Design and development of nanostructured co-delivery of artemisinin and chrysin for targeting hTERT gene expression in breast cancer cell line: possible clinical application in cancer treatment

Leila Khoshravan Azar  
Tabriz University of Medical Sciences

Mehdi Dadashpour  
Tabriz University of Medical Sciences

Akram Firouzi-Amandi  
Tabriz University of Medical Sciences

Nosratollah Zarghami (✉ Zarghami@tbzmed.ac.ir)  
Tabriz University of Medical Sciences  https://orcid.org/0000-0002-4236-4537

Primary research

Keywords: Breast cancer, Nanotechnology, Artemisinin, Chrysin, hTERT

DOI: https://doi.org/10.21203/rs.3.rs-623122/v1

License: © This work is licensed under a Creative Commons Attribution 4.0 International License.  
Read Full License
Abstract

Background: Breast cancer is one of the most significant causes of female cancer death worldwide. To explore the possibility of a novel chemo-preventive strategy for improving breast cancer treatment, the anticancer effects of a combination two natural compounds, Artemisinin (Art) and Chrysin (Chr), against T47D breast cancer cells were investigated.

Methods: For this purpose, Art and Chr were co-encapsulated in PEGylated PLGA nanoparticles (NPs) and evaluated for their therapeutic efficacy. The morphology and dynamic light scattering (DLS) analyses were carried out to optimize the Nano formulations. Drug release study was performed using the dialysis method and then the cytotoxic and inhibitory effect of individual and combined drugs on the expression level of hTERT in the T47D breast cell line was evaluated using MTT assay and qPCR, respectively.

Results: The results showed that pure drugs and formulations exhibited dose-dependent cytotoxicity against T47D cells and especially, Art/Chr–PLGA/PEG NPs had a more synergistic anti-proliferative effect and significantly arrested the growth of cancer cells than the other groups. Real-time PCR results revealed that Art, Chr and combination of Art–Chr in pure and encapsulated forms inhibited hTERT gene expression.

Conclusions: It was found that Art–Chr–PLGA/PEG NPs relative to pure combination could further decline hTERT expression in all concentrations. Our study demonstrated that Art–Chr–PLGA/PEG NPs based combinational therapy holds promising potential for the treatment of breast cancer.

Introduction

Cancer is one of the most problematic health issues world wild [1]. Breast cancer is the second rates in incidence and mortality in both sexes. Breast cancer is a serious global health concern for women in developed and developing nations[2, 3]. Breast cancer has several subtypes. Most patients with breast cancer belong to the group that express estrogen receptors (ER) and progesterone (PR). The other subgroup is TNB, which lacks ER, PR, and HER-2 expression[4].

Due to the heterogeneity of breast cancer, it is difficult to treat breast cancer in some patients with conventional therapies such as radiation therapy, chemotherapy, surgery, immunotherapy, and hormone therapy. Despite new methods of treatments and chemotherapy medicine, issues such as relapse, a significant reduction in quality of life, and serious side effects resulted in this type of treatment cannot be ignored[4, 5]. Therefore, the search for new compounds with anti-cancer potential is essential.

Phytochemicals are natural compounds derived from medicinal plants as secondary metabolites. They have various biological functions, including anti-inflammatory and anti-cancer effects (through cell cycle involvement, apoptosis, angiogenesis, and metastasis), enhanced immunity, and antioxidant effects[6, 7]. According to numerous studies, phytochemicals are applied as chemo-preventive and chemotherapeutic compounds in various types of cancer [2]. According to the WHO, herbs will be the best source for
obtaining a variety of medicines[8]. A wide range of phytochemicals is showing promising results in the treatment of cancer due to their ability to target several cancer cell pathways and their limited toxic effect on normal cells. The use of phytochemicals in combination with other cytotoxic drugs can increase the anti-cancer effect and reduce the side effects on normal cells and also combined treatment can delay resistance onset[7]. There are different types of phytochemicals, of which Artemisinin and Chrysin are two types.

Chrysin is a natural flavonoid that found in some plants, especially chamomile, Pleurotus ostreatus, and honeycomb [9]. Among the biological properties, the anti-cancer and cancer-preventive effects of chrysin are significant [10]. Chr exerts its anti-cancer effects by blocking and inhibiting cancer cells in a variety of ways, including apoptosis, autophagy, angiogenesis, and cell proliferation. The cytotoxic effects of Chr on cancer cells are greater than normal cells [11]. The potential effects of apoptosis on various cancer cells, including breast, prostate, thyroid, pancreas, NSCLC, and intestine, have been tested and documented [12]. Chrysin has been tested for its anti-cancer effects in in vivo and in vitro states. This also has been applied in different cell lines, like T47D, SW480, PC3, SKOV-3 and MCF-7 [11, 13]. Due to its molecular structure, Chrysin is also more concentrated in the T47D cell line [14].

Phytochemical Artemisinin is derived form of the Chinese plant Artemisia annua. Art is registered as the standard protocol for the treatment of malaria based on its anti-malaria effects [15]. Beyond its anti-malarial effects; Art has shown anti-cancer and significant cytotoxic effects against a wide range of cancer types [16]. The biological Artemisinin effects and its derivatives in cancerous cells are inhibition of cancerous cells growth through cell cycle arrest, apoptosis, inhibition of angiogenesis, perturbation of angiogenesis and nuclear receptor responsiveness [16, 17]. Its anti-cancer properties in various cancer cells including breast, prostate, ovary, cervix, leukemia cells in in vivo and in vitro states have been studied and recorded [6, 18].

Nanotechnology is a technology in nanometer dimensions whose functional properties originate more than its size. With the development of this technology, scientists have paid special attention to the use of this technology for the controlled transmission, discharge and release of drugs, especially in the cancer treatment [19]. Phytochemicals such as Art and Chr have some disadvantages and limitations that reduce their biological function, but these limitations are significantly offset by the advances in nanotechnology. These limitations include short half-life, low solubility, poor biological availability, low duration of stability in the bloodstream, and rapid metabolism and degradation [20, 21]. One of the most successful polymer nanoparticles is PLGA. It is a degradable polymer nanoparticle approved by the FDA and EMA due to its conversion into natural monomeric metabolites of glycolic acid and lactic acid in the body. To maximize the ability of PLGA used PEG4000. PEGylation increases the half-life of nanoparticles [22]. Therefore, encapsulation of anti-cancer phytochemicals such as Art and Chr in PLGA-PEG biodegradable nanoparticles may have many advantages over other drug delivery systems.

Studies show that more than 90% of cancer cells have changes in telomerase gene expression. Telomerase is an RNA-dependent DNA polymerase enzyme, which is a reverse transcriptase
enzyme. The human telomerase consists of two main subunits and several subunits. Its main subunits include hTERT, a catalytic subunit that acts as a reverse transcript, and hTERC, a functional RNA subunit that acts as a template for telomeric DNA sequences. Telomerase activity is proportional to the expression of the hTERT gene[23, 24]. The hTERC gene is expressed in normal and cancerous cells either, but the hTERT gene is expressed only in stem cells and cancer cells after embryonic development. Due to the high expressed hTERT gene in cancerous cells, targeting and inhibiting the expression of the hTERT gene in the cancer treatment is an acceptable idea for researchers[25, 26]. Knowing the exact mechanisms and pathways in the growth of breast cancer allows us to have a better targeted and more effective pathway in the treatment of breast cancer incorporating new technologies. The goal in Molecular Target Therapy is identifying and having the greatest impact on cancer cells and the least impact on healthy cells. Pathway treatment using anti-cancer compounds is an advancement that has received special attention in recent years[27, 28].

In this study, the anti-cancer effects of Artemisinin and Chrysin on expressed hTERT gene in the T47D cell line of breast cancer cells were investigated in pure form and nanoencapsulated form, both individually and in combination.

**Material And Methods**

**Material**

Fetal bovine serum (FBS) and RPMI 1640 were purchased from Gibco. Penicillin G, streptomycin, DMSO (Dimethylsulfoxide), MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide), DL-Lactide, glycolide, polyethylene glycol (PEG, molecular weight: 4000), stannous octoate [Sn(Oct)₂], PVA (alcoholic polyvinyl), DCM (dichloromethane), Artemisinin and Chrysin were purchased from Sigma-Aldrich. Sodium bicarbonate was purchased from Merk. Termo Fisher. RiboEx (total RNA isolation solution) from Gene All’s company has sold the first strand of cDNA synthesis kit and SYBER Green PCR Master Mix.

**cell culture and cell line**

Pasteur institute cell bank of Iran provided T47D human breast cancer cell line. Cultured T47D human breast cell line in RPMI 1640 complemented with 0.08 mg/ml streptomycin, 0.05 mg/ml penicillin G, 2 mg/ml sodium bicarbonate, 10% heat-inactivated fetal bovine serum (FBS), and cells were grown at 37°C in an incubator with 55% humidity and 5% CO₂.

**Synthesis of PLGA-PEG**

Preparation of PLGA-PEG was through open ringed polymerization of glycolide and DL-lactide followed by PEG additional vacuum. PEG₄₀₀₀ and PLGA were copolymerized in presence of Sn(Oct)₂ as the catalyst in polymerized melt mentioned before. Melting process was through glycolide (0.570 g), DL-lactide (2.882 g) and PEG₄₀₀₀ (1.54 g) bottleneck flask at 140°C under a nitrogen atmosphere. Mixture
reaction comprising a 1:3 proportion of glycolide to DL-lactide and 0.05% (W/W) stannous octoate was made, heated to 175°C and kept for 4 hours. [29]

**Preparation of nanoparticles containing drug**

Generally, encapsulation in PLGA-PEG nanoparticle of all drugs occurred the method of double emulsion (W/O/W) with a minor modification. In sum, 20 mg of Chrysin and 200 mg of PLGA-PEG were dissolved in dichloromethane (DCM) and sonicated for 1 minute to allow Chrysin to be entrapped within the nanoparticle network (the W/O primary mixture). Alcoholic Polyvinyl (PVL) and DMSO 1% (1:1) were added to W/O emulsion then sonicated for 1 minute to produce W/O/W emulsion. Then, DCM was evaporated using a rotary evaporator and the remaining solution was centrifuged at 10000 rpm for 30 minutes.

For the synthesis of Art NPs and Art/Chr NPs according to the mentioned steps and with the following quantities; For the synthesis of Art NPs, 30 mg of Artemisinin and 120 mg of PLGA-PEG and for the synthesis of Art/Chr NPs ... of Artemisinin, ... of Chrysin and ... of PLGA-PEG.

Drug loading efficiency determination in PLGA-PEG is the supernatant was isolated and compared with the drug total amount. Drug amount which was not used in aqueous phase was determined by an ultraviolet spectrophotometer.

\[
(1) \quad \text{EE} = \frac{\text{Weight of drug in NP}}{\text{Weight of the initial drug}} \times 100
\]

\[
(2) \quad \text{DL} = \frac{\text{Weight of drug in NP}}{\text{Weight of NPs}} \times 100
\]

**Characterization of the nanoparticles containing drug**

FT-IR, SEM and DLS methods have been utilized to study and analyze the physicochemical properties of drug-containing nanoparticles.

Using FT-IR spectroscopy, various vibrations of functional groups in the structure of the prepared branched polymers (drug-loaded nanoparticles) such as carbonyl, ether, methylene and other groups have been investigated. Separately, a certain concentration (0.5%) of PLGA-PEG, Art NPs, Chr NPs and Art/Chr NPs copolymers were prepared in KBr disks.

Using scanning electron microscope (SEM) morphology and surface topology of nanoparticles were studied. SEM images are the result of the interaction of electron beams with sample atoms at different depths. In this device, shorter wavelengths create sharper images with more accurate resolution and provided information. The size and morphology of the lyophilized sample of Art/Chr NPs were imaged under vacuum pressure, using a below 30 kW voltage and with various magnifications. Before performing SEM imaging, the sample was coated with a thin layer of gold metal (about a few angstroms).
Analyzing and determining particles size in range of few nanometers, microns or even smaller than nano have been possible by using dynamic light scattering (DLS) which is a physical method. The method mentioned is dependent on interaction of light and particle. Scattered light by nanoparticles in suspension (liquid medium) changes during a period of time that could be related to the particle diameter.

**In vitro evaluation drug release**

Drug release from the PLGA-PEG nanopolymer was determined using dialysis. Shortly, each drug’s 25 mg were loaded with PLGA-PEG nanopolymer, dissolved in 5 mL PBS and transferred to a dialysis membrane tube (MW cut off 3000) and placed in 30 mL PBS which was shaken with a stirrer at 120rpm at the temperature of 37 °C. For 96 hours, the PBS medium was repeatedly replaced at regular intervals, and the studied compounds (Chr NPs, Art NPs and Art/Chr NPs) were taken in the previous medium at a wavelength of 348 nm for chrysin, 203 nm for Artemisinin (maximum wavelength).

**Cell viability and MTT-based cytotoxicity assay**

Effect of Art, Chr and Art/Chr cytotoxicity both in pure and in nanoencapsulated form in PLGA-PEG on the T47D cell line using MTT assay was studied in 24-hour and 48-hour intervals. 3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyl tetrazolium bromide (MTT) is a water-soluble yellow substance that is regenerated in living cells as a result of the action of the enzyme succinate dehydrogenase and turns into a purple substance called Formazan, which is insoluble in the aqueous phase.

In brief, cells (T47D cell line) were cultured in an incubator at 37 °C in a humid atmosphere containing 5% CO2, which is required to strengthen cell binding. After cell culture and passage, the number of cells has reached a sufficient amount and subsequently 3.000 cells were seeded in 96-well plates. In the incubator, the cells were given 24 hours to adhere to the wells. After 24 hours, 4 series of dilutions from each of pure-Art, pure-Chr, pure-Art/Chr, Art NPs, Chr NPs and Art/Chr NPs were prepared from the drug stock and were treated in triplicates in the wells. After 24 hours of incubation, the 24-hour IC$_{50}$ (IC$_{50}$ dose of the drug that kills 50% of living cells) was assessed by MTT assay. For this purpose, the culture medium inside the wells was gently removed and 200 Lambda MTT solution (for every 1 mL of PBS, 2 mg of MTT powder) was poured into each well and the plates were covered with aluminum foil and incubated for 4 hours at 37 °C. After 4 hours, the surface of the wells was gently removed, and the formed insoluble purple crystals of Formazan were dissolved by adding 200 Lambda DMSO to each well by placing it on an orbital shaker for 10 minutes (at this stage the plates are still covered with aluminum foil). In the next step, the absorption rate of each well was read and recorded at 570 nm using an EL X 800 microplate absorbance reader (Bio Tek Instruments, Winooski, VT) with a reference wavelength of 630 nm. To calculate the IC$_{50}$ for 48 hours, 24 hours after trituration, the surface of the wells were gently replaced with fresh culture medium then incubated for another 24 hours. At this stage, which is 48 hours after trituration, the relevant IC$_{50}$ was calculated through MTT assay. Using Graph.Pad Prism 6.01 software the IC$_{50}$ level of the drugs was calculated. Also, in combination therapy, our criterion for evaluating how two drugs interact with each other is the combination index (CI). Calculation of combination index and
isobolograms to quantify drug interactions is in drug combination therapy studies and the classification of drugs is in three groups of synergy, additive or antagonism. Isobolograms and CI analysis are widely used to evaluate drug interaction in cancer combination therapy. CI diagrams were drawn using Compusyn software.

**Quantitative real-time PCR assay**

Real-time PCR was used to evaluate the changes in hTERT gene expression in the T47D cell line after drug treatment. For real-time PCR, RNA\textsubscript{total} was extracted, then cDNA synthesis, and then RT-PCR. For this purpose, 300,000 cells were cultured in 6-well plates. The cells were treated using 6 studied compounds (drug dilution less than IC\textsubscript{50}) for 24 hours and one well was grown as a control measure with culture medium only. Then the RNA\textsubscript{total} of each well was extracted using the effective and fast method of Trizol reagent (Riboc Ex total RNA-Gene All) considering to the manufacturer’s instructions. During the extraction process, isopropanol causes RNA deposition. To analyze the RNA\textsubscript{total} quantity, a nanodrop device was used, and the OD\textsubscript{260} / OD\textsubscript{280} calculation showed the purity of the extracted RNA\textsubscript{total}. To analyze RNA\textsubscript{total} quality 1% agarose gel electrophoresis was used. After RNA\textsubscript{total} is extracted, cDNA (complementary DNA) is synthesized from the RNA pattern by reverse transcriptase (RTs). RTs directly synthesizes the first strand of cDNA using the RNA template and Oligo dT short primer.

For this purpose, the First Strand cDNA synthesis kit made by Thermo Fisher Scientific Company was used. After cDNA synthesis, it is time for RT-PCR. In this study, the analysis of RT-PCR data is a relative evaluation method. This is a more accurate method to examine changes in gene expression. In this method, the studied gene (hTERT) is compared with a reference gene as an internal control which in this study is considered β-actin. The important point in this method is that the efficiency of the studied and reference genes is as equal as possible (by examining Ct\Delta\Delta), otherwise, the results of this method will not be very valid. For RT-PCR, primers related to the hTERT gene were designed by Primer3 software and synthesized by Takpajohan Gene Company. In this RT-PCR research, was based on SYBER Green fluorescent dye, where the SEBER Green qPCR Master Mix kit was prepared by Thermo Fisher Scientific. In statistical analysis, the results were reported as mean ± SD. Raw RT-PCR data were calculated by Mic PCR, ∆Cts software. Then all statistical analyzes were performed using GraphPad Prism 6.01 and Excel 2013 software. Data comparison was performed using two-way ANOVA. The value of p<0.05 was considered as a significant level.

**Statistical analysis**

All experiments were carried out in triplicate and data were represented as mean values ± standard error (SE) of the experiments. GraphPad 6 Prism software (CA, USA) was employed to evaluate statistical significance between study groups using T-test for comparison between two groups and one-way analysis of variance for comparison between more than two groups. P values less than 0.05 were regarded statistically significant.
Results

FT-IR spectroscopy

FT-IR spectroscopy was utilized to confirm the encapsulation of the studied pure compounds i.e. pure-Art, pure-Chr in PLGA-PEG. In the IR spectrum related to PLGA-PEG copolymer, absorption band 3454.52 cm\(^{-1}\) belongs to hydroxyl groups, absorption band 3130.20 cm\(^{-1}\) corresponds to COOH, absorption band 1637.04 cm\(^{-1}\) correlates with the tensile vibrations of the C = O group (related to ester), absorption bands in the range of 1082.21 cm\(^{-1}\) belong to the tensile vibrations of the C-O-C bond (related to PEG), and absorption bands in the range of 1188.19 cm\(^{-1}\) corresponds to C-O. Displayed in (Figure 1-a), the infrared spectroscopy of pure Artemisinin, absorption bands 2913.14 cm\(^{-1}\) and 3453.06 cm\(^{-1}\) correlates with N-H vibrations, absorption band 1739.64 cm\(^{-1}\) belongs to the tensile vibrations of C-O, and range of 1380.96 cm\(^{-1}\) to 1457.38 cm\(^{-1}\) corresponds to C-H. Displayed in (Figure 1-b), the infrared spectroscopy of pure-Chr, absorption band 2884.78 cm\(^{-1}\) belongs to the vibrations of the C-H group, absorption band 1653.07 cm\(^{-1}\) corresponds to the tensile vibrations of the carbonyl group C-O, and absorption bands of 906.42 cm\(^{-1}\) to 1027.48 cm\(^{-1}\) correlate with the C-C, C-O, and C-O-C bonds. Displayed in (Figure 1-c), the IR spectrum of PLGA-PEG copolymer containing Art/Chr, absorption band in range of 1737.53 cm\(^{-1}\) belongs to the hydroxyl groups, absorption band 2916.68 cm\(^{-1}\) corresponds to the vibrations of N-H, absorption band 1737.53 cm\(^{-1}\) correlates with the C-O bond, absorption band 1453.47 cm\(^{-1}\) belongs to the C-H bond, absorption band 1094.44 cm\(^{-1}\) corresponds to C-O-C bond. (Figure 1-d): The matching of the absorption bands in Art/Chr NPs with the pure Artemisinin and pure Chrysin absorption bands indicates that both drugs are encapsulated in nanoparticles (PLGA-PEG).

Size Distribution (Scanning Electron Microscopy)

Evaluating the physicochemical properties of PLGA-PEG and Art/Chr PLGA-PEG NPs scanning electron microscopy (SEM) was utilized. From these micrographs, the observation of PLGA-PEG nanoparticles showed a spherical uniformed shape and their dimensions varied between 20 and 65 nm (Figure 2.B and C). After encapsulating and formatting of Art/Chr-PLGA-PEG NPs, the size of particle changed to about 200 nm.

Dynamic light scanning

Nanoparticle dimension analysis via DLS shows a median dimension of 210 nm with uniform dispersion for PLGA-PEG nanoparticles. It was also observed that Art NPs and Chr NPs have an average dimension of 200 nm with a range between 150 and 250 and Art/Chr NPs have an average size of 250 nm with a range between about 200 to 300. (Figure 2A): The specifications of PLGA-PEG-loaded drugs are reported in Table 2.

Entrapment Efficiency (EE) and drug loading (DL)
According of equation 1 Encapsulation efficiency (EE) and drug loading (DL) of chrysin in PLGA-PEG was 94.7% and 15.16% respectively. ...

**Release rate determination of Artemisinin and Chrysin from PLGA-PEG**

The kinetics of Art and Chr drugs loaded in 160 hours were examined in *in vitro* state. The amount of Art and Chr released in the first 15 hours was almost equal, subsequently, the release of both drugs was increasing, but the amount of Art released was much higher compared to Chr. After the first 24 hours of the rest of the phase, the rate of Art release was noticeably higher than of Chr. Overall, however, both drugs showed a significant increase in release within 160 hours. (Figure 3)

**In vitro cytotoxicity study (MTT assay)**

MTT assay is an essential technique of assessing the biological materials` cytotoxicity in *in vitro* state. In this study, the results of cytotoxicity assay for the treatment of T47D cells with concentrations of 0, 5, 10, 20, 30, 40 μM of pure and nanoencapsulated Art, pure and nanoencapsulated Chr, pure and nanoencapsulated Art/Chr; Shown in Figures 4A and Figure 4B at 24 and 48 hours. The results obtained from the graphs show that all 6 compounds have got an inhibitory effect on the T47D cell line. In all 6 compounds, due to the time-dependent functionality, the inhibition of cancer cells is more effective for 48 hours than 24 hours. It can also be seen from the graphs that each of the drugs in the nano state has a greater degree of inhibition than in their pure state. Among the all samples, Art/Chr NPs had the greatest inhibitory effect at all concentrations treated at both 24 and 48 hours. After Art/Chr NPs, Art NPs have shown the greatest inhibitory effect on all concentrations in both periods. Also, Table 3 shows the mean IC50 values (Mean ± SD) for the T47D cell line of breast cancer after treatment with 6 studied compounds during 24 and 48 hours. The IC50 results indicate the inhibitory performance of all 6 compounds, especially nanoencapsulated compounds, and show the highest performance among nanocapsules, Art/Chr NPs.

Figure 5 shows the CI diagram, which is an indicator for evaluating the interaction activity of Art and Chr drugs in combination therapy. Art/Chr compounds in both pure and nanoencapsulated states have a CI of less than 1, which means that Artemisinin and Chrysin have a synergistic effect in both pure and nanoencapsulated states.

**Real-time PCR and RNA*total* extraction results**

To perform real-time PCR, it is first necessary to extract the RNA*total* of the treated cells. For quantitative and qualitative analysis of the extracted RNAs, nanodrop and 1% agarose gel electrophoresis were used, respectively. Using RNA*total* nanodrop, each showed a ratio of 2 to 1.8 in the OD260/280 ratio, which confirms the purity and amount of RNA*total* for cDNA synthesis. Also, in 1% agarose gel electrophoresis of the samples displayed 18s rRNA, 28s rRNA (mRNA subunits) and 5s bands.
A real-time PCR technique was applied to quantify expression of the hTERT gene in T47D cells treated with concentrations of 5 and 10 μM of Art, Chr and Art/Chr, in both pure and nanoencapsulated form after 24 h of incubation. By increasing the concentration of Art, Chr and Art/Chr in pure and nanocapsules, because of the high effect of these compounds on reducing the expression of hTERT gene in treated samples and high concentrations of drug in real-time PCR due to lower mRNA expression, T47D treatment reach the threshold of Ct in the above cycles. Therefore, high concentrations have not been tested for this study. As Figure 6 shows, all 6 drugs showed a remarkable reduction in hTERT expression of T47D breast cancer. Nanoencapsulated drugs have a greater inhibitory effect on hTERT gene expression than the pure state. The results also show a greater inhibitory effect of combination drugs in both pure and nanoencapsulated states. The difference was statistically significant (p ≤ 0.05).

Discussion

It is reported that global statistics on female breast cancer is one of the most common cancer. Despite the year by year increase in the survival rate and the decrease in mortality rate, the side effects and the negative impact on life quality of most patients during treatment cannot be ignored [30]. Therefore, for preventing and treatment of breast cancer, a more optimal method of treatment without these side effects is a vital action [31]. Due to their low toxicity and a wide range of biological activities, phytochemicals can be utilized as an alternative method of treatment [32]. The anticancer effects of the phytochemical Artemisinin have been proven in numerous studies. It exerts its anti-cancer properties in various ways [16]. The level of iron are remarkably higher in cancerous cells than normal cells, and iron breaks down the endoperoxide structure in Artemisinin, leading to cell death [33]. Studies conducted by Guo-Qing Chen et al. in 2019 demonstrated Art’s antitumor property by direction breast TNCB cancer cells to ferroptosis, which is a type of cell death [34]. Also in studies conducted on normal and cancerous breast cell lines in 24 hours of treatment with Art has shown that it has a cytotoxic and inhibitory effect by reducing proliferation, migration, invasion, and apoptosis on MCF-7, T47D, and MDA-MB-231 and had no effect on normal MCF-10A cells [35]. The biological and anti-cancer properties of the phytochemical Chr have been revealed by researchers and are therefore used in treating of many diseases including cancer. Studies conducted by Sulaiman et al. in 2018 on the MCF-7 breast cancer cell line and SKOV-3 ovarian cancer cell line display the inhibitory effect of Chr NPs and modified Chr NPs (PLGA-PVA) in in vitro state [13]. Using multiple drugs for the same purpose and the same cellular pathways can have an essential and functional role in effectiveness of more and better complete treatment [36]. Several studies have been conducted on the effectiveness of Artemisinin in combination cancer therapies. The synergistic effect of Artemisinin medication in combination with other compounds to increase the therapeutic effect and reduce the side effects of cancer therapies has been documented in cell lines and animal testing. According to research, the anti-cancer effect of Artemisinin is significantly increased when used with flavonoids. Though, in combination therapies, Artemisinin has not always shown a synergistic effect [15, 37]. Studies by Efferth et al. in 2017 displayed the synergistic effect of Art with a wide range of drugs, including some flavonoid compounds. It can even be used to increase the therapeutic effect of radiotherapy and other macromolecules such as recombinant protein and therapeutic nucleic acid [15]. In
this study, Art and Chr showed a synergistic effect in drug combination form, so that Art/Chr had lower IC_{50} than Art and Chr separately with similar concentrations, which indicates the greater cytotoxic effect of the drug combination. Nanotechnology-based approaches offer significant potential for addressing therapeutic vulnerabilities, including cancer. Therefore, in several studies using nanoencapsulation, the limitations of phytochemicals are significantly compensated, and their biological potential is increased [38]. In research, nanoencapsulated phytochemicals have far greater antitumor effects than in pure form. In one study, the effect of metformin nanocapsules showed a greater inhibitory effect than pure metformin by reducing the expression of the hTERT gene in T47D and MDA-MB-231 cell lines in \textit{in vitro}. In another study, the effect of Curcumin and Chrysin in the nanoencapsulated form in the SW480 cell line of colorectal cancer was more than their pure form [39, 40]. In the present study as well; Art NPs, Chr NPs and Art/Chr NPs at concentrations of 5, 10, 20, 30, and 40 µM at 24 and 48 hours had a greater cytotoxic effect that at their pure state and concentrations of 5 and 10 µM at 24 hours had a greater inhibitory effect in T47D cell line.

Much more effective treatment methods have been created by the new Molecular Target Therapy [27]. Alterations in telomerase gene expression are one of the most essential and key factors in cancer cells. In this regard, several studies have been performed on the inhibition of cancer cells by reducing telomerase activity [41, 42]. In these studies, Silibinin-PLGA/PEG at different concentrations for 24 hours treated with reduced expression of the hTERT gene in the T47D cell line had an inhibitory effect on breast cancer cells [43]. With studies on the level of telomerase gene expression, researchers believe that future research centers will focus on regulating telomerase gene expression to present a new strategy for cancer treatment [24, 26, 44, 45]. In confirmation of previous research, in this study, the T47D cell line of breast cancer was inhibited by reducing the expression of the hTERT gene and telomerase activity.

**Conclusion**

In this present study, Art and Chr-loaded PLGA-PEG NPs were successfully prepared by using the w/o/w method in the presence of PVA as a stabilizer. Morphological and particle size analysis displayed that the synthesized Art and Chr-loaded PLGA-PEG NPs displayed approximate spherical shapes with smooth surfaces and an average particle size < 250 nm. Based on our results, combinational nanoformulation of Art–Chr was be capable of killing T47D cancer cells more quickly than either treatment alone, thereby potentially this strategy can reduce cytotoxicity and patient side effects. Moreover, the codelivery of Art and Chr by PLGA/PEG nanocarrier suppresses hTERT expression more efficiently in relative to the delivery of either Art or Chr at the same concentrations, indicating a synergistic manner. These results suggest that the nano-encapsulation of Art and Chr in PLGA-PEG copolymers can provide an attractive drug delivery approach for the treatment of breast cancer. Further in vivo examination should be conducted to clarify the therapeutic efficacy of these Art/Chr-loaded PLGA-PEG NPs.

**List Of Abbreviations**
| Art | Artemisinin          |
|-----|----------------------|
| Chr | Chrysin              |
| ER  | estrogen receptors   |
| PR  | Progesterone receptors |
| FBS | Fetal bovine serum   |
| DMSO| Dimethylsulfoxide    |
| PEG | polyethylene glycol  |
| PVA | alcoholic polyvinyl  |

### Declarations

#### Acknowledgements

The authors would like to thank to the stem cell research center, Tabriz University of Medical Sciences, Tabriz, Iran.

#### Authors’ contributions

Leila Khoshravan and Akram Firouzi-amandi: Methodology, Investigation, Data curation, Original draft preparation. Mehdi Dadashpour: Writing- Reviewing and Editing. Nosratollah Zarghami: Supervision, Conceptualization, funding acquisition, Reviewing and Editing.

#### Funding

This project was supported by Faculty of Medicine, Tabriz University of Medical Sciences. Grant Number: 97007.

#### Availability of data and materials

The analyzed data during the current study are available from the corresponding authors on reasonable request.

#### Ethics approval and consent to participate

The Ethics Committee of Tabriz University of Medical Sciences has approved the current study and written informed consent was obtained from all of the participants (Code of Ethics: IR.TBZ.MEDICINE.REC.1399.492).

#### Consent for publication

Not applicable
Declaration of interest

The authors’ statement has no conflicts of interest in this article content.

Author details

1 Stem Cell Research Center, Tabriz University of Medical Sciences, Tabriz, Iran. 2 Department of Immunology, School of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran. 3 Department of Clinical Biochemistry and Laboratory Medicine, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran.

References

1. Siegel RL, Miller KD, Jemal A: Cancer statistics, 2019. CA: a cancer journal for clinicians 2019, 69(1):7-34.

2. Abbasi BA, Iqbal J, Mahmood T, Khalil AT, Ali B, Kanwal S, Shah SA, Ahmad R: Role of dietary phytochemicals in modulation of miRNA expression: Natural swords combating breast cancer. Asian pacific journal of tropical medicine 2018, 11(9):501.

3. Cai Z, Liu Q: Understanding the Global Cancer Statistics 2018: implications for cancer control. Science China Life Sciences 2019:1-4.

4. Dandawate PR, Subramaniam D, Jensen RA, Anant S: Targeting cancer stem cells and signaling pathways by phytochemicals: Novel approach for breast cancer therapy. In: Seminars in cancer biology: 2016: Elsevier; 2016: 192-208.

5. Júnior RGO, Ferraz CAA, Pereira ECV, Sampaio PA, Silva MFS, Pessoa CO, Rolim LA, da Silva Almeida JRG: Phytochemical analysis and cytotoxic activity of Cnidoscolus quercifolius Pohl (Euphorbiaceae) against prostate (PC3 and PC3-M) and breast (MCF-7) cancer cells. Pharmacognosy Magazine 2019, 15(60):24.

6. Zyad A, Tilaoui M, Jaafari A, Oukerrou MA, Mouse HA: More insights into the pharmacological effects of artemisinin. Phytotherapy Research 2018, 32(2):216-229.

7. Russo M, Spagnuolo C, Tedesco I, Russo GL: Phytochemicals in cancer prevention and therapy: truth or dare? Toxins 2010, 2(4):517-551.

8. Yadav R, Agarwala M: Phytochemical analysis of some medicinal plants. Journal of phytology 2011.

9. Mehdi S, Nafees S, Zafaryab M, Khan M, Rizvi A: Chrysin: A promising anticancer agent its Current trends and future Perspectives. Eur Exp Biol 2018, 8(3):16.
Mohammadian F, Abhari A, Dariushnejad H, Nikanfar A, Pilehvar-Soltanahmadi Y, Zarghami N: Effects of chrysin-PLGA-PEG nanoparticles on proliferation and gene expression of miRNAs in gastric cancer cell line. *Iranian journal of cancer prevention* 2016, 9(4).

Khoo BY, Chua SL, Balaram P: Apoptotic effects of chrysin in human cancer cell lines. *International journal of molecular sciences* 2010, 11(5):2188-2199.

Yin KB: Chrysin in PI3K/AKT and Other Apoptosis Signalling Pathways, and its Effect on HeLa Cells.

Sulaiman GM, Jabir MS, Hameed AH: Nanoscale modification of chrysin for improved of therapeutic efficiency and cytotoxicity. *Artificial cells, nanomedicine, and biotechnology* 2018, 46(sup1):708-720.

Anari E, Akbarzadeh A, Zarghami N: Chrysin-loaded PLGA-PEG nanoparticles designed for enhanced effect on the breast cancer cell line. *Artificial cells, nanomedicine, and biotechnology* 2016, 44(6):1410-1416.

Efferth T: Cancer combination therapies with artemisinin-type drugs. *Biochemical pharmacology* 2017, 139:56-70.

Wong YK, Xu C, Kalesh KA, He Y, Lin Q, Wong WF, Shen HM, Wang J: Artemisinin as an anticancer drug: recent advances in target profiling and mechanisms of action. *Medicinal research reviews* 2017, 37(6):1492-1517.

Firestone GL, Sundar SN: Anticancer activities of artemisinin and its bioactive derivatives. *Expert reviews in molecular medicine* 2009, 11.

Chen T, Li M, Zhang R, Wang H: Dihydroartemisinin induces apoptosis and sensitizes human ovarian cancer cells to carboplatin therapy. *Journal of cellular and molecular medicine* 2009, 13(7):1358-1370.

Farokhzad OC, Langer R: Impact of nanotechnology on drug delivery. *ACS nano* 2009, 3(1):16-20.

Bhandare N, Narayana A: Applications of nanotechnology in cancer: a literature review of imaging and treatment. *J Nucl Med Radiat Ther* 2014, 5(4):1-9.

Hrkach JS, Peracchia MT, Bomb A, Langer R: Nanotechnology for biomaterials engineering: structural characterization of amphiphilic polymeric nanoparticles by 1H NMR spectroscopy. *Biomaterials* 1997, 18(1):27-30.

Spek S, Haeuser M, Schaefer M, Langer K: Characterisation of PEGylated PLGA nanoparticles comparing the nanoparticle bulk to the particle surface using UV/vis spectroscopy, SEC, 1H NMR.
spectroscopy, and X-ray photoelectron spectroscopy. *Applied Surface Science* 2015, **347**:378-385.

23. Alibakhshi A, Ranjbari J, Pilehvar-Soltanahmadi Y, Nasiri M, Mollazade M, Zarghami N: An update on phytochemicals in molecular target therapy of cancer: potential inhibitory effect on telomerase activity. *Current medicinal chemistry* 2016, **23**(22):2380-2393.

24. Leão R, Apolónio JD, Lee D, Figueiredo A, Tabori U, Castelo-Branco P: Mechanisms of human telomerase reverse transcriptase (h TERT) regulation: clinical impacts in cancer. *Journal of biomedical science* 2018, **25**(1):1-12.

25. De Vitis M, Berardinelli F, Sgura A: Telomere length maintenance in cancer: at the crossroad between telomerase and alternative lengthening of telomeres (ALT). *International journal of molecular sciences* 2018, **19**(2):606.

26. Singh Z, Khangotra P: Human telomerase reverse transcriptase as a major therapeutic target in different cancer types.

27. Gasparini G, Longo R, Torino F, Morabito A: Therapy of breast cancer with molecular targeting agents. *Annals of oncology* 2005, **16**(iv):28-iv36.

28. Sawyers C: *Targeted cancer therapy*. *Nature* 2004, **432**(7015):294-297.

29. Santoveña A, Monzón C, Alvarez-Lorenzo C, del Rosario C, Delgado A, Evora C, Concheiro A, Llabrés M, Fariña JB: Structure-performance relationships of temperature-responsive PLGA-PEG-PLGA gels for sustained release of bone morphogenetic protein-2. *Journal of Pharmaceutical Sciences* 2017, **106**(11):3353-3362.

30. Cao X, He Q: Anti-tumor activities of bioactive phytochemicals in Sophora flavescens for breast cancer. *Cancer management and research* 2020, **12**:1457.

31. Parrella P, Poeta ML, Gallo AP, Prencipe M, Scintu M, Apicella A, Rossielo R, Liguoro G, Seripa D, Gravina C: Nonrandom distribution of aberrant promoter methylation of cancer-related genes in sporadic breast tumors. *Clinical Cancer Research* 2004, **10**(16):5349-5354.

32. Israel BeB, Tilghman SL, Parker-Lemieux K, Payton-Stewart F: Phytochemicals: Current strategies for treating breast cancer. *Oncology letters* 2018, **15**(5):7471-7478.

33. Li Z, Wu X, Wang W, Gai C, Zhang W, Li W, Ding D: Fe (II) and Tannic Acid-Cloaked MOF as Carrier of Artemisinin for Supply of Ferrous Ions to Enhance Treatment of Triple-Negative Breast Cancer. *Nanoscale research letters* 2021, **16**(1):1-11.

34. Chen G-Q, Benthani FA, Wu J, Liang D, Bian Z-X, Jiang X: Artemisinin compounds sensitize cancer cells to ferroptosis by regulating iron homeostasis. *Cell Death & Differentiation* 2020, **27**(1):242-254.
35. Kumari K, Keshari S, Sengupta D, Sabat SC, Mishra SK: Transcriptome analysis of genes associated with breast cancer cell motility in response to Artemisinin treatment. *BMC cancer* 2017, **17**(1):1-13.

36. Rasouli S, Zarghami N: Synergistic growth inhibitory effects of chrysin and metformin combination on breast cancer cells through hTERT and cyclin D1 suppression. *Asian Pacific journal of cancer prevention: APJCP* 2018, **19**(4):977.

37. Ferreira JF, Luthria DL, Sasaki T, Heyerick A: Flavonoids from Artemisia annua L. as antioxidants and their potential synergism with artemisinin against malaria and cancer. *Molecules* 2010, **15**(5):3135-3170.

38. Jain V, Kumar H, Anod HV, Chand P, Gupta NV, Dey S, Kesharwani SS: A review of nanotechnology-based approaches for breast cancer and triple-negative breast cancer. *Journal of Controlled Release* 2020.

39. Javidfar S, Pilehvar-Soltanahmadi Y, Farajzadeh R, Lotfi-Attari J, Shafiei-Irannejad V, Hashemi M, Zarghami N: The inhibitory effects of nano-encapsulated metformin on growth and hTERT expression in breast cancer cells. *Journal of drug delivery science and technology* 2018, **43**:19-26.

40. Bagheri R, Sanaat Z, Zarghami N: Synergistic effect of free and nano-encapsulated chrysin-curcumin on inhibition of hTERT gene expression in SW480 colorectal cancer cell line. *Drug research* 2018, **68**(06):335-343.

41. Ganesan K, Xu B: Telomerase inhibitors from natural products and their anticancer potential. *International journal of molecular sciences* 2018, **19**(1):13.

42. Akiyama M, Hideshima T, Munshi NC, Anderson KC: Telomerase inhibitors as anticancer therapy. *Current Medicinal Chemistry-Anti-Cancer Agents* 2002, **2**(5):567-575.

43. Ebrahimnezhad Z, Zarghami N, Keyhani M, Amirsaadat S, Akbarzadeh A, Rahmati M, Taheri ZM, Nejati-Koshki K: Inhibition of hTERT gene expression by silibinin-loaded PLGA-PEG-Fe3O4 in T47D breast cancer cell line. *BiolImpacts: BI* 2013, **3**(2):67.

44. Guterres AN, Villanueva J: Targeting telomerase for cancer therapy. *Oncogene* 2020, **39**(36):5811-5824.

45. Lü M-H, Liao Z-L, Zhao X-Y, Fan Y-H, Lin X-L, Fang D-C, Guo H, Yang S-M: hTERT-based therapy: a universal anticancer approach. *Oncology reports* 2012, **28**(6):1945-1952.

**Tables**

**Table 1.** Characterization of PLGA-PEG and drug-loaded PLGA-PEG NPs. Results are expressed as mean ± SD of three different measurements (n=3).
| Groups                         | Particle size (nm)* | Polydispersity | Zeta potential (mV)* |
|-------------------------------|---------------------|----------------|---------------------|
| PLGA-PEG NPs                  | 180±4.57            | 0.165          | −32.5±2.6           |
| Chr loaded PLGA-PEG NPs       | 233±10.30           | 0.148          | −31.3±3.5           |
| Art loaded PLGA-PEG NPs       | 210±5.55            | 0.090          | −20.9±2.0           |
| Chr/Art loaded PLGA-PEG NPs   | 274±8.52            | 0.123          | −31.2±2.5           |

Table 2. IC$_{50}$ values for T47D cell line treated with pure and nanocapsulation of each drug within 24h and 48h.

| Incubation time (h) | IC$_{50}$ values (µM) Mean ± SD |
|---------------------|---------------------------------|
|                     | Pure-Chr | Chr loaded PLGA-PEG | Pure-Art | Art loaded PLGA-PEG | Pure-Art/Chr | Art/Chr loaded PLGA-PEG |
| 24 h                | 23.73    | 20.61                | 26.36    | 20                  | 18           | 20.51                     |
| 48 h                | 20       | 17.2                 | 26.36    | 17                  | 16.2         | 15                         |

Figures
Figure 1

FTIR spectra of MET, PLGA-PEG and MET-loaded PLGA-PEG NPs.
Figure 2

Characterization of Art/Chr-loaded PLGA-PEG NPs PLGA-PEG. Dynamic light scattering (DLS) (A), field emission scanning electron microscopy (FE-SEM) (B).

Figure 3

Drug release profiles of pure-Art, pure-Chr and Art/Chr from PLGA-PEG NPs in PBS at pH 7.4. Results are presented as mean ± SD (n=3).
Figure 4

In vitro cytotoxicity of pure-Art, pure-Chr, pure-Art/Chr, Art NPs, Chr NPs and Art/Chr NPs against T47D cells. A, incubated for 24h and; B, incubated for 48 h.

Figure 5

Synergistic effects of pure Art/Chr (A) and Art/Chr NPS (B) on T47D cell proliferation. CI<1 means synergistic effect; CI=1 means additive effect; CI>1 means antagonism effect.
All 6 drugs showed a remarkable reduction in hTERT expression of T47D breast cancer.