RESEARCH ARTICLE

Luteolin-loaded Phytosomes Sensitize Human Breast Carcinoma MDA-MB 231 Cells to Doxorubicin by Suppressing Nrf2 Mediated Signalling

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Abstract

Nuclear factor erythroid 2-related factor 2 (Nrf2) has been recognized as a transcription factor that controls mechanisms of cellular defense response by regulation of three classes of genes, including endogenous antioxidants, phase II detoxifying enzymes and transporters. Previous studies have revealed roles of Nrf2 in resistance to chemotherapeutic agents and high level expression of Nrf2 has been found in many types of cancer. At physiological concentrations, luteolin as a flavonoid compound can inhibit Nrf2 and sensitize cancer cells to chemotherapeutic agents. We reported luteolin loaded in phytosomes as an advanced nanoparticle carrier sensitized MDA-MB 231 cells to doxorubicin. In this study, we prepared nano phytosomes of luteolin to enhance the bioavailability of luteolin and improve passive targeting in breast cancer cells. Our results showed that co-treatment of cells with nano particles containing luteolin and doxorubicin resulted in the highest percentage cell death in MDA-MB 231cells ($p<0.05$). Furthermore, luteolin-loaded nanoparticles reduced Nrf2 gene expression at the mRNA level in cells to a greater extent than luteolin alone ($p<0.05$). Similarly, expression of downstream genes for Nrf2 including Ho1 and MDR1 were reduced significantly ($p<0.05$). Inhibition of Nrf-2 expression caused a marked increase in cancer cell death ($p<0.05$). Taken together, these results suggest that phytosome technology can improve the efficacy of chemotherapy by overcoming resistance and enhancing permeability of cancer cells to chemical agents and may thus be considered as a potential delivery system to improve therapeutic protocols for cancer patients.

Keywords: Nrf2 - luteolin - MDA-MB231 - chemoresistance - phytosome

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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 tomur carcinoma identified (Shen et al., 2013)khunluck reported Nrf2 polymorphism

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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-tetrahydroxyflavone) 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 chrysin 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.Ithas 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). Phytosoms 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. Phytosome 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 ccl4 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 for obtain to the luteolin-phospholipid complex we have done common stages respectively. The proper phospholipid among phosphatidylcholin, phosphatidyletanolamine 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

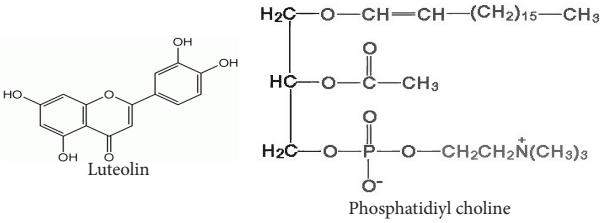

Figure 1. Chemical Structure of Luteolin and Phosphatidiyl Choline

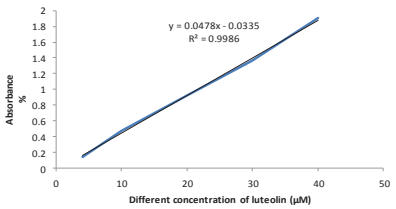

Figure 2. Calibration Curve of Luteolin

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 metanol in 10 ml volumetric flask. Absorbance of each solution was measured at 284 nm taking metanol 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 spectroscopy370 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 electoral 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% CO_2 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 formazoncrystals,and the absorbance at 570 nm after

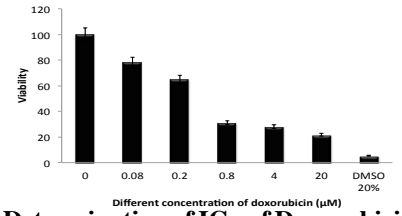

Figure 3. Determination of IC_{50} of Doxorubicin against MDA-MB231. MDA MB231 cells incubated with increasing concentration (0.08-20Mm) of doxorubicin in culture medium 1640

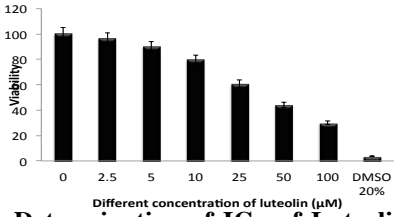

Figure 4. Determination of IC_{50} of Luteolin against MDA-MB231. MDA MB 231 cells were incubated with increasing concentration (2.5-100) of luteolin in RPMI 1640 medium for 24h

shaking for 15 min was measured using a microplate reader (Biotek, ELX 800, USA).

Determination of IC_{50} of doxorubicin and luteolin against MDA-MB231

Plots of cytotoxicity index versus increasing concentration of doxorubicin and luteolin were drawn. IC_{50} 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 231cells 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'-ACTCCCAGGTTGCCCAC-3') and (5'-GTAGCCGAAGAAACCTCATTGTC-3':HO1,(5'- ACGGCTTCAAGCTGGTGATG-3')and (5'- TGCAGCTCTTCTGGGAAGTAG-3')MDR1, (5'-ATGACCAGGTATGCCTATTATTAC-3') and (5'-CACATCAAACCAGCCTATCTC-3') were used for PCR. The PCR product were electrophoresed on agarose gel and normalized with internal control GAPDH.

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 roles 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 phosphatidiyl 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 peaksshown 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

Formulation	PS±SD (nm)	PI±SD	ZP±SD (mv)	EE%±SD
Blank phytosome	62±7.5	0.591±0.01	-25.8±0.2	68.3±2.6
Phyto-luteolin	83±7.9	0.596±0.03	-29.6±0.5	

*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

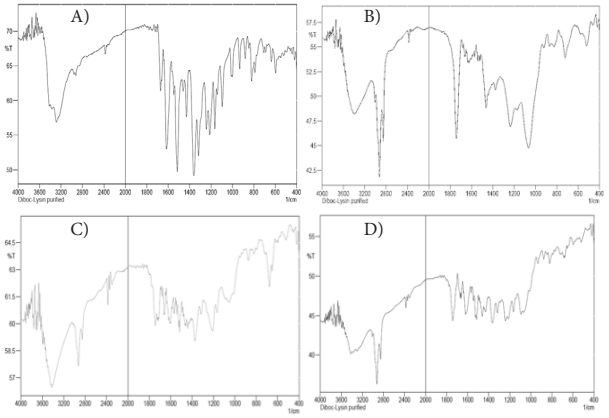

Figure 5. Fourier Transform Infrared Spectra. A) luteolin; B) lecithin; C) phytoluteolin and; D) complex of luteolin and lecithin

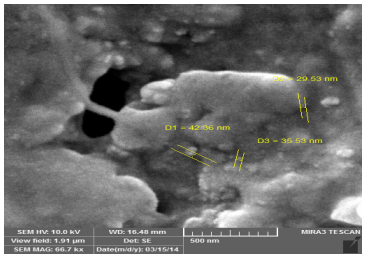

Figure 6. Scanning Electron Microscopy of Phyto-Luteolin in 500 Scale

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 231cells 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.

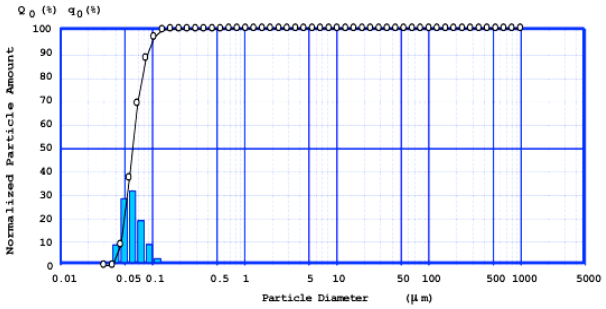

Figure 7. Phyto-luteolin in 63 Nanometer. The ZP of phyto-luteolin was measured at 25° C, under an electoral field of 40V/cm

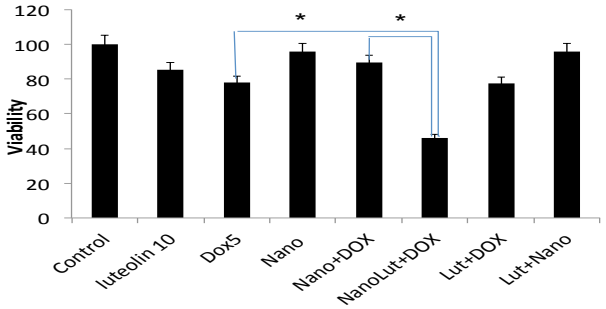

Figure 8. NanoLut+Dox had More Cytotoxicity in Comparison with Nano+Dox and Dox on MDA-MB231 Cells. Cytotoxicity test were conducted by MTS assay. Results had analyzed with one-way ANOVA test ($p<0.05$)

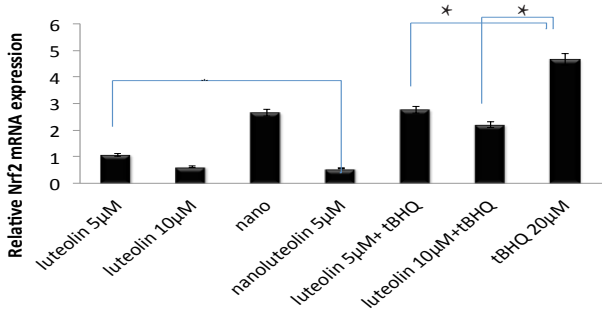

Figure 9. Nanoluteolin Suppressed mRNA Expression Level of Nrf2 More Than Luteolin Alone ($p<0.05$). More over, luteolin could reverse the effect of tBHQ in activating of Nrf2 expression

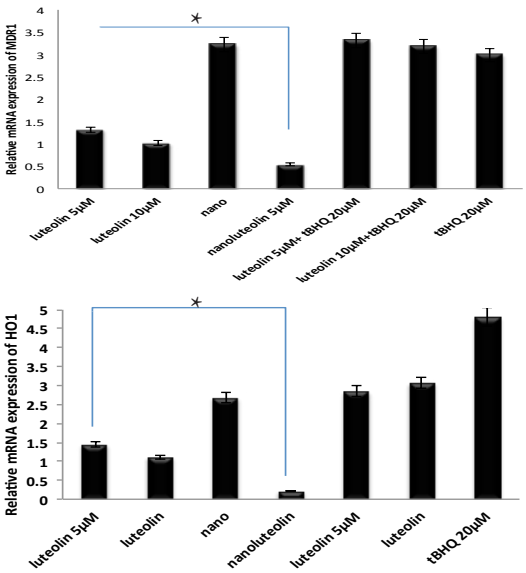

Figure 10. Nanolutolin Could Inhibit the Nrf2 Signal Transduction Pathway by Decreasing Expression of Downstream Genes More Than Luteolin Alone (p<0.05). Nanoluteolin reduced the mRNA level of Nrf2 target genes. MDA-MB 231 cells were treated with different concentration of luteolin and nanoluteolin for 24 h and subjected to qRT-PCR

Phyto-luteolin sensitizes MDA-MB231 cells to doxorubicin by inhibition of Nrf2

To evaluate whether expression of Nrf2 and its downstream target genes were modulated by phyto-luteolin, MDA-MB 231 cells were treated with 5 μM or 10 μM of luteolin, 5 μM nanoluteolin, 20 μM of tBHQ and combination of them for 24h. qRT-PCR analyses shown that nanoluteolin suppressed mRNA expression of Nrf2 more than luteolin alone, dose–dependently ($p<0.05$) Figure 9.

Phyto-luteolin inhibits the Nrf2 signal transduction pathway

It has been revealed that constitutive activation of Nrf2 accompanied by chemoresistance in NSCLC cells. So we determined whether phyto-lutolin influenced the susceptibility of MDA MB231 cells to doxorubicin by inhibiting Nrf2 pathway.nanolutolin could inhibit the Nrf2 signal transduction pathway by decreasing expression of downstream genes HO1 and MDR1 more than luteolin alone ($p<0.05$) (Figure 10).

Discussion

One of the major obstacles to successful treatment of many cancersisdrug resistance in chemotherapy protocol. There are several mechanisms to reach for the drug resistance, for instance increased expression and activity of several ATP-dependent drug efflux pumps, increased xenobiotic metabolism enzymes, and enhanced DNA repair (Samadi et al., 2011). Recently, Nrf2 has been emerged as a potent therapeutic target to overwhelming chemoresistance. Therefore, identification of potent and unique Nrf2 inhibitor for sensitizes a broad spectrum of cancer cells urgently needed (Zhu et al., 2013) Flavonoids act as a free radical scavenger, immune system modulator, and antioxidant agent. These characteristics with low toxicity, making them good option for cancer chemoperventive agent (Lopez-Lazaro, 2009; Xu et al., 2009; Akan and Garip, 2013).

Several flavonoid 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 Nrf2in the gastric cancer samples and gastric cancer cell lines (Hu et al., 2013) Luteolin (2-(3, 4-Dihydroxyphenyl) -5, 7-dihydroxy-4-chromenone) is one of the most common flavonoid that is found in celery, green pepper, honey, proposes 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 Azoxymethane (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 Apoptosisin 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 thatsensitize 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.

Acknowledgements

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