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# Electrospun Poly Vinyl Alcohol-Chitosan Fiber Containing Punica Granatum: Promising Effects on Malignant Melanoma Cell Line

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## Abstract

**Background:** Melanoma is the deadliest type of cancer affecting the skin's deeper layers and spreading rapidly to other tissues. The use of biodegradable scaffolding is widely used nowadays.

**Methods:** In this study the anticancer activity against Malignant Melanoma (MM) of different concentrations of *Punica granatum* (PG) extract loaded on electrospun Poly Vinyl Alcohol- Chitosan fiber was evaluated. The antitumor effects of these compounds were studied against MM cell lines (A375).

**Results:** The experimental data indicate that the cell cycle arrest occurs at different phases for the species analyzed (G2 checkpoint and G0/G1), suggesting a potential involvement of cell-cycle regulatory mechanisms.

**Conclusion:** Since metastasis is very important in cancer cells, the results of this study showed that the use of this plant extract prevents cell migration. The results of this study have promising effects on use of PG versatility in biological systems and into its role as a potential platform for further melanoma-related studies.

**Keywords:** Cell cycle checkpoints, Cell line, Cell movement, Chitosan, Melanoma, Plant extracts, Pomegranate
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## Introduction

Skin cancer is one of the most common types of cancer worldwide and an important public health problem, accounting for almost half of the cancer population among whites [1]. Among the major types of skin cancer, melanoma is the deadliest type affecting the skin's deeper layers and spreading rapidly to other tissues [2,3]. A prominent environmental risk factor for the spread of melanoma appears to be prolonged exposure to sunlight. UV rays produce free radicals that damage DNA [4]. Melanomas are often resistant to treatment and exhibit high rates of metastasis. Major clinical signs of melanoma include asymmetric shape, reddish-brown discoloration, and irregular edging, which are accompanied by itching and bleeding [2,3].

Surgery, chemotherapy and radiotherapy are currently the most common treatments for melanoma. However, due to the high side effects of these treatments, researchers are looking for alternative, complementary, or even combination therapies that, while having high efficacy, also have fewer side effects [2,3,5]. Innovative treatments like tissue engineering and cell therapy are considered highly promising solutions. The design of scaffolds is crucial in this context. Recently, the electrospinning technique has been extensively utilized as a method for fiber production, capable of mimicking conditions found in the extracellular matrix.

Besides the significance of developing a structure that closely resembles the extracellular matrix, the choice of polymeric biomaterials used to fabricate these fibrous scaffolds is also critically important [6]. Natural polymers such as chitin and chitosan are widely used in biomedical applications due to their suitable biological properties such as biocompatibility [7]. Chitosan is a linear polysaccharide composed of acetylated units (N-acetyl-D-glucosamine and N-glucosamine). Thus, chitosan facilitates the delivery of polar drugs beyond the epithelial surface [8].

Chitosan is a hydrophilic polymer; however, due to its high molecular weight and intrinsic viscosity, blending with another hydrophilic polymer is often required to reduce solution viscosity [9]. Also, chitosan has polycationic properties in an acidic environment and in an

electric field, which causes the adjacent chains to break, so that it is not possible to electrify it alone [10]. Polyvinyl Alcohol (PVA) is incorporated as a second polymer component into the chitosan matrix, reducing the electrostatic repulsion between like charges and preventing the rupture of chitosan chains [11]. This combination increases the electrospinning capacity of chitosan [12].

Herbal medicines play an important role as an effective and reliable source of anti-cancer agents [13]. More than half of the currently available anti-cancer agents are extracted from natural sources such as plants [14]. Pomegranate with the scientific name *Punica granatum* L. (PG), belongs to the Punicaceae family and is one of the oldest edible fruits that are widely used in folk medicine of many cultures [8,15]. The pomegranate tree is native to Iran. This plant has been cultivated not only in the Mediterranean region but also in Asia, Africa and Europe since ancient times [16]. Pomegranate is one of the plants that has attracted considerable attention due to its valuable secondary metabolites in various parts such as fruits, seeds, fruit skins, leaves, flowers, tree bark and roots. Each of these components has significant pharmacological activity [17]. Pomegranate flowers are widely used in Greek, Chinese and Indian medicine [18]. Pomegranate flowers are rich in bioactive phytochemicals, including polyphenols, flavonoids, tannins, and triterpenoids, which are considered responsible for many of their biological activities. Different parts of pomegranate flowers have very strong antioxidant activity and a protective effect against oxidative damage to DNA molecules [19,20].

Pomegranate flowers contain different kinds of secondary metabolites. The most common of these are polyphenols, which include gallic acid, ellagic acid, and punicalagin. Secondary metabolites include triterpenes such as oleanolic acid, ursolic acid, and maslinic acid [21]. Each of these compounds has shown significant biological and pharmacological activity. Anti-inflammatory, anti-proliferative, anti-metastatic, and apoptosis-inducing activities of ursolic acid has been reported in cancer models *in vivo* and *in vitro* [22]. Ursolic acid is able to induce apoptosis in cancer cells, prevent tumor formation, and inhibit cancer cell proliferation [23,24]. Therefore, pomegranate can be used in clinical practice due to the presence of ursolic acid in pomegranate flowers. This compound plays a crucial role in the synthesis of a wide range of new bioactive molecules and has considerable potential as an anti-inflammatory and anti-cancer drug [25]. Despite the development of novel treatments, acquired resistance to targeted therapies limits long-term remission. Therefore, PG flower extract was selected for this study because of its rich content of bioactive compounds, particularly polyphenols and ursolic acid, which have demonstrated antioxidant, antiproliferative, pro-apoptotic, and anti-metastatic activities in different cancer models. Incorporation of this extract into electrospun nanofibers may enhance its local delivery and therapeutic potential against melanoma cells. The aim of this study was to investigate the anti-cancer effect of hydroalcoholic extract of pomegranate flowers incorporated in PVA/chitosan electrospun scaffolds on the A375 human melanoma cell line. The novelty of this study lies in evaluating PG flower extract-loaded PVA/chitosan electrospun fibers as a potential platform for inhibiting melanoma cell growth and inducing apoptosis, cell-cycle arrest, and migration suppression in A375 cells.

## Materials and Methods

***Preparation of pomegranate extract***

Whole pomegranate flowers were dried for 15 *min* at 105°*C* and then for two days at 65°*C*. The dried flowers were then ground. To prepare the extract, dried pomegranate powder was mixed with 90% ethanol solvent in a ratio of 1:20 *ml/g*. The solvent was removed using rotary evaporator to obtain an extract (100 *ml*) with 28.4% dry matter. Subsequently, the samples were placed in a freeze dryer to obtain a powder, and then were stored at 4°*C* until analysis [26,27].

***Preparation of the fiber***

The solutions of chitosan (2% wt.) in acetic acid (2%) and PVA (10% wt.) in distilled water were prepared. Pomegranate extract in amounts of 3 and 6% wt. were dissolved in PVA solution. The prepared solutions were electrospun through two separate 5 *ml* syringes with a 22 gauge needle and the feed rate of the solutions was set at 0.1 *ml*/*hr* while the distance from the syringe tip to the collector was 10 *cm*, and an applied voltage of 20 *kV* was used during the electrospinning process [28]. This experiment was performed at room temperature.

***Characterization of electrospun fibers***

***Mechanical properties characterization:*** The tensile mechanical characterization of fibers in PVA/Chitosan, PVA/Chitosan/3% PG and PVA/Chitosan/6% PG groups were determined using SANTAM mechanical testing machine (Iran). The PG content (3 and 6% wt.) was calculated relative to the total mass of the polymers (PVA+Chitosan) used in the electrospinning solution. For this purpose, the specimens were prepared in a rectangular shape. Five specimens were randomly cut from different regions of each electrospun mat and tested (n=5). The uniaxial tensile test was performed at a constant tensile speed of 1 *mm*/*min* until rupturing the sample. Tensile test continued from the start to the rupture of the specimens and the Young's moduli, elongation at break, and ultimate tensile strength were determined.

***Scanning electron microscopy (SEM):*** The produced PVA/chitosan electrospun fibers with and without PG were examined in terms of morphology and fiber diameter by scanning electron microscopy (SEM, TESCAN-Vega 3, Czech Republic). Five SEM micrographs were acquired from different regions of each

sample to ensure representative morphological evaluation. ImageJ software (Wayne Rasband, National Institutes of Health, USA) was used to measure the diameter of the fibers. At least 15 randomly selected fibers were measured from each micrograph, and the results were expressed as mean ± standard deviation.

The porosity of the electrospun PVA/chitosan scaffolds were quantified from SEM micrographs using MATLAB software (MathWorks Inc., USA). For each sample, at least five randomly selected fields from different regions of the scaffold were analyzed. SEM images were converted to grayscale and then transformed into binary images using threshold-based image segmentation to distinguish fiber and pore regions. Porosity (%) was calculated as the ratio of the pore (void) area to the total image area. The reported values represent the average porosity obtained from the analyzed images.

***In vitro release study***

Bag diffusion technique was conducted to represent the release profile of PG from the PVA/chitosan fibers. In this regard, 0.025 *g* of PG-loaded fibers were placed into dialysis membrane bags with a molecular weight cutoff of 14 *kDa*. Each bag was submerged in 10 *mL* of the Phosphate Buffer Solution (PBS, pH=7.4). The release study was carried out at 37°*C* under continuous shaking at 120 *rpm*. At predetermined time intervals (0.5, 1, 3, 6, 9, 12, 24 *hr*, 2 days, 3 days), one *ml* of release medium was withdrawn and the fresh solution was replaced and the UV-absorbance of samples was obtained by an ultraviolet-visible spectrometer. The drug release value was calculated following preparation of calibration curve of PG in PBS.

***Cell seeding and culture***

A375 human melanoma cancer cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich; Germany) with 10% fetal bovine serum, penicillin 100 $\mu g/ml$ and streptomycin 100 $\mu g/ml$ at 37°*C*, 5% $CO_2$ and 95% humidity for three days. Electrospun scaffolds were prepared as a disk shape with a diameter of 10 *mm* and sterilized under UV light for 3 *hr* and then with 70% ethanol for 30 *min*. After three times washing out with sterile PBS, the scaffolds were immersed in a cell culture medium overnight and then transferred to a 96-well tissue culture plate. A375 cells were detached from the bottom of cell culture flasks after adding 1 *ml* of 0.25% trypsin containing 0.1% EDTA. After centrifugation, the cells were cultured on each electrospun scaffold at a density of $10^4$ cells per square centimeter and then incubated at 37°*C*, 5% $CO_2$ and 95% humidity. A375 cells cultured on standard tissue culture plates without scaffolds were used as the control group. Cells cultured on PVA/chitosan scaffolds without PG served as the scaffold control group.

***Cell viability assay***

The viability of A375 cells seeded on the scaffolds was assessed by Dimethylthiazol-2-yl -2, 5-difenyltetrazolium bromide (MTT, Sigma, USA) assay. The MTT assay was performed in three independent experiments (n=3). For this purpose, after cell culture for 24, 48, and 72 *hr*, the culture media in each well was substituted with 100 $\mu l$ of MTT solution (0.5 *mg/ml*) and then the wells were placed at 37°*C* for 4 *hr*. Afterwards, the solution in the wells was discarded and 100 $\mu l$ of DMSO (Sigma, USA) was added to each of them for two *hr* at room temperature. DMSO dissolves formazan crystals and produces varying intensities of purple color depending on the rate of living cells. ELISA reader (Hyperion MPR4, Germany) was used to read the light absorption of each well at 570 *nm*.

***Apoptosis assay***

The percentage of apoptotic and necrotic cells was determined by flow cytometry method using a flow cytometer (BD FACSCALIBUR, USA) (n=3). In this regard, annexin and Propidium Iodide (PI) are two combinations used to distinguish apoptotic cells from necrotic cells. Phosphatidylserine in the outer layer of apoptotic cells membrane is detectable by combining non-transplanted annexin with Fluorescein Isothiocyanate (FITC). On the other hand, fluorescence combination of PI only enters necrotic cells and can make a distinction between apoptotic and necrotic cells. In this method, A375 cells on the PVA/chitosan electrospun fibers with/without PG and the cells in the control group were trypsinized after 24 *hr* of culture and separated from the bottom of the plate, and then centrifuged at 1200 rpm for 5 *min*. Isolated cells were mixed by binding buffer (100 $\mu l$) in a microtube, and then 10 $\mu l$ of PI dye and 5 $\mu l$ of annexin were added to the microtube. Obtained samples were incubated in the dark at 25°*C* for 10 *min*. Finally, cell analysis was performed by flow cytometry [29].

In the diagram reported by the flow cytometer, the X-axis (FL-1) represents the fluorescence logarithm of the V-FITC and the Y-axis (FL-3) represents the fluorescence PI [30].

***Cell cycle assay***

Cell cycle analysis was performed using a cell cycle kit, which contains a solution of propidium iodide, a red fluorescent dye that binds to DNA after cell permeability, thus determining the amount of cellular DNA during cell cycle progression. Cell cycle assays (n=3) were performed on an A375 cell line treated with 3 and 6% PG. Depending on the test time, A375 cells were seeded on plates of 6 wells with optimal density ($10^4$×2 cells/well). The cells were then washed with PBS and fixed with 1 *ml* of 70% cold ethanol. After incubation at 20°*C* overnight, the samples were centrifuged, resuspended in 200 $\mu l$ of cell cycle solution and kept in the dark at room temperature for 30 *min* [31,32].

Table 1. Tensile Properties of PVA/Chitosan and PVA/Chitosan/PG electrospun fibers

| Samples | Young's modulus (MPa) | UTS (*MPa*) | Elongation at break (%) |
|---|---|---|---|
| PVA/Chitosan | 0.46±0.11 | 0.78±0.17 | 18.03±0.18 |
| PVA/Chitosan/3%PG | 0.57±0.14* | 1.16±0.12 | 24.1±0.5* |
| PVA/Chitosan/6% PG | 0.59±0.11* | 1.08±0.16 | 25.3±0.18* |

* Significantly different from corresponding parameters of fibers ($p<0.05$). UTS: Ultimate Tensile Strength.

Table 2. The mean diameter and porosity of electrospun fibers

| Sample | Mean diameter (*μm*) | Porosity (%) |
|---|---|---|
| PVA/Chitosan | 0.38±0.1 | 76.2±3.2 |
| PVA/Chitosan/3%PG | 0.41±0.08 | 77.1±4.1 |
| PVA/Chitosan/6%PG | 0.43±0.07 | 80±4 |

PVA: Poly (vinyl alcohol), PG: *Punica Granatum.*

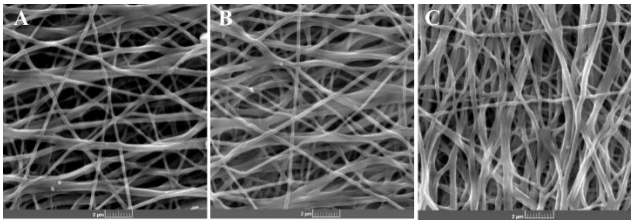


Figure 1. Scanning electron microscopy (SEM) images of electrospun fibers from different experimental groups: (A) PVA/chitosan, (B) PVA/chitosan containing 3 wt% pomegranate extract (PG), and (C) PVA/chitosan containing 6 wt% PG. Scale bar = 2 *μm*.

***Migration assay***

Cell migration was evaluated after culturing A375 cells on PVA/chitosan electrospun fibers, with or without PG, in 96-well plates until they reached approximately 80% confluency, using the Scratch assay. A vertical scratch was made in the center of the well using a sterile pipette tip, and the plate was washed with PBS to remove any detached cells. Images were captured at the time of the scratch and again 48 *hr* later using an inverted light microscope (Nikon Eclipse TE2000-S, Germany) and the groove filling was examined [33].

***Statistical analysis***

Presented results were shone as mean±standard deviation (SD). Statistical analyses were performed using SPSS software (version 22.0). Differences among groups were evaluated using one-way analysis of variance (ANOVA) followed by the Least Significant Difference (LSD) post hoc test. A p-value ≤0.05 was considered statistically significant.

## Results and Discussion

***Mechanical measurement***

An ideal three-dimensional scaffold can provide an alternate artificial extracellular matrix for cell adhesion and growth in order to evaluate the cells' behavior and activities [34]. In this study, PVA/chitosan nanofibers were prepared as alternate artificial extracellular matrix. There are some studies that prove that PVA in blending with chitosan can potentially create scaffolds with appropriate mechanical properties for tissue engineering [35,36]. However, incorporating agents in scaffolds can change the mechanical properties of the electrospun nanofibers that must be evaluated. Therefore, the mechanical properties of fibers, including Young's modulus, Ultimate Tensile Strength (UTS), and elongation percentage at break were investigated before and after loading pomegranate extract and the results are presented in table 1.

According to the results, the ultimate tensile strength of PVA/Chitosan/3% PG and PVA/Chitosan/6% PG groups was higher than PVA/Chitosan group fibers. The Young's modulus of PVA/Chitosan/ PG 3 and 6% fibers was found to be 0.57±0.14 and 0.59± 0.11 *MPa*, respectively. These values were significantly higher compared to PVA/Chitosan fibers (0.46± 0.11, $p<0.05$). Moreover, the elongations at break point (also known as fracture strain) were found to be 25.1 and 24.3%, respectively which were significantly compared with those of the PVA/chitosan group. Therefore, the tensile strength was enhanced upon the inclusion of PG in the PVA/Chitosan fiber. The improved mechanical properties may enhance the structural stability of the scaffold during cell culture and drug release. Such stability is important for maintaining scaffold integrity and supporting sustained biological performance [35,36].

***Morphology of fibrous matrices***

Figure 1 shows the SEM images and the diameter distribution of fibers. It could be observed that incorporating the PG content into fibers led to an increase the fiber diameter.

The increase in the diameter of the PG loaded fibers can be attributed to the conductivity changes of the hybrid PVA/PG solution. Moreover, it was observed that increasing the content of PG incorporated into the PVA solution had increased the viscosity of the solution and enhanced its spinnability as well. Measurement of porosity of fibers using MATLAB software showed that the percentage of porosity in fibers containing PG was increased. The results are shown in table 2. It has been shown that highly porous scaffolds having interconnected pores and large surface in the presence of adhesive and growth factors can potentially provide an appropriate environment for cells growth. In addition, the increased fiber diameter and porosity may influence the diffusion of the encapsulated extract and contribute to the sustained release behavior observed in the release study [37].

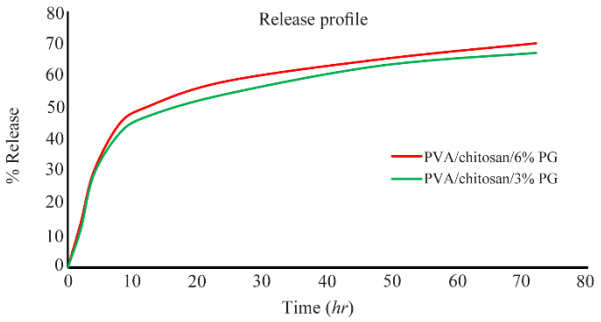


Figure 2. *In vitro* release profile of PG from PVA/chitosan electrospun nanofibers over a period of 72 *hr*. The release study demonstrated a sustained release behavior, with approximately 70% of the loaded PG being released from the nanofibers within 72 *hr*.

***Release assessment***

The release of PG from electrospun PVA/Chitosan fibers was evaluated at 37*°C* and illustrated in figure 2. A sustained release of PG was observed over a period of 72 *hr*, with an initial burst release of more than 50% occurring during the first 10 *hr*. The release profile exhibited an initial burst release followed by a more sustained release phase. The rapid release during the early hours may provide an effective initial concentration of PG to exert anticancer activity against melanoma cells, while the subsequent sustained release could help maintain therapeutic levels over an extended period. Such a biphasic release pattern may be advantageous for reducing the need for repeated administration and supporting prolonged biological activity.

***Cell viability and proliferation***

Evaluation of the cytotoxicity of the prepared fibers is an important aspect to evaluate the effectiveness of these fibers. The viability and proliferation of cells on PVA/Chitosan, PVA/Chitosan/3% PG and PVA/Chitosan/6% PG groups were assessed at 24, 48 and 72 *hr* after cell seeding using MTT assay. Monolayer culture was used as a control group. The A375 cells were active on the surface of the PVA/Chitosan fibrous mats making them suitable to be applied as effective/potential biocompatible scaffolds for cell proliferation. It was reported that mats with highly fibrous topographies and porosity are similar to ECM and are able to enhance the cellular responses [38]. However, cells viability on the surface of fibers containing PG was lower compared to the PVA/Chitosan as well as control group with increasing incubation time. Moreover, with the increase in the weight percentage of PG in the structure of fibers, the rate of cell proliferation decreased. As figure 3 illustrates, PVA/Chitosan/6% PG nanofibers significantly lead to reduction in melanoma cells viability after 24 *hr* ($p<0.05$). These reductions were also significant in PVA/Chitosan/3% PG ($p<0.05$) and PVA/Chitosan/6% PG groups ($p<0.01$) after 48 and 72 *hr* in comparison with control and PVA/Chitosan groups. In addition, the A375 melanoma cells viability rate on PVA/Chitosan/6% PG scaffold was

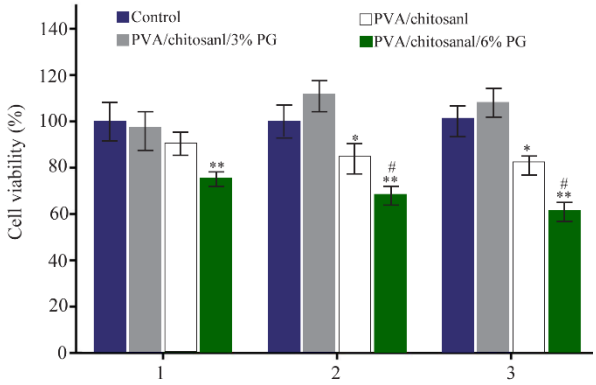


Figure 3. MTT assay results showing the viability of A375 melanoma cells cultured on PVA/chitosan electrospun fibers containing different concentrations of PG after 24, 48, and 72 *hr* of incubation. Data are presented as mean ± SD (n=3). *p<0.05 and **p<0.01 *vs.* control and PVA/Chitosan groups. #p<0.05 *vs.* PVA/Chitosan/ 3% PG group.

significantly lower than PVA/Chitosan/3% PG after 48 and 72 *hr* ($p<0.05$). The results showed that pomegranate flower extract has significant cytotoxic effects on the A375 melanoma cell line. Pomegranate flower extract showed a concentration-dependent and time-dependent inhibitory effect on melanoma cell proliferation rate. This result can be associated with the presence of some anti-proliferative compound such as triterpenes and polyphenols in Pomegranate flower extract [39]. Anti-proliferative effect of ursolic acid on M4Beu human melanoma cells was demonstrated in previous study [40,41]. Furthermore, purified polyphenols, punicalagin and ellagic acid have been previously shown to exhibit anti-proliferative activity on oral, colon and prostate cancer cell lines [42].

***Apoptosis assay***

To determine if the cytotoxic activity observed in A375 melanoma cells was due to the ability of electrospun PVA-chitosan fibers containing different concentrations of PG extract to induce apoptosis, the Annexin V assay, as outlined in Section 2-6 was utilized. The results are summarized in figure 4. The percentage of apoptotic cells on PVA/Chitosan scaffold containing 3 and 6% PG was significantly increased. These observations allow us to consider the PG extract as a good candidate for the development of new therapeutic agents against melanoma. In a study it was demonstrated the antiproliferative effect of ursolic acid on M4Beu human melanoma cells [43]. They showed that ursolic acid induced apoptosis in cancer cells by overexpression of Bax with decreased Bcl-2 expression and subsequent collapse of the mitochondrial membrane [44]. This apoptotic pathway has also been identified by the Caspase cascade and the activation of Caspase 3. Liu *et al* investigated the preventive properties of several phytochemicals, including grape seed extract, and ursolic acid, on the mechanism of inhibition of skin cancer in mice [45]. They found that ursolic acid significantly reduced the thickness of the epidermis. Whether similar molecular events occur in A375 melanoma

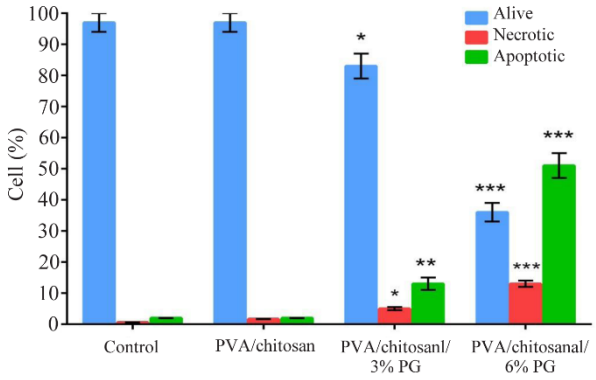


Figure 4. Apoptosis induction on A375 cell line 24, 48 and 72 *hr* before analyzing samples with the Apoptosis Kit - Annexin V and Propidium Iodide (PI) as described in section2. Results represent the mean (± SD) of three independent experiments and are expressed as the percentage of cells fluorescently labeled (green+green/red = apoptotic; red = necrotic) or unlabeled (alive) on the total number of cells analyzed.
*p<0.05, **p<0.01 and ***p<0.001 *vs.* control and PVA/Chitosan groups.

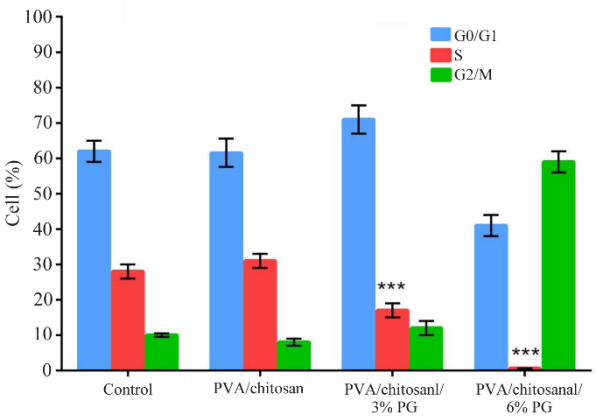


Figure 5. Cell cycle arrest in A375 cells. Cells were treated with 3% and 6% extract for 24 *hr*. Flow cytometric analysis was used to determine the percentages of cells in the G0/G1, S, and G2/M phases of the cell cycle. Histograms represent the mean±SD of three independent experiments (n = 3).
***p<0.001 *vs.* control and PVA/Chitosan groups.

cells was not investigated in the present study. Further investigations will be necessary to highlight the specificity of the apoptotic mechanisms induced.

***Cell cycle analysis***

To better delineate the mechanisms of anti-cancer effect of PG extracts loaded in electrospun fiber on A375 cells we analyzed the cell cycle phase distribution after treatments, as described in the Section 2-7. PI cell staining after treatments allowed the quantification of cellular DNA, indicating the cell cycle phase. Cells treated in 48 and 72 *hr* did not allow the graphical representation of the cell cycle phases because most of them were dead, showing DNA content lower than diploid. As illustrated in figure 5, cells entered the cell cycle but they did not go through the G2/M phase, before dying through apoptosis. These results suggest that administration of 3% PG extracts disrupts the progression of the normal cell cycle. The observed cell-cycle arrest was associated with increased apoptosis and reduced cell viability in treated cells. Some previous studies demonstrated that compounds like ellagic acid and ursolic acid present in pomegranate plant, have anticarcinogenic properties such as induction of cell-cycle arrest, which can inhibit tumor formation in animals [46]. The observed apoptosis induction and cell-cycle arrest suggest that PG-loaded nanofibers may influence intracellular pathways involved in melanoma progression; however, the underlying molecular mechanisms were not investigated in the present study and require further research.

***Migration assays***

Migration and invasion are two important events in cancer metastasis. In this study, cell migration was measured by scratch assay and the microscopic image on day 2 in the different groups studied is shown in figure 6. The yellow dashed line shows the border of

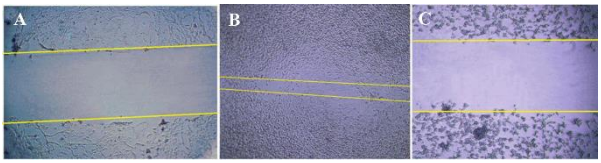


Figure 6. Analysis of migration by in vitro scratch assay. A) PVA/Chitosan 3%, B) PVA/Chitosan, C) PVA/Chitosan 6%. The yellow line defines the areas lacking cells. PG-loaded scaffolds (3 and 6%) significantly reduced A375 cell migration compared with the PVA/Chitosan scaffold alone (p<0.001, n=3).

the cell-free area. As illustrated in figure 6, when PVA/Chitosan scaffold was used with PG (3 and 6%) cell migration decreased compared to the PVA/Chitosan scaffold alone and reduced the invasion power of the cells. Previous studies demonstrated that pomegranate extract can decrease the invasion power of cancer cells. It has been previously shown that, pomegranate extract has the potential to inhibit the migration of cancer cells. For instance, Chaves and colleagues indicated that migration capacity of prostate cancer cells decreased in presence of pomegranate peel extract [47]. Moreover, pomegranate extract has been shown to suppress the migration in breast cancer cells [48,49]. Recently, Peng and colleagues reported that pomegranate extract through changing gene expression are able to inhibit the migration and invasion of oral cancer cells [50].

## Conclusion

In the field of regenerative medicine, both *in vitro* and *in vivo* tests have highlighted the efficacy of electrospun scaffolds incorporating plant extracts to stimulate cell proliferation, modulate inflammation response, and prevent bacteria colonization. In this study, the antitumor activity of pomegranate flower (PG) extract-loaded electrospun Poly Vinyl Alcohol- Chitosan fibers were characterized and their ability to inhibit the

growth of A375 melanoma cells *in vitro* through the induction of apoptosis, cell cycle arrest and inhibition of migration was demonstrated. The developed fiber composite exhibited favorable structural stability and significant anticancer activity against melanoma cells. Further, the high porosity of the electrospun suggests their potential application as a drug delivery system for localized cancer therapy. The present findings are consistent with previous reports and further support the potential of PG extract-loaded electrospun fibers as a promising strategy for melanoma treatment. Although the present findings demonstrate significant biological activity, the molecular pathways responsible for these effects remain to be investigated.

It is anticipated that future research will integrate these findings with emerging advances in polymer fabrication technologies to develop multifunctional scaffold architectures capable of harnessing the therapeutic potential of diverse medicinal plant extracts. Overall, the results of this study suggest that pomegranate flower extract, which contains bioactive compounds such as ursolic acid, represents a promising candidate for further preclinical investigation as a potential therapeutic approach for melanoma.

***Limitations***

One limitation of the present study is that the biological evaluations were conducted using only a single melanoma cell line (A375). Although the results demonstrated promising anticancer effects of the pomegranate extract-loaded nanofibers, the response may vary among different melanoma cell lines and other cancer models. Therefore, further studies using additional melanoma cell lines, normal skin cells, and *in vivo* models are required to confirm the generalizability and therapeutic potential of the findings. Another limitation of this study is that the molecular mechanisms underlying the observed anticancer effects were not investigated and require further evaluation. In addition, the loading/encapsulation efficiency of the pomegranate extract within the electrospun nanofibers was not quantitatively determined. Furthermore, the reproducibility of the electrospinning process was not systematically assessed through batch-to-batch comparisons. Future studies should address these aspects to further validate and optimize the developed nanofibrous system.

***Ethics statement***

This study was performed entirely *in vitro* using a commercially available human melanoma A375 cell line. No human participants, patient-derived samples, animal subjects, or identifiable personal data were involved.

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## Conflict of Interest

The authors declare that they have no conflict of interest.

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