RESEARCH ARTICLE

In Vivo Study of New Oral Docetaxel -Loaded Nanomicelles

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ABSTRACT

Poor solubility, low bio-stability, and the high toxicity of docetaxel medicine result in limited consumption, common side effects, and low efficacy. The current commercial form of the product, Taxotere®, with intravenous administration caused hypersensitivity due to hemolysis by separating hemoglobin and red blood cells. In addition, the patient suffers from a severe treatment regime through prolonging medicine injection. The most important advantages of medicine in cancer treatment are to consider patients' comfort, and acceptance during treatment, as well as to choose the most effective medicine to achieve the highest improvement in cancer cells. Following our previous study, in this study stabilized docetaxel loaded nanomicelles were used for the treatment of mice with C26 colon carcinoma. The synthesized nanomicelles have satisfying results on animal trials and adequate characters such as an oral form of medicine, particle size of less than 15 nm, proper PDI, sufficient zeta potential for physical stability and maintaining particle size, non-toxicity of career, and high efficacy than the commercial product Taxotere®. In addition, lower side effects of synthesized oral form medicine on the treatment of C26 colon carcinoma can be named as the other advantage of this study.

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INTRODUCTION

Cancer constitutes the second cause of mortality in the United States and causes 22.9% of global mortality annually [1]. Recently, several studies and researches have been performed to treat cancer and eliminate cancer cells, mostly focused on decreasing medicine side-effects. Cancer is a cell disease that is caused by altering the control processes of cell differentiation and proliferation [2]. The current cancer treatments usually contain aggressive methods, such as primary chemotherapy to reduce the size of a tumor, possible surgeries to remove a tumor, and eventually radiotherapy and complete chemotherapy [3]. The common target of all cancer treatments is removing the maximum amount of cancer cells and damaging the minimum amount of normal cells simultaneously. The quality improvement in patient life and his life expectancy is directly related to the mentioned target [4].

Although anti-cancer medicines have more effect on tumor cells than on normal cells, severe side-effects caused by prescribed high dosage make patients stop taking medicine before it has time to effect [5, 6]. The advantages of nanotechnology on quality improvement of drug delivery systems and targeted treatment have been discovered by scientists over the recent decades. Improving drug delivery technics, which leads to lower toxicity and higher efficacy of medicine has various benefits for the patients and create new markets for pharmaceutical companies [7]. Using an oral drug delivery system and nanoparticles in drug formulation are considered as the new technics. The particle size and particle size distribution are important characters in the new nanoparticle

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Table 1. Material list for synthesis and formulation

Material	Company	Country
Tween 20,40,60,80	Merck	Germany
MCT OIL	SHS	UK
Sesame Oil	Youthing Strategies	USA
Docetaxel	Knowshine Pharmachemical Inc	China
Taxotere	Sanofi Aventis	France
Methanol, ethanol,	Merck	Germany
Acetonitrile, Chloroform	Sigma Aldrich	Germany

systems [8]. The factors have effects on in vivo distribution of nanoparticles, bio-destiny, toxicity, and the ability of nanoparticle to reach to the targeted point [9].

Nanoparticles administrated intravenously, are determined by the defense system simply and are eliminated by macrophages from the bloodstream. In addition to the particle size of nanoparticles, its hydrophobic surface defines the content of blood opsonins on nanoparticles [10,11]. In order to increase targeting, the retention time of nanoparticles in the bloodstream needs to be increased which can be achieved mostly by minimizing opsonization. The following actions can be done in this regard [12, 13]:

- Nanoparticles coated with polymers or hydrophilic surfactants.
- Nanoparticles with copolymer containing hydrophilic parts e.g. polyethylene oxide, polyethylene glycol, poloxamine, poloxamer, Tween 80.

Currently, polymeric nanoparticles are widely used for oral drug delivery and have several advantages compared to other technologies [14, 15]. They are stable in the gastrointestinal tract and can secure loaded drugs in counter with enzymatic reaction, pH, and drug pump inhibitors. Moreover, a successful nanoparticle system should have a high capacity to load the drug which leads to the necessity of a lower amount of matrix material for administration.

Nanomicelles have recently been used more in oral drug delivery. The micelles categorize in the nanoparticles group and have two parts hydrophobic internal part and a hydrophilic external part. Therefore, it can encapsulate hydrophobic drugs in its core and can simply deliver the hydrophobic drug to the target point via hydrophilic liquid media of the body [16].

Docetaxel, an anti-cancer medicine belongs to the toxoid group, has a similar molecular shape like paclitaxel, but it acts more effectively to inhibit the depolymerization of microtubules in the G2/M phase [17, 18, 19].

Docetaxel administered in locally advanced breast cancer, metastatic breast cancer, ovary cancer, lung cancer, uterus cancer, prostate, head and neck cancer, and gastrointestinal tract cancer [12, 20, 21, 22].

In the commercial product, Taxotere®, low solubility character (4/13 μg/ml) of docetaxel into water is increased by using polysorbate 80 nonionic surfactant (Tween 80) [10, 12, 19]. The medicine is administrated intravenously via one hour of injection in the hospital [23]. Drug dosage can be altered depending on patient conditions every three weeks. Its side-effects can be mentioned as bone marrow suppression including neutropenia, thrombocytopenia, and anemia, sensitivity reactions like redness, asthma, blood pressure drop, oedema including weight gain, edema, and ascites, alopecia, monocyte, diarrhea, nausea, and vomiting [24,25,26]. Currently, most of the medicine is only administrated by intravenous injection. The oral method can be one of the best alternative methods to its simplicity, and better acceptance of patients, especially for chronic diseases that need frequent medicine administration.

As mentioned in the previous study, synthesized oral docetaxel nanomicelles have a size of less than 15 nm, proper polydispersity index (PDI), sufficient zeta potential for physical stability and maintaining particle size, high drug encapsulation capacity, non-toxicity of career, and high efficacy than the commercial product Taxotere®. The synthesized nanomicelles have been used for the animal trials. In this study, the results of the animal trials will be evaluated and discussed for synthesized nanomicelles with different Tween and surfactants.

EXPERIMENTS

Materials and methods

The materials used to prepare nanomicelles are summarized (as in Table 1).

Table 2. List of equipment for synthesis and formulation

Equipment	Model	Country
Probe Sonicator	Soniprep 150	UK
Zeta sizer	Nano-ZS	UK
Rotary Evaporator	Heidolph Laborota 4000	Germany
Centrifuge	Hettich-Micro 120	Germany
HPLC	Shimadzu LC- 10 AVP	Japan
Electron Microscope	TEM-LEO 910- Zeis	Germany
ELISA reader	Synergy H4- Hybrid reader	USA

Table 3. Variables list

Variable	Variations
Surfactant weight percentage	40-45-50-55-60-65-70 (%)
Surfactant type	Tween 20-40-60-80
Stabilizer weight percentage	5-10-15-20-25-30-35-40-45 (%)
Stabilizer type	MCT Oil- Sesame oil
Water weight percentage	14-24 (%)
Dissolution temperature	45-50-55-60 (°C)

Table 4 . The details of the formulation

Formulation	Composition
I	DTX, Tween80, MCT Oil, Water
II	Tween80, MCT Oil, Water
III	DTX, Tween20, MCT Oil, Water
IV	Tween20, MCT Oil, Water
V	DTX, Tween80, Sesame Oil, Water
VI	Tween80, Sesame Oil, Water
VII	DTX, Tween20, Sesame Oil, Water
VIII	Tween20, Sesame Oil, Water

A list of equipment that has been applied to prepare nanomicelles is summarized (as in Table 2).

DTX-Loaded Nanomicelles Preparation

To prepare DTX-Loaded Nanomicelles, different compositions of different surfactants (Tween) and stabilizers (MCT oil and Sesame oil), distilled water, and dissolution temperature have been used considering one factor at a time. Studied variables are summarized (as in Table 3).

Transparency, size, and surface charge of each sample were studied to choose the optimized synthesis method and determine composition. Verified results (nanomicelles under 500 nm, negative surface charges, and complete clarity) lead to materials and composition selection. The optimized samples were determined including 55% wt. of surfactant types Tween 20 and 80, 20% wt. of stabilizers, and 55% wt. of distilled water [27].

Components were measured in 15 mL Falcon tubes and were warmed up to 50˚C in a hot purified water bath. The solution was agitated by a vortex with a sequence of 4 minutes' intervals. The solution was chilled to 37 ˚C after 30 minutes. One percent of DTX was added and it was kept in 37 ˚C for 40 minutes. Mixing of the solution was performed continuously. Agitation was continued for 30 minutes, and ultra-sonicated for 1~2 minutes to obtain homogenous liquid [27].

Formulations with the Tween 80 and 20 and DTX free were synthesized similarly and were considered as the control.

Nanomicelles Evaluation

The details of the formulation are shown as below (as in Table 4).

TEM photos showed the spherical shape of the synthesized formulations [24]. The spherical

Table 5. Particle size (Z-average (nm)), PDI, and zeta potential (mV) of formulations (mean ± SD, n = 3).

Formulation	Z-Average (nm)	PDI	Zeta Potential (mV)
I	14.03±1.23	0.132±0.02	-9.45
II	9.89±2.68	0.125±0.07	-5.67
III	132.55±12.88	0.256±0.02	-6.09
IV	126.2±19.39	0.224±0.06	-2.97
V	145.2±11.32	0.532±0.03	-7.21
VI	130.4±9.89	0.511±.0.01	-6.14
VII	289.75±15.37	0.65±0.04	-1.12
VIII	312.4±16.63	0.78±0.05	-0.2

shape of the synthesized nanomicelles enabled it to have the maximum ability to control the release and protection of encapsulated drugs which has been discussed in detail in our previous study [24]. The spherical shape has the longest path for encapsulated drug migration and has the minimum contact surface with the aqueous phase compared to the other nanoparticle shapes [27, 28, 29, 30, 31, 32].

Zeta potential

The particle size, polydispersity index (PDI), and zeta potential are defined (as in Table 5). The mean particle size of the Tween 80 nanomicelles was 14.03 ± 1.23 nm, and that of the Tween 20 nanomicelles was 132.55 ± 12.88 nm using MCT oil as the stabilizer, while the mean particle size of the Tween 80 nanomicelles was 145.2 ± 11.32 nm, and that of the Tween 20 nanomicelles was 289.75 ± 15.37 nm using sesame oil as the stabilizer. The nanomicelles containing sesame oil were greater than 100 nm for all compositions and the sesame stabilizer PDI was not adequate (PDI>0.5). According to the study's purpose for having proper sized DTX-loaded nanoparticles, other reviews continued on formulation I to IV.

The negative amount of zeta potential in all formula provided sufficient electrostatic repulsion for physical stability and preventing particle accumulation. The average size and PDI of the formula I and II was less than that of formula III and IV. It revealed that the dimension and uniformity of the Tween 80 were better.

The prepared nanomicelles need to be diluted in 5% dextrose to be used for the animal trials. The size and zeta potential of the formulation I in dextrose were also investigated. The sterilized 5% dextrose was used instead of distilled water in Formulation I. Besides, the zeta potential in MOPS buffer was measured. The mean particle size was 15.2±1.45 with PDI= 0.23±0.031. The zeta potential was -10.1 mV. The results showed no significant differences between using dextrose and water.

The size and surface characters dictate the extension and the drug absorption rate in the gastrointestinal tract. The small dimension of nanomicelles increases specific area, nanomicelles contact surface with epithelial increases, and therefore there will be more possibility of nonspecific drug absorption into cells or endocytosis via receptor [33, 34]. The studies reveal that the nano-careers smaller than 50 nm absorb enterocytes, thus the small size of Formulation increases the enterocyte absorption. The drug travels through the lymphoid system, therefore, it inhibits liver metabolic first-pass effects. It increases the specific area in the blood and decreasing cancer cell growth [35]. Based on the results, the prepared Tween 80 nanomicelles had all the particle size requirements for oral absorption in the gastrointestinal tract.

The surfactants are divided into two groups of the ionic and the nonionic. The ionic group is applied to solve partly soluble materials while the nonionic for severely insoluble materials [36]. The critical concentration (CMC) and aggregation number (N_{ag}) are the main parameters to evaluate the quality. Tween was used in many types of research due to its low CMC (nanomicelles stability in diluted media and ability to administer low dosage) and proper aggregation number [30]. The nanoparticles of 10 to 100 nm were used in the study. Moreover, its low toxicity allowed to be used in oral form, and the nonionic character increased solubility of nonionic hydrophobic docetaxel.

The clarity is one of the important characters. The hydrophil-lipophil balance (HLB) in preparing fat-water emulsions needs to be in the range of 10 < HLB < 18. Tween 80 with HLB=15 and Tween 20 with HLB=16.6 provided a transparent solution [37,38,39,40]. Both of the surfactants had

Fig. 1. Mice during anesthesia after subcutaneous injection of C-26 cells

the maximum hydrophilic character, did not act as the stabilizer, which made the nanomicelles process reversible to perform on-time drug release [41,42,43,44].

The negative zeta potential for prepared nanomicelles inhibited the particle accumulation and provided better physical stability by electrostatic repulsion. The DTX-loaded Formulations showed higher zeta potential than those without which was caused by the docetaxel negative OH group.

In vivo studies

Twenty-five female BALB/c mice were bought form the Pasture Institute of Iran for the study. The mice were 4-5 weeks old and had 13-17 gr weight. All the animal trial was performed in Buali (Avicenna) Research Institute, Mashhad, Iran under laboratory animals' rights and laws.

The C-26 tumor grew on the mice back, and efficacy of the new DTX-loaded Formulations compared to the commercial product has been reviewed.

Mice back were shaved as it is shown in Fig. 1, and 100-150 µl of anesthetic solution (Xylazine 10 mg/kg dosage, Ketamine 10 mg/kg) was injected into each mouse peritoneum. The anesthetic solution contained 4.5 ml of Ketamine and 1.5 ml of Xylazine in 25 ml of 5% dextrose. Mice became unconscious after 20 min, 300,000 C-26 cells in 50 µl of PBS was injected subcutaneously to the shaved areas (Fig. 1).

A tangible tumor was created at mice backs after 7 days (Fig. 2). The drug treatment started in this phase, mice with the proper size, tumor condition, and weight were selected and painted by picric acid. Each group included 5 mice, and marking was done on neck, tail, left hand, right hand, and no-color. Each mouse was weighed separately and its drug dosage was defined. Finally, 10 mg/kg of the commercial drug Taxotere® was injected, the optimized prepared DTX-loaded nanomicelles was forced fed to mice in two dosages of 50 and 100 mg/kg, dextrose and empty nanomicelles with the dosage of 100 mg/kg were forced fed to mice. To control mice's condition, a 5% dextrose oral solution was used for the witness group. The drug dilution was done in a 5% dextrose injectable solution.

The mice were placed in their boxes after registration. The tumor size and mouse weight was evaluated every other day for 60 days. The tumor volume was measured as bellow:

$$Tumor\ Volume\left(mm^3\right) = L \times W \times H$$

L, W, and H were the tumor length, width, and height respectively.

According to the laboratory animals' rights and laws, the mouse life should be terminated by concentrated chloroform inhaler to avoid suffering in case of following conditions: a) the mouse weight becomes less than 15% of its primary weight, b) the

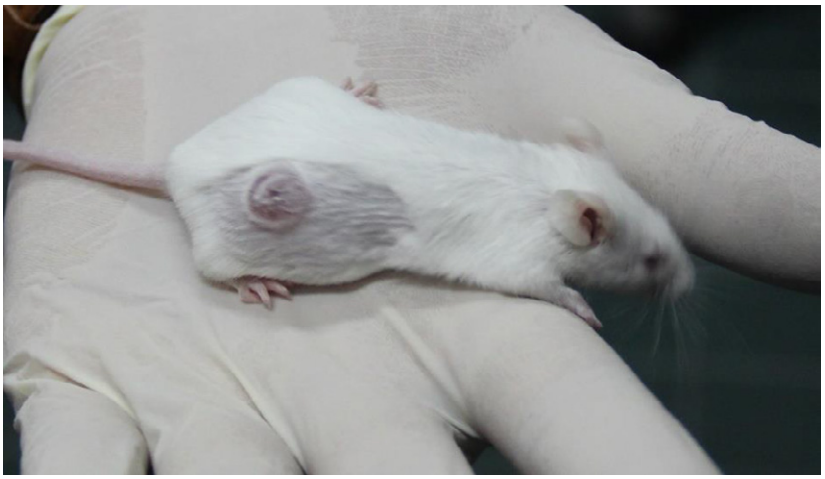

Fig. 2. The tumor created on a mouse back after 7 days

tumor size becomes more than 2,000 mm^3, c) one dimension of tumor becomes more than 20 mm.

RESULT AND DISCUSSION

The stability of nanocarrier is the key point in drug delivery [41]. The drug oral administration has been limited by the gastrointestinal tract condition such as epithelium adsorption limitations, pH, and enzymatic reaction. The pH value varies from one to eight in the stomach and in the intestine respectively. The variation results in oxidation, deamination, or hydrolysis of drug proteins, and decreases the activity. The drug may reach the epithelial cell to be absorbed if it can overcome those obstacles. DTX intakes by a transcellular mechanism. DTX enters the cell by a vesicle-like substance [27, 28].

The stability of prepared micelles must be adequate enough to inhibit rapid dissolution and to avoid dilution in counter with the gastrointestinal tract. The stability of the prepared Formulation was conducted at 37 ˚C for 12 hours, in simulated gastric fluid (SGF, pH 1.6) and simulated intestinal fluid (SIF, pH 6.5). Results demonstrate that in the first 2 h, the particle size changes are minor in the SGF, and the particle size in SIF during the second 6 h increased. Nanomicelles were kept less than 6 h in the intestine [27, 30], and the physical stability is proper. Also, formula I shows more stability in the gastrointestinal media [27].

In vivo tumor inhibition efficacy

In vivo tumor inhibition efficacy was evaluated for all the formulations. Fig. 3 demonstrates in vivo tumor inhibition efficacy of DTX-loaded nanomicelles, Formulation I, with two dosages of 50 and 100 mg/kg compare to the commercial product Taxotere®, with a dosage of 10 mg/kg, and the nanomicelles without DTX, M-BLK. The inhibition efficacy of the Formulation I, both dosage, and the commercial product, Taxotere®, can be evaluated compared to M-BLK and Dextrose. It demonstrates that Formulation I with a dosage of 100 mg/kg inhibited tumor growth significantly, and Formulation I with a dosage of 50 mg/kg is better than the commercial product, Taxotere®, however, it was not significant.

Fig. 4 demonstrates the weights of the mice with C-26 tumors during the treatment. The figure shows no significant weight changes.

Fig. 5 demonstrates survival rates after 50 days of study. As it shows, 60% of the mice under treatment with the Formulation I, 100 mg/kg, were survived after 44 days, while 80% of the mice under treatment with the commercial product, Taxotere®, 10 mg/kg, gone after 30 days, and the only survival last 42 days, 40% of the mice under treatment with the Formulation I, 50 mg/kg, were survived after 44 days. The control group under treatment by dextrose survival rate was 40% in day 42, and only 20% of mice that were administrated M-BLK survived in day 42. The most convenient rate of survival was for Formulation I, 100 mg/kg.

The animal trial was conducted with two dosages of 50 and 100 mg/kg. DTX-loaded nanomicelles, 100 mg/kg, compared to the commercial product, 10 mg/kg could inhibit tumor growth for 26 days significantly. Summarizing animal trial and all the lab results, Formulation I was defined as the best-

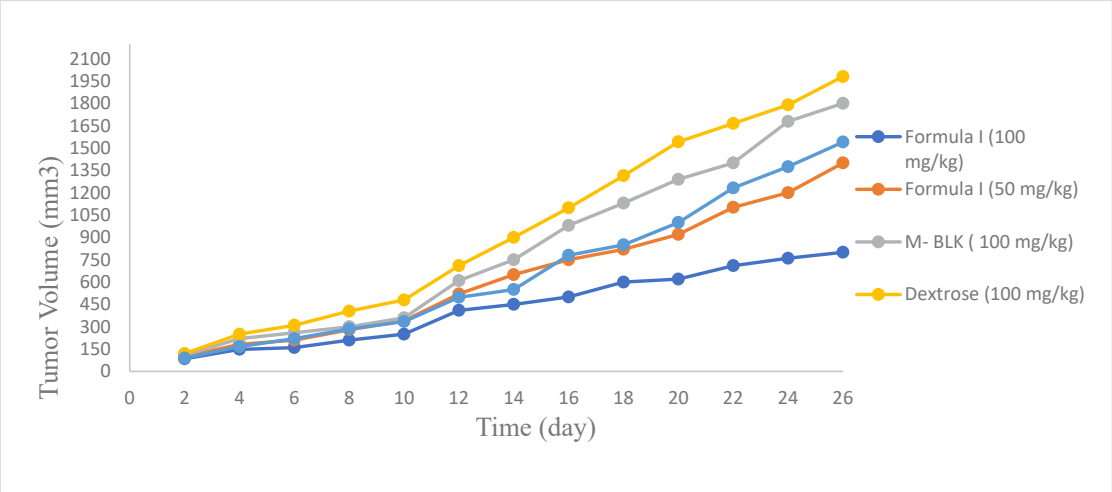

Fig. 3. In vivo tumor inhibition efficacy for the prepared Formulation compare to TXT

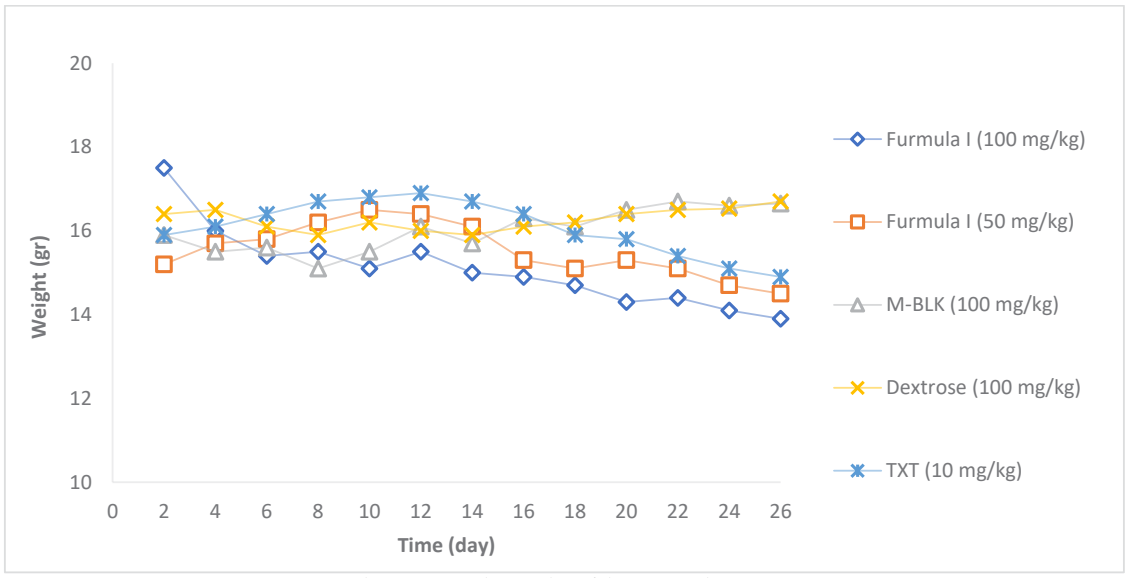

Fig. 4. demonstrates the weights of the mice with C-26 tumor

optimized nanomicelle. The higher dosage was not used due to the drug high metabolism. When the tumor faced with high drug dosage, multidrug resistance (MDR) mechanisms will be activated and efficacy will decrease [45].

The cancer tumors have a more permeable artery with larger pores due to its abnormal angiogenesis, therefore nanoparticles intend to gather in the tumor tissues much more than they do in normal tissues. It is called the enhanced permeability and retention (EPR) effect [45]. The prepared nanomicelles in this study have passive targeting character, accumulate in the tumors, and showed better efficacy than the commercial product. The Formulation I, 100 mg/kg, demonstrated the best efficacy. Moreover, the under-treatment mice with the prepared nanomicelles had better and more stable than the commercial product, 60% of the group survived within the 44 first days out of 50 days' animal trial duration. It means that the Formulation could satisfyingly inhibit the mortality caused by the tumor compared to the commercial product. As it is shown in Fig. 5, the commercial

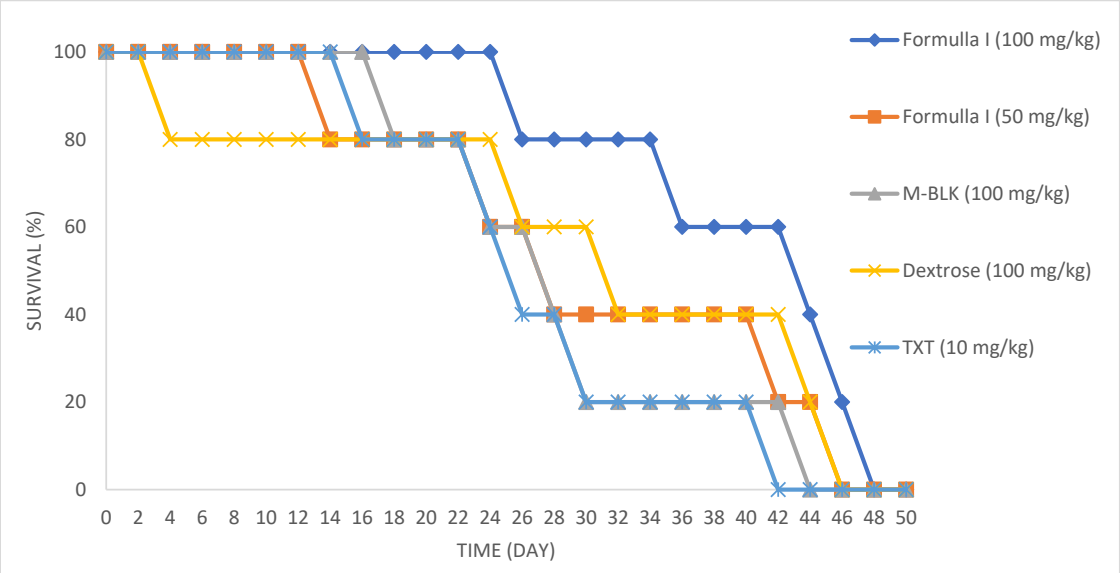

Fig. 5 . Survival rates after 50 days of study

product, registered numerous death rates, while Formulation I registered the maximum survival rate that can prove its low toxicity.

CONCLUSION

Docetaxel nanomicelles were successfully synthesized and administrated in the animal trial compared to the commercial product. Synthesized nanomicelles have satisfying results on animal trials and adequate characters such as an oral form of medicine, size of less than 15 nm, proper polydispersity index (PDI), sufficient zeta potential for physical stability and maintaining particle size, non-toxicity of career, and high efficacy than the commercial product. Also, the lower side effects of synthesized oral medicine on the treatment of C26 Colon Carcinoma can be named as the other advantage of this study. The outcome of the study showed that the proposed DTX nanomicelle could be administrated in oral form and deserves subsequent researches to determine the pharmacokinetics, tissue distribution, and tissue pathology study in the next studies.

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CONFLICT OF INTEREST

Authors declare no conflict of interest in present work.

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