Original Article

EFFECT OF POMEGRANATE EXTRACT AND ITS NANOPARTICLES ON HUMAN TONGUE SQUAMOUS CELL CARCINOMA CELL LINE

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Abstract

Aim: The present study would be directed to evaluate: The effects of Pomegranate Extracts (PE) and PE loaded with polyethylene glycol-poly lactic acid-co-glycolic acid (-PE-PEG-PLGA) on human tongue squamous cell carcinoma cell line.

Methodology: The Human tongue carcinoma cell line (HNO-97) cells were propagated and maintained under basic culture media. Cells were grouped according to culture media to control group A and experimental groups (PE group (B1), PE - PEG-PLGA group (B2), was tested for cell viability, Bcl-2-associated X protein (BAX) expression, caspase-3 expression, and DNA fragmentation were all examined using (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) real-time polymerase chain reaction (RT-PCR) and diphenylamine (DPA) respectively.

Results: Regarding cell viability, a higher mean value at different concentrations was recorded in groups B2 and B1, respectively. In aspects of BAX expression, caspase-3 expression, and DNA fragmentation, groups B2, and B1, had higher mean values than control group A with a highly significant difference ($p = 0.000$). Regarding inhibitory concentration (IC50%) doses, a lower dose needed to show the same effect was recorded in groups B2 and B1(1.21ug/ml and 6.41ug/ml, respectively.

Conclusions: The pomegranate plays a role in antitumor activity as alone or with nanoparticles.

Keywords: anticancer, Oral cancer, HNO-97, POMX, nanoencapsulation, nanotechnology PEG-PLGA.

1-Introduction

Worldwide, more than 350 000 oral cancer cases are diagnosed yearly; moreover, the prevalence differs according to geographic distribution[1]. In Egypt, ~4500 cases are diagnosed yearly, with a 50% mortality rate[2]. Through advances in surgical resection, proper chemotherapy and radiotherapy, the worldwide 5- year survival rate was less than 65%. Consideration should therefore be given to preventing oral cancer through the study of its etiology and therapy [3].

Surgical intervention, radiotherapy, and chemotherapy are the primary therapeutic techniques in cancer treatment[3]. Chemotherapeutic drugs play a valuable role in cancer treatment by killing malignant cells. However, they cause cytotoxic damage to the together cancer and normal cells, on the other hand, cause resistance of cancer cells to interact with chemotherapeutic treatment[4]. Thus, it is incumbent upon the scientific community to identify a naturally occurring medicine that may be used for cancer therapy with fewer adverse effects and more effectiveness[5].

Traditional medical herbs have been important in developing complementary medicine in cancer treatment. As a result of the evolution and recognition pathophysiology of cancer cells, researchers are currently focusing on separating bioactive substances from plant components and employing them in cancer cell therapy. The number of studies focusing on the therapeutic effects of plants and their components as potential anticancer medicines is rising[6].

Pomegranate is an important source of bioactive compounds and has been demonstrated to have anticancer effects[7]. Pomegranate juice, peel, and oil have been proven to have anticancer activities, including reduced cell proliferation and cell cycle, anti-metastatic effects, and anti-inflammatory effects[8]. Pomegranate characteristics' pharmacological and phytochemistry actions revealed various clinical applications for cancer prevention and therapy[7].

Recent research has shown that PE loaded on a nanoparticle delivery system has more anticancer efficacy than PE alone, even though it still has to overcome the challenges of intestinal hydrolyzing sensitivity, limited absorption, poor systemic biodistribution, and a short half-life [9,10].

2. Materials and Methods

2.1. Cell Culture and Drug Source

HNO-97 was obtained from Veterinary Serum & Vaccine Research Institute (VACSERA); it was stored in liquid nitrogen containers at -196 C°.

Dako company provided POMx, which is a commercial pomegranate (Punica granatum L.)-derived polyphenols-rich extract powder. Previous reports have already described the constituent components of this POMx powder extract[10], such as ellagitannins (punicalagin and punicalin) and ellagic acid. POMx was immediately prepared in dimethyl sulfoxide (DMSO) before experiments and used in experiments at different concentrations (0.01ug/ml-100ug/ml) to calculate the IC50%.

2.2. PE PEG-PLGA nanoparticles (pomegranate nanoparticles prepared using ultra sonication nanotechnology).

2.3. Preparation and characterization of PEG-PLGA Nanoparticles

PEG-PLGA nanoparticles were prepared in the advanced materials and nanotechnology department at the National Research Centre (Cairo, Egypt). The double emulsion–solvent evaporation method was used to formulate PEG-PLGA nanoparticles[11].

TEM were performed on JEOL JEM-2100 high resolution transmission electron microscope at an accelerating voltage of 200 kV. Samples were prepared by evaporating dilute suspensions of the nanoparticles in ethanol on a copper grid coated with an amorphous carbon film. Field emission Scanning electron microscope (FESEM, Quattro S, Thermo Scientific) was used for the purpose of imaging, finally Zeta potential measurements were performed with a Zetasizer ZS90 instruments (Malvern Instruments, Malvern, UK) by suspending the nanoparticles in deionized water.

2.4. Cell viability MTT assay

The MTT assay is a colorimetric assay in which the number of viable cells is determined by mitochondrial nicotinamide adenine dinucleotide phosphate (NADPH)- dependent cellular oxidoreductase enzymes. The activity

level measures cells' viability because MTT reduction can only occur in metabolically active cells[12]. These mitochondrial reductase enzymes can reduce the yellow-soluble MTT stain to insoluble purple-colored formazan. The insoluble purple formazan result is dissolved into a colored solution using an MTT solubilization solution (acidified isopropanol). According to the MTT assay, we used a spectrophotometer to measure the degree of light absorption of this colored solution at a certain wavelength [13].

2.5. IC50 Value Calculation

The half-maximal inhibitory concentration (IC50) represented the concentration of the tested extracts required to inhibit a given biological activity by 50%[14].

2.6. Real-Time Polymerase Chain Reaction (RT-PCR)

RT-PCR, also known as quantitative polymerase chain reaction (qPCR, RT- qPCR), is a laboratory technique of molecular biology based on the principle of PCR. RT-PCR is commonly used to measure gene expression of target genes of interest with a known sequence, and it is highly sensitive in detecting small changes in expression. Besides this benefit, RT-PCR allows the accurate quantification of nucleic acids with superior reproducibility[15]. We used PCR to quantify the expression of CASP3, BAX and GAPDH Gene.

2.7. DNA Fragmentation

Diphenylamine (DPA) assay Protocol: This approach is based on the idea that centrifugal sedimentation can distinguish substantially fragmented double-stranded DNA from chromosomic DNA. The process includes cell lysis and nuclear DNA release, centrifugation with two fractions (corresponding to intact and fragmented DNA, respectively), DNA precipitation, hydrolysis, and colorimetrical quantification after staining with DPA, which binds to deoxyribose [16]. DPA was used to determine the effect of the tested extracts on DNA fragmentation.

2.8. Statistical methodology:

IBM SPSS (Statistical Package for the Social Sciences) Version 21 will be used for the data analysis (SPSS Inc., Chicago, IL). Mean, standard deviation, median, and range were used to describe numerical data. Quantitative and percentage-based descriptions are for describing the categorized information.

3. Results

3.1. Characterization of PLGA- PEG Nanoparticles

The TEM and SEM images were revealed that formation of PLGA–PEG nanoparticles encapsulating PE in spheroidal shape with average particle size 170±20nm with high homogeneity in size distribution and negative charge by ZETA (fig 1).

3.2. Cell viability (cytotoxicity) assay

The HNO-97 viability was evaluated using the MTT assay. Cell viability was tested in (HNO-97) in control groups (group A) and experimental groups (B1, B2).

Among all groups, the (B2 group) recorded the lowest cell viability at a dose of 100 ug/ml (4.36%); however, the control group A recorded the highest % viability (100%) and the group treated with 0.01 ug/ml with pomegranate B1 group (100%) (figure 2.A, B and C).

However, the different investigated groups showed a considerable dose-dependent reduction in their viabilities when treated with increasing the dose from (0.01 ug/ml - 100 ug/ml). In all groups, the lowest cell viability value was recorded by B2 and B1 groups, whereas group A (control) showed the highest value.

3.2.1. Statistical analysis

We utilized mean and standard deviation to characterize the data using descriptive statistics. Two groups were compared using the percentage of live cells in the concentration (100 ug/ml), and an independent t-test was utilized for the analysis (tables (2) and fig. (3 A), which demonstrated a highly significant difference between groups.

3.3. IC50 Results

The investigations of the current study showed that the PE group presented the highest dose needed to inhibit 50% of cancer cells (6.41ug/ml); meanwhile, the least dose needed to do the same action (1.36u/ml) was offered by PE-PEG-PLGA group (B2). The summary of results of the different IC50% values of different groups is shown in table (3) and fig (3B).

3.4. DNA Fragmentation

Cleavage of chromosomal DNA into oligonucleosomal fragments is a biochemical hallmark of apoptosis, so the nuclear DNA fragmentation of (HNO-97) cells after 24 h of exposure to the interventions in a complete culture medium was done. It is generally known that the caspase family of cysteine proteases is responsible for the chromatin condensation and DNA fragmentation that define apoptosis.

This study demonstrated that DNA fragmentation increased after treatment of (HNO97) cells with various treatments. The apoptotic ratio, an indicator of the rate of DNA degradation, differed significantly between the treated and untreated cells.

The PE-PEG-PLGA group showed the highest percentage of DNA fragmentation (20.51%) compared to the PE treatment group (9.81%) and the control group (4.03%).

3.5. Apoptosis by RT-PCR

Various methods in the present study evaluated apoptosis; the detection of caspase3 and BAX were used. The apoptosis was evaluated in (HNO-97) cells after 24 h of exposure to the intervention in a complete culture medium by caspase 3 and BAX. It is well known that apoptosis is mediated by the cysteine protease family called caspases.

At different IC50% doses, all treated groups recorded upregulation of caspase -3 gene and BAX gene expression relative to control. The highest folds change of caspase -3 and BAX expression was revealed in HNO cells treated with PE- PEG-PLGA (B2) group (3.04 folds, 2.22 folds respectively), followed by the PE group (2.19 folds, 1.65 folds respectively) meanwhile the lowest folds-change of caspase-3 and BAX were found in HNO cell control (1) (A).

3.4.1. Statistical analysis

We utilized mean and standard deviation to characterize the data using descriptive statistics. One-way ANOVA was used to compare groups through the DNA fragmentation, caspase-3 and BAX, which showed a highly significant difference between groups table (4) and fig (3 C).

4. Discussion

Cell cytotoxicity, apoptosis, and DNA damage were all seen in oral cancer cells cultured in the PE and PE- PEG-PLGA groups. Further discussion is provided below on how exposure to PE or PE PEG-PLGA causes cell cytotoxicity.

4.1. The cell viability (cytotoxicity) of PE group

The PE group showed a higher decrease in cell viability than the control group. This finding concludes that PE interacts with tumor cells and downregulates specific carcinogenic proteins. The present work's findings corroborate with a prior study showing that specificity protein (Sp) transcription factor downregulation underlies the cytotoxicity of numerous botanicals (Sp1, Sp3, Sp4). These transcription factors, which control essential

functions including cell proliferation, survival, and angiogenesis, are frequently amplified in malignant tumors[17–19].

The reduction in cell viability of cancer cell treated PE group explained by the polyphenols from pomegranate has demonstrated anti-inflammatory and anticarcinogenic activities through enhancement of the cytotoxic activities of cancer[20,21]. PE group expressed selective cytotoxicity of cancer cells compared to normal cells[8] . In addition, PE significantly reduced cell viability and blocked the cell-cycle progression of a mouse mammary cancer cell line versus control [22].

The cytotoxicity effect of PE has been demonstrated in different reports with different mechanisms and pathways. Still, all of them confirm the therapeutic benefits of pomegranate against different types of cancers.

4.2. The apoptotic effect of PE

PE induce apoptosis in human oral cancer cells through upregulation of the caspase-3 and BAX.

4.2.1. The expression of caspase-3

The gene expression of caspase-3 is measured by RT-PCR and showed the PE-treated group was higher expressed of proapoptotic gene caspase 3 with the IC 50% of PE than the control group (A). The available data conclude that PE induced the apoptosis pathway by activating the caspase-3 gene. The present results are consistent with previous studies that revealed pomegranate-induced apoptosis through the activation of caspase 3[23,24].

4.2.2. The expression of BAX

The PE-treated group expressed a higher proapoptotic genes BAX with IC 50% of PE than the control (A). This finding concluded that Pomegranate extracts induce apoptosis by activating BAX proteins. This agrees with previous studies that revealed the pomegranate extract induces apoptosis through BAX activation[25,26].

4.3. The DNA fragmentation percent of PE

DNA fragmentation percent of PE treated group was measured using Diphenylamine assay; the DNA fragmentation effect of PE treated group (B1) was higher than control (A) at IC 50% concentration of PE. These results are consistent with studies that reported that pomegranate could inhibit the proliferation of breast cancer cells by influencing morphological changes and DNA fragments[27]and inducing Prostate cancer cell apoptosis by induction and DNA fragmentation[28].

The effect of pomegranate may be attributed to polyphenols contents that may interfere with the genome of cancer cells and entrance them to degradation[29].

Another reason is that actin is cleaved during caspase-3 activation, rendering it unable to control deoxyribonuclease (DNase) activity and the subsequent DNA fragmentation it causes. Apoptosis, the most common form of cell death, is mediated by caspase-3, which cleaves proteins necessary for DNA repair and cellular stability[30].

4.4. The cell viability of PE- PEG-PLGA group.

The PE- PEG-PLGA treated group showed higher cell viability decreased (cytotoxicity) than PE and control. The higher cytotoxic effect of PE-PEG-PLGA could be related to the smaller overall dimension of the nano-formulation, thereby facilitating its passive transport into the cell. This finding is inconsistent with a recent study that anticancer drug febuxostat (FBX)-loaded PEG-coated PLGA nanoparticles showed more cytotoxicity than the febuxostat itself due to the nanoparticle size of PEG-coated PLGA nanoparticles[31] .

The enhancement; is the cytotoxicity of pomegranate loaded with PEG-coated PLGA nanoparticles[32] or solid lipid nanoparticles[33] These results indicate that nanoparticles can transport more drugs into the cells than free drugs, leading to decreased cell viability and increased cytotoxicity after 24 hours.

4.5. The apoptotic effect of PE-PEG-PLGA group

PE-PEG-PLGA group induce apoptosis in human oral cancer cells through the upregulation of caspase-3 and BAX.

4.5.1. The expression of Caspase-3 and BAX

The caspase 3 and BAX detected through RT-PCR expressed the highest expression folds. The enhancement of intracellular drug concentration could explain these findings through an increase in the cellular uptake of drugs loaded by the PE-PEG-PLGA group [32]. Moreover, the improvement of apoptosis potential of anticancer drugs loaded with the PEG-PLGA due to drug entrapped may be attributed to diblock copolymer[34]**.**

Adding hydrophilic polyethylene glycol (PEG) to nanoparticles has been found to boost their capacity to overcome the biological barrier, as was shown in a previous explanation. As expected, PEGylation of PLGA led to a greater uptake of larger nanoparticles than smaller, non-PEGylated nanoparticles[35].

4.6. The DNA fragmentation PE- PEG- PLGA group

DPA assay Protocol in cancer cells treated with PE- PEG- PLGA expressed a higher percent of DNA fragmentation than other groups. The DNA fragmented in cancer cells treated with PE- PEG- PLGA group due to intracellular accumulation of polyphenols contents in pomegranate that may interfere with the genome of cancer cells and induce DNA degradation[29].

The increase of DNA fragmentation in cancer cell treated PE- PEG- PLGA group may be due to the promotion of interaction of the nanoparticles with the cells and thus augments the rate and extent of internalization[32] Another explanation may be related to the nanoparticles themselves due to the surface charges or related to the enhancement of cell uptake [36].

In addition, other investigators explained the superior effect of nanoparticle pomegranate regarding metabolism (bioavailability) so that the encapsulation of pomegranate polyphenols into biodegradable sustained-release nanoparticles may enhance the bioavailability[11]. Different binding sites inside the nanoparticles' well-defined structure (spherical shape with excellent particle size) may enable significant quantities of medication to be integrated within the particle[37].

The potent cytotoxic effect of PE against cancer cells cannot ignore. From this point, using pomegranate loaded by PEG-PLGA nanoparticles could provide a compound utilizing their benefits, as it could fight the cancer cell more efficiently.

In conclusion, pomegranate is a rich source of bioactive compounds and has been demonstrated to have anti-cancer properties. Pomegranate is high in ellagitannins, anthocyanins, and hydrolysable tannins and has high antioxidant activity. Studies have shown pomegranates to be a natural alternative to chemical treatment because of their capacity to fight a wide spectrum of infections.

Furthermore, it has been hypothesized that the anticancer effect of nanoparticle pomegranate (nanoparticle delivery of pomegranate) may be related to benefits superior to pomegranate extract by overcoming sensitivity to gastrointestinal hydrolysis, poor absorption, and limited systemic bioavailability.

References

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2018;68(6):394–424.

2. Radwan A, Higazy A, Metwally M, Mousa W, Kandil M, Gamal MA. Single-incision mid-urethral mini slings versus standard transobturator slings in management of women with stress urinary incontinence : a randomized controlled trial. 2020;920–5.

3. Nandini DB, Rao RS, Hosmani J, Khan S, Patil S, Awan KH. Novel therapies in the management of oral cancer: An update. Disease-a-Month [Internet]. 2020;66(12):101036. Available from: https://doi.org/10.1016/j.disamonth.2020.101036

4. Cui C, Yang J, Li X, Liu D, Fu L, Wang X. Functions and mechanisms of circular RNAs in cancer radiotherapy and chemotherapy resistance. Mol Cancer. 2020;19(1):1–16.

5. Lagoa R, Silva J, Rodrigues JR, Bishayee A. Advances in phytochemical delivery systems for improved anticancer activity. Biotechnol Adv [Internet]. 2020;38(March 2019):107382. Available from: https://doi.org/10.1016/j.biotechadv.2019.04.004

6. Gan QX, Wang J, Hu J, Lou GH, Xiong HJ, Peng CY, et al. Modulation of Apoptosis by Plant Polysaccharides for Exerting Anti-Cancer Effects: A Review. Front Pharmacol. 2020;11:792.

7. Usha T, Middha SK, Sidhalinghamurthy KR. Pomegranate Peel and Its Anticancer Activity: A Mechanism-Based Review. Plant-derived Bioact. 2020;223–50.

8. Keta O, Deljanin M, Petković V, Zdunić G, Janković T, Živković J, et al. Pomegranate (Punica granatum L.) peel extract: Potential cytotoxic agent against different cancer cell lines. Rec Nat Prod. 2020;14(5):326–39.

9. Wang B, Guo C, Liu Y, Han G, Li Y, Zhang Y, et al. Novel nano-pomegranates based on astragalus polysaccharides for targeting ERα-positive breast cancer and multidrug resistance. Drug Deliv. 2020;27(1):607–21.

10. Yusefi M, Shameli K, Ali RR, Pang SW, Teow SY. Evaluating Anticancer Activity of Plant-Mediated Synthesized Iron Oxide Nanoparticles Using Punica Granatum Fruit Peel Extract. J Mol Struct [Internet]. 2020;1204:127539. Available from: https://doi.org/10.1016/j.molstruc.2019.127539

11. Shirode AB, Bharali DJ, Nallanthighal S, Coon JK, Mousa SA, Reliene R. Nanoencapsulation of pomegranate bioactive compounds for breast cancer chemoprevention. Int J Nanomedicine. 2015;10:475–84.

12. Stockert JC, Horobin RW, Colombo LL, Blázquez-Castro A. Tetrazolium salts and formazan products in Cell Biology: Viability assessment, fluorescence imaging, and labeling perspectives. Acta Histochem [Internet]. 2018;120(3):159–67. Available from: http://dx.doi.org/10.1016/j.acthis.2018.02.005

13. Buranaamnuay K. The MTT assay application to measure the viability of spermatozoa: A variety of the assay protocols. Open Vet J. 2021;11(2):251–69.

14. Aykul S, Martinez-Hackert E. Determination of half-maximal inhibitory concentration using biosensor-based protein interaction analysis. Physiol Behav. 2017;176(5):139–48.

15. Adams G. A beginner's guide to RT-PCR, qPCR and RT-qPCR. Biochem. 2020;42(3):48–53.

16. Gendimenico GJ, Bouquin PL, Tramposch KM. Diphenylamine-colorimetric method for DNA assay: A shortened procedure by incubating samples at 50°C. Anal Biochem. 1988;173(1):45–8.

17. Kang D, Zuo W, Wu Q, Zhu Q, Liu P. Inhibition of Specificity Protein 1 Is Involved in Phloretin-Induced Suppression of Prostate Cancer. Biomed Res Int. 2020;2020.

18. Jutooru I, Chadalapaka G, Lei P, Safe S. Inhibition of NFκB and pancreatic cancer cell and tumor growth by curcumin is dependent on specificity protein down-regulation. J Biol Chem. 2010;285(33):25332–44.

19. Mertens-Talcott SU, Noratto GD, Li X, Angel-Morales G, Bertoldi MC, Safe S. Betulinic acid decreases ER-negative breast cancer cell growth in vitro and in vivo: Role of Sp transcription factors and microRNA-27a: ZBTB10. Mol Carcinog. 2013;52(8):591–602.

20. Adhami VM, Siddiqui IA, Syed DN, Lall RK, Mukhtar H. Oral infusion of pomegranate fruit extract inhibits prostate carcinogenesis in the TRAMP model. Carcinogenesis. 2012;33(3):644–51.

21. Jonathan Posner and Bradley S. Peterson JAR. Cytotoxicity of Pomegranate Polyphenolics in Breast Cancer Cells in Vitro and Vivo - Potential Role of miRNA-27a and miRNA-155 in Cell Survival and Inflammation. Bone [Internet]. 2008;23(1):1–7. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3624763/pdf/nihms412728.pdf

22. Dai Z, Nair V, Khan M. Pomegranate extract inhibits the proliferation and viability of MMTV-WNT-1 mouse mammary cancer stem cells in vitro. Oncol Rep. 2010;24(12):1087–91.

23. Abdul Ghani R, Nurasyikin Nik Abdul Malek N, Abdullah N. Pomegranate Juice Induced Cell Cycle Arrest and Apoptosis via Mitochondrial Pathway in Human Lung Adenocarcinoma A549 Cells. Int J Eng Technol. 2018;7(3.30):309.

24. Bishayee A, Mandal A, Bhattacharyya P, Bhatia D. Pomegranate exerts chemoprevention of experimentally induced mammary tumorigenesis by suppression of cell proliferation and induction of apoptosis. Nutr Cancer [Internet]. 2016;68(1):120–30. Available from: http://dx.doi.org/10.1080/01635581.2016.1115094

25. Dikmen M, Ozturk N, Ozturk Y. The antioxidant potency of punica granatum L. fruit peel reduces cell proliferation and induces apoptosis on breast cancer. J Med Food. 2011;14(12):1638–46.

26. Deng Y, Li Y, Yang F, Zeng A, Yang S, Luo Y, et al. The extract from Punica granatum (pomegranate) peel induces apoptosis and impairs metastasis in prostate cancer cells. Biomed Pharmacother [Internet]. 2017;93:976–84. Available from:

http://dx.doi.org/10.1016/j.biopha.2017.07.008

27. Manasathien J, Indrapichate K. Apoptosis of mcf-7 cancer cell induced by pomegranate (Punica Granatum L.) Peel extract. Suranaree J Sci Technol. 2017;24(1):63–74.

28. Amri Z, Kharroubi W, Fidanzi-Dugas C, Leger DY, Hammami M, Liagre B. Growth Inhibitory and Pro-Apoptotic Effects of Ornamental Pomegranate Extracts in Du145 Human Prostate Cancer Cells. Nutr Cancer [Internet]. 2020;72(6):932–8. Available from: https://doi.org/10.1080/01635581.2019.1660385

29. González-Sarrías A, Espín JC, Tomás-Barberán FA, García-Conesa MT. Gene expression, cell cycle arrest and MAPK signalling regulation in Caco-2 cells exposed to ellagic acid and its metabolites, urolithins. Mol Nutr Food Res. 2009;53(6):686–98.

30. Larsen BD, Rampalli S, Burns LE, Brunette S, Dilworth FJ, Megeney LA. Caspase 3/caspase-activated DNase promote cell differentiation by inducing DNA strand breaks. Proc Natl Acad Sci U S A. 2010;107(9):4230–5.

31. Alfaifi MY, Shati AA, Elbehairi SEI, Fahmy UA, Alhakamy NA, Md S. Anti-tumor effect of PEG-coated PLGA nanoparticles of febuxostat on A549 non-small cell lung cancer cells. 3 Biotech [Internet]. 2020;10(3):1–10. Available from: https://doi.org/10.1007/s13205-020-2077-x

32. Afshari M, Derakhshandeh K, Hosseinzadeh L. Characterisation, cytotoxicity and apoptosis studies of methotrexate-loaded PLGA and PLGA-PEG nanoparticles. J Microencapsul. 2014;31(3):239–45.

33. Badawi NM, Hteaima MH, El-Say KM, Aattia DA, Ael-Nabarawi MA, Elmazar MM. Pomegranate extract-loaded solid lipid nanoparticles: Design, optimization, and in vitro cytotoxicity study. Int J Nanomedicine. 2018;13:1313–26.

34. Alhakamy NA. Development and Evaluation of Icariin-Loaded PLGA-PEG Nanoparticles for Potentiation the Proapoptotic Activity in Pancreatic Cancer Cells. AAPS PharmSciTech [Internet]. 2021;22(8):1–11. Available from: https://doi.org/10.1208/s12249-021-02111-w

35. Luo L, Tam J, Maysinger D, Eisenberg A. Cellular internalization of poly(ethylene oxide)-b-poly(ε-caprolactone) diblock copolymer micelles. Bioconjug Chem. 2002;13(6):1259–65.

36. Mirakabad FST, Nejati-Koshki K, Akbarzadeh A, Yamchi MR, Milani M, Zarghami N, et al. PLGA-based nanoparticles as cancer drug delivery systems. Asian Pacific J Cancer Prev. 2014;15(2):517–35.

37. Bronze-Uhle ES, Costa BC, Ximenes VF, Lisboa-Filho PN. Synthetic nanoparticles of bovine serum albumin with entrapped salicylic acid. Nanotechnol Sci Appl. 2017;10:11–21.

Figure caption

Fig (1).

A. Photomicrograph showing the size of PE-loaded in PEG-PLGA through TEM.
B. Photomicrograph showing the shape of PE-loaded in PEG-PLGA through SEM.
C. Photomicrograph showing the zeta potential of PE through ZETA.
D. Photomicrograph showing the zeta potential of PE-loaded in PEG-PLGA through ZETA

Fig (2).

A. Photomicrograph showing many malignant epithelial cells with prominent cell outlines or boundaries on HNO-97 oral squamous cell line without any treatment. (x10 inverted phase Contrast Microscope filter).

B. Photomicrograph showing the cell viability of on the (HNO-97) cell line that was treated with different concentrations of PE t for 24 h. the reduction in cell numbers loss cell outline (cell activity) is increase with PE dose increase. (X10 magnification of inverted phase contrast microscope filter)**.**

C. Photomicrograph showing the cell viability of on the (HNO-97) cells were treated with different concentrations of PE- PEG-PLGA for 24 h. The reduction in cell numbers loss cell outline (cell activity) is increase with dose increase. (X10 magnification of inverted phase contrast microscope filter)

Fig (3)

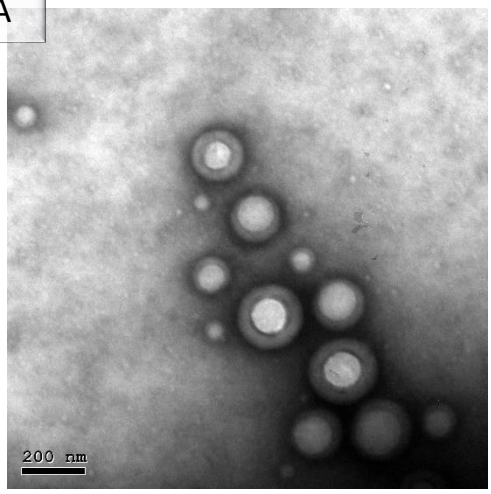
A. A clustered column chart comparing the viability % across the PE group and PE-PEG-PLGA group.

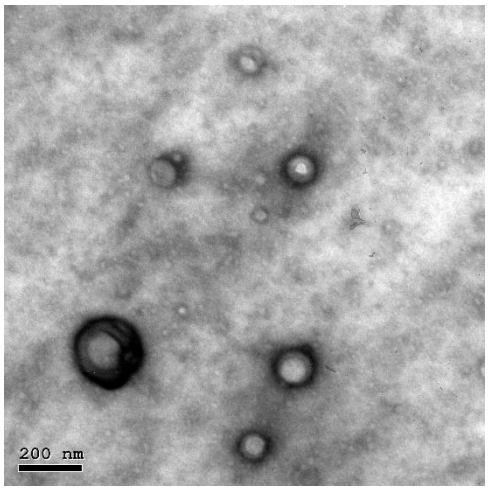
B. bar chart illustrated the different IC50% values of PE group and PE-PEG-PLGA group.

C. bar chart illustrated the DNA fragmentation percent, caspase-3 and BAX among the PE group and PE-PEG-PLGA group.

Figure 1
A,B,C and D

A





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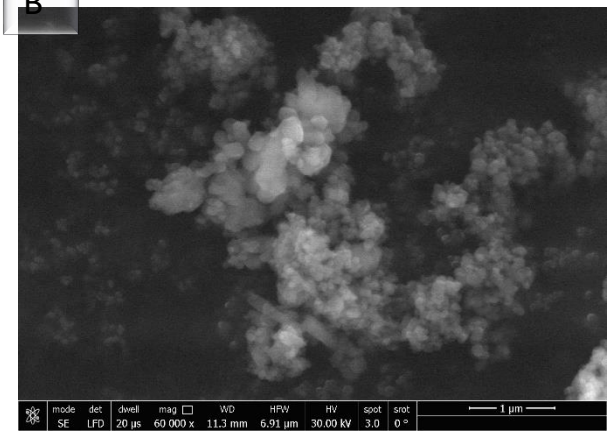
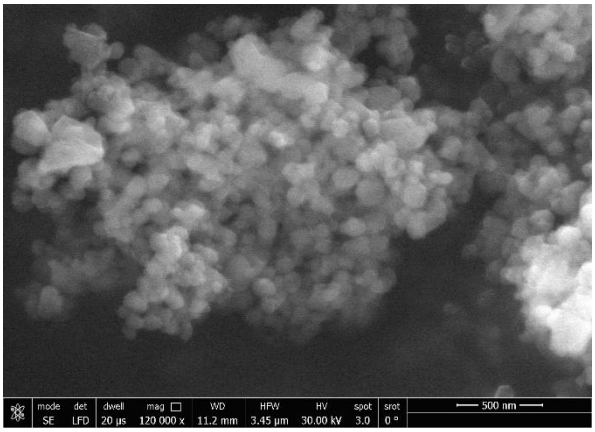
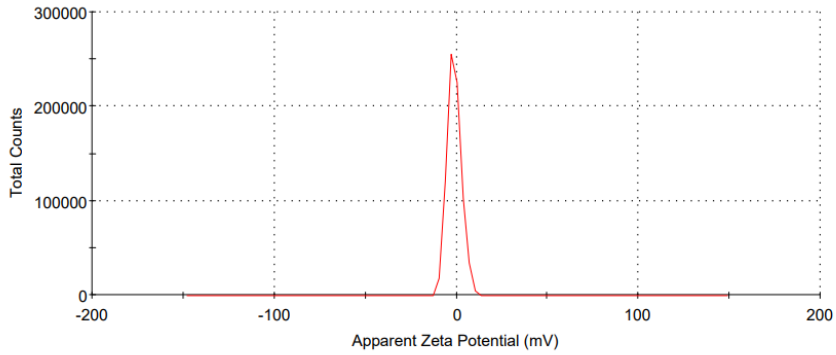




C



D

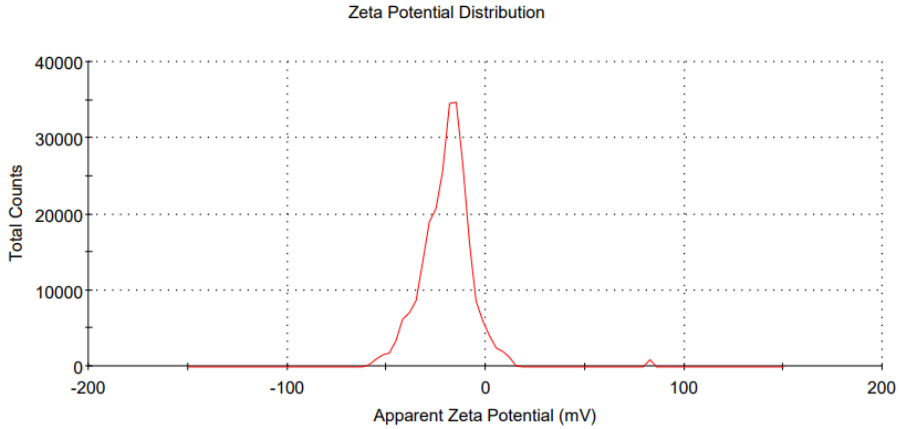
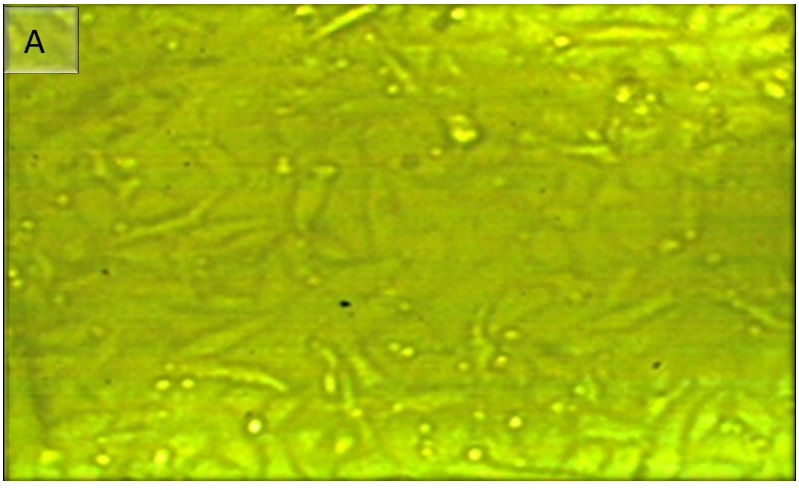
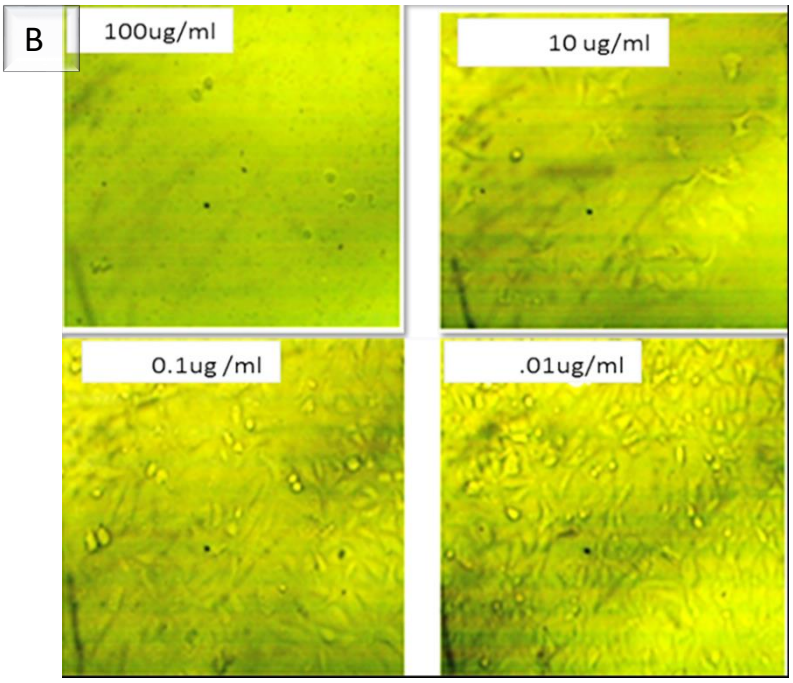


Figure 2

A, B and C

A




B
100ug/ml
10 ug/ml
0.1ug /ml
.01ug/ml

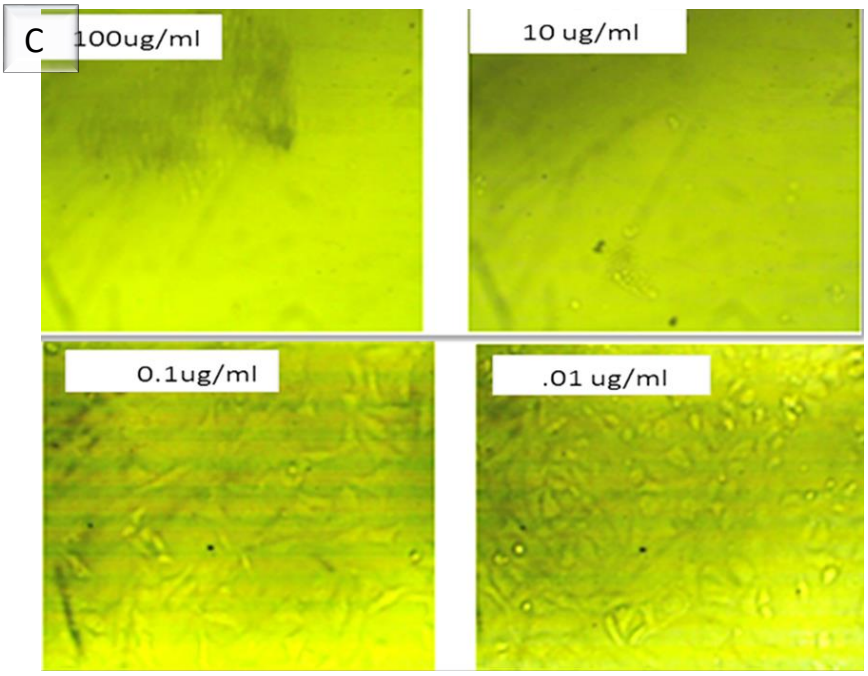
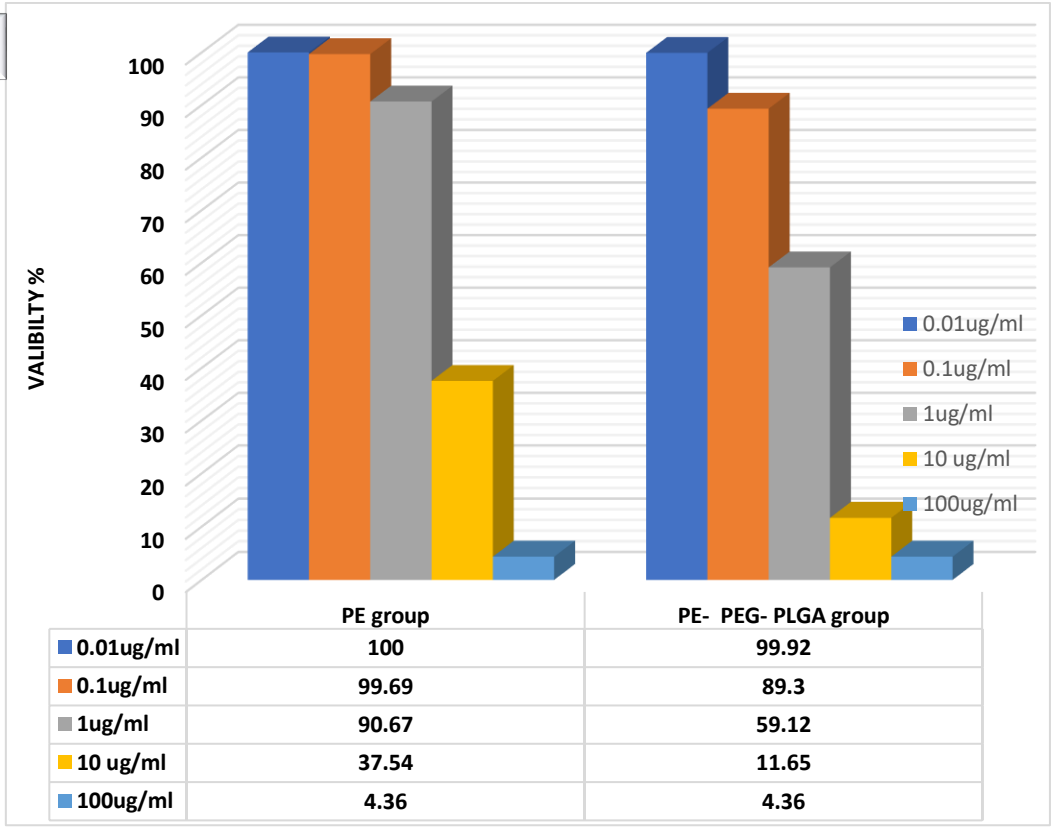

C
100ug/ml
10 ug/ml
0.1ug/ml
.01 ug/ml

Figure 3

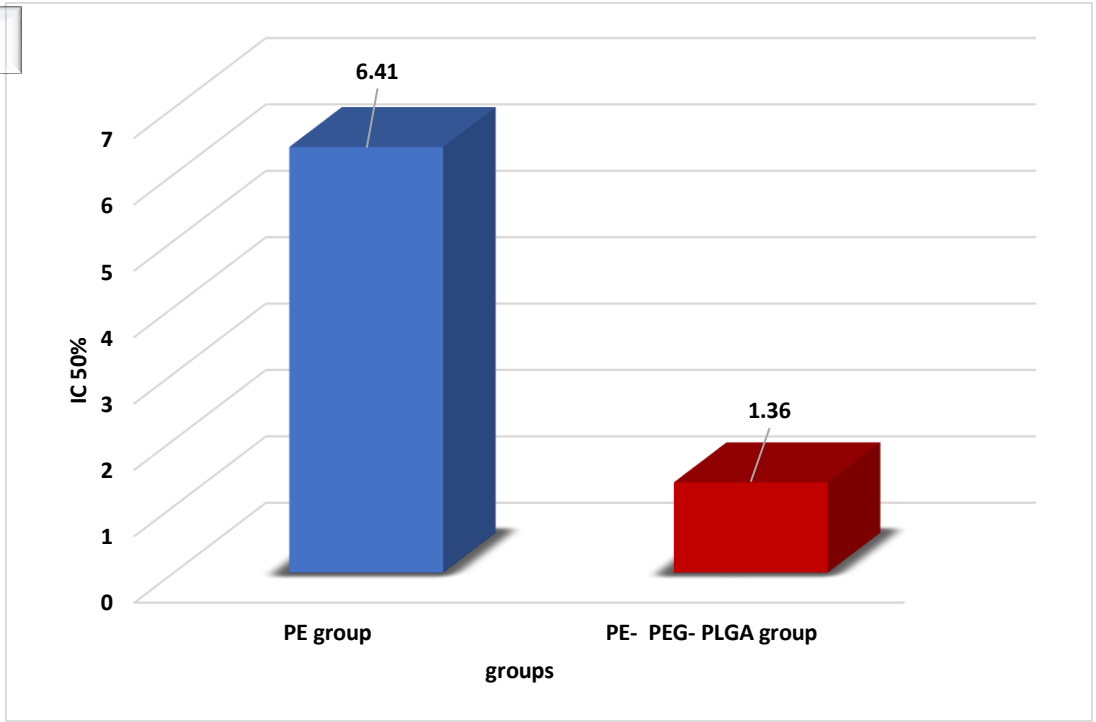
A, B and C

A



	PE group	PE- PEG- PLGA group
0.01ug/ml	100	99.92
0.1ug/ml	99.69	89.3
1ug/ml	90.67	59.12
10 ug/ml	37.54	11.65
100ug/ml	4.36	4.36

B



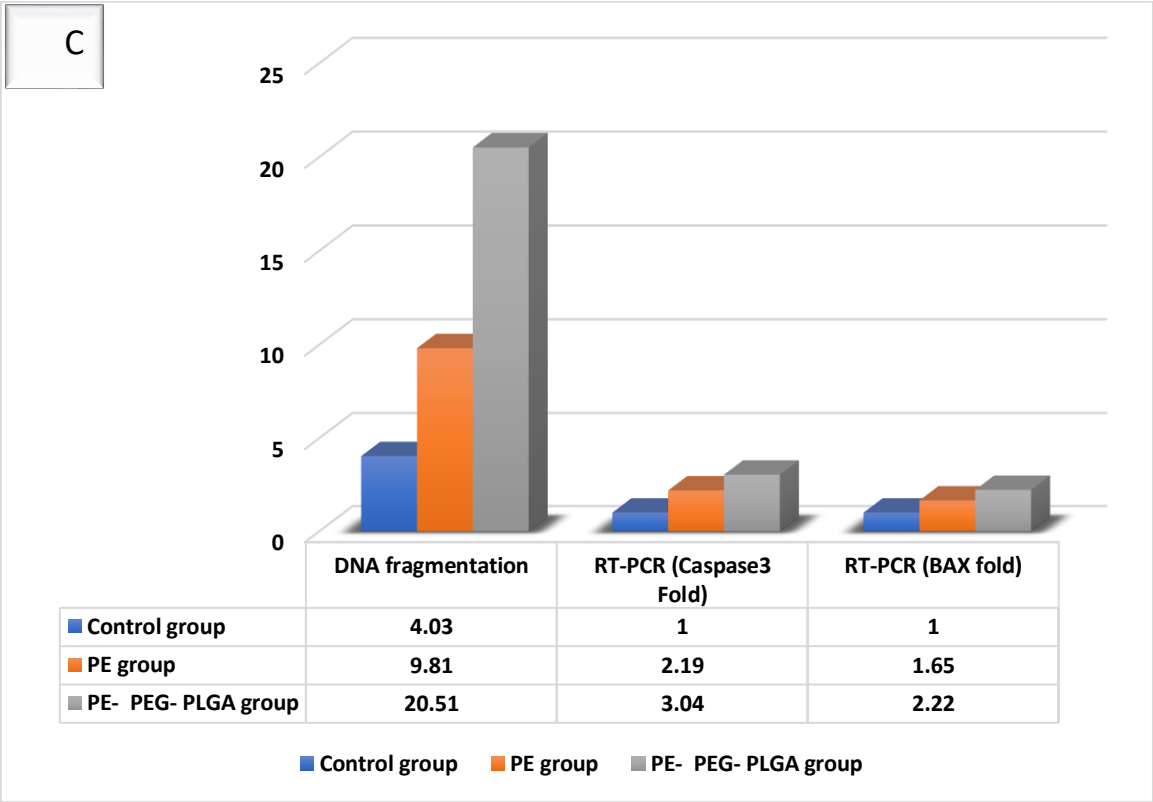


Table (I). Primers sequence of CASP3, BAX and GAPDH Gene

Primer	Sequence from 5'- 3'
CASP3	F 5′-CTCGGTCTGGTACAGATGTCGA-3′
	R 5′-CATGGCTCAGAAGCACACAAAC-3
BAX	F 5′-GTGTATGAGCGTGTTCGT-3′
	R 5′-GAGTCGGCTGAAGATTAGAG-3′
GAPDH	F 5'-GCAAGTTCAACGGCACGATCAAG-3'
	R 5′-CTACTCAGCACCAGCATCACC-3’

Table (II). Mean and SD of cell viability of cancer cell treated with 0.01-100 ug/ml of different drugs (independent t - test).

Viability Test at 0 .01-100 ug/ml				
Concentration	**PE**	**PE-PEG-PLGA**	Independent t-test	
	Mean (%) ±S.D	Mean (%) ±S.D	t	p-value
0.01	100±1.99	99.92±1.41	0.080	0.938
0.1	99.69±1.19	89.30±1.16	15.215	0.000**

1	90.67±1.16	59.12±1.15	47.312	0.000**
10	37.54±0.92	11.65±0.22	67.068	0.000**
100	4.36±0.12	4.36±0.12	0.0001	1.000

(Table III): Mean and SD of IC50% in different groups (Independent t-test).

IC 50%			
Groups	Mean ±S. D	Independent t-test	
		t	p-value
PE group	6.41±0.06	34.834	0.000**
PE-PEG-PLGA group	1.36±0.35		

Table (VI): Mean and SD of DNA fragmentation, caspase-3 and BAX different groups (One-way ANOVA - test).

DNA fragmentation					
Groups	Mean (%) ±S. D	M.W ug/mol	One way ANOVA		Multiple comparison (Tuckey test)
			F	p-value	
Control group	4.03±0.22	-------	1569.999	0.000**	Control vs. PE → 0.000**
PE group	9.81±0.56	6.41			Control vs. PE-PEG-PLGA →0.000**
PE-PEG-PLGA group	20.51±1.16	1.36			PE vs. PE-PEG-PLGA →0.000**
RT-PCR (Caspase3 Fold)					
Control group	1	-------	803.528	0.000**	Control vs. PE → 0.000**
PE group	2.19±0.14	6.41			Control vs. PE-PEG-PLGA →0.000**
PE-PEG-PLGA group	3.04±0.06	1.36			PE vs. PE-PEG-PLGA →0.000**
RT-PCR (BAX fold)					
Control group	1	-------	231.275	0.000**	Control vs. PE → 0.000**
PE group	1.65±0.15	6.41			Control vs. PE-PEG-PLGA →0.000**
PE-PEG-PLGA group	2.22±0.08	1.36			PE vs. PE-PEG-PLGA →0.000**

There is a significant at P-value< 0.05 (*), and highly significant at P-value< 0.000 (**).