Everolimus loaded NPs with FOL targeting: preparation, characterization and study of its cytotoxicity action on MCF-7 breast cancer cell lines

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ABSTRACT

Background and objective: The mortality cases of breast cancer (BC) are probably caused by inadequate of the benefits of treatment and early detection, moreover the lack of appropriate facilities for diagnosis and detection, also high cost effective of the treatment. The preparation of Everolimus (EV) loaded PLGA-TPGS NPs (nano-EV) targeting with folate (PLGA-TPGS-EV-FOL) and investigate their toxicity effect on human MCF-7 BC cell lines may be given an appropriate lines in BC treatment. **Methods:** EV loaded NPs were prepared by combination of sonication and emulsification/solvent evaporation method with slight modifications into four formulations A, B, C , and D. EV loaded NPs were characterized by FESEM and TEM for particle size (PS) and zeta potential (ZP), FT-IR, drug loading (DL%), encapsulation efficiency (EE%), In vitro drug release, inhibition concentration IC_{50} and cell viability%, apoptosis, angiogenesis, glucose transporting, and HIF-1A mRNA gene expression. **Results:** Formulation B of EV loaded NPs was found to be lowest PS 100±12.7 nm, PDI 0.152, and have ZP, -23.2.5. The higher DL% content of EV was detected as 7.32±1.1% and EE% was 87± 2.6% in formulation B. The results suggested that the free EV dissolution was 80% within 4h but release profile of A, B, C, and D formulations within 4h were 34, 23, 26, 30%, respectively. EV formulated in NPs showed better effects against the MCF-7 BC cell line than EV free and EV loaded NPs formulation B achieved even better therapeutic effect than A, C, and D formulations. HIF-1A mRNA gene expression was decreased when treated the MCF-7 BC cells with EV loaded NPs formulation B compare to expression in untreated cells. **Conclusion:** EV loaded NPs may be useful in lowering the cost of treatment and may be involved in the decline of chemotherapy side effects.
Keywords: Breast cancer, PLGA NPs, MCF-7, Everolimus, mTOR inhibitors.

Abbreviations: BC: breast cancer, HIF-1A: hypoxia inducible factor-1 alpha, PLGA: Poly lactic-co-glycolic acid, TPGS: D-α-Tocopheryl Polyethylene Glycol Succinate, Ev: everolimus, FOL: folate, PS: particle size, ZP: zeta potential, PDI: polydispersity index DL: drug loading, EE: encapsulation efficiency, PBS: phosphate buffer saline, MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, AC: acetone, AN: acetonitrile, DCM: dichloromethane, EA: ethyl acetate, DMSO: dimethyl sulphoxide, FESEM: Field Emission Scanning Electron Microscopy.

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1-Introduction

BC is a highly prevalent and mortality type of cancer among women worldwide (1). The mortality cases are probably caused by an inadequate of the benefits of treatment and early detection, moreover the lack of appropriate facilities for diagnosis and detection, also high cost effective of the treatment (2). The particle materials with various shape, and size (diameters between 1-100 nm) and prepared from organic or inorganic materials have been called nanoparticles (3). Hydrophilic small drugs, hydrophobic small drugs, vaccines and biological macromolecules are a wide variety of drugs that can be delivered by using nanoparticles (4). The maximizing drug efficacy and minimizing cytotoxicity are the most things that focuses by nanoparticle drug delivery system (5). The one of the most common synthetic polymeric nanoparticles is Poly (lactic-co-glycolic acid) (PLGA), its successfully used as biodegradable polymer because its hydrolysis leads to metabolite molecules, lactic acid and glycolic acid (6). TPGS (D-α-tocopheryl polyethylene glycol succinate) is a water-soluble derivative of natural Vitamin E, which is formed by esterification of Vitamin E succinate with polyethylene glycol (PEG). As such, it has advantages of PEG and Vitamin E in application of various nanocarriers for drug delivery. TPGS has been widely used in wetting, emulsification, solubilization, spreading, and detergency as one of the novel nonionic surfactants (7). High drug encapsulation efficiency, sustained release behavior and improved therapeutic effects are factors that can be achieve when used PLGA-TPGS as a polymer for drug delivery system (8). Serine/ threonine protein kinase that belongs to the phosphoinositide 3-kinase (PI3K)-related kinase family and interacts with several proteins are named mTOR complex to form two distinct complexes (mTORC1) and 2 (mTORC2) (9). The mTOR inhibitor that recently approved by the Food and Drug Administration (FDA) and European Medicines Agency (EMA) for treatment of renal cell carcinoma (RCC) is everolimus (10). The aims of this study to assessment of

PLGA- EV-TPGS-FOL as EV loaded NPs for inhibition of both, mTOR and gene expression of HIF-1A mRNA and investigate the effects of low dose of EV loaded NPs in the angiogenesis, glucose transporting, and apoptosis in MCF-7 BC cell lines.

2. Materials and Methods

2.1. Materials

PLGA (50:50), TPGS, and TPGS-FOL were purchased from Beijing (China). Everolimus (purity ≥95%, CAS No: 159351-69-6), folic acid, PBS, and tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (for MTT test) were bought from Sigma-Aldrich. Other solvents such as acetone(AC), acetonitrile (AN), dichloromethane (DCM), ethyl acetate (EA), dimethyl sulphoxide (DMSO) and media were bought from BDH (England).

2.2. Preparation of EV loaded NPs

EV loaded NPs were prepared by combination of sonication and emulsification/solvent evaporation method with slightly modifications (17). The summary of protocol that used in 1:20 drug /polymer ratio (DPR)(W/W%) of PLGA-EV-TPGS-FOL NPs formulation A preparation is in briefly, 100 mg of PLGA was dissolved in 10 ml of acetone (AC). Polymer was letting to complete dissolving for overnight. Five mg of Everolimus (EV) was dissolved in 1 ml of AC and added to encapsulant directly to the polymer solution with stirring until encapsulant is homogenously dispersed. The polymer and drug solution was then added to 20 ml of 0.03% w/v aqueous solution of vitamin E-TPGS and TPGS-FOL complex with covering and stirring to 300 rpm for 12 h. Immediately the emulsified polymer was transferred to the ultra-sonication under ice water for 10 sec. Folate was prepared by dissolving 0.03 g in 1 ml AC and add to above solution drop by drop with stirring for three hours. The emulsion polymer was then evaporated overnight to remove any AC. The solid NPs was centrifuged, filtered, dried, and then kept the NPs in the fridge. Five mg of dry and solid EV loaded NPs were re-suspended in 10 ml deionized water

(DW) for different types of analysis in this study.

2.3. Characterization of the EV loaded NPs

2.3.1. Particle size (PS), Zeta potential (ZP), and Polydispersity Index (PDI)

The re-suspension PLGA-EV-TPGS-FOL NPs were mixing well and sonication. EV loaded NPs solution were analyzed by dynamic light scattering (DLS) technique for determination of ZP, PS, and PDI.

2.3.2. DL% and EE%

DL% and EE% of the prepared PLGA-EV-TPGS-FOL NPs were quantified by measuring the absorbance at 356 nm by using spectrophotometer (Analytikjena/ Germany). One mg of freeze and dried EV loaded NPs samples were dissolved in DCM (10ml) for spectrophotometric measurement. EV loaded NPs solutions of various formulations in DMSO (5–50 μg/ml) were prepared, and the absorbance at 356 nm was measured at different concentrations to generate a standard calibration curve ($R2 = 0.963$). DL% and EE% were calculated from following equations:

EE %= Wt. of EV for preparing formulation - Wt. of EV in supernatant/ Wt. of EV for preparing formulation × 100

DL %= Wt. of EV for preparing formulation - Wt. of EV in supernatant/polymer wt. – Wt. of EV for preparing formulation × 100

2.3.3. FESEM analysis

The surface morphology of EV loaded NPs was observed using field emission scanning electron microscope FESEM. The NPs samples were sputter-coated with platinum (4–5 nm) at a current intensity of 40 mA for 40 s. The images were captured keeping the accelerating voltage between 1–5 kV.

2.3.4.TEM analysis

A one drop (10 μl) of EV loaded NPs suspension was placed carefully on a 300 mesh carbon coated copper TEM grid. The excess solution on the grid was removed using a fine piece of filter paper and the samples were air-dried for 10 h and the dried sample was then examined at 120 kV under a microscope.

2.3.5. FT-IR analysis

PLGA, EV, and TPGS-FOL were scanned over a wave number range of 400–4000 cm^{-1} in an inert atmosphere in an FT-IR spectrophotometer (without drug). PLGA-EV-TPGS-FOL NPs were scanned over a wave number range of 4000–400 cm−1 in an inert atmosphere in an FT-IR spectrophotometer (with drug).

2.4. In vitro drug release

One mg of NPs were weighted and resuspended in 20 ml of phosphate buffered saline (PBS) (pH 7.4) with dialysis bag and stirring on a magnetic stirrer (100 rpm) at 37 °C. At preselected time intervals, 3 ml of the release media was taken out and centrifuged (6000 rpm) to collect of supernatant. To the pellet containing NPs with unreleased EV, an equal volume of fresh media was added (3ml PBS was added to replace 3 ml that taken). The collected supernatants containing released EV were then analyzed by HPLC. The EV release % at sample time was calculated depending on the following equation:

EV release %= [EV] initial – [EV] sample/ [EV] initial ×100

HPLC conditions were: Stationary Phase: C18 column, 5μm, 4.6 ×150mm and Mobile phase: water: acetonitrile (30:70)(pH 4.8 ammonium acetate solution, flow rate was 0.8 ml/min, and detector type: UV at λ= 365 nm.

2.5.Long term stability study

In closed glass vials, 1 mg of samples of EV loaded NPs formulations A, B, C, and D were stored for 30 days at RT (25-26 C°) away from direct sunlight exposure by covering with appropriate piece of aluminum foil. The formulations were assayed periodically, at the time points of 0, 10, 20, 30,40, 50, and 60 days, for particle size (PS) and drug encapsulation efficiency (EE%).

2.6. Blood hemolysis study

Freshly collected 0.5 ml of blood sample from control group were taken in heparinized tube and centrifuged (5 min at 1000 g) at 4C°. The supernatant was discarded and

erythrocytes were washed three times with PBS (pH 7.4) to form suspension.190 µl of the suspension was added to each well of a 96-well plate and treated with 10 µl of EV loaded NPs (A,B,C, and D formulations). The negative control was prepared by adding 10 µl of PBS to 190 µl of erythrocyte suspension. The positive control was prepared by adding 10 µl of TritonX-100 (10%) to 190 µl erythrocyte suspension. The plate was incubated at 37 °C for 1 h and 24 h with gentle stirring, the unlysed erythrocytes were separated by centrifugation at 10,000 g for 5min and the optical density (OD) of the supernatant was measured at 570nm. Hemolysis effect of EV NPs was calculated by the following equation:

Blood Hemolysis (BH)%= (Abs sample - Abs negative control)/(Abs positive control - Abs negative control) *100

2.7. Cytotoxicity studies of EV loaded NPs

In vitro cytotoxicity study of free EV and EV-loaded NPs was quantitatively measured by employing on MCF-7 breast cancer cells. MCF-7 cells were cultivated in RMPI 1640 medium without FOL, which was supplemented with 10% FBS and 1% antibiotics at 37 C° in humidified environment of 5% CO2. The cells were seeded in 96-well plate and then the media were changed with various concentrations of free EV (5mg/dl) or EV-loaded NPs (PLGA-TPGS-EV-FOL) in different four formulations A, B, C, and D and treated for 24, 48, and 72 h. In vitro cell viability was determined by using the MTT assay. At predetermined time intervals, the media were removed and the wells were washed two times with PBS. The cells were incubated with 90 µl medium and 10 µl MTT (5 mg/ml in PBS) for 3–4 h and the precipitant was dissolved in 100 µl isopropanol before analyzed by micro plate reader and reading the absorbance at 570 nm. Untreated cells represent the control cells (not exposed to the PLGA NPs). Percentage of viability, cytotoxicity, and IC_{50} were calculated by the following equations:

Cell viability(%) = (Abs treated cells/Abs control cells)×100

Cytotoxicity (%)= 100- cell viability%

IC_{50}= {EV dose(w/w)*100/cytotoxicity%}/2

2.8. Apoptosis, Angiogenesis and Glucose Transporting study

This part was performed by assessment of CD44, VEGF, and GLUT-1 as a markers of apoptosis, angiogenesis, and glucose transporting, respectively. The procedures using here were described by ELISA protocol kits and the samples were homogenized cells.

2.9. GEF of HIF-1A mRNA in MCF-7 BC cell lines after incubation with EV loaded NPs.

This part was performed by assessment of GEF of HIF-1A mRNA in cell lines after incubation with PLGA-EV-TPGS-FOL NPs formulations in different time 24, 48, and 72 h. The cells then were harvested to RNA extraction and cDNA synthesis and the amplification of HIF-1A (gene of interest GOI-1) , GAPDH (housekeeping gene HKG), and FOLR (gene of interest GOI-2) mRNA genes were done by qRT-PCR by using specific designed primers.

2.10. Statistical Analysis

Statistical analysis were applied when possible by using SPSS version 20 and data were expressed as (mean ± SD). The normality of the distribution of all variables was assessed by the student's ANOVA test and person correlation analyses that have been used to determine the significant difference between the groups. Graphs were prepared using Microsoft excel 2010. P-values less than (0.05) was considered significant and less than (0.001) was considered highly significant.

3. Results

3.1. Particle size (PS), Zeta potential (ZP), and Polydispersity Index (PDI)

Table 1, showing mean ±SD of the Zeta Potential (ZP), particle size (PS) , and Polydispersity Index (PDI) of PLGA-TPGS-EV-FOL NPs of A,B,C, and D formulations:

Table (1): ZP, PS, and PDI of EV loaded NPs of A,B,C, and D formulations

Formulation type	PDR	Organic phase	PS (nm) mean±SD n=3	PDI mean±SD n=3	ZP(mV) mean±SD n=3
A	1:20	AC	108±13.8	0.213±0.014	-22±2.2
B	1:20	DCM	100±12.7	0.152±0.018	-23±2.5
C	1:10	AN	177±12.2	0.293±0.023	-34±2.4
D	1:5	EA	196±13.5	0.562±0.018	-38±2.9

3.2 Drug Lauding (DL%) and Encapsulation Efficiency(EE%):

The higher DL% content of EV in PLGA-TPGS-EV-FOL NPs was detected as 7.32±1.1% and EE% was 87±2.6% in formulation B, as shown in table 2:

Table (2): (DL%) and (EE%) of EV in A,B,C, and D formulations

Formulation type	DPR	EV mg	Organic phase	DL (%) mean±SD n=3	EE (%) mean±SD n=3
A	1:20	5	AC	6.72±1.2	79±3.8
B	1:20	5	DCM	7.32±1.1	87±2.6
C	1:10	10	AN	5.71±1.3	77±2.9
D	1:5	20	EA	5.11±1.6	66±3.6

3. 3. EV ,PLGA, and TPGS-FOL interaction study

The effects of EV encapsulation on the chemical group of the formed components and the interaction between the components were studied by FT-IR. The FT-IR spectra of PLGA, TPGS-FOL complex, and PLGA-TPGS-EV-FOL NPs as EV loaded polymer indicated that there was no change in the position of absorption peaks, as shown in figure 1.
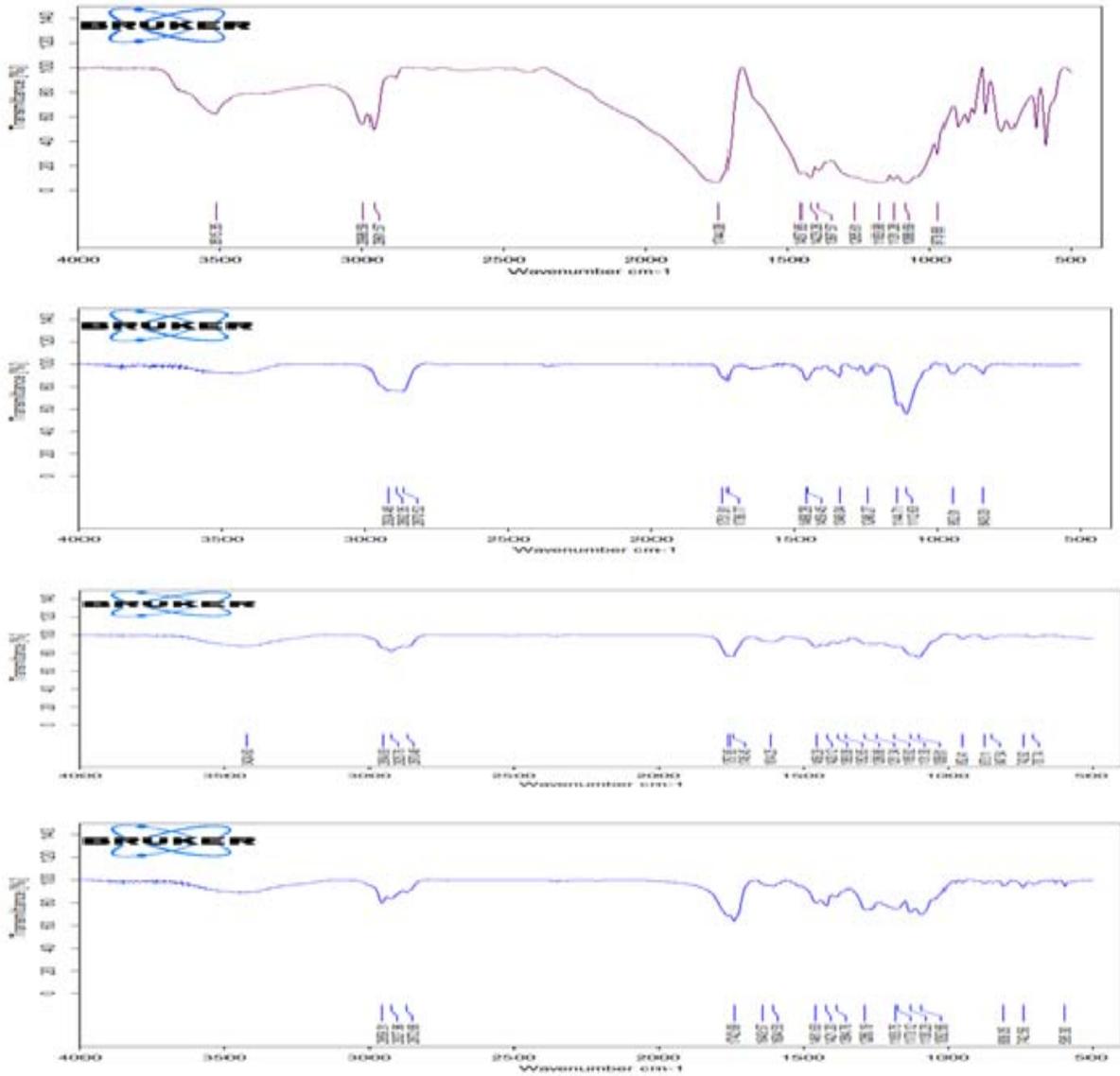
Figure 1. FT-IR spectrum of PLGA, free EV, TPGS-FOL, and PLGA-TPGS-EV-FOL NPs, respectively.

3. 4. Field Emission Scanning Electron Microscopy (FESEM) and TEM

Figure 2, showing the FESEM TEM images that revealed that PLGA-TPGS-EV-FOL NPs shapes were approximately spherical.
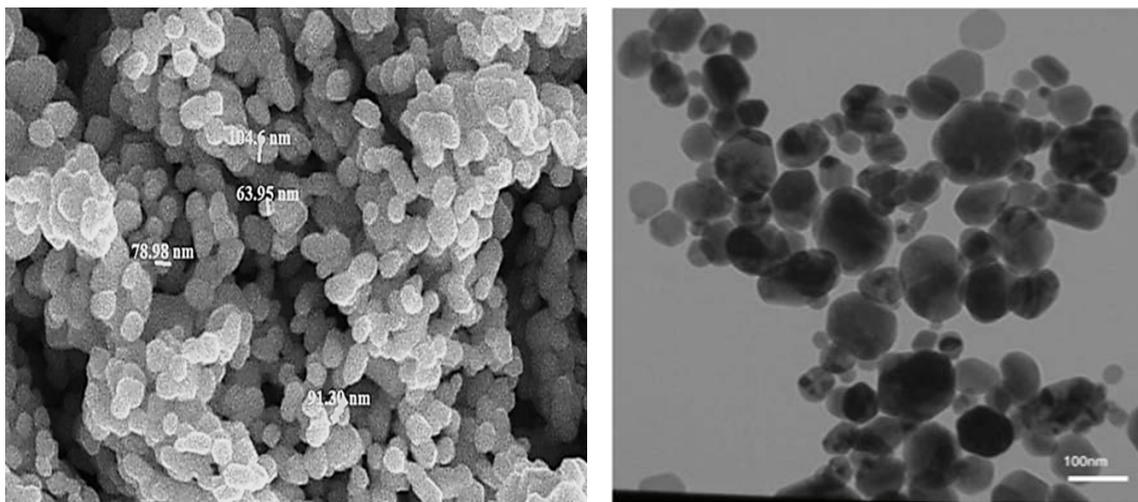
Figure 2. FESEM and TEM images of EV loaded PLGA NPs of B formulation

3.5. *In vitro* drug release

Figure 4, shows the EV release profile from the EV loaded PLGA-TPGS-EV-FOL NPs of all formulations, the release profile were compared with the dissolution profile of the free EV in the same pH media and an equal concentration. The free EV dissolution was 80% within 4h but release profile of A, B, C, and D were within 4h were 34,23,26,30%, respectively.
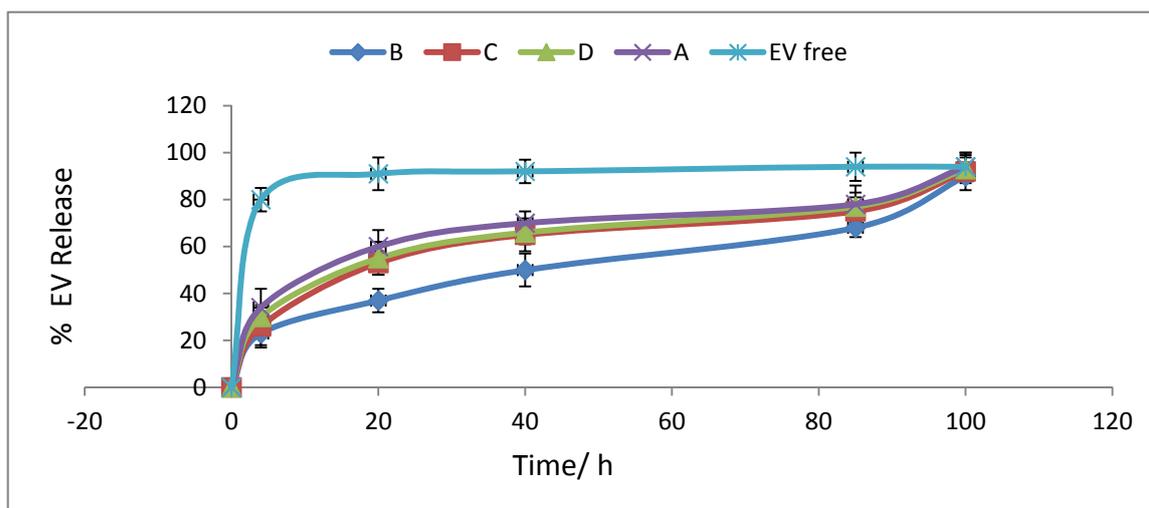

Figure3. *In vitro* drug release profile of encapsulated EV from PLGA NPs at 37Cº and each data point represents the mean ± SD (n = 3).

3.6. Long term stability study

Figure 4 , represents the influence of long term storage on stability of PLGA-TPGS-EV-FOL NPs in the four formulations in different storage time (0,10.20,30,40,50, and 60 days) at RT 25-26 Cº.
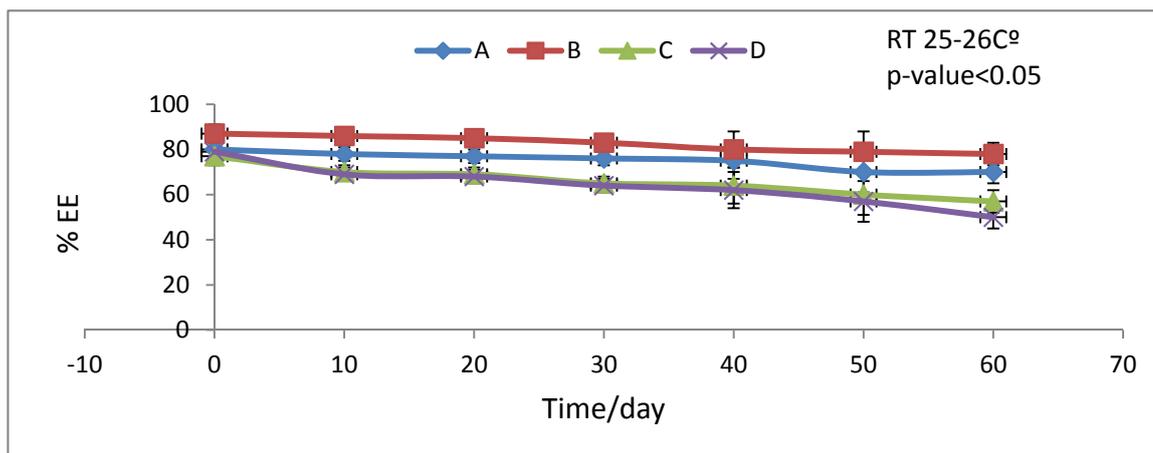
Figure 4. Effects of storage time on EE% of NPs

3.7 *In vitro* blood hemolysis study

To explain the impact of PLGA-TPGS-EV-FOL NPs on blood components, in vitro hemolysis study was done on fresh blood samples of control and women with BC and the results were same on both samples. Intravenous injection or orally administration of nanoparticles for drug delivery targeting system needs to performed this study. Table 3, shows the hemolysis percent of EV loaded NPs of all formulations on blood samples of women with BC:

Table (3): Blood hemolysis BH% of EV free and loaded NPs of all formulations on blood samples of women with BC

Sample N=3	1h incubation Hemolysis % mean±SD	24h incubation Hemolysis % mean±SD	CI (95%)	p-value
EV free	0.022±0.0001	2.112±0.002	2.07-2.08	0.0001
A	0.017±0.002	0.0195±0.002	-0.0049-0.0004	0.404
B	0.023±0.001	0.022±0.001	-0.0078-0.0058	0.760
C	0.024±0.002	0.0249±0.002	-0.0043-0.0003	0.070
D	0.023±0.002	0.031±0.002	-0.0025-0.0065	0.287

Figure 5, shown the BH % profile of PLGA-TPGS-EV-FOL NPs of A, B, C, and D formulations. From figure the B formulation seems to be the best one because of no change in blood hemolysis % at end of 60 min of time.
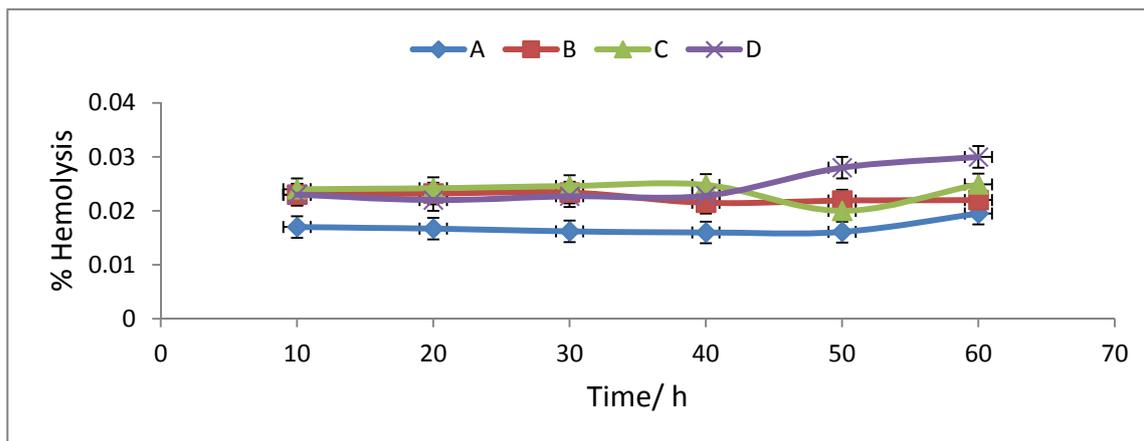
Figure 5.Hemolysis profile of EV loaded NPs formulations

3.8. *In vitro* cytotoxicity of PLGA-EV-TPGS-FOL NPs on MCF-7 BC cell lines.

It can be concluded from figure 7, that in general the EV formulated in PLGA–TPGS-EV-FOL NPs showed better effects against the MCF-7 BC cell lines than EV free and PLGA–TPGS-EV-FOL NPs formulation B achieved even better therapeutic effect than A, C, and D formulations, as shown in figure 6.
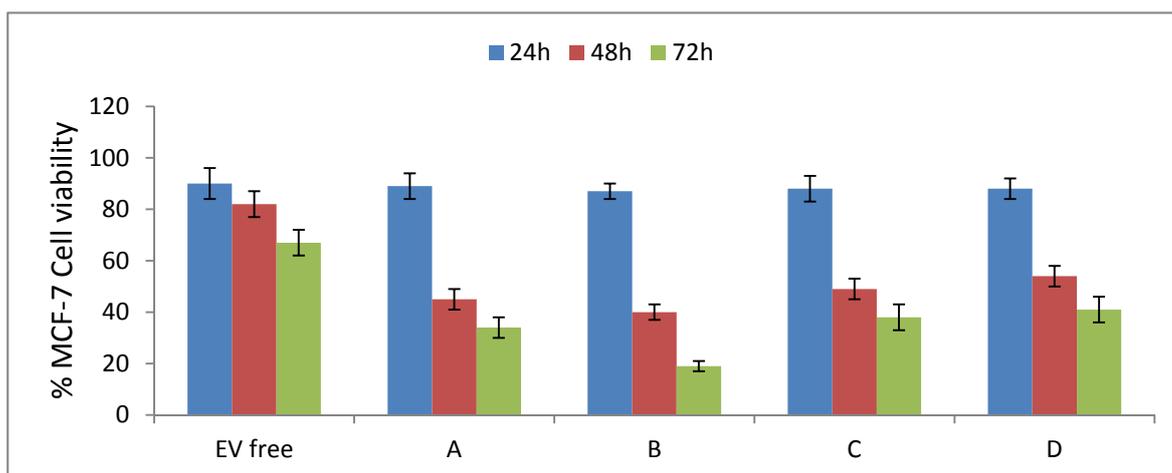

Figure 6. Viability of MCF-7 BC cell lines cultured with PLGA–TPGS-EV-FOL NPs and EV free at 24, 48, and 72h incubation time, the values are mean (n=3) ± SD.

The inhibition concentration IC_{50} can be calculated from figure 7, which was listed in Table 4 to make comparison between EV free and the PLGA–TPGS-EV-FOL NPs formulations A, B, C, and D.

Table (4): IC$_{50}$ of MCF-7 BC cell lines incubated with EV free and EV loaded PLGA NPs formulations

Incubation time	IC$_{50}$ EV free mean±SD	IC$_{50}$ A mean±SD	IC$_{50}$ B mean±SD	IC$_{50}$ C mean±SD	IC$_{50}$ D mean±SD
24h	25.7±4	19. 9±3	19.1±3	20.8±4	83.3±6
48h	13.8±3	4.5±1.5	4.1±1.8	9.8±2.4	21.7±5
72h	10.8±2	1.4±0.9	1.04±0.3	8.06±2.1	16.1±3.6

3.9   Apoptosis,   Angiogenesis   and   Glucose Transporting study

MCF-7 BC cell lines after 72h incubation time with PLGA-TPGS-EV-FOL NPs formulation B were harvested to explain the expression rate of CD44, VEGF, and GLUT-1 as apoptotic, angiogenesis, and glucose transporting markers, respectively. Figure 7, show the statistically decreasing in levels (ng/ml) of these markers (p-value<0.05) in treated compare to untreated cells that investigated by ELISA technique.
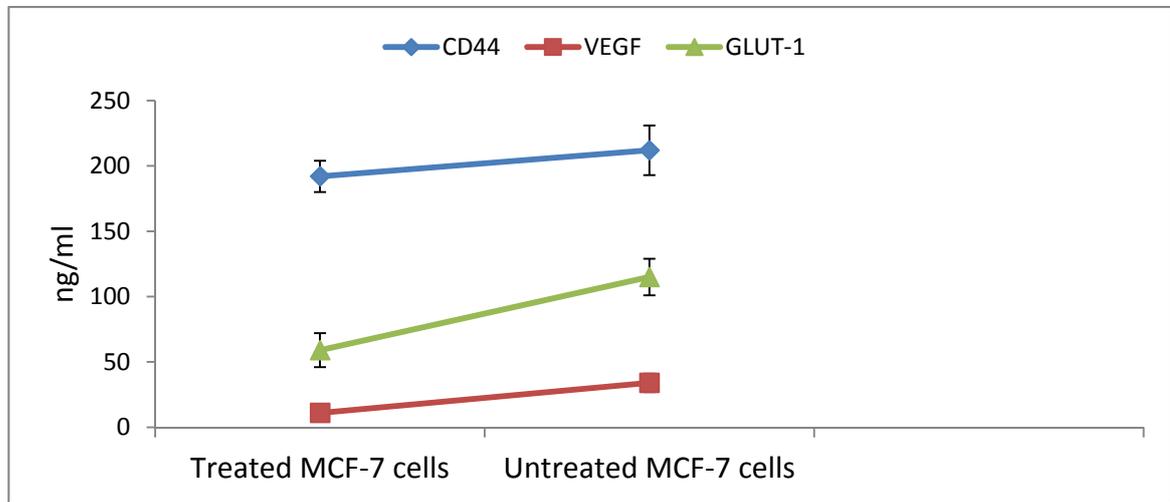

Figure 7. Levels of CD44, VEGF, and GLUT-1 (ng/ml) in treated and untreated MCF-7 cell lines 3.10 Gene expression fold of mRNA HIF-1A

When treated with EV free and PLGA-TPGS-EV-FOL NPs formulation B, fold change in expression (GEF) of HIF-1A mRNA was normalized to GAPDH and then compared to the untreated values for that MCF-7 cell line using the formula: $2^{-\Delta\Delta CT}$ (treated and untreated cells). The amplification and melting curves of HIF-1A mRNA in treated MCF-7 BC cell lines are shown in figure 8.
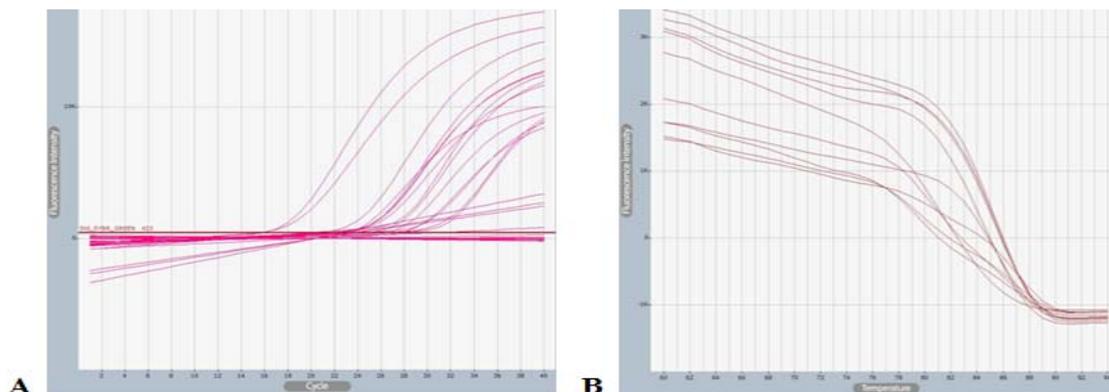
Figure 8. A: Amplification curve B: Melting curve measured as -dF/dT versus temperature (Cº) of HIF-1A mRNA in treated MCF-7 BC cells

HIF-1A mRNA gene expression fold in MCF-7 BC cells treated by PLGA-TPGS-EV-FOL NPs formulation B (n=12) and untreated cells (n=12) compare to expression of reference gene, GAPDH is show in figure 9.
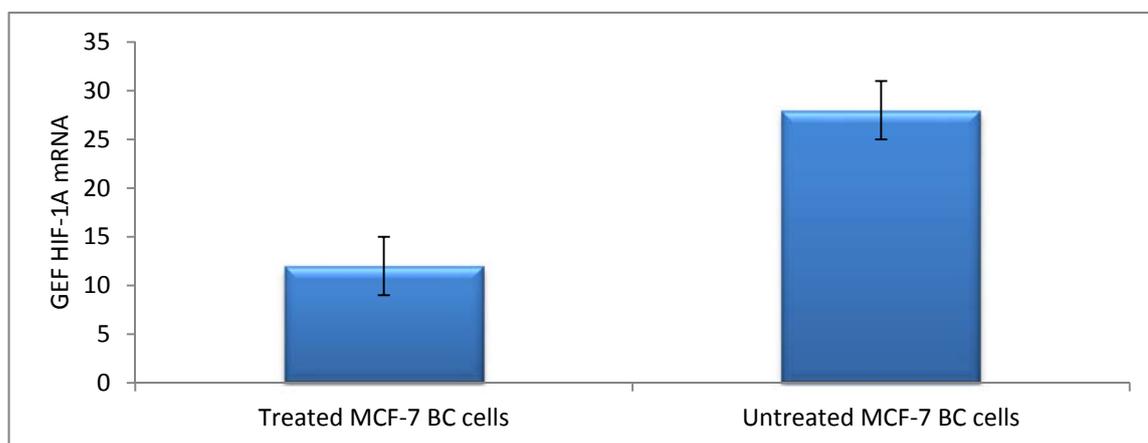

Figure 9.HIF-1A mRNA gene expression fold in MCF-7 BC cells

4. Discussion

To the best of the present study knowledge, this is the first study reporting the inhibition role of EV loaded NPs on HIF-1A mRNA gene expression (GEF) and its related proteins such as GLUT-1 and VEGF as glucose transporting and angiogenesis markers in progression of BC. The effect of drug concentration on the particle size, size distribution and zeta potential was investigated by preparing formulations A,B,C, and D. The larger size of NPs with very high PDI and very low zeta potential are indicative of particle growth or aggregation which could be due to absence of polymer (11). This mean that formulation B with DCM as organic solvent and the EV loading amount (5 mg) and DPR at 1:20 is optimal formulation to targeting of EV to BC cells. The smaller nanoparticles are known to be absorbed or up taken by mucosal cells better than their larger counterparts (12). The present study give a novel EV loaded PLGA based NPs drug delivery system using vitamin E- TPGS as emulsifier and FOL as targeting receptor. *Felix L et al, 2013* suggested that EE% of mycophenolate mofetil NPs was increases with

increasing drug-polymer ratios(DPR) (13). The EV loading percentage in formulation B was 87% in 100 mg of PLGA. The results of this study not agree with above study because of PDR in formulation B that give higher EE% was 1:2 and this not highest DPR. It can be explained that the increase in particle size is probably due to changes in viscosity and surface tension of the organic phase as well as increase in content of the solid mass of oil droplets which is similar to several studies (14,15). The FT-IR spectra demonstrated that there was no chemical interaction between functional groups of EV and FOL with PLGA-TPGS polymer but there is may be only entrapment of drug polymer and this may be due to ionic interactions between functional groups of molecules and this explain the sustain release of EV loaded NPs after pH changing. Likewise, the in vitro drug release profiles of NPs revealed that EV release rate can be controlled by the type of organic phase such as DCM , DPR (1:2), and EE % in preparation of PLGA-TPGS-EV-FOL NPs formulations for an extended period of time carriers. As previously observed by *Janes et al;* 2001, drug release is governed by the degradation of the HA/CS-TPP NPs' hydrophilic carrier, which depends on the intensity of the interaction between the polymers and the medium ionic strength (16). *Yuandong M et al*; 2010 found an advantage of the PLGA-TPGS nanoparticles versus the traditional PLGA nanoparticles, which were found to release the drug too slowly to meet the therapeutic needs (17). *Shuqian W et al;* 2018 reported that the PLGA-NPs showed miniscule variation in the formulation parameters during six months of storage at the stability conditions of 4C° and protected from light and this meant that the NPs were stable enough with high encapsulation ability (18). The results of this study not in agreement with above study because of EE% showed declines in all formulations in stable temperature (RT 25-26 C°). The results of this study are in accordance with results of (19,20) in which they showed that encapsulated drug have more stability and anti-cancer activity against cancer cells compared to the free drug. The results suggested by *Ma Y et al*; 2010 showed that PLGA-TPGS–based nanoparticles were biocompatible, and the

docetaxel-loaded PLGA-TPGS nanoparticle had significant cytotoxicity against Hela cells. The cytotoxicity against HeLa cells for PLGA-TPGS nanoparticles was in time- and concentration-dependent manner (21). *Maleki H et al*; 2017 reported that the increasing in the antitumor activity of MTX loaded PLGA NPs is due to the protection of the drug using PLGA from hydrolysis and decomposition as well as maintenance of the drug activity in a sustained release manner (22). In tumor cells, the endocytic activity will enhance and higher amount of NPs can be internalized into the cells which is consequently provided the greater concentration of drugs and increasing the concentration gradient near to cell surface may be cause interring the drug to inside cells and this in agreement with explanations that suggested by (23). *Al-Hajj M et al*; 2003 suggested that CD44 regulates critical aspects of metastatic disease, including transformation, growth, cell invasion and motility, and chemoresistance, and it is a marker of breast cancer stem cells (24). Moreover, the results of present study might help identify MCF-7 BC cells for CD44-based targeting therapy by decreasing chemoresistance may be due to chemical interactions of EV loaded NPs with amino acids of CD44 and blocking it and decreasing the levels of CD44 may be leads to declines in cell-cell interactions and in result decline in cells proliferations. *Jun et al*; 2011 documented that patients with higher GLUT-1 expression demonstrated poor overall survival and disease‑free survival (25). In addition to GLUT-1, expressions of other glycolytic enzymes have also been demonstrated to be induced by HIF-1A (26). Moreover, GLUT-1 is one of the targeting of HIF-1A mRNA gene expression and EV loaded NPs inhibited GLUT-1 expression through mTOR pathway inhibition. The chemical structure of EV has more than one hydroxyl groups and this may be facilitated of binding with HIF-1A leading to inhibitory effects. It was reported by *Greenberger L et al*; 2008 that inhibition of HIF-1A by EZN-2698 decline the HIF-1A protein levels and tumor progression in various in vitro (human prostate and glioblastoma cell lines) and in vivo studies (27). *Georgina N et al*; 2015 were reported anthracyclines are the potent well-known chemotherapeutic

agents, also act as HIF-1A inhibitors by preventing to binding with DNA (28). Many studies of the novel anticancer drugs that target specific pathways have been shown to have an effects that appear to be inhibition of its targets (29-31). Declines of toxicity for the formulation B were observed in vitro study, indicating the EV loaded NPs can be safely dosed and have potential for application in future treatment strategies of BC. In conclusion the treatment of women with BC by EV loaded NPs may be useful in decreasing of cost and side effects of classical chemotherapy.

Conclusion

The results of this research showed that EV loaded NPs may be useful in lowering the cost of treatment and may be involved in the decline of chemotherapy side effects by targeting to inhibition of both HIF-1A and mTOR pathway.

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دواء الإيفروليمس النانوي و نقل الدواء الى الخلايا الهدف و ارتباطه بمستقبلات الـFOL: تحضيره، تشخيصه، ودراسة التأثير السمي له على خطوط الخلايا MCF-7 لسرطان الثدي

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ملخص

الخلفية والهدف: من المحتمل أن تكون حالات الوفاة من سرطان الثدي (BC) ناجمة عن عدم كفاية فوائد العلاج والكشف المبكر، علاوة على عدم وجود مرافق مناسبة للتشخيص والكشف، فضلاً عن ارتفاع تكلفة العلاج. الهدف من هذا العمل هو ايجاد طرق عمل لتحضير النانو الذي يعمل بنظام نقل الدواء الى الخلايا الهدف مناسبة في لتحضير PLGA- TPGS NPs (nano-EV) المحمّل بـ Everolimus (EV) والذي يستهدف حمض الفوليك (PLGA-TPGS-EV- FOL) والتحقيق في تأثير السمية على خطوط الخلايا البشرية MCF-7 **طرق العمل:** تم تحضير دواء الايفروليمس NPs المحملة بالفوليت مع الامواق فوق الصوتية مع عن طريق الجمع مع الاستحلاب و طريقة تبخر المذيبات مع تعديلات طفيفة وقسمت الى أربعة تركيبات A و B و C و D. تم تشخيص الـ NPs المحملة بعدة طرق منها الـ FESEM و TEM لحجم الجسيمات (PS) وجهد زيتا(ZP)، FT-IR، وتم قياس المعايير الاتية: تحميل الدواء (DL٪)، كفاءة التغليف (EE٪)، تحرر الدواء في المختبر، تركيز تثبيط IC_{50} وحيوية الخلية٪، موت الخلايا المبرمج، تكوين الأوعية، نقل الجلوكوز، والتعبير الجيني HIF-1A mRNA. **النتائج:** تم العثور على التركيب B من NPs المحملة EV لتكون أقل PS 100 ± 12.7 نانومتر ، PDI 0.152، ولها ZP ،23.2.5-. تم الكشف عن نسبة DL٪ الأعلى من EV بـ 7.32 ± 1.1 ٪ و EE٪ 87 ± 2.6 ٪ في الصياغة B. واقترحت النتائج أن انحلال EV الحر كان 80٪ خلال 4 ساعات لكن ملف تعريف تحرير A و B و C كانت تركيبات D خلال 4 ساعات 34، 23، 26، 30٪ على التوالي. أظهرت EV المصممة في NPs تأثيرات أفضل ضد خط الخلايا MCF-7 BC من تركيبة NPs الخالية من EV والمحملة EV والتي حققت تأثيرًا علاجيًا أفضل من تركيبات A و C و D. انخفض التعبير الجيني HIF-1A mRNA عند معالجة خلايا الـ MCF-7 بتركيبة الـ NPs B المحملة EV مقارنة بالتعبير في الخلايا غير المعالجة. الخلاصة: قد تكون NPs المحملة بـ EV مفيدة في خفض تكلفة العلاج وقد تشارك في انخفاض الآثار الجانبية للعلاج الكيميائي.

الكلمات الدالة: سرطان الثدي، بوليمرPLGA النانوي، MCF-7، الايفروليمس، مثبطات الـ mTOR.