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RESEARCH ARTICLE

Punicic Acid Inhibits Glioblastoma Migration and Proliferation *via* the PI3K/AKT1/mTOR Signaling Pathway

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Abstract: ***Background*:** Punicic Acid (PA) is a polyunsaturated fatty acid that accounts for approximately 70%-80% of Pomegranate Seed Oil (PSO). PA possesses strong antioxidant, anti-inflammatory, anti-atherogenic effects, and anti-tumorigenic properties. Pomegranate extracts have been shown to have anticancer activity in many studies. However, there is no evidence for the effect of PSO on T98 glioblastoma cells. Therefore, the present study was the first to investigate the mechanisms induced by PA on T98 cells, which is one of the major compounds extracted from PSO.

***Methods*:** The effects of PA on cell viability; oxidative stress; and migration, proliferation, and apoptosis at the IC_{50} dose were studied.

Results*:** The proliferation and migration were inhibited in the treated group compared to the non-treated group by 9.85µl/ml PA. The difference was statistically significant (p<0.001). Furthermore, PA-induced apoptosis in the T98 glioblastoma cells compared to non-treated group and the difference was statistically significant (***p<0.001). Apoptosis was determined *via* immunocytochemistry staining of caspase-3, caspase-9 and TUNEL methods. Apoptosis was checked by flow cytometry (using caspase 3 methods) and Scanning Electron Microscopy Analysis. We also investigated the potential signaling pathway underlying this apoptotic effect. The immunocytochemical stainings of PI3K/ Akt-1/ mTOR-1 demonstrated that Akt-1 staining was increased with PA treatment similar to mTOR-1 and PI3K staining (***p<0.001). These increases were statistically significant compared to the non-treated group.

***Conclusion*:** PA exhibited exceptional abilities as an anticancer agent against GBM cells. The use of punicic acid in combination with other drugs used in the treatment of glioblastoma may increase the efficacy of the treatment. This study provided a basis for future investigation of its use in preclinical and clinical studies.

ARTICLE HISTORY

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1. INTRODUCTION

Glioblastoma Multiforme (GBM) is a neuroepithelial tumor which is the most common and aggressive malignant tumor of the central nervous system. Based on properties such as cell infiltration, rapid spread, high recurrence rate, and very poor prognosis, the World Health Organization has defined GBM as a grade IV glioblastoma [1, 2]. Tumor infiltration in perivascular spaces and along with white matter tracts, genetic heterogeneity, complex molecular pathology, and an inability of therapeutic agents to cross the blood-brain barrier contribute to the local recurrence of GBM, making it the most difficult-to-treat brain cancer [3]. Conventional treatment for GBM includes postoperative radiation with adjuvant temozolomide administration followed by maximum resection of the tumor. Despite this treatment protocol, the median overall survival rate for GBM is approximately 18 months. [1]. This has necessitated the use of other treatment methods such as gene therapy, immunotherapy, and stem cell therapy [1, 2, 4] and various pharmacological agents such as embelin, suberoylanilide hydroxamic acid, and tumor necrosis factor-related apoptosis-inducing ligands [3].

The beneficial effects of Pomegranate Seed Oil (PSO) have been demonstrated repeatedly. Punicic Acid (PA) is a polyunsaturated fatty acid that accounts for approximately 70%-80% of PSO [5]. PA possesses strong antioxidant, anti-inflammatory, and antiatherogenic effects, and some studies have suggested that it also has antitumorigenic properties [6-8]. Further, it shares structural similarity with conjugated linoleic acid and α-linolenic acid that have been found to have beneficial effects for health [9, 10]. In recent years, considering the health benefits associated with these fatty acids, investigators are showing substantial interest in exploring the functional and nutraceutical properties of PA against various diseases such as neurodegenerative disease, necrotizing enterocolitis, and breast and prostate cancer [6, 7, 9, 11-13]. However, there is no evidence for the effect of PSO on T98 glioblastoma cells. Therefore, the present study was the first to investigate the mechanisms induced by PA on T98 cells, which is one of the major compounds extracted from PSO. In this study, we investigated the effects of PA on migration, proliferation, and cell death using human T98 glioblastoma cells. In addition, the mechanisms and potential pathways of PA were investigated. The identification of the mecha-

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nisms of action of PA may provide a better understanding of how PA affects cancer cells.

2. MATERIALS AND METHODS

This study was approved by the Ethics Committee of Manisa Celal Bayar University School of Medicine (No: 20.478.486). T98 glioblastoma cells were divided into two groups: cells treated with PA (PA-treated cells) and cells with no treatment (non-treated cells). Pomegranate seed oil was kindly provided by the company of Mavi Deniz (Izmir, Turkey) [5]. The effects of PA on cell viability; oxidative stress; and migration, proliferation, and apoptosis at the IC_{50} dose were studied using MTT analysis (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), immunocytochemical staining, and TUNEL method (terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling) respectively. In addition, the cells in both groups were immunocytochemically stained with anti-iNOS and anti-eNOS antibodies for oxidative stress; VEGF (anti-vascular endothelial growth factor) for angiogenesis; anti-MMP20 and anti-TGFbeta-1 for proliferation; anti-Akt-1, anti-PI3K, and anti-mTOR-1 for metastasis; and anti-p53, anti-caspase-9 and anti-caspase-3 for apoptosis. Further, apoptosis was determined by caspase-3 staining using flow cytometry and the TUNEL method.

2.1. Cell Lines

The human T98 GBM cell line was acquired from the American Type Culture Collection Company. The cell line was maintained in Eagle's minimal essential medium (L0430, Biowest, France) supplemented with 10% fetal bovine serum (S0113, Biochrom, Berlin, Germany), 100 U/mL penicillin, and 100mg/mL streptomycin (A2213, Biochrom, Berlin, Germany). All cells were cultured at 5% CO_2 and 37°C. The cells were switched to fresh supplemented medium every third day, passaged fewer than six times for all experiments, and used at a confluency of less than or equal to ~65%.

2.2. MTT Assay

To detect the IC_{50} dose of PA, MTT assay (M6494, Invitrogen) was performed. The cells were seeded in 96-well plates at a density of 1×10^4 cells/well/200mL and incubated for 24h at 37°C and 5% CO_2. Then, the cells were treated with different concentrations of solvent extracts (0, 1, 5, 10, 50, 100µl/ml) and 0.2% (v/v) dimethyl sulfoxide (DMSO; A3672, AppliChem, Darmstadt, Germany) as a negative control. Following 24h treatment, 10mL MTT stock solution was added to each well. The plates were then incubated at 37°C and 5% CO_2 for 4h. Then, 100mL DMSO was added to each well, followed by a 10min incubation at 37°C to dissolve formazan crystals. Finally, absorbance was measured using a spectrophotometer multi-plate reader (ELx800UV, BioTek) at 490nm and the IC_{50} dose of PA was calculated [14].

2.3. Cell Migration Assay

The human T98 GBM cells were trypsinized and plated (1×10^4 cells/cm^2) on coverslips coated with poly-L-lysine. After reaching sub-confluency, the cells were subjected to scratching. A cell-free area was prepared by scratching the coverslips with a pipette tip, and cells were treated with the IC_{50} dose of PA for 24h. Then migration of cells from the surrounding area to the cell-free area was analyzed. A camera was used to photograph the migrating T98 cells with bright-field microscopy, and the migrated cells were counted [15].

2.4. Scanning Electron Microscopy (SEM)

After the administration with the IC_{50} dose of PA for 24h, the cells were fixed with 2.5% glutaraldehyde (w/v) in 0.1M cacodylate buffer (pH 7.4) for 30min, postfixed in 1% (w/v) osmium tetroxide in distilled water for 30min, dehydrated through a graded ethanol series, desiccated for 5min in hexamethyldisilazane, air dried for 30min, and incubated overnight in a desiccator with phosphorus pentoxide. Then, the samples were coated with a thin layer of gold by ion sputtering [16] and examined using an LEO EVO 40 (Cambridge) scanning electron microscope [17].

2.5. Immunocytochemical Staining

Following PA treatment, the cells were fixed in 4% paraformaldehyde for 30min and washed with Phosphate-Buffered Saline (PBS, 00-3002, Invitrogen, Camarillo, USA) thrice for 5min. Permeabilization was performed using 0.1% Triton X-100 (A4975; AppliChem, Darmstadt, Germany) at 4°C for 15min, and the cells were washed with PBS. Next, the cells were treated with 3% hydrogen peroxide solution for 5min to inhibit endogenous peroxidase activity and washed with PBS. Then, the cells were incubated with monoclonal anti-eNOS (RB-1711-P1; Neomarkers), anti-iNOS (RB-1605-P; Neomarkers), anti-MMP20 (orb101641; Biorbyt), anti-TGFbeta-1 (sc-146; Santa Cruz Biotechnology), anti-VEGF (sc-7269; Santa Cruz Biotechnology), anti-Akt-1(sc-5298; Santa Cruz Biotechnology), anti-PI3K (sc-1637; Santa Cruz Biotechnology), anti-mTOR-1(sc-1549; Santa Cruz Biotechnology) anti-p53 (sc-6243; Santa Cruz Biotechnology), anti-caspase-3 (sc-56053; Santa Cruz Biotechnology), and anti-caspase-9 (RB-1205-P; Neomarkers) antibodies for 18h at 4°C. Further, the cells were washed thrice for 5min with PBS and treated with biotin-streptavidin hydrogen peroxidase secondary antibody (85-9043; Invitrogen®-Histostain Plus Bulk Kit, CA, USA) for 30min. After three 5-min washes with PBS, the cells were incubated in diaminobenzidine (00-2020; Zymed, CA, USA) for 5min for immunolabeling and subsequently stained with Mayer's hematoxylin (72804E; Microm, Walldorf, Germany). The cells were covered with a mounting medium (AML060; Scytek, Logan, Utah, USA) and viewed under an Olympus BX40 (Tokyo, Japan) light microscope. On the other hand, the control samples were identically processed except that the primary antibody was omitted. Immunostaining was repeated thrice [18].

2.6. TUNEL Assay

To detect apoptosis, an in situ apoptosis detection kit (Dead End Colorimetric) TUNEL system (Promega) was used, according to the manufacturer's instructions. After PA treatment, the cells were fixed in 4% (w/v) paraformaldehyde for 30min and washed with PBS for 5min. The cells were then incubated with 20µg/ml proteinase K for 10min and washed again with PBS. Endogenous peroxidase activity was inhibited by incubating the cells with 3% hydrogen peroxide for 5min and washing them with PBS. The cells were then treated with an equilibration buffer for 5min and incubated with Tdt-enzyme for 60min at 37°C. TdT was omitted in reactions that served as a negative staining control for TUNEL. After incubation with Tdt-enzyme, the cells were treated with 2× SCC solution for 15min and washed thrice with PBS. Streptavidin-peroxidase treatment was performed for 45min, after which the cells were rinsed with PBS. Apoptotic cells were labeled with diaminobenzidine (00-2020; Zymed, CA, USA), and counterstaining was performed using Mayer's hematoxylin (72804E; Microm, Walldorf, Germany). The cells were washed in distilled water and mounted using a mounting medium. TUNEL-positive cells were stained as brown nuclei and quantified by counting 1000 cells from random fields. Counting was performed by a blinded observer under an Olympus BX40 light microscope [19].

2.7. Annexin V Assay

Annexin V method was also used to determine the apoptotic effect of PA. Monolayer culture cells were centrifuged at 1000 rpm for 10 minutes after collection into microcentrifuge tubes by the trypsinization process. The pellet was dissolved with PBS and cen-

trifuged again at 2000 rpm for 5 minutes. The supernatant is shed. 100 µl of medium containing 1% FBS were added to the pellets in the tubes and the cell suspension was generated by pipetting. 100µl Muse® Annexin V and Dead Cell Kit solution were added to each tube, which was brought to room temperature, and pipetted slowly. Incubated for 20 minutes at room temperature in the dark. The sample was analyzed in the Muse cell analyzer (Millipore, Billerica, MA, USA, MCH100105) according to the Muse® Annexin V and Dead Cell Kit protocol [20].

2.8. Western Blot

Cells were plated, lysed in lysis buffer with protease inhibitor cocktail (Sigma-Aldrich) for 30min on ice. After centrifugation at 15,000 × g for 5min at 4°C, the supernatants were collected and protein concentrations were determined by Qubit machine. Then, 50µg of protein from each lysate was resolved by SDS-PAGE, transferred to nitrocellulose membranes, blocked for 1,5h, incubated with primary antibodies against Akt and IP3K from Santa Cruz Biotechnology (Santa Cruz, CA) or β-actin (Sigma-Aldrich) and incubated with horseradish peroxidase-linked secondary antibodies (Amersham, GE Healthcare, Buckinghamshire, UK). Proteins were detected by the ECL system (Amersham, GE Healthcare). Densitometric analyses were performed using the ImageJ software [21].

2.9. Flow Cytometry

The human T98 GBM cells at passage 6 were planted on two T25 flasks. After achieving 100% confluency, group-1 cells were treated with the IC_{50} dose of PA and incubated for 24h at 37°C. The other flask served as the control and was treated with an equal volume of a feeding medium. Following incubation, the cells were digested with 0.05% trypsin with ethylenediaminetetraacetic acid (25300; Gibco) and collected (digestion time was shortened as much as possible to avoid false-positive results). Then, the cells were rinsed twice with PBS, centrifuged at 400g for 5min, and the pellet resuspended with FACs buffer containing 1:6400 cleaved caspase-3 (Asp175) antibody (Cell Signaling Technology, #9661) as the primary antibody and 1:10 fluorescein (FITC)-conjugated affinipure goat anti-rabbit IgG, Jackson ImmunoResearch 111-095-003) as the secondary antibody. The fluid was mixed well and allowed to react at room temperature in the dark for 5-15min. The sample was measured using a flow cytometer (BD FACSCanto II). The green fluorescence of cleaved caspase-3 was detected in the FITC channel (FL1). Statistical analysis was performed using the BD FACSDiva™ software [22].

2.10. Statistical Analysis

During the evaluation of results, the percentage and intensity of immunostaining were obtained using H-scoring and determined as the ratio of positively labeled cells to all cells in selected fields [19]. The apoptotic index was similarly determined as the ratio of positively labeled cells to all cells in selected fields [19]. However, in the graphical representation, grading was used to demonstrate the relationship of oxidative stress and apoptosis for immunochemical intensity and TUNEL staining, as described previously [19]. Immunocytochemical intensity was scored by two blinded observers as follows: 0, no staining; 1, weak staining; 2, moderate staining; 3, moderate-strong staining; 4, strong staining; and 5, very strong staining [23]. The respective score was then calculated using the formula H-score = Pi (intensity of staining + 1), where Pi is the percentage of stained cells for each intensity, varying from 0% to 100% [23].

For TUNEL staining, the total number of cells was counted in 10 randomly selected microscopic fields and apoptotic index was calculated as follows: Apoptotic index = (number of apoptotic cells / total number of cells) × 100 [24].

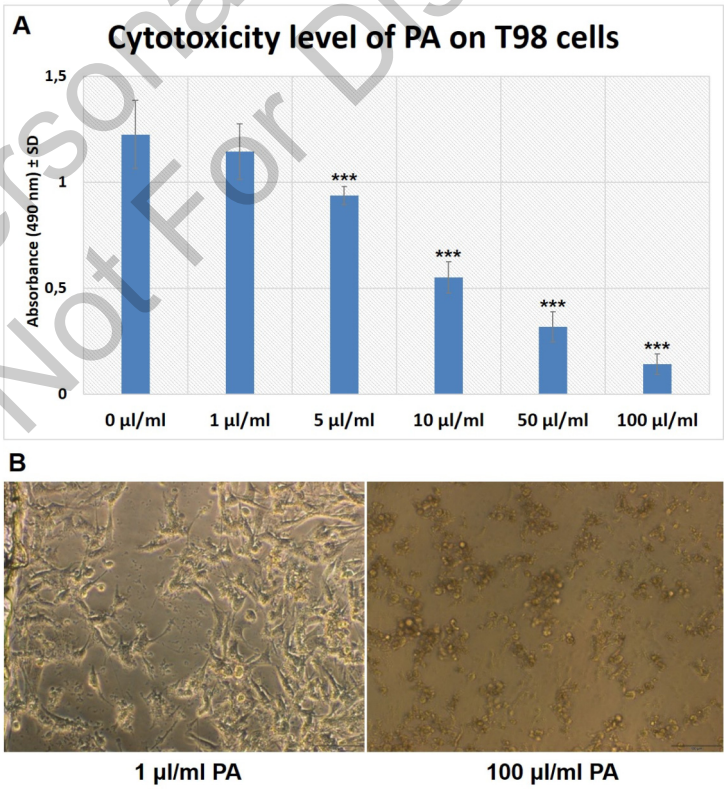

Fig. (1). Cytotoxicity level of PA was analyzed using MTT (**A**). The toxic effect of PA on T98 cells at doses of 1 and 100µl/ml was observed (**B**). At 24h, the IC_{50} dose was 9.85µl/ml.

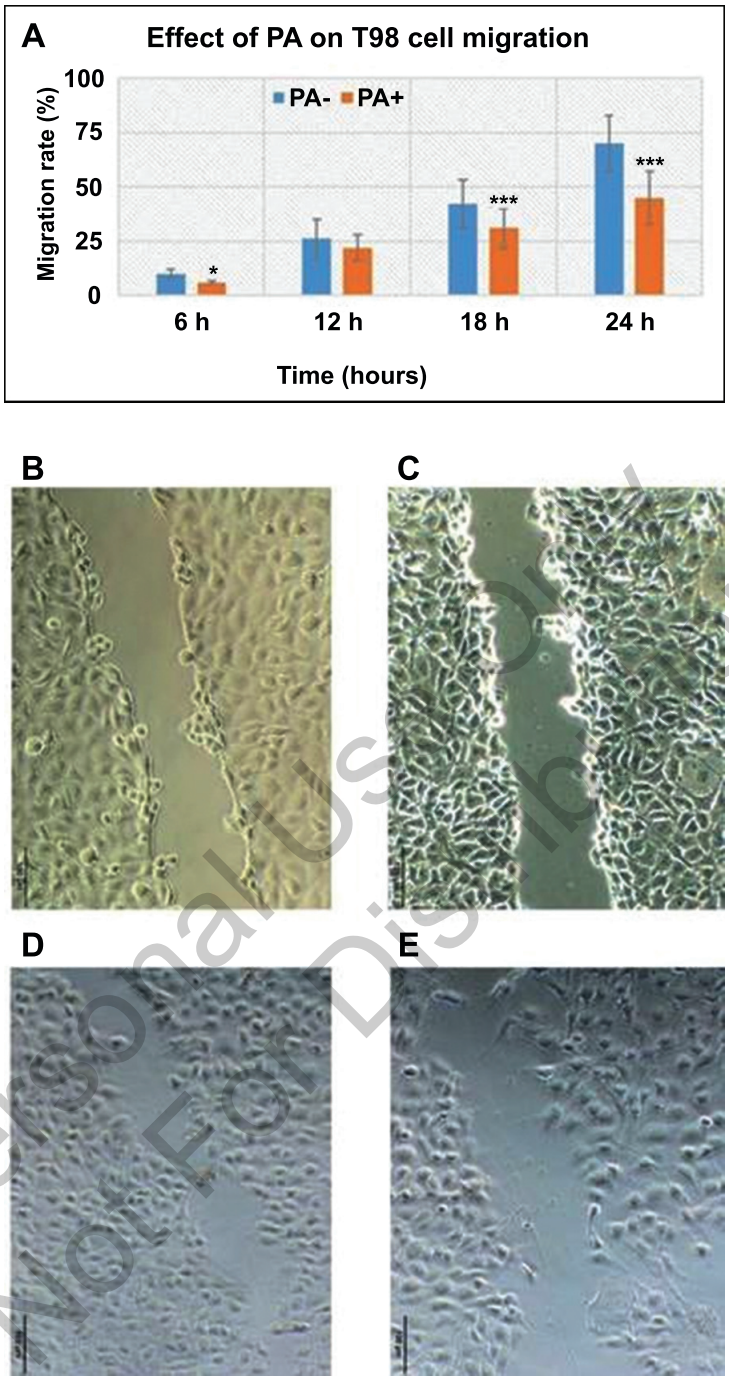

Fig. (2). Effect of PA on proliferation and migration of T98 glioblastoma cells following 24h treatment at the IC50 dose was observed (**A**); * denotes $p < 0.05$, *** denotes $p < 0.001$). (**B**) Wound site in confluent cultures by scratch method prior to PA treatment (**C**) after PA treatment, (**D**) proliferation and migration of non-treated cells, (**E**) and inhibition of migration and proliferation of PA-treated T98 glioblastoma cells.

The results were calculated in GraphPad (GraphPad Software, San Diego, CA, USA) using one-way ANOVA and presented as mean ± SD values. Statistical significance was defined as $p \leq 0.05$ (18).

3. RESULTS

The human T98 glioblastoma cells were replicated and allowed to reach confluence. PA was applied at increasing concentrations to determine the IC_{50} dose. Further, the toxic effect of PA on T98 cells following 24h treatment at doses of 1, 5, 10, 50, and 100µl/ml was evaluated, and the IC_{50} dose was found to be 9.85µl/ml (Fig. **1**).

3.1. PA Treatment Inhibits the Proliferation and Migration of T98 Glioblastoma Cells

To examine the effect of PA treatment on proliferation and migration, we used the scratch assay, an *in vitro* wound model for confluent cultures. PA at the IC_{50} dose was added to the wound site (Fig. **2B**-**E**). Cell migration and proliferation were observed in the non-treated group, but they were found to be inhibited following PA treatment. The difference between the PA-treated and non-treated groups was statistically significant ($p < 0.001$; Fig. **2A**).

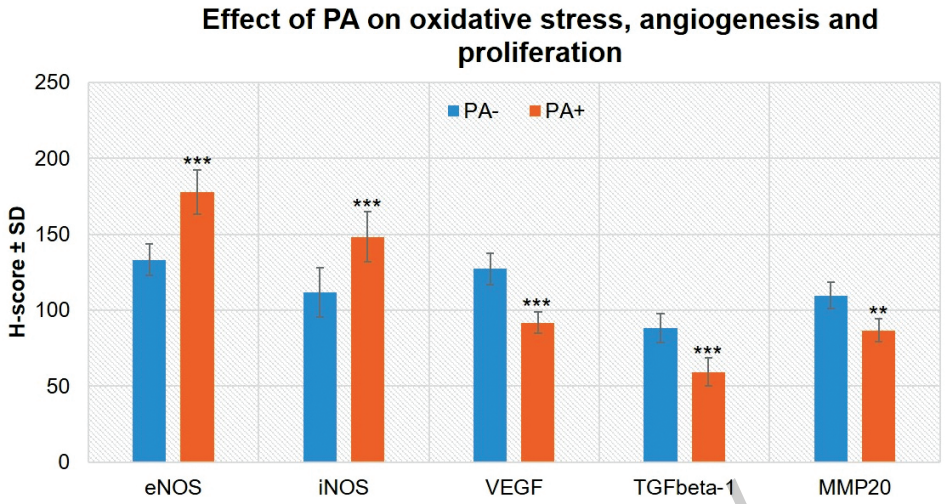

Fig. (3). H-score evaluation of iNOS and eNOS, VEGF, TGFbeta-1, and MMP20 immunostainings with (PA+; orange) or without (PA−; blue) 24h punicic acid treatment. ** $p < 0.01$, *** $p < 0.001$.

3.2. PA Increases the Marker Expressions of Oxidative Stress but Decreases Angiogenesis and Tumor Proliferation Marker Expressions in T98 Glioblastoma Cells

Oxidative stress and angiogenesis are important factors in tumor progression. We evaluated the effect of PA on oxidative stress, vascularization, angiogenesis, and matrix proteins in T98 glioblastoma cells by immunostaining for iNOS (inducible nitric oxide synthase) and eNOS (endothelial nitric oxide synthase), VEGF, tumor growth factor (TGF-beta-1), and matrix metalloproteinase-20 (MMP20; Figs. **3** and **4**). In comparison with the non-treated group, the PA-treated group had significantly higher expression of the oxidative stress markers iNOS and eNOS ($p < 0.001$). Expression of the angiogenesis and vascularization marker VEGF significantly reduced in the PA-treated group compared with that in the non-treated group ($p < 0.001$; Fig. **3**). Moreover, TGF-beta-1 ($p < 0.001$) and MMP20 ($p < 0.01$) expressions were significantly lower in the PA-treated group than in the non treated group (Fig. **4**). These results indicate that although PA treatment increased oxidative stress, it decreased angiogenesis and tumor progression.

3.3. PA Induces Apoptosis in T98 Glioblastoma Cells

Apoptosis is a programmed cell death pattern regulated by proteins such as caspase. In this study, we performed anti-caspase-3 and anti-caspase-9 immunostaining (Fig. **5A** and **C**) to determine whether PA induces apoptosis *via* the intrinsic pathway. We also performed anti-p53 and TUNEL stainings to quantify apoptosis (Fig. **5B**). We demonstrated that PA treatment significantly increased p53 expression ($p < 0.001$; Fig. **5A** and **C**). Similarly, caspase-3 and caspase-9 stainings significantly increased in the PA-treated group compared with those in the non-treated group ($p < 0.001$). Increase in TUNEL staining also supports PA-mediated apoptosis in T98 glioblastoma cells (Fig. **5B**). In order to further investigate, we used flow cytometry with Muse Annexin V & Dead Cell Assay to confirm apoptosis. The assay is based on the detection of the phosphatidylserine appearance on the cell surface by Annexin V binding. Distribution of punicic acid-treated T98 glioblastoma cells shifted from living (%94.60) to early (%14.10) and late (%19.60) apoptotic state. Treatment with punicic acid significantly induced the rate of apoptosis, as compared with the control group. Flow cytometry of Annexin V-stained cells was used to quantify the apoptotic rate of the T98 cells. The percentage of apoptotic cells was significantly higher in the punicic acid treatment groups, as compared with the control group (Fig. **6**).

In addition, Caspase-3 immunocytochemistry results were confirmed by flow cytometry (Fig. **7**). Increase in caspase-9 indicates that PA induces apoptosis *via* the intrinsic pathway. Caspase-9 causes activation of caspase-3, leading to apoptosis.

3.4. PA Inhibits Glioblastoma Cell Proliferation *via* the PI3K/Akt/mTOR Signaling Pathway

The PI3K / Akt / mTOR signaling pathway is a well-known pathway in the pathogenesis of GBM. To better understand the signaling pathway underlying the apoptotic effects of PA, we performed immunocytochemical staining for Akt-1, mTOR-1, and PI3K. Akt-1, mTOR-1, and PI3K expressions were found to be significantly increased in the PA-treated group compared with the non-treated group ($p < 0.001$; Fig. **8A** and **B**), indicating that PA prevents T98 glioblastoma cell proliferation *via* the PI3K/Akt/mTOR signaling pathway.

Western blot analysis was performed to confirm PI3K and AKT1 protein expressions analyzed by immunohistochemistry. Beta-actin was used as a loading control for western samples to assure that protein loading is the same across the gel. Western blot of T98 glioblastoma cells revealed that the PA treatment significantly reduced the PI3K and AKT1 levels in T98 cells (Fig. **9**) as compared with a non-treated group, which is the most important metastatic signaling pathway.

3.5. SEM Analysis of the Apoptotic Effect of PA Treatment on T98 Glioblastoma Cells

SEM showed that the cellular morphology of T98 glioblastoma cells substantially varied following PA treatment (Fig. **8**). The cells were shrunken and rounded with predominantly raised nuclear regions. Other visible changes included cellular shrinkage and alterations of the cytoplasmic structure. Some cells also showed formation of apoptotic bodies in the PA-treated group.

4. DISCUSSION

Despite novel therapies being reported, therapeutic success in the treatment of glioblastoma remains drastically low. This study is the first to report on direct inhibition of glioblastoma cell migration and proliferation by PA. Pomegranate extracts have been shown to have anticancer activity in many studies [7, 9, 10, 25]; however, no study has evaluated the specific effects of PA in relation to glioblastoma. In this study, we demonstrated that PA not only ceased cell

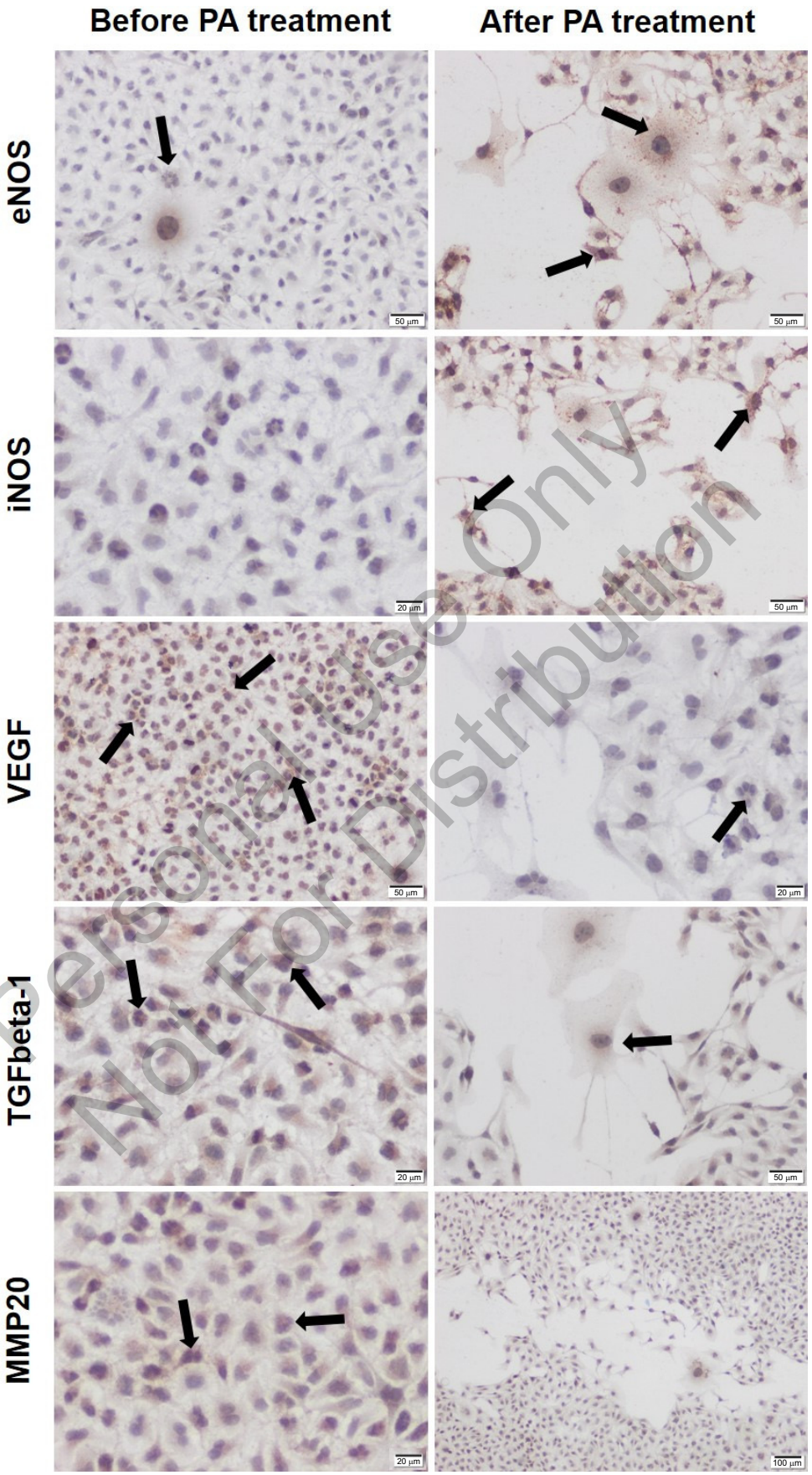

Fig. 4. Immunocytochemical staining results for iNOS and eNOS, VEGF, TGFbeta-1, and MMP20 before and after 24h Punicic Acid (PA) treatment. Arrows, immunopositive cells.

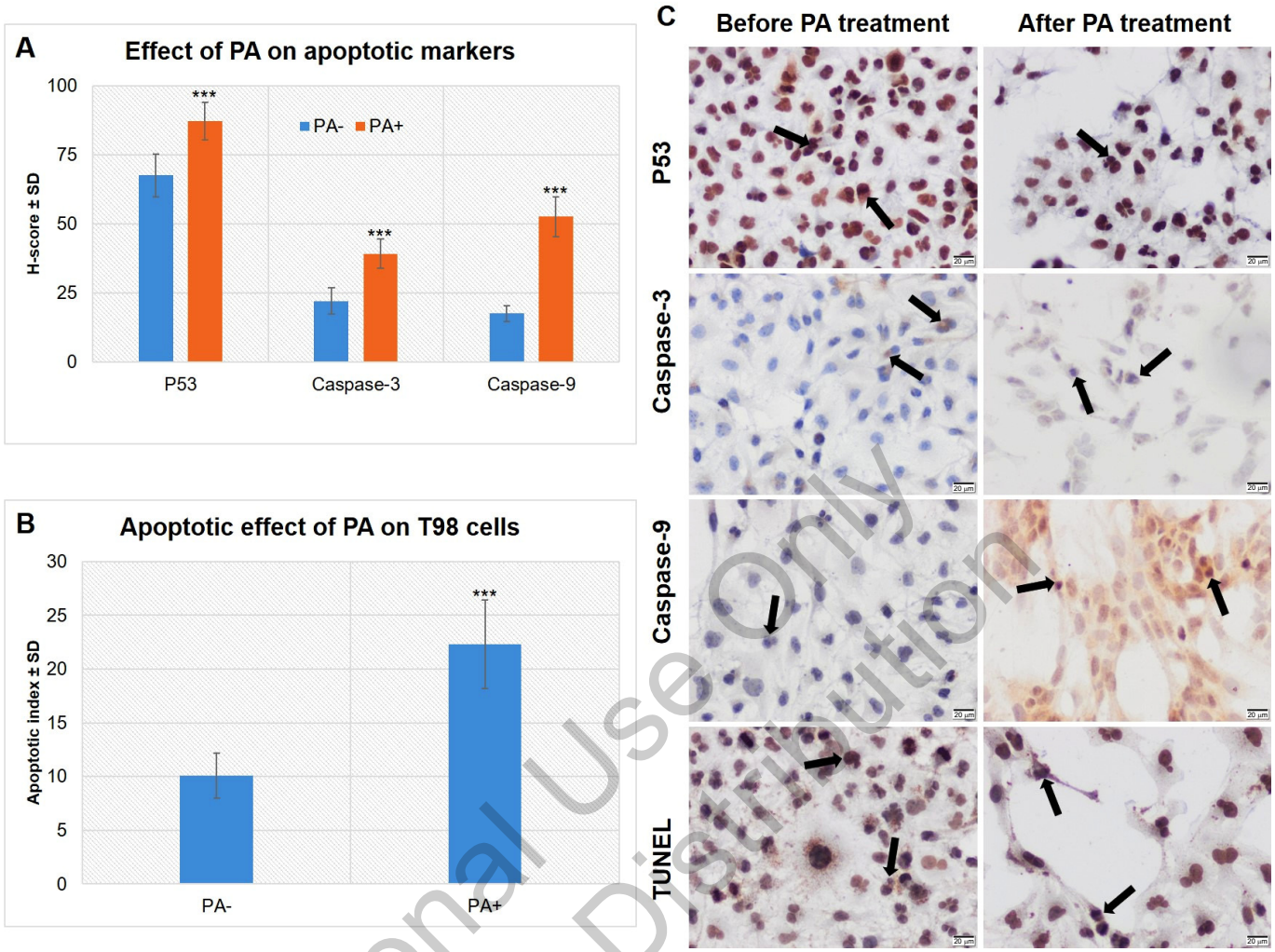

Fig. (5). The IC_{50} dose of punicic acid (PA) was evaluated in T98 glioblastoma cells. Using immunocytochemistry, cells were stained for the apoptotic markers p53, caspase-3, caspase-9 (**A** and **C**), and TUNEL staining (**B** and **C**). Results were compared for the cells treated with (PA+) or without (PA−) PA. Arrows, immunpositive cells; *** $p < 0.001$.

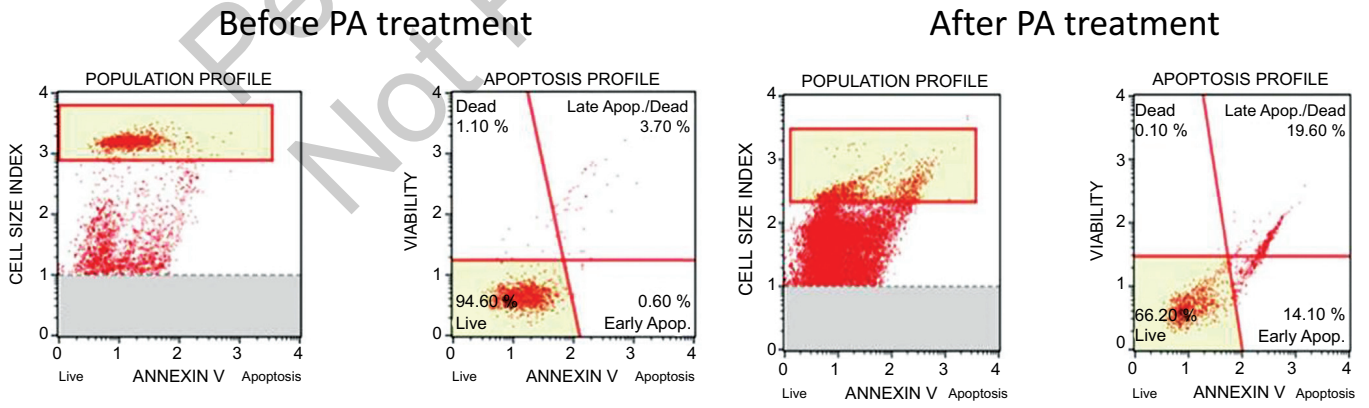

Fig. (6). Muse Annexin V & Dead Cell Assay demonstrate the distribution of punicic acid-treated T98 glioblastoma cells shifted from living (%94.60) to early (%14.10) and late (%19.60) apoptotic state.

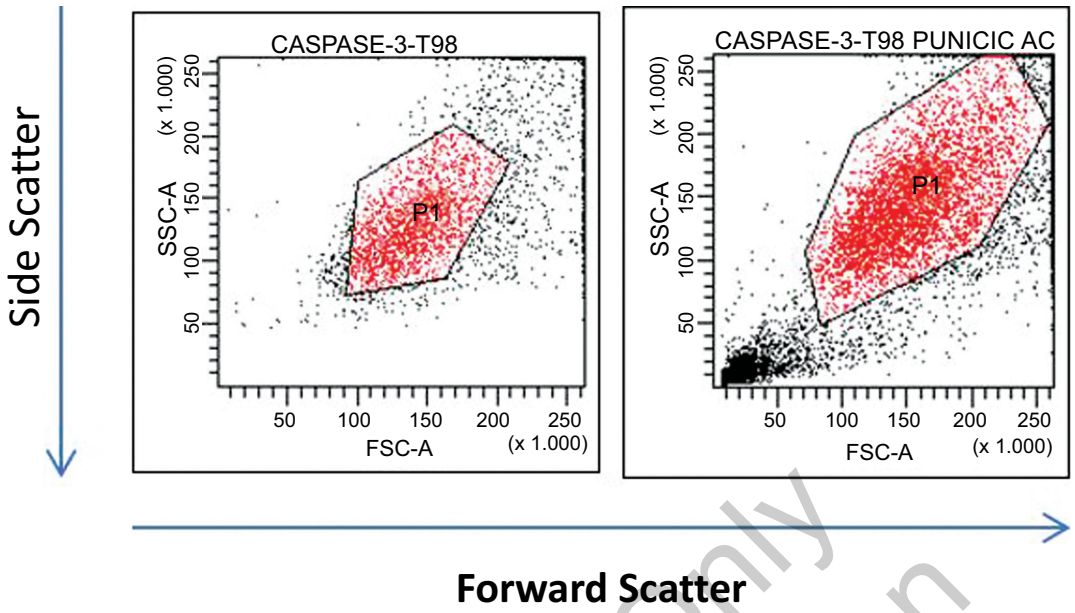

Fig. (7). Caspase-3-positive apoptotic T98 glioblastoma cells were detected using flow cytometry. Punicic acid treatment increased caspase-3-mediated apoptosis (right panel). Side scatter and forward scatter were used to determine cell granulation and cell size, respectively.

migration and proliferation but also stimulated apoptosis *via* the PI3K/Akt/mTOR metastatic signaling pathway.

PA was first observed in pomegranate seed oil by Melo [26]. In the past decade, numerous studies have been published on PA. These studies demonstrate that PA has anticancer activity by affecting tumor cell proliferation, cell cycle and angiogenesis [9, 27]. Gasmi *et al*. demonstrated that PA had growth inhibitory effects and pro-apoptotic activities in androgen-dependent LNCaP human prostate cancer cells [27]. When PA was applied to LNCaP cells at increasing concentrations (0, 3, 10, 30, and 100μM), it induced cell death at doses of 10μM and above, with an IC_{50} dose of 18 ± 4μM. Concurrently, it caused inactivation of Akt at doses of 30 and 100μM. They reported that PA induced intrinsic apoptosis *via* a caspase-dependent pathway [27]. In another study, Grossman *et al*. [9] investigated the potential ability of PA inhibiting the growth of an estrogen-insensitive (MDA-MB-231) and estrogen-sensitive (MDA-ERα7) breast cancer cell line. The authors concluded that PA inhibited breast cancer proliferation at a dose of 40μM *via* lipid peroxidation and the protein kinase C pathway [9]. In our study, we determined that PA decreased cell mobility at an IC_{50} dose of 9.85μl/ml. Exposure of T98 glioblastoma cells to PA led to decrease cell motility as demonstrated by scratch assays and tracking of individual cells. Comparison between the non-treated group and the treated group at 24h indicated that PA has a certain inhibitory effect on the migration ability of T98 glioblastoma cells.

Glioblastoma accounts for approximately 15% of primary CNS tumors and 45% of all malignant CNS tumors. In the United States, 12,390 cases were reported in 2017 [2]. The major genetic causes of GBM include gene mutation, amplification, modification, and rearrangement. These changes inactivate cancer suppressor genes or activate oncogenic genes, leading to the activation of multiple signaling pathways [1]. Three major, well-known signaling pathways have been identified in the pathogenesis of GBM to date: 1- dysregulation of growth factor signaling *via* amplification and mutational activation of receptor tyrosine kinase genes [10], 2-activation of the phosphatidylinositol-3-OH kinase (PI3K) pathway [11], and 3- inactivation of the p53 and retinoblastoma tumor suppressor pathways [28]. The RAS/RAF/MEK/MAPK, Wnt, TGFβ, and UPR pathways are other signaling pathways identified in the pathogenesis of GBM [1]. In recent years, various novel agents have been used against T98 glioblastoma cancer cells with antitumor mechanisms such as the release of Reactive Oxygen Species (ROS), downregulation of cyclin-dependent kinase 6, and targeting of the ROS/JNK signaling pathway [29-31]. In this study, we used PI3K/Akt/mTOR, p53, and TGFbeta-1 immunostaining to demonstrate the potential apoptotic pathway by which PA acts on T98 glioblastoma cells to inhibit their proliferation.

ROS are required to sustain normal metabolism. Oxidative stress, *i.e*., disturbance in the balance between ROS production and antioxidant defenses, plays a pivotal role in apoptosis [32]. In particular, overproduction of ROS such as Nitric Oxide (NO) causes cell death. The presence of NO can be detected by the NO synthase enzyme. Reportedly, low levels of NO trigger cancer onset, whereas overexpression of NO causes tumor cell death [33-35]. Several studies have showed that most plant extracts have anticancer properties and cause cell death in glioblastoma by increasing oxidative stress [36-38]. In our study, we used PA from pomegranate seeds on T98 glioblastoma cells and found an increase in the immunoreactivities of eNOS and iNOS. This increase suggests that PA mediates cell death *via* oxidative stress.

VEGF has been identified as a regulator of tumor angiogenesis and possibly plays an essential role throughout the tumor cycle by facilitating the establishment, growth, and survival of tumor vessels. VEGF is the key mediator of angiogenesis in cancer, wherein it is upregulated by oncogene expression, various growth factors, and hypoxia [39]. Oxidative stress and VEGF are important in the course of apoptosis. In this study, oxidative stress markers such as iNOS and eNOS were found to be significantly elevated in the PA-treated group compared within the non-treated group. However, VEGF staining was found to be significantly reduced following PA treatment in the PA treatment group compared with that in the non-treated group.

The underlying mechanisms of apoptosis are highly complex and involve an energy-dependent cascade of molecular events. Primarily, there are two apoptotic pathways: extrinsic and intrinsic. Intrinsic apoptosis is a mitochondrion-centered cell death mediated

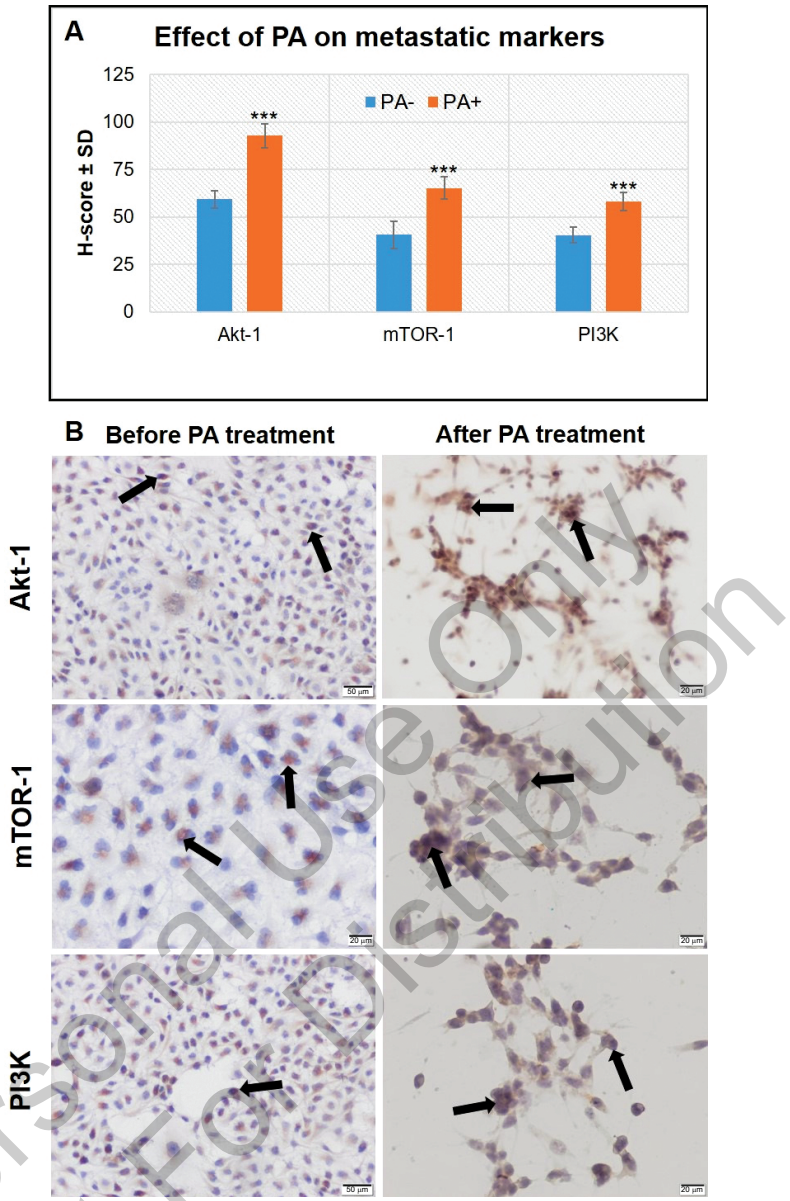

Fig. (8). Evaluation of the effect of Punicic Acid (PA) treatment at the IC_{50} dose on the expression of the metastatic signaling pathway markers Akt-1, mTOR-1, and PI3K in T98 glioblastoma cells by quantifying the H-score (**A**) of immunocytochemical staining (**B**). Arrows, Immunpositive cells.

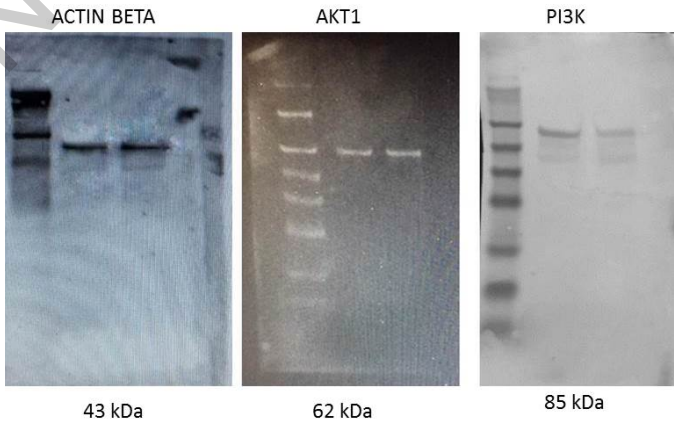

Fig. (9). Beta actin was used as a loading control for western samples to assure that protein loading is the same across the gel. Western blot analyses revealed that the PA treatment significantly reduced the PI3K and AKT1 levels in T98 glioblastoma cell.

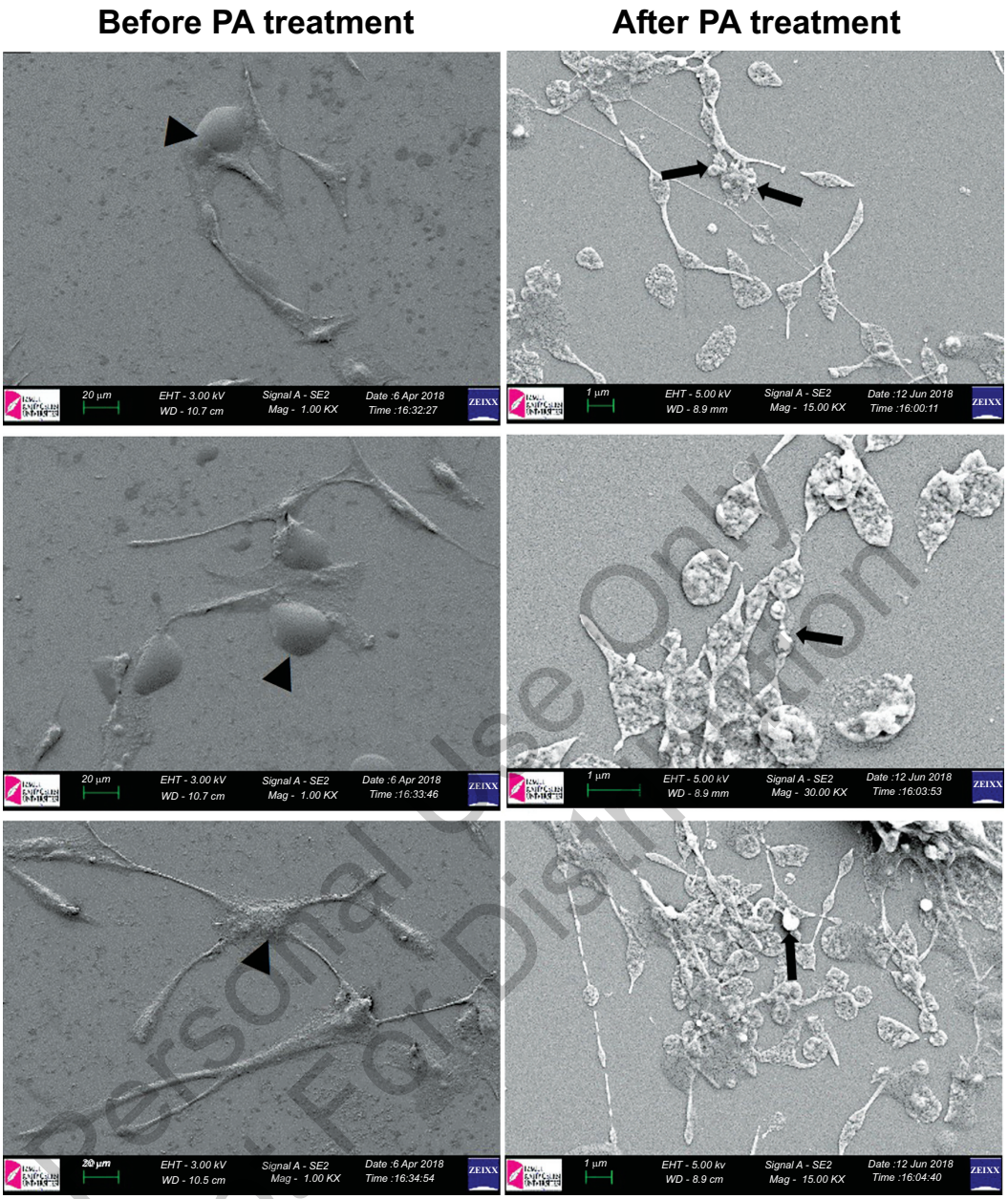

Fig. (10). Scanning electron microscopy of T98 glioblastoma cells before and after PA treatment. Arrow heads: healthy cells, Arrows, apoptotic cells.

by mitochondrial outer membrane permeabilization, caspase-9 activation, and subsequent effector caspase activations [40, 41]. Caspase-9 activates caspase-3 by proteolytic cleavage, following which caspase-3 cleaves vital cellular proteins [40, 41]. PA cleaved caspase-9 and PARP while inhibiting Bcl2 expression in LNCaP human prostate cancer cells [27]. Here, we demonstrated that caspase-9 and caspase-3 immunostaining intensities increased following PA treatment, indicating that PA mediates cancer cell death *via* the intrinsic pathway. In addition to caspase proteins, we confirmed apoptosis using TUNEL staining and Annexin V & Dead Cell Assay. TUNEL staining was significantly higher in the PA-treated group than in the non-treated group. Similar to TUNEL staining, Annexin assay demonstrated that PA treatment significantly induced the rate of apoptosis, as compared with the non-treated group. Also, SEM analyses demonstrated the morphological changes of apoptotic bodies on T98 glioblastoma cells following PA treatment.

The p53 suppressor protein is an important component of the apoptosis pathway. p53 is frequently inactivated by genetic alterations in ~50% of all types of human cancer [42] and in 87% of GBM patients [28]. Previously, Lin *et al*. reported that p53 expression was higher in high-grade glioblastoma than in low-grade glioblastoma, and the expression was not associated with sex, age, or tumor size, demonstrating that p53 serves an important role in the occurrence and development of glioblastoma [43]. Another important factor affecting apoptosis in the cell cycle is TGFβ. TGFβ is a multifunctional polypeptide that controls cell proliferation, differentiation, and angiogenesis. In normal cells, TGFβ inhibits hemostasis and tumor progression by inducing differentiation or apoptosis. However, cancer cells lose the TGFβ tumor suppressor property, and tumor cells may then use TGFβ to their advantage to produce growth factors, differentiate into an invasive phenotype, or metastasize. Therefore, cancer cells, in general, secrete larger amounts of TGF-β than their normal counterparts [44]. In this

study, p53 immunoreactivity was prominently higher whereas TGF-β1 immunostaining intensity was significantly lower in PA-treated cells than in non-treated cells. This suggests that PA treatment causes the death of T98 glioblastoma cells, resulting in high p53 expression and low TGF-β1 secretion.

The PI3K/Akt/mTOR pathway is a well-known signaling pathway involved in tumorigenesis and development, migration, and invasion of tumors [45]. This pathway is frequently activated in cancer, driving cell division and influencing the activity of other signaling pathways, such as the MAPK, JAK-STAT and TGFβ pathways, to enhance tumor growth, metastasis, and therapy resistance [46]. This pathway is altered in approximately 88% of GBM patients [28]. Chakravarti *et al*. examined the prognostic value of activation of this signaling pathway in glioblastomas. They reported that the frequency of the expression of activated proteins in this pathway was significantly higher in GBM tumors than in non-GBM tumors, suggesting that GBM patients with an activated PI3K/Akt/mTOR pathway also have a poorer prognosis than those without oncogenic activation of the pathway [47]. Considering this information, inhibitors targeting PI3K/Akt/mTOR, p53, and TGFβ have emerged as potential treatment options against GBM. Therefore, we investigated whether PA acts on these pathways. In our study, immunocytochemistry staining of PI3K/Akt-1/mTOR demonstrated that PA mediates its apoptotic effect on T98 glioblastoma cells *via* this signaling pathway. In addition, Western blot revealed that the PA treatment significantly reduced the PI3K and AKT1 levels in T98 cells.

CONCLUSION

PA exhibited exceptional abilities as an anticancer agent against GBM cells. Overall, our results highlight the potential of PA in the treatment of glioblastomas. The use of punicic acid in combination with other drugs used in the treatment of glioblastoma may increase the efficacy of the treatment. This study provided a basis for future investigation of its use in preclinical and clinical studies.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study was approved by the Ethics Committee of Manisa Celal Bayar University School of Medicine (No: 20.478.486), Turkey.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are base of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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