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Short-Communication

Anticancer potential of *Amygdalus scoparia* bee pollen extract against prostate (pc-3) cancer cell *in vitro*

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Abstract

Objective: Prostate cancer has been diagnosed as the second most common cancer in men around the world. The present study was conducted with the aim of utilizing the cytotoxic function of *Amygdalus scoparia* against prostate cancer.

Materials and Methods: Bee pollen loads from *A. scoparia* were collected from pollen traps, purified and identified, and then, its methanolic extract was obtained and, [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] MTT, flow cytometry, real time PCR, and ELISA assays and (Gas Chromatography) GC methods were used. The protein and saponin contents and specific catalase activity of *A. scoparia* pollen extract was determined. Data were analyzed by SPSS V.22 software.

Results: *A. scoparia* pollen extract had a concentration- and time-dependent cytotoxic effect on PC-3 cancer cell line. Eight fatty acids were found in the pollen extract, with the highest amount related to palmitoleic acid (28.51%). The cytotoxic effect on Human foreskin fibroblasts (HFF) cell line was significantly lower than that on PC3 cancer cells (1.9-fold), which caused cell cycle arrest in the sub-G1 phase and induced apoptosis in cancer cells. The expression levels of *p21*, *p53* and *Bax* genes was significantly increased and the expression level of *Bcl-2* gene was significantly decreased. Our findings introduced *A. scoparia* pollen extract as an anticancer compound against prostate cancer cells.

Conclusion: Many anticancer drugs damage healthy cells, but *A. scoparia* had good antitoxic effects only against prostate cancer cells, not healthy cells.

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Introduction

One of the most important limitations of cancer treatment is the elimination of tumor cells in the presence of normal cells in the body, without causing cytotoxicity to normal cells. Therefore, targeted therapies for cancer cells or the use of herbal medicines with fewer side effects is one of the main concerns of researchers to provide therapeutic strategies (Yousefi et al. 2021; Pande et al. 2018).

Prostate cancer has been diagnosed as the second most common cancer and the sixth leading cause of death in men around the world (Rawla 2019). The global prevalence of prostate cancer is estimated

to reach 2.3 million new cases and 740,000 deaths by 2040 (Momayyezi et al. 2022). Hormone therapies can be considered treatment strategies, although long-term administration of these drugs increases the risk of thromboembolism and leaves other side effects such as breast enlargement and decreased sexual potency (Mohammadi et al. 2020). Other treatments such as radiotherapy, chemotherapy and surgery also have their own problems. However, the treatment prospects for prostate cancer have improved significantly in recent decades with new treatments and better use of existing treatments (Teo et al. 2019). In addition, the World Health Organization (WHO) emphasis on the gradual replacement of natural materials with chemicals has led various countries to invest, plan to plant, and mass-produce medicinal plants for use in the pharmaceutical, health, and food industries (Tang et al. 2020). Due to the increasing resistance of cancers to conventional therapies, many efforts have been made to find compounds, especially natural ones, with anti-cancer properties (Benchikh and Louailèche 2014). In recent years, alternative medicine has turned to traditional and herbal compounds because of the many side effects of synthetic drugs, and these natural compounds have found their way into modern medicine (Taheri et al. 2011; Soltani et al. 2022).

Amygdalus scoparia Spach. is a plant belonging to the Rosaceae family (Deardorff et al. 2015). The medicinal and therapeutic effects of this medicinal plant, especially its pollen, have not yet been studied intensively enough. Some studies have pointed to the proapoptotic effects of bee pollen, suggesting the potential use of these compounds or their active ingredients as a treatment for human tumors (Yusuf et al. 2007).

The literature review reveals that there have been no studies so far on *A. scoparia* pollen extract. Accordingly, the present *in vitro* study aimed to evaluate the anti-cancer activities of bee-collected *A. scoparia* pollen extract against prostate cancer cells.

Materials and Methods

Establishment and preparation of the hives to collect pollen and collection of *A. scoparia* bee pollen samples

In 2020, the hives were set up by-traps in March in Fars province (Iran). The pollens were collected using pollen traps. The shape and morphological characteristics of *A. scoparia* pollen were consistent with the research of Vafadar et al. (2010) and Sawyer & Pickard (2006). *A. scoparia* was identified, registered, and preserved at the Imam Khomeini Higher Education Center in Karaj with the HIKHEC-3436 herbarium number.

Measurement of the protein, saponins content, and catalase activity

Three grams of pollen was crushed. The protein content was measured by the Bradford protein assay (Bradford 1976). Saponin extraction was performed using the method of Zeb et al (Zeb et al. 2014). In order to measure catalase activity:1 g of pollen was poured and added 1500 µl of potassium phosphate buffer. Then, 20 µl of enzymatic extract was mixed with 400 µl of 25 mM sodium phosphate buffer solution and 300 µl of H_2O_2. The rate of H_2O_2 elimination for two minutes was measured at a wavelength of 240 nm by spectrophotomete.

Catalase enzyme activity= Absorbance difference/39.5×100

Catalase specific activity= Catalase volume activity/ Extract protein concentration

Cell culture

Prostate cancer cell line, PC-3 (CA24), and human foreskin fibroblasts, HFF (C163) as normal cell line were purchased from the Cell Bank of Pasteur Institute of Iran. The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) medium

with 10% Fetal bovine serum (FBS) and 1% penicillin/streptomycin and incubated at 37°C with 5% CO_2 and 95% humidity.

Evaluation of cytotoxic effect of *A. scoparia* pollen

Ten grams of pollen was crushed and added with methanol at a ratio of 1:10. It was placed on a shaker at 150 rpm for 24 hr. The cells treated with pollen methanolic extract at the different concentrations.

Methanol extraction

10 g of *A. scoparia* pollen was crushed and transferred to a flask and added with methanol at a ratio of 1:10 (100 ml of 70% methanol). The lid of the flask was sealed with parafilm and covered with foil. The flask was then placed on a shaker at 150 rpm for 24 hours at room temperature. The supernatant was filtered through Whatman filter paper and 100 ml of 70% methanol was re-added to the precipitate. After 72 hours, the filtered solution was concentrated by a rotary evaporator at a pressure of 180-150 Pa and a temperature of 35°C. The vial containing the extract was kept at -4°C until further testing. The sterile MTT dye solution with 0.5 mg/ml concentration was added and incubated at 37°C for 4 hr. The absorbance was read at 570 nm and the reference wavelength of 630 nm using an ELISA reader. The cell survival rate was calculated according to the following equation (Bikhof Torbati et al. 2017).

$$\text{Cell survival rate} = (\text{mean OD}_{\text{sample}} / \text{mean OD}_{\text{control}}) \times 100$$

Induction of apoptosis in *A. scoparia*-treated cells

The apoptosis rate of PC-3 cells was calculated by flow cytometry and Annexin V/PI double staining kit (Affymetrix kit). The cells were treated by IC_{50} concentrations of pollen extract and evaluated for apoptosis after 24 hr. The cells were trypsinized, washed with PBS and centrifuged. The cell deposition was suspended by 500 µl of binding buffer and incubated after adding 3 µl of Annexin V-FITC dye. The cells were washed and centrifuged. Next, 250 µl of binding buffer and 5 µl of bioscience Propidium Iodide (PI) solution were added to the cell deposition and the fluorescent stained cells were evaluated using a flow cytometer at an excitation wavelength of 488 nm and a reading filter of 515 nm for Fluorescein isothiocyanate (FITC) and a filter of 600 nm for PI. Early and late apoptotic cells respectively, positive only for Annexin-FITC and both stains, was seen in the bottom and top right quadrant, necrotic cells positive only for propidium iodide in the top left quadrant. lived cells are negative for both stains in bottom left (Rieger et al. 2011).

Evaluation of cell cycle arrest of *A. scoparia* pollen-treated cells

To evaluate the effect of the extract on the cell cycle, 10^6 cells were cultured in two 25-cm^2 flasks. After cell monolayer formation, one of the flasks was treated with IC_{50} concentration of the extract and the other was considered a control. After 24 hr, the cells were trypsinized, and to resulting cell deposition was added 250 µl of RNase A-containing PI/Triton X-100 staining solution. The cells were incubated at room temperature for one hour in a dark place and then analyzed by flow cytometry and FlowJo v.7.6.1 software (Behdarvand et al. 2020).

Evaluation of gene expression exposed to *A. scoparia* pollen extract

After the construction of cDNA from RNA using a kit (Thermo Scientific, USA), the real-time PCR process was performed using the primers listed in Table I. Then, 10 µl of CyberGreen (Amplicon Bio, Korea), 2 µl of cDNA sample, 0.5 µl of forward and reverse primers (10 pmol) and 7 µl of nuclease-free water were mixed. The comparative C_t ($2^{-\Delta\Delta Ct}$) method was applied

to analyze the relative changes in the gene expression level. The β-actin, a housekeeping gene, was considered the control gene.

GC Analysis

One gram of *A. scoparia* pollen was powdered and was mixed with 10 ml of hexane solvent. The preparation was done with the help of boron trifluoride derivatizing compound and incubation at 60°C for one hour. After cooling and adding of hexane, it was centrifuged at 4000 rpm for 10 min. The top layer of hexane was separated and ready to be injected into the device. The standard was purchased from American Nu-Chekprep company with the code GLC-462, which includes 28 saturated and unsaturated fatty acids (cis and trans).

Table 1. The sequence of primers designed to assess gene expression by real-time PCR

Gene	Primer sequence
p21	Forward: 5'- F: GCTTCATGCCAGCTACTTCC-3' Revers: 5'- CCCTTCAAAGTGCCATCTGT-3'
p53	Forward: 5'- CATACTCCACAGCACCTGGTTA -3′ Revers: 5'- ACTCAAATTCTGTTGCCACCTT -3'
β-actin	Forward: 5'- TCCTCCTGAGCGCAAGTAC-3' Revers: 5'- CCTGCTTGCTGATCCACATCT-3'
bax	Forward: 5'- GAGCTGCAGAGGATGATTGC-3′ Revers: 5'- AAGTTGCCGTCAGAAAACATG-3'
bcl2	Forward: 5'- ATTGGGAAGTTTCAAATCAGC-3′ Revers: 5'- CAGTCTACTTCCTCTGTGATGTTG-3'

Data were analyzed by SPSS V.22 software. Due to the normal distribution of data, the one-way analysis of variance (ANOVA) was used to compare the treatment and control groups at the significance level of p-value<0.05, followed by Tukey's post hoc test.

Results

Pollen morphological results

A. scoparia pollen was rounded-triangular in shape and tri-lobed at the corners. The pollen loads were olive-colored.

Results of protein, saponin and catalase levels

The mean protein content of pollen extract was 23.33mg/g of pollen, saponin level was 1.0266 g/10g of pollen, and the catalase activity level 0.006 µM/mg/min was calculated (Table 2).

Table 2. Protein and saponin contents and catalase activity level of *A. scoparia* pollen

Protein content	23.33 mg/g of pollen
Saponin content	1.0266 g/10g
Specific activity of catalase	0.006 µM/mg/min

Cytotoxic effect of different concentrations of *A. scoparia* pollen extract

The results of MTT assay showed the concentration- and time-dependent cytotoxic effect of *A. scoparia* pollen extract on PC-3 cells. In fact, the survival rate of PC-3 cells was significantly reduced ($p<0.05$) over time and with increasing the extract concentration (Figure 1-A). The cytotoxic effect of *A. scoparia* pollen extract on HFF normal cells was not significantly dependent on concentration and time (Figure 1-B). The IC_{50} values of the extract were obtained for each of PC-3 and HFF cells at 48 and 72 hr (Figure 2). At the incubation time of 72 hr, the decrease in IC_{50} value of the extract on PC-3 cells compared to HFF cells indicates a 1.9-fold increase in the cytotoxicity of the extract on prostate cancer cells compared to healthy cells. These values did not differ significantly at 48 hr.

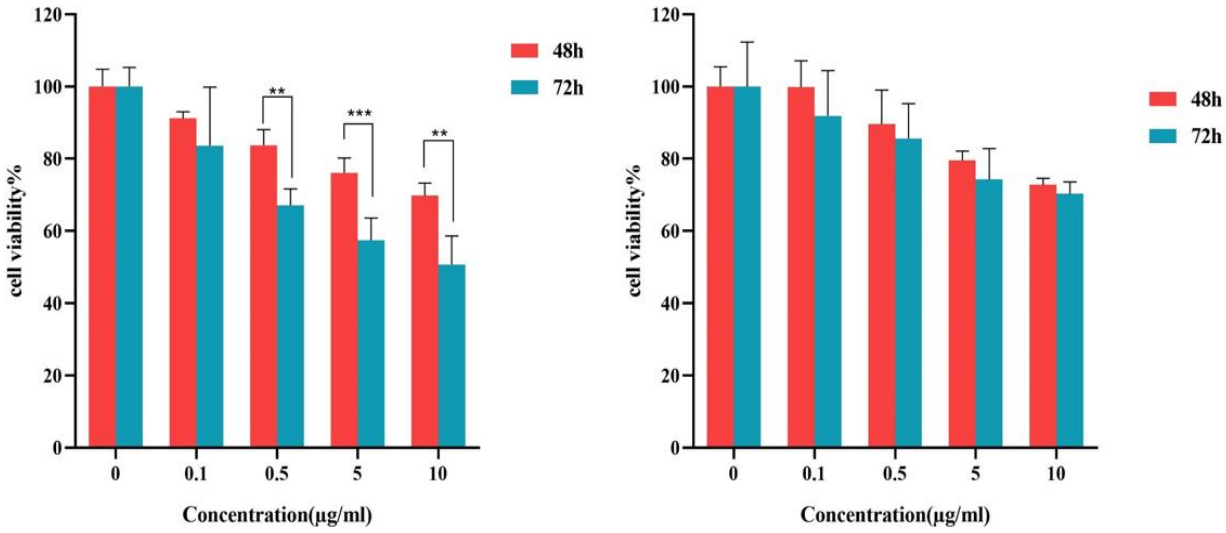
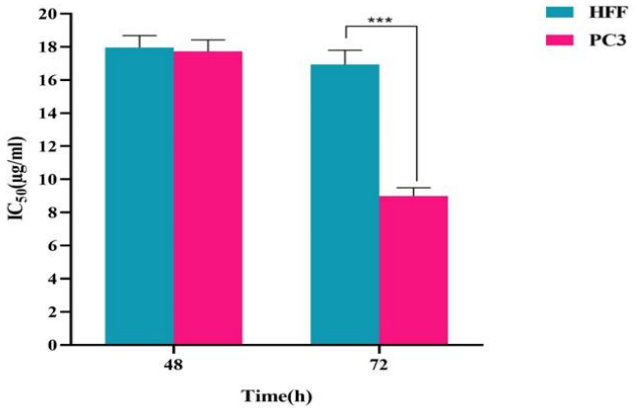


Figure 1. Cytotoxic effect of *A. scoparia* pollen extract on PC-3 (A) and HFF (B) cell lines at 48 and 72 hr, indicating concentration- and time-dependent cytotoxicity of the extract (*p<0.05, **p<0.01, and ***p<0.001).



IC50 (μg/ml)	48h	72h
HFF	17.966±0.720	16.931±0.860
PC3	17.718±0.705	8.995±0.500

Figure 2. IC_{50} values of *A. scoparia* pollen extract against PC3 and HFF cell lines at 48 and 72 hr. (***p<0.001)

Effect of *A. scoparia* pollen extract on apoptosis

The cytograms present Q1 as the necrotic cells, Q2 as the late apoptotic cells, Q3 as the early apoptotic cells, and Q4 as the live cells. Accordingly, the results indicated that the treatment of PC-3 cells with *A. scoparia* pollen extract caused a significant increase in early and late apoptotic cells. As the treatment time was longer, the number of apoptotic cells increased significantly. However, no significant difference was observed in the number of necrotic cells between the treatment and control groups (Figure 3).

Effect of *A. scoparia* pollen extract on cell cycle arrest, gene expression and apoptosis

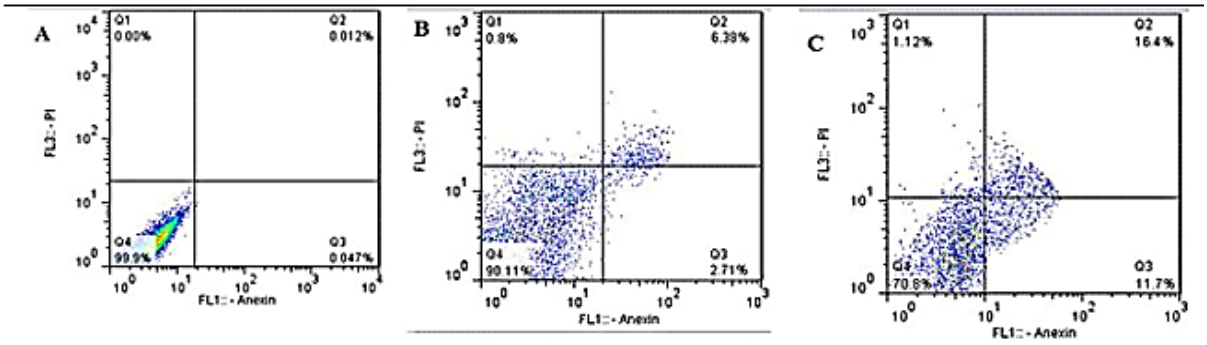
The results showed that the treatment of PC-3 cells with a concentration equivalent to the IC_{50} value of *A. scoparia* pollen extract arrested a significant percentage of the cell population in the sub-G1 phase of the cell cycle and prevented cells from entering the next phases, indicating the induction of apoptosis.

These changes were time-dependent so that the number of cells arrested in the sub-G1 phase showed a significant increase with prolonging the treatment time (Figure 4). Examination of gene expression

revealed that this extract significantly increased the expression of *bax*, *p53* and *p21* genes in PC-3 cells compared to the control sample. The *Bcl-2* gene expression was significantly reduced in these cells following treatment with *A. scoparia* pollen extract. These changes were time-dependent in all cases, with more changes observed after 72 hr than after 48 hr. Therefore, the expression of any of these genes in HFF cells was not significantly different from the control sample (Figure 5).

Results of GC method

Eight fatty acids were found in the pollen extract, among which the highest amount was related to palmitoleic acid (Figure 6).



D

	control	48h	72h
Early apoptosis	0.067 ± 0.01	3.88 ± 1.65	9.86 ± 2.60
Late apoptosis	0.036 ± 0.01	5.54 ± 1.18	16.25 ± 0.2
Total apoptosis	0.103 ± 0.02	9.42 ± 2.83	26.11 ± 2.8
Necrosis	0.023 ± 0.00	0.63 ± 0.24	1.23 ± 0.15
Living cells	99.9 ± 0.07	89.95 ± 0.22	72.65 ± 2.6

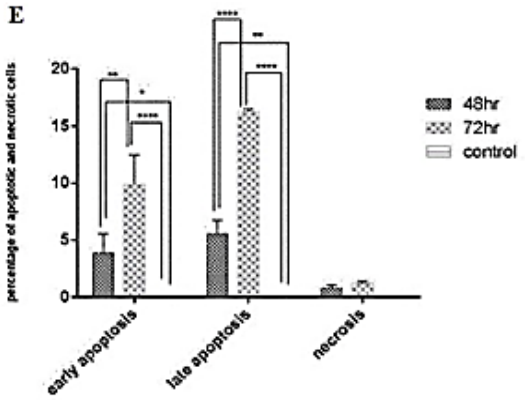
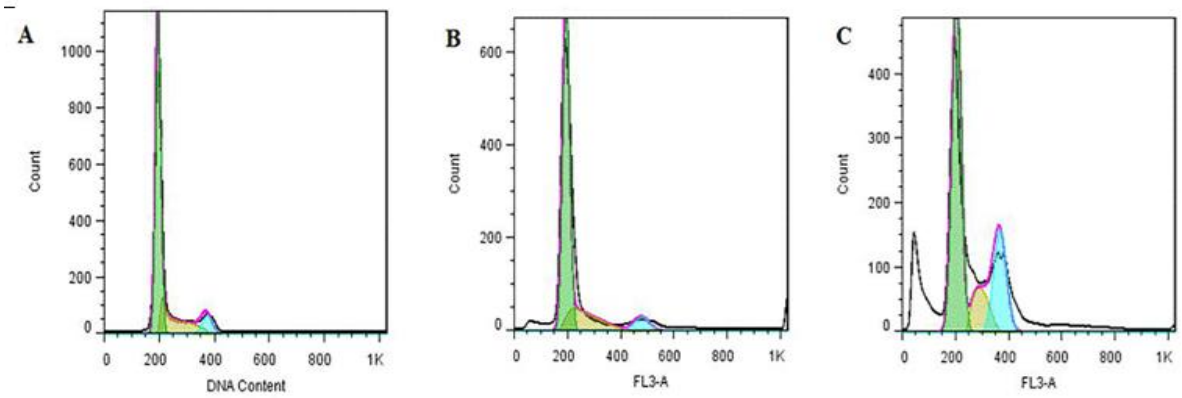


Figure 3. The ability of *A. scoparia* pollen extract to induce apoptosis in PC-3 cell line at 48 and 72 hr. A: control, B: 48-hr treatment, C: 72-hr treatment, D and E: induction of cell death (*p<0.05, **p<0.01, and ***p<0.001).



D

	control	48h	72h
Sub G1	2.27 ± 0.02	11.72 ± 1.19	21.16 ± 1.47
G1	69.67 ± 0.09	63.15 ± 6.16	43.5 ± 3.08
S	21.44 ± 0.40	16.09 ± 0.55	9.075 ± 3.62
G2	8.37 ± 0.24	9.1 ± 4.59	21.96 ± 3.42

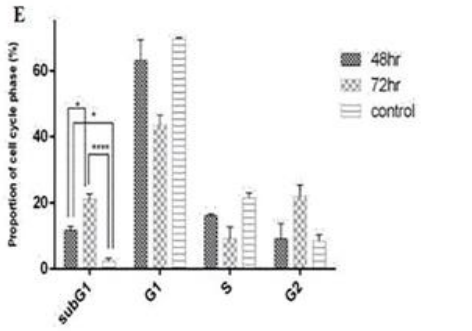


Figure 4. Effect of *A. scoparia* pollen extract on cell cycle arrest in PC-3 cell line at 48 and 72 hr. A: control, B: 48-hr treatment, C: 72-hr treatment, D and E: cell cycle (*p<0.05 and ***p<0.001).

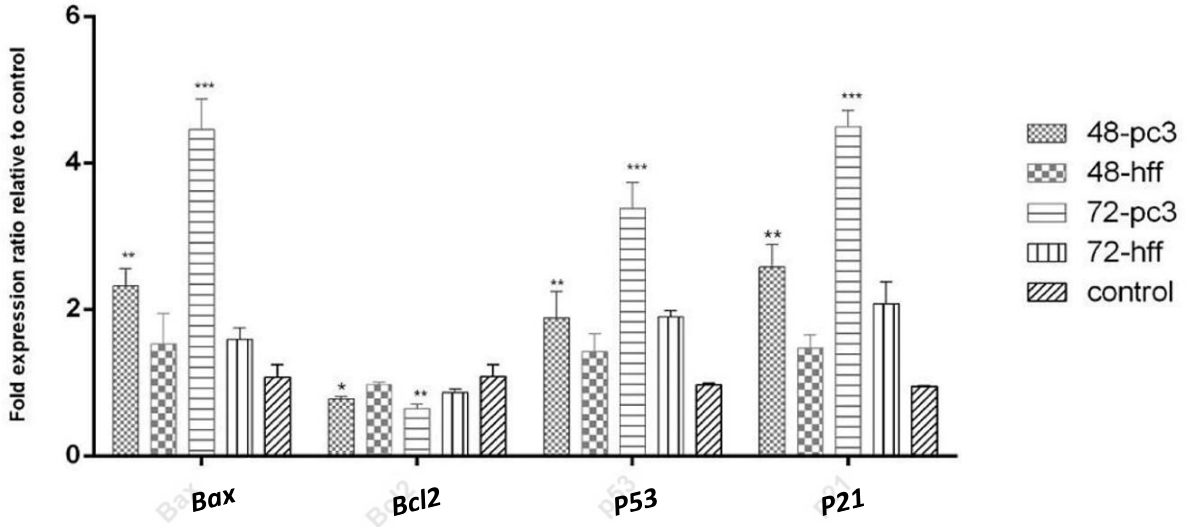
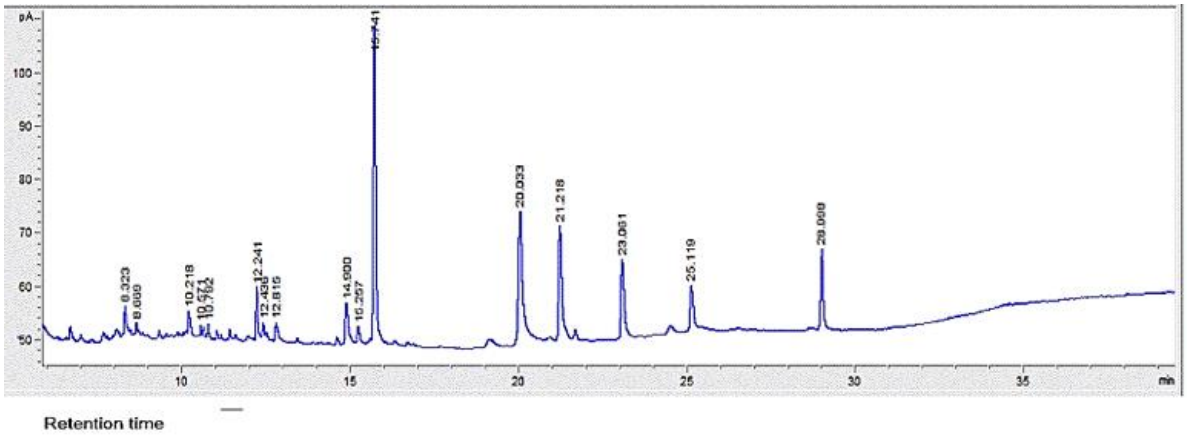


Figure 5. Gene expression levels in HFF and PC-3 cell lines exposed to *A. scoparia* pollen extract at 48 and 72 hr (*p<0.05, **p<0.01, and ***p<0.001).



Name	Formula	Retention time	Area%
METHYL MYRISTATE	C14:0	12.815	1.275
METHYL MYRISTOLEATE	C14:1	14.900	4.708
METHYL PALMITOLEATE	C16:0	15.741	28.51
METHYL STEARATE	C18:0	20.033	18.018
METHYL OLEEATE	C18:1	21.218	13.352
METHYL LINOLEATE	C18:2	23.061	9.327
METHYL ALPHA LINOLEATE	C18:3	25.119	5.459
METHYL ARACHIDONATE	C20:4	28.998	7.929

Figure 6. Analysis of fatty acids of *A. scoparia* pollen extract using gas chromatography

Discussion

The pollen protein content in the current study was relatively high (23.33%). The presence of relatively high protein in pollen samples collected by bees in this study was an important feature of these pollens, which is most likely due to the presence of catalase.

Protein and amino acids have been introduced as the main source of nutrition for bees and it was reported that the pollen protein content was often between 13.9 and 25.5% (Liolios et al. 2015). It has also been identified that pollen protein is an indicator of the flower origin of honey (Nazarian et al. 2010).

The catalase is one of the most important proteins in honey, part of which is secreted by a gland in bees and added to honey when honey is diluted, and the other part enters the honey through the pollen that bees had previously stored in the hive cells to feed their babies (Sahin et al. 2020). The anti-cancer role of *A. scoparia* bee pollen may be due to its high catalase level.

Research on plant pollen is very difficult due to its characteristics such as stickiness and high viscosity; therefore, less research has been done. In the present study, we made many changes to many of the preparations and detection of specific catalase activity that had not been done before. For example, these differences are clearly evident in the Cakmak and Marschner research (Cakmak and Marschner 1992).

the IC_{50} value of the extract for HFF cell line was significantly higher than that for PC-3 cells. The number of apoptotic cells affected by this extract was significantly increased. According to the results of real time PCR, the expression level of *bax*, *P53* and *P21* genes was significantly increased in PC-3 cells exposed to *A. scoparia* pollen extract whereas the expression level of *Bcl-2* anti-apoptotic gene was significantly reduced, in line with the results of analyzing the cell cycle and apoptosis.

Bax protein is a member of the Bcl-2 family that releases cytochrome C and induces apoptosis following mitochondrial damage (Peña-Blanco and García-Sáez 2018). Therefore, due to the increased expression of *bax* under the effect of *A.*

scoparia pollen extract, a significant increase in apoptotic cells treated with this extract was also observed in the flow cytometry results. The results of the present study demonstrated that the *A. scoparia* pollen extract could significantly reduce the *bcl-2* -expression and thus eliminate its inhibitory effect on *bax*. Increased expression of *p21* accordingly leads to cell cycle arrest; our results of flow cytometry showed the arrest of PC-3 cells in the sub-G1 phase and thus inhibition of the cell cycle. The effect of rape pollen extract on the PC-3 cells has been investigated with IC_{50} value of 1.4 mg/ml (Zhang XingHai et al. 2010). In the current research microscopic examination revealed the induction of cell apoptosis. These findings were in line with the results of the present study which showed the potential of some plant pollens to induce apoptosis in PC-3 cancer cells.

Saponins increased *bax* expression and decreased *bcl-2* expression, thereby inducing apoptosis in cancer cells (Wang et al. 2019). The anti-cancer effect of saponin extracted from *Albizia lebbeck was* investigated and the IC_{50} value of saponin-rich extracts for MCF-7 breast cancer cell line was calculated to be 1 µg/ml (Desai and Joshi 2019). Increased caspases lead to the induction of apoptosis. Therefore, part of the anti-cancer effects observed in the current study might be attributed to the saponins present in *A. scoparia* pollen extract.

The *A. scoparia* pollen extract appears to trigger antioxidant activity against high concentrations of ROS due to the presence of high concentrations of catalase. Decreased catalase concentration has been suggested as a specific feature of cancer tissues (Díaz et al. 2012). In this regard, it was reported that the catalase of bee-collected pollen could regulate hydrogen peroxide formation, antioxidant effect and antibacterial activity through the degradation of excess H_2O_2 (Alygizou et al. 2021). The present study reports for the first time the anti-cancer potential of *A. scoparia* pollen extract on PC-3 prostate cancer cell line.

In this research, palmitoleic acid was extracted in a large amount (28.51%) from the analysis of *A. scoparia* pollen extract (Fig. 6). Palmitoleic acid was described as a lipokine able to prevention of endoplasmic reticulum stress (Bermúdez et al. 2022). high levels of stress are necessary to maintain the rapid proliferation and metastasis of tumor cells (Zhang et al. 2024).

The cytotoxicity of *A. scoparia* bee pollen extract on HFF cell line was less than that on the cancer cell line. This property can make *A. scoparia* pollen extract a potential anti-cancer agent with low side effect. Also, the analysis of cell cycle and apoptosis showed that *A. scoparia* pollen extract was able to arrest the proliferation of PC-3 cells in the sub-G1 phase. The results of analyzing gene expression in exposure to *A. scoparia* pollen extract documented that the *p53* expression levels were significantly increased in PC-3 cells. Also, the expression of bcl-2 decreased and the expression of bax and p21 increased. The presence of saponins, palmitoleic acid and, catalase activity in pollen extract at a high level is probably a factor in inducing cell death and changes in the expression of the studied genes

On the other hand, Amygdalus scoparia pollen in the Zagros region can be easily available for medicinal use because, according to researchers, honey bees are very interested in collecting its pollen and the pollen can be collected through a pollinator trap. The attractive features of the Amygdalus scoparia flower include having a white corolla, triangular-shaped and olive color pollen grains (Taghavizad et al. 2007, 2009).

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Conflicts of interest

The authors declare no conflicts of interest.

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