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Enhancement of the brain delivery of methotrexate with administration of mid-chain ester prodrugs: *In vitro* and *in vivo* studies

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ABSTRACT

In the present study, with the aim of improving the permeability of methotrexate (MTX) to the brain, the lipophilic MTX prodrugs containing the ester functional moiety were synthesized. The chemical structure of synthesized prodrugs was characterized and confirmed by FT-IR, NMR and mass spectral studies. Based on the results of *in vitro* cytotoxic studies, all of the synthesized prodrugs led to decrease in the IC50 in 72 h on U87 cancer cell line and the best result was observed for dihexyl methotrexate (MTX-DH) in comparison with free MTX, which led to decrease the IC50 amount up to 6 folds. In addition, *in vivo* toxicity on Artemia salina (A. salina) showed that the lipophilic MTX prodrugs have been able to partially mask the toxic profile of free MTX, at the same concentrations. These findings were also in compliance with hemolysis assay results, which confirm that the conjugates has not made the drug more toxic. Furthermore, *in vivo* study in rat model, was employed to determine the simultaneous drug concentration in brain and plasma. According to the obtained results, the brain-to-plasma concentration ratios (Kp values) of MTX-DH and dioctyl methotrexate (MTX-DO) groups were significantly higher compared with free MTX. Moreover, the uptake clearance of MTX by brain parenchyma increased significantly (3.85 and 9.08-time increased for MTX-DH and MTX-DO prodrugs, respectively). These findings indicate that the synthesized lipophilic MTX prodrugs are non-toxic and able to enhance brain penetration of MTX.

1. Introduction

In spite of extensive advances in brain research, the central nervous system (CNS) and brain diseases, still remain the world's leading cause of disability (Hosokawa et al., 2007). The main challenge in CNS disease treatment, is the presence of blood-brain barrier (BBB) that is a functional and structural barrier between neural tissue and circulating blood. BBB is comprised of brain capillary endothelial cells that are surrounded by astrocytes, neurons, and pericytes which create a microenvironment that is crucial to BBB function (Abbott et al., 2010; Hawkins and Davis, 2005). Therefore, owing to its particular features, penetration across the BBB, is the major limiting factor in the delivery of therapeutics to diseased sites for the treatment of CNS disorders or brain tumors. Thus, CNS therapeutic agents should be designed and developed with suitable

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brain penetration attributes (Liu et al., 2008).

Recently, various drug delivery vehicles are being developed in order to achieve enough CNS penetration. Among them, prodrug strategy is one of the most effective and promising approaches to attain good BBB permeability and low brain non-specific tissue binding, without losing the pharmacological efficiency (Cacciatore et al., 2018; Pavan et al., 2008; Rautio et al., 2008a; Rautio et al., 2008b; Sutera et al., 2017).

Nowadays, the fabrication and design of novel prodrugs are proposed as one of the most promising and significant strategies for both the time-controlled and site-specific drug delivery. Prodrugs are chemically modified derivatives of drugs that will convert to active drug molecules in biological systems. The main goal of utilizing prodrugs in drug delivery is to overcome various pharmaceutical, physicochemical, biopharmaceutical, and/or pharmacokinetic limitations of the free drug, which otherwise would prevent its optimum clinical use (Najjar and Karaman, 2019; Rautio et al., 2018; Zawilska et al., 2013).

It is noteworthy that ester bonds are among the most fundamental and numerous chemical linkages in nature that can be found in bulk polymers, natural products, pharmaceuticals, and many other substances (Ahankar et al., 2018; Fattahi et al., 2019). This chemical bond can be utilized in the conjugation of therapeutic agents to the various promoieties (Fattahi et al., 2020b; Irby et al., 2017).

MTX, a folate analogue, functions as a strong inhibitor of dihydrofolate reductase, a key enzyme involved in tetrahydrofolate synthesis. Thus, it can interfere in the synthesis of RNA, DNA, and consequently proteins (Bertino, 1993; Chen et al., 2007; Fattahi et al., 2020a). MTX is a hydrophilic anticancer drug (MW, 454.5 and log P, −1.8) widely utilized in the treatment of autoimmune diseases and various cancers especially brain cancer. However, some drawbacks limit the use of MTX such as high toxicity, low specificity, short plasma half-life, low solubility, drug resistance by target cells and rapid diffusion throughout the body (Abolmaali et al., 2013). In addition, its brain permeability at conventional doses is weak and should be applied in high-doses (1–8 g/m^2) to achieve the desired amount in the brain (Batchelor et al., 2003).

Considering limitations of MTX and complexities of BBB, various methods have been developed for improving its brain delivery, including osmotic BBB disruption (NEUWELT et al., 1981), cetuximab dendrimer bioconjugates(Wu et al., 2006), transnasal delivery (Shingaki et al., 2010) and intracarotid administration of short-chain alkylglycerols (Erdlenbruch et al., 2003). In continuous of our research on MTX brain delivery (Azadi et al., 2013; Azadi et al., 2015), herein, with the goal of demonstrating the full potential of the prodrug approach in drug delivery, lipophilic MTX prodrugs were synthesized via an esterification reaction and characterized by FT-IR, ^{1}HNMR, ^{13}C NMR and mass spectroscopy. The synthesized prodrugs were examined for their *in vitro* stability in phosphate buffer (pH 7.4), and in the presence of rat serum. Furthermore, their cytotoxic effects against U-87 glioma cells as well as *in vivo* on A. salina were investigated. In addition, *in vivo* capability of prodrugs in MTX brain delivery was examined in the rat model.

2. Materials and methods

2.1. Materials

Methotrexate sodium (MTX) was kindly provided by Loghman Pharmaceutical Co. (Tehran, Iran). Butyl bromide, hexyl bromide (HB), octyl bromide, and cesium carbonate were purchased from Merck Chemical Co. All cell culture media and their supplements, including RPMI cell culture medium, fetal bovine serum, penicillin/streptomycin, phosphate buffer saline (PBS), L-glutamine and non-essential amino acids were obtained from Atocel Co. (Hungry). Cell culture flasks and cell culture plates were obtained from Nest Co. (China). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was obtained from Sigma-Aldrich Co. (Germany). All other solvents, reagents, and chemicals utilized were of analytical grade or chemically pure grade in quality.

2.2. Synthesis of lipophilic MTX prodrugs

MTX was chemically attached to alkyl halide based on the literature reported method with slight modification (Moura et al., 2011). In brief, 0.3 mmol of MTX was dissolved in 5 mL of dimethyl sulfoxide (DMSO). Then, cesium carbonate (0.32 mmol) and alkyl halide (0.73 mmol) were added to the reaction vessel and the progress of the reaction was checked by thin-layer chromatography. After completion of the reaction, 4 mL of water was added in order to quench the reaction. The final product was extracted with (3 × 5 mL) of chloroform and purified by preparative layer chromatography plates (Merck silica gel (F254) powder); methanol–chloroform (10:90, v/v). The chemical structure of conjugates was characterized by the Fourier transform infrared spectroscopy on a Jasco 6300 FTIR spectrometer in the range of 400–4000 cm^{-1} and nuclear magnetic resonance spectroscopy in $CDCl_3$ on a Bruker DRX-250 Avance spectrometer at 250.13 MHz ^{1}H NMR and 62.90 MHz ^{13}C NMR. Mass spectra were recorded on a FINNIGAN-MAT 8430 mass spectrometer operating at an ionization potential of 70 eV. The melting points were measured on an Electrothermal 9100 apparatus and reported without correction.

2.2.1. Dibutyle methotrexate (MTX-DB)

Yellow solid, m.p: 154–157 °C, IR (KBr) 3386, 3179, 2959, 2855, 1736, 1630, 1511, 1441, 1206, 1016, 828 cm^{-1}; ^{1}H NMR (250.13 MHz, $CDCl_3$) δ 0,85 (t, J = 6.5 Hz, 6H), 1.24–1.66 (m, 8H), 2.07–2.15 (m, 1H), 2.25–2.34 (m, 1H), 2.43–2.61 (m, 2H), 3.17 (s, 3H), 4.02 (t, J = 6.00 Hz, 2H), 4.16 (t, J = 6.5 Hz, 2H), 4.72 (s, 2H), 4.77–4.80 (m, 1H), 5.49 (s, 2H), 6.73 (2H, d, J = 8.25 Hz), 6.90 (d, J = 6.5 Hz,1H), 7.70 (d, J = 8.0 Hz, 2H), 8.63 (s, 1H) ppm; ^{13}C NMR (62.90 MHz, $CDCl_3$) δ 13.6, 19.0, 27.5, 30.5, 39.2, 52.2, 55.9, 64.6, 65.5, 111.5, 121.8, 128.8, 147.2, 149.7, 151.5, 162.9, 166.8, 172.5, 173.4 ppm; MS *m/z*: calculated for $C_{28}H_{38}N_8O_5$, 566.30; found 566.50.

2.2.2. Dihexyl methotrexate (MTX-DH)

Yellow solid, m.p: 152–155 °C, IR (KBr) 3328, 3200, 2929, 2857, 1736, 1632, 1509, 1448, 1200, 1098, 827 cm^{-1}; ^{1}H NMR (250.13 MHz, $CDCl_3$) δ 0.86 (t, J = 6.0 Hz, 6H), 1.24–1.27 (m, 12H); 1.54–1.62 (m, 4H), 2.12–2.14 (m, 1H), 2.26–2.28 (m, 1H), 2.31–2.46 (m, 2H), 3.13 (s, 3H), 4.00 (t, J = 6.5 Hz, 2H), 4.13 (t, J = 6.5 Hz, 2H), 4.68 (s, 2H), 4.77–4.79 (m, 1H), 5.73 (bs, 1H), 6.70 (d, J = 5.5 Hz, 2H), 6.96 (bs, 1H), 7.68 (d, J = 5.5 Hz, 2H), 8.59 (s, 1H) ppm; ^{13}C NMR (62.90 MHz, $CDCl_3$) δ 14.0, 22.5, 25.5, 27.4, 28.4, 30.6, 31.3, 39.2, 52.2, 55.8, 64.9, 65.7, 111.4, 121.6, 128.8, 147.1, 151.4, 162.9, 166.8, 172.5, 173.3 ppm; MS *m/z*: calculated for $C_{32}H_{46}N_8O_5$, 622.36; found 622.50.

2.2.3. Dioctyl methotrexate (MTX-DO)

Yellow solid, m.p: 139–141 °C, IR (KBr) 3458, 3318, 2953, 2855, 1736, 1644, 1509, 1448, 1201, 1099, 827 cm^{-1}; ^{1}H NMR (250.13 MHz, $CDCl_3$) δ 0,85 (t, J = 6.5 Hz, 6H), 1.23 (s, 20H); 1.56–1.60 (m, 4H), 2.12 (m, 1H), 2.26 (m, 1H), 2.44–2.47 (m, 2H), 3.13 (s, 3H), 3.98 (t, J = 6.5 Hz, 2H), 4.11 (t, J = 6.5 Hz, 2H), 4.67 (s, 2H), 4.77 (m, 1H), 5.83 (bs, 1H), 6.70 (2H, d, J = 5.5 Hz), 6.98 (bs, 1H), 7.68 (d, J = 5.5 Hz, 2H), 8.57 (s, 1H) ppm; ^{13}C NMR (62.90 MHz, $CDCl_3$) δ 14.0, 22.6, 25.8, 27.5, 28.5, 29.1, 30.6, 31.7, 39.1, 52.2, 55.9, 65.0, 65.8, 111.4, 121.6, 128.9, 151.4, 162.4, 162.9, 166.9, 172.6, 173.3 ppm; MS *m/z*: calculated for $C_{36}H_{54}N_8O_5$, 678.42; found 678.60.

2.3. Stability studies in phosphate buffer solution

The synthesized prodrugs were investigated for their chemical stability in phosphate buffer solution at 37 °C (0.05 M, pH 7.4). The kinetic studies were performed by adding 50 μL of a stock solution of the prodrugs (1.0 mg/mL in DMSO) to 1.95 mL of the buffer solution. After vortexing, the samples were maintained in a 37 °C water bath. At appropriate time intervals, 200 μL samples were withdrawn, filtered (0.22 μm), and then analyzed by HPLC. Experiments were performed in

triplicate, and the pseudo-first-order rate constants were calculated from the slopes of the linear plot of the logarithms of the residual concentrations of compounds against time.

2.4. Stability studies in rat serum solution

The *in vitro* stability of synthesized prodrugs was also performed in physiological medium containing 50% v/v of rat serum. 100 μL of the stock solution of prodrugs in DMSO (1 mg/mL) was added to 1.9 mL of serum solution. After mixing, the samples were kept in a 37 °C water bath. At different time points over a period of 0–120 min, aliquots of 100 μL of each sample were removed and added to 500 μL of cold acetonitrile for deproteinization of serum. Then, the samples were centrifuged (4000*g*, 15 min) and the supernatant (20 μL) was analyzed by HPLC.

2.5. Evaluation of safety

2.5.0.1. Hemolysis assay

The blood samples were received from healthy volunteers and placed in EDTA-containing tubes, then centrifuged during 5 min at 3000 × g. After then, plasma was separated, and erythrocytes were washed with PBS solution (pH 7.4) three times, followed by resuspending them in the PBS, a ratio of 20: 1 PBS and pure erythrocyte was utilized for preparing final hematocrit of 5% (HCT 5%). The erythrocyte solution with the desired HCT value was then placed into a 2 mL microtube in triplicate. A two-fold serial dilution of MTX, MTX-DB, MTX-DO, and MTX-DH were tested, starting at concentration of 1000 μg/mL. The deionized water and PBS were utilized as positive (100% hemolysis) and negative (0% hemolysis) controls, respectively. Then the microtubes with rotator shaker were incubated at 37 ± 1 °C for 4 h, and were centrifuged at 3000 × g for 5 min. Finally, 100 μL of supernatant was taken and introduced to a 96-well microtiter plate and the content of free hemoglobin was analyzed at a wavelength of 540 nm by UV–Vis spectrophotometry. The percent of hemolysis was determined using the following formula:

$$\text{Hemolysis (\%)} = [(A_{sample} - A_{negative})/ (A_{positive} - A_{negative})] \times 100$$

In the above formula, A_{sample} stands for the samples absorbance, and $A_{positive}$ and $A_{negative}$ represent the absorbance of the positive and the negative control samples, respectively.

2.6. In vitro cytotoxicity and anticancer activity

Effects of the lipophilic MTX prodrugs and free MTX on cell viability were investigated on U87 glioblastoma and cancer cells using MTT assay. Firstly, the cells were cultured in RPMI supplemented with 1% penicillin/streptomycin and 10% PBS and incubated at cell culture conditions (5% CO_2, 37 °C). U87 cells were then seeded in 96-well plates at 10^4 cells/well. After 24 h, different concentrations of free MTX, MTX-DB, MTX-DH, and MTX-DO prodrugs (0.625, 1.25, and 2.5 μg/mL) were added to the cells. Cell culture medium was preferably utilized to achieve favorable concentrations, if necessary, DMSO (up to 1% V/V) was added. Cell viability was determined using MTT reagent after 72 h. Briefly, MTT solution (5 mg/mL in PBS, 20 μL) was added into each well and the plates were incubated for next 4 h. After that, the MTT solution was carefully aspirated and DMSO (100 μL) was added to each well for dissolving formazan crystals and eventually the absorbance was determined at 570 nm by plate reader. The reference wavelength was set to 690 nm. The cell viability (percent) was measured as the ratio of the samples to the control group.

2.7. In vivo study

2.7.1. Artemia test

The general toxicity of MTX and synthesized lipophilic MTX prodrugs on A. salina was performed according to our previously reported method (Rajabi et al., 2015). A. salina eggs were kindly gifted by Aquatic Animal Research Center, Urmia University, Urmia, Iran. Briefly, in a bottle containing artificial sea water (35 g/L NaCl solution), dried cysts were collected. After incubation at room temperature during 36–48 h under the direct light and air conditioner, the larvae hatched and the Nauplii are ready for the test. The experiment was conducted on larvae of brine shrimp (A. salina Leach.). A stock solution of 10 mg of MTX and synthesized prodrugs in distilled water (1 mL) was made. Next, various concentrations of MTX and synthesized prodrugs were prepared via serial dilution of the stock samples of synthesized prodrugs and free MTX in artificial sea water instantly before use. Then, 20 μL of test samples were added at various concentrations to each well of the 96-well plates which contained 160 μL RPMI (with a concentration of 35 g/L NaCl). Then, 10 nauplii were transferred into each well of the 96-well plates using Pasteur pipet followed by incubation at 25 °C for 24 h. After then, the numbers of surviving nauplii were counted in each well using a stereoscopic microscope. For each concentration, the test was carried out in triplicate. Also, the negative control wells contained only artificial sea water and 10 nauplii. The percentages of deaths were estimated by counting the number of surviving nauplii in both control and test wells.

The lethality was obtained using Abbott's formula as follows:

$$\text{\% Lethality} = [(\text{Test} - \text{Control})/\text{Control}] \times 100$$

2.7.2. Neuropharmacokinetic study

2.7.2.1. Animals. Male Sprague-Dawley rats weighing from 250 to 300 g were procured from Pasteur Institute, Iran Branch (Karaj, Iran). The rats were placed in individual standard cages (54 × 33 × 20 cm) at 25 ± 2 °C under 12-hours light–dark period and relative humidity of 60 ± 5%. The care of animals was in compliance with the guidelines established by the ethics committee of Zanjan University of Medical Sciences (Zanjan, Iran).

2.7.2.2. Experimental procedure and sample collection. The rats were anesthetized one day before the experiments, by an intraperitoneal injection of xylazine (10 mg/kg) and ketamine (100 mg/kg) mixture, and a polyethylene-silicone rubber cannula was implanted in the right jugular vein of rats according to a standard surgical protocol (Harms and Ojeda, 1974; Waynforth, 1992). Details of the surgery protocol have been previously described (Azadi et al., 2015).

On the day of the experiments, 48 rats were divided randomly into three groups (n = 16). Each group received a dose of MTX (1 mg/mL) from the following formulations: MTX (dissolved in deionized water) was injected intravenously to group 1 (control) via the cannula, while to groups 2 and 3 was injected the equi-dose of the MTX-DH and MTX-DO prodrugs dissolved in absolute ethanol, respectively. During the entire drug administration and sampling, animals remained unrestrained in their cages. At time intervals of 0.25, 0.5, 1, 2 and 4 h followed by the injection, a 1-mI blood sample was taken via the cannula and then animals were immediately anesthetized and decapitated. The whole brain samples were harvested, washed externally with saline and stored frozen until the time of drug assay. Plasma was separated from the rest of the blood components by centrifuging blood samples for 20 min at 2000*g*. Brain and plasma samples were stored frozen at −80 °C until drug analysis by high performance liquid chromatography (HPLC). The protocol of the animal experiment was approved by the Ethics Committee of Zanjan University of Medical Sciences (Zanjan, Iran).

2.7.2.3. Drug assay. The concentrations of MTX in both plasma and brain tissue homogenates were measured by HPLC technique developed in our previous work [21] adopted and modified for this project. The brain samples were weighed and homogenized in normal saline by high-speed homogenizer (Heidolph, Silent Crusher M, Schwabach, Germany). To 225 μL of brain homogenates or plasma samples, trichloroacetic acid

(40 μL, 2 N in ethanol) and *para*-amino acetophenone (25 μL, 2 μg/mL aqueous solution; internal standard) were added; the mixture was then shaken during 30 min and centrifuged (12,000*g*, 15 min). Finally, the clear supernatant (50 μL) was injected to HPLC system which was composed of a Rheodyne injector (Rheodyne, Model 7725, USA) equipped with a 20 l loop and a pump-controller unit (Knauer, Wellchrom®, model k-1001, Berlin, Germany). A C18 column (Eurospher 100–5 C18, 150 mm × 4.6 mm, Knauer, Germany) as the stationary phase and a mixture of 0.01 M phosphate buffer (pH 4.0) and acetonitrile (90:10, v/v) as the mobile phase were used with the flow rate of 1.0 mL/min and a detection wavelength of 307 nm (UV-detector; Knauer, model k-2600, Berlin, Germany). The limits of detection (LOD) and quantitation (LOQ) of the proposed method for plasma analysis were 18 and 50 ng/ml, and for brain analysis were 10 and 25 ng/ml, respectively.

2.8. Statistical analysis

All data were presented as mean ± standard deviation. Data were analyzed by Kruskal–Wallis and Mann–Whitne U tests. Outcomes were considered statistically significant at P value ≤ 0.05.

3. Results and discussion

3.1. Synthesis and characterization of lipophilic MTX prodrugs

The lipophilic MTX prodrugs were prepared by esterification of the α- and γ-carbonyl moieties of the glutamic acid of MTX with alkyl halides in the presence of cesium carbonate (Fig. 1). The isolated yields were found to be over 97%. The chemical structure of obtained prodrugs was characterized and confirmed by ^{1}H NMR and ^{13}C NMR. For example, the ^{1}H NMR and ^{13}C NMR spectra of MTX-DH are shown in Figs. 2 and 3, respectively. As clearly seen, the characteristic signals of MTX and HB could be clearly observed in MTX-DH prodrug. As indicated in Fig. 2a, the typical ^{1}H NMR spectrum of HB gives a triplet signal for methyl protons at 0.86 ppm ($J = 6.00$ Hz) and signals for the methylene groups at the range of 1.25–3.34 ppm. MTX gives two doublet signals at δ 7.61 ($J = 8.00$ Hz) and 6.81 ($J = 8.75$ Hz) ppm for protons on benzene ring and the singlet signal at 8.55 ppm, which is assigned to the proton on the pyrimidine ring (Fig. 2b). On the other hand, Fig. 2c indicates the ^{1}H NMR of MTX-DH prodrug. The presence of characteristic signals of both HB and MTX confirms the synthesis of prodrug. In addition, from the ^{13}C NMR spectra in Fig. 3, the specific signals of MTX and HB could all be found in MTX-DH prodrug.

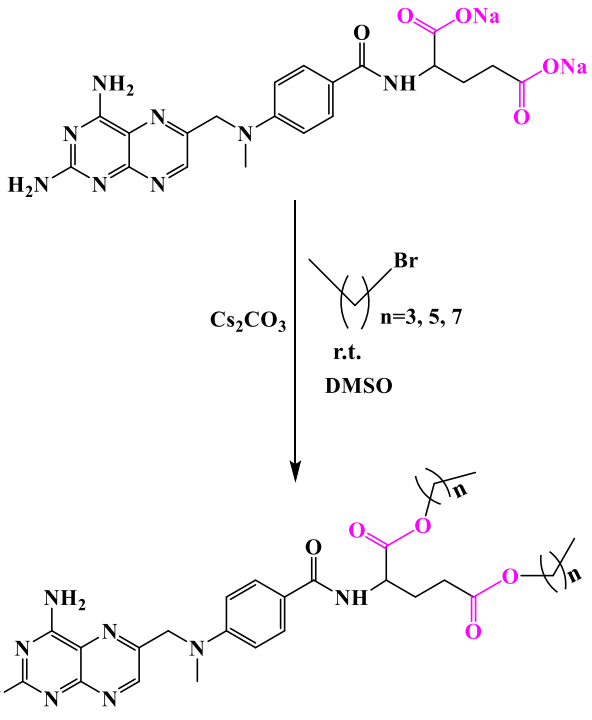

Fig. 1. Schematic representation of chemical synthesis of lipophilic MTX prodrugs.

3.2. Lipophilicity study

Lipophilicity is a fundamental physicochemical parameter of bioactive compounds, related to the ability of molecule to be transported through biological membranes. Lipophilicity parameters like LogP and CLogP of synthesized prodrugs as well as free MTX were calculated using ChemBiodraw ultra 14.0 software and the values are presented in Table 1. It was found that all synthesized compounds having values of LogP and CLogP are in acceptable range and appeared to be suitably lipophilic to cross the BBB.

3.3. Stability studies

The chemical stability of synthesized prodrugs was investigated in phosphate buffer solution (0.05 M, pH 7.4) at 37 °C; the physiological stability was also carried out using a dilute rat serum solution (50% *v/v*) at 37 °C. The half-lives were computed by the disappearance of the starting compounds and are presented in Table 1. It was found that prodrugs were stable enough in pH 7.4 phosphate buffer solution. But the stability in serum was decreased, most likely owing to the presence of enzymes in serum which are active in hydrolyzing ester prodrugs. The obtained results also indicated that increasing the molecular weight of the alkyl chain, decreased the hydrolysis of the prodrug. However, it needs further investigation to find out the pattern of enzymatic degradation for the synthesized prodrugs.

3.4. Hemolysis assay

In order to utilize diverse drugs and drug conjugates in biomedicine, it is necessary to evaluate its hemocompatibility. Hemolytic toxicity is based on the disruption of erythrocytes' membrane which resulted in release of hemoglobin to surrounded media. To get a clue of the hemolytic toxicity of the drugs and drug conjugates that are directly administrated and exposure to the human blood, hemolysis test is of significant importance. The MTX and lipophilic MTX prodrugs were well tolerated as suggested by the toxicity studies done. The major limitation of drugs and drug conjugates for using in biomedicine is its hemolytic toxicity. Therefore, hemolytic toxicity was carried out in terms of percent RBC hemolysis to monitor toxicity of the free drug and its conjugates. As presented in Fig. 4, both free MTX and lipophilic MTX prodrugs exhibited does-dependent hemolysis. Moreover, the lipophilic MTX prodrugs exhibited relatively less hemolytic toxicity in comparison with free MTX at same concentrations. Indeed, these results indicated that the lipophilic MTX prodrugs have an ability to partially reduce the toxic profile of free MTX, at the same concentration. Since the hemolysis value of lipophilic MTX prodrugs were close to that of free MTX at low concentrations (<250 μg/mL), the hemolytic toxicity profiles can be considered similar to that of free MTX, at the same time conjugate has also not made it more toxic.

3.5. Cell viability assay

The obtained data from MTT cell viability assays on U87 cells is presented in Fig. 5. Results were reported compared to the control group. The IC50 values of MTX-DB, MTX-DH, MTX-DO, and free MTX were 0.609, 0.254, 1.51 and 1.7 μg/mL, respectively. As observable, in comparison with free MTX, all of the synthesized prodrugs led to

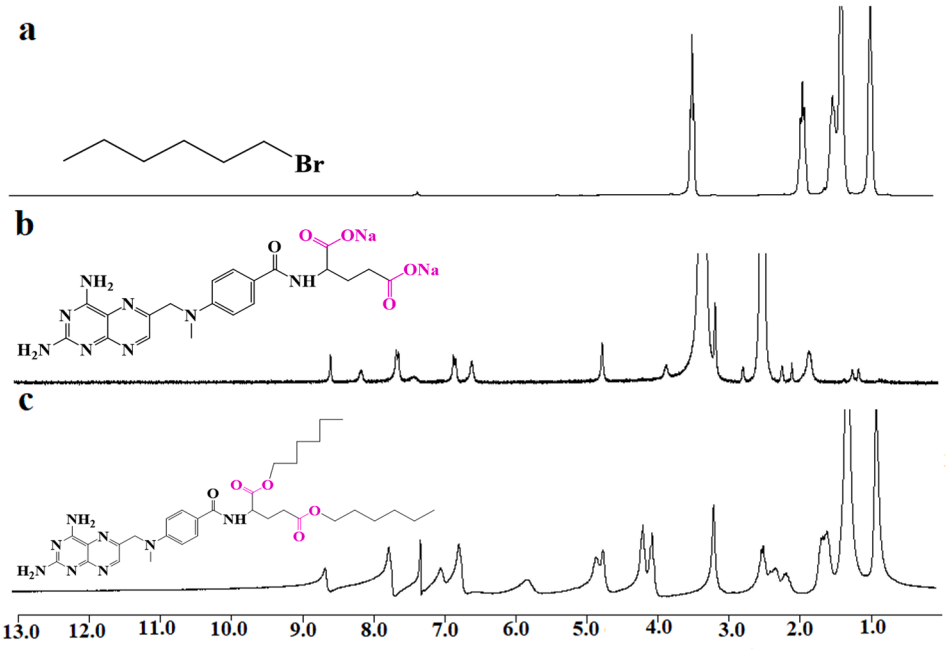

Fig. 2. ^{1}H NMR (250.13 MHz) spectra of (a) HB in $CDCl_3$, (b) MTX sodium in DMSO and (c) MTX-DH prodrug in $CDCl_3$.

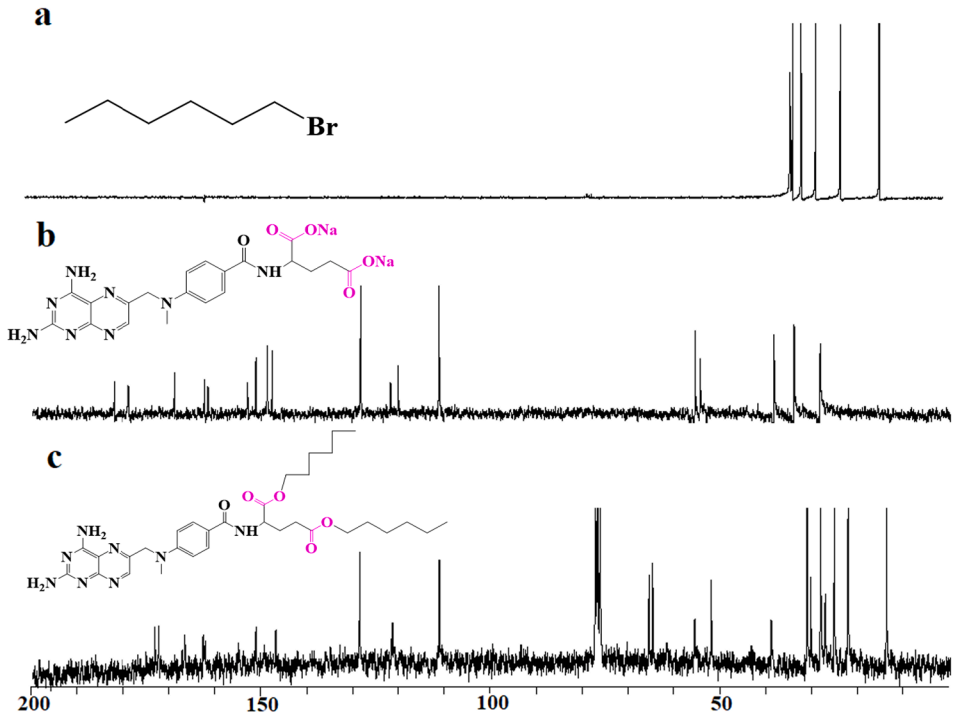

Fig. 3. ^{13}C NMR (62.90 MHz) spectra of (a) HB in $CDCl_3$, (b) MTX sodium in DMSO and (c) MTX-DH prodrug in $CDCl_3$.

Table 1
LogP and ClogP values and stability studies in phosphate buffer solution and in Diluted Rat Serum.

Compound	LogP	ClogP	$t_{1/2}$(h) PBS	$t_{1/2}$(min) Diluted Rat Serum
MTX-DB	3.95	3.23	41 ± 0.11	46 ± 0.11
MTX-DH	5.62	5.34	64 ± 0.21	59 ± 0.21
MTX-DO	7.71	7.46	79 ± 0.18	67 ± 0.13
MTX	0.94	−0.52	–	–

LogP and ClogP calculated by using ChemBioDraw Ultra version 14.0.

decrease the IC50 in 72 h on U87 cancer cell line and the best result was observed for MTX-DH prodrug which led to decrease the IC50 amount up to 6 folds.

3.6. *Artemia assay*

Since the A. salina is characterized by ease of handling, low cost, high sensitivity, and the fact that it can be observed with the naked eye and without requiring additional or advanced equipment, the use of this microcrustacean as an animal model for the evaluation of acute toxicity

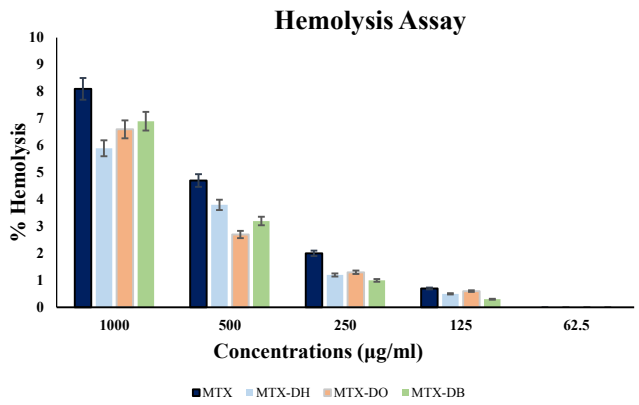

Fig. 4. *In vitro* hemolysis of synthesized prodrugs and free MTX at different concentrations.

has been unexpectedly increased. So, in this study, inexpensive and reliable acute toxicity of lipophilic MTX prodrugs and free MTX were carried out against A. salina. The percentage of lethality can be utilized as a bioassay indicator for the acute toxicity of MTX and lipophilic MTX prodrugs. The acute toxicity was evaluated by measuring the number of dead *nauplii* after 24 h exposure to MTX and lipophilic MTX prodrugs and the mortality rates of A. salina nauplii are shown in Fig. 6. As indicated, at high concentrations of MTX and lipophilic MTX prodrugs values from 1000 to 250 µg/mL, the concentration-dependent toxicity was observed, whereas no mortality was observed at lower concentration) < 250 µg/mL) for both MTX and lipophilic MTX prodrugs. In addition, there was no significant difference of mortality was observed among the lipophilic MTX prodrugs. Also the percentage of dead nauplii reduced from 13.3% at 1000 µg/mL of MTX to a little more than 3% at 250 µg/mL and the percentage of dead nauplii for lipophilic MTX prodrugs reduced from 8% at 1000 µg/mL to a little more than 0.9% at 250 µg/mL. Indeed lipophilic MTX prodrugs in all cases exhibited less toxicity in comparison with free MTX, but the values were close to that of free MTX. These results suggest that the lipophilic MTX conjugate has been able to partially mask the toxic profile of free MTX, at the same concentration. These results were also repeated in the hemolysis assay, which confirms that the conjugates has not made the drug more toxic.

3.7. Neuropharmacokinetic study

In order to obtain the simultaneous drug concentrations in brain and plasma and the corresponding Kp values, a cross-sectional pharmacokinetic study was performed by drug administration to rats via the catheter inserted intravenously followed by brain and plasma sampling. The pharmacokinetic approach used was such that a total of 4 rats were utilized to collect the data for each time point since the need for the decapitation of animals does not allow any of the animals to survive for the further time points. Fig. 7a and b show the plasma and brain concentrations of MTX at various time points following intravenous (IV) injection of MTX-DH, MTX-DO, as well as the equi-dose free MTX. As presented in Fig. 7a, the MTX plasma concentration at 15 min was significantly different between the free drug and the synthesized prodrugs. Also, during all test times, the MTX plasma concentration in both status, MTX-DH and MTX-DO, is less than the free drug. This is probably due to the different tissue distribution of the precursors due to their higher lipophilicity.

The concentration of MTX in the free drug mode at 15, 30, and 60 min is higher than in both MTX-DH and MTX-DO modes. Also, during 120 min, the brain concentration of MTX in both MTX-DH and MTX-DO modes is significantly higher than in the free drug, indicating that despite the low plasma concentrations of MTX in both MTX-DH and MTX-DO cases, the brain concentration has been increased. It shows that MTX-DH and MTX-DO prodrugs increase the ability of MTX to pass through BBB due to their lipophilic properties. During 240 min, medullar concentration of MTX was higher in both MTX-DH and MTX-DO modes than the free drug. At this point, there is no significant difference in brain concentration in MTX-DH and MTX-DO prodrugs (Fig. 7b).

In addition, by considering the Kp values for three groups, it is clear that MTX in the free drug state has a lower shelf life in brain than plasma, which is probably due to the limited entry into the brain owing

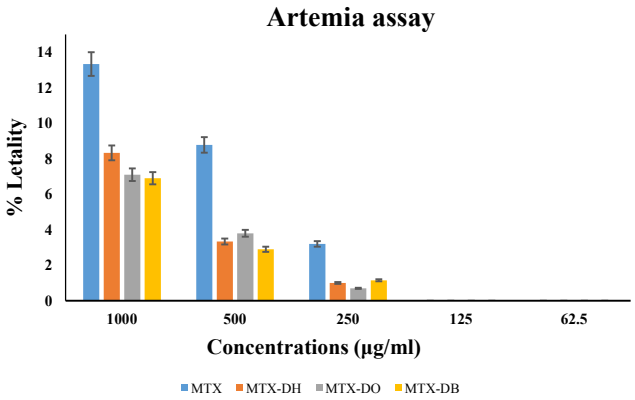

Fig. 6. General toxicity of various formulations to A.salina in sea water.

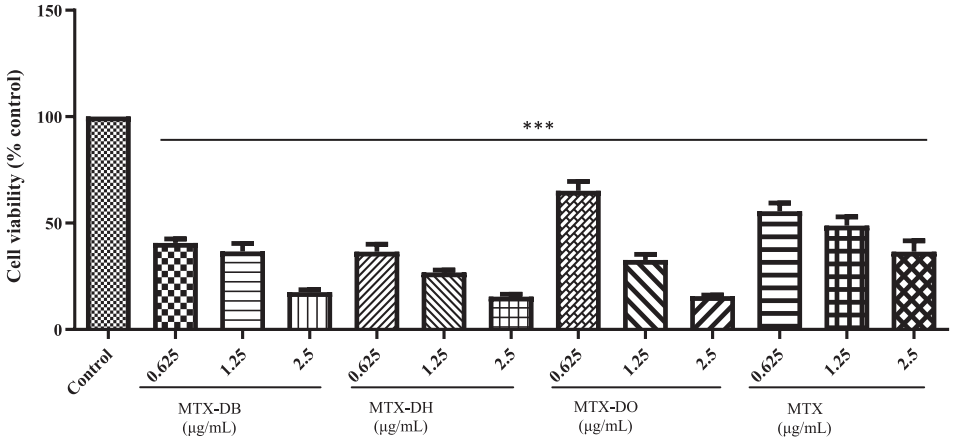

Fig. 5. Effect of lipophilic MTX prodrugs on viability of U87 cells which were exposed with appropriate concentrations of the various formulations and incubated for 72 h at cell culture conditions.

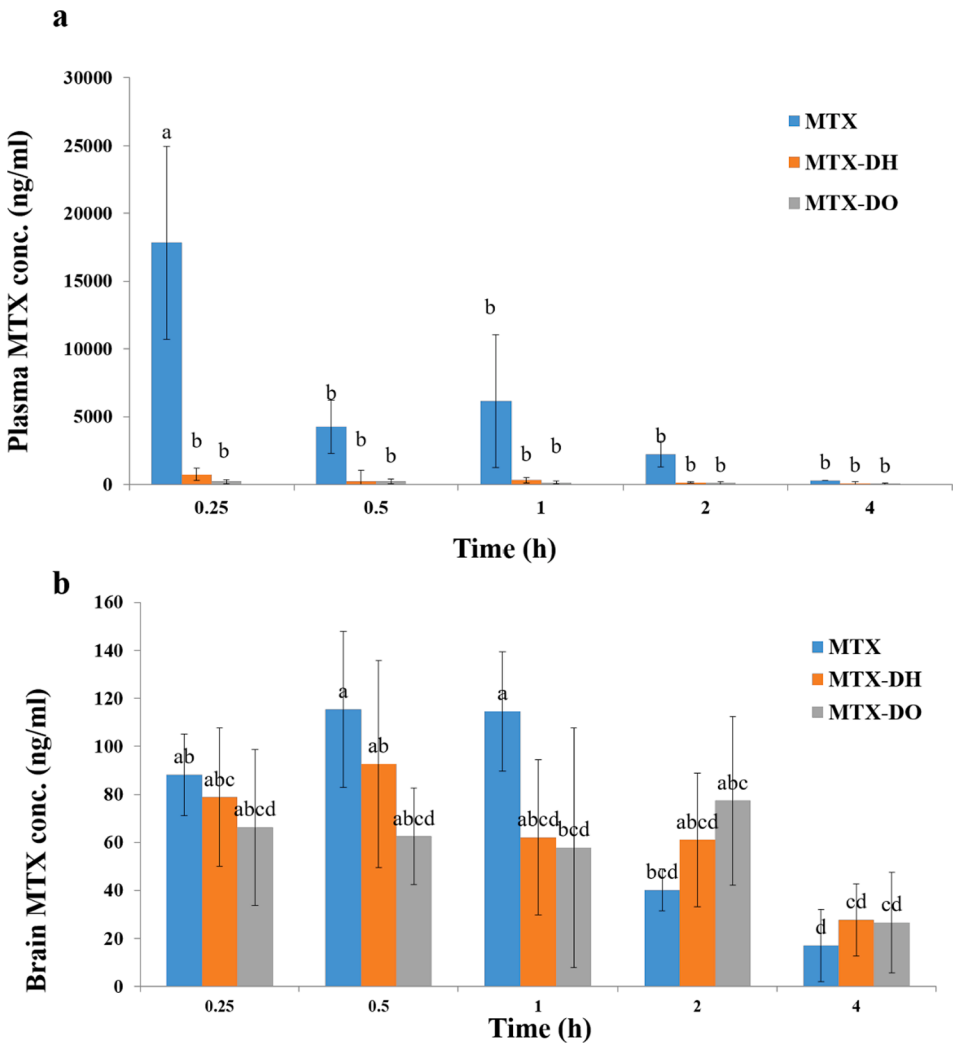

Fig. 7. (a) Plasma and (b) brain concentration-time profiles of MTX after 1 mg IV administration of free MTX and equi-dose of the MTX-DH and MTX-DO prodrugs in male Sprague-Dawley rats.

to its hydrophilicity. In addition, higher Kp is observed in both MTX-DH and MTX-DO compared to the free drug (Fig. 8).

Integration plot analysis was carried out for the calculation of brain uptake clearance of free MTX and synthesized formulations. The theoretical basis for this analysis was illustrated in previous reports (Azadi et al., 2015; Hamidi, 2006; Karami et al., 2019; Nasiri et al., 2019).

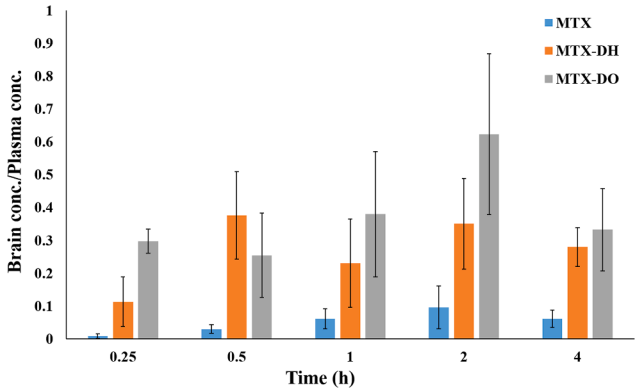

Fig. 8. Kp (Brain-to-plasma concentration–time) profile of MTX after 1 mg IV administration of free MTX and equi-dose of the MTX-DH and MTX-DO prodrugs in male Sprague-Dawley rats.

Briefly, Kp was plotted against AUC/Cp (the ratio of the AUC in the plasma to the corresponding plasma concentrations) from zero to a given time. The slope of the plot, which shows the apparent brain uptake clearance, was computed (Fig. 9 and Table S1 in Supporting Information).

Brain uptake clearance shows clearly the efficiency of MTX-DH and MTX-DO in increasing MTX levels in the brain. In this case, MTX-DH and MTX-DO have reached 3.85 and 9.08 times the clearance value of brain uptake in comparison with the free drug. These data besides the plasma AUC of the free drug, which is probably 16.76 and 28.84 times higher than the MTX-DH and MTX-DO, respectively, which is due to the different tissue distribution of the prodrugs, can confirm the scenario of the MTX drug delivery system to the brain. Considering the lipophilic properties of MTX-DH and MTX-DO, the role of ester-prodrugs in penetration through BBB could be the main reason for the increasing the level of MTX.

4. Conclusion

In summary, the lipophilic MTX prodrugs containing the ester functional moiety were synthesized and characterized by FT-IR and NMR analyses. *In vitro* cytotoxic studies showed that all of the synthesized prodrugs led to decrease in the IC50 in 72 h on U87 cancer cell line. In addition, *in vivo* toxicity on A. salina indicated that the lipophilic MTX prodrugs have been able to partially mask the toxic profile of free MTX,

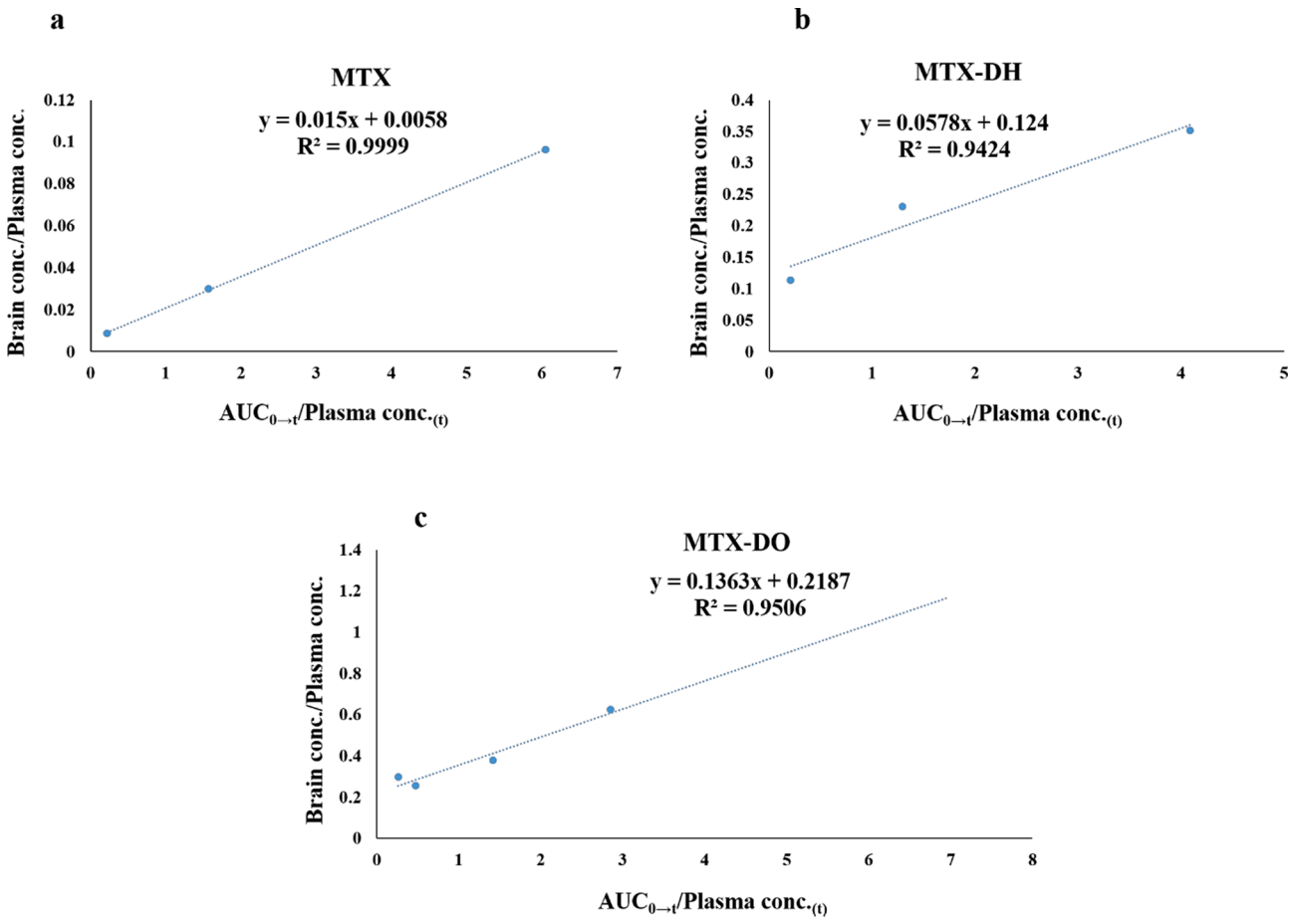

Fig. 9. Brain uptake plot of MTX after 1 mg IV administration of (a) free MTX and equi-dose of the (b) MTX-DH and (c) MTX-DO prodrugs; in male Sprague-Dawley rats.

at the same concentrations. These findings were also in compliance with hemolysis assay results, which confirm that the conjugates has not made the drug more toxic. Meanwhile, results from *in vivo* study on rats showed that the Kp values of MTX-DH and MTX-DO groups were significantly higher compared with free MTX. Moreover, the uptake clearance of MTX by brain parenchyma increased significantly (3.85 and 9.08-time increased for MTX-DH and MTX-DO prodrugs, respectively). Taken together, our results indicate that the synthesized lipophilic MTX prodrugs are non-toxic and able to enhance brain penetration of MTX.

CRediT authorship contribution statement

Nadia Fattahi: Methodology, Investigation, Visualization, Writing - original draft. **Ali Ramazani:** Project administration, Writing - review & editing. **Mehrdad Hamidi:** Conceptualization, Project administration, Writing - review & editing. **Maliheh Parsa:** Formal analysis, Methodology, Software. **Kobra Rostamizadeh:** Methodology. **Hamid Rashidzadeh:** Validation, Methodology.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpharm.2021.120479.

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