Fabrication and Characterization of a Three-Dimensional Fibrin Gel Model to Evaluate Anti-Proliferative Effects of Astragalus hamosus Plant Extract on Breast Cancer Cells

Mozaffar Mahmoodi¹, Somayeh Ebrahimi–Barough¹, Shaghayegh Kamian², Mahmoud Azami³, Mozhgan Mehri³, Mohammad Abdi⁴,⁵*, Jafar Ai¹*

Abstract

Background: Breast Cancer (BC) is a malignancy with high mortality among women. Recently, scaffold-based three-dimensional (3D) models have been developed for anti-cancer drug research. The present study aimed to investigate the anti-proliferative effects of Astragalus hamosus (A. hamosus) in 3D fibrin gel against MCF-7 cell line. We have also evaluated anti-proliferative effect of A. hamosus differences between 3D and 2D cultures. Methods: The fibrin gel formulation was first optimized by testing the structural and mechanical properties. Then the cytotoxic effect of A. hamosus extract was assessed on MCF-7 cells by MTT assay. Cell apoptosis was evaluated using TUNEL method and flow cytometry. Cell cycle and proliferation were analyzed by flow cytometry. Apoptosis-related gene expression such as Bcl-2, caspase-3, -8 and -9 were quantified by real time-PCR. Results: TUNEL staining showed a significant damage accompanied with cell apoptosis. Flow cytometry analysis revealed that apoptosis increased after treatment with A. hamosus extract in 3D culture model compared to 2D culture. The A. hamosus extract arrested cell cycle in the S and G2/M phases in 3D model while in the 2D culture G0/G1 phase was affected. Treatment with A. hamosus extract led to upregulation of the caspase-3, -8 and -9 genes and downregulation of the Ki-67 in the 3D-culture compared with the 2D culture. Conclusion: These results indicated that A. hamosus extract could be used as a therapeutic candidate for BC due to its anti-proliferative effects. Furthermore, 3D fibrin gel could be better than 2D-cultured cells in simulating important tumor characteristics in vivo, namely, anti-proliferative and anti-apoptotic features.

Keywords: Astragalus hamosus- breast cancer- fibrin gel- three dimension cell culture model

RESEARCH ARTICLE

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Introduction

Breast Cancer (BC) is the most common cancer in women worldwide and the second cause of cancer death among them (Abbastabar et al., 2013; Ginsburg et al., 2017). Like other malignancies, the breast cancer undergoes genetic changes that alter the molecular mechanism of healthy cells and upset the balance between immune cells in breast tissue and mammary gland cells, leading to chronic inflammation and eventual malignancy (Montazeri et al., 2008; Pollard, 2009; Menbari et al., 2020). Disruption in family life, worrying about the future, fearing of death, decreased performance, mental disorders, and sexual problems are some social and familial complications for BC-suffering patients which induce a variety of stresses and cause mental and psychological disorders in their lives. Chemotherapy, radiation therapy, surgery, and hormone therapy are common BC treatments (Chie et al., 1999). Long-term chemotherapy causes many physical, sexual and psychological problems for patients due to mastectomy-related negative changes in the mental image of women (Fobair et al., 2006). Inappropriate therapeutic effectiveness, many side effects (Ginsburg et al., 2017), and resistance of some BC tumors to chemotherapy drugs need alternative treatments (Zhang et al., 2017) or new drugs having suitable protective or therapeutic effect on breast cancer (Abbastabar et al., 2013). Although two-dimensional (2D) in vitro cytotoxicity models are traditional in pre-clinical studies for drug development, they could not simulate three-dimensional (3D) tumor environment (TME) and do not reflect
actual tumor responses to drugs. The cells cultured in 2D monolayers poorly retain their original phenotype (Bahcecioglu et al., 2020), and do not recapitulate the complexity of tumor microenvironment (Leonard and Godin, 2016) resulting in different cell-cell and cell-material interactions from actual in vivo behaviors (Duval et al., 2017; Noori et al., 2017). On the other hands, animal models are extremely useful to understand drug activity and metabolism; however, they are most costly and time-consuming (Bahcecioglu et al., 2020), have ethical concerns (Huang et al., 2013), and are most importantly do not reflect human responses to drug treatments (Bahcecioglu et al., 2020). Since tissue microenvironment can play an important role in cellular functions, we need to develop different laboratory models that can show how the structural and biochemical properties of tissue microenvironment have effect on tumorigenesis suppression. The 3D culture plays an important role in tumor biology and responses to therapeutic drugs by providing in vivo like microenvironment (Gurski et al., 2010; Huang et al., 2013). Huang (2013) (Huang et al., 2013) reported that the encapsulated MCF-7 cells in the hydrogel matrix during hydrogelation and cells residing in 3D have better growth than those 2D cultures. Treatment by an anti-cancer drug (cisplatin) causes a significant decrease of cell viability in hydrogels leading to provide evidence that the hydrogel is a promising 3D culture for drug testing. Consequently, 3D in vitro engineered models mimicking TME (Bahcecioglu et al., 2020), providing ethical and economic benefits, and bridging the gap between 2D and in vivo studies (Leonard and Godin, 2016; Yakavets et al., 2020) could be developed due to advancements in biomaterials and tissue engineering (TE) (Bahcecioglu et al., 2020).

Tissue engineering by its innovations in scaffold designs for engineering TME (Lequeux et al., 2012) provides a new approach to evaluate anti-BC therapy (Li et al., 2019a). Three-dimensional scaffold-based cell cultures having better morphology and function of differentiated cells as well as ability to select and control various culture factors including bioreactors and culture methods can be performed at a higher scale (Afewerki et al., 2019). Scaffold-based models developed for anti-cancer drug researches can be divided by origin into natural or synthetic and by structure into solid scaffolds or hydrogels (Lequeux et al., 2012; Carvalho et al., 2017; Mahmoodi et al., 2020). Polyurethane foam (Angeloni et al., 2017), nanoclays (Kar et al., 2019), silk fibroin (Wang et al., 2010), human decellularized adipose tissue (Dunne et al., 2014), multi-walled carbon nanotubes (Akinoglu et al., 2017), polycaprolactone (PCL) fibers (Guiro et al., 2015), PCL-chitosan electrospun nanofibers (Sims-Mourtada et al., 2014), and agarose hydrogel (Vantangoli et al., 2015) are some natural and synthetic materials which have been used to make suitable scaffolds. Fibrin gel, among various natural and synthetic polymers used to design and simulate TME for breast tumor cells, is more desirable due to its salinity properties (Ahmed et al., 2008), high cell seeding efficiency, adhesive property, and improved cellular interaction (Malafaya et al., 2007).

Since anti-cancer chemotherapeutic agents damage normal cells, significant efforts have been made recently to identify natural compounds and related synthetic agents to prevent the development and recurrence of cancers (Greenwell and Rahman, 2015). Medicinal plants have been discovered and used in traditional medicine practices (Ahn, 2017), and remain as the basis of modern medicine (Shkondrov et al., 2019; Berezutskii et al., 2020; Borah and Banik, 2020; Darvishi et al., 2020). Numerous phytochemicals with potential or established biological activity have been identified (Ahn, 2017), and several anti-cancer chemotherapeutic drugs have been identified and isolated from medicinal plants (Borah and Banik, 2020). Traditional Persian medicine (TPM) as one of the most ancient categories of complementary and alternative medicine with the unique medical texts can be a good help in introducing new anti-cancer drugs (Bahramsoltani et al., 2017). The pterygium, scientifically named Astragalus hamosus (A. hamosus), is one of the traditional drugs which has been shown to have analgesic properties, acute anti-inflammatory properties, and antioxidant activity, possibly by inhibiting prostaglandin synthesis (Lysiuk and Darmohrav, 2016). Anti-cancer activity of A. hamosus having different mechanisms such as immune response modulation, inhibition of angiogenesis, and decytotoxicity has been well established (Krasteva et al., 2008; Shojaei et al., 2015). The active ingredients of this plant include unsaturated fatty acids, free amino acids, polyphenols, triterpenes, flavonoid glycosides and glycolipids (Hamedi et al., 2016). The flavonoid glycoside rhamnoglucin 4’-β-D-galactopyranoside extracted from the leaves of the crown plays a significant role in the prevention of carcinoma by inhibiting free radicals and reducing the production of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione S-transferase (GST) and lipid peroxidation (LPO) (Saleem et al., 2013).

According to the consumption of the fruit of A. hamosus in Iranian medicine sources, we engineered a 3D human BC model using MCF-7 cells in fibrin gel to mimic TME. A. hamosus was used as a kind of herbs as anti-proliferative agent in 3D fibrin gel against BC cell line. Moreover, the total hydroalcoholic extract containing flavonoids and saponins was used for the first time to investigate its anti-tumor effects in a three-dimensional environment.

Materials and Methods

Tissue collection and mechanical analysis of BC tissue

Breast cancer tissue specimens were obtained from the Iran Tumor Bank (ITB), and confirmed pathologically. The present study was approved by the Ethics Committee of Tehran University of Medical Sciences. To analyze mechanical properties of obtained tissues, tumor tissues were punched into cylindrical form at the thickness of 5 mm and diameter of 10 mm. Then, the samples were fixed between the DMTA parallel plates, and fully immersed in phosphate-buffered saline (PBS) in a standard submersion-compression clamp configuration at 25 °C. Two series of compressive tests were run on each tumor sample following an initial application of 9% pre-strain to ensure full sample engagement and homogeneous compression.
before measurement. A controlled compressive force was applied at constant rate of 0.003 N/min in all tests. The mean Young’s modulus (E) was obtained for each sample from the slope of the stress-strain curve in the low-strain elastic regime.

Preparation and mechanical characterization of fibrin gels

In order to achieve the viscoelasticity similar to natural tissue, different factors involved in gel formation were used according to the Table 1. Considering the Hasanzadeh (2019) method (Hasanzadeh et al., 2019) to make the fibrin gel, fibrinogen (Sigma-Aldrich, USA) was dissolved in 1 ml M199 solution (Sigma-Aldrich, USA) and transferred to a 6-well culture plate. Afterward, thrombin solution (120 U/ml in 1 M sodium buffer, Sigma-Aldrich, USA), calcium chloride (CaCl2, 1% (w/v)), and fetal bovine serum (FBS, Gibco BRL, Germany) were added to each well. The culture plates were placed in an incubator at 37 °C for one hour to form a 3D network structure. After leaving from the incubator, the consistency (no degradation) of prepared gels were examined by placing at 25 °C for three hours. The samples 3 and 6 could be taken out of the laboratory for mechanical testing, and other samples were degraded and removed due to lack of necessary strength. The mechanical properties of the gels were investigated by the method used for BC tissue analysis.

Preparation of the A. hamosus extract

The A. hamosus extract was obtained from fruit of A. hamosus. After preparing the fruit of A. hamosus plant, the scientific name of the plant (Astragalus hamosus L.) was approved by the Herbarium of Pharmacy of Tehran University of Medical Sciences and its voucher specimen number was assigned as PMP-3610. The hydro-alcoholic extract was prepared by massaging the fruit in 70% ethanol and the extract was then filtered and concentrated with vacuum evaporator. Then, the dried extract was suspended in deionized water to yield different fractions (Saleem et al., 2013).

Determination of total steroid by Lieberman-Buchard test

To standardize the product, the amount of total unsaturated sterols in the dried A. hamosus extract was measured by the quantitative Lieberman-Buchard test. Lieberman-Buchard reagent (LBR) was prepared according to the process described in literature (Adu et al., 2019). Different concentrations of standard cholesterol (0.5, 1, 1.5, 2 and 2.5 mg/ml) were prepared in chloroform, the absorption optical densities were measured at a wavelength of 640 nm, and the linear calibration curve was plotted. One gram of the extract was dissolved in 30 mL of 70% methanol and then 20 mL of water was added to it. The mixture was poured into a decanter funnel and extracted three times with 10 mL of chloroform. The bottom solution was collected in a concentrator containing chloroform, and dried. After dissolving the dried powder in 10 mL chloroform, three test tubes containing 1mL of the LBR, 1.5 mL of the sample and 1mL of chloroform were prepared, and the optical absorptions were measured at 640 nm to determine total steroids. A test tube consisting of chloroform (2 mL) and the sample (1.5 mL) was used as control sample.

IC\textsubscript{50} determination by MTT assay

The MCF-7 cell line was prepared from the International Centre for Genetic Engineering and Biotechnology (ICGEB; Tehran, Iran) were grown in DMEM/Ham’s F12 culture medium (Invitrogen, Carlsbad, CA) supplemented with 10% FBS (Gibco BRL, Germany) under common culture conditions (37 °C, 95% humidity, and 5% CO\textsubscript{2}). After cell seeding in a 96-well plate at density of 5000 cells/well for 24 h, the supernatant was removed and 200 μL of different concentrations of the A. hamosus extract (62.5 μg/ml, 125μg/ml, 250μg/ml, and 500μg/ml) (Csupor-Löffler et al., 2009; Li et al., 2019b) and paclitaxel (0.005 μM/mL, 0.01 μM/mL, 0.05 μM/mL, and 0.1 μM/ml) (Zuo et al., 2010; Vali et al., 2015) were added in any well separately, and incubated for 72 hours. To calculate IC50 (the half maximal inhibitory concentration) by MTT assay, 10 μL of MTT solution (5 % (w/v)) was added at the predetermined time, and incubated for four hours. After removing supernatants, 100 μl of isopropanol was added to each well, incubated for 20 min to dissolve deposited formazan salt, and the absorbance at 570 nm with reference to 630 nm was determined using Biotek Synergy HTX microplate reader (Biotek, USA). Finally, cell viability was calculated using Eq.1 and IC50 was found by curve fitting on the viability data. Paclitaxel as a cytotoxic drug for BC treatment (Foglietta et al., 2018) was used as a positive control. Also, untreated MCF-7 cell was considered as negative control.

\[
\text{Cell viability(\%) } = \frac{\text{Average absorbance of sample at 570nm}}{\text{Average absorbance of negative control at 570nm}} \times 100 \quad (\text{Eq.1})
\]

Spheroid formation and three-dimensional (3D) cell culture in fibrin gel

Fibrin gel was used as a hydrogel scaffold for encapsulation of MCF-7 spheroid cells. Due to the fact that the MCF-7 cells were adherent cells and had high tendency to adhere to the bottom of common culture plates, non-adherent plate was used to form cellular spheroids. The MCF-7 cell line was cultured in non-adherent plate. Briefly, enzymatic dissociated MCF-7 cells were suspended in the culture medium and the cell suspension (2×10\textsuperscript{5} cell/ml) was transferred to a sterile, non-adherent 8-well plate and incubated under common culture conditions for 72 hours. The tumor spheroid formation was assessed visually after 24, 48, and 72 hours by taking optical images to calculate spheroid size. Finally, the MCF-7 spheroids were encapsulated in fibrin gel.

Treatment of cancer cells under 2D and 3D conditions

For 2D and 3D treatments, a total of 1×10\textsuperscript{5} MCF-7 cells were seeded in a 6-well plate and total of 1×10\textsuperscript{5} MCF-7 cells in spheroid shape were encapsulated in 3D fibrin gel for 24 hours, respectively. Then, the supernatant was removed and the samples were treated with A. hamosus extract and paclitaxel at their IC\textsubscript{50} concentrations for 72
Mozaffar Mahmoodi et al  

Molecular and biological assays to evaluate treatment efficiency

Apoptosis evaluation by TUNEL assay and flow cytometry

The terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay was used for apoptosis evaluation by applying a available kit (In situ Cell Death Detection kit, Roche, Germany). MCF-7 spheroids were encapsulated in fibrin gel in 96-well flat-bottom plate and treated with IC$_{50}$ concentrations of $A. hamosus$ extract for 72 hours. Briefly, the spheroids were washed in PBS, fixed in 4% (w/v) paraformaldehyde (PFA), washed in PBS, and stored in 70% ethanol at 4°C overnight. After being washed with 10 mM Tris-HCl (pH 7.6), the spheroids were incubated in methanol containing 0.3% H$_2$O$_2$ for 10 min to quench endogenous peroxidase activity. Then, the cells were treated with proteinase K at 37°C for 30 min. The spheroids were then incubated in the TUNEL reaction mixture at 37°C for 60 min followed by incubating in horseradish peroxidase (Santa Cruz, Germany) solution for 30 min. The color reaction was developed in 3, 30-diaminobenzidine (DAB, Roche; 0.5μl DAB and 1.5μl peroxyde buffer) for 10 min. A set of cells was incubated in the absence of TUNEL as a negative control. Using TUNEL procedure, apoptotic cell nuclei will be colored dark brown and were observed under a light microscope.

Moreover, apoptotic cells were assessed by flow cytometry (CyFlow SL, Partec-Germany) using Annexin V-FITC Apoptosis Detection Kit (Biovision, USA) according to the manufacturer’s instructions. Data from independent triplicate experiments were calculated to analyze the number of apoptotic cells.

Proliferation assay

The MCF-7 cell was fixed with 4% PFA and permeabilized with 0.1% Triton X-100 at room temperature. The cells were incubated with anti-Ki-67 antibody (ab16667, Abcam, Cambridge, MA, USA) overnight, and fluorescein-conjugated goat anti-rabbit IgG (A-21428, Life Technologies) as secondary antibody.

Cell cycle analysis

The MCF-7 cells were harvested in ice-cold PBS, fixed in ice-cold 70% ethanol to synchronize the cell cycle, resuspended in cold PBS. RNAase (2μg/ml) was added and incubated at 37°C for 30 min, followed by cultivation in 400 μL propidium iodide (PI) for 40 min at room temperature. The DNA content was determined by a FACS Caliber flow cytometer (CyFlow SL, Partec-Germany) and the cells were analyzed in different phases of G0, G1, S, and M. All experiments were performed in triplicates.

Real-time quantitative polymerase chain reaction (RT-qPCR)

After 72 hours exposure to IC50 concentrations of $A. hamosus$ and paclitaxel, the expression of cell apoptosis markers was investigated by real time-PCR. The total RNA was extracted using RNX-Plus Solution kit (Sinaclon, Iran), converted to cDNA by First Strand cDNA Kit (Sinaclon, Iran). RT-qPCR was performed using the Rotor-Gene 6000 instrument (Corbett Life Science, USA). The comparative Ct method, 2$^{-ΔΔCt}$, was used for relative gene expression analysis. Results were reported as the relative expression normalized to the β-globin housekeeping gene, used as endogenous control. In each experiment, there were at least three different samples. Each stage was done in duplicates. The primers were designed using Primer 3 software and are listed in Table 2.

Statistical analysis

All data were expressed as means ± standard errors of three independent experiments. After analysis of normality and homogeneity of variance using Kolmogorov–Smirnov test and Levene’s test, Student’s t-test, one-way ANOVA, and Tukey’s post-hoc were used to determine the differences at p <0.05 significant level.

Figure 1. Dynamic- Mechanical Thermal Analysis (DMTA) behavior of BC Tissue and 3D Fibrin Gel. E’ and E” denote elasticity and viscosity, respectively. Also, N and S stand for natural breast cancer and synthetic 3D fibrin gel
Results

Dynamic mechanical behaviors of breast cancer tissue and 3D fibrin scaffold

Since the natural BC tissue demonstrated better mechanical properties compared to fibrin scaffold, the viscoelastic behavior of tissue and fibrin scaffold was evaluated using DMTA at the temperature between 37°C and 40°C. Figure 1 shows DMTA results related to sample 6 whose viscoelastic behaviors was closer to that of BC tissue. The BC tissue and fibrin gel sample had higher elasticity (E') than viscosity (E''). Also, in the frequency range studied which indicates different body movements, the behaviors of both samples were almost the same.

Total steroid value in terms of cholesterol in *A. hamosus* extract

The results of Lieberman-Bouchard test was done to determine the amount of steroids and cholesterol in the extract and 0.166% (w/w) was recorded as total steroids weight percent in *A. hamosus* extract.

IC50 of *A. hamosus* extract and paclitaxel

MCF-7 cells were treated with different concentrations of *A. hamosus* extract and paclitaxel for 72 hours to determine their IC50. The MTT results (Figure 2) showed that *A. hamosus* extract and paclitaxel decreased cell viability of MCF-7 cells by increasing the concentration of treating agents. By curve fitting on the MTT data, the calculated IC50 of *A. hamosus* extract was 253.2±5.1 µg/ml while paclitaxel had IC50 at concentration of 0.097±0.008 µM/ml after 72 hours.

Considering nearest concentration to the IC50 amounts (250 µg/ml for *A. hamosus* and 0.1 µM/ml for Paclitaxel, respectively), the growth index (ratio of cell number at a certain time of culture period to the inoculation density) was determined from MTT assay at the end of 72-hours incubation period, which was ~0.5 for both drugs.

Size and morphology of MCF-7 spheroids

During 72-hours observation and at 24-hours interval, optical microscopic images were taken and average size of formed spheroids was calculated using ImageJ v1.50c. Figure 3 shows the morphologies of MCF-7 spheroids after 24, 48, and 72 hours. By time increasing, the average diameter of the MCF-7 spheroids increased from 33.59±0.22 µm for 24-hours to 73.51±59 µm for 48-hours and 88.04±0.21 µm for 72-hours incubation. Considering the average diameter of 20 µm for MCF-7 [50] and assuming spherical shape for MCF-7 cells and spheroids, the average number of cells consisting of a spheroid are about 5, 50, and 85 cells for 24-hours, 48-hours, and 72-hours incubation, respectively.
Figure 3. Optical Microscopic Images of MCF-7 Spheroid after (A) 24 hours (B) 48 hours and (C) 72 hours Incubation.

Figure 4. MCF-7 Cells Stained by the TUNEL Technique. (A) MCF-7 control (B) MCF-7 treated with paclitaxel (C) MCF-7 treated with A. hamosus showing abundant brown-stained cells. The brown color indicates apoptotic cells at IC<sub>50</sub> of A. hamosus (253 µg/ml) and paclitaxel (0.097 µM/ml). Original magnifications=40x.

Figure 5. (I), Cell Apoptosis by Flow Cytometry Analysis of Treated MCF-7 Cells in 3D Fibrin Gel and 2D Culture (A, C, E: control, paclitaxel-, and A. hamosus-treated in 2D culture and B, D, F: control, paclitaxel-, and A. hamosus-treated in fibrin gel); (II), (A) viable intact cells (B) early apoptotic cells (C) late apoptotic cells. Data show the means ± standard deviation. Different groups were compared statistically by One-way ANOVA. (a: p<0.001 compared to control, b: p<0.001 2D paclitaxel-A. hamosus, c: p<0.0001 3D paclitaxel-A. hamosus, d: p<0.0001 2D-3D paclitaxel, e: p<0.0001 2D-3D A. hamosus, f: p<0.05 2D-3D A. hamosus).
Apoptosis analyses

The exposed cell to IC_{50} concentrations of A. hamosus extract and paclitaxel for 72 hours were harvested and examined by TUNEL assay. Figure 4 shows that nuclei of apoptotic cells absorb DAPI stain and become brown. The results indicated that both A. hamosus extract and paclitaxel induced morphological changes including cell shrinkage, nucleus fragmentation and typical apoptotic bodies.

The apoptosis index of MCF-7 cells in all groups was determined with Annexin V using flow cytometry analysis (Figure 5I). There was a significant difference between the 2D and 3D control cells and other groups in terms of early apoptosis index. The highest early apoptosis index was belonged to 3D paclitaxel group followed by 2D paclitaxel, 2D A. hamosus extract, 3D A. hamosus extract, 2D-3D paclitaxel, 2D-3D A. hamosus.

Figure 6. Percentage of Ki-67 Expression in MCF-7 Cells in 2D Culture and MCF-7 Spheroids Encapsulated in Fibrin Gel. Data show the means ± standard deviation. Different groups were compared statistically by One-way ANOVA. (a: p<0.0001 compared to control, b: p<0.0001 2D paclitaxel-A. hamosus, c: p<0.0001 3D paclitaxel-A. hamosus, d: p<0.0001 2D-3D paclitaxel, e: p<0.0001 2D-3D A. hamosus).

Figure 7. Cell Cycle Phases in MCF-7 Cell in 2D Culture and MCF-7 Spheroids Encapsulated in Fibrin Gel. Data show the means ± standard deviation. Different groups were compared statistically by One-way ANOVA. (a: p<0.0001 compared to control, b: p=0.05 compared to control, c: p<0.0001 2D paclitaxel-A. hamosus, d: p<0.0001 3D paclitaxel-A. hamosus, e: p<0.0001 2D-3D paclitaxel, f: p<0.05 2D-3D paclitaxel, g: p<0.0001 A. hamosus, h: p<0.01 A. hamosus).
and control groups, respectively (Figure 5II, B). The highest late apoptosis index was belonged to 2D paclitaxel followed by 3D A. hamosus and 2D A. hamosus groups. In contrast the lowest viable MCF-7 cells were belonged to 2D paclitaxel followed by 3D paclitaxel, 3D and 2D A. hamosus extract, and control groups (Figure 5II, C).

**Proliferation analyses**

Figure 6 shows Ki-67 area percent (presenting proliferative index) for MCF-7 cells and spheroids treated with paclitaxel and A. hamosus at IC_{50} concentrations under 2D and 3D culture conditions. The results showed significant decrease in Ki-67 expression after treatment with paclitaxel and A. hamosus in 3D fibrin gel compared to 2D culture. Moreover, the Ki67 expressions in the A. hamosus-treated MCF-7 cells under 2D and 3D conditions were lower than those of the paclitaxel-treated MCF-7 cells and spheroids.

**Cell cycle phases**

Extended investigation indicated obvious changes in percentages of cell cycle phases, where G2/M and S phase showed significant reduction with marked elevation of G0/G1 phases in A. hamosus-treated MCF-7 spheroids encapsulated in fibrin gel compared to control group and paclitaxel-treated MCF-7 cells and spheroids (Figure 7); however, the percentage of S phase in 2D A. hamosus treatment was not significant compared to control group. Although three-dimensional treatment of MCF-7 spheroids with paclitaxel significantly increased the percentage of G0/G1 and decreased the percentages of S and G2/M compared to the 2D treatment, A. hamosus could keep its advanced position under 2D and 3D conditions.

**qRT-PCR**

Real-time PCR analysis showed that the relative expression level of caspase-3, -8 and -9 in 3D fibrin gel was significantly higher than the 2D treatment. The mean expression levels of Bcl-2 were similar for both 3D fibrin gel and 2D treatment (Figure 8). By 2D and 3D treatment of MCF-7 with paclitaxel, Bcl-2 expression decreased in comparison with A. hamosus, but not significantly.

**Discussion**

Promoting tumor cell apoptosis is one of the most important methods towards tumor treatment. Three-dimensional culture system using fibrin gel is a good method for analysis of drug effects on tumor cells (Bayat et al., 2016). Biomaterials sometimes mimic tumor microenvironment allowing 3D space to investigate the behavior, response and progression of cancer cells (Hassan et al., 2021). A. hamosus is commonly used herb in traditional medicine. In the present study, a 3D fibrin gel was designed to investigate the apoptotic potency and anti-proliferative effect of A. hamosus extract on cell morphology, apoptosis, proliferation, and cell cycle of MCF-7 cells. To evaluate apoptotic effect of A. hamosus extract on BC cells in 3D and 2D culture, TUNEL and Annexin V assays as well as expression analysis of caspase-3, -8, -9, and Bcl-2 genes were investigated. Similar to BC tissue, DMTA results showed that the amount of elasticity (E’) was higher than the viscosity (E”) in the fibrin gel. The higher level of E’ and E” in the BC tissue than those of fibrin gel was due to stiffer structure of the BC tissue as a result of the presence of proteins and biomolecules as well as connections between the filaments and intermolecular bonds.

Evaluating cytotoxic activity by MTT assay revealed that the IC_{50} of paclitaxel was lower than that of A. hamosus extract which was expected due to intense effect of paclitaxel as a pure substance. However, growth indexes (ratio of cell number at a certain time of culture period to the inoculation density) for both substances were
similar. Since A. hamosus extract is a mixture of effective and non-effective constituents, it is expected that isolation of effective components could apply more strong and controlled cytotoxic effect on MCF-7 cells at lower and safer concentrations.

Our findings indicated that the A. hamosus extract increased apoptosis in 3D fibrin gel compared to 2D culture. Flow cytometry analysis showed that the highest early and late apoptosis index as well as the lowest viable cells were belonged to 2D and 3D paclitaxel groups followed by 3D and 2D A. hamosus groups confirming apoptosis induction by A. hamosus extract. In addition, we found that S and G2/M phases of cell cycle were arrested in the A. hamosus-treated cells in 3D fibrin gel compared to control group. We found that caspase-3, -8 and -9 upregulated and the percentage of Ki-67 decreased after treatment with A. hamosus extract in 3D fibrin gel compared to 2D culture.

The A. hamosus extract consists of different flavonoid compounds such as rutin, astragalin, hyperoside, and isoquercitrin (Ionkova, 1995) having antioxidant, anti-cancer, and anti-inflammatory properties (Ghodrati Azadi et al., 2008, Ghahremani-majd et al., 2012; Krejčová et al., 2014). It was proved that rutin component had an anti-apoptotic activity against the MCF-7 cells, upregulated caspase gens significantly, and decreased volume of tumor, and level of carcinoembryonic antigen (CEA) (Saleh et al., 2019). Isoquercitrin is another important ingredient of A. hamosus and has strong activity against MCF-7 cells which could suppresses cell proliferation (Yang and Liu, 2009). Hyperoside is one of the flavonoid glycosides with anti-inflammatory, antidepressant, and anti-cancer effects. Investigating the effect of hyperoside on MCF-7 cells has been indicated that it acted as an anticancer drug through ROS-related apoptosis through activation of the Bax-caspase-3 axis and the inhibition of the NF-κB signaling pathway (Qiu et al., 2020).

Although the expression of caspase-3, caspase-8, and especially caspase-9 increased significantly confirming apoptotic effect of A. hamosus under 2D and 3D conditions, its effect was not as great as the paclitaxel. Three-dimensional fibrin gel caused increase in expression of caspase-9 in A. hamosus-treated MCF-7 spheroids indicating activation of intrinsic apoptosis pathway (Elmore, 2007). To eliminate cells by executing apoptotic death early in development stage, caspase-9 is essential to inhibit proliferative diseases through the continuous removal of irreparable cells in the lifecycle (Li et al., 2017). Meanwhile activation of intrinsic apoptosis pathway, paclitaxel caused little increase in expression of caspase-8 in 3D condition compared to 2D treatment which proved enhanced extrinsic apoptosis pathway (Elmore, 2007). The expression of caspase-3 is the common route of apoptosis which increases by paclitaxel 3D treatment compared to A. hamosus. Caspase-3, as the most important of the executioner caspases which could be activated by other initiator caspases such as caspase-8 and caspase-9 (Elmore, 2007), had higher expression level for paclitaxel compared to A. hamosus. Bayat (2016) (Bayat et al., 2016) provided a 3D model of glioma in fibrin gel with different concentrations of atorvastatin, and showed that the most probable mechanisms are up-regulation of caspase-8 and 3. They suggested that this biomimetic model with fibrin may provide an appropriate 3D culture system to study the effect of anti-cancer drugs.

The anti-proliferative effect of A. hamosus can be attributed to its steroids content that was detected in the present study. Saponins are found in a wide range of plants. They have different carbon backbones that classify them as either triterpenes or steroids (Aung et al., 2017). Saponins alone or in combination with other conventional therapies have potent biological functions such as anti-tumor activity against several tumors by triggering apoptosis and causing cell cycle arrest (Man et al., 2010). Krasteva (2008) (Krasteva et al., 2008), evaluated the anti-proliferative effect of a saponins mixture and of a new flavonoid, isolated from A. hamosus against a panel of human tumor cell lines. The saponin mixture has been demonstrated to show inhibitory effects against a multi-drug resistant cell line HL-60/Dox, and IC50 value was lower in the resistant sub-line in comparison with the chemo sensitive parent cell line HL-60. Dineva (2010) (Dineva et al., 2010) confirmed the antineoplastic activity of the saponin mixture, derived from A. hamosus, in two BC cell lines, including MDA-MB 231 estrogen receptor negative and MCF-7 estrogen receptor positive. Moreover, the saponin mixtures decrease the expression level of the mitochondrial protein BclxL that outlines its influence on the cell death signal transduction.

In conclusion, our data clarified the base for the pharmacological use of A. hamosus extract in breast cancer. The anti-proliferative effect of this extract justified the further investigation of its antineoplastic potential. Furthermore, fibrin gel provided a suitable 3D scaffold to improve drug response in tissue engineering. This developed scaffold could be potentially used in future investigations of anti-cancer drugs.

**Abbreviations**

BC: Breast Cancer  
A. hamosus: Astragalos Hamosus

**Author Contribution Statement**

Conceptualization: [Mozaffar Mahmoodi] and [Jafar Ai]; Methodology: [Somayeh Ebrahimi-Barough], [Mahmoud Azami], [Mozhgan Mehri], [Shagayegh Kamian], and [Mozaffar Mahmoodi]; Formal analysis and investigation: [Mozaffar Mahmoodi], [Mohammad Abdi], and [Somayeh Ebrahimi-Barough]; Writing-original draft preparation: [Mozaffar Mahmoodi]; Writing-review and editing: [Mozaffar Mahmoodi]; Funding acquisition: [Jafar Ai]; Resources: [Jafar Ai], [Mohammad Abdi]; Supervision: [Jafar Ai]. The authors read and approved the final manuscript.

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**Ethics approval and consent to participate**

This study was conducted under a protocol approved by the Ethical Committee of Tehran University of Medical Sciences (IR.TUMS.VCR.REC.1397.980).

**Consent for publication**

All the authors have approved the manuscript and agree with submission to your journal.

**Availability of data and material**

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

**Competing interests**

The authors declare that there is no conflict of interest.

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