Introduction

Oxidative stress has a pivotal role in the initiation and progression of chronic inflammatory diseases and many types of cancers. Nuclear factor erythroid 2-related factor 2 (Nrf2), is a member of the cap n collar (CNC) subfamily transcription factors, that regulate intracellular antioxidant response and also maintains cellular redox homeostasis. Under oxidative stress Nrf2 induces cellular protective genes by binding antioxidant response element (ARE) to get rid of carcinogenic reactive intermediate. Many Nrf2 target genes identified that can be classified into three categories, including (i) redox–balancing proteins: hemeoxygenase-1 (HO1) and thioredoxin (Trx) (ii) phase II detoxifying enzymes: NADPH quinone oxidoreductase-1 (NQO1), glutathione S transferase (GST) and (iii) ATP-dependent drug efflux pumps: multi drug resistance-associated protein (MRP) (Li et al., 2012; Samadi et al., 2011; Zucker et al., 2014).

Under basal condition Nrf2 is anchored in cytosol by Kelch-like ECH-associated protein 1 (keap1), a master regulator protein of Nrf2. In this situation, keap1 acts as a molecular switch that facilitates the degradation of Nrf2 through ubiquitin–mediated proteasomal systems. Upon exposure to electrophilic abrogates, and carcinogenic molecules, keap1 can modify cysteine residues, resulting in a reduction of the E3 ubiquitin ligase activity, stabilization of Nrf2 and translocation to the nucleus and activation the regulatory regions of antioxidant response element (ARE)-target genes. Therefore Nrf2 pathway can eliminate carcinogenic reactive intermediates and boost cellular defense response (Taguchi et al., 2011; Crunkhorn 2012) Paradoxically, recent findings have revealed the “dark” side of Nrf2. Overexpression of Nrf2 during development of many tumor carcinoma identified (Shen et al., 2013) Khunluck reported Nrf2 polymorphism
associated with Cholangiocarcinoma cancer (Khunluck et al., 2014). Additionally in other study cav-1 introduced as a tumor suppressor genes that used for restriction cancer via arrested Nrf2 (Hart et al., 2014). Studies reported luteolin (3’, 4’, 5, 7-tetrahydroxylflavone) inhibited selectively the Nrf2 signaling pathway and sensitized NSCLC A549 cell lines to anticancer drugs (Tang et al., 2011). Brusatol introduced as a compound that have ability to inhibit Nrf2 pathway in dose dependent manner in MDA-MB 231 cell line (Ren et al., 2011). In a similar study Gao et al. (2013) revealed chrys in as a potent and selective Nrf2 inhibitors that can enhances sensitivity of BEL-7402/ADM cells to chemotherapy agent (Gao et al., 2013). In another study was shown with high concentration of EGCG can inhibit Nrf2 activity and reduce HO-1 expression in A549 cells (Kweon et al., 2006). Collectively these finding demonstrate that Nrf2 contributes to acquiesced drug resistance. Nrf2 is now considered as a molecular target to overcome drug resistance in a variety of cancers, including: neuroblastoma, breast, ovarian, prostate, lung, and pancreatic. Luteolin (3, 4, 5, 7-tetrahydroxy flavone), as a flavonoid has antioxidant, anti-inflammatory, cardiovascular protection and anti-cancer effects. It has been proved luteolin act as a potential Nrf2 inhibitor. Luteolin could cause the degradation of Nrf2 mRNA, down-regulation of the antioxidant response element (ARE)-gene battery and leading to sensitivity of A549 cells to anti-cancer drugs (Hwang et al., 2011; Zhao et al., 2011).

On the other hand, the goal of any drug delivery system is to modification of drug release profile, and improving product efficacy achieve to a desire therapeutic effect. Toxicity, low solubility, and excellent molecular size for absorption are major limiting factor for molecules to be a good candidate for therapeutic protocols Many research attempts had done to get an ideal drug delivery system which facilities these problems (Freag et al., 2013; Mathur, 2013; Tang et al., 2013). Phytosomes are novel advanced technique applied for the enhancement of bioavailability and improvement of molecular size that can produce better profile for drugs to pass the biological membrane of tumor cells after administration. Phytosmes is a novel target delivery for herbal medicine and also can be used for therapeutic purposes like cancer and health purposes as nutraceutical (Manthena and Srinivas, 2010). Pierro et al. (2009) studied phytosome formulation of green tea in obese subjects (n=100). After treatment, significant weight loss was reported in the cluster taking phytosome and no adverse effects observed during consuming (Pierro et al., 2009). Hesperetin phytosome has a sustained release property for antioxidant activity in cc14 intoxicated rats and revealed that the phytosome had higher bioavailability than hesperetin at the same dose level (Mukherjee et al., 2010). Pharmacokinetic and pharmacological parameter have improved in phytosome technology, which in results can be good option in treatment of acute liver disease, anti-inflammatory, immunomodulator and anticancer agents.

In this study luteolin and lecithin complex was prepared as phytosome. Then nanoparticles were characterized in terms of molecular size, polydespresity index and drug loading. Luteolin inhibited Nrf2 pathway with reduction cellular HO1, MDR1 levels and sensitized MDA-MB 231 cells to therapeutic drugs. Collectively, these results demonstrate luteolin - phytosome is a potential drug delivery system for enhancement of passive targeting and increasing the efficacy of chemotherapy agent in cancer therapeutic protocols. Further, it is necessary to identify compounds that inhibit the Nrf2 pathway especially and develop them into targeted delivery systems to enhance bioavailability and selectivity tumor cells.

Materials and Methods

Materials
Luteolin, tBHQ, and Doxorubicin was purchased from (Sigma –Aldrich Corporation, St. Louis, MO, USA). Roswell park Memorial Institute 1640 medium, penicillin-streptomycin and Fetal bovine serum (FBS) were provided (Invitrogen Life Technologies, Auckland, New ZEALAND). Primers were supplied from MWG Biotech (Ebersberg, Germany). RNA isolation Kit (TRIZOL) was obtained from (Sigma-Aldrich).Power SYBER Green PCR Master Mix (5 ml) was obtained from Applied Bio systems (Warrington, UK). Methylthiazoltetrazolium (MTT) was purchased from (Santa Cruz, CA, USA).

Preparation of phytosome formulation
At first to obtain to the luteolin-phospholipid complex we have done common stages respectively. The proper phospholipid among phosphatidylcholine, phosphatidyl ethanolamine and phosphatidylserin was selected. After choosing the best ratio between phospholipid, and component, luteolin and phospholipid was dissolved in appropriate organic solvent. After that the thin layer was dried by using of evaporator in result formation of thin film and finally, hydration of thin layer to formation of phytosomal suspension was done Figure 1.

Preparation of standard curve of luteolin
1 mg of luteolin was accurately weighed and dissolved in 1 ml of metanol to give a concentration of 1 mg/ml. 1 ml of the above solution was pipette out into a 100 ml volumetric flask and made into 50 ml of distilled water to

Figure 1. Chemical Structure of Luteolin and Phosphatidyl Choline

Figure 2. Calibration Curve of Luteolin
give concentration of 20 μg/ml. From resultant solution dilutions of standard concentration in Beer-Lamberts range of 2-20 μg/ml was prepared with 10 ml methanol in 10 ml volumetric flask. Absorbance of each solution was measured at 284 nm taking methanol as blank by using UV visible spectrophotometer. Statistical test (Linearity test) was applied to authenticate the standard curve Figure 2.

Characterization of phytosome

Determination of particle size: the particle size and size distribution of loaded nanoparticles characterized by laser light scattering Particle size Analyzer (WING Sald 2101 SHIMADZU JAPAN).

Determination of luteolin entrapment efficiency in phytosome complex: the proportion of encapsulated luteolin was determined by centrifuging (MC01/s.n.740 models, spin win) a certain volume of formulation at 15000 “g” for one hour at room temperature. The phytosome Complex was separated from supernatant and estimating of the supernatant for detection of drug loading by UV-Visible spectroscopy at 370 nm (λmax). The percentage entrapment efficiency was calculated by following formula: Loading efficiency% = ([percentage of loaded drug in mg]/[percentage of added drug in mg]) ×100%

SEM analysis

The investigation of surface morphology is often essential in detecting the entrapment behavior. The SEM (Scanning Electron Microscopy) provides photomicrograph of the phytosomes at appropriate magnification after covering it with a very thin layer of gold. Photomicrographs were taken using SEM Scanning Electron Microscopy to study the morphology.

Determination of zeta potential

The ZP of phytosome was measured at 25˚C, under an electrical field of 40V/cm (Malvern Instruments Ltd. Zeta sizer 2000 Malvern UK).

Cell culture

The human MDA-MB 231 breast cancer cells were purchased from Pasteur Institute cell bank Tehran, Iran. Cells were cultured in RPMI 1640 medium (Sigma-Aldrich St. Louis, MO, USA) containing 10% fetal bovine serum FBS (Invitrogen, Auckland, New Zealand), 100 U/ml penicillin and 100μg/ml streptomycin at 37˚C in a humidified 5% CO₂ atmosphere. Cells of passages from 2-5 were used in the experiments after reaching 70% confluence, MDA-MB 231 cells were seeded in a 96-well micro plate that each well containing 12000 cells and 200μl growth medium. Cells were exposed with increasing concentration of doxorubicin, luteolin and phyto-luteolin.

MTT assay

Following overnight incubation MDA-MB231 cells were treated with deferent concentration of drugs. Then, cells were replaced with 200 μl fresh media containing 20 μl of MTT solution (2 mg/ml) and incubated for 4h at 37˚C. Then, media was removed and 200 μl of DMSO plus 25 ml of Sorenson, s glycin buffer was added to dissolve the formazan crystals and the absorbance at 570 nm after shaking for 15 min was measured using a microplate reader (Biotek, ELX 800, USA).

Determination of IC₅₀ of doxorubicin and luteolin against MDA-MB231

Plots of cytotoxicity index versus increasing concentration of doxorubicin and luteolin were drawn. IC₅₀ was determined for each agent by calculating the slope and intercept Figure 3 and Figure 4.

Real-time quantitative PCR (RT-PCR)

The total RNA was isolated from cultured MDA MB 231 cells using Trizol reagent (Invetrogen, Carlsbad, USA) the protocol was established in our previous study (Samadi et al., 2009) according to the manufacturers protocol. Then, the cDNA was synthesized using the RT-PCR kits (Fermentas). PCR amplification was carried out for 35 cycles using the following protocol: 95˚C for 1 min, 94˚C for 15 s, 52.5˚C for 20 s, 72˚C for 20s and 72˚C for 5 min. specific primers for Nrf2 (5’-ACTCCCAGGTTGCCCCAC-3’) and (5’- TGCAGCTCTTCTGGGAAGTAG-3’) MDR1, (5’- HO1,( 5’- ACGGCTTCAAGCTGGTGATG-3’) and (5’- GTAGCCGAAGAAACCTCATTGTC-3’): the proportion of encapsulated drug in mg)/(percentage of added drug in mg) ×100%

Statistical analysis

Data were conducted and expressed as ±standard deviation from three independent experiments. Statistical analysis was used by ANOVA and the significance level was considered as 95% p<0.05.

Results

Characterization of phytosomes

The behavior of phytosomes in vivo and in vitro
systems is governed by physical properties such as particle size, zeta potential, membrane permeability, percentage of entrapped solutes, and etc. In drug delivery study, it was reported that PS and ZP have a key role in bioavailability and penetration properties of nanosystems. The result in terms of PS, PI, ZP, and EE% of phytosome prepared with and without luteolin incorporation are shown in Table 1.

According to DSC analysis that Lasonder reported, luteolin had been absolutely dispersed in phosphatidyl choline. The components have some interaction through hydrogen bonds between the polar head of phospholipid and the polar functional group of luteolin or van der waals force.

**FT-IR spectroscopy of phyto-luteolin and Size and morphology of phyto-luteolin (SEM)**

FTIR spectroscopy was used to declare the attachment of phospholipid and luteolin. While the strong peak shown in area around 1100-1300 cm\(^{-1}\) (p=O stretch 1320-1140 cm\(^{-1}\) strong in FT-IR) indicated to p=O stretch, the peak was weaken in the product due to interacting of phosphate group in lecithin and Luteolin. Also weaken peak in 1600-1800 cm\(^{-1}\) the stretch bond of C=O in esters group of lecithin is the other reason to synthesizing the product Figure 5.

**Table 1. PS, PI, ZP, and EE% of Blank and Luteolin-loaded Phytosome**

<table>
<thead>
<tr>
<th>Formulation</th>
<th>PS±SD (nm)</th>
<th>PI±SD</th>
<th>ZP±SD (mv)</th>
<th>EE%±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank phytosome</td>
<td>62±7.5</td>
<td>0.591±0.01</td>
<td>-25.8±0.2</td>
<td>68.3±2.6</td>
</tr>
<tr>
<td>Phyto-luteolin</td>
<td>83±7.9</td>
<td>0.596±0.03</td>
<td>-29.6±0.5</td>
<td></td>
</tr>
</tbody>
</table>

*The results are the means±SD (n=7); **Abbreviations: PS: particle size; PI: polydispersity index; ZP: zeta potential; EE%: entrapment efficiency; SD: standard deviation; Phyto-luteolin: luteolin loaded phytosome

Having no obvious changes in FT-IR of the product and in its nano form FT-IR and also showing the same peaks in both FT-IRs beside dimensions below 50nm in the SEM picture of product demonstrated that the nano particle was synthesized Figure 6 and Figure 7.

**Cell growth inhibition**

Phyto-luteolin sensitizes MDA-MB 231 cells to doxorubicin. MTT assay were done to determine cell viability and cytotoxicity rate of MDA MB 231 breast cancer cells after being incubation with different concentration of luteolin, phyto-luteolin, and doxorubicin for 24 hours. As shown in Figure 8 results demonstrated that nano-luteolin+Dox could inhibit growth of MDA-MB231 cells more effectively than nano+dox and dox (p<0.05).

Additionally, there was no significant difference between phytosome alone and control group (p>0.05), which confirmed their low toxicity Figure 8.

**Figure 5. Fourier Transform Infrared Spectra.** A) luteolin; B) lecithin; C) phytoluteolin and; D) complex of luteolin and lecithin

**Figure 6. Scanning Electron Microscopy of Phyto-Luteolin in 500 Scale**

**Figure 7. Phyto-luteolin in 63 Nanometer.** The ZP of phyto-luteolin was measured at 25°C, under an electoral field of 40V/cm

**Figure 8. NanoLut+Dox had More Cytotoxicity in Comparison with Nano+Dox and Dox on MDA-MB231 Cells.** Cytotoxicity test were conducted by MTS assay. Results had analyzed with one-way ANOVA test (p<0.05)

**Figure 9. Nanoluteolin Suppressed mRNA Expression Level of Nrf2 More Than Luteolin Alone (p<0.05).** Moreover, luteolin could reverse the effect of tBHQ in activating of Nrf2 expression
Flavonoids act as a free radical scavenger, immune system modulator, and antioxidant agent. These characteristics with low toxicity, making them good option for cancer chemopreventive agent (Lopez-Lazaro, 2009; Xu et al., 2009; Akan and Garip, 2013).

Several flavonoids have been suggested to be selective Nrf2 inhibitor. Nrf2 plays dual roles in cancer, but more emerging data had shown it performs its role as a protooncogene. Studies was shown overexpression of Nrf2 in the gastric cancer samples and gastric cancer cell lines (Hu et al., 2013) Luteolin (2-(3,4-Dihydroxyphenyl))-5, 7-dihydroxy-4-chromone) is one of the most common flavonoid that is found in celery, green pepper, honey, propolis and chamomile tea. Many studies have showed that luteolin play an important role in prevention of the human body against inflammation, reactive oxygen species and cancer. Luteolin can inhibit Nrf2 signaling activation in drug-resistant cells and suppress expression of Nrf2-targeted genes in small intestinal cells of wild type mice, but had no significant effects in knockout mice (Chian et al., 2014). Furthermore, luteolin decreased activity of mitochondrial enzyme significantly in mice with colon cancer induced with Azoxy methane (Pandurangan et al., 2012). Tang et al. (2011) reported luteolin is a unique molecule inhibitor of Nrf2 that can sensitize NSCLC cells to anticancer drugs. And also luteolin indicated a potential role in inducing Cell Cycle Arrest and Apoptosis A375 human melanoma cells (George et al., 2013). But the low solubility and poor penetration across the cells is the biggest obstacle, which restricts the application of luteolin in therapeutic protocols (Bhattacharya and Ghosh, 2009).

So using of the new and potent drug delivery systems that can ameliorate the side effects of these components and enhancing the efficacy of chemotherapy are the main purposes in the development of cancer treatment. Phytosome as a new advanced formulation technology can be an attractive candidate for enhancement of bioavailability of luteolin and also improvement better absorption of the cells (Suryawanshi, 2011). To amplification of luteolin function numerous laboratories loaded luteolin in new formulation, such as phytosome (Agarwal et al., 2012). So we employed phytosome system for enhancement activation of luteolin that sensitize cancer cells to anti-cancer drugs (Outcomes in the present study indicated that luteolin loaded phytosome can inhibit the growth of MDA-MB 231 when combined with doxorubicin.

In conclusion, the present study demonstrated that phytosome not only increase the water solubility of luteolin but also enhances its therapeutic efficiency. Pharmacokinetic and pharmacological parameter have improved in phytosome technology, which in results can be good option in therapeutic aims like cardiovascular, anti-inflammatory, immunomodulator and anticancer agents.

Our finding indicate that the combination of nano luteolin and doxorubicin can provide a promising strategy for breast cancer therapy, especially for tumors with constitutive activation of Nrf2 and further studies in animal model is necessary to exploit this nano carrier.
as a potent drug delivery system for the prevention and treatment of cancer.

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References


