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RESEARCH PAPER

A Novel Nanoformulation for Reducing the Toxicity and Increasing the Efficacy of Betulinic Acid Using Anionic Linear Globular Dendrimer

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ABSTRACT

Betulinic acid (BA) is an antileishmanial herbal drug with low solubility and high toxicity. To our knowledge, this is the first study in which betulinic acid is loaded into Anionic Linear Globular Dendrimer (ALGD) in order to resolve the toxicity and insolubility problem. In order to solve mentioned problems, BA was loaded into ALGD nanocarrier. The formulation was characterized in terms of chemical bonds and morphology using Fourier Transform Infrared (FTIR) Spectroscopy, Atomic Force Microscopy (AFM) and Proton Nuclear Magnetic Resonance (^{1}HNMR) methods. According to our study insoluble BA could loaded well into ALGD. This loading caused an increase in the solubility rate of BA by more than 700-fold and a decrease in the toxicity effects to zero in vivo environment. Overall, BD at a dose of 40 mg/kg caused a significant decreased in the number of parasite (leishmania major (L. major)) in vitro and in vivo without inducing any toxic effect.

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INTRODUCTION

Leishmaniasis is a parasitic disease which is transmitted to human through sandfly bite. The main problem for treatment of this disease is the limited number of therapeutics and their limited clinical application, thus development of novel therapeutics seems to be required [1]. The antileishmanial effects of betulinic acid (BA) have been shown in various studies [2-4].

BA (Fig. 1) is a pentacyclic triterpenoid compound derived from various natural plants such as bark of white birch (Betula species) [5]. It exists in different plant organs in form of aglycon and glycosyl derivatives [6]. Various pharmacological effects have been reported for this triterpenoid including anticancer, hepatoprotective, antimalarial, anti-inflammatory, anthelmintic, antileishmanial, and antioxidant effects [7]. However, it suffers from poor biopharmaceutics owing to low solubility and permeability, resulting

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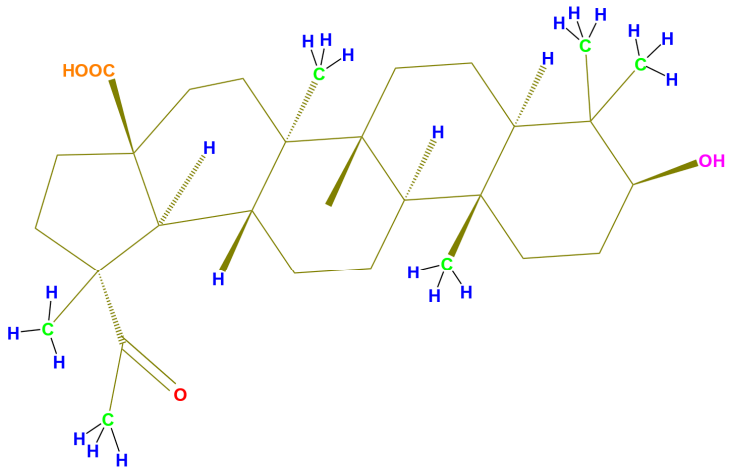

Fig 1. Chemical structure of betulinic acid

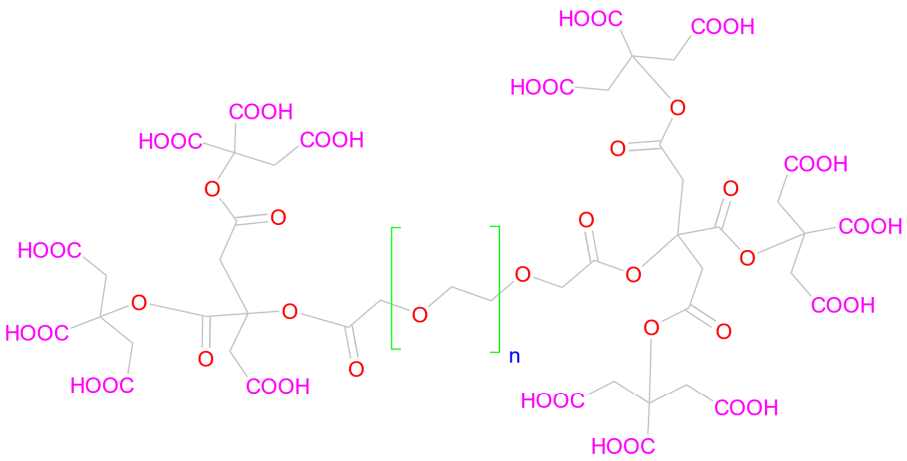

Fig 2. Chemical structure of ALGD

in its low dosing for treatment [3].

There are various approaches to resolve the problem of low water solubility of therapeutics, such as using nanocarriers. In this regard, dendrimer nanoparticles have shown promising results [8]. Development of nanosized delivery systems causes fundamental changes in the field of pharmaceutical technology by improving the therapeutic effects and bioavailability of most drugs. In this context, nanoparticles have received increasing attention as drug carrier. They can cause an increase in the therapeutic effects and simultaneously cause a decrease in the side effects of drugs [9]. ALGD is a negatively charged nanoparticle which can be used as a powerful vehicle for drug delivery due to water solubility, biodegradability, biocompatibility, and low polydisprsity index. The negative charge of this molecule reduces the drug repulsion and cell surface junction leading to decreased intraction rate, enhanced physiological activity of cells to toxic material, and increased viability of cells [10].

To the best of our knowledge, no studies have been conducted to load BA into ALGD (Fig. 2) nanocarriers in order to decrease its toxicity and increase the solubility. Thus in the present study, we are going to use ALGD as nanocarrier to improve the solubility rate and toxicity of BA in order to provide an effective therapeutic dose. The formulation is being tested for the first time for the treatment of *leishmania major* (L. major) infected Balb/c mice.

MATERIALS AND METHODS

Materials

BA was purchased from Baoji Guokang Bio-Technology Co. Ltd (China). Chloroform, polyethylene glycol 600 (PEG600), N,N'-Dicyclohexylcarbodiimide (DCC) , MTT and acetic acid were purchased from Merck Company (Germany). The RPMI1640 medium, fetal bovine serum (FBS), and penicillin–streptomycin antibiotics were bought from Gibco Inc. (USA). Schneider culture medium, dimethyl sulfoxide (DMSO), dialysis membrane (cut off 6 KDa) and Polyethyleneglycol (PEG) were provided from Sigma Aldrich Company (USA). Iranian strain of L. Major (MRHO/IR/75/ER) was purchased from Pasteur Institute of Iran. All other materials used were of analytical grade. The double distilled water was used throughout the study.

Synthesis and drug loading into anionic linear globular dendrimer

ALGD was synthesized according to the method described by Zadeh Mehrizi *et al.* [11] using PEG, DMSO, DCC and citric acid. BA was loaded into dendrimer using adsorption procedure according to a literature method [11]. In addition, drug loading efficiency was measured using standard curve according to the method described in the literature [12] using the following formula:

$$Drug\ loading\ efficiency\ (\%) = \frac{ED}{TD} \times 100$$

In this formula, ED is equivalent to the primary drug used (mg) subtracted to the drug in supernatant (mg) and TD is the primary drug used.

Thin-layer chromatography (TLC)

The purity of BA and BD was evaluated using thin-layer chromatography (TLC). For this purpose, 2 µL of the samples were poured on a silica plate and left to dry at room temperature. Next, the plate was incubated at 45 ℃ in a chamber containing 10% methanol in chloroform for 45 min. Then, the plate was picked up, dried and analyzed to identify the components by means of iodine vapors to visualize the spots.

Determining the size, size distribution and zeta potential

Size, size distribution, and zeta potential of nanoparticles were determined using dynamic light scattering (DLS) technique according to a literature method [11]. In brief, the absorbance of nanoparticles suspension was read at 620 nm and then the suspension was introduced into the Zetasizer instrument (ZEN 3600, Malvern Instruments Ltd., Worcestershire, UK).

Morphology evaluation of Betulinic acid loaded dendrimer nanoparticles using Atomic Force Microscopy

BD was morphologically evaluated by Atomic Force Microscopy based on previously explained method [11]. For this purpose, a drop of the nanoparticle suspension was poured on a mica surface and left at room temperature to dry. Then Atomic Force Microscopy (AFM) imaging (NanoWizard II AFM, JPK Instruments, Berlin, Germany) was performed using a tapping-mode in air at room temperature on a silicon-nitride cantilever with a spring constant of Nm^{-1}. The obtained images were analyzed by a JPK information processing software (Germany).

Betulinic acid loaded dendrimer nanoparticles evaluation by Fourier Transform Infrared Spectroscopy

Fourier Transform Infrared (FTIR) Spectroscopy was used to investigate the BD composition and determine the type of drug loading according to the previously described method [12]. Briefly, the suspension of drug-loaded nanoparticles was centrifuged (13000 r.p.m, 4 ℃, 30 min) and the pellet was dried at room temperature and mixed with potassium bromide at room temperature. The mixture was pressed to form a tablet and examined by the FTIR (Nicolet 740SX) device.

Betulinic acid loaded dendrimer nanoparticles evaluation using H-Nuclear Magnetic Resonance

BD was investigated in terms of the structure, molecular properties and chemical bonds using Proton Nuclear Magnetic Resonance (^{1}HNMR) method, according to the procedure published elsewhere [11]. For this purpose, one mg of the powder form of BD nanoformulation was dissolved in DMSO deuterium and ^{1}HNMR spectroscopy was performed in FT pulse mode at a resonance frequency of 300 MHz.

Cellular uptake and kinetic of drug release from betulinic acid loaded dendrimer nanoparticles

Cellular uptake of BD was determined using BD at the dose of 20 µg/mL according to the method

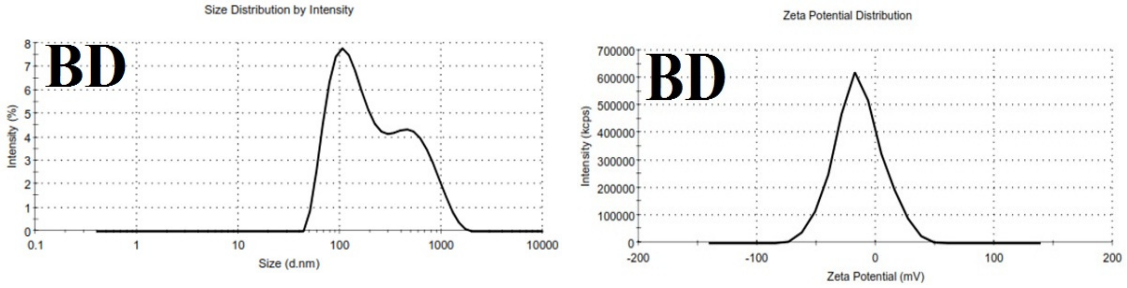

Fig 3. Diagrams of size, size distribution (left) and zeta potential (right) of BD formulation, obtained by Zetasizer instrument.

defined in another study [11] using peritoneal macrophages obtained from the Balb/c mice. Pattern of BA release from ALGD nanoparticles was determined using dialysis membrane method according to a literature method [12].

In vitro viability and toxicity effects of the formulations

Peritoneal macrophages were obtained from Balb/c mice (from peritoneal lavage using cold RPMI-1640 medium) and *in vitro* viability effects of the formulations were evaluated according to the previous study [12]. Briefly, the peritoneal macrophages were cultured in 96-well plate (10^4 cells/well) containing 90% RPMI-1640 culture medium, 10% FBS and 1% Penicillin/Streptomycin. After 24 hours of incubation in a 5% CO2 incubator at 37 ℃, the culture medium was replaced with medium containing various concentrations of dendrimer, BA and BD formulations. The plate was then transferred to the incubator and after 48 hours, the culture medium containing different formulation was replaced with MTT solution (0.5 mg/mL in complete RPMI-1640 medium) and incubated for three hours in the incubator. Next, to dissolve the prepared formazan crystals, the MTT solution was replaced with 100 µl of acidic isopropanol and incubated for 15 minutes. After 15 minutes, the absorbance was read at 570 nm using microplate scanning spectrophotometer (ELISA reader; Organon Teknika, Boxtel, the Netherlands) and then the viability was measured by the formula below. The cells treated only with medium were considered as control.

$$Viability = \frac{Absorbance\ of\ the\ cells\ treated\ with\ drug}{Absorbance\ of\ control} \times 100$$

In vivo toxicity of the formulations

Toxicity effects of the formulations were determined *in vivo* environment using female Balb/c mice according to previously described method in terms of mortality rate and enzymatic toxicity [11]. For enzymatic toxicity evaluation, the blood samples of the mice were obtained and serums were prepared. Next, serum concentrations of alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), blood urea nitrogen (BUN) and creatinine were measured.

Statistical analysis

After data evaluation and confirming their normal distribution, parametric statistics including two-way analysis of variance (ANOVA) and Tukey's tests were used under the supervision of a statistician. Graphs were plotted using Graphpad Prism software version 6.

RESULTS AND DISCUSSION

Nanoparticles characterization

The drug loading efficiency was measured using the standard curve of BA and spectrophotometer at 254 nm, which was equal to 96%. Also, the results of DLS method demonstrated that monodispersed nanoparticles with the negative zeta potential were synthesized (Fig. 3, 156±15 nm, size distribution of 0.31±0.14 and zeta potential of -14.6±1.7 mV).

TLC

There are various methods for evaluating the purity of compounds such as TLC method [13]. TLC is the most effective method for the low-cost analysis of those samples that require minimal sample clean-up or sample preparation steps [14]. In the present study, the R_f values for BA and BD were 0.84 and 0.49, respectively, indicating the purity of the samples.

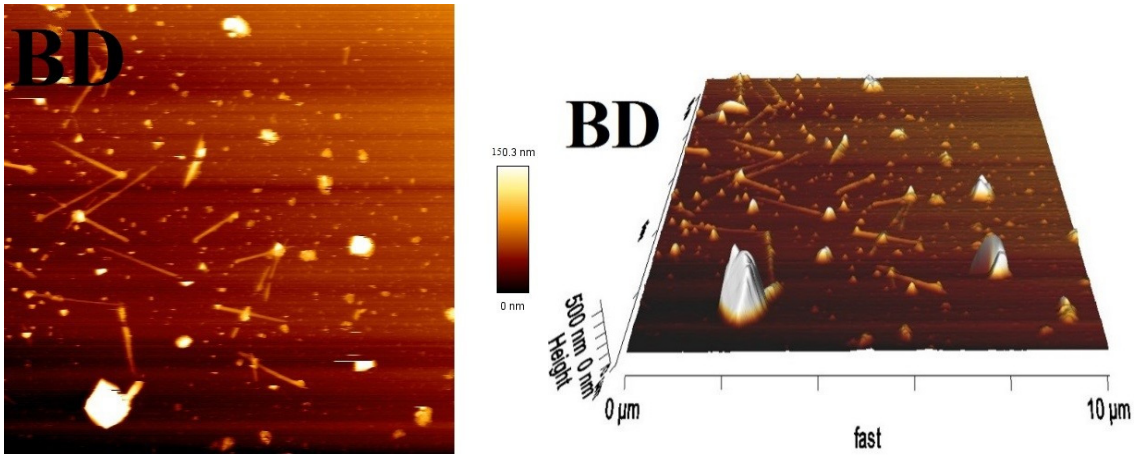

Fig 4. AFM micrographs of BD nanoformulation in 2 (left) and 3 (right) dimensions.

Table 1. FTIR data of BD formulation

		Functional group	Frequency (cm^{-1})
Formulations	BA	CH and OH	2900-3500
	BA	C=O	1700
	D	C=O	1600
	D	OH	3400
	BD	OH of BA	3400
	BD	C=O of BA	1700

Morphology evaluation of betulinic acid loaded dendrimer nanoparticles using Atomic Force Microscopy

Results of AFM Microscopy indicated that BD nanoparticles were formed as swollen particles owing to BA loading into nanoparticles, resulting in a novel topology (Fig. 4). Also, results demonstrated that dendrimer nanoparticles were formed as spherical particles (Fig. S1, supplemental file). Both BD and dendrimer nanoparticles were formed as smooth shapes which can cause a decrease in the tissue irritation compared to the crystalline and irregular particles [15]. Overall, AFM results depicted that BA was loaded well into nanoparticles.

Betulinic acid loaded dendrimer nanoparticles evaluation by Fourier Transform Infrared Spectroscopy

FTIR results of BD showed the presence of common spectra related to BA (regions of 1451, 1043 and 855 cm^{-1}) and ALGD (regions of 3380, 2920, 1250 cm^{-1}) into formulation and confirmed the loading of BA into ALGD nanoparticles (Table 1, Fig. 5). The band at 1451 is assigned to symmetrical deformation of -CH2 of the cyclopentane functional group of BA, the peak at 1043 cm^{-1} is related to the C–O stretching of alcohol group of BA and the peak at 855 cm^{-1} is attributed to the $R_2C{=}CH_2$ functional group of BA. Overall, results illustrated the decent loading of BA into nanoparticles.

Betulinic acid loaded dendrimer nanoparticles evaluation using H-Nuclear Magnetic Resonance

ALGD nanoparticles were synthesized using divergent method and BA was loaded into the nanoparticles. The particles were evaluated using ^{1}HNMR method. Results demonstrated that BD showed the peaks related to BA and ALGD in which the peaks of carboxylic acid groups appeared at 12 ppm and double bonds at 4 and 5 ppm. In the obtained result, the peak of 2.5 ppm was related to the functional group of citrate, while the peak of 3.5 was corresponded to the PEG molecule (Table 2, Fig. 6). Overall, results of ^{1}HNMR indicated that BA was loaded well into the nanoparticles.

Cellular uptake and kinetic of drug release from betulinic acid loaded dendrimer nanoparticles

Results of cellular uptake measurement using flow cytometry demonstrated that BD had proper cellular uptake equal to 94.6% (Fig. 7) [16]. BA

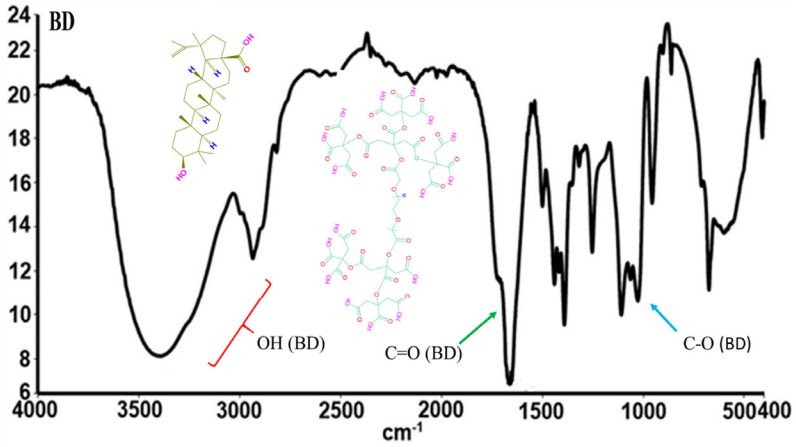

Fig 5. FTIR spectrum of BD, confirming physically BA loading into dendrimer nanocarrier.

Table 2. ^{1}H NMR data of BD formulation

Functional groups		CH2, CH3 (parts per million; ppm)	OH (ppm)	CH=CH (ppm)	CH-O (ppm)
Formulations	BA	0.7-1.2	4.1	4.3	-
	ALGD	2-2.25	-	-	-
	BD	-	-	-	3.5

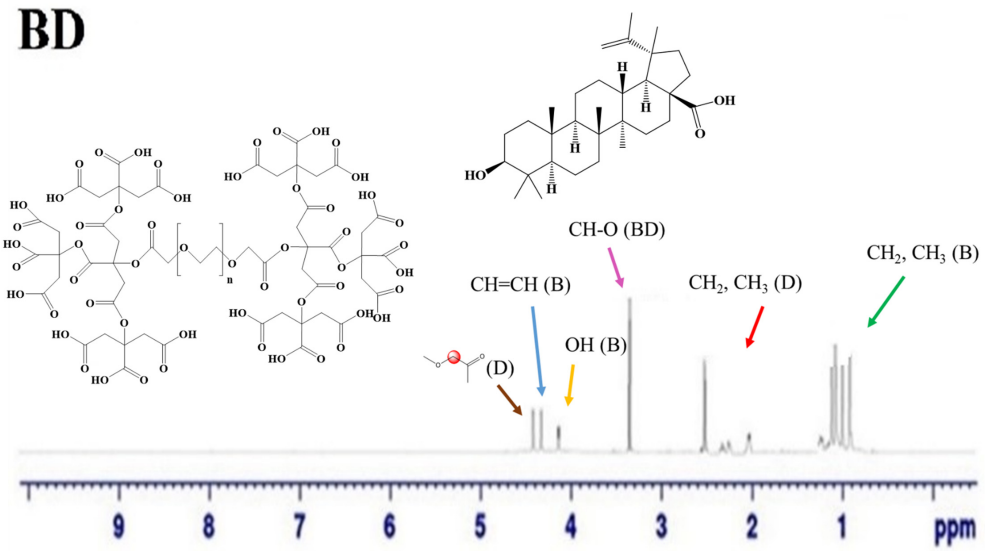

Fig 6. ^{1}HNMR spectrum of betulinic acid dendrimer (BD), indicating that BA was loaded into dendrimer nanocarrier.

was released from BD formulation in a slow and sustained manner over a period of 48 hours in which 90% of the loaded drug was released in this period of time, confirming that BD released BA in a controlled manner (Fig. 8) [16]. These results indicated that BD nanoparticles could be utilized for BA release in a prolonged and controlled manner, resulting in a reduction in the fluctuations of the BA plasma concentrations and improvement of BA therapeutic effects [17].

In vitro viability and toxicity effects of the formulations

The viability effects of BD and BA formulations

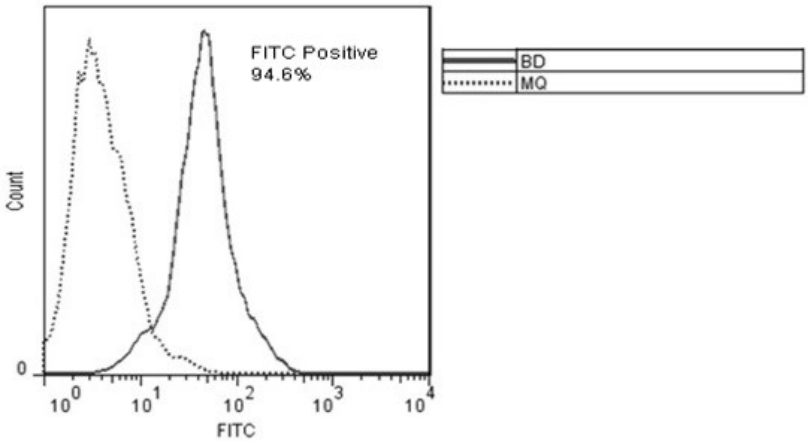

Fig 7. Cellular uptake of betulinic acid dendrimer (BD) into macrophages, measured by flow cytometry mehtod which was equal to 95%.

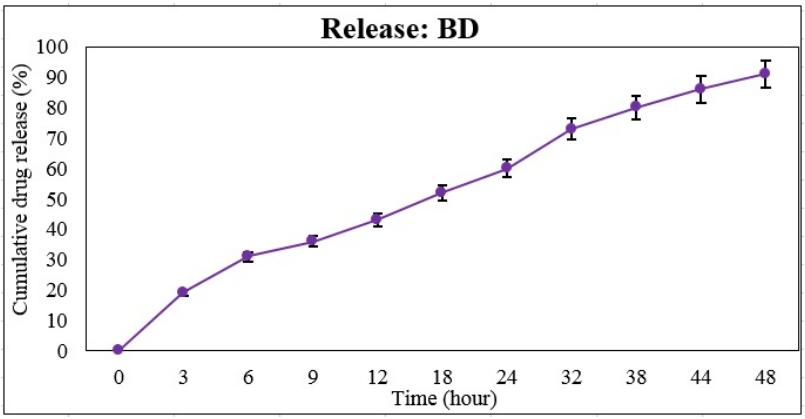

Fig 8. Profile of BA release from BD formulation, indicating that BA was released from the carrier in a controlled manner.

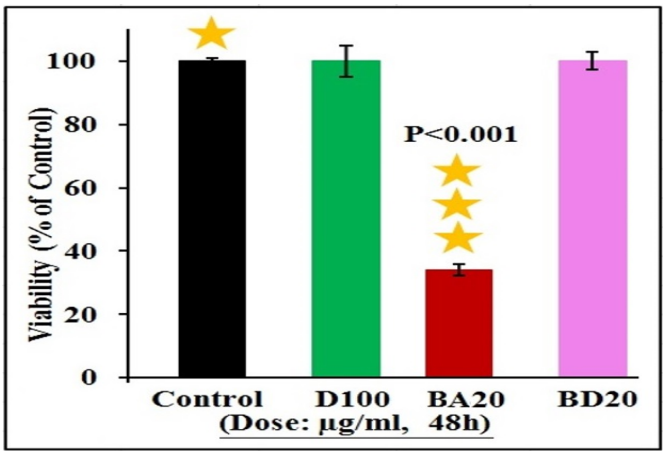

Fig 9. Viability effects of various formualtions, measured by MTT assay after 48 h incubation. Results were shown as Mean±SD from three independent experiments.

were compared to that of control using MTT test [16]. In the present study, BD at a dose of 20 µg/ml was found to be perfectly non-toxic compared to that of BA (P< 0.001) (Fig. 9). Then the therapeutic efficacy of BD was evaluated on the promastigote and amastigote of L. major showing that BD was

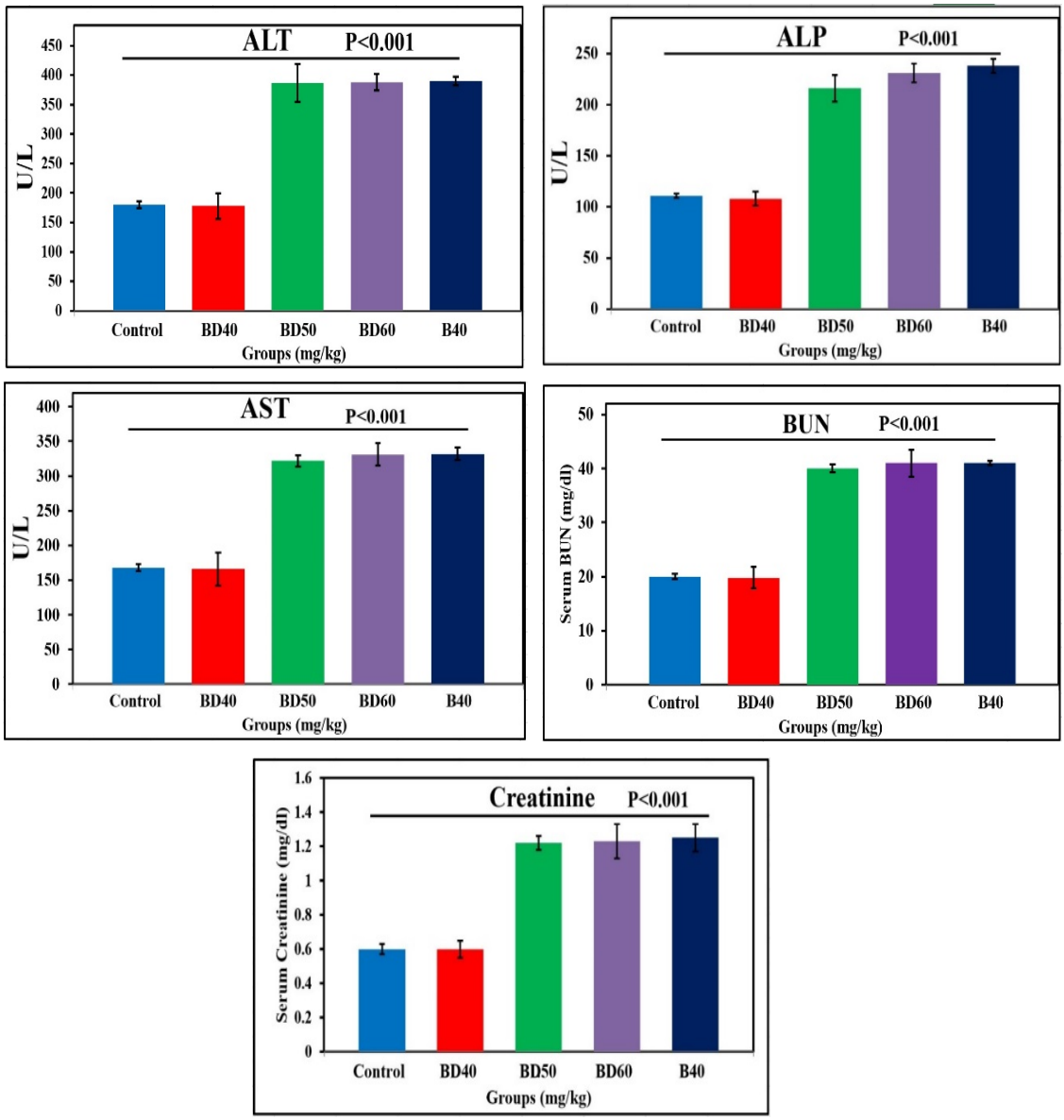

Fig 10. Dose determination and in vivo toxicity evaluation of BA40, BD40, BD50 and BD60 mg/kg formulations by measuring liver related enzymes including ALP, ALT and AST and kidney related factors including BUN and creatinine. Enzymatic toxicity of BA40 mg/kg, BD50 mg/kg, BD60 mg/kg were confirmed, while BD40 mg/kg was nontoxic. Also, BA40 mg/kg, BD50 mg/kg and BD60 mg/kg compared to BD40 mg/kg caused a significant increase in blood concentrations of BUN and creatinine.

more potent than BA to kill the parasite. One of the main feature of nanoparticles is their ability to improve the efficacy of drugs which was demonstrated in this study [18-21].

In vivo toxicity of the formulations

BD at a dose of 40 mg/kg was found non-toxic in terms of liver enzymes (AST, ALT and ALP) and kidney markers (BUN and creatinine) compared to BA at the same dose which was toxic and increased the enzymes and markers compared to the control group (Fig. 10). Also, the mortality rate in BD (40 mg/kg) receiver mice was found to be 0%, while BA (40 mg/kg) was toxic and caused the mortality rate of 30% (Table 3). BD at the dose of 40 mg/kg increased the solubility rate of BA by more than 700-fold which cause an increase in the efficient dose, resulting in efficient treatment of L. major infected mice [13, 16, 18, 22]. Overall, the results indicated that BD at the dose of 40 mg/kg

Table 3. Mortality rate (%) of BA and BD formualtions in Balb/c mice

		Mortality rate (%)
Formulations	BA (40 mg/kg)	30%
	BD (40 mg/kg)	0

Table 4. comparison of in vivo and in vitro effects of BA with or without nanocarrier in lishmaniasis treatment

		In vitro effects	In vivo effects	Ref.
Formulations	BA	40% inhibition of parasite	0% inhibition of parasite due to toxicity	[2]
	BA-ALGD	60% inhibition of parasite	75% inhibition of parasite	Present study

did not cause any toxicity compared to same dose of BA. Table 4 represents the promising results of our study in terms of *in vivo* and *in vitro* effects compared to other published articles.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

REFERENCES

1. de Souza A, Marins DSS, Mathias SL, Monteiro LM, Yukuyama MN, Scarim CB, et al. Promising nanotherapy in treating leishmaniasis. International Journal of Pharmaceutics. 2018;547(1-2):421-31.
2. Domínguez-Carmona DB, Escalante-Erosa F, García-Sosa K, Ruiz-Pinell G, Gutierrez-Yapu D, Chan-Bacab MJ, et al. Antiprotozoal activity of Betulinic acid derivatives. Phytomedicine. 2010;17(5):379-82.
3. Halder A, Shukla D, Das S, Roy P, Mukherjee A, Saha B. Lactoferrin-modified Betulinic Acid-loaded PLGA nanoparticles are strong anti-leishmanials. Cytokine. 2018;110:412-5.
4. Sousa MC, Varandas R, Santos RC, Santos-Rosa M, Alves V, Salvador JAR. Antileishmanial Activity of Semisynthetic Lupane Triterpenoids Betulin and Betulinic Acid Derivatives: Synergistic Effects with Miltefosine. PLoS ONE. 2014;9(3):e89939.
5. Liu Y, Gao D, Zhang X, Liu Z, Dai K, Ji B, et al. Antitumor drug effect of betulinic acid mediated by polyethylene glycol modified liposomes. Materials Science and Engineering: C. 2016;64:124-32.
6. Ríos J, Máñez S. New Pharmacological Opportunities for Betulinic Acid. Planta Medica. 2017;84(01):8-19.
7. Tan JM, Karthivashan G, Arulselvan P, Fakurazi S, Hussein MZ. Sustained Release and Cytotoxicity Evaluation of Carbon Nanotube-Mediated Drug Delivery System for Betulinic Acid. Journal of Nanomaterials. 2014;2014:1-11.
8. Shafiee Ardestani M, Assadi A, Sharifi Najafabadi V, Shandiz AS, Shayeq Boroujeni A, Ashrafi S, et al. Novel chlorambucil-conjugated anionic linear-globular PEG-based second-generation dendrimer: in vitro/in vivo improved anticancer activity. OncoTargets and Therapy. 2016;Volume 9:5531-43.
9. Casa DM, Scariot DB, Khalil NM, Nakamura CV, Mainardes RM. Bovine serum albumin nanoparticles containing amphotericin B were effective in treating murine cutaneous leishmaniasis and reduced the drug toxicity. Experimental Parasitology. 2018;192:12-8.
10. Hashempour Alamdari N, Alaei-Beirami M, Sadat Shandiz SA, Hejazinia H, Rasouli R, Saffari M, et al. Gd^{3+}-Asparagine-Anionic Linear Globular Dendrimer Second-Generation G2 Complexes: Novel Nanobiohybrid Theranostics. Contrast Media & Molecular Imaging. 2017;2017:1-19.
11. Mehrizi TZ, Ardestani MS, Khamesipour A, Hoseini MHM, Mosaffa N, Anissian A, et al. Reduction toxicity of Amphotericin B through loading into a novel nanoformulation of anionic linear globular dendrimer for improve treatment of leishmania major. Journal of Materials Science: Materials in Medicine. 2018;29(8).
12. Mehrizi TZ, Ardestani MS, Molla Hoseini MH, Khamesipour A, Mosaffa N, Ramezani A. Novel nano-sized chitosan amphotericin B formulation with considerable improvement against Leishmania major. Nanomedicine. 2018;13(24):3129-47.
13. Zadeh Mehrizi T, Khamesipour A, Shafiee Ardestani M, Ebrahimi Shahmabadi H, Haji Molla Hoseini M, Mosaffa N, et al.
Comparative analysis between four model nanoformulations of amphotericin B-chitosan, amphotericin B-dendrimer, betulinic acid-chitosan and betulinic acid-dendrimer for treatment of *Leishmania major*: real-time PCR assay plus. International Journal of Nanomedicine. 2019;Volume 14:7593-607.
14. Poole CF. Thin-layer chromatography: challenges and opportunities. Journal of Chromatography A. 2003;1000(1-2):963-84.
15. Kang B-S, Choi J-S, Lee S-E, Lee J-K, Kim T-H, Jang WS, et al. Enhancing the in vitro anticancer activity of albendazole incorporated into chitosan-coated PLGA nanoparticles. Carbohydrate Polymers. 2017;159:39-47.
16. Zadeh Mehrizi T, Mosaffa N, Haji Molla H, lrm, Mostafa, Shafiee Ardestani M, et al. In Vivo Therapeutic Effects of Four Synthesized Antileishmanial Nanodrugs in the Treatment of Leishmaniasis. Archives of Clinical Infectious Diseases. 2018;In Press(In Press).
17. Porta-i-Batalla M, Eckstein C, Xifré-Pérez E, Formentín P, Ferré-Borrull J, Marsal LF. Sustained, Controlled

and Stimuli-Responsive Drug Release Systems Based on Nanoporous Anodic Alumina with Layer-by-Layer Polyelectrolyte. Nanoscale Research Letters. 2016;11(1).
18. Zadeh Mehrizi T, Shafiee Ardestani M, Haji Molla Hoseini M, Khamesipour A, Mosaffa N, Ramezani A. Novel Nanosized Chitosan-Betulinic Acid Against Resistant Leishmania Major and First Clinical Observation of such parasite in Kidney. Scientific Reports. 2018;8(1).
19. Zadeh Mehrizi T. Adjuvanticity effects of selenium chelate nanocomplexes on the immunogenicity of hepatitis B vaccine. Thesis. 2013;School of Medicine, Shahid Beheshti University of Medical Sciences(Department of Medical Immunology).
20. Shahabi J, Shahmabadi HE, Alavi SE, Movahedi F, Esfahani MKM, Mehrizi TZ, et al. Effect of Gold Nanoparticles on Properties of Nanoliposomal Hydroxyurea: An In Vitro Study. Indian Journal of Clinical Biochemistry. 2013;29(3):315-20.
21. Fatemeh DRA, Ebrahimi Shahmabadi H, Abedi A, Alavi SE, Movahedi F, Koohi Moftakhari Esfahani M, et al. Polybutylcyanoacrylate Nanoparticles and Drugs of the Platinum Family: Last Status. Indian Journal of Clinical Biochemistry. 2013;29(3):333-8.
22. Zadeh Mehrizi T, Pirali Hamedani M, Ebrahimi Shahmabadi H, Mirzaei M, Shafiee Ardestani M, Haji Molla Hoseini M, et al. Effective materials of medicinal plants for leishmania treatment in vivo environment. Journal of Medicinal Plants. 2020;19(74):39-62.