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The Nanosuspension Formulations of Daidzein: Preparation and *In Vitro* Characterization

Daidzein Nanosüspansiyon Formülasyonları: Hazırlanması ve İn Vitro Karakterizasyonu

İngilizce Kısa Başlık: Daidzein Nanosuspensions
Türkçe Kısa Başlık: Daidzein Nanosüspansiyonları

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ABSTRACT
INTRODUCTION: Daidzein (DZ), a water-insoluble isoflavone, has many beneficial effects (anti-inflammatory, antioxidant and anticancer effects, etc.) on human health. DZ has a very low oral bioavailability related to its physicochemical properties (low solubility, intense metabolism of DZ in the intestine and liver). The aim of this study was to prepare and in vitro characterize the nanosuspension formulations of DZ to improve the poor solubility and efficacy of DZ.
METHODS: DZ nanosuspension formulations were prepared with media milling technique using zirconium oxide beads as a milling media. Pluronic F127 and PVP K30 (Formulation A; F-A) and SDS (SDS+Pluronic F127+PVP K30; Formulation B; F-B) were used as stabilizers. The nanosuspension formulations were evaluated for morphological properties, particle sizes, zeta potential, DZ content, saturation solubility, dissolution, and their cytotoxic effects on RG2 glioblastoma tumor cells.
RESULTS: F-A and F-B formulations were nano-sized (in the range of about 181-235 nm) and also had negative zeta potential values before and after lyophilization. The DZ content of F-A and F-B formulations were found to be 93.68±0.78% and 89.75±0.49%, respectively. FTIR analysis showed that there was no significant interaction between DZ and the excipients. DSC and XRD analyses confirmed no change in the crystal structure of DZ in F-A

and F-B formulations.
DISCUSSION AND CONCLUSION: In this study, the nanosuspension formulations were successfully prepared and characterized in vitro. Nanosuspension formulations increased the saturation solubility, dissolution rate, and cytotoxic effect of DZ.
Keywords: Cytotoxicity, daidzein, FTIR analysis, nanosuspension, media milling

ÖZ
GİRİŞ ve AMAÇ: Suda çözünmeyen bir izoflavon olan daidzein (DZ), insan sağlığı üzerinde pek çok faydalı etkiye (antiinflamatuar, antioksidan ve antikanser etkileri vb.) sahiptir. DZ, fizikokimyasal özelliklerine (düşük çözünürlük, bağırsakta ve karaciğerde DZ'nin yoğun metabolizasyonu) bağlı olarak çok düşük bir oral biyoyararlanıma sahiptir. Bu çalışmanın amacı, DZ'nin zayıf çözünürlüğünü ve etkinliğini iyileştirmek üzere DZ'nin nanosüspansiyon formülasyonunu hazırlamak ve in vitro olarak karakterize etmektir.
YÖNTEM ve GEREÇLER: DZ nanosüspansiyon formülasyonları, öğütme ortamı olarak zirkonyum oksit boncukları kullanılarak yaş öğütme tekniği ile hazırlandı. Stabilizan olarak Pluronic F127 ve PVP K30 (Formülasyon A; F-A) ve SDS (SDS+Pluronic F127+PVP K30; Formülasyon B; F-B) kullanıldı. Nanosüspansiyon formülasyonları, morfolojik özellikleri, partikül boyutları, zeta potansiyel, DZ içeriği, doygunluk çözünürlüğü, çözünme ve RG2 glioblastoma tümör hücreleri üzerindeki sitotoksik etkileri açısından değerlendirildi.
BULGULAR: Liyofilizasyon öncesi ve sonrası, F-A ve F-B formülasyonları nano-boyutluydular (yaklaşık 181-235 nm aralığında) ve ayrıca negatif zeta potansiyel değerlerine sahiptiler. F-A ve F-B formülasyonlarının DZ içeriği sırasıyla % 93.68±0.78 ve % 89.75±0.49 olarak bulundu. FTIR analizi, DZ ve yardımcı maddeler arasında önemli bir etkileşim olmadığını gösterdi. DSC ve XRD analizleri, F-A ve F-B formülasyonlarında DZ'nin kristal yapısında hiçbir değişiklik olmadığını doğruladı.
TARTIŞMA ve SONUÇ: Bu çalışmada, nanosüspansiyon formülasyonları başarıyla hazırlandı ve in vitro olarak karakterize edildi. Nanosüspansiyon formülasyonları DZ'nin doygunluk çözünürlüğünü, çözünme hızını ve sitotoksik etkisini artırdı.
Anahtar Kelimeler: Daidzein, FTIR analizi, nanosüspansiyon, sitotoksisite, yaş öğütme

INTRODUCTION

It is known that about 10% of the drugs in the clinical use and 40% of the newly developed drugs are poorly water-soluble. The poor solubility of active substances leads to poor bioavailability and limits their potential pharmacological effects. Therefore, increasing the aqueous solubility of poorly soluble active substances is very important. There are many approaches such as the use of co-solvents, salt formation, pH adjustment and the preparation of solid dispersions, inclusion complexes, nano-sized dosage forms (nanosuspension, micelles, nanoliposome, microemulsion etc.) to overcome the problem.[1,2] Nanosizing is a promising and popular approach to improve the solubility and bioavailability of hydrophobic active substances.[3,4] According to the Noyes-Whitney equation, the dissolution rate and bioavailability of a hydrophobic active substance increases with reducing the particle size and hence increasing the surface area of the particle.[1] In addition, as theoretically confirmed by the Ostwald-Freundlich equation, the surface area and the saturation solubility of the particle increase with decreasing the particle size to the nanometer range.[2]
Nanosuspensions are colloidal dispersions of nanosized-particles (generally, the mean particle size: 200-600 nm), stabilized with stabilizers (surfactants, polymers, or their combination).[3,5] Especially, nanosuspensions are convenient formulations for the active substances with high Log P value, high dose, and high melting point to increase the bioavailability of such active substances, reduce their dose and, obtain stable formulations as the selection of proper

stabilizers.[6,7] Nanosuspension formulation leads to reduce the administered dose of active substance and its side effects/toxicity by improving the bioavailability of the active substances. Nanoscale size and hence greatly increased surface area of particles are responsible for the physical instability of nanosuspensions. The increased surface area leads to high interfacial tension, resulting in an increase in the free energy of the system. Therefore, nanosuspension is not essentially thermodynamically stable system.[7,8] To reduce the system's free energy by decreasing interfacial tension, stabilizers are used in the formulation. The stabilizers (polyvinylpyrrolidone (PVP), Tween 80, polyvinyl alcohol (PVA) sodium lauryl sulfate, poloxamers, etc.) prevent nanoparticle aggregation by steric or electrostatic stabilization.[8] Currently, there are many nanosuspension products of poorly soluble active substances in the market, and many nanosuspension formulations are also under development.[9]

DZ (7-hydroxy-3-(4-hydroxyphenyl)-4H-chromen-4-one) mainly present in soy bean and soy products. DZ, a water-insoluble isoflavone, is a potent antioxidant and enzyme inhibitor. It also inhibits cytokines, cell adhesion proteins and platelet aggregation, induces nitric oxide production, and reduces low density lipoprotein (LDL) cholesterol levels.[10,11] DZ has many beneficial effects (anti-inflammatory activities, anticancer effect, prevention of the onset of diabetes, prevention and treatment of cardiovascular diseases and prevention of bone loss after menopause, etc.) on human health as mentioned in previous studies.[10–13] The cytotoxic effects of DZ were investigated in different cancer cells (neuroblastoma, glioma, melanoma, and pancreatic carcinoma cells, colon, prostate, cervical cancer cells, etc.) and it has been reported to have anti-carcinogenic properties.[10,14–17] DZ has a very low oral bioavailability related to its physicochemical properties (low solubility, intense metabolism of DZ in the intestine, and liver), limiting its potential bioactivities for human health. It was reported that the absolute bioavailability of DZ was 6.1% after the oral administration of the suspension of DZ to rats.[11,18] DZ-loaded poly(lactide-co-glycolide) nanoparticles, or lipid nanocarriers, or chitosan microspheres for different application routes, DZ-cocrystals, and DZ-cyclodextrin-polymer complexes were prepared to resolve the problems such as the poor solubility and low bioavailability of DZ.[11–13,19–21]

Glioblastoma (GBM), the most common primary malignant tumor, accounts for about 30% of all CNS tumors. GBM constitutes 2.3% of all cancer-related deaths each year. In spite of some clinical trials in the past decade, improvement in the therapy of GBM has been insufficient. Although there is a multimodal approach consisting of surgery followed by radiotherapy and chemotherapy for GBM treatment, the average overall survival time of all patients with GBM is 12-15 months; only, the survival time of <5% of patients with GBM is longer than 5 years.[22]

In a study, the antitumor effects of DZ on neuroblastoma cells were investigated, and it was found that DZ inhibits cell proliferation by preventing cell cycle progression.[14] The intrinsic apoptotic pathway is modulated by DZ, and Bcl-2 plays a fundamental role in malignant glioma cell death mediated by the combination of TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) and DZ.[23]

Zhang et al.[24] evaluated the inhibitory effects of daid002, a novel DZ derivative, on glioblastoma cells (U87MG) proliferation. They reported that glioblastoma growth was inhibited by daid002, and it induced G0/G1 phase arrest.

The aim of this study was to prepare and in vitro characterize nanosized-DZ for nanosuspension formulation to improve the poor solubility and efficacy of DZ. Also, its cytotoxic effect was evaluated using RG2 glioblastoma tumor cells.

MATERIAL AND METHODS

Materials

In this study, DZ (LC Laboratories, USA), Pluronic F127 (BASF, Brenntag Canada Inc., Canada), PVP (Polyvinylpyrrolidone)-K30 (Santa-Farma İlaç Sanayi A.Ş., Turkey), sodium dodecyl sulfate (SDS) (Santa-Farma İlaç Sanayi A.Ş., Turkey), thiazolyl blue tetrazolium bromide (MTT) (AppliChem GmbH, Darmstadt, Germany), N,N-Dimethylformamide (DMF) (Sigma-Aldrich, Taufkirchen, Germany), dimethylsulfoxide (DMSO) (Lab-Scan, Ireland), Dulbecco's Modified Eagle's Medium (DMEM)/Ham's F12 (Biochrom, Berlin, Germany), L-glutamine (Biochrom, Berlin, Germany), fetal bovine serum (Biochrom, Berlin, Germany), penicillin (Biochrom, Berlin, Germany) and streptomycin (Biochrom, Berlin, Germany) were used.

Preparation of Formulations

DZ nanosuspension formulations were prepared with media milling technique from coarse DZ using zirconium oxide beads as a milling media and Pluronic F127 and PVP K30 (Formulation A; F-A) and also SDS (SDS+Pluronic F127+PVP K30; Formulation B; F-B) as stabilizers (Table 1).

Coarse DZ was dispersed in a 10 mL glass vial containing the stabilizers' aqueous solution and zirconium oxide beads (diameter: 0.3-0.4 mm). Comminution was carried out on a magnetic stirrer at 1200 rpm for 24 hours at room temperature as determined by preliminary studies. After the beads were removed by decantation, the obtained nanosuspensions were centrifuged at 12500 rpm for 40 minutes. Then, the prepared DZ nanosuspension formulations were lyophilized for 24 hours (-55 °C, 0.021 mbar; Martin Christ, Alpha 1-2 LD Plus).

Characterization of the Prepared Formulations

Particle Size and Zeta Potential

The mean particle size, polydispersity index (PDI) and, zeta potential of the formulations (F-A and F-B) were determined by Malvern Zetasizer ZS (Malvern Ins. Ltd, UK). Measurements were performed before and after lyophilization at 25°C. Samples (n=6) were diluted with ultrapure water to obtain a suitable concentration for measurement. Moreover, Mastersizer Hydro 2000 MU (Malvern Ins. Ltd., UK) was used to determine the particle size of coarse DZ due to its micron size. The results were expressed in mean ± standard error (SE).

Morphological Analysis

The morphological features of coarse DZ and the lyophilized nanosuspension formulations (F-A and F-B) were examined using scanning electron microscope (SEM, Zeiss Sigma 300, Germany) at an acceleration voltage of 5 kV and different magnifications. Before analysis, samples were fixed to metal plates and coated with gold under vacuum to increase conductivity.

FT-IR Analysis

FT-IR analyzes of coarse DZ, stabilizers (Pluronic F127, PVP K30, and SDS), the lyophilized nanosuspension formulations (F-A and F-B) were carried out in the region of 4000-400 cm^{-1} and under vacuum by using Fourier transform infrared spectroscopy (Perkin Elmer Spectrum One FT-IR Spectrometer, Germany).

DSC analysis

DSC analyzes of coarse DZ, stabilizers (Pluronic F127, PVP K30, and SDS), the lyophilized nanosuspension formulations (F-A and F-B) were performed at 25-400°C with a heating rate of 10°C/min in air atmosphere using differantial scanning calorimetry (Netzsch STA 409 PC Luxx®, Germany) to determine their thermal properties. Alumina pans were used for samples.

X-Ray Diffraction (XRD) Analysis

XRD analyzes of coarse DZ, stabilizers (Pluronic F127, PVP K30, and SDS), the lyophilized nanosuspension formulations (F-A and F-B) were carried out using Rigaku Miniflex

diffractometer (Japan) using Cu Kα radiation (1.5406 Å) with a divergence slit 1.25°. The XRD data for the samples were collected between 5° and 90°. Percentage crystallinity of the materials were calculated by XRD deconvolution method using origin software.

DZ Content

The lyophilized formulations (F-A and F-B) was dissolved in DMSO by mixing for 15 min to determine the DZ content. Later, the samples were filtered using a membrane filter (PTFE; pore size: 220 nm), and DZ content was determined using a validated HPLC-UV method [HPLC conditions: stationary phase: C18 column (Diamonsil 5 μm, 200 x 4.6 mm) and guard column (EasyGuard C18 10 x 4 mm), mobile phase: methanol: ultrapure water (60:40), flow rate: 1mL/min, UV detection: 249 nm, injection volume:10 μL].

Saturation Solubility

Saturation solubilities of coarse DZ and the lyophilized nanosuspension formulations (F-A and F-B) were evaluated in six different media [HCl-pH 1.2; PB-pH 6.8, and 7.4; HCl+5% Tween 80-pH 1.2; PB+5% Tween 80-pH 6.8, and 7.4 (PB: phosphate buffer)]. An excess amount of coarse DZ and the formulations (F-A and F-B) were dispersed in the suitable medium and shaken continuously for 24 h in a water bath at 37±0.5°C. After centrifugation at 12500 rpm for 15 minutes, the obtained supernatants were filtered using a membrane filter (PVDF; pore size: 220 nm). Then, DZ concentration in samples was determined using a validated HPLC-UV method (at 249 nm).

Dissolution Studies

Dissolution studies for coarse DZ and the lyophilized nanosuspension formulations (F-A and F-B) were performed in 500 mL of three different dissolution media (HCl+5% Tween 80-pH 1.2; PB+5% Tween 80-pH 6.8, and 7.4) using USP Dissolution Apparatus 2 (paddle method) (Pharma Test PTWS IIIE/CE, Germany). During the dissolution experiment, the temperature was maintained at 37 ± 0.5 °C, using a paddle speed of 100 rpm. 5 mL of samples were withdrawn at predetermined time intervals, and an equal volume of fresh dissolution medium was added to the dissolution vessel to maintain Sink condition. After centrifugation at 12500 rpm for 15 minutes, the obtained supernatants were filtered using a membrane filter (PVDF, pore size: 220 nm). Then, DZ concentration in samples was determined using a validated HPLC-UV method (at 249 nm).

Cell culture study

To evaluate the effects of coarse DZ and the nanosuspension formulations (F-A and F-B) on the viability of rat glioma 2 (RG2) cell line (American Type Culture Collection, Manassas, VA, USA), MTT assay was used. 1:1 mixture of Dulbecco's Modified Eagle's Medium (DMEM)/Ham's F12 supplemented with 2.5 mM L-glutamine, 10% fetal bovine serum, penicillin (50 units/mL), and streptomycin (50 μg/mL) was used as culture medium. RG2 cells were seeded in 96-well plates (5×10^3cells per well) and were incubated overnight at 37 °C in 5% CO2. After incubation, the cells were treated with DZ solution in a culture medium containing 0.5% DMSO (AppliChem GmbH, Darmstadt, Germany), the suspension of coarse DZ and the nanosuspensions of F-A, and F-B formulations prepared in the culture medium, and also the solutions of the excipients (Exp F-A: pluronic F127, and PVP K30) used in formulation A and formulation B (Exp F-B: pluronic F127, PVP K30, and SDS) prepared in the culture medium. In this experiment, DZ concentrations were used in the range of 50-400 μM. After 24 and 48 h incubation, 25 μL of MTT solution (5 mg/mL) was added per well. 4 hours later, 80 μL of 23% SDS solution in DMF:water (45:55, v/v) was added to each well and the plates were incubated overnight at 37°C in 5% CO_2. After incubation, absorbance (at 570 nm) was measured using a microplate reader to assess cell viability.

Statistical Analyses

SPSS Statistics Version 22.0 (SPSS Inc., Chicago, USA) was used to perform statistical analysis. An independent t-test was used to evaluate the significance of the difference between two independent groups. The difference was accepted to be significant if $p<0.05$.

RESULTS AND DISCUSSION

Nanosuspension formulation has been developed to improve the poor solubility and low bioavailability of poorly water-soluble active substances/compounds by reducing their particle size. Consequently, the formulation alters the pharmacokinetics of these active substances/compounds and improve their efficacy and safety.[7,25] In our study, we prepared and characterized DZ nanosuspension formulations (F-A and F-B).

The Particle Sizes of Coarse DZ, F-A, and F-B Formulations

The mean particle sizes, and PDI values of F-A and F-B are shown in Figure 1. Also, the d_{10}, d_{50}, d_{90}, and span values of coarse DZ are given in Table 2. Coarse DZ was micron in size, and d_{50} and d_{90}, which correspond to the particle diameter at 50% and 90% of the total volume, were found to be 55.545±1.473 and 164.561±7.941 μm, respectively (Table 2). On the other hand, it was found that both formulations (F-A and F-B) were nano-sized (in the range of about 181-235 nm) (Figure 1a). The mean particle size of the F-A formulation was smaller than those of the F-B formulation. Before lyophilization, the difference between particle sizes of F-A and F-B was significant ($p<0.05$), but after lyophilization, the difference was not significant ($p>0.05$). Also, the particle sizes of both formulations increased significantly after lyophilization ($p<0.05$), but their particle sizes were still nano-size range (Figure 1a). Lyophilization without cryo- and lyoprotectant or in the presence of a low concentration of cryo- and lyoprotectant leads to an increase in particle size due to aggregation.[26,27]

Furthermore, the PDI values of formulations (F-A and F-B) were less than 0.2 before lyophilization and approximately 0.3 after lyophilization; therefore, the prepared formulations (F-A and F-B) have narrow particle size distribution. The particle size, and PDI are very critical factors for the physical stability of colloidal dispersions. PDI value less than 0.3 is acceptable and indicates monodispersity for colloidal dispersions.[23]

The Zeta Potentials of F-A and F-B Formulations

Zeta potential is an essential parameter for the physical stability of colloidal dispersions. Nonionic surfactants/stabilizers and negative zeta potential prevent the aggregation of the particles by creating steric and electrostatic hindrances. Hence, the physical stability of nano-sized dispersions is increased.[28,29] In the case of a combined steric and electrostatic stabilization, a zeta potential of at least about ±20 mV is acceptable.[30]

In this study, it was found that F-A and F-B had negative zeta potential values (in the range of (-) 17.23- (-) 22.53 mV) before and after lyophilization (Figure 1b). Due to the presence of SDS (anionic surfactant) in the F-B formulation, the zeta potential of F-B was greater than the zeta potential of F-A ($p<0.05$). Besides, lyophilization did not cause a significant change in the zeta potential values of both formulations ($p>0.05$).

The Morphological Analyzes of Coarse DZ, F-A and F-B Formulations

SEM images were obtained for the morphological analysis of coarse DZ and F-A and F-B formulations (Figure 2). It was observed that the coarse DZ particles were non-uniform and rod-like micron-sized particles (Figure 2a), in contrast, the F-A and F-B formulations had an approximately uniform shape and nano-sized distribution (Figure 2b and 2c).

The results of FTIR, DSC, and XRD analyzes

FTIR analysis is performed to identify the compound's structural properties by determining the vibration characteristics of functional groups. It is also used to determine the interactions among the active compound (s) and other formulation components.[29]

FTIR spectra of coarse DZ, Pluronic F127, PVP K30, SDS, and F-A and F-B formulations were given in Figure 3. In the FTIR spectrum of DZ, several characteristics peaks at about 3225 cm^{-1} (assigned to –OH group (intermolecular) stretching vibration), 2834 cm^{-1} (due to –CH stretching vibrations), 1630 cm^{-1} (assigned to -C=O stretching vibrations) and 1598 cm^{-1} (corresponding to -C=C vibration) (Figure 3). Similar data were reported by Bhalla et al.[21] When the FTIR spectra of coarse DZ, F-A and F-B formulations were examined, the FTIR spectra of F-A and F-B formulations (characteristic peaks related to DZ with different intensities were seen) were similar to the spectrum of coarse DZ. It showed that there was no significant interaction between DZ and the excipients. Consequently, the chemical structure of the DZ was preserved in the F-A and F-B formulations.

In this study, DSC analysis was performed to determine the thermal properties of the active compound and to examine the possible interactions among active compound and excipients in the formulation. The thermograms of the coarse DZ, Pluronic F127, PVP K30, SDS, F-A, and F-B formulations were given in Figure 4. A sharp endothermic peak at about 338 °C was seen in the thermogram of DZ (Figure 4). This characteristic peak is related to DZ's melting point (in the range of 330–340 °C).[10,31,32] The thermograms of F-A and F-B formulations exhibited the characteristic peak related to the melting point of DZ (Figure 4); as a result, DSC analysis showed that the crystallinity of DZ was maintained in both formulations.

Besides, XRD analyzes of the coarse DZ, Pluronic F127, PVP K30, SDS, F-A, and F-B formulations were performed, and the results were given in Figure 5. This analysis was used to identicate the structure at the crystalline lattice level.[33] There were peaks at 2θ values of 6.9°, 8.5, 10.4, 12.9, 15.9, 17.0, 24.6, 25.3, 26.5, 28.1, and 28.8 in the XRD patterns of coarse DZ (Figure 5). These results are consistent with a previously published study.[33] The XRD patterns of F-A and F-B formulations were similar to the XRD patterns of coarse DZ (Figure 5). In our study, it was shown that the crystal structure of DZ was preserved in both formulations.

As a result, DSC and XRD analyzes confirmed no change in the crystal structure of DZ in F-A and F-B formulations.

DZ Content of F-A and F-B Formulations

The DZ content of F-A and F-B formulations were found to be 93.68±0.78% and 89.75±0.49% (mean±SE, n=6), respectively. The negligible loss of DZ might be associated with the loss occurring during the preparation process of nanosuspension.[34] There was a slight reduction in the DZ content of F-B formulation compared to that of F-A formulation ($p<0.05$). This reduction in F-B formulation can be attributed to the presence of SDS, which likely causes a slight increase in the solubility of DZ during the preparation of nanosuspension.

The Saturation Solubility of Coarse DZ, F-A, and F-B Formulations

DZ, which belonging to Biopharmaceutical Classification System class IV, has low solubility and poor bioavailability.[35] Saturation solubility studies for coarse DZ, F-A and F-B formulations were carried out in the buffer solutions with different pH (pH 1.2, 6.8, and 7.4) and with/without 5% Tween 80. The solubility results, which were given in Table 3, indicated that coarse DZ, F-A, and F-B formulations have a pH-dependent solubility. Panizzon et al.[35] reported that DZ has a higher solubility in alkaline pH compared to acidic and neutral pH; thus, its solubility is pH-dependent. In our study, the solubilities of coarse DZ in the buffer solutions with different pH (HCl-pH 1.2; PB-pH 6.8; and PB-pH 7.4) were found to be just 0.99±0.15 μg/mL, 1.81±0.06 μg/mL and, 3.21±0.24 μg/mL, respectively (Table 3). In F-A and F-B formulations, the solubility of DZ in the different buffer solutions (HCl-pH 1.2; PB-pH 6.8; and PB-pH 7.4) increased in the range of about 6-14-fold in compared to coarse DZ ($p<0.05$; Table 3). The saturation solubility of DZ increased in F-A and F-B formulations due

to the large specific surface area as a result of the particle size decreasing to the nano-size range.

Moreover, it is significant to ensure a sink condition in the dissolution/release medium. In sink conditions, the saturation solubility of an/a active substance/compound is at least 3 times more than its concentration in the dissolution/release medium.[36] In the literature, to achieve sink condition in the study of dissolution/release, which was performed for the formulations genistein or DZ, the buffer solutions (PB or PBS pH 7.4) with ethanol (30%) or SDS (5%) or methanol (50%) or aqueous solutions with SDS (3%) or Tween 80 (0.5%) were used as release/dissolution medium.[10,37–40] Oliveira et al.[38] evaluated the solubilities of genistein and daidzein in several different mediums (sodium acetate buffer pH 4.5, water, water with 3% Tween 80, water with 3% Tween 20; water with 3% SDS) to properly design the dissolution test.

In our study, the solubility of DZ was also evaluated in the different buffer solutions with Tween 80 (5%) to properly design the dissolution study. The solubilities of coarse DZ in the buffer solutions (HCl-pH 1.2; PB-pH 6.8; and PB-pH 7.4) with Tween 80 (5%) were found to be 80.68±6.80 μg/mL, 107.06±5.54 μg/mL and, 128.77±3.66 μg/mL, respectively (Table 3). Furthermore, the solubility of DZ in F-A and F-B formulations was higher than that of coarse DZ ($p<0.05$; Table 3).

Dissolution Studies of Coarse DZ, F-A, and F-B Formulations

The dissolution studies of coarse DZ and nanosuspension formulations (F-A and F-B) were carried out in the different dissolution media (HCl+5% Tween 80-pH 1.2; PB+5% Tween 80-pH 6.8, and 7.4), and the dissolution profiles for coarse DZ, F-A, and F-B formulations were shown in Figure 6. In HCl+5% Tween 80-pH 1.2, about 14% (for coarse DZ), 65% (for F-A), and 84% (for F-B) of DZ dissolved within 5 min. Besides, in PB+5% Tween 80-pH 6.8, and 7.4, 16-17% (for coarse DZ), 66-68% (for F-A) and, and 85-86% (for F-B) of DZ dissolved within 5 min. At 60th min, about %60 (for coarse DZ) and %100 (for F-A and F-B) of DZ dissolved in all three dissolution media (Figure 6). As a result, it can be concluded that the dissolution of DZ can be improved and significantly increased by preparing a nanosuspension formulation of DZ. Wang et al.[41] prepared and evaluated the nanosuspension formulations of DZ using various stabilizers (soy lecithin, D-alpha-Tocopherol polyethylene glycol 1000 succinate, hydroxypropyl-β-cyclodextrin, sodium dodecyl sulfate, hydroxypropyl methylcellulose E5, sulfobutyl ether-β-cyclodextrin, or their combinations) to improve the solubility and oral bioavailability of DZ. They used the stabilizers to stabilize the small particles in colloidal dispersion by electrostatic repulsion or steric hindrance to overcome the physical instability problem caused by small particle size and increased free energy in the colloidal dispersion. They reported that the DZ nanosuspension formulations with particle sizes of 360–600 nm and regular shapes. The authors also evaluated the saturation solubility and the dissolution of coarse DZ and DZ nanosuspensions in distilled water and 900 mL of dissolution medium with 0.1% Tween-80, respectively. They found that the saturation solubility of crude DZ was very low (about 3 μg/mL), however, the saturation solubility (about 8–21 μg/mL) of DZ in nanosuspension formulations was higher compared to that of coarse DZ. Furthermore, they showed that less than 10% and 75% of coarse DZ dissolved in the dissolution medium with 0.1% Tween-80 within 5 min and 240 min, respectively; however, more than 80% and 90% of DZ in nanosuspension formulations dissolved within 5 min and 240 min, respectively. As a result of the preparation of nanosuspension formulations of poorly soluble active substances/compounds, the particle surface area of these active substances/compounds increases significantly by reducing the particle size to the nano-range. The increased surface area provides a significant increase in the dissolution rate of the active substances/compounds according to the Noyes-Whitney equation.[42] In addition, the use of the appropriate amount of Tween-80 causes an increase in the dissolution rate of DZ.[43]

Moreover, Bhalla et al.[21] prepared co-crystals of DZ with isonicotinamide, theobromine, and cytosine by solvent-assisted grinding. They evaluated the solubility and intrinsic dissolution of co-crystals of DZ in PB-pH 6.8. They stated that co-crystals of DZ showed an almost 2-fold improvement in the solubility and dissolution of DZ compared to pure DZ.

Cell culture study

The cytotoxic effect of DZ on cancer cells is dose-dependent.[17,44] In our study, the cytotoxic effects of DZ solution in the culture medium with 0.5% DMSO, and the suspensions of coarse DZ, and the nanosuspension formulations (F-A and F-B) in culture medium on RG2 cell lines were evaluated using MTT assay. Control cells were treated with only a culture medium (C1) or a culture medium with 0.5% DMSO (C2). The maximum DMSO concentration to be used for cell culture studies should be 0.5%.[17,45] There was no significant difference in the cell viability between C1 and C2 for 24 h and 48 h incubation ($p>0.05$; Figure 7). The cell viability was over 93% after incubation with Exp F-A or Exp F-B for 24 h and 48 h incubation (Figure 7). This suggests that Exp F-A and Exp F-B exhibited no significant cytotoxicity compared to C1 ($p>0.05$). After 24 h and 48 h incubation, high concentrations (200 and 400 µM) of DZ solutions had a significant cytotoxic effect on RG2 cells compared to C2 ($p<0.05$) and coarse DZ ($p<0.05$). Besides, coarse DZ showed a cytotoxic effect on cancer cells at high concentrations (100-400 µM; $p<0.05$ compared to C1; Figure 7) for 24 h and 48 h incubation.

After 24 h and 48 h incubation, the results of the cytotoxicity study indicated that F-A and F-B caused a significant decrease in cell viability of RG2 cells at all concentrations ($p< 0.05$; except 400 µM for DZ solution) compared to C1, coarse DZ, and DZ solution. After 24 h incubation, F-A and F-B formulations reduced the viability of RG2 cells by about 28% and 20% (at 50 µM concentration) and by approximately 53% and 54% (at 400 µM concentration), respectively (Figure 7). After 48 h incubation, the decrease in the viability of RG2 cells was approximately 48% (at 50 µM concentration for both formulations F-A and F-B) and about 67% (at 400 µM concentration for both formulations F-A and F-B) (Figure 7). Coarse DZ, F-A and F-B formulations decreased RG2 cell viability in a dose-dependent manner,

CONCLUSION

In our study, DZ nanosuspension formulations were successfully preprared and characterized in vitro. The results of characterization studies showed that the prepared nanosuspension formulations significantly increased the saturation solubility and dissolution rate of DZ as well as its cytotoxic effect on RG2 glioblastoma tumor cells.

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TABLES

Table 1. Content of the formulations.

Ingredients	Formulation A (F-A)	Formulation B (F-B)
DZ	50 mg	50 mg
Pluronic F127	1% (w/v)	1% (w/v)
PVP K30	1% (w/v)	1% (w/v)
SDS	-	0.5% (w/v)
Ultrapure water	5 mL	5 mL

Table 2. Mean droplet size and span values of coarse DZ (n=3, Mean ± SE; SE: Standard error)

Mean Diameter (μm)			Span
d_{10}	d_{50}	d_{90}	
18.490±0.617	55.545±1.473	164.561±7.941	2.633±0.162

Table 3. The results of saturation solubility study (Mean±SE; n=3).

	Coarse-DZ (μg/mL)	F-A (μg/mL)	F-B (μg/mL)
pH 1.2 HCl	0.99±0.15	7.94±1.00	13.83±0.27
pH 6.8 PB	1.81±0.06	11.95±1.00	21.23±0.33
pH 7.4 PB	3.21±0.24	17.39±1.16	30.89±0.39
HCl+5% Tween 80-pH 1.2	80.68±6.80	102.16±6.36	118.99±7.65
PB+5% Tween 80-pH 6.8	107.06±5.54	143.80±5.34	159.53±7.02
PB+5% Tween 80-pH 7.4	128.77±3.66	150.04±2.01	188.69±4.36

FIGURES

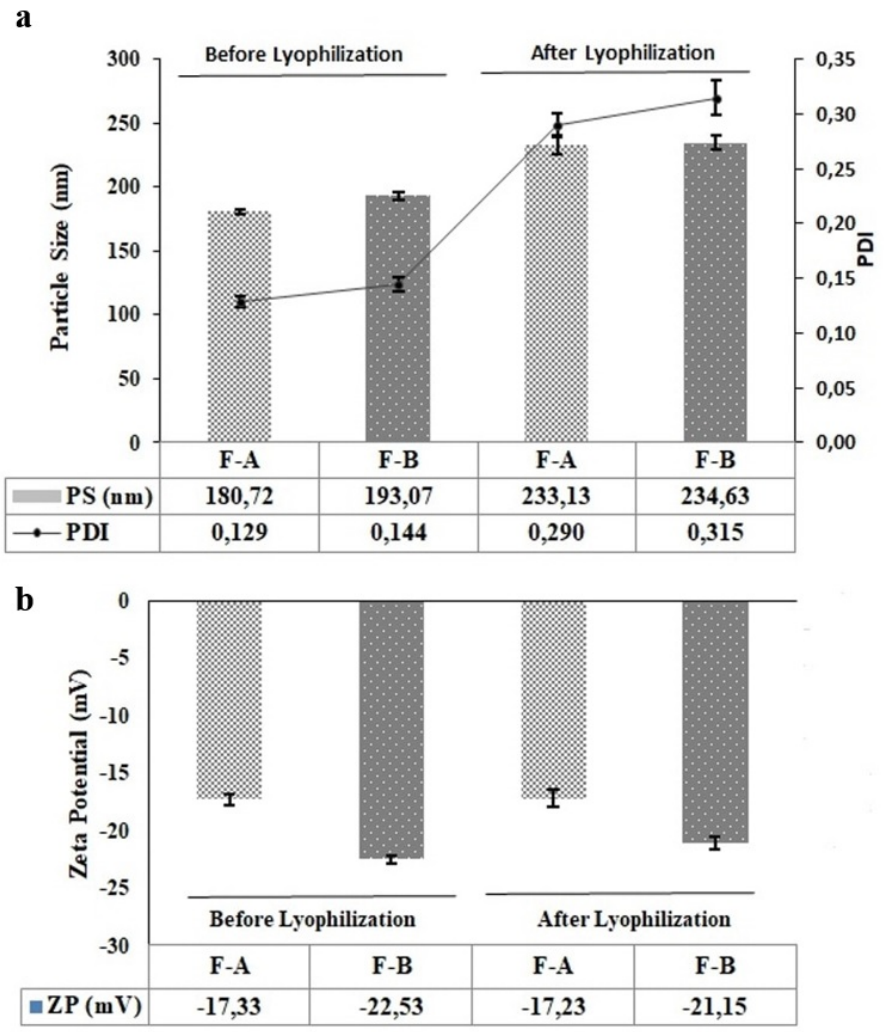

Figure 1. The particle size (PS), PDI and zeta potential (ZP) values of F-A and F-B formulations (n=6, Mean±SE; SE: Standard error)

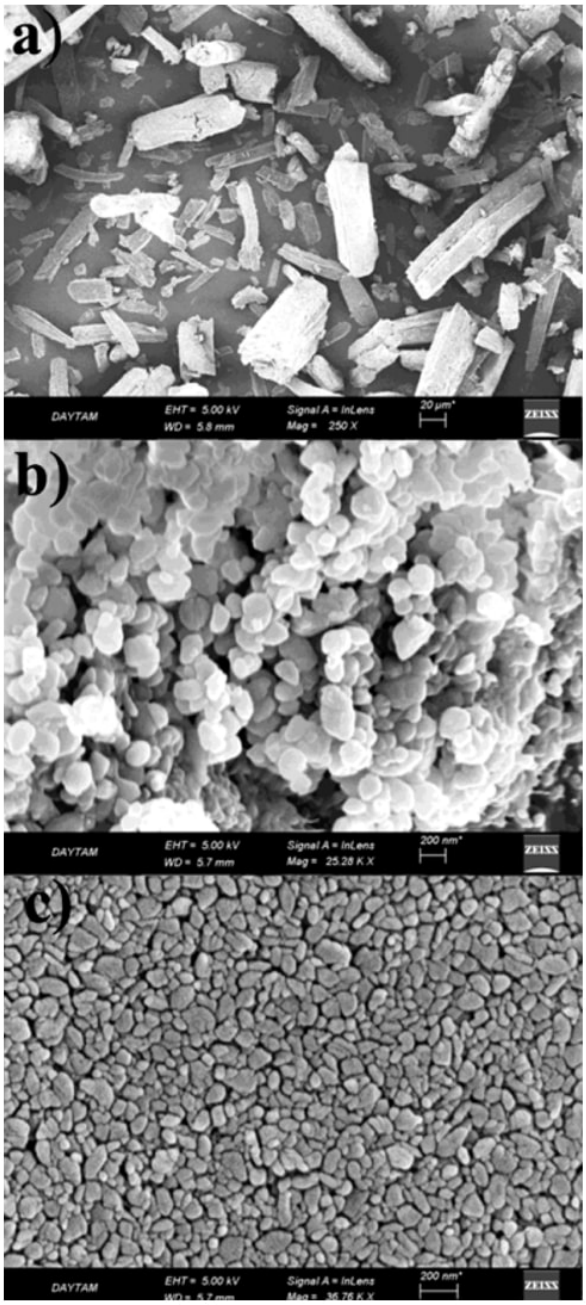

Figure 2. SEM images of coarse DZ (a), F-A (b) and F-B (c) formulations.

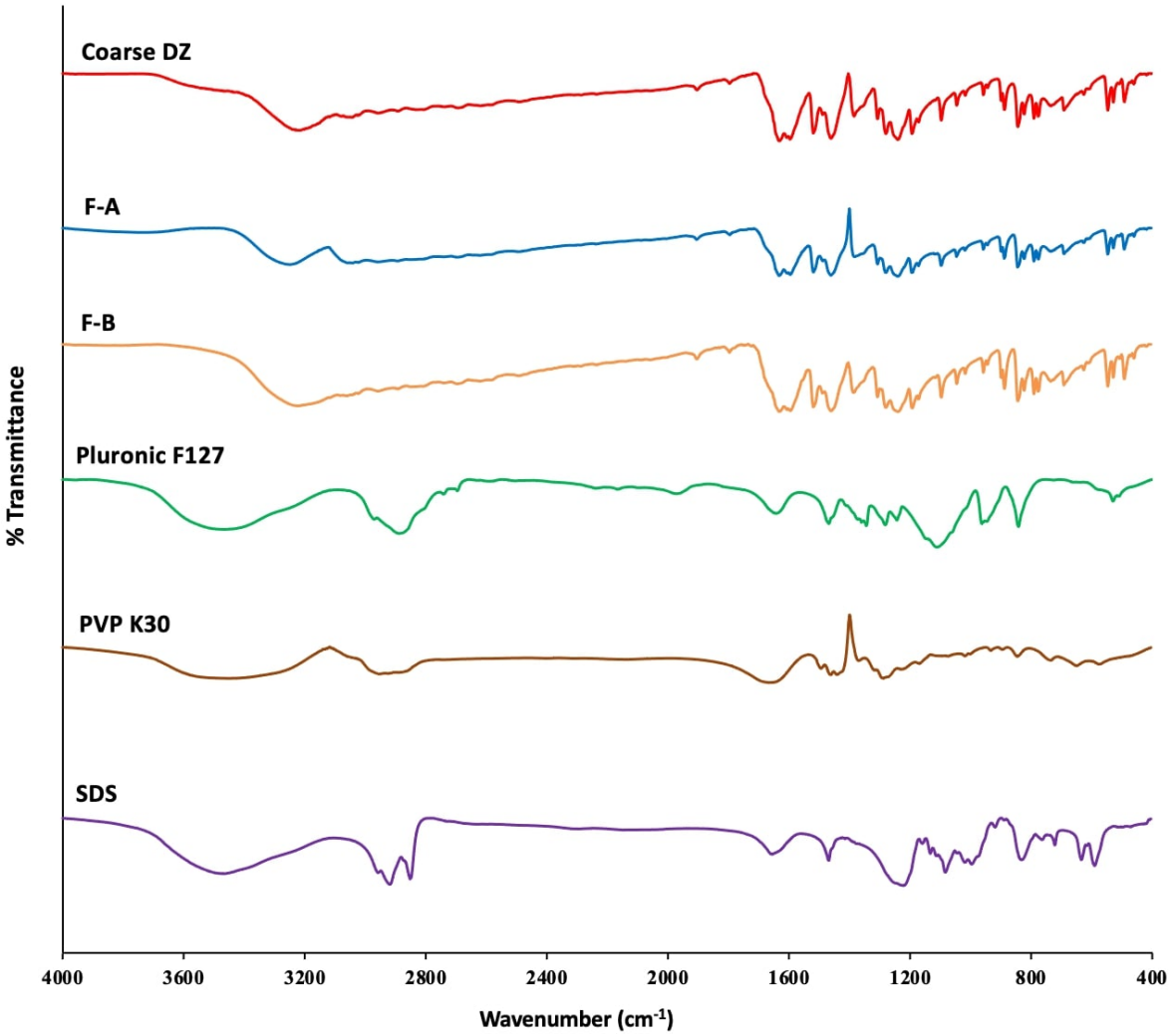

Figure 3. FTIR spectra of coarse DZ, F-A and F-B formulations, and the excipients in the formulation (Pluronic F127, PVP K30, and SDS)

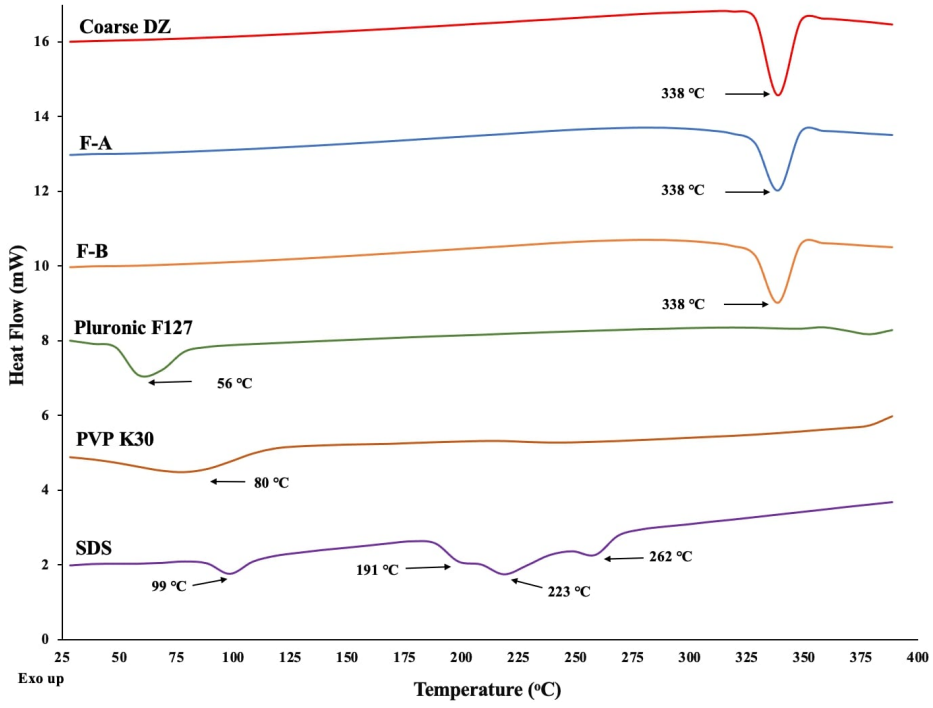

Figure 4. DSC thermograms of coarse DZ, F-A and F-B formulations, and the excipients in the formulation (Pluronic F127, PVP K30, SDS)

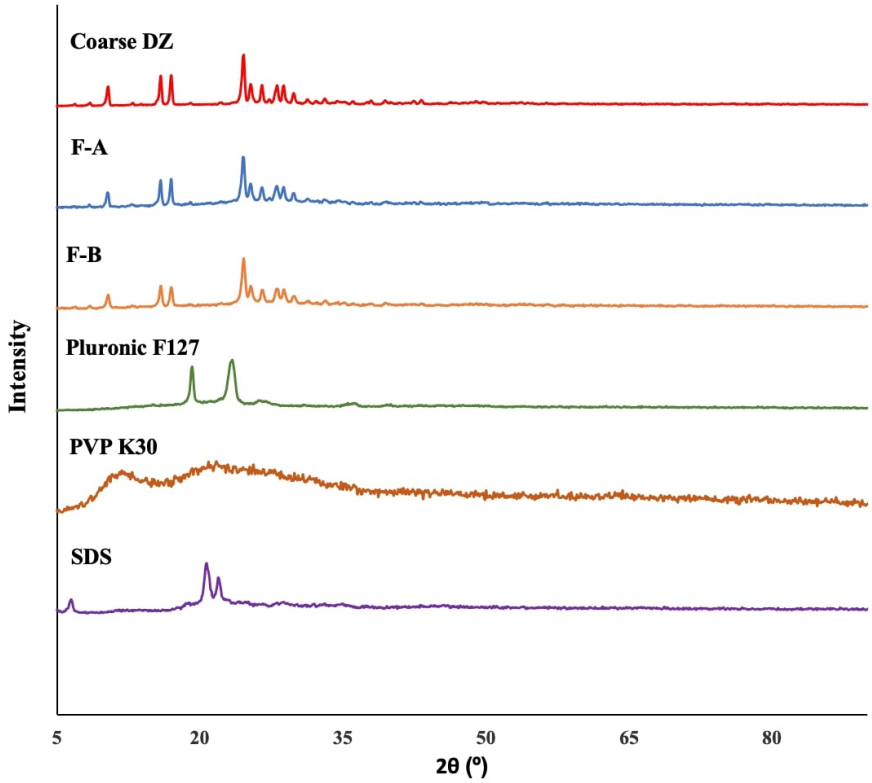

Figure 5. X-ray patterns of coarse DZ, F-A and F-B formulations, and the excipients in the formulation (Pluronic F127, PVP K30, and SDS).

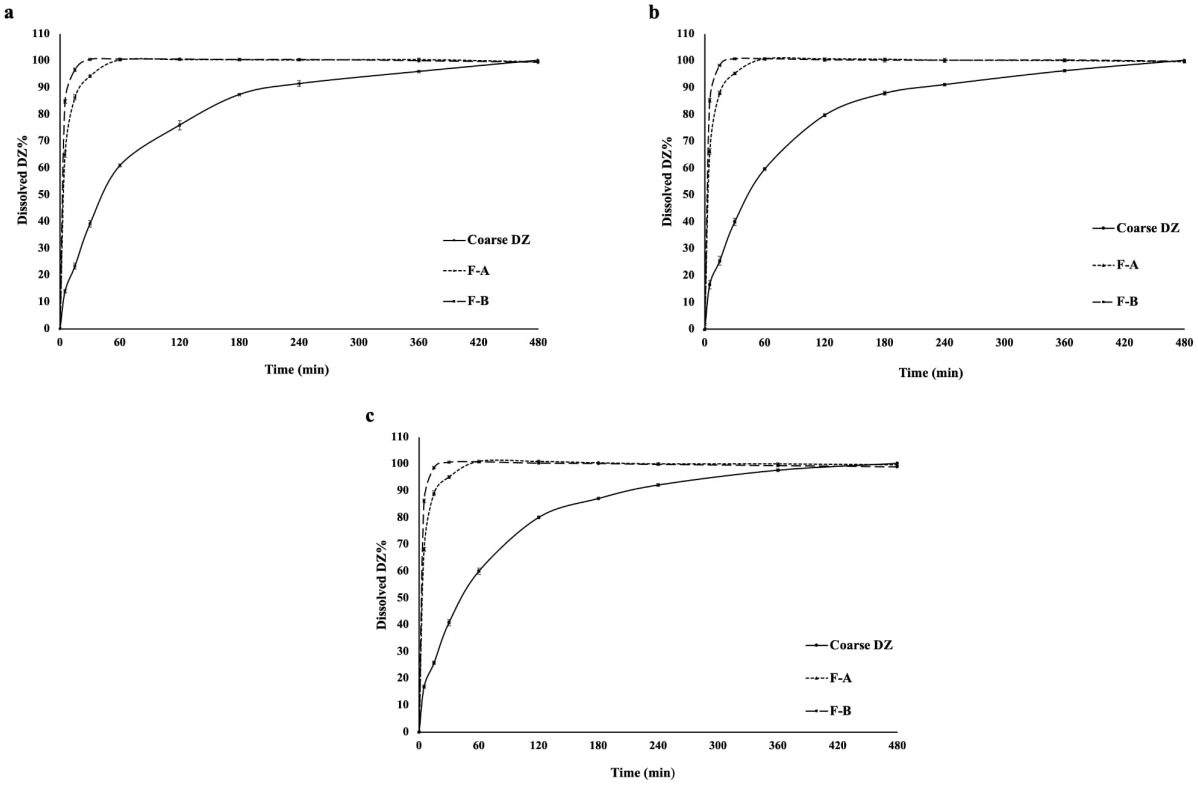

Figure 6. The dissolution profiles of coarse DZ, F-A and F-B formulations in the different dissolution media (a). HCl+5% Tween 80-pH-1.2; (b). PB+5% Tween 80-pH-6.8; (c). PB+5% Tween 80-pH-7.4 (Mean±SE; n=3)

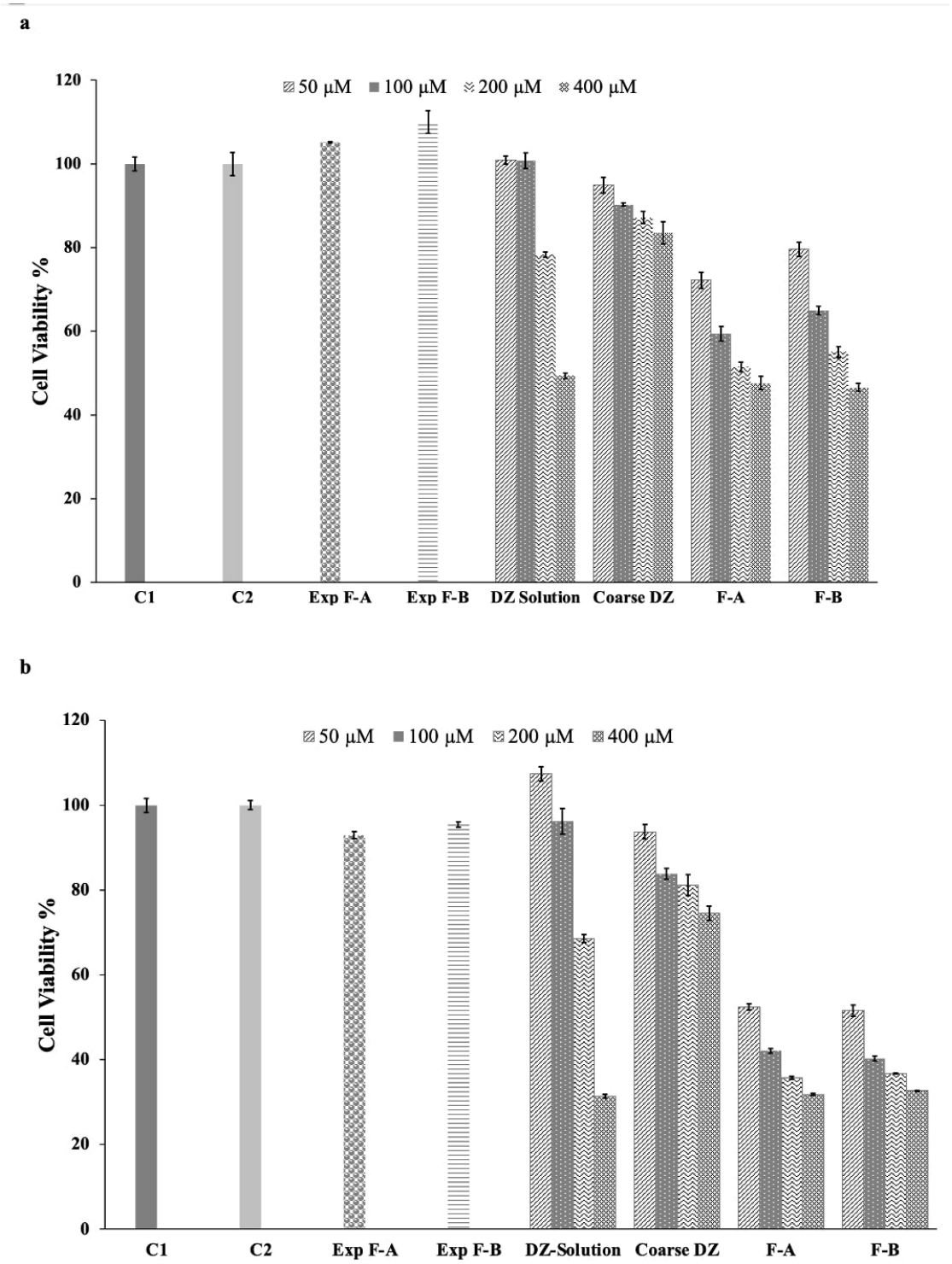

Figure 7. Cytotoxic effects of DZ solution, coarse DZ and the nanosuspension formulations (F-A and F-B) for 24 h (a) and 48 h (b) incubation.