Preparation and In Vitro Evaluation of Daidzein-Loaded Nanoparticulate Systems

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Abstract: Daidzein is a water-insoluble phytoestrogenic isoflavone of the Leguminosae family, mainly found in soy and soy-derived foods. Daidzein has a broad spectrum of physiological and pharmacological functions. It has anti-cancer, anti-inflammatory, antioxidant, enzyme inhibitor properties and causes a decrease in low-density lipoprotein levels. Glioblastoma Multiforme (GBM) is the most common, rapidly progressing, and fatal primary brain tumor. It accounts for more than 51% of all gliomas. For this reason, much scientific research is being done to treat brain tumors. This study aims to prepare daidzein-loaded PLGA and PLGA-Gelucire® 44/14 nanoparticles for the treatment of GBM and to evaluate their characteristics, neurotoxicity, and cytotoxicity in vitro. The PLGA and PLGA-Gelucire® 44/14 nanoparticles were prepared by using the non-modified and modified emulsion-solvent diffusion method. The surface morphology, particle size, zeta potential (ZP), encapsulation efficiency (EE) and in vitro release characteristics of nanoparticles were investigated. Furthermore, FT-IR, DSC and SEM were used to characterize these systems. The neurotoxicity on neurons and cytotoxicity against the U-87 MG cell line of the chosen optimum nanoparticle formulations were evaluated. The mean particle size and ZP values of all prepared nanoparticles were a range of 198.52±7.04-672.78±70.95 nm (p≥0.05) and -14.70±0.36--0.50±0.34 mV (p≥0.05), respectively. SEM images of nanoparticles revealed their approximately spherical shape. The EE % values of nanoparticles were a range of % 35.79±3.43-84.85±2.20 (p≥0.05). The cumulative daidzein release from daidzein-loaded nanoparticle formulations was up to about 100 % at 19-37 days of release. It was found that the prepared nanoparticulate systems reduced the neurotoxic effects of daidzein and showed similar cytotoxic effects to those of daidzein (p>0.05). The prepared nanoparticles are useful and promising systems for sustained release of daidzein, reduction of the neurotoxic effects of daidzein at high doses (200 μM and 300 μM) and maintaining of its cytotoxic activity against cancer cells.

Keywords: Cytotoxicity, Daidzein, Gelucire® 44/14, Neurotoxicity, PLGA, U-87 MG

Introduction

Drug delivery systems are developed for many reasons, such as controlling the release rate/location of a pharmaceutical compound, increasing its effectiveness and stability in order to create a therapeutic effect on humans and animals (Felice et al., 2014). Nanoparticles have become extremely attractive for their applications.
in the fields of health sciences in recent years as drug delivery systems. The main goal in the design of nanoparticles as drug delivery systems is to control the particle size and surface properties (passive targeting) or to target them with a special design (active targeting) and/or to provide the therapeutically optimum dose and speed effectiveness in the desired region as a result of controlling the release rate (Mirakabad et al., 2014).

Biodegradable polymers have been applied as carriers for drug delivery systems and are frequently preferred in the preparation of nanoparticulate systems due to their advantages, such as low toxicity and not requiring removal after the release of the active substance (Gandhi et al., 2012; Joshi & Patel, 2012). Poly lactic-co-glycolic acid (PLGA), which is constantly used in the preparation of nanoparticles, is a biopolymer approved by the US FDA (Food and Drug Administration) and the EMA (European Medicine Agency). PLGA is one of the most successfully used biodegradable polymers as it is hydrolyzed into lactic acid and glycolic acid monomers in the body. These monomers are endogenous and easily metabolized via the Krebs cycle; that’s why the use of PLGA for drug delivery or biomaterial applications causes minimal systemic toxicity. The degradation time can vary from several months to several years, depending on the molecular weight and copolymer ratio (Danhier et al., 2012). Gelucire® 44/14 is an inert, amphiphilic, semi-solid waxy excipient that are generally regarded as safe (GRAS) and identified by their melting points and HLB (hydrophilic–lipophilic balance) values. For Gelucire® 44/14, 44 is the melting point, and 14 is the HLB value. Gelucires with different compositions have been added to the formulations in order to increase the colloidal stability and biocompatibility of nanoparticles and to obtain high encapsulation efficiency. Therefore, it can be used as an emulsifier, wetting and stabilizing agent in many formulations and carrier systems (Wehrung et al., 2012).

Flavonoids, which are abundant in plants, are natural phenolic compounds that have positive effects on human health because of their high pharmacological activities like anti-inflammatory, anti-diabetic, hepatoprotective, and gastro-protective, and antiviral. These compounds are potent antitumor agents. Due to its antioxidant (radical scavenging effect) and antiproliferative functions, it has apoptosis-inducing, cell differentiation and cell cycle modulating effects (Romagnolo & Selmin, 2012; Yao et al., 2004). According to their chemical structure, flavonoids are classified as flavanols (e.g., quercetin, myricetin), flavones (e.g., apigenin, luteolin), flavanones (e.g., hesperidin, naringenin), catechins (e.g., catechin, epicatechin), anthocyanins (e.g., pelarycin) and isoflavones (e.g., genistein, daidzein) (Yao et al., 2004). Daidzein is a water-insoluble isoflavone belonging to the Leguminosae family, found mainly in soy and soy-derived foods (Zhang et al., 2012).

Daidzein has a broad spectrum of physiological and pharmacological functions, including the chemoprevention of cardio-cerebrovascular and cancer diseases. It can prevent and treat osteoporosis in postmenopausal women with bone loss and diabetes. Daidzein, which has been shown to have antioxidant properties in vitro and in vivo, is seen as a promising agent for treating many diseases due to its low cost, widespread availability, and clinical safety. US FDA has approved daidzein’s health claim for soy-based clinical trials (Dwiecki et al., 2009; Gao et al., 2008). Daidzein inhibits the growth of various cancer cells in vitro and in vivo by inducing apoptosis and inhibiting the growth of cancer cells through the modulation of genes that control cell cycle progression. The inhibitory effect of daidzein on cancer cells may vary depending on the cell type, daidzein concentration, and exposure time (Adjakly et al., 2013). Daidzein can exert a biphasic effect on cancer cells; that is, it may have an inhibitory effect at high concentrations and an activating function at low concentrations. Sometimes, it may show a more apoptosis-inducing effect at low concentrations than at high concentrations (Guo et al., 2004; Xiao et al., 2011). Brain tumor treatment is still difficulty in oncology. Gliomas represent a broad spectrum of malignancies ranging from slowly to highly growing aggressive tumors. The World Health Organization (WHO) graduates gliomas into four grades: grade I (pilocytic astrocytoma), grade II (diffuse astrocytoma), grade III (anaplastic astrocytoma), and grade IV (glioblastoma multiforme, GBM). The last two grades are considered high-grade gliomas or “malignant gliomas” and regarding poor prognosis (Germano et al., 2010). GBM is the most common, very rapidly progressing lethal primary brain tumor in adults. It accounts for more than 51% of all gliomas (Adamson et al., 2009). In high-grade glioma, surgery is the most crucial treatment option and is the most effective treatment option in the patient’s life. None of the single or combination use of chemotherapeutic agents has shown any superiority in prolonging life expectancy (less than one year). For this reason, new approaches in treatment are being tried in addition to conventional chemotherapy options. For this reason, nanoformulations are often preferred in cancer treatment (del Burgo et al., 2014). The aim of this study was to prepare daidzein-loaded PLGA and PLGA-Gelucire® 44/14 nanoparticulate drug delivery systems for the treatment of malignant brain tumors, to perform in vitro characterization studies, and to evaluate their cytotoxicity in neuron cells and U-87 MG cell line. Characterization studies include determination of particle size and distribution, evaluation of surface morphology, encapsulation efficiency, loading capacity, yield, and release properties. In addition, DSC and FTIR analyze of the daidzein, physical mixtures, and formulations were made.
Method

Development and Validation of Quantification Method for Daidzein

The quantification method for daidzein was studied using UV-VIS Spectrophotometry. Standard solutions containing daidzein were prepared in DMSO:phosphate buffer (PB, pH 7.4, USP 30/NF 25) (1:1, v/v) at a concentration of 100 µg/mL, and a calibration curve was drawn from 6 points by making the necessary dilutions (2-6.5 µg/mL). Within the scope of the validation study, linearity, accuracy, precision, the limit of detection and quantification, and specificity were evaluated.

Preparation of Nanoparticulate Systems

The modified and non-modified emulsion-solvent diffusion methods were used for the PLGA and PLGA-Gelucire® 44/14 nanoparticles. In the modified emulsion-solvent diffusion method (Hariharan et al., 2006), PLGA (75:25) was dissolved in dichloromethane:ethyl acetate (DCM:EA, 1.5:1, v/v) mixture and daidzein in DMSO was added. Then, this organic solution was dropped into PVA solution (% 1, w/v, in pH 7.4 PB). After mixing for 3 hours to form nanoparticles, it was homogenized at 15000 rpm using a high-speed homogenizer (TH-02, Omni International, USA). To this nanoemulsion, 5 mL ultrapure water was added with constant stirring to facilitate diffusion, and then the organic solvent was removed with the help of a rotavapor. At the end of this process, the nanoparticles were centrifuged (Kubota, 3780, Japan) and lyophilized after freezing (Martin Christ, Alpha 1-2 LD Plus, Germany). The nanoparticles with Gelucire® 44/14, prepared by adding 50 mg of Gelucire® 44/14 to 5 mL organic solvent, and the rest of the process was the same.

In the non-modified emulsion-solvent diffusion method (Sah & Sah, 2015), PLGA (75:25 and 50:50) was dissolved in EA, and daidzein in DMSO was added. Then, this organic solution was dropped into PVA solution (% 3, w/v, in pH 7.4 PB) and homogenized at 15000 rpm using a high-speed homogenizer (TH-02, Omni International, USA). For the diffusion of EA to ultrapure water, nanoparticle suspension was poured into 100 mL ultrapure water at a magnetic stirrer and stirred for 5 hours. After, the organic solvent residue was removed with the help of a rotavapor. At the end of this process, the nanoparticles were centrifuged (Kubota, 3780, Japan) and lyophilized after freezing (Martin Christ, Alpha 1-2 LD Plus, Germany). The nanoparticles with Gelucire® 44/14, prepared by adding 25 mg of Gelucire® 44/14 to 5 mL organic solvent, and the rest of the process was the same.

For blank nanoparticles, daidzein was not added to the organic solvents, and the rest of the process was the same for the modified and non-modified emulsion-solvent diffusion method.

In Vitro Characterization Studies

Determination of Morphological Properties

The morphological properties of the PLGA and PLGA-Gelucire® 44/14 nanoparticles were investigated using a scanning electron microscope (SEM, LEO 440, England). The lyophilized nanoparticles were placed on a metal plate and coated with 100 Å gold, and then SEM images were taken.

Determination of Particle Size and Zeta Potential

Particle size and zeta potential of the PLGA and PLGA-Gelucire® 44/14 nanoparticles were investigated using a zeta sizer (3000 HS, Malvern Instruments, UK). Zeta potential is an indicator of surface charge, which determines particle stability in dispersion. All measurements of nanoparticles were performed in triplicate after the nanoparticles were diluted in ultrapure water at 25 °C.

Determination of Encapsulation Efficiency (EE), Loading Capacity (LC) and Yield

The percentage of daidzein encapsulated in the nanoparticles was determined with the lyophilized PLGA and PLGA-Gelucire® 44/14 nanoparticles. The nanoparticles containing daidzein were mixed with EA and stirred for 1 hour on a multi-point magnetic stirrer (2mag, MIX 15 eco, Germany) at 750 rpm for PLGA degradation,
and then DMSO:PB (1:1, v/v) mixture was added. After a while of stirring, the organic solvent was evaporated, and the mixture was centrifuged. Then the daidzein in the supernatant was analyzed by using the validated UV-VIS spectrophotometric method (min. n=3). The yield of daidzein-loaded nanoparticles was calculated by comparing them with the final amount of lyophilized nanoparticles.

**Determination of In Vitro Release**

The release of daidzein from the PLGA and PLGA-Gelucire® 44/14 nanoparticles was determined by an incubation method. The blank and daidzein-loaded nanoparticles (10 mg) containing 20 mL pH 7.4 PB were placed in a horizontal shaker water bath (Memmert, WNB 14, Germany) set at 37 °C/50 rpm, and the release was carried out for the daidzein release was ended. An appropriate amount of samples were withdrawn at certain time intervals (0.5 h, 1 h, 2 h, 4 h, 8 h, 12 h, 24 h,...), and subsequently, the same amount of fresh buffer medium was added to the samples to maintain the sink conditions. Samples were centrifuged at 12500 rpm, and the daidzein in the supernatants was determined by a validated UV-VIS spectrophotometric method.

**Differential Scanning Calorimetry (DSC) Analysis**

The DSC method is frequently used to examine the thermal properties and stability of materials. It is generally used to obtain information about the interaction of the pure active substance with various excipients in the formulation, especially the polymer, as well as to determine the thermal properties of the active substance before and after the nanoparticle formulation. Powder samples of daidzein, PLGA, Gelucire® 44/14, physical mixtures, and nanoparticle formulations were prepared to determine by differential scanning calorimeter (Netzsch, STA 409 PC, Luxx®, Germany) in the range of 0-450 °C.

**Fourier Transmission Infrared Specroscopy (FT-IR) Analysis**

FT-IR spectroscopy is an important technique used to elucidate the chemical composition and bond arrangements in the structure. Vibration frequencies of various bonds in the structure are measured with this technique, and information about the functional groups in the structure is obtained. Powder samples of daidzein, PLGA, Gelucire 44/14, physical mixtures, and nanoparticle formulations were prepared to determine if there were any unwanted interactions between the formulation ingredients and the active ingredient. Infrared spectra were taken by FT-IR spectrophotometer (Perkin-Elmer, Spectrum One, USA) via KBr disks in the range of 4000–400 cm⁻¹ wavenumber.

**Cell Culture Studies**

**Neurotoxicity Test**

Primary cultures of cerebellar granular cells were prepared from few-hour-old newborn Sprague-Dawley rats (Gepdiremen et al., 2001). Briefly, newborn rats were decapitated, and their brain cortex was taken and kept waiting in the neurobasal medium (NBM, Gibco® by Life Technologies, USA) till ending the operation. Cortex was dissected out and suspended in calcium-free Hank’s balanced salt solution (HBSS, Sigma-Aldrich®, USA) containing 1/3 ratio of trypsin (Sigma-Aldrich®, USA) and incubated at 37 °C, 5% CO₂ for 15 min. Trypsin digestion was ended by the addition of HBSS. After 5 min of centrifugation at 1200 rpm, the sediment was suspended with a culture medium that contained 10 % (v/v) of fetal bovine serum (FBS, Sigma-Aldrich®, USA), 2 % (v/v) of B-27 supplement (Gibco® by Life Technologies, USA) and 0.1 % (v/v) of antibiotics (10000 IU penicillin, 10 mg streptomycin, 25 µg Amphotericine B/mL, Sigma-Aldrich®, USA) and filled the culture wellplates that containing each well as 1x10⁵ cell (300 µL). Following a 30-min period, media were changed to eliminate non-adhered cells. The culture dishes were kept at 37 °C in humidified 95 % air and 5% CO₂. Culture media were changed once in three days (3 times); neurons were tested for neurotoxicity in experiments after 10 days in vitro. Each experimental group was tested in 7 culture mediums (n=7). Blank and daidzein-loaded nanoparticles and daidzein were prepared at different concentrations (200-300 µM) in the culture medium and applied to culture wellplates for 2 days. After incubation at 37 °C, MTT solution was added and incubated for 4 hours. Following this period, DMSO was added, and the wellplates were analyzed by using a plate reader (μQuantTM, BioTek® Ins., USA) at 570 nm.
Cytotoxicity Test

U-87 MG GBM cells were purchased from American Type Culture Collection (ATCC®, USA) and used up to passage 7. Cells were grown to ~70 % confluence in 25 cm² of flasks with a culture medium containing 10 % (v/v) FBS, 1 % (v/v) L-glutamate, 1 % (v/v) antibiotics and RPMI 1640 (Sigma-Aldrich®, USA) in an incubator with 37 °C and % 5 CO₂. Cells were filled the culture wellplates that containing each well as 2x10⁵ cell (300 µL) and incubated at 37 °C, in an atmosphere of 85 % relative humidity and 5 % CO₂, and the culture medium was changed once in three days. Each experimental group was tested in 7 culture mediums (n=7). After 5 days, blank and daidzein-loaded nanoparticles and daidzein were prepared at different concentrations (200-300 µM) in the culture medium and applied to culture wellplates for 2 days. After incubation, MTT solution was added, and incubated for 4 hours. Following this period, DMSO was added and the wellplates were analyzed by using a plate reader (μQuantTM, BioTek® Ins., USA) at 570 nm.

Statistical Analysis

Statistical analysis of the obtained data was made with SPSS Statistics 20.0 (SPSS Inc., Chicago, Illinois, USA) program. While the data of cell culture studies were evaluated using the One-Way ANOVA test, it was determined whether there was a significant difference between the data of nanoparticle characterization using the Mann-Whitney U test (the difference was considered significant when p<0.05).

Results and Discussion

Development and Validation of Quantification Method for Daidzein

UV spectra of the solutions of daidzein prepared in DMSO:PB mixture in the wavelength range of 200-400 nm was taken, and λmax was determined as 259 nm. The calibration curve and the equation of daidzein are given in Figure 1. Inter-day and intra-day accuracy and precision values of daidzein are given in Table 1. The limits of detection and quantification were determined as 0.104 µg/mL and 0.316 µg/mL, respectively. In addition, by evaluating the specificity of the method, it was determined that the excipients and medium components in the formulation did not absorb at the wavelength where daidzein gave the maximum absorbance.

![Figure 1. The calibration curve and the equation of daidzein.](image)

| Theoretical Concentration (µg/mL) | Practical Concentration (µg/mL) | Accuracy (% Relative Error) | Precision (Variation Coefficient) |
|----------------------------------|---------------------------------|-----------------------------|----------------------------------|
| Inter-Day² | 2.5 | 2.529±0.030 | 1.159 | 1.202 |
| Day | 4.5 | 4.501±0.017 | 0.016 | 0.367 |
| 5.5 | 5.540±0.022 | 0.735 | 0.400 |
| Intra-Day² | 2.5 | 2.496±0.016 | -0.166 | 0.651 |
| Day | 4.5 | 4.441±0.026 | -1.322 | 0.593 |
| 5.5 | 5.471±0.026 | -0.528 | 0.467 |

a: For each concentration n=6; b: For each concentration n=6/day; c: Mean±Standard deviation (SD).
Preparation of Nanoparticulate Systems

The PLGA and PLGA-Gelucire® 44/14 nanoparticles were developed by the modified and non-modified emulsion-solvent diffusion method successfully. Formulation components of PLGA and PLGA-Gelucire® 44/14 nanoparticles are given in Table 2.

Table 2. Formulation components of the PLGA and PLGA-Gelucire® 44/14 nanoparticles.

| Formulation Code | PLGA | Organic Solvent (mL) | Gelucire® 44/14 (mg) | Aqueous | Phase (mL) |
|------------------|------|----------------------|----------------------|---------|------------|
|                  |      | DCM:EA               |                      |         | % 1 PVA    |
| F1-B             | 75:25| 2.5                  | -                    | 5       |
| F2-D             | 75:25| 2.5                  | -                    | 5       |
| F3-BG            | 75:25| 5                    | 50                   | 10      |
| F4-DG            | 75:25| 5                    | 50                   | 10      |
|                  |      | EA                   |                      |         | % 3 PVA    |
| F5-B             | 75:25| 5                    | -                    | 10      |
| F6-D             | 75:25| 5                    | -                    | 10      |
| F7-BG            | 75:25| 5                    | 25                   | 10      |
| F8-DG            | 75:25| 5                    | 25                   | 10      |
| F9-B             | 50:50| 5                    | -                    | 10      |
| F10-D            | 50:50| 5                    | -                    | 10      |
| F11-BG           | 50:50| 5                    | 25                   | 10      |
| F12-DG           | 50:50| 5                    | 25                   | 10      |

In Vitro Characterization Studies

Determination of Morphological Properties

SEM images of the daidzein-loaded PLGA and PLGA-Gelucire® 44/14 nanoparticles are given in Figure 2. The surface properties of PLGA and PLGA-Gelucire® 44/14 nanoparticles were examined, and it was observed that nano-sized, approximately spherical structures were obtained.

Determination of Particle Size and Zeta Potential

Particle size and zeta potential data of the PLGA and PLGA-Gelucire® 44/14 nanoparticles are given in Table 3. When the particle size data of the PLGA and PLGA-Gelucire® 44/14 nanoparticles were evaluated, the empty and daidzein-loaded nanoparticle groups were evaluated among themselves, and it was determined that there was no statistical difference (p≥0.05); that is, the loaded daidzein had no effect on the particle size. However, when all of the nanoparticles were evaluated in terms of size, it was observed that smaller particles were prepared after the addition of Gelucire® 44/14 except F12-DG, but there was no statistically significant
difference between the groups (p=0.05). When the formulations prepared by modified and non-modified emulsion-solvent diffusion methods were evaluated, it was determined that the particle sizes of the nanoparticles prepared by the non-modified emulsion-solvent diffusion method were smaller. When the statistical evaluation was made, it was determined as p≥0.05, and no significant difference could be detected.

Table 3. Particle size and zeta potential data of the PLGA and PLGA-Gelucire® 44/14 nanoparticles.

| Formulation Code | Particle Size (nm) | Zeta Potential (mV) |
|------------------|--------------------|---------------------|
| F1-B             | 398.57±55.98       | -0.55±0.61          |
| F2-D             | 672.78±70.95       | -3.83±0.28          |
| F3-BG            | 228.13±14.72       | -3.22±0.88          |
| F4-DG            | 409.12±51.69       | -14.70±0.36         |
| F5-B             | 291.42±9.04        | -0.50±0.34          |
| F6-D             | 306.65±5.67        | -1.92±0.13          |
| F7-BG            | 198.52±7.04        | -4.92±0.53          |
| F8-DG            | 220.65±3.43        | -5.21±0.49          |
| F9-B             | 259.90±7.44        | -0.88±0.32          |
| F10-D            | 270.40±10.11       | -14.57±1.91         |
| F11-BG           | 234.85±8.16        | -0.56±0.34          |
| F12-DG           | 276.37±13.72       | -12.13±1.53         |

a: n=6; b: n=3.

Determination of Encapsulation Efficiency, Loading Capacity and Yield

The encapsulation efficiency, loading capacity and yield data of the PLGA and PLGA-Gelucire® 44/14 nanoparticles are given in Table 4. The % EE values obtained for the PLGA and PLGA-Gelucire® 44/14 nanoparticles prepared by the modified emulsion-solvent diffusion method were found to be 45.00±0.19% and 84.85±2.20%, respectively. It was determined that an increase in % EE was achieved with the addition of Gelucire® 44/14 (p=0.05). The % EE values obtained for the PLGA nanoparticle formulations prepared by the non-modified emulsion-solvent diffusion method were found in the range of 35.79±3.43-44.01±1.92% (p≥0.05). It was determined that the polymer (PLA:PGA ratio) used for the nanoparticles prepared by the non-modified emulsion-solvent diffusion method and the addition of Gelucire® 44/14 to the formulation (although there was a slight decrease) did not have a significant effect on the % EE. %LC values in the range of 3.41±0.41%-4.59±0.19% were obtained (p≥0.05), and the yield values were above 60% for all formulations.

Table 4. Encapsulation efficiency, loading capacity and yield data of the PLGA and PLGA-Gelucire® 44/14 nanoparticles.

| Formulation Code | % EE    | % LC    | % Yield   |
|------------------|---------|---------|-----------|
| F1-B             | -       | -       | 95.92±4.41|
| F2-D             | 45.00±0.19 | 4.23±0.01 | 96.79±0.19|
| F3-BG            | -       | -       | 95.05±3.17|
| F4-DG            | 84.85±2.20 | 4.20±0.12 | 96.21±2.19|
| F5-B             | -       | -       | 95.11±2.90|
| F6-D             | 41.94±0.88 | 3.91±0.11 | 97.48±1.02|
| F7-BG            | -       | -       | 63.52±2.14|
| F8-DG            | 35.79±3.43 | 3.41±0.41 | 65.79±2.25|
| F9-B             | -       | -       | 96.54±1.70|
| F10-D            | 44.01±1.92 | 4.59±0.19 | 87.11±0.48|
| F11-BG           | -       | -       | 69.69±2.93|
| F12-DG           | 40.00±2.38 | 4.00±0.46 | 62.78±4.55|

Determination of In Vitro Release

The in vitro release profiles of the daidzein-loaded PLGA and PLGA-Gelucire® 44/14 nanoparticles are given in Figure 3. Release studies for daidzein-loaded PLGA and PLGA-Gelucire® 44/14 nanoparticles were performed in PB. The 50% daidzein release occurred on day 3 from F2-D and on day ~12 from F6-D and F10-D. For the same formulations, 100% daidzein release occurred on days 25, 19, and 21 of release, respectively.
Release of 50% daidzein from F4-DG, F8-DG, and F12-DG prepared using Gelucire® 44/14 occurred on days 7 and 1, and 12 hours of release, respectively. The release of 100% daidzein occurred on the 37th, 21st and 20th days of release, respectively. The release data we obtained in our study were found to be compatible with the literature, and it was determined that polymer molecular weight, PLA:PGA ratio and nanoparticle size were effective on drug release. The PLA:PGA ratio affects the hydrophilicity and degradation of the prepared carrier system and consequently causes a change in the release rate. However, with increasing PLGA molecular weight, the polymer chain length increases, and degradation occurs more slowly due to the chain length. In addition, factors such as the size of the prepared nanoparticles, the type and concentration of the active substance, the physical properties of the polymer-active substance matrix, the pH of the release medium are also effective on the release of the active substance from the carrier system.

![Figure 3. The in vitro release profiles of the daidzein-loaded PLGA and PLGA-Gelucire® 44/14 nanoparticles.](image)

**DSC Analysis**

DSC thermograms of daidzein, PLGA 75:25, PLGA 50:50, Gelucire® 44/14 and daidzein-loaded nanoparticles are given in Figure 4. When all thermograms are examined, it is seen that there is no deterioration when daidzein and the formulation components are together, and daidzein is dispersed at the molecular level in the polymeric matrix.

![Figure 4. DSC thermograms of daidzein, PLGA 75:25, PLGA 50:50, Gelucire® 44/14 and daidzein-loaded nanoparticles.](image)
FT-IR Analysis

FT-IR spectrums of daidzein, PLGA 75:25, PLGA 50:50, Gelucire® 44/14 and daidzein-loaded nanoparticles are given in Figure 5. When all spectrums are examined, it is seen that there is no deterioration when daidzein and the formulation components are together, and daidzein is loaded to the nanoparticles.

![Figure 5. FT-IR spectrums of daidzein, PLGA 75:25, PLGA 50:50, Gelucire® 44/14 and daidzein-loaded nanoparticles.](image)

Cell Culture Studies

The cell viability of the PLGA and PLGA-Gelucire® 44/14 nanoparticles is given in Figure 6. In our study, the MTT method was used to evaluate cell viability. Neurotoxic and cytotoxic effects of active substance solutions and nanoparticle suspensions were investigated using neuron and U-87 MG cells. In the study examining the neurotoxic effect, it was determined that pure daidzein (200 μM and 300 μM) and F12-DG (200 μM and 300 μM) showed statistically significant neurotoxic effects when compared to the control group (p<0.05). Other nanoparticle formulations (blank and daidzein-loaded) did not show a statistically significant neurotoxic effect compared to the control group (p>0.05). When the neurotoxic effects of pure daidzein doses (200 μM and 300 μM) were compared statistically, it was observed that there was no significant difference between them (p>0.05).

According to the cytotoxicity test performed on the U-87 MG cell line, daidzein (200 μM and 300 μM) and all nanoparticle formulations loaded with daidzein (at a dose equivalent to 200 μM and 300 μM daidzein) were found to have a significant cytotoxic effect compared to the control group (p<0.05). Pure daidzein showed a slightly higher cytotoxic effect than all daidzein-loaded nanoparticle formulations in terms of both doses, but the statistical analysis did not reveal a significant difference (p>0.05).

The difference in cell uptake mechanisms between pure active substance and drug-loaded nanoparticle formulations may occur via the factors that increase the solubility of the active substance and the necessity of the active substance release from nanoparticles. When the blank nanoparticle formulations were compared with the control group, there was no significant difference between them (p>0.05). It was determined that there was no significant difference in cytotoxic effect between the pure daidzein and nanoparticle formulations in terms of different doses of daidzein (p>0.05). It was observed that the polymer structure [when PLGA (50:50)] and the addition of Gelucire® 44/14 had a slight positive effect on the cytotoxic effect, but when statistical comparison was made, it was determined that these two factors did not make a significant difference in terms of cytotoxicity (p>0.05).
Conclusion

Daidzein-loaded nanoparticulate systems have been successfully prepared using PLGA and PLGA-Gelucire® 44/14, and in vitro characterization studies have been performed. The prepared nanoparticles are useful systems for sustained release of daidzein, reduction of the neurotoxic effects of daidzein at high doses (200 μM and 300 μM) and maintaining of its cytotoxic activity against cancer cells. However, in vivo studies of the prepared nanoparticle systems should be done.

Acknowledgements or Notes

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Scientific Ethics Declaration

The authors declare that the scientific ethical and legal responsibility of this article published in EPHELS journal belongs to the authors.

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