Improving the Therapeutic Performance of Glycyrrhiza Glabra Hydroalcoholic Extract Using Liposomal Nano-carriers and Their Characterization

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Abstract
Background: The use of plants as therapeutic drugs has long been common among human beings. The Glycyrrhiza glabra is one of the medicinal plants with many therapeutic properties. However, using this herb in traditional methods faces some challenges. The use of pharmaceutical nano-carriers such as liposomes is one of the new strategies to overcome these challenges. In this regard, the current study aimed to synthesize and characterize liposomal nanocarriers containing the G. glabra hydroalcoholic extract to improve its therapeutic effects.

Materials and Methods: After the extraction of the G. glabra root by the Soxhlet method, nano-liposomes containing G. glabra extracts were synthesized by the thin-film preparation method. Then, the encapsulation efficiency (EE) rate and drug release pattern of nanoliposome were examined using the spectrophotometry method. Next, physicochemical properties such as size, zeta potential, morphology, and non-interaction of the nano-system with the extract were investigated by dynamic-light-scattering (DLS), atomic force microscope (AFM), and Fourier transform infrared spectroscopy (FTIR) methods, and finally, the toxicity of the nano-system on human foreskin fibroblast cells was assessed using the MTT method.

Results: Nano-liposomes containing licorice extracts with the EE of 2.3 ± 75.32% were from the type of slow release and controlled release, having a size of 111.4 ± 1.2 nm, a surface charge of -53.6 ± 6.3, and a dispersion index of 0.210 ± 0.13, and they had no interaction with the loaded extract. The results of the MTT test also demonstrated that the synthesized nano-liposomes were non-toxic on normal cells.

Conclusion: Overall, the findings proved that synthesized nano-liposomes with proper physicochemical properties can be a suitable carrier for the G. glabra extract and thus cause stability and improve the therapeutic effects of this herbal extract as a medicinal plant.

Keywords: Liposome, Glycyrrhiza glabra, Encapsulation efficiency, MTT assay, Slow-release

Introduction
Herbs have different compounds that can be used for treating various diseases. Medicinal use of plants dates back to more than 2600 BC (1). Today, medicinal plants have attracted the attention of the developed countries of the world due to their less complications as safe drugs. Iran is also one of the richest countries in terms of the source of medicinal plants (2). Numerous studies confirmed the beneficial effects of herbal in the prevention and treatment of diseases such as diabetes, neoplasm, cancer, various neurological diseases, and atherosclerosis (3-7). The abundance and availability of medicinal plants, in addition to low side effects, are among the reasons for attracting attention to these natural drugs for clinical use in recent years (8). In addition, the proper compatibility of these compounds with the human body and other living organisms originating from their natural nature is another advantage of using these compounds over chemical drugs (9).

Loricic (Glycyrrhiza glabra) is one of the most popular...
medicinal plants that belongs to the Fabaceae family (also known as Leguminosae and Licorice) and is commonly applied orally. This species is native to the Mediterranean region, but it is now found in India, Russia, and China as well. The extract of this plant is employed in the pharmaceutical and food industries, as well as food supplements. Licorice is one of the oldest and most popular herbal worldwide. Medicinal use of licorice dates back before the Persian and Greek Empires. For example, licorice was used as a common drug for gastrointestinal tract problems, cough, bronchitis, and osteoarthritis in traditional Chinese medicine. It is still widely applied for the treatment of gastritis, peptic ulcer, and respiratory infections. Dried licorice root is also referred to as a tooth cleaner. The most important industrial application of G. glabra is the production of food additives such as flavorings and sweeteners (10). Moreover, the antioxidant activity of G. glabra is one of the main reasons for its use. The phenolic compounds of this plant are probably responsible for its strong antioxidant activity (11). The root of the plant is used in the prevention and treatment of various diseases such as microbial/viral infections, cancer, skin inflammation, bronchitis, depression, kidney diseases, and gastric and intestinal ulcers (12-19). Various chemical materials have been identified in this plant, including glycyrrhizin, 18β glycyrrhizic acid, glabrin A and B, or isoflavones, which are associated with biological properties such as antioxidant, antiviral, antimicrobial, anti-cancer, and anti-inflammatory activities (20).

The use of medicinal plants in traditional ways faces many challenges. For example, the oxidation of some active substances in the plant or its extract and the essential oil and improper effects on the target and non-target tissues are among the problems in the traditional use of medicinal plants. The use of drug delivery nano-carriers (e.g., liposome) is one of the newest strategies for improving the therapeutic effects of herbs and their extracts (21, 22).

Liposomes are lipid nano-carriers that are formed by the accumulation of lipids in aquatic environments, which were first discovered by Alec Bangham in 1960. Similar to natural cell membranes, these nano-carriers are spherical vesicles and are made of two phospholipid bilayers. The ability of these nanoparticles (NPs) to encapsulate large amounts of drugs, their resemblance to cell membranes, their ability to reduce unwanted side effects of drugs, high effectiveness on the target tissue, and low side effects on normal body tissues have led to widespread use and attraction of researchers’ attention to these NPs. Proper adaptability of these particles in terms of electrical charge and chemical composition is also another advantage of using these NPs (23, 24).

Considering the widespread and proven use of the licorice extract in the treatment of many diseases, as well as the benefits of liposomes as a drug delivery carrier, the present study sought to synthesize liposomal nano-carriers containing the G. glabra extract by the thin-film preparation method and characterize their physicochemical properties and examine their non-toxicity on the normal cells of the human body.

Materials and Methods
Phosphatidylcholine (SPC80) and cholesterol were purchased from Lipoid-Gmbh (German) and Sigma-Aldrich (USA), respectively. Cell culture materials such as DMEM (Dulbecco's Modified Eagle Medium), Fetal Bovine Serum (FBS), streptomycin, penicillin, trypsin, amphotericin B, DAPI dye, and 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl-tetrazolium bromide (MTT) were all purchased from Thermo Scientifics Gibco, USA. Finally, human foreskin fibroblast (HFF) cells were prepared from Pasteur Institute, Iran.

Extraction
Before extraction, the type and species of licorice were approved by the botanical experts of Yazd University. To extract the roots of the licorice plant, first, its roots were pulverized away from sunlight at dry ambient temperature and by an electric mill. Extraction was performed by the Soxhlet method, which is one of the most common extraction methods in laboratories. The Soxhlet machine consists of a balloon, heater, extraction chamber, and condenser. For this purpose, 50 grams of licorice root powder was poured into the extraction thimble, and the extraction thimbles were transferred to the Soxhlet column. Then, 500 mL of 70% alcohol was poured into a balloon, which was connected to the Soxhlet. Extraction began after installing the condenser and the water outlet inlet. After finishing extraction, the extract inside the balloon was filtered through filter paper to dry and evaporate the solvent at ambient temperature. The extract was maintained in the refrigerator until use.

Maximum Wavelength Determination and Standard Curve Drawing
The spectrophotometric method was used to determine the maximum wavelength of the licorice extract. In this method, first, the stock solution of the licorice extract was made with a concentration of 1 mg/mL in the phosphate-buffered saline (PBS) solvent (Sigma, USA) and isopropyl (Merck, Germany). Then, using the stock solution, different concentrations of the extract (500, 250, 125, 62.5, 30, 15, and 7.5 mg/mL) were made using dilution methods in the PBS solution and isopropyl. The absorption spectrum was then read by a spectrophotometer (Epoch, USA) in the range of 200-800 nm for all dilutions. The maximum wavelength was the wavelength at which the highest amount of absorption was obtained in all dilutions. Next, using the obtained adsorbents from different dilutions at the maximum wavelength, the standard curve of the G. glabra extract was drawn in PBS and isopropyl buffer, and the linear equation of the extract in PBS and isopropyl
was calculated using the obtained diagram. At this stage, the experiments were conducted with three repetitions.

**Synthesis of Liposomal Nano-carriers**

Liposomal nano-carriers were synthesized by phosphatidylcholine and cholesterol with a molar ratio of 70/30% (Spc = 0.1101 g and Col = 0.022 g) and lipid/drug ratio of 10:1 by the thin-film preparation method. This method was performed in organic and aqueous phases.

**Organic phase:** In this phase, phosphatidylcholine and cholesterol were dissolved in a balloon by the chloroform solvent, and after 20 minutes, the solvent was removed using a rotary device (Heidolph, Germany) and at 45°C and 150 rpm and under vacuum situation.

**Aqueous phase:** The licorice extract with a concentration of 2 mg/mL dissolved in the PBS buffer was loaded by the rotary at 60°C for 1 hour (the inactive method).

**Sonication:** Sonic Probe (ChromeTech, UH1200B) was used to reduce NP size (60% amplitude, 10 seconds on, and 15 seconds off).

**Separation of the Un-encapsulated Extract**

To separate the amount of the un-encapsulated extract, the dialysis bag method (Mw cut off=12000 Da) was applied based on the diffusion process. First, to feed the dialysis bag, the dialysis bag was boiled for 10 minutes with a dialysis bag buffer (1 mM EDTA, 2% sodium bicarbonate) at 80°C, and then the bag was boiled for another 10 minutes in distilled water to remove the buffer. Next, the synthesized system was transferred to the bag, which was placed in a Becher containing the PBS buffer (150 times the sample volume) and sterilized at 4°C. The existing PBS buffer was replaced with the fresh buffer every 30 minutes until fixing drug absorption.

**Evaluation of the Encapsulation Efficiency of the Synthesized Nano-system**

To evaluate the encapsulation rate of the extract by the synthesized nano-liposomes, nano-systems with concentrations of 1-10, 1-20, and 1-40 were diluted with isopropyl in order to break the lipid wall around the liposomes and release the extract. Then, using a spectrophotometer, the absorbance of the samples was measured at the maximum wavelength of the licorice extract, and the system encapsulation rate was measured using a standard licorice extract curve in isopropyl and Eq. (1).

\[
\text{Encapsulation efficiency} = \frac{\text{The amount of the encapsulated extract}}{\text{The amount of initial extract}} \times 100
\]

Eq. (1)

**Investigation of the Release Pattern of the Extract From the Synthesized Nano-liposomes**

To investigate the release pattern of the licorice extract from the synthesized nano-liposomes, 1 mL of the synthesized nano-system was poured into the dialysis bag and transferred to a falcon containing 10 mL of the PBS buffer with a pH rate of 7. Then, to evaluate the release process, the Falcon was stirred in a bath with a temperature of 37°C and a pH rate of 7 (physiological conditions of the body), and sampling from the PBS buffer around the dialysis bag was performed at different intervals (0.5, 1, 2, 3, 4, 5, 6, 7, 8, 12, 24, and 48 hours). At each stage, after sampling, a new buffer was replaced with the same conditions. Next, the absorption rate of each sample was read at the maximum wavelength of the licorice extract, and the release pattern of the extract from the system was investigated using the standard licorice extract curve in the PBS buffer.

**Investigation of Size, Dispersion Index, and Zeta Potential of Nano-systems**

The dynamic-light-scattering (DLS) device was used to measure the size and surface charge (zeta potential) of the particles. Zeta sizer (Malvern Instrument, Nano zeta sizer, model ES, UK) was employed to measure NPs. In addition, 600 µL samples with a concentration of 0.5-0.1 mg/mL were applied to determine the size. For this purpose, nano-liposomes were performed at an angle of 90 degrees and irradiated with laser light at a wavelength of 657 nm at 25°C for 30 seconds. The experiments of this step were repeated three times. Eventually, 1500 µL of the sample with a concentration of 0.1 mg/mL was used to measure the surface charge.

**Atomic Force Microscope**

Using microscopic methods is one of the best alternatives for evaluating the morphology of particles (i.e., shape, smoothness, and lack of mass). In this study, the atomic force microscope (AFM) was utilized to evaluate particle topology at the nanometer scale. For this purpose, the samples were examined by a microscope with a needle with a length of 2 microns and a diameter of less than 10 nm.

**Evaluation of the Interaction of the System With the Extract by Infrared Spectroscopy (Fourier Transform Infrared Spectroscopy)**

Infrared spectroscopy was used to evaluate the interaction between the system and the extract. To this end, The IR spectra of the licorice extract, empty nano-liposomes, and the extract-containing system were obtained by the Fourier transform infrared spectroscopy (FTIR) device (Model 8300, Shimadzu Company, Japan). The presence of each peak indicates the active factor groups in the samples.

**Evaluation of the Cytotoxicity of Nano-liposomes**

MTT method was applied to evaluate the lack of cytotoxicity of empty nano-liposomes and liposomes containing the extract to normal cells. The MTT assay is a colorimetric test that aims to reduce tetrazolium yellow...
crystals (MTT) by living cell mitochondrial reductases. The studied cells in this experiment were the normal cells of the human foreskin fibroblasts (HFF), which were prepared from Pasteur Institute of Tehran and were cultured in the DMEM culture medium with (10%) FBS and penicillin and streptomycin antibiotics (W/V1%) in an incubator (Memmert GmbH Co. KG, Germany, 37°C, 5% CO₂, and 95% humidity).

After 48 hours of culturing HFF cells in plate 96 wells, HFF cells reached a concentration of 1×10⁴ per well. After changing the culture medium, the cells were separately exposed to different concentrations of empty nano-liposomes (0.1, 1, 10, 100, and 1000 µg/mL) and the liposomal extract (10, 25, 50, 100, 200, 400, and 800 µg/mL) for 48 hours. Then, 20 µL of the MTT salt with a concentration of 0.5 mg/mL dissolved in PBS was added to the well and incubated for 4 hours. After removing the solution in the wells, 150 μL of DMSO was added to each well to remove the formed formazan crystals, and pipetting was performed as well. After 30 minutes of incubation, the absorption of each well at the wavelength of 570 and 630 nm was performed by the ELISA reader (Synergy HTX, Bio Tek; USA), and the cell survival was calculated by Eq. (2) as follows.

\[
\text{Survival rate} = \frac{\text{Absorbance of experiment group}}{\text{Absorbance of control group}} \times 100
\]

**Results**

*Maximum Wavelength of the Licorice Extract and Standard Curve Drawing*

Based on the obtained results from the absorption spectra examinations of the licorice root extract, the highest absorption of the extract was in the range of 200-600 nm at a wavelength of 320 nm (Figure 1).

Moreover, the examination of the spectrophotometric data of the licorice extract in different dilutions in the isopropyl buffer demonstrated that the standard curve of the licorice extract in this buffer is straight linear with equation \( Y = 0.0017X - 0.0067 \) and a determination coefficient of 0.9996 (\( R^2 \)) (Figure 2). This curve in the PBS buffer is also a straight line with equation \( Y = 0.0012X - 0.0053 \) and a determination coefficient of 0.999 (\( R^2 \)) (Figure 3). The experiments of this stage were performed with three repetitions, and error bars indicate variations.

*Encapsulation Efficiency in Synthesized Nano-vesicles*

After examining the obtained absorption spectra from the spectrophotometer and using Eq. (1) and the standard curve of the licorice extract in the isopropyl buffer (Figure 2), the loading rate of the extract in the synthesized nano-liposomes was calculated as 75.32 ± 2.3%, indicating the high efficiency of synthesized nano-liposomes.

**Release Process of the Licorice Extract From Lipid NPs**

The release process of the licorice extract was determined in 24 hours and after 3 repetitions according to Figure 4. Studies also showed that the highest release rate of this extract from the synthesized nano-liposomes under the physiological conditions of the body was 65.35%. Additionally, it was found that the release was faster (more slope of the diagram in Figure 4) in the first 10 hours, and the difference in high concentrations of the drug with the buffer around it can be a logical reason for this speed. However, drug release became slower after 10
hours (reducing the slope of the diagram in Figure 4), and the decline in the concentration difference is a reason for a reduction in the release rate. Therefore, the synthesized liposomal system is of slow-release and semi-controlled type, and the release of the drug under physiological conditions is slow.

**Size and Surface Charge of Lipid Nano-carriers Containing the Licorice Extract**
The examination of the obtained data from the DLS device represented that the mean size of the synthesized nano-system is equal to 111.4 ± 1.2 nm. Further, the scattering index in these particles equals 0.210 ± 0.13, indicating that the particles are not stuck together (Figure 5).

The mean surface zeta potential of nano-liposomes containing the licorice extract determined by DLS was -53.6 ± 6.3, implying that the particles have negative loads and are of anionic type (Figure 6).

**Investigation of Particle Morphology Using an AFM**
The investigation of the results of atomic force microscopy images (Figure 7) revealed that the particles have a suitable size distribution and a spherical structure in terms of shape and structure. As shown, the particles are uniform and homogeneous, and there is no cohesion between them. These images confirm the obtained results from DLS, indicating that the particle size is appropriate.

**Investigation of the Extract and System Interaction by FTIR**
The graphs depicted in Figure 8 are obtained from the FTIR machine. It shows (A) extract-free liposome, (B) licorice extract, and (C) the system containing the extract. The comparison of the three diagrams of Figure 8 demonstrates that neither the new peak nor the index peak has been removed, thus there is no link and no interaction between the extract and the liposomes. In fact, the extract retained its chemical nature and was stable at the time of formulation, representing that the extract still has its biological activity. The presence of the index peaks of diagrams A and B in diagram C indicates that the
system does not interact with the extract. For example, the 3440 index peak represents the OH group with a slight difference in the system containing the extract at 3430 cm$^{-1}$. The peak 2925 cm$^{-1}$, which denotes the alkane group and is formed by the rotation around the C-H axis, is also created in diagram C with a slight difference at 2931 cm$^{-1}$. Additionally, the peaks of 1710, 721 cm$^{-1}$, demonstrating the bioactive groups in the licorice extract, are created with a slight difference in 1637, 661 cm$^{-1}$ in the diagram of the system containing the extract, confirming the stability of the extract in the system.

**Evaluation of the Toxicity Effect of Blank Nano Lipid -Systems on the Normal Cells of the Body for Assessing the Non-toxicity of Nano-systems**

To evaluate the non-toxicity of nano-systems on normal cells, normal cells of the HFF cell line were treated with different concentrations (0.1, 1, 10, 100, and 1000 μg/mL) of the empty synthesized liposome and the liposomal extract (10, 25, 50, 100, 200, 400, and 800 μg/mL) for 48 hours, separately. According to Figure 9, after performing an MTT assay on these cells, the survival rate of cells (100, 99, 99, 98, and 97%, respectively) indicates the non-toxicity of the empty synthesized liposomes on HFF class cells. After the treatment of HFF cells with different concentrations (10, 25, 50, 100, 200, 400, and 800 μg/mL) of liposomes containing the extract, the cell survival rate was 99, 98, 97, 97, 96, and 96% (Figure 10), respectively, implying the non-toxicity of synthesized nano-systems on normal cells.
Improving the anti-cancer effects of Glycyrrhiza Glabra extract by using nanoparticles as drug carriers

Figure 8. Spectra Obtained From the Study by the FTIR Device: (A) Empty Synthesized Liposome, (B) Licorice Extract, and (C) Liposome Containing the Licorice Extract. Note: FTIR: Fourier-transform infrared spectroscopy.
Discussion

In this study, the *G. glabra* hydroalcoholic extract was successfully loaded into nano-liposomes with appropriate physicochemical properties. Numerous studies have reported that encapsulation of herbal extracts in NPs increases their therapeutic properties. For example, Bashiri et al. found that the cytotoxicity effect of the *Nepeta persica* extract increased by loading it into nanoliposomes compared to its free form on the MCF-7 breast cancer cell line (25). In another study, Taebpour et al. concluded that encapsulating the *Artemisia absinthium* hydroalcoholic extract in nanoliposomes would increase its anti-cancer properties (26). Therefore, the use of drug delivery nanocarriers as a new tool for delivering the drug to the target tissue can increase the therapeutic effects of herbal extracts, but the choice of suitable NPs for drug delivery relies on several factors. Encapsulation efficiency (EE%), which depends on various factors such as the type of the applied lipids and the molar percentage of each lipid, the type and nature of the loaded material, manufacturing method, and the like, is one of the important and effective factors in the selection of nanosystems as drug delivery carriers (27). The EE% of the licorice extract in the synthesized nano-liposomes in this study was 75.32 ± 2.3%, which is acceptable. In general, as the saturation rate of the membrane constituents increases, the fluidity rate of the membrane decreases, but its rigidity represents an increase. In the present study, SPC80, along with cholesterol was applied to synthesize liposomal systems. Due to the higher saturation rate of SPC80, compared to SPC60, the membrane fluidity of synthesized liposomes decreases, while its rigidity increases, which can be a reason for an increase in the EE% of the liposome. Using the proper molar rate of cholesterol is also one of the factors influencing EE%. Excessive use of cholesterol reduces stability, controlled release, and EE%. In other words, the use of cholesterol has a dual effect and the use of cholesterol in extremely large and small ratios can have negative effects on the EE% and release of the drug from the NPs (26, 28, 29).

Zeta potential (surface charge) is one of the important and influential indicators on the stability of lipid systems and depends on the type and molar percentage of the applied lipids in the structure of the liposome, the type of the loaded material, and the temperature. This physical characteristic is directly related to particle stability. The higher surface charge of the particles leads to a greater repulsive force between them, preventing the accumulation and deposition of particles and thus increasing the stability of the lipid nanosystem. Although more positive and negative zeta potential leads to theoretically more stable NPs, in practice, particles with different electrical charges than normal cells in the body are quickly eliminated by the immune system. Negative zeta potential also improves the cellular uptake of NPs, while reducing their removal by macrophages through reducing nonspecific interactions with body cells (30, 31).

Based on the report by Zhang et al, NPs with zeta potential in the range of MCF-7 zeta potential cells (-20 mV) were more facilely uptaked by these cells, and absorption, which was mediated by proteins, could be the cause of this process (32). The synthesized nano-liposomes in this study had a zeta potential of -53.6 ± 6.3 mV, confirming their appropriate stability. However, their differences with the zeta potential of body cells can be one of the defects of this study.

The size of liposomes affects their half-life, distribution, stability in the circulatory system, and biodegradation, along with their effect on the target tissue, thus it could be mentioned as an important factor in the design and fabrication of drug delivery nanocarriers (33). Phospholipids, which are the main constituents in the structure of liposomes, play an important role in their size. Wu et al (34) stated that decreasing the concentration of phosphatidylcholine reduces the size of liposomes. In fact, liposomes with lower contents of phospholipids (50% phosphatidylcholine) were smaller.
than liposomes with higher contents of phospholipids (90% phosphatidylcholine). In addition, cholesterol, which stabilizes the structure of liposomes, is one of the factors influencing their size. An increase in the concentration of cholesterol increases the distribution of this molecule in the phospholipid bilayer, which, in turn, increases the mean diameter of liposomes (35). The size of the synthesized liposomes and their polydispersity index (PDI) were 111.4 ± 1.2 nm and 0.210 ± 0.13, respectively. The size in the range of 100 nm and PDI below 0.3 indicate the appropriate size of these NPs, which can be mentioned as an advantage of this study.

Targeted and controlled release is one of the goals of the encapsulation of drugs into NPs. The results of this study revealed that the synthesized liposomes had a slow release so that 65.35% of the encapsulated extract was released within 24 hours. Kinetic studies also showed that the release of the extract from the nanoliposomes followed a 2-phase pattern, and release in the first phase was at high speed, while it had a slow speed rate in the second phase. Drug release is also a factor that depends on the chemical composition of the liposome. Maritim et al. demonstrated that increasing the amount of cholesterol can increase the drug release from liposomes. They noticed that higher contents of cholesterol lead to competition between cholesterol and drug at packing space in the bilayer of the liposome which, in turn, causes an increase in the excretion of the drug. Furthermore, increased membrane rigidity due to increased cholesterol contents can increase the rate of drug release (36, 37).

As mentioned earlier, infrared spectrum studies showed that the extract and the synthesized nanoliposomes did not interact with each other, and the extract retained its chemical nature after encapsulation. Further, the liposomes could enclose the extract within itself without making significant changes in its structure.

Conclusion
In this study, the researchers succeeded in synthesizing liposomal nanocarriers to deliver the _G. glabra_ Hydroalcoholic extract with high EE, slow release rate, and proper physicochemical properties. This is a testament to the suitability of this nanosystem as a drug delivery carrier, and therefore, additional research is recommended for clinical and commercial use of this nanosystem. Moreover, although the researchers could fabricate and synthesize nanoliposome containing the _G. glabra_ hydroalcoholic extract with appropriate physicochemical properties, similar to other studies, this study had its drawbacks. The lack of the evaluation of characteristics such as the release pattern of the extract from the nanosystem in the conditions of cancerous cells, the stability of nanoliposomes in different cellular conditions, and the uptake of nanosystem by normal and cancer cells are among the disadvantages of this study. Accordingly, it is suggested that other interested researchers focus on this field.

Acknowledgments
The authors thank the participants of this study. Special thanks also go to the staff of the NBTF Company, Yazd, Iran for helping in data collection.

Authors’ Contribution
MA and MT contributed to designing the study, writing the manuscript, and conducting statistical analyses. BFH supervised the study. Other authors played a role in data collecting and manuscript editing.

Conflict of Interest Disclosures
There are no conflicts of interests.

Consent for Publication
Not applicable.

Ethical Statement
The study received ethics approval from the Ethics Committee of Shahid Sadoughi University of Medical Sciences, Yazd (IR.SSU.RSI.REC.1398.039).

Funding/Support
The current study was funded by NBTF Company, Yazd, Iran and Shahid Sadoughi University of Medical Sciences, Yazd, Iran.

Acknowledgments
The authors thank the participants of this study. Special thanks also go to the staff of the NBTF Company, Yazd, Iran for helping in data collection. The authors thank the participants of this study. Special thanks also go to the staff of the NBTF Company, Yazd, Iran for helping in data collection.

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Dis Diagn. Vol 11, No 2, 2022
174/1871520619666181224212004.