Anticancer potential of *Pistacia vera* L. pollen shell *in-vitro* using human osteosarcoma cell line MG63

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ABSTRACT

Pollen shells (PShs) are natural polymeric microcapsules that can be used as medical devices such as drug carriers. In the present study, for the first time, the anticancer potential of *Pistacia vera* L. PSh was investigated *in-vitro* using cell line MG63. Optical microscopy was used to study the PSh-cell interface. MTT assay was performed to assess cell cytotoxicity on the PSh microcapsules. The apoptotic activity of PShs was examined using the qRT-PCR technique. DAPI staining was benefited to visualize nuclear morphological changes in cells cultured on the PShs. Optical imaging indicated a bioactive PSh-cell interface and MTT assay results showed an anti-proliferative effect of PShs on the MG63 cells. Moreover, from the expression analysis of apoptosis-related genes (*BAX* and *BCL2*) and DAPI staining, PShs was found to be an apoptosis-inducing delivery vehicle against MG63 cells. Hence, such a microcapsule is proposed to be used as a drug carrier with anti-bone tumor activity.

Keywords: Pollen, *Pistacia vera* L., Anticancer, Bone-targeted drug delivery

INTRODUCTION

Chemotherapy (chemo) is a drug treatment using anticancer agents to kill cancer cells or stop them from growing and spreading [1, 2]. Drug therapy is an important part of the treatment plan for many bone cancers [3]. The side effects of anticancer agents can be significant. An effective cancer therapy requires adequate intracellular concentration of drugs to destroy the cancer cells. In traditional administration methods, anticancer agents do not reach therapeutic dosages in the bone cancer cells and over-ingestion of a drug leads to toxicity in healthy cells. To improve drug efficiency, bone-targeted delivery systems have been developed [4-6]. There are various types of materials used as drug delivery vehicles such as polymers, liposomes, lipids and inorganic carriers [7, 8]. Among them, natural polymers have attracted a great deal of attention for their inherent biocompatibility, biodegradability, nontoxicity and ready availability [9, 10].

Pollen grain (PG) is the male reproductive organ of higher flowering plants, consists of various nutrients and therapeutic phytochemicals [11, 12]. Pollens exhibit a wide range of properties including low density and high surface area, renewability, biocompatibility, biodegradability, radical scavenging, antimicrobial, antimutagenic, antitumor, antiradiation, antiinfection, growth-promoting, longevity and also boost the immune system [13-18]. The grains of pollen have core-shell microstructure, the core cytoplasm contains nuclei for fertilization and the shell is made up of two main layers: an interior layer of the intine and an exterior layer of the exine. The intine is composed of cellulose, hemicellulose and

pectin and the highly robust exine is composed mainly of a biopolymer called sporopollenin. The chemical structure of sporopollenin has been considered as a highly cross-linked copolymer of long-chain fatty acids and phenolic compounds [19-21]. Phenolic acids and flavonoids, the major types of phenolic compounds, are responsible for a wide range of pollen biological properties [22-24]. The core part can easily undergo decomposition, while the shell can be preserved to use as medical devices such as drug carriers [25]. Recent studies have indicated potent anticancer activities of the pollen shells [22].

The current study was performed to evaluate the anticancer activity of *Pistacia vera* L. (*P. vera*) pollen shell (PSh) against MG63 human osteosarcoma cells to design drug carrier microcapsules with anti-bone tumor activity. In this regard, the pollens cytoplasmic contents were first expelled in order to obtain nonallergic PShs. Then, cell function (attachment, proliferation and apoptosis) on the shells was investigated *in-vitro* using the technique of optical microscopy imaging, MTT assay, expression analysis of apoptosis-related genes and DAPI staining.

METHODOLOGY

Materials

The PGs of *P. vera* were collected in spring in Qazi Jahan, Iran. The MG63 cell line was obtained from the National Cell Bank of Iran, Pasteur Institute (Tehran, Iran). DMEM Low Glucose medium containing L-Glutamine was purchased from biowest (Nuaillé, France). Other cell culture components were supplied from Gibco, Life Technologies (Paisley, UK). Cell culture dishes (flasks, pipettes and well plates) were bought from SPL Life Sciences (Pocheon, South Korea). Trypan blue solution (0.4%), MTT salt and 4',6-Diamidino-2-phenylindole (DAPI) were provided from Sigma-Aldrich (Poole, UK). Trizol solution and reverse transcriptase reagent kit were obtained from Thermo Fisher Scientific (Waltham, MA, USA). Oligonucleotide primers were procured from Takapo Zist (Tehran, Iran). Maxima SYBER Green qPCR master mix was received from BioFACT (Daejeon, Korea). All other chemicals were purchased from Merck (Kenilworth, NJ, USA).

Preparation of PSh Microcapsules

PShs were extracted using alkaline lysis method. PGs were refluxed with stirring in acetone for 4 h while refreshing the solvent after 2 h. Grains were filtered, washed with acetone and left in open air overnight. The defatted PGs (3 g) were suspended in 15 ml aqueous solution of potassium hydroxide (5% w/v) and gently stirred at room temperature for 24 h. The suspension was repeatedly washed with hot water and then ethanol, filtered by vacuum filtration and dried.

Characterization of PSh Microcapsules

Field Emission Scanning Electron Microscopy (FE-SEM) Analysis

The surface characteristics of the PGs and PSh microcapsules were analyzed by field emission scanning electron microscopy (FE-SEM; MIRA3, Tescan Company, Brno, Czech). Before imaging, samples were coated with gold using a sputter coater (Emitech K550, Kent, UK). Imaging was performed at an accelerating voltage of 20 kV.

Bio-Content Staining

The PGs and PShs were fixed in FAA solution consisting of 50% formalin-acetic acid-ethyl alcohol (90:5:5, v/v/v) for 24 h, passed through a graded series of dehydrating ethanol (50-100%), immersed in clearing agent of xylene and embedded in paraffin. After that, paraffin blocks were cut into slices of 10 µm using a rotary microtome (R. JUNG, HEIDELBERG, Germany), followed by deparaffinization with xylene, rehydration with a graded ethanol series, staining with Basic Fuchsin [26] and Light-Green as the DNA- and protein-binding dyes, respectively [27]. Finally, the slices were sealed using the mounting medium Entellan, analyzed by a biological fluorescent microscope (N800-F, Jiangsu, China) and photographed with a microscope camera (TrueChrome Metrics, Tucsen, China).

***In-Vitro* study**

In this study, the effect of *P. vera* PSh on the behavior of human osteosarcoma cell line MG63 was examined *in-vitro.* Two groups of PShs-free and PShs were assigned to the control and experimental group, respectively. PShs (2 mg) were put into 24-well plate and immersed in ethanol solution (70% v/v) for 1 h to sterilize. After sterilization, shells were dried and rinsed twice in phosphate-buffered saline solution (PBS) for 30 min. Then, 5000 cells were seeded per well containing complete medium of DMEM low glucose (+ l-glutamine, sodium pyruvate, 10% FBS and 1% antibiotics) and incubated in a humidified atmosphere of air/CO_2 (19:1) at 37°C. The medium was replaced with a fresh one every 48 h.

Cell Adhesion to PShs

To evaluate cell adhesion behavior of MG63 cells on the PShs, Olympus CKX41 inverted light microscope equipped with a DP20 camera (Olympus Corp., Tokyo, Japan) was used. After culturing for 1 day, cell attachment were depicted at the cell-PShs interface.

Cell Cytotoxicity Analysis

The cytotoxic effect of PShs on MG63 cells was quantified via mitochondrial dehydrogenase activity using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. On days 1, 3 and 7 following cell seeding, MTT reagent (5 mg/ml in DMEM low glucose, 50 µl) was added into each well to a final volume of 500 µl. After 3 h incubation, the supernatant was removed and replaced with 200 µl isopropanol to solubilize purple formazan crystals formed by metabolically active cells. Finally, the

resulted solution was transferred to a 96-well plate to read the color intensity at 570 nm by microplate spectrophotometer (Elx808, BioTek, USA). The background absorbance of PShs was omitted.

Gene Expression Analysis of Apoptosis

RNA Isolation and cDNA Synthesis

On days 7 and 21 after seeding, total RNA was isolated from the cells using Trizol reagent followed by purification steps of chloroform-initiated phase separation, ethanol precipitation and DEPC water dissolution. The quality and quantity of the extracted RNAs were confirmed by measuring the ratio of the absorbance at 260 and 280 nm (A260/280) using NanoDrop 1000 Spectrophotometer (NanoDrop, Wilmington, USA). First-strand complementary DNA (cDNA) was synthesized using the cDNA Synthesis Kit. Briefly, a reaction mixture consisting of 1 µl random hexamer (2.5 ng/µl), DEPC water and extracted RNA in a total volume of 12 µl was incubated at 65ºC for 5 min, then added to a mixture of 4 µl 5X RT Buffer, 1 µl dNTP mix, 1 µl RNase inhibitor (20U/µl), 1 µl M-MuLV reverse transcriptase (100U/µl) and 1 µl of DEPC water. Reverse transcription was accomplished with SimpliAmp Thermal Cycler (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) under the following condition: 25°C for 10 min, 42°C for 60 min and 75°C for 10 min.

Quantitative Polymerase Chain Reaction (qPCR) Assay

The transcriptome expression of *BCL2*-associated X protein (*BAX*), B-cell lymphoma 2 (*BCL2*) and Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*, endogenous control) was evaluated using Bio-Rad iQ5 real-time PCR amplification system (Bio-Rad Laboratories Inc., Hercules, USA). The qPCR was fulfilled in the final volume of 20 µl, containing 10 µl 2X Power SYBR Green PCR Master Mix, 1 µl cDNA, 8 µl DEPC water and 0.5 µl of each forward and reverse primer (200 nM). The cycling conditions were initial denaturation at 94°C for 5 min, 35 cycles of 94°C for 30 s, annealing at temperature mentioned in Table 1 for 30 s, followed by extension at 72°C for 30 s and amplification at 72°C for 10 min.

Table 1 Primer sequences used in qPCR experiments

Gene	Forward sequence (5′-3′)	Reverse sequence (5′-3′)	Annealing Temperature (°C)
BAX	GATGCGTCCACCAAGAAG	AGTTGAAGTTGCCGTCAG	52
BCL2	GTTCCCTTTCCTTCCATCC	TAGCCAGTCCAGAGGTGAG	57
GAPDH	CCTGCTTCACCACCTTCTTG	CCATCACCATCTTCCAGGAG	58.5

Nucleus Morphological Analysis

The DAPI staining was performed for the characterization of nuclear morphological alterations in apoptotic cells. Briefly, on day 21 of culture, incubated cells were fixed in ice-cold 4% formaldehyde and permeabilized by 0.1 % Triton X-100 in PBS for 10 min each. Then, the nuclei of cells were stained with 1 μg/ml of DAPI in PBS for 5 min in the dark. Finally, the cells were washed with PBS to remove excess dye and images were taken with a fluorescence microscope system (Cytation 5, BioTek, USA).

Statistical Analysis

The results were presented as mean ± standard deviation (SD) run in triplicate. Statistical analyses were evaluated using the Mann-Whitney U test. The difference $p<0.05$ was designated to indicate statistically significant.

RESULTS AND DISCUSSION

Over the past decades, natural polymers have been widely explored as potential materials for drug delivery applications due to their versatile characteristics such as biocompatibility, biodegradability, bioactivity and encouraging higher cellular attachment [28, 29]. Sporopollenin is a biological polymer forming the outer cell wall of pollens (exine) [30]. Concerning the anticancer property of pollens, the current *in-vitro* study was performed to evaluate the performance of the *P. vera* PSh as an anti-bone tumor drug carrier using MG63 osteosarcoma cells.

PShs Extraction and Characterization

To use *P. vera* PSh as a drug carrier, PGs were washed in acetone and alkaline solution to clean external cement and internal cytoplasm for obtaining allergen-free PShs. Apertures placed at the wall allow the pollen insides to leak out easily [31]. Basic Fuchsin and Light-Green staining are commonly used methods for displaying DNA and protein content of plant cells, respectively. Evaluating the staining results of raw pollens and PSh microcapsules demonstrated a clean inner cavity, reflecting the non-allergenic characteristic of prepared shells. Optical microscopy images of raw PGs stained with Basic Fuchsin demonstrated magenta color at DNA-binding sites, deeply in the condensed appeared nucleus (Figure 1a), whereas PSh microcapsules produced no color (Figure 1b). The comparison between Light-Green stained raw and processed PGs showed complete removal of proteins from the PGs producing nonallergic PShs (Figures 1c,d).

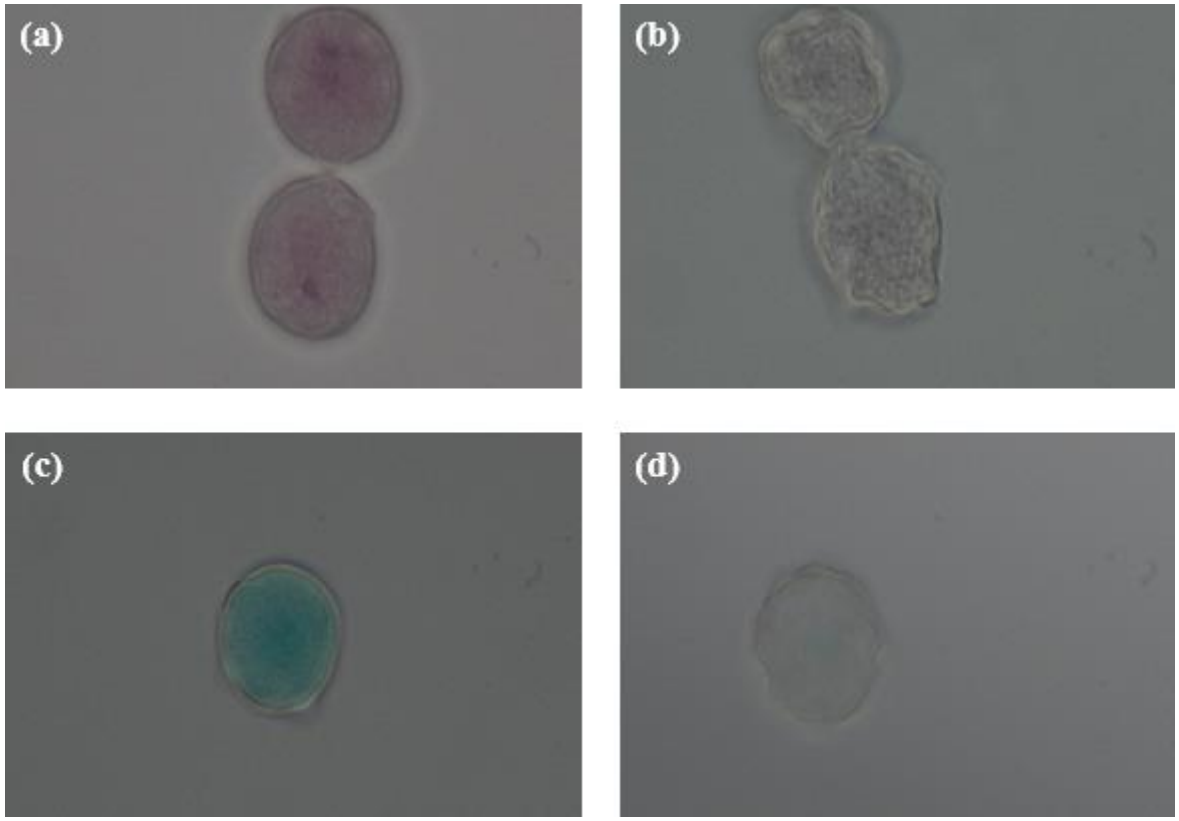

Figure 1. Genetic materials staining by Basic Fuchsin in *Pistacia vera* L. (a) PG and (b) PSh and proteins staining by Light-Green in *Pistacia vera* L. (c) PG and (d) PSh. Magnification= 100X. PG: pollen grain. PSh: pollen shell

Structural variation for native and treated PGs was investigated by FE-SEM morphological analysis. As revealed, *P. vera* microparticle pollens possess uniform spherical shape and sizes with several germinative apertures in the outer wall (Figure 2a). At a larger magnification, the exine surface presented reticulate and homobrochate ornamentation patterns (Figure 2b). Removing cytoplasmic contents using alkaline treatment resulted in PShs with well-preserved nanostructure (Figure 2c). The obtained results revealed successful removal of pollen interiors without detrimentally affecting the wall structure and surface ornamentation pattern.

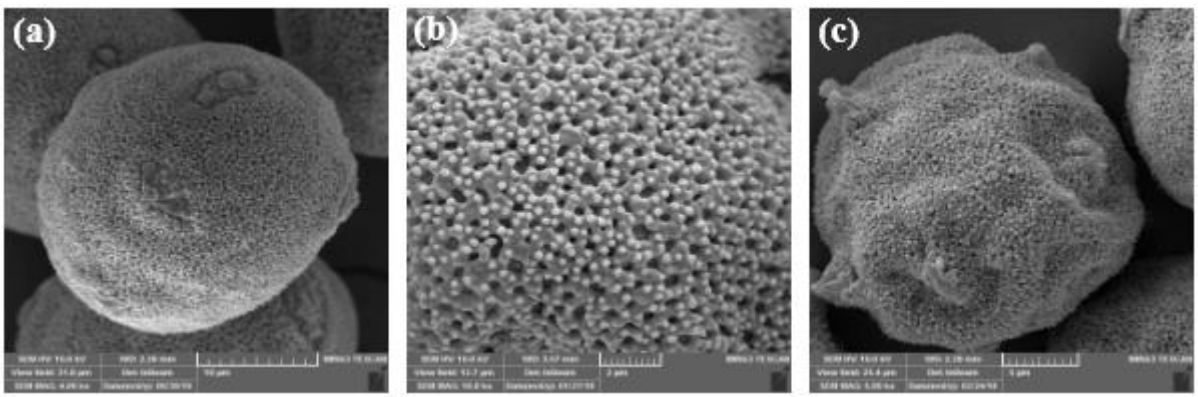

Figure 2. FE-SEM images of (a) PG (b) PG at a larger magnification (c) PSh. PG: pollen grain. PSh: pollen shell

Cell Adhesion and Metastasis Countering

Surface properties of the underlying substrate strongly influence cell-substrate interactions [32]. Monitoring the interface of the PSh-MG63 cells using an optical microscope demonstrated good cell adhesion and maintaining osteosarcoma cells localized to their initial positions (Figure 3). According to the obtained image, the prepared shells could provide a desirable adsorption surface for the attachment of cells. Surface physical nano-topography and chemical functionality have a great effect on protein adsorption and subsequent cell adhesion [32, 33]. The surface characteristics of *P. vera* PG offer high cell-anchoring points through the dense brochi nanostructures distributed over the exine as well as oxygenated functional groups (i.e. OH and COOH), which promote desirable PSh-cell interactions. As well, the physical porosity of the shell provides more surface area and capillary forces to promote cell attachment [34, 35]. The tendency of cancer cells to adhere to PShs can be vital in bone tumor treatments. Once adhered to designed shells, detached motile cancer cells would be prevented from spreading during tissue regeneration, therefore promoting substantially local cancer therapy.

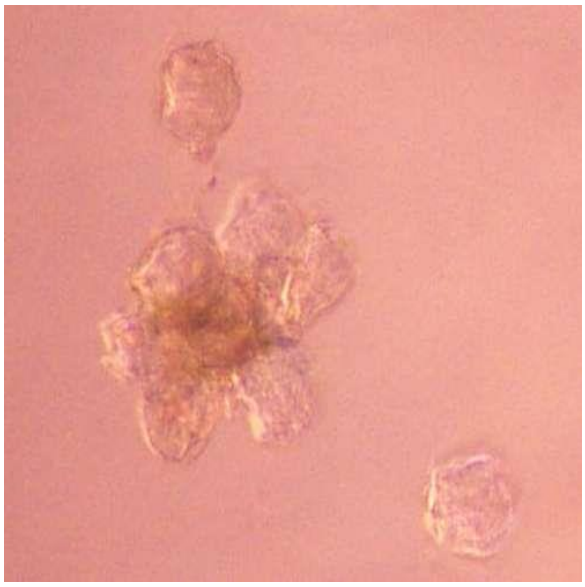

Figure 3. Optical image of PSh-MG63 cells interface on day 1. PSh: pollen shell

Cell Cytotoxicity

In-vitro cell cytotoxicity assay was performed to determine the impact of PShs on the viability of osteosarcoma cells over 7 days. From Figure 4, it can be seen that the ability of proliferation was significantly (p<0.05) reduced in the presence of PShs in all days, predominantly for longer culture periods, which leads to the elimination of tumor cells. The observed reduction

indicated cytotoxicity and anti-proliferative activity of the PShs against MG63 cells, confirming their anticancer characteristic. The positive impact of pollens on cancer prevention is associated with the existence of anticarcinogenic agents such as phenolic compounds, steroids, unsaturated fatty acids and polysaccharides [22, 36]. The observed anti-proliferative effect of shells on the cancer cells may owe to active anticancer compounds that are abundantly found in the pollen shells. Furthermore, reactive oxygen species (ROS) play an important role in the carcinogenesis process and function as intra/intercellular messenger molecules regulating cell growth [37, 38]. Therefore, the moderate level of ROS is essential for cancer cells survival, proliferation and migration [39, 40]. The outer walls of pollens contain a large number of strong antioxidants such as flavonoids and phenolic acids [22, 41]. It seems that PShs can also fulfill an inhibition effect on the development of cancer cells through powerful ROS scavenging activities and subsequent disruption of cancer redox balance [40, 42, 43].

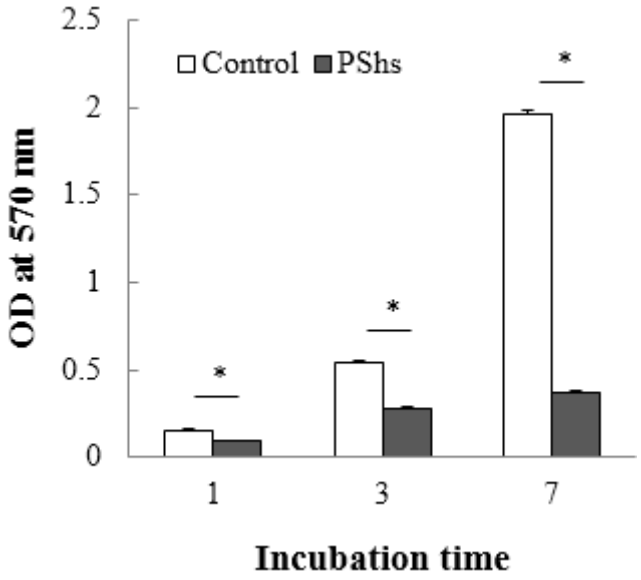

Figure 4. Viability of MG63 cells on days 1, 3 and 7. Asterisks (*) denote a significant difference of $p<0.05$. PShs: pollen shells

Apoptosis-Related Gene Expression

Apoptosis plays an important role in the inhibition of cancer progression [44]. The pro-apoptotic gene *BAX* and anti-apoptotic gene *BCL2* are key regulators of the apoptosis pathway and the balance between them determines the cellular fate for survival or death [45]. The examination of apoptotic activity using qRT-PCR technique indicated that PShs are capable of inducing cell death in MG63 bone cancer cells through disruption in the balance of counteracting pro- and anti-apoptotic genes expression. According to the results, the expression of pro-apoptotic gene *BAX* was increased, while the expression of anti-apoptotic gene *BCL2* was reduced in the presence of shells, resulting in the upregulation of *BAX*/*BCL2* ratio ($p<0.05$, Figure 5). This augmentation leads to loss of mitochondrial membrane potential, cytochrome c release and eventually cell apoptosis [46]. The most apoptotic activity was observed when

the exposure time enhanced up to 21 days. Research findings of the impacts of pollen steroids on the viability of nine human cancer cell lines have represented strong cytotoxic effects for 24h. The steroid fraction triggered apoptosis in PC-3 cancer cells via elevation of caspase-3 activity and reduction of *BCL2* expression [47].

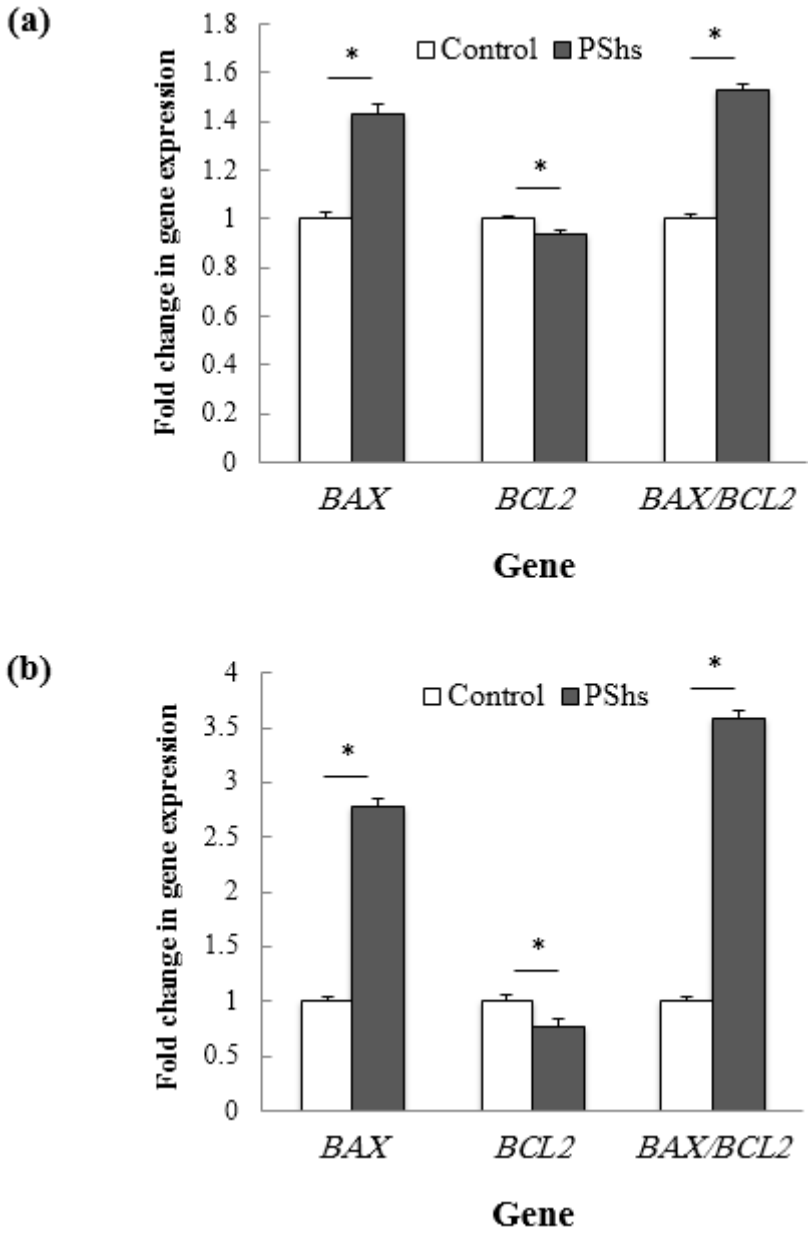

Figure 5. Gene expression of *BAX*, *BCL2* and *BAX*/*BCL2* ratio using qRT-PCR on days (a) 7 and (b) 21. *GAPDH* was used as an endogenous control. Asterisks (*) denote a significant difference of $p<0.05$. PShs: pollen shells

Nuclear Morphology

The induction of apoptosis by PShs in MG63 cells was further confirmed by morphological-based analysis, DAPI staining. DAPI is a fluorescent DNA-specific stain that is used to

visualize nuclear changes [48]. In apoptosis, cell membrane permeability changes and the nucleus becomes very condensed hence, more DAPI pass through to induce stronger blue fluorescence in the apoptotic cells [49]. As shown in Figure 6, prepared shells can potently exert apoptotic effects in osteosarcoma cells *in-vitro*. In comparison with the control group, the cells seeded on PShs demonstrated condensed and more brightly stained nuclei, reflecting anticancer activity of PShs against MG63 cells by inducing apoptosis. Increased fluorescence indicates that the cell membrane was compromised by cell death. Therefore, the finding presents the cancer-fighting characteristic of a designed carrier in bone-targeted drug delivery systems.

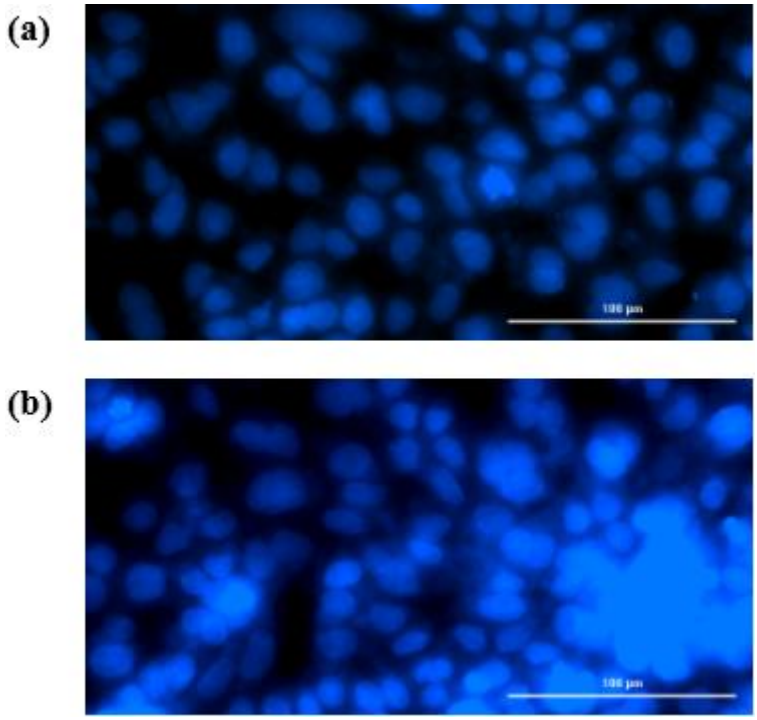

Figure 6. DAPI staining of MG63 cells (a) in a PShs-free condition and (b) on the *Pistacia vera* L. PShs on day 21. PShs: pollen shells

In summary, PSh of *P. vera* can be an ideal drug delivery vehicle for the efficient treatment of bone cancers due to the high content of various anticarcinogenic compounds and strong antioxidants. It could maintain MG63 bone cancer cells localized to their first position, suppress cell proliferation and trigger apoptosis through the upregulation of *BAX*/*BCL2* ratio.

ACKNOWLEDGEMENT

This paper was extracted from a Ph.D. thesis supported by the Drug Applied Research Center, Tabriz University of Medical Sciences. Also, the authors would like to express their gratitude to the Research Center for Pharmaceutical Nanotechnology, Tabriz University of Medical Sciences for technical and financial support.

ETHICAL STATEMENT

The paper is exempt from ethical committee approval. There are no ethical issues with human or animal subjects.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

FUNDING SOURCES

This work was supported by the Drug Applied Research Center, Tabriz University of Medical Sciences (grant No. 58781) and Research Center for Pharmaceutical Nanotechnology of the same university.

AUTHOR CONTRIBUTIONS

These authors contributed equally.

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