RESEARCH ARTICLE

Development of PEGylated PLGA Nanoparticles Co-Loaded with Bioactive Compounds: Potential Anticancer Effect on Breast Cancer Cell Lines

Sina Mohammadinejad[1], Davoud Jafari-Gharabaghlou[2], Nosratollah Zarghami[3]*

Abstract

Objective: The incidence of breast cancer continues to rise despite decades of laboratory, epidemiological and clinical research. Breast cancer is still the leading cause of cancer death in women. Cyclin D1 is one of the most important oncoproteins associated with cancer cell proliferation and is overexpressed in more than 50% of cases. Curcumin and chrysin are plant-derived components that are believed to assist in inhibiting the viability of breast cancer cells. These agents are involved in cancer cells' growth and reducing cyclin D1 expression. In this study, the hypothesis of combining curcumin and chrysin is applied to analyze the potential synergistic effect in inhibiting cancer cell proliferation and down-regulation of cyclin D1. Furthermore, applying PLGA-PEG NPs could improve the bioavailability of free curcumin and chrysin components and at the same time increases the anti-cancer potential of this compound. **Methods:** PLGA-PEG NPs were synthesized via the ring-opening polymerization technique and characterized with FT-IR and FE-SEM for chemical structure and morphological characteristics, respectively. Next, curcumin and chrysin were loaded in PLGA-PEG NPs and MTT assay was performed to assess the cytotoxic effect of these agents. T-47D cells were treated with appropriate concentrations of these agents and cyclin D1 expression level was evaluated by real-time PCR. **Results:** The obtained results from FT-IR and FE-SEM techniques illustrated that curcumin and chrysin were efficiently encapsulated into PLGA-PEG NPs. Curcumin, chrysin, and curcumin-chrysin in free and nano-encapsulated forms exhibited an anti-cancer effect on T-47D cells in a time- and dose-dependent manner, especially in a combination of free and encapsulated forms demonstrated synergistic anti-cancer effects. Compared to free form, Nano-curcumin, Nano-chrysin, and Nano-combination remarkably down-regulated cyclin D1 gene expression. (p-value < 0.05). **Conclusion:** Our results revealed that the curcumin-chrysin combination has a synergistic effect and the encapsulated form of this nano-component has more inhibition on cyclin D1 expression.

Keywords: PLGA-PEG Nano-particles- breast cancer- curcumin- chrysin- cyclin D1

Introduction

Cancer is becoming a universal health issue in the modern world. Recent data shows fatality rate for cancer is getting close to that of heart and artery disease making it the second major cause of death in the modern world. Lung cancer as well as bronchus, colorectal and prostate in men and bronchus, breast and colorectal cancer in women account for most common fatal cancers among all types while breast cancer by itself constitutes a proportion of one-third in women with cancer in the U.S in 2012 (Siegel et al., 2012; Musika et al., 2021).

For years, scientists have been trying to retard or stop cancer progression by making disturbance in cell cycle of malignant tissues (Yan et al., 2021). Cyclin dependent kinases (cdks) are known as key regulating factors in cell cycle. Thus, playing important role in the maintenance of a living cell (Wang, 2021). Growth factors are inducers of cyclins, which are modulators of cdks (Gutierrez-Chamorro et al., 2021). D cyclines when bound to cdk6 and 4, trigger the phosphorylation of retinoblastoma protein (Rb), in consequence deactivate the repressing effect of Rb on E2F cluster of transcription factors leading to expression of genes that move the cell from G1 to S phase, the result is cell proliferation (Roufayel et al., 2021). Cyclin D1 is reported to be related with invasiveness of cancer when overexpressed (Alao, 2007). It is extensively mentioned in several reports on human breast cancer, the overexpression of cycline D1 gene in up to 50% of the breast cancer cases. Furthermore,

*[1]Department of Medical Biotechnology, Faculty of Advanced Medical Sciences, Tabriz University of Medical Sciences, Tabriz, Iran. [2]Department of Clinical Biochemistry and Laboratory Medicine, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran. [3]Department of Medical Biochemistry, Faculty of Medicine, Istanbul Aydin University, Istanbul, Turkey. *For Correspondence: zarghamin@gmail.com*

antiestrogenic drugs appear to be inefficient toward the accumulation of cyclin D1. So, ablation of cyclin D1 appears to be a strong proposes for future research in breast cancer treatment (Mohammadinejad et al., 2015a; Kashyap et al., 2021a).

The present strategy in treatment of breast cancer including Chemotherapy, radiation, and surgical resection seems unstable, invasive and expensive. In case of radiotherapy and chemotherapy, normal gene functions are also affected. In conclusion, there is a growing attitude about developing strategies with less complexity for future breast cancer research (Bagheri et al., 2018). Plants have always been sources for investigation of news compounds that have potency to be used in pharmaceutical industry. In many civilizations, plant base products are widely used in their intact form either as syrup or other edible forms. In case of cancer, many uninvestigated plant compounds can provide potential cheaper, safer and in some cases more effective ways to treat cancers of various types. To date, only a few of these compounds have been studies comprehensively in terms of activity, mechanisms of action and their adverse side effects. Despite this, as such compounds constitute roughly 70 percent of all anticancer drugs. Curcumin and chrysin are two of plant compounds that are under focus these days in many anticancer researches (Mohammadinejad et al., 2015b; Bhatia et al., 2021; Alagheband et al., 2022; Salmani Javan et al., 2022).

Curcumin (1, 7-bis (4-hydroxy 3-methoxy phenyl)-1, 6-heptadiene-3, 5-Dione) is an active constituent isolated from Curcuma longa Linn (turmeric) has been recorded to be consumed as medicine since ancient world. Different types of curcuminoids such as curcumin I (or curcumin, ≈77%), curcumin II (de-methoxy-curcumin, ≈17%) and curcumin III (bis-demethoxy-curcumin, ≈3%) are derived from the yellow fraction of turmeric (Goel and Aggarwal, 2010; Pulido-Moran et al., 2016; Abadi et al., 2022). It is widely concluded from modern researches that curcumin acts in a pleiotropic way and it is a result of its ability in modulating multiple signaling molecules (Gupta et al., 2012; Badrzadeh et al., 2014; Kumar et al., 2021). Several reports introduce curcumin as a considerable anticancer agent in many tumor cell lines and animal models while it acts both as a blocker of initiation step of the cancer and an inhibitor of cell proliferation and promotion and progression step of carcinogenesis (Khameneh et al., 2018; Sarkhosh et al., 2019; Kumar et al., 2021). It is mentioned, curcumin acts in various ways most popularly, by means of inhibition of several cell signaling pathways, cell cycle arrest, antiproliferating effect, inactivation of oncogene, and induction of apoptosis which results in downregulation of determining cancer genes for example cyclin D1, c-Myc, N-Myc and antiapoptotic factors (Bangaru et al., 2010; Rami and Zarghami, 2013; Shetty et al., 2021). This is in consistence with decreased amount of cyclin D1 mRNA levels after curcumin treatment reported in some other experiments (Gupta et al., 2012; Rahmani et al., 2014).

Flavonoids are referred to as a diversity of plant base poliphenolic compounds found in honey as well as some fruits and vegetables. Flavonoids are affirmed to be of low toxicity and this lack of medical issues make them good candidates to be used as anticancer agents. Such compounds are classified into flavonols, flavanones, flavanols and flavans (Khan and Uddin, 2021). Chrysin (5,7-dihydroxyflavone) is a by-product from honey showing a high degree of biological activity and mostly known as a convenient anticancer, anti-inflammatory, anti-oxidant and anti-allergic metabolite (Eatemadi et al., 2016; Mohammadian et al., 2017; Ganai et al., 2021). Rendering cell cycle arrest, chrysin conclusively brings about cell death. Chrysin effectively inhibits cell proliferation while it induces apoptosis in a several cancer cell lines including breast cancer (Middleton et al., 2000; Maasomi et al., 2017; Khazei et al., 2021). It is reported sporadically that this anticancer activity may come from down regulation of cyclin D1 and causes G1 cell cycle arrest (Mohammadinejad et al., 2015a; Dias et al., 2021).

The concept of combined therapeutics has resulted in development of several types of new cancer drugs with higher efficacy, lower therapeutic dosage, and less drug resistance emergence. Furthermore, combining plant derivate compounds may result in compositions with higher chemopreventive effects (Wagner, 2011; Lotfi-Attari et al., 2017; Bagheri et al., 2018; Adlravan et al., 2021; Lin et al., 2022).

Despite showing strong anticancer effect on many cancer cell lines, curcumin and chrysin still have issues regarding their poor bioavailability which is primarily a result of poor absorption, high metabolism rate and systemic elimination (Walle et al., 2001; Sadeghzadeh et al., 2017; Rasouli et al., 2020). To overcome these issues, scientists are trying to improve their bioavailability using nanoparticles (NP), micelles, liposomes, phospholipids complexes and in some cases, structural analogs (Tiyaboonchai et al., 2007; Sohn et al., 2021). A great amount of research is being undertaken to utilize Polymeric nanoparticle-based drug delivery in order overcoming complications associated with delivery of free drugs like curcumin and chrysin (Mohammadian et al., 2016; Khameneh et al., 2018; Gowtham, 2021). The use of PLGA (Polylactide-co-glycolide) provides benefits in regard to its biodegradability and biocompatibility and its approvals the U.S. Food and Drug Administration (Mukerjee and Vishwanatha, 2009). Modification of PLGA surface by PEG (poly-ethyleneglycol) give raise to better permeability and bioavailability as well as elongation of circulation time (Khalil et al., 2013; Zohre et al., 2014).

In this study, the hypothesis of combining curcumin and chrysin is applied to analyze the synergistic potential effect in inhibiting cancer cell proliferation and down-regulation of cyclin D1. Furthermore, applying PLGA-PEG NPs improves the bioavailability of free curcumin and chrysin components and at the same time increases the anti-cancer potential of this compound.

Material and Methods

Material

T47-D breast cancer cell line (code: c203) was purchased from Pasteur Institute of Iran. Curcumin powder was obtained from Merck (Germany). Chrysin, 3(4,

5-dimethylthiazol-2-yl) 2, 5-diphenyl-tetrazolium bromide (MTT), streptomycin, penicillin G, stannous octoate (Sn (Oct) 2), PEG (6000), glycolide, polyvinyl alcohol (PVA), dimethyl sulphoxide (DMSO), dichloromethane (DCM) and D, L-lactide all were obtained from Sigma-Aldrich (USA). Trypsin-EDTA, Fetal bovine serum (FBS), and RPMI-1640 were from Gibco, Invitrogen (UK). RNX-Plus kit and Hot Taq EvaGreen qPCR Mix purchased from CinnaGen (Iran) and were used for total RNA extraction and real time PCR respectively. 2-step RT-PCR kit was obtained from vivantis. Primers were purchased from Takapouzist. Nanodrop spectrophotometer was Bio Photometer. Real-time PCR was done using Corbett (Rotor Gene 6000).

Synthesis of PLGA-PEG NPs

PLGA-PEG NPs were synthesized via Ring-opening polymerization technique. First, the drying process of PEG was performed by stainless steel reactor at 150 °C under vacuum (5 mmHg) for 2 h. Then, D, L-lactide (2.882 g), and glycolide (0.270 g) were added to dry PEG (1.44 g) and the process was followed at 150 °C under vacuum conditions for another 30 min. After that, the heating process at 160 ± 5°C under vacuum conditions was followed in the presence of the stannous 2-ethylhexanoate (0.04 g), as a catalyst, for 8 h. The synthesized copolymer was dissolved in dichloromethane and the precipitation process followed in the ice cold diethyl ether. Finally, the obtained product was dried in a vacuum oven.

Preparation of curcumin and chrysin loaded PLGA-PEG

S/O/W manner applied for encapsulation the drugs in PLGA-PEG NPs. For each component 200 mg of the polymer, PLGA-PEG, was dissolved in dichloromethane (DCM). Separately, 20 mg pure curcumin and chrysin were added to the PLGA/DCM solution and sonicated for 1 minute to produce the s/o primary emulsion. Ethanol and polyvinyl alcohol (PVA) 1% (1:1) solution was added to curcumin emulsion while chrysin emulsion was mixed by DMSO (dimethyl sulphoxide) and PVA 1% (1:1) solution then these two mixtures were sonicated for 1 minute to form the final s/o/w emulsion. Afterward, these s/o/w emulsions were vacuum evaporated to eliminate the organic solvent, separately. The final s/o/w emulsions were centrifuged at 10,000×g for 30 minutes to assist the removal of remaining solvents. The encapsulation efficiency of the nanoparticles was investigated by assessing the supernatant of the final emulsions. For the evaluation of curcumin and chrysin present in the supernatants, absorbances were measured by spectrophotometer at 450 nm and 348 nm respectively. The amount of the drug encapsulated and the percentage of encapsulation (E %) in the NP was calculated by E (%) = ([Drug] total - [Drug] free)/ [Drug] total × 100 (Khalil et al., 2013).

Scanning electron microscopy (SEM) and FTIR

The surface morphology and the size of PLGA-PEG NPs were measured by scanning electron microscopy (SEM) and characterization of the functional groups was carried out by FTIR analysis.

Cell culture

Breast cancer cell line (T47-D) were obtained from the Cell Bank of Pasteur Institute of Iran and retained in RPMI-1640 medium supplemented with 10% FBS, Penicillin G (100 u/mL), Streptomycin (50 μg/mL), and NaHCO$_3$ (2 mg/mL) incubated at 37°C and in 5% CO$_2$.

MTT Assay

Cell viability measurement was carried out through MTT assay. T47-D cells at exponential phase were seeded on 96-well plate at density of (10×10^3 per well) and incubated for 24 h. Then cells were treated with different concentrations of curcumin (10-80 μM) and chrysin (5-640 μM) separately, each for 24, 48, 72 h and combination of curcumin and chrysin (5-640 μM) for 24 h. The same series of treatments were repeated by Nano encapsulated drugs. Controls received solvents alone (ethanol, DMSO and PLGA) with no curcumin and chrysin. For each concentration triplicate were allotted. After different exposure time, medium was discarded and 200μl fresh medium and 50 μL MMT solution (2mg/ml in PBS) were added to all wells, the plate were wrapped with aluminum foil, and incubated for 4 h in a humidified atmosphere at 37°C. Afterward, the medium and MTT solution were removed from the wells and the remaining MTT-formazan crystals was dissolved by adding 200 μL of DMSO and 25 μL Sorenson's buffer to all wells. Finally, the absorbance measurement was determined at 570 nm using an ELISA plate reader. The viability was calculated by this formula:

$$\text{percent of viable cells} = \left(\frac{\text{absorbance of experimental wells}}{\text{absorbance of control wells}}\right) \times 100$$

The combination index (CI) for two agents were calculated by CompuSyn synergism/antagonism analysis software (Version 1.0, ComboSyn, Inc., Paramus, NJ, USA) CI values of <1, =1 and >1 indicated synergism, additivity and antagonism in combined agent action respectively (Chou and Talalay, 1984).

Total RNA extraction and cDNA synthesis

After 24 h treatment with different concentrations of curcumin, chrysin and combination of curcumin and chrysin total RNAs were extracted by RNX-Plus according to the instructions of the manufacturer. The same series of treatments were repeated by Nano encapsulated drugs. Quantity and purity of total RNA were defined using the Nanodrop. Total RNAs integrity was examined using electrophoresis on a 1% agarose gel. Complementary DNA (cDNA) synthesis was brought about by 2-step RT-PCR kit from each sample, following the instructions of the manufacturer.

Real-time PCR

After cDNA synthesized, real-time PCR technique was utilized to determine cyclin D1 mRNA expression level. Real-time PCR was processed by Hot Taq EvaGreen qPCR Mix for each sample following the manufacturer instruction. The sequences of forward and reverse primers were used for cyclin D1, F: 5'- TGC CCT CTG TGC CAC

AGA TG-3', R: 5'-TCT GGA GAG GAA GCG TGT GA-3'. The reaction mixture was incubated in following: first step denaturation at 95∘C for 10 min, followed by cycles of denaturation at 95 C for 15 s (1 cycle), annealing step at 60∘C for 30 s (40 cycles), extension step at 72∘C for 30 s (40 cycles) and melting step at 65–95 ∘C (1 cycle). Relative expression of cyclin D1 mRNA was normalized by β-actin mRNA levels and relative cyclin D1 mRNA expression was calculated by this formula:

$$\text{Relative gene expression} = E(\text{target})^{\Delta CP\,(\text{target})}/E(\text{reference})^{\Delta CP\,(\text{refrence})}$$

E target and E reference are referred to real-time efficiency of target and reference gene transcript respectively; ΔCP target and ΔCP reference are refer to CP deviation of control minus sample of the target and reference gene transcript, respectively (Pfaffl, 2001).

Statistical analysis

All data are shown as mean ± SD from at least three independent experiments. Graphs were plotted by GraphPad.Prism.6.01. Statistical analysis of the data was achieved by ANOVA test. The examining importance was showed if p value < 0.05.

Results

Characterization of PLGA-PEG NPs

Fourier Transformed Infra-Red Spectroscopy (FTIR) results confirm the presence of drug in nanoparticles (Figure 1) that prepared in KBr disks and recorded on a Bruker Tensor 270 spectrometer (from 4,000 to 400 cm^{-1}). The surface morphology and shape of NPs was evaluated by SEM. The obtained SEM images from the drug-loaded NPs have shown their spherical and uniform shapes (Figure 2).

MTT-assay

Anti-proliferative effects of pure curcumin, chrysin and combination of curcumin and chrysin were assessed by MTT-assay. Separately, the same test was performed for encapsulated drugs. Our data exhibited that different concentrations of pure and Nano curcumin in 24, 48, and 72 h led to repression cell progression both in time- and dose-dependent manner but in case of free and Nano chrysin, inhibiting effect was only observed in dose-dependent manner. The IC_{50} value of free and Nano curcumin in 24, 48, and 72 h of incubation were 31.53, 25.37 and 17.66 (for free curcumin) and 24.80, 20.77 and 15.73 (for Nano curcumin), respectively. While the
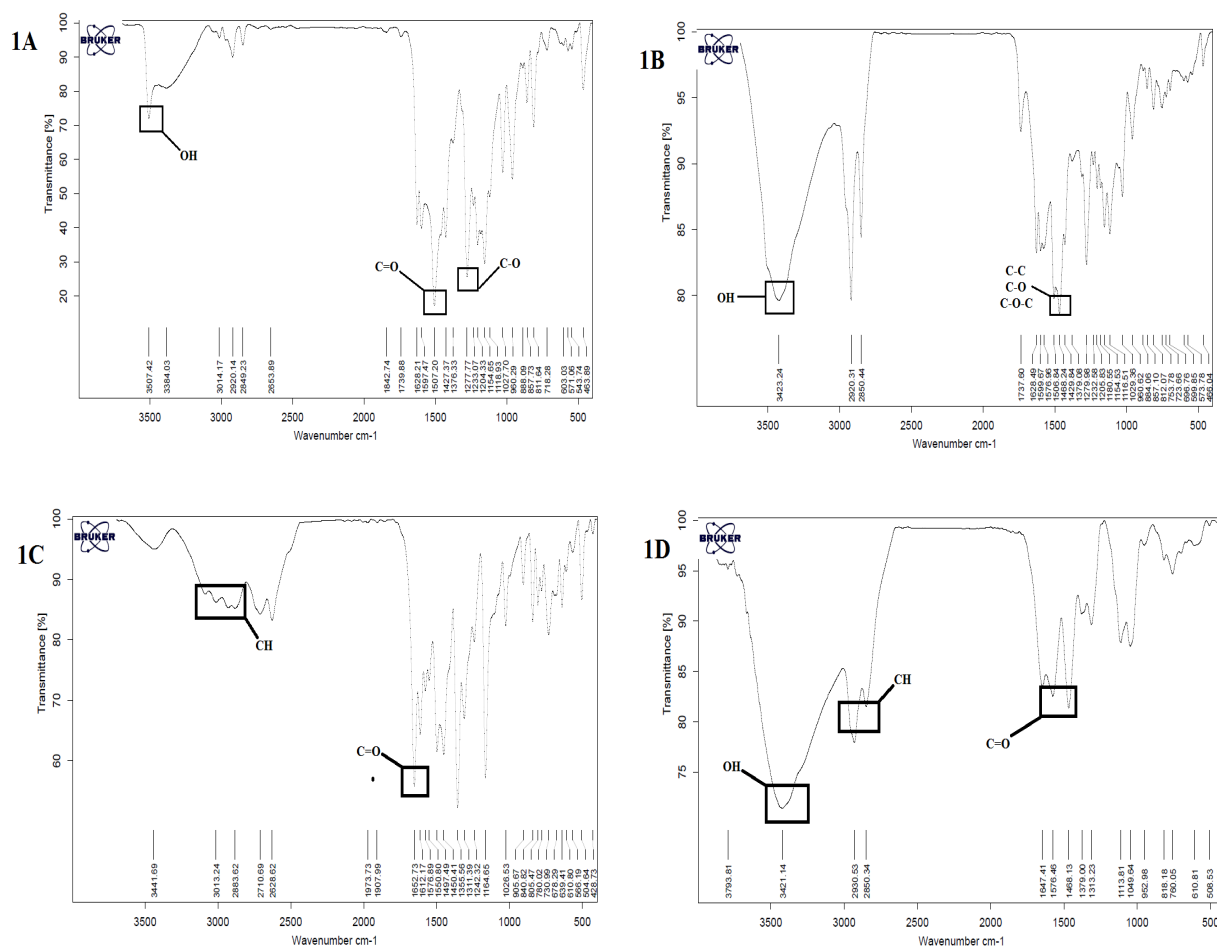

Figure 1. A) FT-IR curcumin. B) FT-IR curcumin-PLGA-PEG: results of FT-IR demonstrated the presence of curcumin loaded in PLGA-PEG Nano-particle. C) FT-IR chrysin. D) FT-IR chrysin-PLGA-PEG: results of FT-IR demonstrated the presence of chrysin loaded in PLGA-PEG Nano-particle.

Table 1. IC$_{50}$ Value (μM) for Different Drug.

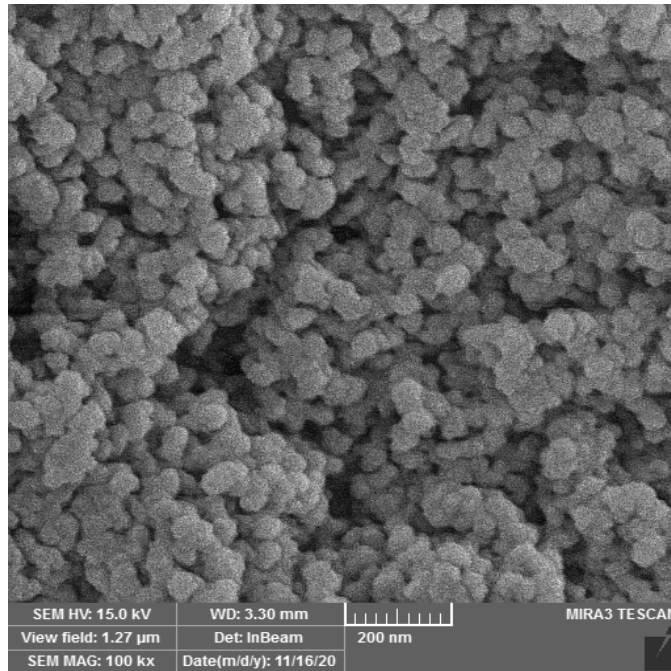| Drug (μM)	Incubation time		
	24 h	48 h	72 h
Free curcumin	31.53±1.04	25.37±1.67	17.66±0.94
Nano curcumin	24.80±1.38	20.77±0.76	15.73±1.42
Free chrysin	54.56±2.31	46.74±2.18	40.28±1.59
Nano chrysin	48.72±1.36	44.78±1.93	40.28±0.91
Free combination (curcumin-chrysin)	19.83±.074	-	-
Nano combination (curcumin-chrysin)	9.77±.082	-	-

Figure 2. SEM of Curcumin- Chrysin Loaded in PLGA-PEG Exhibits the Nano-Particle Sizes Range between 70-200 nm.

IC$_{50}$ value for pure and Nano chrysin, were 54.56, 46.74 and 40.28 (for pure chrysin) and 48.72, 44.78 and 37.54 (for Nano chrysin) respectively. These results shows that anti-proliferation effects of curcumin and chrysin improved when loaded in PLGA-PEG nanoparticles (Figures 3-4). Pure and Nano combination of curcumin-chrysin for 24 hours caused a significant inhibition of cell proliferation in IC$_{50}$ value of 19.83 and 9.77 respectively (Figure 5). Although CI (combination index) indicated that, free combination possesses strong synergistic effect to inhibit cell proliferation, CI of Nano combination has much more synergistic effect in inhibition of cell proliferation even
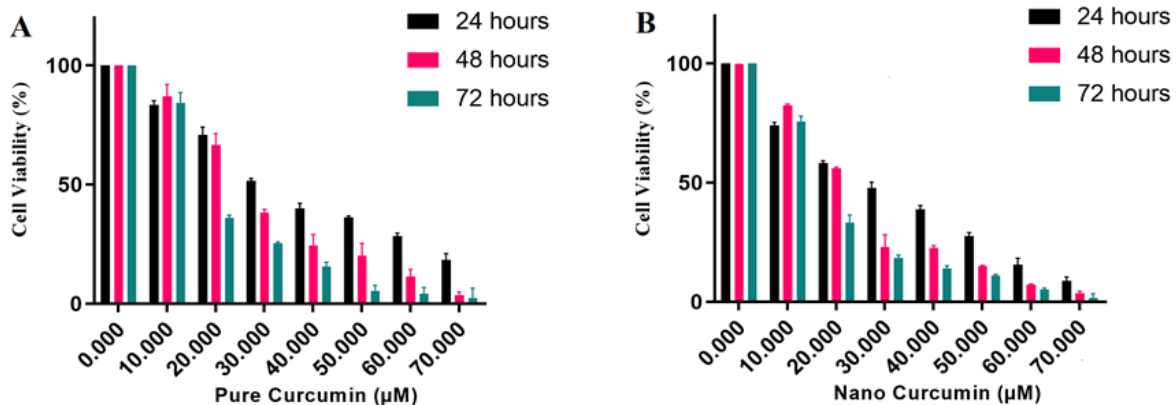

Figure 3. Figures A and B Show the Cytotoxic Effect of Pure Curcumin and Curcumin Encapsulated with PLGA-PEG in 24 h, 48 h, and 72 h Respectively.
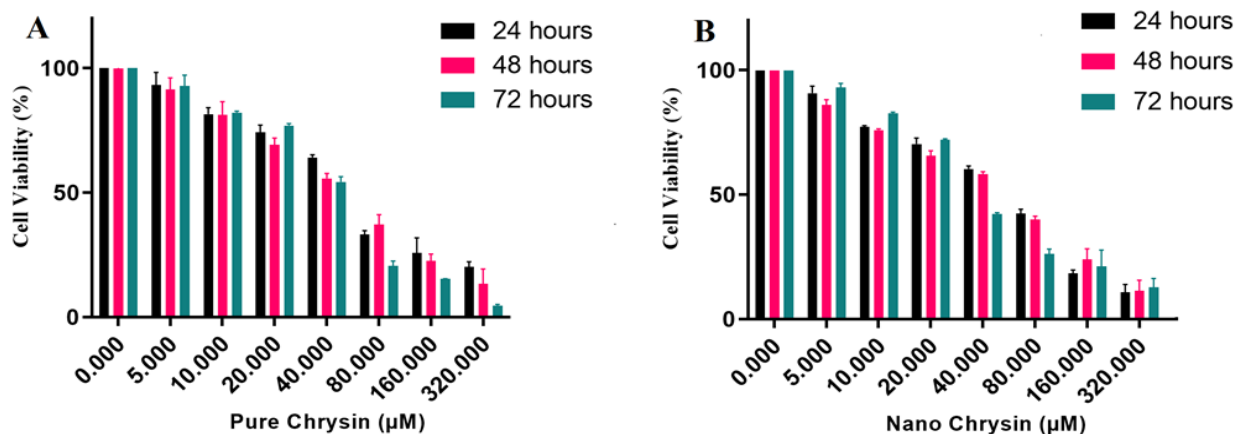
Figure 4. Figures A and B Show the Cytotoxic Effect of Pure Chrysin and Chrysin Encapsulated with PLGA-PEG in 24 h, 48 h, and 72 h Respectively.

at lowest dose (Figure 6 and Table 1).

Quantitative real-time PCR

Cyclin D1 mRNA levels were measured by real-time PCR and the levels of cyclin D1 mRNA expression was normalized by β-actin mRNA levels. The outcome of this analysis revealed that curcumin and chrysin treatment cause down-regulation in cyclin D1 mRNA levels, further more approximately same result was observed in encapsulated drug. Different concentration of free curcumin 21, 31 and 41 μM Compared to the control were decline relative cyclin D1 expression in 0.73±0.06, 0.41±0.03 and 0.30±0.03, while same concentration of Nano curcumin was decreased relative cyclin D1 expression in 0.62±0.02, 0.36±0.03 and 0.21±0.02 respectively (Figure 7A). Different concentration of free and Nano chrysin in 34, 54, and 74 μM were affected in 0.78±0.04, 0.37±0.03 and 0.24±0.02 (for free chrysin) and 0.56±0.04, 0.23±0.03 and 0.16±0.03 (for Nano chrysin) (Figure 7B). Free and Nano combination of these plant constituents (curcumin-chrysin) significantly decreased relative cyclin D1 expression compared to control in following order: 9 μM 0.44±0.03, 0.26±0.05 and 19 μM 0.32±0.03, 0.18±0.02 and 29 μM 0.13±0.01, 0.07±0.02 respectively for free and Nano combination (Figure 7C).

Discussion

Cancer may occur if the balance between cyclin/cdk activation and inhibition is distorted (Laphanuwat et al., 2018; Najm et al., 2019). Cyclin D1 play roles in progression of the cell from G1 phase in which, it interacts with its specific kinases and ultimately deactivate retinoblastoma protein and stopping cell division. Several reports have shown the notable role of cyclin D1 overexpression in breast cancer tissues (Sherr, 1996; Montalto and De Amicis, 2020). Development of drugs that selectively target cyclin D1 gene sure is a unique trait of inhibition of cancer since Cyclin D1 over expression is proved to be in direct association with cancer
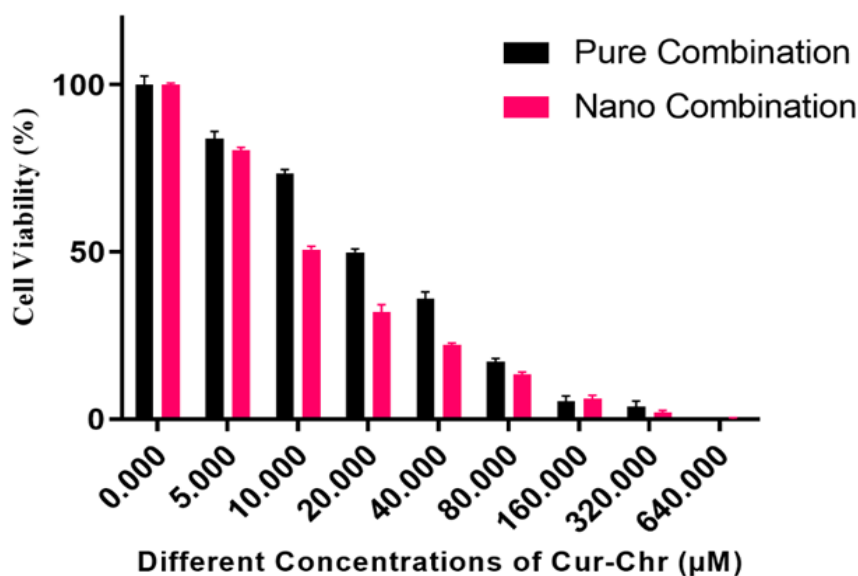

Figure 5. Cytotoxic Effect of Free and Encapsulate Curcumin and Chrysin Combination within 24 h of Exposure.
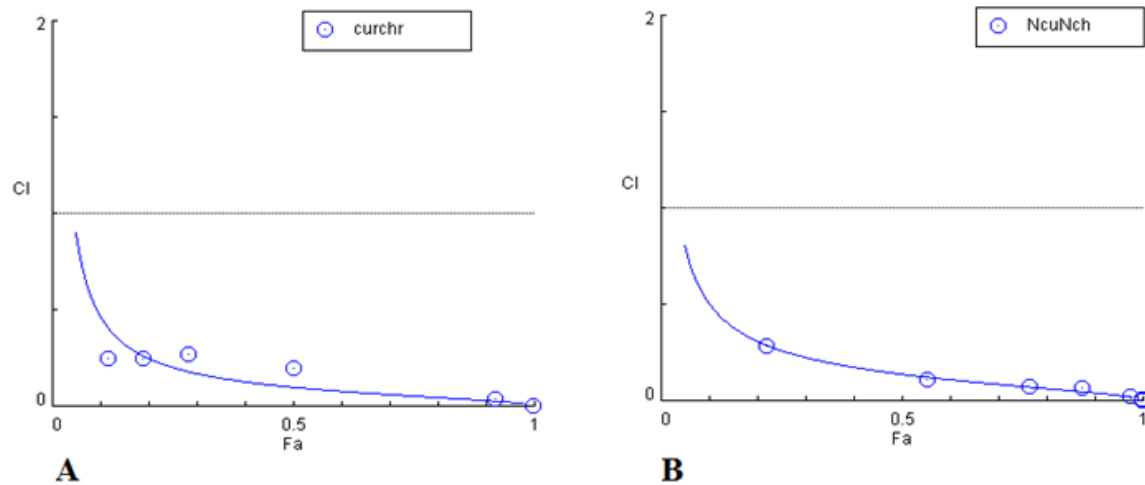
Figure 6. Combination Index (CI) A) CI of Free Curcumin and Chrysin Reveals that, these Two Components had Synergistic Effect on Cell Growth Inhibition. B) CI of curcumin and chrysin encapsulated with PLGA-PEG also reveals that, these two components had synergistic effect on cell growth inhibition.

development typically in case of breast cancer (Kashyap et al., 2021b). Chemoprevention is the major approach applied all around the world to treat types of cancer. In a chemoprevention study the favorable result is the best one with least amount of chemopreventive compound (Zhou et al., 2013). Curcumin and chrysin in their pure form show great anticancer effect due to modulation multiple signaling molecules. The drawback is the fact that their low bioavailability *in vivo* (Jaganathan and Mandal, 2009; Gupta et al., 2012). To mitigate this problem, we utilized PLG-PEG nanoparticle technology to deliver our drug in this study.

In another study with chrysin in its pure form, flowcytometry assisted seeing an increase in number of G1 arrested cells and consequently a fall in the number of cells in S and G2/M phase cells. They reported that increasing the duration of incubation leaded to suppression of cyclin D1 protein levels along with increase in G0 and G1 phase cell count. This *in vitro* anti-cancer property of chrysin is referred to its capability in suppressing proliferation, induction of G1 cell cycle arrest, up regulation of p21 and reducing in cyclin D1 and cdk2 protein levels (Pal-Bhadra et al., 2012). In consistence with our work, they showed that cell lines treated with
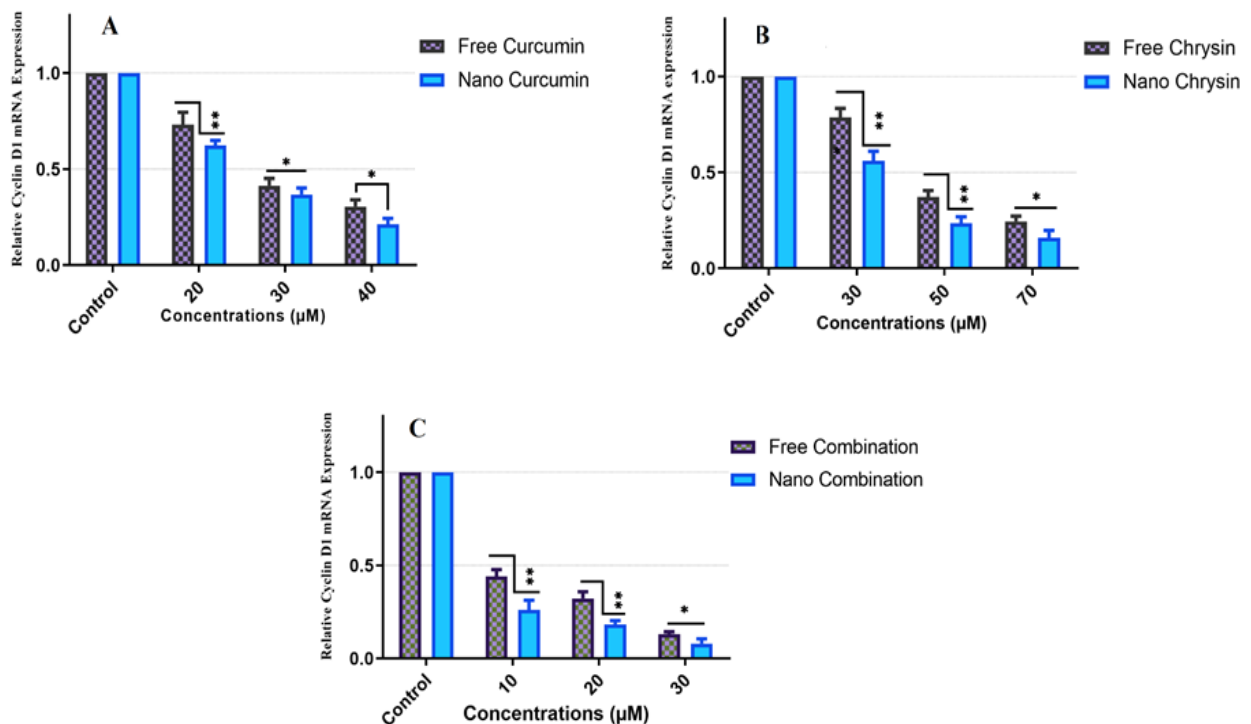

Figure 7. Quantitative Real-Time PCR of Cyclin D1 mRNA Expression. A, Free and Nano capsulated curcumin; B, Free and Nano capsulated chrysin; C, Combination of free and Nano capsulated curcumin and chrysin.

chrysin, to a great extent tend to stop in G1 and cyclin D1 expression is reduced considerably.

A few studies by Cao et al., (2014) on colon and gastric cancer cell lines, it is shown the low bioavailability of chrysin is a serious obstacle in the use of chrysin as a cancer chemotherapeutic and then BrMC (8-Bromo-7-methoxychrysin) analog of chrysin is introduced instead because of its more intense effect and greater capability in induction of apoptosis. In our experiments, we have tried to benefit from nanoparticle PLGA PEG delivery system to overcome the bioavailability problem of free chrysin. This results in a better chemopreventive effect. Furthermore, addition of curcumin to chrysin exponentially increases the inhibition of cancer cell proliferation and suppresses cyclin D1 expression.

The induction of G0-G1 arrest has clearly seen as a property for curcumin in consistence with other works on esophageal, colon and gastric cancers as well as mantle cell lymphoma. The real-time PCR results makes it clear to see the suppression of expression of cyclin D1 at 24h. It must be in correlation of G0-G1 cell cycle arrest due for this protein is accepted as responsible for progression of cell through G0-G1 transition (Su et al., 2006; Subramaniam et al., 2012).Our results are in parallel with several data which have previously shown that curcumin treatment has suppressive effects on cyclin D1 and brings about cell cycle arrest in G1. In addition, using PLGA-PEG delivery system apart from its complexities, can lead to a greater suppressive effect on the expression of cyclin D1. It is shown that using curcumin and chrysin in combination, fruitfully increases this anticancer effects even in much smaller doses giving raise to the idea of possible synergism between these two components.

Another study by Huang et al., (2008) on breast cancer MDA-MB-231 suggests the possibility of using chrysin as an additive to other anticancer drugs. On the contrary, their experience refuses any possible synergistic correlation between curcumin and chrysin when used as combination while our data approve the synergism effect either when used for free co-treatment or in PLGA-PEG nanoparticles on T47-D. This might be due to the fact that Huang et al., **(????)** only made a single dose in a low concentration and because of the low bioavailability, the combinated drug might have quickly metabolized. As a result of this, this combination might not show the synergism in very low concentrations. We also observed the synergistic effect in very low doses when using nanoparticle delivery technology.

Some other studies parallel to our work, have tried to investigate the efficacy of nanoparticle PLGA-PEG and NIPAAm-MAA delivery systems on some cancers, show that curcumin in this form has a sharper and more durable effect relative to the pure form (Khalil et al., 2013; Badrzadeh et al., 2014; Tabatabaei Mirakabad et al., 2014).

Other data shows that combination of anti-cancer components can improve the effect of cancer prevention and progression (Zhou et al., 2013). Nasiri et al., (2013) also studied curcumin in combination with silibinin and announced the synergy between these in compared to when used separately. This study also emphasizes the significance of cucumin dosage in the efficacy of the drug

in suppressing T47-D cancer cell line. Lai et al., (2019) also have shown that γ-polyglutamic acid-Gefitinib/Cur NPs dramatically repressed tumor size when comparing to free Gefitinib/Cur-treated group. In parallel to our study, Lotfi-Attari et al., (2017) shows that NP combination of cur-chr is promising manner to inhibit Caco-2 colorectal cancer cell line rather than combination of these free components alone.

In conclusion, our data demonstrated that combination of these plant components strongly suppresses cell proliferation and synergistic effects of these combinations were manifested when applied to the cells in lower doses. Either chrysin or curcumin are powerful inhibitors of cell cycle kinases and cell cycle related proteins (5-22, 13). Combination of the bioactive compounds may concurrently target various cell cycle related pathways. mRNA analysis revealed that curcumin and chrysin separately decreased cyclin D1 expression levels but their combination could dramatically decline cyclin D1 mRNA levels.

Author Contribution Statement

Writing - original draft preparation: Sina Mohammadinejad and Davoud Jafari-gharabaghlou; editing: Davoud Jafari-gharabaghlou; Conceptualization, Supervision: Nosratollah Zarghami

Acknowledgements

Not applicable.

Ethical Approval
Not applicable.

Availability of supporting data
The data that support the findings of this study are available from the corresponding author, upon reasonable request.

Conflict of Interest
No potential competing interest was reported by the authors.

References

Abadi AJ, Mirzaei S, Mahabady MK, et al (2022). Curcumin and its derivatives in cancer therapy: Potentiating antitumor activity of cisplatin and reducing side effects. *Phytother Res*, **36**, 189-213.

Adlravan E, Nejati K, Karimi MA, et al (2021). Potential activity of free and PLGA/PEG nanoencapsulated nasturtium officinale extract in inducing cytotoxicity and apoptosis in human lung carcinoma A549 cells. *J Drug Deliv Sci Tec*, **61**, 102256.

Alagheband Y, Jafari-gharabaghlou D, Imani M, et al (2022). Design and fabrication of a dual-drug loaded nano-platform

for synergistic anticancer and cytotoxicity effects on the expression of leptin in lung cancer treatment. *J Drug Deliv Sci Tec*, **73**, 103389.

Alao JP (2007). The regulation of cyclin D1 degradation: roles in cancer development and the potential for therapeutic invention. *Mol Cancer*, **6**, 24.

Badrzadeh F, Akbarzadeh A, Zarghami N, et al (2014). Comparison between effects of free curcumin and curcumin loaded NIPAAm-MAA nanoparticles on telomerase and PinX1 gene expression in lung cancer cells. *Asian Pac J Cancer Prev*, **15**, 8931-36.

Bagheri R, Sanaat Z, Zarghami N (2018). Synergistic effect of free and nano-encapsulated chrysin-curcumin on inhibition of hTERT gene expression in SW480 colorectal cancer cell line. *Drug Res*, **68**, 335-43.

Bangaru MLY, Chen S, Woodliff J, et al (2010). Curcumin (diferuloylmethane) induces apoptosis and blocks migration of human medulloblastoma cells. *Anticancer Res*, **30**, 499-504.

Bhatia M, Bhalerao M, Cruz-Martins N, et al (2021). Curcumin and cancer biology: Focusing regulatory effects in different signalling pathways. *Phytother Res*, **35**, 4913-29.

Cao XZ, Xiang HL, Quan MF, et al (2014). Inhibition of cell growth by BrMC through inactivation of Akt in HER-2/neu-overexpressing breast cancer cells. *Oncol Lett*, **7**, 1632.

Chou T-C, Talalay P (1984). Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Adv Enzyme Regul*, **22**, 27-55.

Dias MC, Pinto DC, Silva A (2021). Plant flavonoids: Chemical characteristics and biological activity. *Molecules*, **26**, 5377.

Eatemadi A, Daraee H, Aiyelabegan HT, et al (2016). Synthesis and characterization of chrysin-loaded PCL-PEG-PCL nanoparticle and its effect on breast cancer cell line. *Biomed Pharmacother*, **84**, 1915-22.

Ganai SA, Sheikh FA, Baba ZA (2021). Plant flavone Chrysin as an emerging histone deacetylase inhibitor for prosperous epigenetic-based anticancer therapy. *Phytother Res*, **35**, 823-34.

Goel A, Aggarwal BB (2010). Curcumin, the golden spice from Indian saffron, is a chemosensitizer and radiosensitizer for tumors and chemoprotector and radioprotector for normal organs. *Nutr Cancer*, **62**, 919-30.

Gowtham P (2021). Advances in targeted drug delivery in melanoma. *Asian Pac J Cancer Biol*, **6**, 331-7.

Gupta SC, Patchva S, Koh W, et al (2012). Discovery of curcumin, a component of golden spice, and its miraculous biological activities. *Clin Exp Pharmacol P*, **39**, 283-99.

Gutierrez-Chamorro L, Felip E, Ezeonwumelu IJ, et al (2021). Cyclin-dependent kinases as emerging targets for developing novel antiviral therapeutics. *Trends Microbiol*, **29**, 836-48.

Huang Q, Shen HM, Ong CN. Chrysin inhibits tumor cell migration and invasion through disruption of the membrane lipid rafts-associated integrin signaling pathway. Cancer Research. 2008 May 1;68(9_Supplement):5700-.

Jaganathan SK, Mandal M (2009). Antiproliferative effects of honey and of its polyphenols: a review. *Biomed Res Int*, **2009**.

Kashyap A, Umar SM, JR AD, et al (2021). Dihydrotanshinone-I modulates epithelial mesenchymal transition (EMT) thereby impairing migration and clonogenicity of triple negative breast cancer cells. *Asian Pac J Cancer Prev*, **22**, 2177.

Kashyap D, Garg VK, Sandberg EN, et al (2021). Oncogenic and tumor suppressive components of the cell cycle in breast cancer progression and prognosis. *Pharmaceutics*, **13**, 569.

Khalil NM, Nascimento TCFd, Casa DM, et al (2013). Pharmacokinetics of curcumin-loaded PLGA and PLGA–PEG blend nanoparticles after oral administration in rats. *Colloids Surfaces B Biointerfaces*, **101**, 353-60.

Khameneh ZR, Mohammadian M, Rasouli MA, et al (2018). Effects of curcumin in combination with doxorubicin in human colorectal cancer cell line. *Asian Pac J Cancer Biol*, **3**, 89-92.

Khan AQ, Uddin S (2021). Anticancer activity of natural compounds. *Asian Pac J Cancer Prev*, **22**, 1-2.

Khazei K, Mohajeri N, Bonabi E, et al (2021). New insights toward nanostructured drug delivery of plant-derived polyphenol compounds: cancer treatment and gene expression profiles. *Curr Cancer Drug Tar*, **21**, 689-701.

Kumar A, Harsha C, Parama D, et al (2021). Current clinical developments in curcumin-based therapeutics for cancer and chronic diseases. *Phytother Res*, **35**, 6768-801.

Lai K-C, Chueh F-S, Hsiao Y-T, et al (2019). Gefitinib and curcumin-loaded nanoparticles enhance cell apoptosis in human oral cancer SAS cells *in vitro* and inhibit SAS cell xenografted tumor in vivo. *Toxicol Appl Pharm*, **2019**, 114734.

Laphanuwat P, Likasitwatanakul P, Sittithumcharee G, et al (2018). Cyclin D1 depletion interferes with oxidative balance and promotes cancer cell senescence. *J Cell Sci*, **131**, jcs214726.

Lin W, Wu L, Zhang Y, et al (2022). An enhanced cascade-based deep forest model for drug combination prediction. *Brief Bioinform*, **23**, bbab562.

Lotfi-Attari J, Pilehvar-Soltanahmadi Y, Dadashpour M, et al (2017). Co-delivery of curcumin and chrysin by polymeric nanoparticles inhibit synergistically growth and hTERT gene expression in human colorectal cancer cells. *Nutr Cancer*, **69**, 1290-99.

Maasomi ZJ, Soltanahmadi YP, Dadashpour M, et al (2017). Synergistic anticancer effects of silibinin and chrysin in T47D breast cancer cells. *Asian Pac J Cancer Prev*, **18**, 1283.

Middleton E, Kandaswami C, Theoharides TC (2000). The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. *Pharmacol Rev*, **52**, 673-751.

Mohammadian F, Abhari A, Dariushnejad H, et al (2016). Effects of chrysin-PLGA-PEG nanoparticles on proliferation and gene expression of miRNAs in gastric cancer cell line. *Iran J Cancer Prev*, **9**.

Mohammadian F, Pilehvar-Soltanahmadi Y, Zarghami F, et al (2017). Upregulation of miR-9 and Let-7a by nanoencapsulated chrysin in gastric cancer cells. *Artif Cells Nanomed Biotechnol*, **45**, 1201-06.

Mohammadinejad S, Akbarzadeh A, Rahmati-Yamchi M, et al (2015a). Preparation and evaluation of chrysin encapsulated in PLGA-PEG nanoparticles in the T47-D breast cancer cell line. *Asian Pac J Cancer Prev*, **16**, 3753-58.

Mohammadinejad S, Akbarzadeh A, Rahmati-Yamchi M, et al (2015b). Preparation and evaluation of chrysin encapsulated in PLGA-PEG nanoparticles in the T47-D breast cancer cell line. *Asian Pac J Cancer Prev*, **16**, 3753-58.

Montalto FI, De Amicis F (2020). Cyclin D1 in cancer: a molecular connection for cell cycle control, adhesion and invasion in tumor and stroma. *Cells*, **9**, 2648.

Mukerjee A, Vishwanatha JK (2009). Formulation, characterization and evaluation of curcumin-loaded PLGA nanospheres for cancer therapy. *Anticancer Res*, **29**, 3867-75.

Musika W, Kamsa-Ard S, Jirapornkul C, et al (2021). Lung cancer survival with current therapies and new targeted treatments: A Comprehensive Update from the Srinagarind Hospital-Based Cancer Registry from (2013 to 2017). *Asian Pac J Cancer Prev*, **22**, 2501.

Najm MA, Oudah KH, Alyaseen FF (2019). Cyclin-dependent kinase inhibitors as targeted therapy in breast cancer. *Biochem Cell Arch*, **19**, 7-12.

Nasiri M, Zarghami N, Koshki KN, et al (2013). Curcumin and silibinin inhibit telomerase expression in T47D human breast cancer cells. *Asian Pac J Cancer Prev*, **14**, 3449-53.

Pal-Bhadra M, Ramaiah MJ, Reddy TL, et al (2012). Plant HDAC inhibitor chrysin arrest cell growth and induce p21WAF1 by altering chromatin of STAT response element in A375 cells. *BMC Cancer*, **12**, 180.

Pfaffl MW (2001). A new mathematical model for relative quantification in real-time RT–PCR. *Nucleic Acids Res*, **29**, 45.

Pulido-Moran M, Moreno-Fernandez J, Ramirez-Tortosa C, et al (2016). Curcumin and health. *Molecules*, **21**, 264.

Rahmani AH, Al Zohairy MA, Aly SM, et al (2014). Curcumin: a potential candidate in prevention of cancer via modulation of molecular pathways. *Biomed Res Int*, **2014**.

Rami A, Zarghami N (2013). Comparison of inhibitory effect of curcumin nanoparticles and free curcumin in human telomerase reverse transcriptase gene expression in breast cancer. *Adv Pharm Bull*, **3**, 127.

Rasouli S, Montazeri M, Mashayekhi S, et al (2020). Synergistic anticancer effects of electrospun nanofiber-mediated codelivery of Curcumin and Chrysin: Possible application in prevention of breast cancer local recurrence. *J Drug Deliv Sci Tec*, **55**, 101402.

Roufayel R, Mezher R, Storey KB (2021). The role of retinoblastoma protein in cell cycle regulation: An Updated Review. *Curr Mol Med*, **21**, 620-29.

Sadeghzadeh H, Pilehvar-Soltanahmadi Y, Akbarzadeh A, et al (2017). The effects of nanoencapsulated curcumin-Fe3O4 on proliferation and hTERT gene expression in lung cancer cells. *Anticancer Agents Med Chem*, **17**, 1363-73.

Salmani Javan E, Lotfi F, Jafari-Gharabaghlou D, et al (2022). Development of a magnetic nanostructure for co-delivery of metformin and silibinin on growth of lung cancer cells: Possible action through leptin gene and its receptor regulation. *Asian Pac J Cancer Prev*, **23**, 519-27.

Sarkhosh H, Mahmoudi R, Malekpour M, et al (2019). The effect of curcumin in combination chemotherapy with 5-FU on non-malignant fibroblast cells. *Asian Pac J Cancer Care*, **4**, 7-10.

Sherr CJ (1996). Cancer cell cycles. *Science*, **274**, 1672-77.

Shetty NP, Prabhakaran M, Srivastava AK (2021). Pleiotropic nature of curcumin in targeting multiple apoptotic-mediated factors and related strategies to treat gastric cancer: A review. *Phytother Res*, **35**, 5397-416.

Siegel R, Naishadham D, Jemal A (2012). Cancer statistics, 2012. *CA Cancer J Clin*, **62**, 10-29.

Sohn S-I, Priya A, Balasubramaniam B, et al (2021). Biomedical applications and bioavailability of curcumin—An updated overview. *Pharmaceutics*, **13**, 2102.

Su C-C, Lin J-G, Li T-M, et al (2006). Curcumin-induced apoptosis of human colon cancer colo 205 cells through the production of ROS, Ca2+ and the activation of caspase-3. *Anticancer Res*, **26**, 4379-89.

Subramaniam D, Ponnurangam S, Ramamoorthy P, et al (2012). Curcumin induces cell death in esophageal cancer cells through modulating Notch signaling. *PLoS One*, **7**, e30590.

Tabatabaei Mirakabad FS, Akbarzadeh A, Milani M, et al (2014). A Comparison between the cytotoxic effects of pure curcumin and curcumin-loaded PLGA-PEG nanoparticles on the MCF-7 human breast cancer cell line. *Artif Cells Nanomed Biotechnol*, **2014**, 1-8.

Tiyaboonchai W, Tungpradit W, Plianbangchang P (2007). Formulation and characterization of curcuminoids loaded solid lipid nanoparticles. *Int J Pharm*, **337**, 299-306.

Wagner H (2011). Synergy research: approaching a new generation of phytopharmaceuticals. *Fitoterapia*, **82**, 34-7.

Walle T, Otake Y, Brubaker J, et al (2001). Disposition and metabolism of the flavonoid chrysin in normal volunteers. *Br J Clin Pharmacol*, **51**, 143-6.

Wang Z (2021). Regulation of cell cycle progression by growth factor-induced cell signaling. *Cells*, **10**, 3327.

Yan Y, Yao S, Jia Z, et al (2021). Iso-suillin-induced DNA damage leading to cell cycle arrest and apoptosis arised from p53 phosphorylation in A549 cells. *Eur J Pharmacol*, **907**, 174299.

Zhou D-H, Wang X, Yang M, et al (2013). Combination of low concentration of (−)-epigallocatechin gallate (EGCG) and curcumin strongly suppresses the growth of non-small cell lung cancer *in vitro* and in vivo through causing cell cycle arrest. *Int J Mol Sci*, **14**, 12023-36.

Zohre S, Kazem N-K, Abolfazl A, et al (2014). Trichostatin A-induced Apoptosis is mediated by Krüppel-like factor 4 in ovarian and lung cancer. *Asian Pac J Cancer Prev*, **15**, 6581-8.