Original Article: Preparation, Description and Evaluation of the Lethality Acid loaded Liposomal Nanoparticles against in Vitro Colon and Liver Cancer

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ABSTRACT

Today, the use of nanoparticles as carriers in the science of drug delivery is of particular importance. Improving drug performance and reducing side effects due to changes in the pharmacokinetic properties of the drug are special benefits of drug nano-carriers. Monolayer and bilayer nano-liposomes were synthesized by reverse phase evaporation and thin film hydration with HEPES buffer and Sonoike operation, respectively. The results of the scanning electron microscopy (SEM) and transmission electron microscopy (TEM) revealed that pegylated nano-phytosomes have a spherical shape and much less aggregation was observed compared to non-pegylated nano-phytosomes. To evaluate the rate of drug release and the effect of peeling on the quality of drug release, dialysis bag method was utilized. The results of drug release indicated a rapid release of the free drug compared to the release of the drug from Glycyrrhizic acid loaded Nano-Phytosomes. The results of apoptosis test also demonstrated equal distribution of DNA of healthy cells and sphericity of cell nucleus. To investigate the effect of cell mortality in the monolayer nano-liposomes section, two HepG2 and KATO III cell lines were used and, in the bilayer, nano-phytosomes section, two cell lines DLD-1 and LIM-2405 related to colon cancer were used.

Introduction

The most important disadvantages of the old methods of drug delivery are drug wastage, high cost of raw materials, the occurrence of side effects related to the dose, physical and chemical incompatibilities [1-5], as well as clinical drug interactions. To prevent and reduce these disadvantages, the new pharmaceutical industry took steps to produce and use modern drug delivery systems [6-9].

In modern drug delivery methods, small amounts of the active ingredient can be delivered to the target point by appropriate carriers that have been produced in order to minimize the drug to the target cells with minimal side effects and maximum efficiency [10-12].
One of the important practical methods to reduce side effects in cancer treatments is the use of Nano-carriers as a drug delivery system [13-15]. Important applications of drug delivery systems include maintaining the drug concentration in the treatment range at the appropriate time, controlled drug release, and finally specific drug delivery to the target tissue [16-18]. The most important new drug delivery systems are dendrimers. Micelles, hydrogels, metal, organic and inorganic nanostructures, polymers, and Nano-liposomes noted that the performance of each of these carriers varies depending on size, shape, and other physical and chemical properties [19-21].

Dave et al. (2019) investigated, synthesized and described Celecoxib-loaded Peggy liposomal nanoparticles for biomedical applications. These nano-liposomes were prepared by thin film hydration method using different molar ratios of the drug to lipids. Celecoxib causes problems for the stomach when administered orally, but liposomes have been able to provide a continuous combination of drugs and, after overcoming drug-induced problems, can be easily administered by injection [22-25].

Cheng Chi et al. (2019) investigated the synthesis of nano-phytosomes for the synthesis of silymarin phospholipids with increased bioavailability and protective effect on the liver. They found that the homogenization process could significantly improve digestion and absorption in the gastrointestinal tract without causing molecular interaction. By combining phytosome and Nano-suppression technologies, a drug delivery system called silymarin phospholipid nanoparticles was developed using advanced bioavailability of silymarin in both in vitro and in vivo conditions and has been shown to improve performance. [26-28].

New aspect and innovation in research:

Innovations in the production and testing of targeted drug system of Pegylated Nano-liposomes include:

- Loading of anti-cancer drugs with appropriate efficiency
- Production of pharmaceutical system with two chemotherapeutic drugs and herbal medicine.
- Production of drug system with slow release of drug and proper stability of nanoparticles.
- Type of cancer studied, liver and bowel cancer.
- Type of materials used to produce nanoparticles.

Specific Objectives of the Study: The main purpose of this study was to evaluate the effect of lipid Nanoparticles loaded with glycyrrhizic acid anticancer drug on the treatment of liver and intestinal cancer, as well as the effect of stability of these formulations and their lethality on selected cancer cell lines [29-31].

Materials and Methods

Herbal Medicine Glycyrrhizic Acid

Glycyrrhizic acid is an herbal medicine extracted from licorice root. It has a molecular mass of 839.969 g/mol, a boiling point of 971.4° C at a pressure of 760 mm Hg, a solubility of 1 to 10 g / l in water at 20 °C and a chemical formula of C42H62O16. The drug used in this project is manufactured by Sigma Aldrich-USA [32-35].

Distirville Phosphoethanol Amine Methoxy Polyethylene Glycol DSPE - mPEG2000

Increasing the circulation time of liposomes in the body is currently of great interest to scientists. This is one of the most important reasons for using polyethylene glycol in drug delivery systems. Distyril phosphoethanolamine methoxy polyethylene glycol is a phospholipid-bonded PEG (PEG-phospholipid-bonded) in which the PEG layer usually acts as an ester barrier to stabilize molecular assemblies. Nanostructures based on DSPE - PEG2000 have played an important role in drug delivery systems, especially in the Taxol product (FDA approved). However, the concentration of PE-PEG derivatives can affect the structure and properties of liposomes [35-38]. In PEG-containing phospholipids, the larger the size of the bonded polyethylene glycol, the more wedge-like the phospholipids will be. The chemical formula of this substance is...
C45H87NNaO11P and its molecular weight is 892.1 g / mol. The DSPE-mPEG2000 material used in this study is made by Sigma Aldrich-USA [39-41].

**Ultrasonic Homogenizer**

One of the most widely used methods for homogenization is ultrasonic. This device works by creating strong pressure waves in the liquid environment [42-45]. Compression waves cause flow in the liquid and under suitable conditions cause the rapid formation of micro-bubbles, which grow and unite these bubbles until they reach their maximum size and eventually burst, causing intense heat [46-49].

**Freeze Dryer**

The freezer is used to remove solvents from the nanoliposomes and to dry the samples by creating a vacuum at very low temperatures without changing the composition of the composition. Also, the temperature range of the device is from about -40 °C to -58 °C. The process of removing moisture from a frozen sample or piece by creating a vacuum is called Lyophilization or Freeze Drying [50-52].

**Centrifuge**

A centrifuge is a device that uses centrifugal force to separate the components of a solution that have different densities. The centrifugal force pushes heavier materials out of the container. In this study, a hettich-UK centrifuge model MIKRO 220R located in Sheikh Baha'i Laboratory of Tehran University of Science and Research was used to separate the supernatant from nanodrates at 4 °C and 17,000 rpm [53-57].

**ICP-EOS Induction Pair Plasma Spectrometer**

The ICP device is one of the most widely used laboratory equipment in the analysis of a wide range of elements, the function of which is based on the emission spectrum. This device is more sensitive than other similar devices and offers a more accurate result [58-60]. Inductively coupled plasma spectrometers are widely used in material analysis laboratories, especially for the analysis of heavy metals. One of the main features of this equipment is that each element emits energy at a certain wavelength according to the specific characteristics of the same element and the intensity of energy radiated at a certain wavelength is directly proportional to the concentration of the presence of that element in the sample [61-64].

**TEM Transmission Electron Microscope**

Transmission electron microscopy, or TEM, the first type of electron microscope, is a special tool for determining the structure and morphology of materials. These microscopes can be used to study crystal structure, symmetry and orientation, and crystal defects [65-67]. In order to study the morphology of the particles and to confirm the size of the nanoparticles, a drop of a sample of liposomal nanoparticles was placed on a carbon film and after drying at laboratory temperature, it was imaged using a passing electron microscope. A transmission electron microscope from Philips-UK model CM 120 located at Pasteur Institute Iran-Tehran was used [68-70].

Zetasizer Nanoseries: The size of liposomes depends on the composition and method of preparation and can vary from 50 nanometers to 1 micrometer. However, as mentioned, for therapeutic applications, sizes of 100 to even 400 nm have been reported so that nano liposomes can perform best in vivo. To investigate this, the synthesized samples were assayed with a DLS device [70-72].

**Check the amount of drug loaded**

To evaluate the amount of drug loaded, the standard curve will be used using spectrophotometric method. Thus, 6 drug concentrations of cisplatin equal to 0.05, 0.1, 0.3, 0.5, 0.8 and 1 mg / ml are prepared in phosphate buffer, then the amount of absorption of each concentration relative to the buffer is prepared [74-76]. Drug-free phosphate is calculated as blank three times at a wavelength of 301 nm, thus obtaining an absorption and concentration curve. In the next step, the suspension containing the drug is centrifuged (17,000 rpm, 4 ° C for half an hour) and the supernatant is removed [71].
By reading the absorption of the supernatant and using the standard curve, the amount of drug in the supernatant is determined. Then, the amount obtained is subtracted from the amount of drug used, and thus the amount of drug available or loaded into the nanoparticles is obtained. The load and the amount of encapsulation are obtained using two equations (1) and (2).

\[
EE(\%) = \left( \frac{\text{amount of drug carrier}}{\text{amount of feed initially}} \right) \times 100
\]

\[
\text{Loading efficiency (\%)} = \left( \frac{\text{weight of drug in nanoparticle (mg)}}{\text{weight of nanoparticle (mg)}} \right) \times 100
\]

**Investigation of drug release from nanoparticles**

The dialysis bag technique was used to evaluate the release kinetics of the drug. For this purpose, after calculating the drug loaded in the nanoparticles, some precipitate of nanoparticles containing the drug was poured into 5 ml of phosphate buffer to be suspended again and used for other tests. Then, to check the release of the drug, 1 ml of the suspension was poured into a dialysis bag with 13 KDA cut-off (made by Sigma) and after closing both sides of the bag, it was immersed in 20 ml of phosphate buffer solution and then on the magnetic stirrer was placed at 37 °C for 120 h at 120 rpm [80].

In the measurement stage, ICP-OES was used to measure the amount of drug released in phosphate buffer solution by removing 2 mL of ambient phosphate buffer at different intervals (from 1 to 48 hours) and 2 ml of buffer. Fresh phosphate was substituted and then the drug release pattern from nanoparticles was calculated using the cumulative release curve. Finally, using the mathematical model in accordance with the geometric shape of the nanoparticles produced, which are based on the structure of the shell and sphere (Shell and Core), as well as the relationships in the field of drug release rate, the necessary calculations were performed. After performing the relevant calculations, the drug release rate and penetration coefficient were investigated.

**Cytotoxicity of formulations**

In order to evaluate cell viability, cisplatin-free drug formulations, cisplatin-containing nanoliposomes and cisplatin-containing peptide nano liposomes were used by MTT assay and two Hep-G2 liver cancer cell lines and KATO III stomach. In summary, these two cell lines were poured at a concentration of 10,000 cells per well in 96-well plates. Then in RPMI 1640 medium with 5% carbon dioxide, 10% fetal calf serum, 1% sodium pyruvate and / or. Percentage of penicillin, antibiotic and glutamine were cultured at 37 °C. After 24 h, the culture medium was replaced with culture medium containing formulations in different concentrations and incubated for 24, 48 and 72 h. After this period, MTT solution with a concentration of 4 mM per well was added. After 3 h, the MTT solution was poured out and 100% isopropanol was added 100%. After 15 minutes and dissolution of formazan crystals, the amount of dye adsorption produced at 570 nm was read by Alizairder and the percentage of cell viability was calculated by dividing the amount of sample adsorption by the amount of control adsorption [1].

**Check the stability of formulations**

The stability of drug-containing nano formulations in both pegylated and non-pegylated cases was investigated by examining changes in size distribution, zeta potential, retention percentage and the amount of drug loaded in one month after production, compared to the day of manufacture [8].

**Experiments Related to the Synthesis of Bilayer Nano Phytosomes**

In the next step, supernatant was collected to determine the amount of encapsulation. On the other hand, the collected mixture was placed in a freeze dryer for 72 h at -48 °C to be completely free of any moisture. Finally, 5 mL of phosphate buffer solution (pH = 7.4) was added to the nanoparticles and the resulting mixture was
stored at 4 °C for subsequent experiments. Due to the fact that knowledge of the percentage of E.E. indicates the content of nanoparticles, so in the next step, as described in the section on monolithic nano liposomes, the concentration of both drugs in the supernatant was determined separately. Thus, the concentration of cisplatin was determined by ICP-OES and the exact concentration of glycyrrhizic acid was determined by HPLC and finally, using equation (3), the retention rate of each drug in bilayer nano liposomes was reported.

\[
E.E\% = \frac{\text{amount of drug loaded in nano-phytosome (mg/ml)}}{\text{initial concentration of drug (mg/ml)}} \times 100
\]

**Evaluation of drug release from nanoparticles and stability of nano Phytosomes after three months**

A dialysis bag (cut-off of 13 KDA) made by Sigma was used to determine the drug release rate, similar to the method described in monolayer nano liposomes. One ml of the formulation was poured into a dialysis bag placed in 10 mL of phosphate buffer solution (pH 7.4) and placed on an immersed magnetic stirrer at 120 rpm at 37 °C for 24 h. Then, at different time intervals from 1 h to 24 h, samples were collected and the amount of drug released in phosphate buffer solution was measured using ICP-EOS and HPLC, and then the relevant release profile was extracted. In addition, the stability of nano Phytosomes was evaluated by re-examining the zeta potential, mean particle size and encapsulation percentage after 3 months (storage at 4 °C).

The dialysis bag method was used to evaluate the effect of PEG on the quality of drug delivery from nanoparticles. The cumulative release rate of cisplatin from nano drates is demonstrated in figure (2). As can be seen, the free drug cisplatin showed a rapid release of about 98% in fifteen hours. The slope of drug release from nanoparticles is significantly lower than that of free drug. The drug release profile lasted for 36 h. In fact, in the first 15 h, the rate of consecutive release of a small amount of drug from the nanoparticles was observed compared to the same period for the free drug, which continued with a slight upward slope. The drug release rate from non-pelleted nanoparticles after 36 h was also reported to be about 38%. Drug release profile of nanoparticles is one of the most important factors because it determines the biological effects of carriers. In the first hour of the experiment, consecutive release of cisplatin was observed, which is most likely due to the release of the drug adhering to the surface of the nanoparticles. A sustained release pattern was then highlighted with a gradual increase during the experiment that confirmed the carrier’s ability to retain the drug. The release of cisplatin from pelleted and non-pelleted nanoparticles involves a slow and rapid initial expansion phase, which is related to the PEG coating and its inhibitory effect on drug release from nanoparticles. Release studies showed high ability of pegylated nanoparticles with a release of 24.85% after 36 h, while after the same period for non-pegylated nanoparticles, 37.7% release of the drug was reported. These results are also related to the increase in surface potential in this study.

**Figure 1.** The cumulative release rate of cisplatin to free drug and nanoparticles loaded with cisplatin. (* (P <0.05) shows a significant difference compared to nanoparticles)
Investigation of Particle Size Distribution and Morphology

In this research study, the prepared nanoparticles were utilized as carriers of cisplatin and glycyrrhizic acid. In addition, PEG was used in the formulation of Phytosomes nanoparticles to enhance the pharmacokinetic properties of drugs. Morphological studies performed by SEM and TEM revealed that pegylated nano-Phytosomes are spherical in shape and show much less aggregation compared to non-pegylated nano-Phytosomes (figure 2).

**Figure 2.** a) TEM micrograph of cisplatin and glycyrrhizic acid-loaded pegylated nano-Phytosomes, b) SEM micrograph of nano-Phytosomes loaded with cisplatin and glycyrrhizic acid

**Evaluation of glycyrrhizic acid retention in nano Phytosomes**

The results show that the addition of PEG to Phytosomes nanoparticles increases the retention efficiency of the drug. In addition, the use of PEG increases the surface potential of nano Phytosomes, and surface modification by polyethylene glycol can significantly affect the surface potential of nanoparticles.

In a previous study, the use of PEG as a booster factor for the rate of drug retention in liposomal nanoparticles was reported. As can be seen, the use of PEG has a pivotal effect on the properties of nanoparticles and pellletization improves the stability of nanoparticles. This development is important to increase the effectiveness of the drug in the drug delivery system. The higher loading rate and the higher drug retention percentage in pelleted nanoparticles compared to other formulations confirmed this fact. Therefore, the loading rate in pegylated nano-Phytosomes has increased. The pelletizing results in this study are consistent with another study on polybutylene cyanocrylate (PBCA) nanoparticles. Hassanzadegan et al. Loaded carboplatin into PBCA nanoparticles and investigated the properties of polymer nanoparticles. The researchers found that the use of PEG significantly increased loading efficiency and increased drug retention efficiency in nanoparticles [4].

**Figure 3.** Results of zetasizer for cytoplatin and glycyrrhizic acid-loaded pegylated nano photosomes
Surface modification plays an important role in drug productivity. Physicochemical properties such as mean Phytosomes nanoparticle size, mean surface potential and retention efficiency percentage are summarized in Table (1). In addition, the results showed that cisplatin retention efficiency was 26.7% in pegylated nano-Phytosomes and 20.4% in non-pegylated nano-Phytosomes.

Table 1. The characteristics of loaded nano-Phytosomes of cisplatin and glycyrrhizic acid (* (P <0.05) in comparison with ungranulated nano-Phytosomes)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Average nanoparticle size (nanometers)</th>
<th>Average surface potential (mV)</th>
<th>Percentage of cisplatin retention efficiency</th>
<th>Percentage of glycyrrhizic acid retention efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pegylated nano-Phytosomes loaded with cisplatin and glycyrrhizic acid</td>
<td>81/6±3/3*</td>
<td>-30/7±1/2*</td>
<td>26/7±1/7</td>
<td>71/4±2/5*</td>
</tr>
<tr>
<td>Nano Phytosomes loaded with cisplatin and glycyrrhizic acid</td>
<td>92/1±7/2</td>
<td>-26/5±2/5</td>
<td>23/4±3/1</td>
<td>63/5±2/7</td>
</tr>
</tbody>
</table>

Evaluation of stability of glycyrrhizic acid-loaded nano Phytosomes

In this study, the physical and chemical properties of the prepared nanoparticles after 3 months of storage at 4 °C were also investigated. The results of the stability study of nano Phytosomes are reported in Table (2). Findings show that the use of PEG has significantly affected the physicochemical properties of nanoparticles. Observing the results in the table, it can be seen that non-pelleted nanoparticles in all formulations show less stability than pelleted nanoparticles.

Table 2. Specifications of nano-Phytosomes loaded with cisplatin and glycyrrhizic acid after 3 months

<table>
<thead>
<tr>
<th>Sample</th>
<th>Average nanoparticle size (nanometers)</th>
<th>Average surface potential (mV)</th>
<th>Percentage of cisplatin retention efficiency</th>
<th>Percentage of glycyrrhizic acid retention efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pegylated nano-Phytosomes loaded with cisplatin and glycyrrhizic acid</td>
<td>95/5±10/5</td>
<td>-23/7±2/75</td>
<td>22/5±2/2</td>
<td>52/5 ± 4/7</td>
</tr>
<tr>
<td>Nano Phytosomes loaded with cisplatin and glycyrrhizic acid</td>
<td>119/2±15/2</td>
<td>-16/9±3/9</td>
<td>17/1±3</td>
<td>41/1 ± 5/9</td>
</tr>
</tbody>
</table>

Evaluation of drug release rate from glycyrrhizic acid-laden nano Phytosomes

The study of drug release characteristics is known as an important parameter in the biological effects of nanoparticles. A dialysis bag was used to evaluate the drug release behavior of the nanoparticles. As expected, cisplatin showed rapid release and was about 94% released after 15 hours. However, the release of the drug from the nano Phytosomes, which is loaded with
cisplatin and glycyrrhizic acid, has a lower slope than the free form of the drug. The study of drug release profile continued for 24 hours. This can be attributed to the inhibitory effect of PEG coating on drug release profile. Evaluation of drug release showed the ability of high retention of pegylated nano-Phytosomes, which released 29.85% of cisplatin in 24 hours [5].

**Investigation of cell death of glycyrrhizic acid-loaded nano Phytosomes**

The effect of cytoplasm free effect of cisplatin and pegylated and non-pegylated nano Phytosomes loaded with cisplatin and glycyrrhizic acid on two colon cell lines DLD-1 and LIM-2405 was performed by MTT assay. The findings in figures (4) and (5) show that the cell lethality of nanoparticles is higher in equal concentration concentrations compared to cisplatin. It can also be seen that the cytoplasm of nano cytostomes loaded with cisplatin and glycyrrhizic acid is stronger than the free state of cisplatin. The results indicate that pigmentation of bilayer nano Phytosomes has improved cell killing compared to other formulations.

![Figure 4](image1.png)

**Figure 4.** Cell lethal effects of cisplatin-free drug, cisplatin-loaded nano-Phytosomes and glycyrrhizic acid as well as pegylated nano-Phytosomes loaded with these two drugs on LIM-2405 cell line after 24 hours of incubation (* (P <0.05) shows significant changes compared to nanoparticles and # (P <0.05) compared to non-pegylated nano Phytosomes.)

![Figure 5](image2.png)

**Figure 5.** IC50 of free cisplatin, cisplatin and glycyrrhizic acid-loaded nano Phytosomes and pegylated nano Phytosomes loaded with these two drugs on DLD-1 and LIM-2405 cell lines. ( * (P <0.05) # (P <0.05) shows a significant difference compared to nanoparticles and pegylated nanoparticles)

The results of this study are consistent with previous findings on nano Phytosomes loaded with herbal drugs such as silybinin and glycyrrhizic acid (Ochi et al. 2016). They trapped silybinin and glycyrrhizinic acid inside the nano Phytosomes and examined the properties of the nanoparticles in a laboratory study. The findings showed that nanoencapsulation significantly increases the cell death of drugs. Similarly, hammers et al. (2006) reported the effect of enhanced cell viability of trapped platinum-based drug chemotherapy in lipid formulations.
1000 times compared to free drug. In addition, the findings of this project are related to a study conducted by Alavi et al. (2019). Findings have been reported on the use of particle nanoparticles for cisplatin, which can significantly increase the chemical lethality of a drug.

**Investigation of DNA damage**

DNA damage was also assessed by immunohistochemistry. This test was performed in the presence of cisplatin free drug and pegylated nanophytosomes loaded with cisplatin and glycyrrhizic acid at a concentration of 100 μM.

![Image of DNA damage assessment](image)

**Figure 6.** Fluorescent intensity in LIM-2405 cells using mAb to evaluate DNA damage after 8 hours of treatment with cisplatin free drug as well as pegylated nano-phytosomes loaded with cisplatin and glycyrrhizic acid and untreated group (control)

This study has shown that the anti-proliferative effect of cisplatin-loaded pegylated nanophytosomes and glycyrrhizic acid with cisplatin is very different and using nanof ormulation can provide higher efficiency of cancer treatment compared to cisplatin free drug [86].

**Conclusion**

Due to the numerous side effects of using chemotherapy drugs, the need to develop new strategies for cancer treatment in a way that can increase the effectiveness of the drug in the tumor and also reduce the harm of the drug was felt. One of the most important achievements in the development of drug delivery systems is the use of nanoparticles as drug carriers that can penetrate through the bloodstream and be used to transport drugs to different parts of the body.

The definition of nanoparticles is not really based on particle size, but depends on having new properties that differentiate them from other materials or the same material in the usual sizes. Therefore, different nanoparticle sizes from one to one thousand nanometers are defined for drug delivery systems. Nanoparticles have a larger surface area than microparticles or other materials and can increase drug dissolution and provide better bioavailability. Drug delivery systems are defined as being able to spatially control the distribution of drugs in the body to improve immunity and be effective in treating cancer. Concomitant administration of multiple drugs to the same cancer cells is expected to not only increase efficacy but also have synergistic effects. Also, in combination therapy, multiple drugs with different mechanisms are used to reduce the severity of side effects to the extent that the burden of the disease on the patient is reduced by simultaneous drug delivery.

In many cases, at least one potent drug will be selected as the main ingredient in the compound, and other elements will be identified as strong agents with different drugs or natural products that contribute to the effectiveness of chemotherapy. Previous studies have shown that the use of glycyrrhizinic acid as a pretreatment drug can affect the rate of cisplatin administration in terms of its effectiveness on antioxidant enzymes.
So that when using cisplatin as the only therapeutic drug, the amount of antioxidant enzymes is less and gradually with the presence of glycyrrhizic acid and then with increasing amount, the number of enzymes is increased. Therefore, in this study, the possibility of designing and manufacturing a drug system using the above two drugs was investigated simultaneously and the effect of designed nanocarriers on cancer cell lines was evaluated. In this study, a hypothesis was proposed to investigate the simultaneous presence of glycyrrhizic acid as an adjunct therapy as a chemotherapeutic agent in the drug delivery system. In addition, in this study, various features such as drug loading efficiency, size distribution of designed nanoparticles and the lethal effects of innovative nano formulations on different cell lines, etc. were investigated and significant effects were observed.

**Phytosomal nanoparticles loaded with cisplatin and glycyrrhizic acid (two layers)**

- It was found that the thin-layer hydration method combined with HEPES and sonic operation is a suitable method for the production of nano phytosomes. Morphological study performed by SEM and TEM showed that pegylated nano phytosomes had a spherical shape and compared to non-pegylated nano phytosomes, much less accumulation was observed.

- Physicochemical properties such as average nanoparticle size and surface potential and retention efficiency showed that retention efficiency in pegylated nano-phytosomes was about 14% higher than non-pegylated nano-phytosomes.

- Examination of the cell lethality of free drug pegylated and non-pegylated nanophytosomes loaded with glycyrrhizic acid on two cell lines DLD-1 and LIM-2405 related to colorectal cancer showed that the cell lethality of nanoparticles in equal consumption concentrations, compared with more free medicine. Also, the cell killing of glycyrrhizic acid-loaded nanophytosomes is stronger than the free state of the drug. The results indicate that peeling of bilayer nanophytosomes has improved cell killing compared to other formulations.

- The pegylated nanophytosome showed a lower IC50 than other formulations and, as noted, presented higher cell lethality compared to the standard state of the non-pegylated nanophytosome.

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