prostate cancer cell line.

Acknowledgments

This research was part of a master's thesis performed in the genetic and stem cell lab, department of Cell and Molecular Biology, Faculty of Life Sciences and Biotechnology, Shahid Beheshti University.

Conflict of Interest

None of the authors have a conflict of interest.

Author Contributions

R. Sh. was responsible for the Conception and Idea. Sh. F. supervised the research, methodology, and critical revision. MR. R. was the consultant of this project as the urologist specialist. M. H. was responsible for conducting the experiments, data analysis, and interpretation. M. H. and A. Sh. performed drafting, preliminary editing of the article, submission, and tracking.

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[DOI: 10.61186/jmp.23.89.57]

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How to cite this article: Hakimi M, Shiari R, Shirkavand A, Razaghi MR, Farivar Sh. Expression of Apoptotic genes induced by Flaxseed extract in human Prostate cancer cell line. ***Journal of Medicinal Plants*** 2024; 23(89): 57-67.
doi:

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[DOI: 10.61186/jmp.23.89.57]

فصلنامه گیاهان دارویی 1402؛ 23(89): 57-67

فصلنامه گیاهان دارویی

Journal homepage: www.jmp.ir

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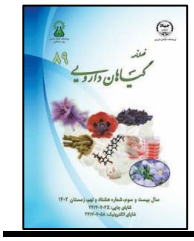

مقاله تحقیقاتی

بیان ژن‌های آپوپتوز ناشی از عصاره بذر کتان در رده سلولی سرطان پروستات انسان

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اطلاعات مقاله

گل‌واژگان:

سرطان پروستات

رده سلولی PC-3

بذر کتان

آپوپتوز

بیان ژن

چکیده

مقدمه: سرطان پروستات به عنوان دومین سرطان شایع در بین مردان شناخته می‌شود و به عنوان پنجمین عامل مرگ و میر معرفی می‌شود. آپوپتوز معروف به "مرگ برنامه‌ریزی شده سلولی" می‌تواند به عنوان یک مکانیسم دفاعی در پاسخ به آسیب سلولی، ناشی از بیماری‌ها یا قرار گرفتن در معرض مواد سمی اندازه‌گیری شود و نقش مهمی در کنترل جمعیت سلولی دارد. برای این منظور، محققان علاقه مند به مطالعه درمان سرطان برای القای آپوپتوز هستند. دانه کتان، *Linum usitassimum*، دارای فواید بی‌شماری در میان سایر دانه‌های روغنی است. حاوی مقدار زیادی اسید-α لینولئیک (ALA)، فیبر غذایی، پروتئین و فیتواستروژن است. **هدف:** هدف اصلی این تحقیق بررسی بیان ژن‌های آپوپتوز ناشی از عصاره بذر کتان در سلول‌های سرطانی پروستات انسانی به منظور تعیین مسیر مرگ سلولی ناشی از عصاره بذر کتان بود. **روش بررسی:** در این مطالعه سلول‌های سرطانی پروستات PC-3 کشت داده شدند و تیمارها با استفاده از عصاره بذر کتان انجام شد. تست MTT، محاسبات IC_{50}، فلوسیتومتری و Real-Time PCR برای ژن‌های آپوپتوز انجام شد و تمامی نتایج مورد تجزیه و تحلیل قرار گرفتند. **نتایج:** دوز 809 IC_{50} میکروگرم بر میلی‌لیتر ($P < 0/05$) به دست آمد. تست فلوسایتومتری برای استخراج 600، 800 و 1000 میکروگرم بر میلی‌لیتر انجام شد و نتایج نشان داد که اکثر سلول‌ها دچار نکروز شدند. نتایج فلوسایتومتری با آزمون qPCR تایید شد و بیان بیش از حد ژن TNF را نشان داد ($P < 0/05$). **نتیجه‌گیری:** نتایج بیانگر بیان بیش از حد تمامی ژن‌های مورد مطالعه پس از تیمار با عصاره بذر کتان بود. نتیجه فلوسایتومتری توسط qPCR تایید شد و نشان داد که ژن TNF بیش از حد بیان شده است.

مخففها: AML، لوسمی حاد میلوئیدی؛ ALA، α-لینولنیک اسید؛ DMEM، محیط کشت؛ IC_{50}، نیمه حداکثر غلظت بازدارندگی؛ MTT، 3-(4،5-دی‌متیل تیازول)-2، 5 دی‌فنیل تترازولیوم بروماید؛ EDTA، اتیلن دیامین ترا استیک اسید

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تاریخ دریافت: 19 فروردین 1403؛ **تاریخ دریافت اصلاحات:** 10 مرداد 1403؛ **تاریخ پذیرش:** 16 مرداد 1403

doi:

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