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Anti-Proliferative Effects of *Pistacia atlantica* Gum Extract on the Human TC1 Cell line: In Vitro Testing

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Abstract

Background: Cervical cancer is one of the most common cancers in women throughout the globe. According to reports, currently used cervical cancer therapies are harmful to patients. These treatments have also revealed certain adverse effects that may have a severe impact on the lives of women who are afflicted with this kind of cervical cancer. To combat cancer, fewer hazardous anti-tumor therapies are required. Natural products, such as herbal medicine, have been revealed to have a variety of biological impacts. *Pistacia atlantica* gum extract was tested on the TC1 cell line to see whether it had any anti-proliferative effects.

Objectives: This study aimed to determine the anti-proliferative effect of *Pistacia atlantica* gum extract on the TC1 cell line.

Methods: Gum extract from dried and milled *Pistacia atlantica* was analyzed using Gas Chromatography-Mass Spectrometry. The cells were divided into five groups at random, including one for the negative control, one for the positive control, and three for the therapy. Cells from TC1 mice were given the extracts of *Pistacia atlantica* gum at three different doses (100, 200, and 400 mg/mL). To determine the viability of the cells, reactive oxygen species (ROS) generation, and the concentrations of caspases-3, -8, and -9, researchers used a commercial enzyme-linked immuno-absorbent assay kit.

Results: Results showed that, in comparison to the negative control and most other treatment groups, the *Pistacia atlantica* gum extract group (400 mg/ml) significantly decreased cell viability (70.138±8.464) and significantly increased apoptosis percentage (71.66±4.041), produced significantly more ROS (15.69±0.799), and significantly increased lactate dehydrogenase release (17.83±0.772) in TC1 cells ($P<0.05$). In addition, the activity of caspase-8 (0.097±0.007) and caspase-9 (0.065±0.004) increased significantly ($P<0.05$) in this study.

Conclusion: Our findings indicated that *Pistacia atlantica* gum extract could drastically decrease cell viability and increase apoptosis in the TC1 cell line, similar to the anti-cancer medication doxorubicin.

Keywords: Cell proliferation, Cervical cancer, *Pistacia atlantica* gum, TC1 Cells

1. Background

Cervical cancer is the fourth most frequent female disease globally, accounting for 7.5% of all cancer deaths in women. The human papillomavirus (HPV) is the viral etiological agent of cervical cancer, responsible for almost all cervical malignancies (1). A subset of HPVs that may be spread by oral, vaginal, or anal sexual contact has been identified as carcinogenic. Annually, over 33,700 malignancies, including cervical, oral, penile, and anal cancers, are caused by HPV infection (2). The common clinical difficulties in the treatment of numerous types of cancer are severe adverse effects of almost all chemotherapy medications and the development of resistance to chemotherapy treatments. Additionally, rapidly proliferating cells, such as some normal and malignant cells, are the primary targets of popular cytotoxic therapies, which result in significant morbidity and little therapeutic benefits for distressed persons (3).

Combination treatment is an effective strategy for lowering anticancer medication dosages and associated adverse effects. Natural herbal metabolites have lately garnered substantial interest as potential alternative treatments for a variety of illnesses (4). According to the World Health Organization, approximately 65% of individuals have supplemented their health treatment with plants and traditional pharmaceuticals. In other words, botanicals and traditional medicines have shown a tremendous potential to be used as anticancer treatments due to the discovery of various chemical compounds capable of destroying rapidly proliferating cells (5).

Today, interest in herbal medicine for cancer treatment is increasing due to its low tissue toxicity and precise harmful effects on cancer cells. Herbs may be used as supplemental and alternative medicine. Natural substances have been shown to decrease inflammation and combat treatment resistance (6). *Pistacia atlantica* is a species of the Anacardiaceae family found from the Mediterranean Sea to Central Asia. Pistacia atlantica, often referred to as Baneh, is found in western mountainous areas of Iran. The plant's unripe fruits and seeds are frequently utilized as edible fruits and nuts and in

cuisines (7). The gum resin, fruits, and seeds of this plant are all employed in Persian Traditional Medicine. Various portions of the plant have recently been shown to have antihyperlipidemic (8), antioxidative (8), anti-inflammatory (9), and antibacterial properties (10). Pistacia species have been demonstrated to induce apoptosis in the prostate (11), colon (12), and breast cancer cell lines in previous investigations (13).

2. Objectives

This study aimed to determine the anti-proliferative effect of *Pistacia atlantica* gum extract on the TC1 cell line.

3. Methods

3.1. Pistacia atlantica gum extraction

Pistacia atlantica gum was obtained fresh from supermarkets in Khorramabad, Lorestan, Iran. It was then washed, dried, ground, and vacuum-dried with the use of an electric mill. Subsequently, the powder was soaked for 48 h in distilled water prior to filtering. Following exposure to re-distillation, the extract was incubated at 50°C (14). The Ethics Committee of the Baqiyatallah University of Medical Sciences, Tehran, Iran, oversaw the conduct of the study. The management procedures (IR.BMSU. REC.1397.156) were established in accordance with the norms of the Iranian Ministry of Health and Medical Education and were certified by the Medical Ethics Committee of Baqiyatallah University of Medical Sciences.

3.2. Gas Chromatography-Mass Spectrometry examination

The Gas Chromatography-Mass Spectrometry (GC-MS) experiment was conducted using a Shimadzu GC-17A gas chromatograph (Kyoto, Japan) equipped with a Shimadzu Quadruple-MS type QP5050 mass spectrometer. The mixtures were partitioned using a 0.22 mm 30 m i.d. fused-silica capillary column covered with a 0.25 m layer of BP-5 (Shimadzu) and a split/splitless injector with 1-mm internal glass liners. At a 70-eV ionization potential, a carrier gas (ultra-pure helium) was used. Temperatures at the interface and injector were 260°C and 280°C, respectively, and the mass was between 35 and 450 amu. The oven temperature restriction was comparable to that previously mentioned for the GC. The extract components were identified for the oil on a DB-5 column using retention indices calculated at the settings and based on the temperature for n-alkanes (C8-C20). The genotype chemicals were identified by comparing their mass spectra with those of the internal reference mass spectrometer library (Wiley and NIST08 9.0) (14).

3.3. Experimental design and cell culture

The TC1 papillomavirus and cervical cancer cell line were donated by the National Cell Bank of Iran of the Pasteur Institute in Tehran, Iran. Strains were grown in Modified Dulbecco's Medium with 100 mg/ml of streptomycin, 10% fetal bovine serum, and 100 units/ml of penicillin G (Sigma, St. Louis, MO, USA). A humidified atmosphere was then utilized to incubate the cells at 37°C, with 5% CO2 in the air. Afterward, the cells were categorized into five categories. Doxorubicin at an 800 nM concentration was used as a positive control (PC) in groups 5, 6, and 7. Group 1 was used as a negative control (NC), while groups 2-4 included various concentrations of *Pistacia atlantica* gum extract (PAGE 1, PAGE 2, and PAGE 3, respectively).

3.4. Cell viability

According to Jabbari et al., the MTT test was used to compare the viability of TC1 cells between treatment groups. Cells extracted from 24-well plates were treated with 0.05% trypsin/ethylenediaminetetraacetic acid (EDTA) solution, and then transferred to 96-well plates containing 1106 cells/well for 48 h. After a 48-hour incubation period, MTT (5 mg/ml) was added to each well and incubated at 37°C for 4 h to produce a purple formazan result. A volume of 100 ml dimethyl sulfoxide was added to the plate, and the plate was incubated at 37°C for 10 min to dissolve the purple crystals. Individual wells were examined using an enzyme-linked immuno-absorbent assay plate reader (Dana 3200, Iran) at 492 nm for the optical density of each well.

3.5. Evaluation of TC1 apoptosis

Jabbari et al. employed acridine orange and propidium iodide to measure the proportion of Michigan Cancer Foundation-7 cells in the *Pistacia atlantica* gum treatment groups. To summarize, cells from 24-well plates were treated with a 0.05% trypsin/EDTA solution and *Pistacia atlantica* gum. Phosphate-buffered saline (PBS) was used to wash the cell suspension that was particular to each group. Afterward, 10 ml of acridine orange and propidium iodide in equal proportions were decanted into the cellular pellet and incubated for 30 min. An improved Neubauer rhodium hemocytometer was used to estimate the percentage of apoptotic cells under fluorescent microscopy (Olympus CKX41) (15).

3.6. Lactate dehydrogenase assay

Lactate dehydrogenase (LDH) is a ubiquitous enzyme found in the cytosol that catalyzes the conversion of lactate to pyruvate. When the integrity of the plasma membrane is disrupted, LDH leaks into the culture medium, raising its extracellular level. Cytotoxicity was determined using the LDH Kit (Abcam, ab65393). The release of

the cytoplasm enzyme LDH was determined in this experiment utilizing injured cells. *Pistacia atlantica* gum was used to treat 96-well plates of grown cells. After 48 h of treatment, the culture supernatant was collected. A reaction mixture was used to incubate the culture supernatant. The LDH-catalyzed conversion resulted in a decrease in the tetrazolium salt to Formosan, which could be detected at an absorbance of 490 nm (15).

3.7. Reactive oxygen species production assay

To quantify reactive oxygen species (ROS) formation, dichlorodihydrofluorescein diacetate (DCFH-DA, 2.5 lM; Invitrogen, St Louis, MO, USA) was utilized. Dichlorodihydrofluorescein diacetate enters the cell passively and reacts with ROS to create DCF, a highly fluorescent chemical. The detached cells were temporarily treated with *Pistacia atlantica* gum from 24-well plates using a 0.05% trypsin/EDTA solution, and then, transferred to 6-well plates for 24 h at a density of 1,106 cells/well. After two washes with PBS, the cells were stained with 20 M DCFH-DA and incubated in DCFH-DA for 30 min. After rinsing the cells with PBS, the fluorescence intensities were defined using a control. The treated cells were imaged using a fluorescent microscope (Olympus CKX 41) set to 40X magnification (15).

3.8. Examining the caspase-8 and -9 creation

This study relied on colorimetric test kits (Sigma-Aldrich) and the recommendations of Jabari et al. (St. Louis, MO). Caspase-3, -8, and -9 protein concentrations were determined following the treatment of TC1 cells with multi-agent mixed treatment groups (11).

3.9. Statistical analysis

The results were analyzed using one-way ANOVA, and mean±SD values were used to compare the experimental groups. Tukey's multiple post hoc tests were employed to compare the mean values of various groups, and ANOVA detected the significant variation. The p-values of 0.05 were considered significant.

4. Results

4.1. Gas Chromatography-Mass Spectrometry analysis

As shown in Table 1, α-pinene and β-pinene comprised 77.9% and 3.66% of the aqueous extract of *Pistacia atlantica* gum, respectively.

4.2. Cell cytotoxicity and viability results

MTT (Figure 1) and apoptosis (Figure 2) assays were used to determine the in vitro cytotoxicity of

Table 1. Concentrations (%) of different compounds in aqueous extract of *Pistacia atlantica* gum

Compound	Retention index	Amount (%)	Compound	Retention index	Amount (%)
Alpha-Pinene	939	77.9	Linalool	1103	0.13
Camphene	953	0.88	Chrysanthenone	1146	0.08
Verbenene	967	0.43	Rans-Limonene-Oxide	1149	0.88
Beta-Pinene	980	3.66	Pinocarveol	1152	0.59
Beta-Myrcene	991	6.26	Cis-Sabinol	1149	1.7
Delta-3-carene	1011	0.24	Isopinocamphone	1181	1.93
Alpha-Terpinene	1018	0.03	Pinocarvone	1162	0.92
P-Cymene	1026	0.3	P-Mentha-1,5-dien-8-ol	1185	0.05
L-Limonene	1031	0.76	Terpinene-4-ol	1190	1.51
1,8-Cineole	1033	0.14	P-Cymen-8-ol	1199	0.25
Trans-beta-Ocimene	1050	0.18	Myrtenol	1190	0.55
Gamma-Terpinene	1062	0.07	Cis-Carveol	1241	0.15
Terpinolene	1088	0.21	Isobornyl acetate	1302	0.2

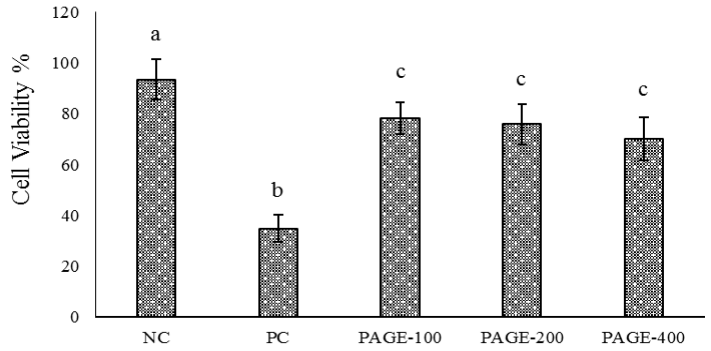

Figure 1. Descriptive statistics (mean±standard deviations) of investigated cell viability percent in the groups under study (The abbreviations are provided in the experimental design section: "Materials and Methods".) Significant statistical differences between groups in each index are indicated by different superscript letters (P<0.05)

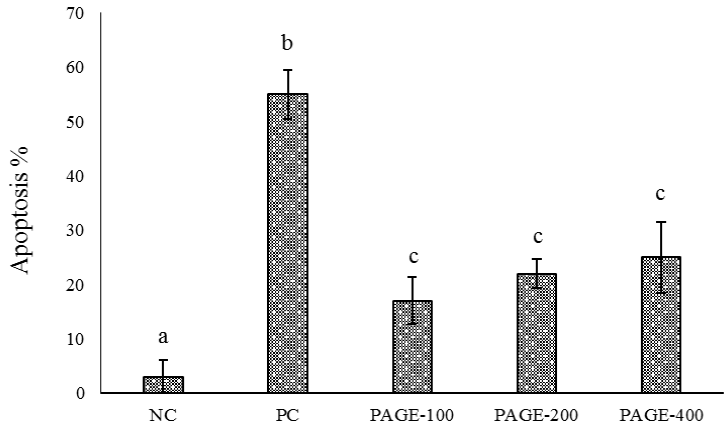

Figure 2. Descriptive statistics (mean±standard deviations) of investigated apoptosis percent in the groups under study (The abbreviations are provided in the experimental design section: "Materials and Methods".) Significant statistical differences between groups in each index are indicated by different superscript letters (P<0.05)

Pistacia atlantica gum extract groups against cervical cell lines TC1. The acquired findings were compared to those of the negative control group and conventional Doxorubicin. In the comparison of cytotoxicity groups with the control and other groups, the greatest value of extract was 400 mg/mL. The effects of extract cytomorphological alterations on TC1 cell lines at various doses (100, 200, and 400 mg/mL) included an intracellular suicide program characterized by oxidative stress, cell shrinkage, biochemical reaction, and coiling, which resulted in apoptosis. The apoptosis percent (viability) of TC1 cell lines significantly increased. The maximal dose (400 mg/ml) was shown to have the most inhibitory effect on cervical cancer cells.

4.3. Reactive oxygen species production

In contrast to the negative control group, ROS generation was significantly enhanced in the treatment groups exhibiting the dose-dependent behavior of *Pistacia atlantica* gum extract (P<0.05) (Figure 3). The concentration of the extract, which ranged from 100 to 400 mg/ml, had an effect on ROS production.

4.5. Lactate dehydrogenase assay

As seen in Figure 4, the extract's effect on membrane integrity was determined by measuring LDH activity 48 h after the treatment of TC1 cells. According to the acquired data, the extract's effect on the membrane integrity of TC1 cells was dose-dependent. The 400 mg/ml group produced the most LDH from cervical cancer cell lines.

4.6. Caspase assay

As presented in Figure 5, the levels of Caspase-8 and -9 in all groups increased considerably after 24 h of incubation (P<0.05).

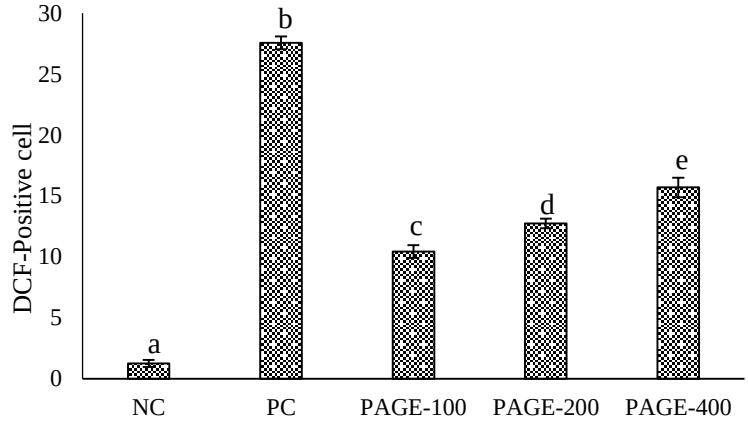

Figure 3. Descriptive statistics (mean±standard deviations) of investigated ROS production in the groups under study (The abbreviations are provided in the experimental design section: "Materials and Methods".) Significant statistical differences between groups in each index are indicated by different superscript letters (P<0.05)

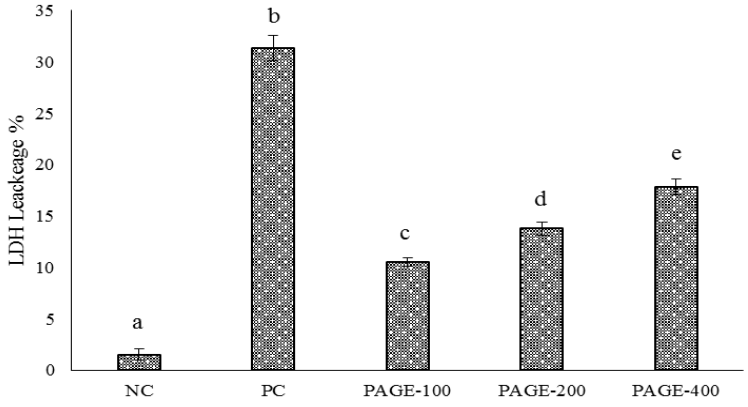

Figure 4. Descriptive statistics (mean±standard deviations) of investigated LDH production in the groups under study (The abbreviations are provided in the experimental design section: "Materials and methods".) Significant statistical differences between groups in each index are indicated by different superscript letters (P<0.05)

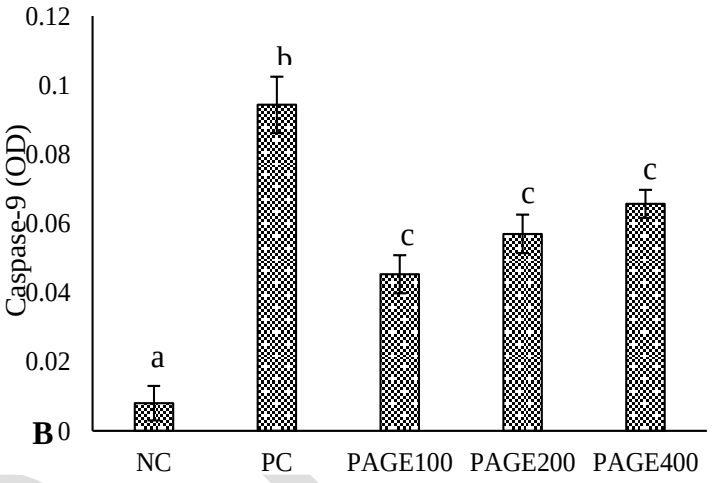

Figure 5. Descriptive statistics (mean±standard deviations) of investigated caspase-8 (A) and -9 (B) activities in the groups under study (The abbreviations are provided in the experimental design section: "Materials and Methods".) Significant statistical differences between groups in each index are indicated by different superscript letters (P<0.05)

5. Discussion

Cancer is a very complex illness in which aberrant intracellular signal transduction plays a role in its onset and progression. Chemotherapy is currently considered one of the primary ways for cancer therapy, and the majority of medicines used for this purpose have a variety of undesirable side effects. As a result, recent research has concentrated on developing effective chemopreventive medicines with a natural basis that exhibit high selectivity and low toxicity to normal cells (16).

Cervical cancer is one of the most common types of cancer in the world. Chemotherapeutic approaches capable of effectively treating colorectal cancer are constantly being evaluated. Inducing apoptosis and cell cycle arrest using chemotherapeutic drugs may be a successful strategy for avoiding malignant cell survival and uncontrolled cell multiplication (17). Natural chemical analysis is a popular and effective technique for developing novel chemotherapeutics. Numerous medications with clinical actions are used in cancer treatment. These medications are either natural compounds or derivatives of natural products. Vincristine, vinblastine, etoposide, paclitaxel, teniposide, camptothecin, and docetaxel are all examples of plant-derived medicines (18).

Numerous anticancer terpene investigations have been conducted earlier. It has been discovered that 37 different monoterpenes found in plant essential oils have anticancer properties (19). Alpha-pinene has been shown to be an anticancer in a variety of cancers and in vivo investigations, including human hepatocellular carcinoma (20) and prostate cancer (21) in xenograft mouse models. Additionally, α-pinene has been shown to trigger apoptosis in human

ovarian and hepatocellular carcinomas by G2/M phage cycle arrest (22). Moreover, α-pinene has been demonstrated to suppress matrix metalloproteinase-9 expression in human breast cancer cells, hence preventing cancer invasion.

The GC-mass data in our study revealed that α-pinene had the highest percentage of components in the extract. As a result, it is possible that this molecule is responsible for its anti-cancer properties. The gum of Baneh was found to reduce the viability of TC1 cells in this investigation. This impact was achieved by inhibiting cell proliferation and inducing apoptosis. Numerous natural compounds have been discovered to be capable of causing apoptosis in a variety of human tumor cells. Compounds with proapoptotic properties may facilitate the removal of started precancerous cells, hence preventing cancer.

In the current study, two corroborative assays for apoptosis induction were performed, including propidium iodide/acridine orange staining of cell DNA following incubation of cells with *Pistacia atlantica* gum aqueous extract. In our investigation, the greatest value for cytotoxicity was 400 mg/mL of extract, compared to the control and other groups. Our findings demonstrated that cytomorphological differences in the extract had an effect on TC1 cell lines at various concentrations, including an intracellular suicide program associated with oxidative stress, cell shrinkage, metabolic reaction, and coiling leading to apoptosis. The 400-mg/ml group produced the highest levels of LDH in cervical cancer cell lines in this study. On the other hand, the concentration of extract increasing from 100 to 400 mg/ml had an effect on ROS generation.

Anticancer drugs are primarily used to promote tumor cell death and regulate the cell cycle. *Pistacia atlantica* has been demonstrated to induce apoptosis in prostate, colon, lung, and breast cancer cell lines in previous research. Due to the variety of components, such as flavonoids and polyphenols, these effects are more likely to neutralize free radicals, chelate metals, and control enzymatic activity (23). Polyphenolic compounds found in plants can influence cell cycle progression by influencing genes involved in cell proliferation and apoptosis. *Pistacia atlantica* gum extract has been reported to inhibit the regulation of Cyclin A and S phase proteins in the human breast cancer cell line (24).

There have been two key signaling channels involved in apoptosis, namely dubbed extrinsic and intrinsic pathways, which are regulated by caspase-8 and -9, respectively. The extrinsic pathway involves the binding of extracellular death ligands to death receptors, such as tumor necrosis factor ligand/tumor necrosis factor-related apoptosis-inducing ligand, which induces the recruitment of adaptor proteins, such as the recruiting caspase-8 Fas-associated death domain protein (25). Caspases-8, when activated, initiates a caspase cascade and subsequent cell death by activating downstream executioner caspases, including caspase-3. Cytochrome c is released from the intermembrane gap via the intrinsic pathway in response to DNA damage, resulting in the loss of mitochondrial membrane potential and adenosine triphosphate generation (26).

Caspase-9 is initiated via cleavage by the apoptotic protease activating factor 1 (Apaf-1), and caspase-9 initiates a signaling cascade that results in direct DNA fragmentation. Cytochrome c is released and interacts with Apaf-1, pro-caspase-9, and deoxyadenosine triphosphate to generate caspases that activate the apoptosome, including caspase-3. (27). Caspase-8 is engaged in the external pathway of apoptosis, which is activated when membrane receptors die, whereas caspase-9 is involved in the internal pathway, which is activated when the mitochondrial membrane is altered.

The results of numerous studies have demonstrated that insufficient apoptosis plays a critical role in cancer progression (28). Based on the findings of a recent study, ethanolic corm extract was found to induce apoptosis in a prostate cancer cell line (PC3) following treatment with ethanolic corm extract (29). The skin extract of Baneh exhibits a substantial antiproliferative effect on T47D cells by activating caspase-3, which is more effective than doxorubicin (30).

Apoptosis is a type of planned cell death triggered by a variety of external or internal stimuli. Two separate mechanisms regulate these signals, including the intrinsic pathway, which involves mitochondria, and the extrinsic pathway, which does not need mitochondria (death receptor pathway). Caspases exert a substantial influence on apoptosis (31). The expressions of apoptotic-related proteins, caspase-8, and caspase-9, in TC1 cells were examined in the present study to elucidate the molecular mechanism underlying apoptosis induced by *Pistacia atlantica* gum aqueous extract. It is notable that both caspase-8 and -9, which are involved in the exterior and interior pathways of apoptosis, increased in our study at 200 and 400 extract concentrations, respectively. This demonstrated that *Pistacia atlantica* gum extracts promoted apoptosis in cervical cancer cells via both internal and exterior signaling mechanisms.

Reactive oxygen species have been shown to have a significant effect on the genesis, progression, and maintenance of tumors. Apart from boosting cell development in non-stress situations, ROS has been demonstrated to modulate and trigger apoptosis in stressed cells. Reactive oxygen species levels have increased in cells subjected to a variety of stressors, such as anticancer treatments, and they have promoted apoptosis by activating pro-apoptotic signaling components (32).

Lactate dehydrogenase leakage into the culture

medium was used to determine the cytotoxicity generated by *Pistacia atlantica* gum extract. The release of LDH, a cytoplasmic enzyme, occurred as a result of a cell membrane breach (33). The ratio of LDH activity in the supernatant of treated cells to the LDH activity generated by control cells was used to determine cell membrane rupture. After 48 h of all treatments in the medium, the levels of the cytosolic enzyme LDH dramatically increased, in comparison to the control. Additionally, when *Pistacia atlantica* gum extract (400 mg/ml) was compared to control, the maximum LDH release occurred.

6. Conclusion

According to the findings of the present study, *Pistacia atlantica* gum extract, like doxorubicin, can drastically reduce cell viability and increase apoptosis in the TC1 cell line. Reactive oxygen species-induced apoptosis was observed in TC1 cells treated with *Pistacia atlantica* gum extract in this investigation. It was found that *Pistacia Atlantica* gum extract activated caspase-3, -8, and -9 in a caspase assay, inducing cell death via the potential of mitochondrial membrane and external pathway with caspase activation.

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Footnotes

Authors' contributions: Masoumeh Bolandian, Hadi Esmaeili Gouvarchin Ghaleh, Ruhollah Dorostkar, and Mahdieh Farzanehpour designed and performed the study. Hadi Esmaeili Gouvarchin Ghaleh and Bahman Jalali Kondori contributed to writing the manuscript.
Ethical Approval: The study protocol was reviewed and approved by the Ethics Committee of the Baqiyatallah University of Medical Sciences (IR.BMSU.REC.1397.156) (Grant Num: 97000265).
Conflicts of Interest: The authors report no conflicts of interest.
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