BJPS

Brazilian Journal of Pharmaceutical Sciences

http://dx.doi.org/10.1590/s2175-97902020000118973

Article

A nanoemulsion-based delivery system for imatinib and *in vitro* anticancer efficacy

Maryam Karimi[1], Kh ashayar Karimian[2], Hossein Heli[1*]

[1]Nanomedicine and Nanobiology Research Center, Shiraz University of Medical Sciences, Shiraz, 7193635899, Iran, [2]Arasto Pharmaceutical Chemicals Inc., Yousefabad, Jahanarar Avenue, Tehran, Iran

A self-nanoemulsifying drug delivery system (SNEDDS) composed of ethyl oleate, Tween 80 and polyethylene glycol 600 was prepared as a new route to improve the efficacy of imatinib. The drug-loaded SNEDDS formed nanodroplets of ethyl oleate stabilized by Tween 80 and polyethylene glycol 600 with a diameter of 81.0±9.5 nm. The nanoemulsion-based delivery system was stable for at least two months, with entrapment efficiency and loading capacity of 16.4±0.1 and 48.3±0.2%, respectively. Imatinib-loaded SNEDDS was evaluated for the drug release profiles, and its effectiveness against MCF-7 cell line was investigated. IC_{50} values for the imatinib-loaded SNEDDS and an imatinib aqueous solution were 3.1 and 6.5 μg mL^{-1}, respectively.

Keywords: Glivec. Nanoemulsion. Anticancer drug. Bioavailability. Breast cancer cell

INTRODUCTION

Uncontrolled proliferation of tumor cells is associated with abnormal activation and aberrant degradation of receptor tyrosine kinases (RTKs). Generally, RTKs induce critical cellular processes, such as cell migration, differentiation, apoptosis and proliferation (Kadivar *et al*., 2017). Several studies revealed that alteration in the activation and or expression of RTKs plays key roles in the oncogenesis and progression of breast cancer (Biscardi *et al*., 2000), and abnormal activity of platelet-derived growth factor receptors and their ligands has also been observed in the breast, ovarian, lung, gastric, and prostatic cancers and melanomas (Rajabi, Mousa, 2017; Sheeba Caroline *et al*., 2014). Since RTKs have been implicated in many aspects of the malignant phenotype, they are the targets used for molecular cures.

Imatinib mesylate is an antineoplastic small molecule used widely in the treatment of chronic myeloid leukemia (Elias *et al*., 2014), gastrointestinal stromal tumours (Din, Woll, 2008), and other human cancers by preventing the signaling of the platelet-derived growth factor receptor, abelson proto oncogene (Bcr-Abl), and cKIT (CD117) tyrosine kinases (Heinrich, *et al*., 2000; Marslin *et al*., 2015). A large amount of imatinib needed to reach sufficient serum concentrations to function as an inhibitor of RTKs signaling, imatinib resistance and intolerance has been detected in many patients. Therefore, controlled local drug delivery reduces the need to daily administrations and side effects including local gastric irritation, skin rashes and oedema, most of which being dose-dependent (Girish Thampi, Godwin, Harish, 2014).

In recent years, much attention has been focused on lipid-based formulations because of the ability to deliver a wide variety of drugs to different parts of the body (Nazari-Vanani *et al*., 2017; Boakye *et al*., 2017). Recently, nanoemulsion-based delivery routes, especially self-nanoemulsifying drug delivery systems (SNEDDSs), have been reported as a suitable approach for enhancing the bioavailability and bioactivity of various drugs or natural bioactive compounds. SNEDDSs include a uniform mixture of oil, surfactant, co-solvent and drug

***Correspondence:** H. Heli. Arasto Pharmaceutical Chemicals Inc. Yousefabad, Jahanarar Avenue, Tehran, Iran. Tel: +98 71 36 12 22 25,6. Fax: +98 71 36 28 15 06. E-mail: hheli7@yahoo.com, heli@sums.ac.ir (H. Heli)

substance and form a nanoemulsion upon dispersion under gentle agitation, when diluted with aqueous solutions (Nazari-Vanani *et al*., 2017; Nazari-Vanani, Moezi, Heli, 2017; Nazari-Vanani, Azarpira, Heli, 2018; Friedl *et al*., 2013). SNEDDSs provide enormous advantages such as production of small-sized droplets of oils, resulting in a high surface area, increased gastrointestinal tract membrane permeability, enhanced drug loading efficiency and protection of the drug from degradation in the gastrointestinal tract (Nazari-Vanani *et al*., 2017, Nazari-Vanani, Moezi, Heli, 2017, Nazari-Vanani, Azarpira, Heli, 2018; Zhang *et al*., 2015).

So far, there have been some reports on the nanocarriers for imatinib, including solid lipid nanoparticles (Vivek, Jose, 2018, Gupta *et al*., 2016), nanoliposomes (Chen *et al*., 2018), PEGylated nanoparticles (Gupta *et al*., 2017), polybutylcyanoacrylate nanoparticles (Hasandoost *et al*., 2017), poly-lactide-co-glycolic acid nanoparticles (Khan *et al*., 2016), and polyvinylpyrrolidone and polyethylene imine coated gold nanoparticles (Labala *et al*., 2015). These nanocarriers led to targeting of the lymphatic system (Vivek, Jose, 2018) or tumor (Chen *et al*., 2018); enhancing the bioavailability (Vivek, Jose, 2018, Chen *et al*., 2018); sustaining the release (Gupta *et al*., 2016), overcoming chemoresistance (Chen, *et al*., 2018, Gupta *et al*., 2017), getting lower IC50 values (Khan, Ahmad, Panda, 2016), and enhancing the skin penetration (Labala *et al*., 2015); they represented anticancer efficacy toward the cell lines of leukemia K562 (Hasandoost *et al*., 2017), MDA-MB-231 and SK-MEL-28 (Gupta *et al*., 2017), glioma U251MG and C6 (Khan *et al*., 2016), and murine melanoma B16F10 (Labala *et al*., 2015).

There has been no report on the evaluation of cytotoxicity of imatinib mesylate-loaded SNEDDSs so far. Therefore, the present study aimed to develop a SNEDDS formulation for imatinib to improve its therapeutic efficacy by reduced drug contact with normal tissue.

MATERIALS AND METHODS

Materials ad biologicals

Imatinib mesylate was received from Arasto Pharmaceutical Chemicals Inc (Iran). Methanol and acetonitrile (high-performance liquid chromatography (HPLC) grade), trypan blue and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (USA). Other chemicals including ethyl oleate, Tween 80, polyethylene glycol (PEG) 600, and dimethyl sulfoxide (DMSO) were purchased from Merck (Germany) or Scharlau (Spain). Roswell Park Memo-rial Institute-1640 (RPMI-1640) and fetal bovine serum (FBS) were purchased from Gibco Laboratories, USA. Breast cancer epithelium-like cell line (MCF-7, NCBI C135) was purchased from Pasteur Institute Cell Bank (Iran). Solutions of trypsin-EDTA and penicillin-streptomycin were prepared from MedChem Express (China) and Danesh Azma, Cell (Iran), respectively. Deionized (DI) water was used throughout the study.

Determination of the solubility of imatinib in the SNEDDS components

Solubility of imatinib was explored in the SNEDDS components of oil (ethyl oleate), surfactant (Tween 80) and co-surfactant (PEG 600) to find the suitable ingredients of the formulation. Determination of saturation solubility was carried out by adding excess quantity of imatinib (approximately 20 mg) to 1.0 mL of each component in glass vials, followed by 24 h shaking at room temperature. Then, the glass vials were centrifuged at 15000 rpm for 20 min in a sealed microcentrifuge (Eppendorf 5424, Germany) to discard the excess insoluble imatinib. The supernatants were carefully taken from each sample and stored at room temperature.

The concentration of imatinib in the supernatants was then quantified by a UV-vis spectrophotometer of Rayleigh UV-2100 (China). The absorption coefficients for imatinib in each component were separately determined by recording the absorbance values of standard imatinib solutions in triplicates. The solubility values were obtained as mean±standard deviation.

Stability of SNEDDS

The blank SNEDDS and imatinib-loaded SNEDDS were kept at room temperature for at least 2 months, and any phase separation in SNEDDSs was visually monitored to evaluate the stability of the SNEDDSs.

Preparation of SNEDDSs

Based on the imatinib solubility data and the phase diagram for the SNEDDS components (Nazari-Vanani

et al., 2017), the ratios of ethyl oleate, Tween 80 and PEG 600 of 15:30:55 (V/V) were selected. To prepare the imatinib-loaded SNEDDS, we premixed the required amounts of ethyl oleate, Tween 80 and PEG 600 in a glass vial and shook it for a period of 5 min in order to obtain a clear mixture. Then, a precisely weighed amount of imatinib mesylate was subsequently added, shaken for 10 min, diluted in 10-fold with DI water under mild agitation, and finally stored at 25 °C until use. The blank SNEDDS was prepared similarly without using imatinib mesylate.

Determination of entrapment efficiency and loading capacity of imatinib

Entrapment efficiency (EE) and loading capacity (LC) were measured based on the following equations:

EE=Weight of imatinib loaded / Weight of imatinib added(1)

LC=Weight of imatinib loaded / Weight of SNEDDS (2)

An imatinib-loaded SNEDDS consisting of 6.0 mg imatinib mesylate and 2.0 mL of SNEDDS was prepared. Then, the loaded imatinib was extracted from the formulation by adding 1.0 mL chloroform followed by vortex mixing for 5 min. The obtained mixture was allowed to stay at room temperature until the two phases were formed; then, the organic phase was carefully separated and its imatinib content was measured in triplicate by HPLC.

Determination of droplet size and zeta potential of imatinib-loaded SNEDDS

The droplet size and zeta potential of imatinib-loaded SNEDDS were measured, using a Malvern Zetasizer Nano ZS™ (UK) over six repeated measurements at room temperature.

HPLC procedure

Imatinib was quantified in various samples by HPLC on a Waters 1525 instrument (USA). Separation was made on a C18 reverse phase 250 mm×4.6 mm (internal diameter) column and particle size of 5mm from Eurospher (Germany); also, detection was done using a UV-vis detector at 256 nm. The mobile phase was a mixture of methanol, acetonitrile and water in volume in ratios of 65:20:15 (Zhuang *et al*., 2017); the retention time was about 7 min, and the injection volume was 60 µL at a flow rate of 1.0 mL min^{-1}. The mobile phase was firstly filtered by a 0.22-µm membrane and degassed by a bath sonicator of Wise Clean (Germany) for 5 min. The samples were diluted with the mobile phase and analyzed, using a previously plotted calibration curve. Standard solutions of imatinib were prepared in the mobile phase as a serial concentration in a range of 0.25 to 500 µg mL^{-1}. From a calibration curve plotted for the imatinib quantitation, the concentration of imatinib in the samples was determined. All samples were analyzed in triplicate, and data were expressed as mean±standard deviation.

In vitro drug release

The release profile of imatinib from SNEDDS was conducted, using the dialysis method. 5.0 mL of imatinib-loaded SNEDD containing 5.0 mg imatinib was transferred to a dialysis bag (1000 MWCO), immersed in 25 mL of a fresh phosphate buffer solution, pH 7.4 (PBS) rotating at 100 rpm, and maintained at 37±0.5 °C. 1.0-mL aliquots were withdrawn at different intervals of 0.5, 1, 2, 3, 5, and 8 hours from the release medium, and PBS of the same amount was replaced. The concentration of imatinib in each sample was determined by HPLC. Determinations were triply performed. A similar procedure was applied to determine and compare the imatinib release from its aqueous solution.

Cytotoxicity assay

In vitro anticancer efficacy of imatinib-loaded SNEDDS toward MCF-7 cells was studied, using the MTT assay as described previously (Gorgizadeh *et al.,* 2018; Sattarahmady *et al.,* 2018; Rahimi-Moghaddam *et al.,* 2018). The cells were grown in cell culture flasks containing RPMI-1640 medium supplemented with 1% penicillin/streptomycin and 10% FBS. The flasks were then incubated at 37 °C in an incubator of 5% CO_2. Confluent cultures were reaped with trypsin solution and the cells were followed for cytotoxicity studies. The cells were seeded in 96-well plates at a density of 3×10^4 cells/well and allowed to adhere for 24 h. Imatinib-loaded SNEDDS and imatinib solution of different concentrations were added to reach final concentrations in a range of 1-40 µg mL^{-1}. Parallel sets using the blank SNEDDS or a buffer solution (without

imatinib) were similarly run as controls. The cells were incubated for 24 h, treated with 100 mL of MTT solution of 0.5 mg mL^{-1} dissolved in RPMI-1640 medium and then for 4 hours. Then, the supernatant was removed, and the sediment (formazan crystals) dissolved in 100 mL DMSO; it was then shaken for 10 min at room temperature, and the absorbance rates were read at 570 nm with a microplate reader of Awareness Technology (USA). The values were reported as mean±standard deviation. For data analysis, mean Optical Density (OD) of each well was calculated and the percentage of the cells' viability was determined.

Statistical analysis

Statistical analyses were performed through SPSS software, using Mann-Whitney test. A p-value <0.05 was considered as statistically significant.

RESULTS AND DISCUSSION

There are selection criteria for the components of a SNEDDS, comprising the following items:

1) Safety (biocompatibility): The most cytotoxic component of a SNEDDS is the surfactant (Gursoy, Benita, 2004; Rebello *et al.*, 2014). Compared to ionic ones, nonionic surfactants are less toxic. Tween 80, as a nonionic surfactant, can, therefore, be a selection although a low amount of this surfactant (30% W/W) was employed in this study.
2) The value of hydrophilic-lipophilic balance (HLB) of the surfactant: This value for the formation of a nanoemulsion should be >10 (Shafiq *et al.*, 2007). HLB for Tween 80 is 15 favoring the SNEDDS formation.
3) A suitable co-surfactant: This component, accompanied by the surfactant, reduces the interfacial tension of the nanoemulsion. Co-surfactant must be solubilized in both oil and surfactant. PEGs are biocompatible species with both hydrophilic and hydrophobic properties. PEG 600, as a co-surfactant in the SNEDDS formulation, is highly hydrophilic and less cytotoxic with a wide use in formulations of pharmaceuticals and foods (Yuan, Williams, Biggs, 2009). It binds a variety of water insoluble drugs with a large HLB value of 12. Therefore, it can stabilize the SNEDDS and cause an increment in the HLB value of SNEDDSs to be >10 (Tadros, 2013), owing to the fact that PEG 600 decreases the surface tension through assembling at the oil droplets/aqueous phase, leading to formation of a single phase system.

Solubility of imatinib in Tween 80, PEG 600 and ethyl oleate was spectrophotometrically measured at room temperature (see also supplementary material S1) and obtained as 2.6 mg mL^{-1}, 5.84 mg mL^{-1} and 1.1 mg mL^{-1}, respectively. The results indicated that PEG 600 showed the highest solubilizing ability for imatinib, compared to the other components.

Based on the already reported results about the miscibility of the PEG 600/Tween 80/ethyl oleate mixtures and their ternary phase diagram (Nazari-Vanani *et al*., 2017; Nazari-Vanani, Moezi, Heli, 2017), emulsification ability of PEG 600/Tween 80 mixtures (Sanka, Suda, Bakshi, 2016), the solubility of imatinib in PEG 600 being the highest (compared to Tween 80 and ethyl oleate), and to keep the toxicity of the formulation low, high amounts of PEG 600 and low amounts of ethyl oleate and Tween 80 were selected to achieve a high imatinib concentration in the SNEDDS with minimum cytyotoxicity. Quantitatively, the ratios of 55/30/15% (v/v) of PEG 600/Tween 80/ethyl oleate were selected to prepare the SNEDDSs. These ratios of the components provide a self-nanoemulsifying system with a minimum free energy and thermodynamic stability, while the amount of the surfactant was very low, and the amount of the co-surfactant was selected as a high value. At the molecular level, imatinib is transported by the oil droplets stabilized by imatinib-dissolved PEG 600/ Tween 80 layer(s).

The droplet size of the nanoemulsions is an important parameter in its performance because it regulates the rate and extent of drug release as well as absorption. We already determined the oil droplet size of the blank SNEDDS to be 29.5±6.3 nm (Nazari-Vanani *et al.*, 2017). However, upon loading the drug, the oil droplet size of the imatinib-loaded SNEDDS reached 81.0±9.5 nm (Figure 1). This can be related to the entrapment of the drug in the oil droplets, attraction forces between the oil and the drug molecules, leading to weakening of the oil-surfactant and oil-co-surfactant attraction. Zeta potential value of the imatinib-loaded SNEDDS was also measured to be -33.8±10.0 mV.

As to the stability of the blank and imatinib-loaded SNEDDSs, visual observations showed no phase separation and significant change in the physical appearance of the formulations at room temperature over a period of 2 months.

To determine EE and LC of the SNEDDS for imatinib, we firstly constructed a calibration curve for imatinib, as shown in Figure 2. Based on the results, imatinib was quantified with the figure of the merit of: 1) the regression equation for the calibration curve was $y=(4.09\pm0.06)x+(74.4\pm23.6)$, $R^2=0.9990$; 2) linear concentration range was 0.25-1000 μg mL^{-1}; 3) reproducibility had a relative standard deviation (RSD) for the concentration of 50 μg mL^{-1} of 3.8%; 4) detection limit was calculated as 3×(standard deviation for the blank signal)/(calibration curve slope) to be 0.05 μg mL^{-1}; and 5) RSDs of intra-day and inter-day assays for the concentration of 50 μg mL^{-1} were 2.2% and 3.7%, respectively. The amounts of loaded/released imatinib into/from the SNEDDS were then measured and EE and LC obtained as 16.4±0.1 and 48.3±0.2%, respectively. These results indicate that although imatinib is a highly water soluble drug, it is deeply loaded into the SNEDDS.

The release profile of imatinib from the imatinib-loaded SNEDDS and an imatinib aqueous solution were evaluated, as shown in Figure 3. The profiles show that the initial rate of drug release from the imatinib-loaded SNEDDS was much slower than the aqueous solution and followed a sigmoid pattern, while the aqueous drug solution was released rapidly. Therefore, SNEDDS caused a slow release of the drug. This can be related to the drug capture into the SNEDDS droplets.

MTT-based cell proliferation assay was employed to evaluate the cytotoxicity of the imatinib-loaded SNEDDS against MCF-7 cells, and the results were compared with an imatinib solution in water, as shown in Figure 4. The MTT assay results indicated that while no cytotoxicity was observed in the control groups of the cells (DI water and the blank SNEDDS with viabilities of 100%), the cell growth was inhibited in a dose-dependent manner in a concentration range of 1-40 μg mL^{-1} by the imatinib aqueous solution and imatinib-loaded SNEDDS. However, the inhibitory effect of the imatinib-loaded SNEDDS was higher in comparison with aqueous drug solution at all concentrations tested in a statistically significant manner. IC_{50} value for the imatinib-loaded SNEDDS was found to be 3.1 μg mL^{-1} at 24 h as compared to an IC_{50} value of 6.5 μg mL^{-1} for the imatinib aqueous solution. Therefore, the SNEDDS formulation led to the enhancement of the imatinib toxicity toward and proliferation blocking in the MCF-7 cancer cells. This can be related to higher imatinib uptake by the cells. It was approved that the imatinib-loaded SNEDDS represents cancer cell cytotoxicity at lower drug concentrations and causes a reduction in the drug dose, enhancing its efficacy.

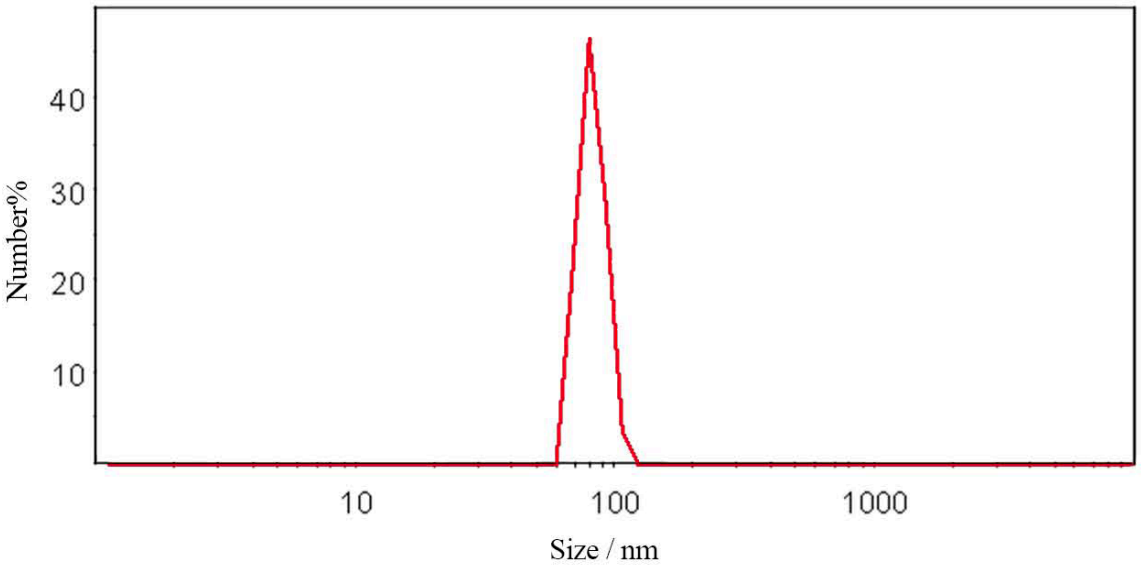

Figure 1 - Dependency of absorbance values on the imatinib concentrations dissolved in tween 80 (A), PEG 600 (B) and ethyl oleate (C) at room temperature. The data was obtained for evaluation of the imatinib solubility in each solvent.

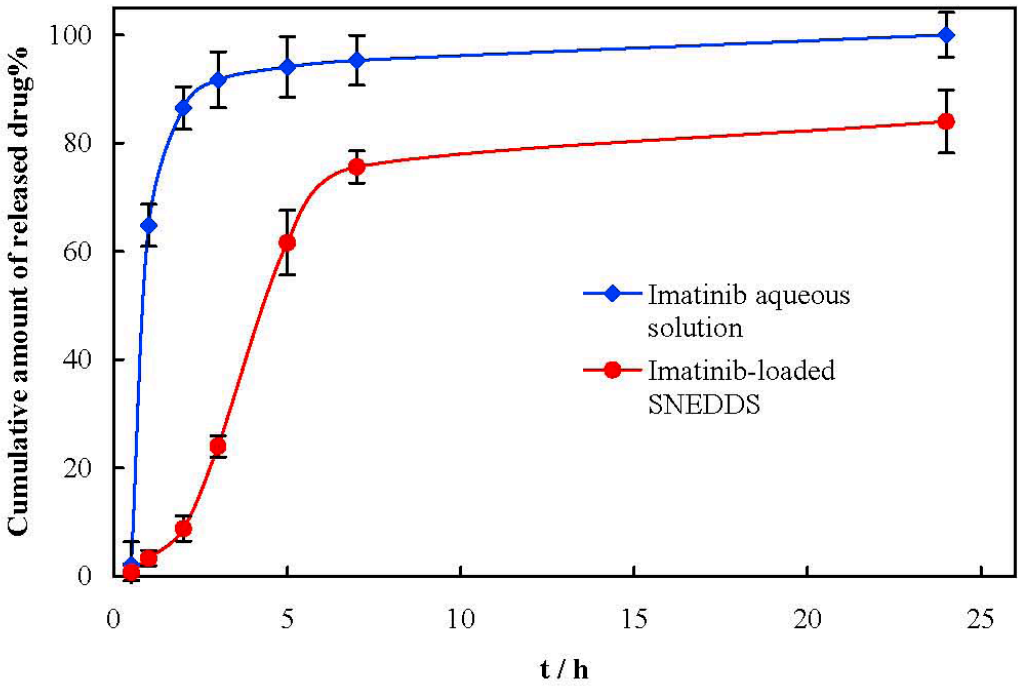

Figure 2 - A calibration curve for imatinib quanitiation by HPLC.

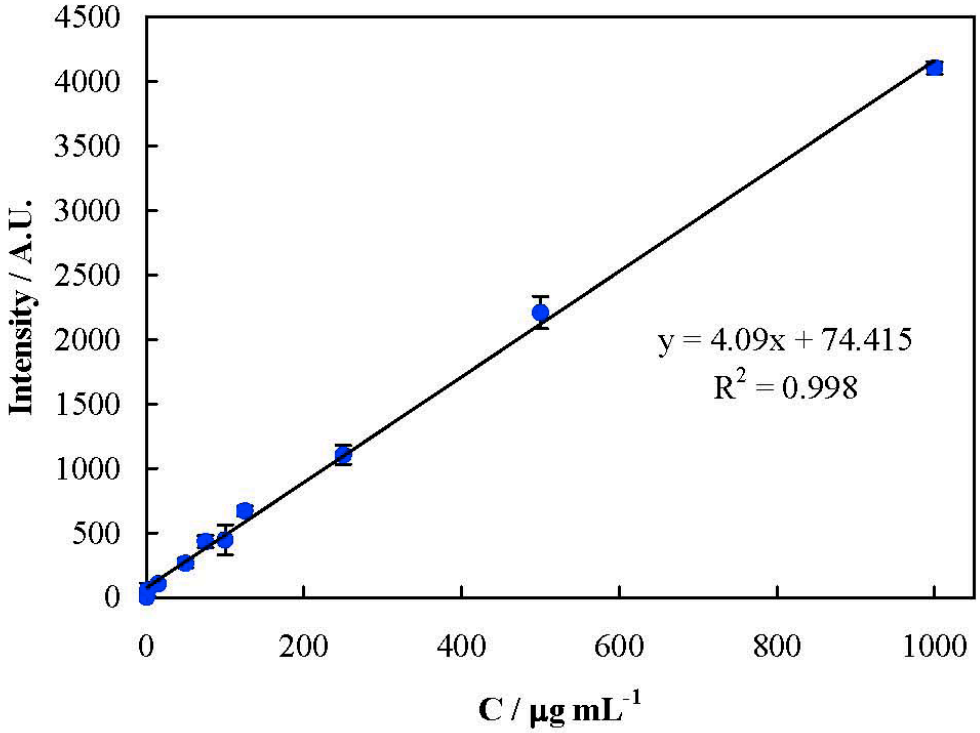

Figure 3 - Release profile of imatinib from the imatinib-loaded SNEDDS and an imatinib aqueous solution.

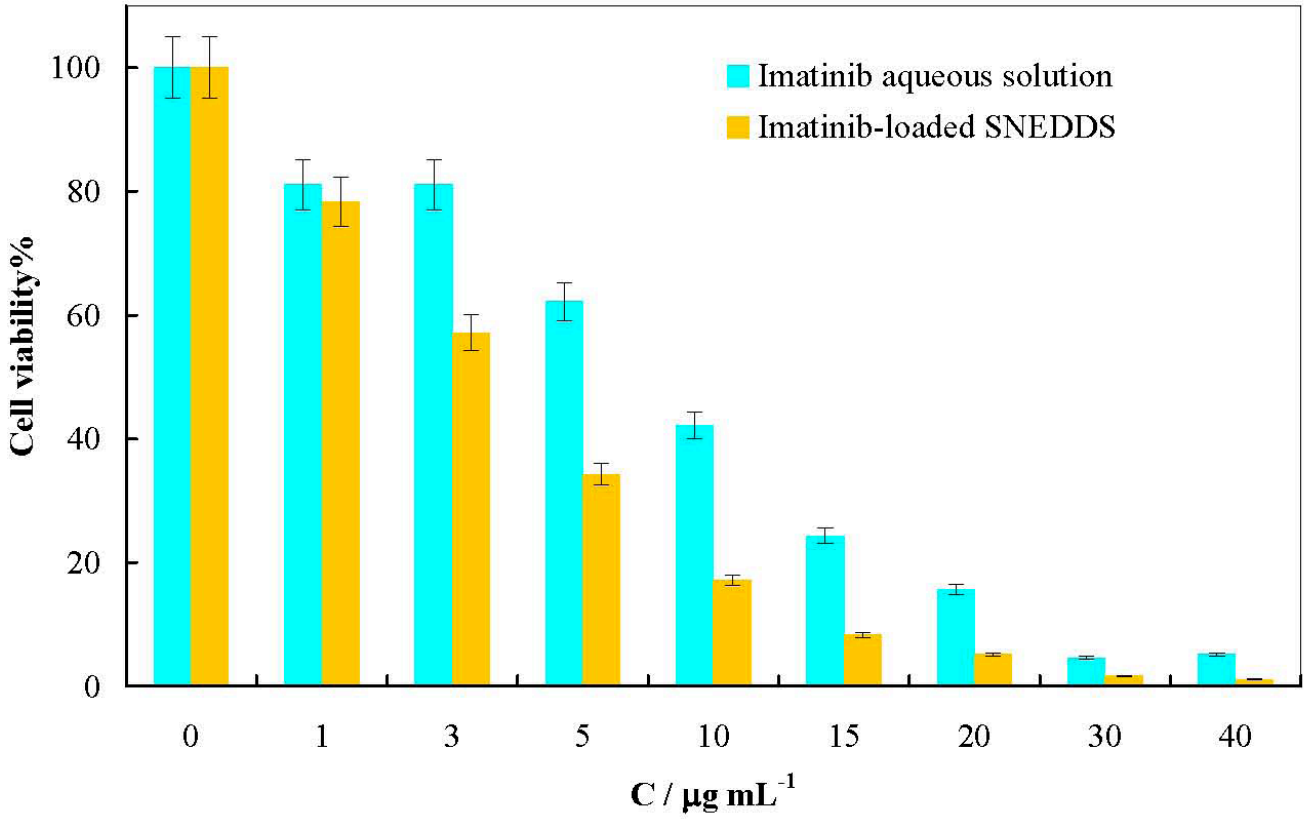

Figure 4 - MCF-7 cells viability upon treatment with different concentrations of imatinib-loaded SNEDDS and an imatinib aqueous solution.

CONCLUSION

SNEDDSs have been widely developed to improve the oral bioavailability of different drugs. In the present study, an optimized SNEDDS was developed where the ethyl oleate was employed as the safe, low-cost, and available oil. The emulsifying ability of the surfactant and co-surfactant was also a key point to stabilize the SNEDDS and anticancer performance of imatinib. The imatinib-loaded SNEDDS contained spherical and homogenous droplets of 81nm with high EE and LC. The imatinib-loaded SNEDDS represented an enhanced toxicity toward the MCF-7 cells, compared to the aqueous solution of the drug. The imatinib-loaded SNEDDS with favorable *in vitro* characters can be employed as a new delivery system for oral delivery of this drug.

ACKNOWLEDGMENTS

We would like to thank the Research Council of Shiraz University of Medical Sciences (15810) for supporting this research.

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Received for publication on 14th November 2018
Accepted for publication on 01st February 2019