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Effect of Polymeric Nano-carrier Loaded with Berberine on Doxorubicin- induced Cardiotoxicity

A dissertation

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Dedication

This work is dedicated to the Prophet of Allah, Mohammad Ibn Abdullah (Peace Be Upon Him).

To My parents, who have consistently provided me with kindness and encouragement throughout my life and have always served as a significant source of strength.

To my husband, Wisam, for his unwavering support and encouragement.

To my children Abdullah, Lobna, and Mujtaba, whose radiant grins serve as a constant source of inspiration in all aspects of my life.

Noora

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Summary

Despite the widespread use of Doxorubicin (Dox) as a chemotherapeutic agent to treat patients with a wide range of cancer types, Dox-induced cardiotoxicity (DIC) has been regarded as a significant challenge to the successful treatment of cancer patients. In spite of extensive research aimed at overcoming this obstacle, there is currently no clinically approved, effective treatment for DIC. Therefore, it is crucial to develop a way to reduce Dox's cardiotoxicity while maintaining the drug's anticancer efficacy. Utilization of phytochemicals for the purpose of mitigating DIC is one potential strategy. Berberine (Ber) is a natural product example. Due to its limited water solubility, low bioavailability, and low cell permeability, Ber's effectiveness as a pharmacological agent is substantially constrained.

The three main parts of this study are: first, the formulation and characterization of a Pluronic F127 polymeric micelle as a nanocarrier for Ber delivery (mBer); second, determining the cardioprotective and chemosensitizing effects of this formulation; and third, evaluating the formulation's cardioprotective effect against a 1 μ M concentration of Dox, which represents the human plasma concentration of Dox following therapeutic doses.

A mBer was formulated using the thin-film hydration technique with the intention of resolving challenging issues associated with Ber delivery. The micelle was analyzed for drug loading and retention efficiency, size, zeta potential, shape by transmission electron microscopy (TEM), in vitro release, and their potential for inducing toxic hemolysis through an in vitro hemolysis assay. The average Ber encapsulation efficiency of the prepared spherical-shaped micelles was 85%, with a hydrodynamic radius of 82.2 ± 10.3 nm, 0.176 ± 0.029 polydispersity index, and -47.4 ± 0.19 mV zeta

potential. The results of fourier transform infrared spectroscopy(FTIR) and X-ray diffraction (XRD) experiments revealed that Ber was physically entrapped and in an amorphous state within the synthesized micelles. Compared to the free Ber solution, the in vitro release of Ber from micelles exhibited both short-term rapid release and sustained release. mBer was shown to be relatively non-toxic and biocompatible via an in vitro hemolysis assay.

In order to determine the cardioprotective and chemosensitizing effects of mBer, the viability of cardiomyocytes (H9c2) and ovarian cancer cells (SKOV-3) in the presence of either free Ber or mBer alone or in combination with Dox was evaluated using the MTT test. Combination index (CI) analyses were also performed in order to evaluate the impacts of the various combinations. In H9c2 cells, co-delivery of mBer and Dox resulted in a significantly higher half maximal inhibitory concentration (IC₅₀) than Dox alone, and they showed an antagonistic effect in combination (CI > 1), indicating the cardioprotective activity of mBer. When Ber is incorporated into the F127 micelle, its cardioprotective activity is enhanced. Conversely, the reduction in cell viability and the CI values (CI < 1) in SKOV-3 cells showed that this combination was synergistic in SKOV-3, indicating the chemosensitizing effect of mBer.

In the last set of experiments for this study, the ability of free Ber and mBer to protect the cultured cardiomyocytes against 1 μ M of Dox, which is the concentration of Dox in human plasma after therapeutic doses, was evaluated. The experimental groups included the control group, the Ber-treated group, the mBer-treated group, the Dox-treated group, the Ber-Dox combination-treated group, and the mBer-Dox combination-treated group, as well as the void micelles-treated group. The alterations in cardiotoxicity markers [lactate dehydrogenase (LDH), creatine kinase

myocardial band(CK-MB), and cardiac troponin I (cTn-1)], lipid peroxidation indicator [malondialdehyde (MDA)], oxidative stress markers [Reduced glutathione (GSH), catalase (CAT), and superoxide dismutase (SOD)], inflammatory cytokines [interleukin-1 β (IL-1 β) and interleukin-6 (IL-6)], and the activity of the apoptosis proteins caspases 3/7 were evaluated.

The Dox group demonstrated significant ($p<0.05$) increases in cardiotoxicity enzyme indices, lipid peroxidation, generation of free radicals, inflammatory cytokines, and caspase 3/7 activity relative to the control group. In contrast to the group of cardiomyocytes treated with Dox, the levels of LDH, CK-MB, cTn-1, and MDA decreased significantly ($p<0.05$), while the activities of SOD and CAT improved significantly ($p<0.05$) when Ber or mBer was co-delivered with Dox, and they reduced the elevation in both IL- β and IL-6 levels as well as the activities of caspases 3 and 7 induced by Dox. Importantly, the utilization of the micellar formulation of Ber in conjunction with Dox significantly enhanced the cardioprotective efficacy of Ber against DIC in H9c2 cells.

The experimental parts of this study supported the conclusion that making polymeric F127 micelles is a fairly simple process that could offer a novel Ber delivery approach and a prospective strategy for the treatment of DIC. In addition, while the administration of mBer has demonstrated cardioprotective effects against DIC in cardiomyocytes, it maintains a chemosensitizing influence when administered in conjunction with Dox in cultured ovarian adenocarcinoma cells, which may hold promise as a prospective therapeutic approach for cancer.

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List of Abbreviations

Abbreviation	Definition
Ber	Berberine
Caspase-3/7	cysteine-aspartic proteases-3/7
CAT	Catalase
CI	Combination index
CK-MB	Creatine kinase myocardial band
CMC	Critical micellar concentration
cTn-I	Cardiac Troponin I
DIC	Doxorubicin -induced cardiotoxicity
DL	Drug loading content
DLS	Dynamic light scattering
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
Dox	Doxorubicin
DR4	Death receptor 4
ELISA	Enzyme-linked immunosorbent assay
FDA	Food and Drug Administration
FTIR	Fourier transform infrared spectroscopy
GSH	Reduced glutathione
H9c2	Rat embryonic cardiomyoblast cell line
HSF-1	Heat shock factor 1
HSP-25	Heat shock protein 25
IC50	Half-maximal inhibitory concentration
IL-1β	Interleukin-1β
IL-6	Interleukin-6
LD50	Lethal dose 50

LDH	Lactate dehydrogenase
mBer	Ber-loaded micelles
MDA	Malondialdehyde
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NF-κB	Nuclear factor kappa B
PBS	Phosphate-buffered saline
PDI	Polydispersity index
PEO	Polyethylene oxide
P-gp	P. glycoprotein efflux transporter
PLGA	Poly lactic- co-glycolic acid
PMMA	Poly(methyl methacrylate)
PPO	Polypropylene oxide
RE	Retention efficiency
ROS	Reactive oxygen species
SD	Standard deviation
SKOV-3	Human Caucasian Ovarian Adenocarcinoma cell line
SOD	Superoxide dismutase
TEM	Transmission electron microscopy
TNFR1	Tumor necrosis factor receptor 1
XRD	X-ray diffraction

Chapter One

Introduction and Literature Review

1-1. General introduction:

Doxorubicin (Dox) is a chemotherapeutic drug that belongs to the anthracycline class of antibiotics that has been extensively and effectively used in clinical practice for the treatment of many malignancies, including lymphoma, leukemia, breast and ovarian cancer, and other various adult and pediatric cancers (1,2). Unfortunately, its clinical use has been hampered by the risk of serious life-threatening cardiotoxicity (3). Owing to their relatively low ability to regenerate, cardiac muscle is highly susceptible to the long-term toxic effects of Dox (3). Doxorubicin-induced cardiotoxicity (DIC) has been considered a major health issue associated with Dox therapy and an important challenge in the successful treatment of cancer patients (4,5). Oncologists and cardiologists face an ongoing problem as they deal with the potential trade-off between mitigating Dox-induced cardiotoxicity (DIC) through dose reduction and the consequent decrease in chemotherapy efficacy [2]. Dexrazoxane has been approved in many countries as a cardioprotective agent to reduce DIC (6). However, its use is restricted due to worries that it may impede Dox's effectiveness, induce suppression of bone marrow and liver toxicity, and increase the risk of future malignancies (7). Although extensive investigations have been done to develop an effective treatment for DIC, there is no clinically approved effective treatment currently available (8,9). Therefore, it is critical to find a way to minimize Dox's cardiotoxicity while simultaneously boosting the drug's anticancer efficacy.

Phytochemicals (plant-derived small molecules) have tested in preclinical studies and showed cardioprotective effects against DIC (10). Among such natural product is berberine (Ber), a plant alkaloid, is extracted from the *Berberis* species' roots and bark (11). It has been stated that over 25 billion Ber pills are consumed annually in various regions of Asia and Africa

(12). A growing number of studies have linked Ber to several pharmacological effects, such as anti-inflammatory, anti-diabetic, antibacterial, neuroprotective, cholesterol-lowering, and, central nervous system, and digestive tract protective actions, as well as antimicrobial and anticancer effects (13). A combination of in-vitro experiments, in-vivo investigations using various animal models collectively provide evidence supporting the capacity of Ber to regulate cardiovascular functions and to safeguard cardiac tissues from the detrimental effects of Dox (14). However, the clinical application of Ber is limited by its very low water solubility, bioavailability, and permeability across biological membranes (15,16). Therefore, Ber's delivery to the target of interest in the body has become quite challenging (12). With the continued development of Ber delivery systems, problems like targeting specific tissues, cellular internalization, and controlling drug release at the targeted site in the body have been challenges (17). Our approach to solve such phytochemical issues related to bioavailability and their respective solubility is to load these molecules into amphiphilic polymeric nanocarriers known as micelles (18).

1-2. Doxorubicin:

Dox (brand name Adriamycin) was approved by the Food and Drug Administration (FDA) in 1974 as one of the most potent chemotherapeutics, it is used to treat a wide range of metastatic malignancies, such as breast cancer, lymphoma, leukemia, and bladder cancer (19). Dox, an example of anthracycline antibiotics, is made by the fungus *Streptomyces peucetius* var. *caesius* (19,20). The chemical formula of Dox is C₂₇H₃₀ClNO₁₁. It possesses both aglyconic and sugar moieties, and its side chain terminates with a primary alcohol group. The anticancer activity of the compound is attributed to the fact that it has several functional groups. These include daunoxamine, which fits into grooves; the

anthraquinone ring, which makes intercalation easier; and the anchor region, which forms hydrogen bonds (Figure 1-1) (5,21).

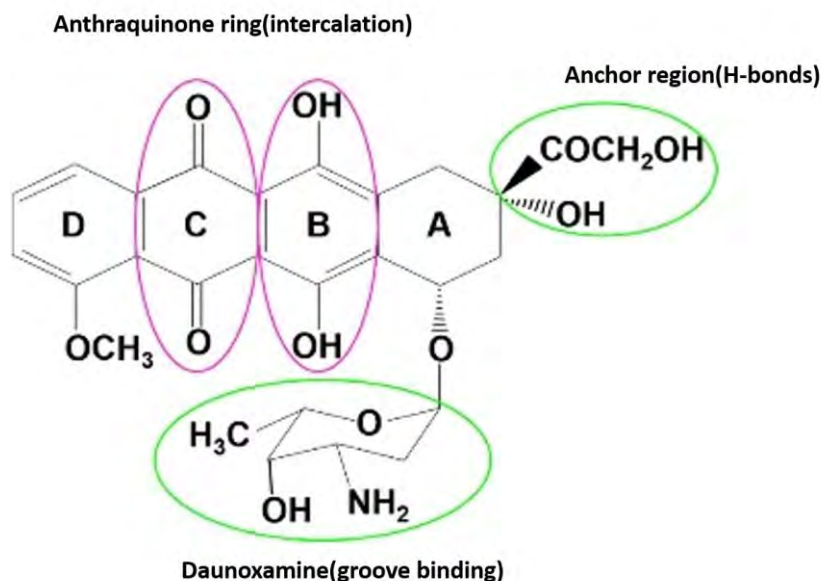


Figure 1-1: The Dox's chemical structure. The marked are the functional groups believed to be crucial to the Dox mechanism of action (5).

Since their discovery in the 1960s, anthracyclines have been generally acknowledged as a class of effective chemotherapeutic medications for treating a variety of cancers (22). Dox's capacity to fight rapidly dividing cells and slow disease development has been widely recognized for decades (23). The potential for dose-dependent cardiotoxicity restricts the therapeutic application of Dox. 5-48% of patients who received cumulative doses of 400-700 mg/m² of Dox had progressive congestive heart failure (24). Dox cardiotoxicity can be either acute or chronic. Acute Dox cardiotoxicity manifests within days of drug exposure, whereas chronic Dox cardiotoxicity appears months or even years later (25,26). However, the biochemical mechanisms underlying Dox cardiotoxicity are obscure, despite the fact that several components have been proposed. As a result, there are no perfect effective therapeutic options for treating Dox cardiotoxicity. Therefore, avoiding Dox

cardiotoxicity is critical for cancer patients. To date, various pharmacological treatments for preventing Dox cardiotoxicity have been utilized or explored in clinical trials (25).

Dexrazoxane was approved as a cardioprotective drug to minimize DIC in the United States, Canada, Europe, and other countries (6). It is administered to patients 30 minutes prior to the administration of the Dox and is distributed to cardiomyocytes. However, its use is restricted due to concerns that it may interfere with the efficacy of Dox and increase the risk of future malignancies (27). In the United States, the only FDA-approved doxorubicin formulation is a pegylated liposomal formulation. Despite the liposomal formulation's attempts to reduce the cardiotoxicity of the Dox formulation, studies have shown that it is just as likely to produce cardiac events (28,29). Thus, Combining Dox with cardioprotective agents is still a pressing necessity

1-2-1. Physiochemical properties of doxorubicin:

The color of the hydrochloride salt of Dox is red. Dox hydrochloride is relatively stable. It can be stored for years at room temperature without degrading (30). Water, physiological saline, methanol, acetonitrile, and tetrahydrofuran all solubilize Dox very well; less polar organic solvents solubilize it just marginally or not at all (30). Dox is best to the stable when dissolved in water at a pH of 4 (pH range of 3.0 to 6.5). It becomes unstable when either the pH is too low (very acidic) or too high (extremely basic) (30,31). However, its stability time in plasma and culture mediums may be shorter (31).

1-2-2. Pharmacokinetics properties of doxorubicin:

Pharmacokinetics is the study of how a drug moves through the body's biological systems. It determines how the drug enters, moves within and exits the body. These processes include absorption, distribution, metabolism, and elimination (32).

1-2-2-1. Absorption:

Due to its instability at stomach pH, Dox is delivered by injection. It has been injected intravenously, intra-arterially (33), and intraperitoneally (34) . The subcutaneous, intramuscular, and intrathecal routes of administration are unsuccessful due to extravasation caused by significant tissue necrosis (35) . A bolus intravenous infusion of Dox creates high plasma concentrations, which rapidly decline due to broad tissue distribution. Due to the rapid uptake of Dox by tissues, its half-life in this instance is approximately 5 minutes. A prolonged tissue clearance time is reflected in the drug's terminal half-life distribution, which ranges from 20 to 48 hours (36) .

1-2-2-2. Distribution in body:

Dox is extensively distributed in plasma and tissues. Fifty to eighty five percent of plasma doxorubicin is bound to protein regardless of the absolute drug concentration (37) , leaving only 15–50% of the entire doxorubicin and doxorubicinol, which is a Dox metabolite, as free drug (37). Seventy-five minutes after treatment, the concentration of Dox in the saliva is between 6 and 26 percent of the levels in the plasma. The drug enters the cell and binds to biological components. Lacking the ability to cross the blood-brain barrier, it is unable to access the high drug concentration in cerebrospinal fluid (38).

1-2-2-3. Metabolism:

Dox is transformed into doxorubicinol via NADPH-dependent aldo-reductases which are responsible for converting Dox into its active metabolite doxorubicinol following its administration. The anticancer action is also provided by doxorubicinol, the active moiety (39) .

After 5 minutes of Dox injection, the metabolite is present in plasma at a concentration of over 20%, with continued increases throughout time.

70 % is reached within 30 minutes, 75 % after 4 hours, and 90 % after 24 hours (1,40).

1-2-2-4. Elimination:

The majority of Dox and its metabolites are eliminated through the bile. More than half are eliminated during the first pass action (33). It is anticipated that 25 to 45 percent of Dox is eliminated in the stools over a seven-day period (41). On average, approximately 5 percent of Dox is eliminated in the urine, with approximately two-thirds of this amount remaining unaltered (42).

1-2-3. Mechanisms of doxorubicin induced cardiotoxicity:

In spite of the fact that a great deal has been discovered about DIC during the past six decades, the precise mechanisms of the toxicity remain unknown. The cardiomyocyte death after Dox treatment appears to be a multistep process involving multiple possible mechanisms (43,44). The most common mechanisms through which Dox induces cardiotoxicity are:

1-2-3-1. Oxidative stress:

Cardiotoxicity from Dox is largely suggested to be due to generation of reactive oxygen species (ROS). One of the hypothesized mechanisms of free radical formation is the redox cycling of the quinolone–semiquinone ring of doxorubicin. Free radical semiquinone donates its unpaired electron to oxygen (O₂), creating the superoxide radical in aerobic conditions(45,46). This redox cycle is especially dangerous since even a small dose of a drug can catalyze the production of many superoxide radicals, which can damage cells, including cardiomyocytes. Cardiolipin is a mitochondrial phospholipid that maintains architecture, performance, cardiac energy metabolism, and cell survival (47). Oxidative stress mediates Dox-induced mitochondrial toxicity (48). Mitochondrial DNA damage from ROS can inhibit electron transport chain proteins, exacerbating mitochondrial dysfunction and ROS formation (49).

Two other mechanisms in the heart that generate ROS when Dox is present are nitric oxide synthases and NADPH oxidases. Oxidative stress is the outcome of interactions between these Dox-interacting enzymes and other molecules promote apoptosis in cardiomyocytes (44,50).

1-2-3-2. Interference with iron metabolism:

In cardiomyocytes, the development of doxorubicin-iron complexes causes mitochondrial inner membrane oxidation, which is thought to lead to the formation of free radicals. Dox and its metabolite doxorubicinol impair iron metabolism by increasing Dox-iron complexes and Fe^{2+} availability to create reactive OH (51). Doxorubicinol has a larger cardiac accumulation and is more effective at disturbing iron homeostasis, leading to increased ROS production which causes oxidative stress and harms cardiomyocytes. (52) .

1-2-3-3. Calcium homeostasis impairment:

It is well known that doxorubicinol accumulates in cardiomyocytes, where it then inhibits Ca^{2+} ATPase function and, as a result, prevents calcium from entering the sarcoplasmic reticulum (53) . In addition, Doxorubicinol also interferes with the sarcolemmal sodium/potassium pump, which is required for calcium to enter a cardiomyocyte's sarcolemma (54) . Overall, calcium homeostasis is disrupted by Dox, leading to elevated intracellular calcium, ROS generation, cardiac cell dysfunction, and induction of death in cardiomyocytes (55).

1-2-3-4. Sarcomeric structure changes:

Titin, a big protein that spans the M-line and Z-discs of cardiac sarcomeres, serves as a vital structural function in the heart. Various cellular activities, ranging from structural to regulatory, depend on this protein (56) .When cardiomyocytes are exposed to Dox, calpain, cysteine protease, is activated quickly and appears to be oriented toward the destruction of titin. cardiomyocytes dysfunction, myofilament instability,

and eventual necrosis of cardiomyocytes could be caused by proteolysis of the titin. Thus, cardiomyopathy results from Dox-induced titin dysregulations via the activation of proteolytic pathways (57)

1-2-3-5. Gene expression modulation:

Dox can downregulate the expression of genes encoding heart muscle-specific proteins, including contractile, mitochondrial, and sarcoplasmic reticulum proteins which causes aberrant contraction and relaxation and leads to cardiac failure (58,43). Dox also suppresses mitochondrial proteins, disrupting myocardial energy production and inducing cardiac failure (58). The Dox-induced decrease of GATA binding protein 4 (GATA-4) expression is related to the activation of apoptosis, indicating that GATA-4 is essential for cell survival (59).

Overall, Dox generates substantial changes in the gene expression of cardiac muscle, leading in remarkable abnormalities in the structure and function of the heart.

1-2-3-6. Apoptosis induction:

Dox induces apoptosis via both intrinsic and extrinsic mechanisms (44,60). Whenever there is an imbalance between oxidants and anti-oxidants, these pathways get activated, leading to the death of cardiac muscle cells (61). Dox-induced oxidative stress activates heat shock factor 1 (HSF-1) and induces heat shock protein 25 (HSP-25), which in turn stabilizes the p53 protein, which is responsible for the production of proapoptotic factors that lead to the death of cardiac muscle cells (61,62). This mechanism is inhibited by antioxidants (63).

Other heat shock proteins, on the other hand, like HSP-27, HSP-10, HSP-20, HSP-22, and HSP-60, are protective and aid in preventing apoptosis and maintaining normal heart function (60).

In vitro investigation with cardiomyocytes showed Dox enhanced death receptors (TNFR1, Fas, DR4, and DR5).

Furthermore, evidence suggests that Dox also affects caspase activation. Both primary cultured cardiomyocytes and whole rat hearts were found to undergo apoptosis after Dox treatment, with the apoptosis being activated by caspase-3 activity (64).

1-2-3-7. Modulation of microRNAs:

Many studies have found that DIC is associated with alterations in microRNAs because of their involvement in all cardiac processes, such as the conduction of electrical signals, and the contraction of the cardiac muscle (65,66). It has been demonstrated that increasing doses of Dox causes an increase in the levels of a number of different microRNAs in the rat heart. These microRNAs include miR-34a, miR-34c, miR-208b, miR-208a, miR-215, miR-216b, miR21, and miR-367 (1,65). Investigations have also shown that treatment with Dox leads to an increase in the expression of miR21 in H9c2 cells. The inhibition of apoptosis that can be seen as a result of the downregulation of microRNAs in response to Dox is indicative of a cardioprotective effect (67). However, therapy with Dox upregulates miR-208a; nevertheless, this results in cardiotoxicity as a consequence of the downregulation of the GATA-4 gene and an increase in cardiomyocyte death (68). On the other hand, Dox administration causes a downregulation of several microRNAs in rat myocardium, including Let-7 g, miR-30a, miR-30c, and miR-30e highlighting Dox's participation in the modulation of microRNAs (65).

1-2-4. Therapeutic approaches against Doxorubicin-Induced Cardiotoxicity:

Because of the complicated mechanisms of the cardiac damage caused by the administration of Dox, a variety of different treatment strategies are being considered in an effort to prevent DIC. Here, we will focus on a phytochemical-dependent strategy.

1-2-4-1. Phytochemicals dependent strategy:

Phytochemicals are the scientific term for compounds that are derived from plants. They have been extensively investigated for their potential as medicinal agents (69). As previously explained, there are multiple mechanisms through which Dox-induced heart toxicity occurs, which precludes the adoption of a single molecule as an effective Dox toxicity prevention target. Since most natural products have several "targets" and may affect multiple signaling pathways, phytochemicals appear to be "suitable remedies" from this perspective. This also mitigates the issue of ensuring that the anticancer effect of Dox is not weakened by co-administration with phytochemicals while minimizing cardiotoxicity. The multi-activity and multi-targeting of phytochemicals provide better therapeutic results when combined with Dox (70). Due to their complexity, phytochemicals have been generically categorized in to many groups such as phenols, carotenoids, quinones, lignans, polysaccharides, terpenoids, and alkaloids (71).

1-2-4-1-1. Challenges for phytochemicals approach:

According to preclinical investigations, plant-derived compounds can reduce Dox cardiotoxicity; nevertheless, the majority of phytochemical compounds that reduce DIC have not yet been adapted for clinical use due to the poor bioavailability of the majority of phytochemical substances (72). This reduces the interest of more pharmacological research and the potential advantages of integrating phytochemicals into clinical therapy as a cardioprotective adjuvant (72). Numerous innovative drug delivery techniques exist for increasing solubility and extending the plasma half-life of phytochemicals and ensuring their targeted distribution (73)

1-3. Berberine:

There has been a surge of curiosity towards therapeutic plants and the chemicals they contain. One of the key causes was the rise of synthetic

drugs, which have grown increasingly connected with adverse side effects, in contrast to plant-based natural resources, which have been historically used for centuries and are typically reliable, abundant, and safe with relatively few documented negative side effects (74). The addition of beneficial phytochemical components has improved a wide range of drugs. As the industry of developing drugs from natural sources continues to expand, there are increasing turning to plants in quest of novel and more effective treatments for diseases (75). Berberine (Ber) is a notable example of phytochemicals because its numerous potential biological and pharmacological effects make it stand out among these substances. Ber has been used to treat a wide variety of illnesses in both traditional Chinese and Indian medicine, both of which have histories stretching back thousands of years (76). In addition to its widespread use in Asia, it is gaining popularity as a dietary supplement in Europe and Africa (77,78). The pharmacological activity of Ber has been successfully utilized to treat a wide variety of health conditions, many of which have been reported to respond favorably to treatment with Ber. Ber's wide-ranging medical applications, including its efficacy in treating a wide range of illnesses, intense chemosensitization, chemoprotective properties, and a significant role in lowering drug resistance and cancer recurrence (79) have made it a remarkable phytochemical in medicinal chemistry.

1-3-1. Berberine nomenclature and sources:

The term berberine is an ancient word that was given to a yellow extract obtained from *Berberis vulgaris* plant by Buchner & Herberger in 1830 (80). Ber, (5,6-dihydro-9,10-dimethoxybenzo-1,3 benzodioxolo[5,6]quinolizinium), is a non-basic and quaternary benzyloquinoline protoberberine alkaloid that has a molecular formula of (C₂₀H₁₇ON₅) and a molecular weight of 336.4 (81). Ber hydrochloride structure as an important molecule in the field of medicinal chemistry is depicted in Figure

1-2. It is mostly found in various medicinal plants belonging to the Berberidaceae, Papaveraceae, Menispermaceae, Annonaceae and Ranunculaceae families. Some examples of these plants are *Arcangelisia flava* (menispermaceae), *Berberis vulgaris* L. (barberry), *B. aristata*, *B. aquifolium*, *B. lyceum*, *B. crataegina*, *Hydrastis canadensis* (Chinese goldthread), *Rhizoma coptidis* (China's Huang Lian), *Berberis pseudumbellata* and *Scutellaria baicalensis* are the main plants that contain berberine and have been used as traditional folk medicines in many countries such as China, India, Iran, and many others (11,82). The *Berberis* genus is widely recognized as the most widespread natural source of Ber. The bark of *B. vulgaris* contains greater than 8% alkaloids, with Ber (approximately 5%) being the most abundant alkaloid (83).

Ber can be found in the barks, leaves, twigs, rhizomes, and stems of several medicinal plant species (84). Different research groups discovered that there is a higher concentration of Ber in roots (1.6-4.3%) and lower altitude plants have a higher concentration of Ber than higher altitude plants (85). Interestingly Ber concentrations vary according to harvest season and timing. *Berberis pseudumbellata*, for example, has the highest concentration of Ber in the summer season, where it is 2.8 percent in the roots, compared to 1.9 percent in the roots when harvested in the winter (86).

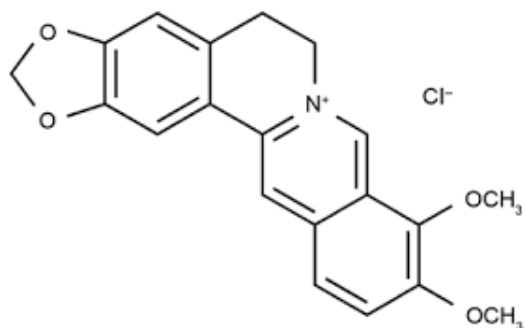


Figure 1-2: Berberine hydrochloride structure (87).

1-3-2. Berberine extraction:

Ber's physicochemical properties contribute to the development of variable extraction and detection methods (81). There are many methods to isolate Ber from its matrix. These methods depend on the principle that Ber belongs to the family of a quaternary protoberberine alkaloid, and because salts are stable in acidic and neutral media, extraction is based on the interconversion reaction of protoberberine salt and base, with organic solvents used to solubilize the base (88).

The most common methods for Ber extraction are maceration, Soxhlet extraction, percolation, and other hot or cold extraction techniques using various solvent systems such as ethanol, methanol, chloroform, and aqueous or acidified mixtures. The temperature must be kept under control during the extraction process (89).

Chromatographic methods are commonly used to separate and quantify Ber after extraction and purification. According to the literature, the most common methods for determining Ber in plant extracts are UV spectrophotometry (90), high-performance liquid chromatography (91), high performance thin layer chromatography, thin layer chromatography (92,93), and capillary electrophoresis (94).

1-3-3. Berberine physiochemical properties:

Ber is an alkaloid that has a bitter taste, a melting point of 145.1–146.7 °C, and a bright yellow color. Ber, sometimes known as "natural yellow 18" has been used as a dye for a long time. For therapeutic uses, it is usually given as chloride or sulfate (95).

In terms of solubility, the salt forms of Ber are relatively better than the pure form, although they are still weakly soluble in water (96). It is

insoluble in some organic solvents such as benzene, ether, and chloroform (97).

Interestingly, when Ber is exposed to UV light, it turns into a bright yellow and exhibits a strong yellow fluorescence (98). Ber has a maximum absorption wavelength of 350 nm and a maximum emission wavelength of 530 nm in a solution of 0.01mol/L sodium dodecyl sulfate (99).

1-3-4. Berberine pharmacokinetic properties:

The absorption of Ber could occur in the gastrointestinal tract. However, it is important to take note of the fact that under physiological settings, Ber changed into the ionized form and that under low pH conditions, it self-aggregated. The process of self-aggregation reduces the solubility of the substance in the gastrointestinal tract as well as its permeability (15) . Generally, Ber is hydrophobic in nature, this property is largely contributing to preventing uptake of the Ber by the human body (100) . In addition, Ber is largely exposed to first-pass effects in the intestinal lumen before it distributes to blood circulation, limiting its therapeutic applicability (101,102). Furthermore, Ber is a substrate for glycoprotein (P-gp) which is responsible for pumping many foreign substances out of the cell leading to a further decrease in Ber concentration in body cells (103). Because of its limited solubility and permeability, Ber in general has a bioavailability of less than 1% in the body (15,104). Specifically, About 0.5% of orally given Ber is absorbed in the small intestine, and an even smaller amount (0.35%) reaches into the bloodstream (12).

Ber is distributed in a wide variety of tissues and organs throughout the body. It is present in almost every part of the body. The levels of Ber and its metabolites that can be detected in tissues are far higher than those that can be found in plasma (105) .

The biotransformation or metabolism of Ber in the body primarily occurs in the liver and the intestines, allowing its metabolites to be eliminated more readily (106). The primary routes through which Ber is eliminated from the body are feces, bile, and urine (15) .

Overall, the pharmacokinetic properties of oral administration of Ber are not promising. It is poorly absorbed and does not persist in the gastrointestinal tracts of animals or humans. As a result, efficient use of Ber as a medicinal drug remains a challenge.

1-3-5. Pharmacological activities of berberine:

1-3-5-1. General pharmacological effects of berberine

The alkaloidal structure of Ber is what's accountable for its diverse array of pharmacological effects. In the numerous experimental studies in animals, cell culture investigations, and clinical trials that have been conducted, Ber has been associated with a wide array of pharmacological effects (80). The Ber has anti-inflammatory properties both in vitro and in vivo, and it also inhibits the transcription of genes that contribute to inflammation, such as IL-1, TNF- α , and IL-6. Ber inhibits the production of both COX-2 and PGE2 (107). Ber is able to suppress NF- κ B signaling, which in turn stops cancer cells from producing IL-8 (108). Ber has been shown to be effective in inhibiting NO and TNF- α (107). In colorectal cancer cells, Ber reduces the activity of the COX-2 transcription factor (109). Because of these properties, Ber can be used to treat a range of inflammatory disorders, including rheumatism (110).

Indirect clinical studies of Ber showed that it lowered the levels of the enzymes alanine and aspartate transaminase in diabetic patients (111). It was thought to be an effective way to treat insulin resistance (112). Improvements in insulin secretion in people with poor β -cell function have been linked to the use of Ber, which has been shown to promote the regeneration of damaged or exhausted islets of Langerhans (113). Like

metformin, Ber can control a number of effectors, like mitogen-activated protein kinase (114). This in turn causes an increase in the initiation of glucose transport by the glucose transporter 1.

It has been established that Ber can reduce lipid levels through a variety of distinct ways. Ber, when mixed with other naturally occurring compounds such as plant stanols, enhanced the efficiency of decreasing cholesterol by limiting cholesterol absorption in a synergistic manner (115). Interestingly, it has been shown that the male diabetic mice who were fed a diet that was high in fat showed a substantial increase in cholesterol level after being exposed to the diet. Ber reduced the amount of total cholesterol found in the blood by 42% when it was administered orally at a dose of 250 milligrams per kilogram of body weight per day for a period of four weeks (116).

Ber has been shown to be effective in the treatment of a number of neurological diseases. it has been found to alter the brain's neurotransmitters and receptors. Ber has been shown to help with central nervous system disorders like Alzheimer's disease, anxiety, and depression (117). Ber is capable of inhibiting monoamine oxidase activity, a key target in the treatment of depression, and reducing depressive symptoms (118).

Ber has also been reported to help with gastric ulcers and other stomach issues. In an ethanol-induced gastric ulcer mouse model, Ber could successfully protect the stomach mucosa from injury (119).

Ber exhibits antibacterial, antifungal, antiviral, and antiprotozoal properties (76). Recent research has revealed that Ber is more effective against gram-positive bacteria than gram-negative bacteria. It has also been demonstrated to inhibit *Helicobacterium Pylori*, the bacteria that account for a large proportion of stomach and small intestine ulcers(120). Ber's antifungal properties have been demonstrated against fluconazole-resistant strains of

Candida and *Cryptococcus neoformans*, as well as the biofilm form of *Candida spp.* (121).

1-3-5-2. Berberine effects on cardiovascular system:

Many in-vitro experiments, in-vivo investigations on different animal models, and human clinical trials all point to Ber's ability to modulate cardiovascular functions. In an in vitro study on canine cardiac Purkinje and ventricular muscle fibers, Ber increases the action potential duration in Purkinje and ventricular muscle fibers in a concentration-dependent manner while having no effect on other action potential parameters. Ber is thought to work by blocking repolarizing currents and therefore increasing the effective refractory period of the myocardium (122). Another in-vitro investigation on isolated coronary arterial rings with ventricular fibrillation generated by an electric stimulus was carried out. Ber proved effective in preventing and treating spasms in the isolated part (123). The pathophysiology of cardiovascular diseases is driven by the aberrant proliferation of vascular smooth muscle cells (124). Ber has also been shown to suppress angiotensin IV-induced proliferation in cultured vascular smooth muscle cells via blocking the peroxisome proliferator-activated receptor α –nitric oxide signaling pathway (125) . As a result, berberine has been recommended as cardiovascular therapy (126).

Eighteen dogs with ischemia and left ventricular failure were used in another study. Ber's positive inotropic activity and modest systemic vasodilation were able to improve left ventricular function that had been compromised (127). It can work as a vasodilator where it has been shown to help patients with severe congestive heart failure(128). Ber has also been reported to have a hypotensive effect, where it efficiently reduces endothelial dysfunction, arterial pressure, and pathological changes in resistance vessel structure, resulting in lower blood pressure. Therefore, it

has been suggested to use it as antihypertensive phytochemical therapy (126).

The effects of Ber on 100 patients with ventricular tachyarrhythmias were investigated utilizing ambulatory monitoring for 24 to 48 hours. According to the data, 62 percent of patients had ventricular premature contractions suppressed by 50% or more, while 38% had suppression of 90% or more. There were no serious side effects, but some patients reported moderate gastroenterological issues (129). Furthermore, Ber has been shown to reduce vascular endothelial damage, aberrant lipid metabolism, chronic inflammation, plaque development, and cell death, all of which are factors in the development of atherosclerosis. As a result, it has the potential of reducing atherosclerosis (126). Cardiovascular and metabolic effects of Ber are now being studied in human clinical trials (77).

Many mechanisms for Ber's actions on cardiovascular system have been postulated. First, Ber's antiarrhythmic properties are related to its potassium channel blocking properties (130). Second, Ber acts as a Ca^{2+} channel agonist (131). Third, Ber also appears to have an inhibitory effect on catecholamine production via lowering tyrosine hydroxylase activity (132). Fourth, Ber's cardiovascular effects can be attributed to the blockage of ATP-sensitive potassium channels and delayed rectifying, as well as the stimulation of $\text{Na}+\text{Ca}^{2+}$ exchange pathways (117). Fifth, Ber also was postulated to modulate the Nitric oxide–cyclic GMP pathway, the angiotensin-converting enzyme system via affecting the peroxisome proliferator-activated receptor signaling pathway, α –nitric oxide signaling pathway, and it might involve in the 1-adrenoceptor blockade (125). Six, Ber can also modulate the ventricular action potential and make it last longer (133). It's worthwhile that Ber can also inhibit platelet function by a number of postulated mechanisms, including increased thrombolysis,

thromboxane generation, calcium influx suppression, and platelet activation via the 2-adreno-receptor (134).

1-3-5-3. Anticancer activity of berberine:

Ber has been shown to have antitumoral efficacy against a range of tumor types (79) . Ber is effective in the treatment of solid tumors and hematological malignancies (135). Ber's anticancer efficacy stems from its ability to affect multiple stages of tumor development, including cell proliferation, invasiveness, and cell death, as well as alteration of cell signaling pathways. Ber has also been shown to improve the efficacy of radiation and chemotherapy. Ber has been shown to help in diminishing the multidrug resistance issues in cancer treatment (79).

1-3-5-4. Chemoprotective effects of berberine:

Ber appears to protect cardiac tissues from DIC in a growing number of in vitro and in vivo studies. In the Dox-treated mice, there was an increase in mortality, an initial fall in body weight, elevated lactate dehydrogenase (LDH) activity, heart rhythm abnormalities, and increased myocardial damage. Pretreatment with Ber considerably reduced these alterations (136). Another study revealed that 20 minutes of pretreatment with Ber (0.06, 0.25, 1.0, 4.0 M) protects rat cardiomyocytes cultured in vitro from acute damage caused by Dox (1 μ M) (137). In addition, it was reported that Ber protects cardiac tissues against DIC damage by suppressing Dox metabolism in the heart cytoplasm and minimizing the accumulation of doxorubicinol, a secondary alcohol metabolite of Dox, in cardiac tissues (138). Likewise, Ber showed protective benefits against Dox-induced cardiac tissue free radical injury in a rat model and cultured cardiomyocytes, likely by suppression of intracellular Ca²⁺ increase and reduction of mitochondrial dysfunction (139).

In vitro and in vivo studies showed that Ber reduces the risk of heart injury induced by Dox through inhibiting p66Shc, a pro-apoptotic protein

that controls oxidative stress and enhances the mitochondrial signaling pathway, by raising sirtuin 1 expression, indicating a novel mechanism for Ber's cardiomyopathy prevention (140).

Ber, on the other hand, successfully protects hepatocytes from the damage caused by common anticancer drugs such as cyclophosphamide and methotrexate which causes oxidative stress, hepatic inflammation, hepatocellular degeneration, and congestion, as well as an increase in liver enzyme production (such as alanine transaminase and aspartate transaminase). Ber, which possesses powerful antioxidant properties, stimulates the production of antioxidant enzymes such as catalase, superoxide dismutase, glutathione peroxidase, and malondialdehyde. As a result, the level of oxidative stress in the liver that is caused by chemotherapy is significantly reduced. Ber also protects the hepatic architecture while lowering liver enzyme levels (141). Ber can also be suggested to have a nephroprotective effect as there is evidence that Ber can successfully protect kidneys from the severe damage produced by the chemotherapeutic medication cisplatin in rats. It improves the activities of antioxidative enzymes in renal tissues, lowering blood urea nitrogen, creatinine, malondialdehyde, nitric oxide, protein carbonyl levels, and myeloperoxidase activity, reducing oxidative stress and nephrotoxicity (142).

1-3-6. Berberine toxicity:

The acute toxicity test is the initial step in assessing harmful effects, and it takes place over the course of 14 days after a single dose has been administered. As a measure of a substance's inherent toxicity, it provides an estimate of the lethal dose 50 (LD50). The mouse and rat are the most popular subjects(143). The LD50, the dose of a substance that kills 50% of test animals, value for Ber varies with the concentration of berberine in a given chemical, route of administration, and organism type (144). The oral

LD50 values for pure Ber in mice and rats are 329 and 1500 mg/kg respectively (144). When administered orally, the LD50 values for the root extract fraction of *B. vulgaris* in rats and mice are 1,280 mg/kg and 520 mg/kg, respectively (144). On the other hand, administering Ber sulfate at a dose of 50 mg/kg causes diarrhea in forty percent of rats, which has a direct impact on the digestive tract (145).

1-4. Nanoformulation:

Nanotechnology was coined in 1959 as the study and application of engineering principles to the creation of useful materials, surfaces, and devices with at least dimension on the order of 1–100 nanometers (146). Because the diameters of biologically important basic components like amino acids, carbohydrates, nucleotides, and proteins are all in the nanoscale range and the majority of biological activities occur at the nanoscale (less than 100 nm), interactions between biological systems and nanomaterials are promoted (146).

When it comes to enhancing the therapeutic efficacy of a certain medication, nanocarriers play a pivotal role in facilitating the delivery of that chemical. Nanocarriers have a wide variety of applications, but the following are among the most significant:

- 1- They help pharmaceutical industry create more effective medicines by increasing the solubility of hydrophobic compounds in water (147).
- 2- They have the capacity to improve the stability of compounds that are particularly susceptible to degradation (148).
- 3- They can permit the development of highly targeted and localized delivery systems (149).
- 4- They have the ability to improve permeability and cellular uptake, enhance drug retention in tissues, and protect against proteolytic enzymes as benefits of this strategy (150).

5- It is possible for an internal stimulus (biomarker, pH, or redox) or an exterior stimulus (temperature, light, radiation, or ultrasounds) to initiate and regulate the controlled release of a chemical loaded in a nanocarrier (151).

6- The most recent use of nanotechnology is in mRNA delivery, as demonstrated by the delivery of an mRNA-based vaccination against SARS-CoV-19 using lipid nanoparticles. The use of nanotechnology in this context is cutting edge(152).

7- Nanocarriers have been the subject of intense research due to their potential as diagnostic agents (153).

Depending on the material employed in their manufacture, nanoscale materials can be categorized as inorganic, lipidic, peptide-based or virus-like nanocarriers, and polymeric nanoparticles (154) . Examples of inorganic nanocarriers that can react to external stimuli are gold nano rods and gold, silver, or metal oxide nanoparticles. Lipid-based formulations such as liposomes, spherical self-assembling colloidal entities with lipid bilayers, and an aqueous center. solid lipid nanoparticles are other examples. Protein structures known as virus-like particles (VLPs) and nanoparticles (NPs) are able to mimic the appearance of wild-type viruses, but they do not possess a viral genome or the ability to cause infection. This makes them, in practice, safer candidates for vaccines (155). Polymeric nano-systems are made from either naturally occurring or synthetic polymers. there are several forms of polymeric nano-systems such as nanocapsules, micelles, and dendrimers each with its own distinct characteristics (154). Typically, polymeric nanoparticles are divided into two kinds: hollow and core shell (156). Drugs are either conjugated with the polymer prior to nanoparticle creation, as is the case with hollow nanoparticles, or they are encapsulated in the very porous polymer matrix of hollow nanoparticles. examples of hollow nanoparticles are poly lactic-

co-glycolic acid (PLGA) and chitosan which are both commonly utilized as polymers (157). However, the drug loading capacity of these nanoparticles is low, and their nanostructures are relatively unstable. On the other hand, core-shell nanoparticles are comprised of two or more distinct polymer components forming the core and the shell. They have better physicochemical stability and decreased cytotoxicity. Silica-coated chitosan nanoparticles and poly (methyl methacrylate) (PMMA) /chitosan core-shell nanoparticles are examples of core-shell nanoparticles (158) . It is possible to enhance therapeutic agent encapsulation by customizing the nanoparticle's core substance.

1-4-1. Polymeric micelles:

Approximately half of the approved active compounds have poor solubility in the physiological aqueous environment, resulting in restricted bioavailability and gastrointestinal absorption (159). In addition to being a challenge for the development of injectable and even topical formulations, poor solubility poses a problem. Since increased solubility is predictive of increased bioavailability, researchers are investigating a variety of nanotechnological approaches to solubilizing drugs in an effective manner (160). One of the nano-oriented tactics studied to increase the solubilization, stability, and bioavailability of medicinal agents is the integration of hydrophobic compounds within polymeric micelles. Polymeric Micelles are usually made of amphiphilic di- or tri-block copolymers with both hydrophilic and hydrophobic parts (161). They are capable of self-aggregation. Below a critical micellar concentration, often known as CMC, these polymers exist in solution as free molecules; however, once they pass CMC, they begin to self-assemble into micelles that have a hydrophilic coating surrounding a lipophilic core (162). These self-assembled micelle structures are capable of preserving insoluble hydrophobic drugs; their structure and function mirror characteristics of

biological transport systems. As a result, systems are formed with specific attributes relative to other nanocarriers, such as reduced size where they have a size on the nanometer scale, often less than 100 nanometers that enables passive targeting to poorly-permeable targets(162).

Due to their tiny size, ease of synthesis, and high solubility, their hydrophobic cores, are well-suited for solubilizing water-insoluble molecules, protecting unstable compounds from chemical and/or biological degradation, and for providing prolonged release in a variety of compositions. Polymeric micelles are attractive carriers for loading drugs. They can increase their bioavailability, prolong circulation durations, and generate a regulated and targeted drug release, which is beneficial for decreasing adverse effects (163). They can also improve permeability and retention where the internalization within cells is improved, allowing for greater efficiency (164). Intravenous (IV) injection or infusion is the preferred method of administration for micelles, for which most studies have been conducted (165). Typically, chemotherapy is administered by this method. However, they can be utilized for practically all routes of administration (166). Polymeric micelles are adaptable, stable, safe, and relatively cost-effective nanovectors thus they are gaining popularity (167).

For the first time, a micelle formulation of the chemotherapeutic drug paclitaxel has been approved by the FDA for use in the treatment of breast, ovarian, and lung malignancies (168). The lipophilic core enhances the solubility of weakly water-soluble molecules like resveratrol and quercetin, as demonstrated in the literature (169). Whereas, the hydrophilic coating protects the encapsulated drug from the external medium, resulting in increased bioavailability and prolonged circulation (168). Among such micelles is the pluronic polymeric micelles which are a type of smart material that exhibits micelle-formation capabilities (170).

1-4-1-1. General properties of polymeric micelles:**1-4-1-1-1 Shape:**

Micelles are typically shown as spherical structures; however, it is possible to observe rod-like, worm-like, and even disk-like shapes (171). The changes in micellar shape are mostly attributable to the polymer structure and to the temperature, pH, and chemical content of the surrounding environment(172).

1-4-1-1-2. Size:

Polymeric micelles can have a size that is greater than 100 nm while still being considered to be micelles (173). It has been demonstrated that micelles in the size range of 30 to 100 nm can readily aggregate in highly permeability tumors. The smaller the size, the greater the ability to penetrate. For example, micelles with a size of 30 nm can penetrate less permeable tumors in an efficient way, demonstrating the importance of size (173).

1-4-1-1-3. Surface properties:

Surface properties affect micelle behavior. Hydrophilic and neutral surfaces diminish interacting of serum proteins with micellar corona and increase intravenous circulation time. They also improve the micellar ability to penetrate mucus (174). However, positively charged nanocarriers are mucoadhesive, meaning that they interact favorably with epithelia (174).It has been reported that micelles with a positive zeta potential have a very low stability in body fluid as a result of non-specific protein complex formation and an enhanced tendency to aggregate in vivo (175).

1-4-1-1-4. Ability to internalize cells:

A micelle is an extremely important component in the process of delivering drugs to their intended subcellular destination. Micelles often enter cells by a process known as endocytosis. This process begins when a

micelle binds to the cellular membrane and continues with uptake and translocation through endosomes to the cytoplasm of the cell that is receiving it (176). Micelles have the ability to be taken into cells in their original state, but they also have the potential to release the drug outside of cells or to be carried into cells as deconstructed unimers and lysed in lysosomes producing drug accumulation in plasma membrane or cellular compartments (177). Because micelles are able to easily bypass the energy-dependent efflux pumps(P-gp) that are a contributing factor in drug resistance and multidrug resistance, endocytosis-mediated uptake has proven to be a viable technique for addressing these issues (178).

1-4-1-2. Pluronics:

Pluronics® is a brand name for a category of water-soluble nonionic triblock copolymers known as poloxamers that were first produced by BASF. Currently, they are utilized extensively in a multitude of fields, including the fabrication of nanoparticles as well as drug delivery (179). The copolymer blocks are composed of chemically different monomers (polar and non-polar) include the polyethylene oxide (PEO) monomers which are water-soluble and the polypropylene oxide (PPO) monomers which are water-insoluble. As a result, the PEO blocks are hydrophilic whereas the PPO blocks are hydrophobic; hence, the block copolymers are amphiphilic, resulting in surface-active characteristics (180) . Pluronics are available in a number of forms, each of which has a unique molecular weight and ratio of hydrophobic to hydrophilic building blocks (181). BASF's nomenclature for Pluronics® consists of a letter representing the physical state (P for Paste, F for Flake, and L for Liquid), one or two digits representing the molecular weight, and a final number representing the weight of the PEO block (182). Mainly, low bioavailability and water insolubility impede the clinical use of many effective medications thus

pharmaceutical biotechnology improves medication absorption and stability, therapeutic effectiveness, and bioavailability by creating optimal polymers such as the development of micellar block copolymer-drug complexes(183). Pluronic F127, also known as Poloxamer 407, is one example of tri-block amphiphilic synthetic copolymers. These copolymers are made up of a chain of PPO that is surrounded by two chains of PEO (Figure 1-3).

Pluronic F127 improves the pharmacokinetics and pharmacodynamics of poorly water-soluble medications by increasing their oral bioavailability and stability and circulation time. The capacity of pluronic F127 to dissolve hydrophobic solutes has sparked a great amount of interest in controlled-release medication administration and other application areas(184). The FDA has given its approval for the use of pluronic F127 as an excipient in oral, ophthalmic, and topical pharmaceutical formulations (185). Due to its low immunogenicity and toxicity, Pluronic F-127 has been the most commonly used in controlled release systems (186).

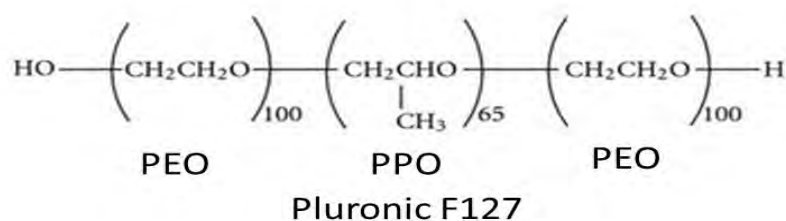


Figure 1-3: Pluronic F-127 chemical structure (180)

1-4-1-2-1. Micellization of pluronic F-127 and chemicals loading:

Micellization process of Pluronic F-127 can be begin when the concentration of PEO-PPO block copolymer in solution exceeds the critical micellization concentration (CMC). Below the CMC and at room temperature, the copolymer is existing in form of unimers. Unimers are block copolymer that are not linked, as opposed to supramolecular chains(187). Reduced solubility induces microphase separation of PPO

blocks from the aqueous environment into the micelle core. PEO and PPO blocks are covalently connected to a copolymer chain, allowing for the self-assembly. Two stages create micelles that are thermodynamically stable: initially, unimers rapidly combine into metastable aggregates (less than millisecond) then, after 1–100 ms, aggregates relax into thermodynamically stable micelles(188). In an aqueous solution, poloxamer micelles are generally spherical and core-shell. The micelle core is hydrophobic and composed of PPO blocks, while the corona is hydrated PEO (188). In order for hydrophobic drug molecules to become encapsulated in hydrophobic PPO cores, it is necessary to raise the temperature of the reaction and combine hydrophobic drug molecules with the quantities needed for pluronic molecules. The nontoxic hydrated PEO coronas prevent the loaded substance from being removed from the core of the micelles. enabling hydrophobic medicines to be more soluble in water, increasing their bioavailability (189). The passive accumulation of pharmaceuticals loaded in Pluronic micelles is more effective than the free drugs. This is due to the prolonged circulation duration and progressive dissociation of drug-encapsulated micelles in the blood. Pluronic F127 micelles can facilitate the transport of drugs through the blood-brain and intestinal barriers (190). The pharmacological properties of pluronic F127 micelles make them an important drug carrier.

1-4-1-2-2.Pluronic F127 improves delivery of low soluble phytochemicals:

Because of their poor water solubility, low lipophilicity, unsuitable molecular size, structural instability in the biological environment, rapid clearance, and high metabolic rate, natural products present significant difficulties when applied to therapeutic settings. In addition, some natural products are inactivated by gastric contents or undergo substantial first-pass action and pre-systemic metabolism in the liver after oral administration before they reach the systemic circulation. Moreover, Reduced

bioavailability is also linked to the accumulation of some natural products in non-targeted tissues, which raises undesired side effects and severely diminishes therapeutic efficacy (168).

Pluronic F127 is frequently utilized because of its ability to increase natural products solubility and stability, it improves their therapeutic bioavailability as well as distribution. Resveratrol is a plant-based phytoalexin with significant biological action, however, it is insoluble in water, reducing its therapeutic use. Emulsion synthesis created stably loaded nanoparticles from Pluronic F127 block copolymer and D-alpha-tocopheryl polyethylene glycol 1000 succinate (Vitamin E-TPGS). Forming a highly soluble formulation. The formed nanoparticle successfully targeted aggressive breast cancer cell lines and reduced cell viability (191). In addition, Pluronic F127 micelles were developed to incorporate resveratrol. Its ability to kill bacteria was tested on *E. coli*, *S. aureus*, and *Candida albicans*. The cytotoxicity was tested on a bladder cancer cell line. The results showed that pluronic F127 micellar nanostructures significantly improve resveratrol's cytotoxic and antibacterial properties of resveratrol (192). The loading of curcumin into Pluronic F127 micelles led to improvements in the solubility, stability, permeability, and controllability of curcumin's release (193).

It is important to note that the combinations of resveratrol and quercetin or curcumin in pluronic F127 micellar not only reduce the cardiotoxicity caused by doxorubicin but also proved to be chemosensitizing combinations (194). Moreover, an increase in oral bioavailability of the genistein, poorly water-soluble phytoestrogen, was observed in rats when the chemical was provided as a loaded substance within Pluronic F127 micelles (195). Thus the Pluronic F-127 micelle is an

interesting candidate for use as a medication delivery tool in the research and development of delivery systems for less soluble phytochemicals.

1-4-2. Nanoformulation-based berberine delivery:

Nanotechnology-based methods of drug delivery have been in use for almost a decade, and nanoformulations containing Ber have been shown to be an effective therapy strategy for a wide range of conditions (196).

Polymeric materials have been utilized in Ber delivery. Natural polymers such as dextran, chitosan, and synthetic polymers such as polylactide glycolic acid (PLGA) nanoparticles have been utilized in Ber delivery (197). Dextran was used to encapsulate Ber, aliphatic hydrocarbon chains were added to amphiphilic dextran in order to encapsulate Ber. Its effect on diabetes was evaluated in rat hepatocytes. Compared to free Ber, these nanoparticles decreased Bcl-2 protein, which is an anti-apoptotic protein, and ROS production significantly(198).

Polymeric micelles are a remarkable delivery strategy for substances that have a low degree of solubility. Micelles for Ber encapsulation were produced by using 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethyleneglycol)-2000] coupled with d-tocopheryl polyethylene glycol 1000 succinate [TPGS]. These micelles were then used to increase the delivery of Ber to tumors. Ber was more soluble in water, and the produced micelles were particularly stable, safe, and they released Ber in a controlled released fashion. These loaded micelles were cytotoxic to human prostate carcinoma cells and caused them to undergo apoptosis. This is because TPGS remarkably improves Ber's ability to internalize treated cells and it enables the block of P-gp efflux pumps, thus eliminating the barrier that prevents drugs from working (199).

Liposomes as nano-delivery carriers were also used to improve Ber pharmacokinetic issues. The antileishmanial activity of the Ber was

significantly increased after the delivery of liposomal Ber ; however, their clearance remained high (200).

Ber can be delivered to solid tumors by bioengineered gold nanoparticles. yielding a high Ber loading capacity (86%) (201).

1-5. Research Problem:

Dox's propensity for heart problems poses a significant barrier to its clinical application (202). Hence, it is imperative to discover a strategy for reducing the cardiotoxic effects of Dox while concurrently enhancing the drug's anticancer effectiveness. Numerous in vitro and animal studies have indicated that Ber may possess the ability to protect cardiac tissues against damage caused by Dox(14). Additionally, other investigations have observed its potential anticancer properties (79). In order to maximize Ber's therapeutic potential, it is necessary to resolve problems with solubility and bioavailability by creating effective dosage forms or implementing novel drug delivery techniques.

1-6. Research Hypothesis:

Given the hydrophobic nature of Ber, we hypothesize that it could be incorporated within the hydrophobic core of Pluronic F127 micelles via hydrophobic interactions, thereby improving its solubility, stability, and cellular permeability. The generated micelles have the potential to be delivered concurrently with Dox to be used as a promising cardioprotective strategy by enhancing the cardioprotective activity of Ber against DIC by reducing oxidative stress and mitigating inflammation and apoptosis that are commonly associated with Dox therapy. In addition, the combined treatment of Ber loaded into Pluronic F127 micelles with Dox will not only minimize the cardiotoxicity caused by Dox but also serve as a novel approach for tumor therapy.

1-7. Aim of the study:

The objectives of the present study include:

- 1-Preperation and characterization of Ber loaded physically entrapped Pluronics F127 micelles.
- 2- Assessment of in-vitro release of Ber from prepared micelles.
- 3- Evaluation of mBer toxicity and biocompatibility via an in vitro hemolysis assay
- 4- Determination of in vitro cytotoxicity of the mBer utilizing cardiomyocytes and ovarian cancer cell lines as research models
- 5- Assessment of the cardioprotective effect of Ber with respect to the concentration of Dox seen in human plasma following the administration of therapeutic doses of Dox.

Chapter Two

Materials and Methods

2-1. Material:**2-1-1. Instruments and equipment used in the experiments:****Table 2-1: The types and sources of laboratory instruments-equipment used in the experiments.**

List	Instruments and equipment	Manufacturer	Origin
1	Sensitive balance	Memmert	Germany
2	Ultrasonic Homogenizer Sonicator	Yuchengtech	China
3	Round-bottom flasks	Quickfit	United Kingdom
4	Rotary evaporator	Labtech	Korea
5	vacuum drying oven	LabsNova	China
6	0.22µm nylon filter.	Thermo Fisher Scientific	USA
7	Zetasizer-3000 instrument	Malvern Instruments	United Kingdom
8	TEM-100CX electron microscope	JEOL	Japan
9	Nicolet 6700 FT-IR Spectrometer	Thermo Fisher Scientific	USA
10	X-ray diffractometer	Bruker	USA
11	Uv-visible Spectrophotometer	Sunny	Japan
12	pH meter	Hanna	Korea
13	Water bath	Termaks	Korea
14	Dialysis cassettes	Thermo Fisher Scientific	USA
15	96 Well Cell culture plates	Thermo Fisher Scientific	USA
16	Deep Freezer	HMG	India

List	Instruments and equipment	Manufacturer	Origin
17	Centrifuge	Eppendorf	Germany
18	Microscope slides	Biomark	Canada
19	cover slip	pearl	China
20	ELISA reader	Micro Lab Instruments	India
21	ELISA washer	Micro Lab Instruments	India
22	CO2 incubator	Vtech	India
23	Cold centrifuge	Eppendorf	Germany
24	Eppendorf tubes	Sigma-Aldrich	USA
25	Incubator	Memmert	Germany
28	Light microscope	Olympus	Japan
29	Micropipettes	Eppendorf	Germany
30	a GloMax® microplate reader	Promega	USA
31	Cold centrifuge	Eppendorf	Germany

2-1-2. Names of Material and Chemical Solution:

Table 2-2: Material and Chemical Solution utilized in the experiments.

List	Name of Material and Chemical Solution	Manufacturer	Origin
1	Berberine hydrochloride	Sigma-Aldrich	USA
2	Pluronic F127	BASF	USA
3	Doxorubicin Hydrochloride	Sigma-Aldrich	USA

List	Name of Material and Chemical Solution	Manufacturer	Origin
4	Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	USA
5	Defibrinated rabbit blood	Thermo Fisher Science	USA
6	Penicillin-Streptomycin	Sigma-Aldrich	USA
7	Fetal Bovine Serum	Sigma-Aldrich	USA
8	Amphotericin B	Sigma-Aldrich	USA
9	Sodium pyruvate	Sigma-Aldrich	USA
10	Glutamine Solution	Sigma-Aldrich	USA
11	Dulbecco's Modified Eagle Medium (DMEM)	Capricorn Scientific	Germany
12	Sodium bicarbonate	BDH chemical Ltd	England
13	Trypsin-EDTA solution	Sigma-Aldrich	USA
14	Methanol	Sigma-Aldrich	USA
15	Phosphate Buffer (PBS)	Elabscience	China
16	Ethanol	Sigma-Aldrich	USA
17	H9c2 cell line	ATCC	USA
18	SKOV-3 cell line	ATCC	USA
19	Glycine	Sigma-Aldrich	USA
20	MTT Cell Growth Assay Kit	Sigma-Aldrich	USA
21	CyQUANT™ LDH Cytotoxicity Assay	Thermo Fisher Scientific	USA
23	Rat CKMB(Creatine Kinase MB Isoenzyme) ELISA Kit	Elabscience	China
24	Rat TNNI3/cTn-I(Troponin I Type	Elabscience	China

List	Name of Material and Chemical Solution	Manufacturer	Origin
	3, Cardiac) ELISA Kit		
25	MDA Content Assay Kit	Sunlong Biotech	China
26	Reduced Glutathione (GSH) Assay Kit (Colorimetric)	Sigma-Aldrich	USA
27	Catalase(CAT) Activity Assay Kit	Sunlong Biotech	China
28	Superoxide Dismutase(SOD) Activity Assay Kit	Sunlong Biotech	China
29	Rat IL-1 β ELISA Kit	Beyotime Institute of Biotechnology	China
30	Rat IL-6 ELISA Kit	Beyotime Institute of Biotechnology	China
31	Caspase-Glo® 3/7 Assay System	Promega	USA

2-2. Preparation of Ber-loaded micelles: Ber in pluronic F127 micelles (mBer) was prepared by a slight modification of the thin film hydration method (203). In order to get better encapsulation efficiency of the mBer, we prepared different micelle compositions by using different weights of Ber (1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 mg), while the other conditions in the micelle preparation are fixed. Shortly, a fixed amount of pluronic F127 (100 mg) and one of the variable weights of Ber mentioned above were both dissolved in 20 mL of methanol (99% purity) in rounded bottom flask, followed by sonication until complete dissolve. Next, to remove alcohol, the flask was placed on a rotary evaporator (45 °C) for a few min until a homogenous film was formed. In order to enhance the elimination of any remaining alcohol, the resulting film was subjected to an overnight vacuum

drying process at ambient temperature. Subsequently, the film was subjected to rehydration using 30 mL of deionized water at a temperature of 37°C for a duration of 1 hour, accompanied by gentle agitation. This was followed by a period of mild sonication lasting 2 minutes. To remove undissolved agents or contaminants, the resultant micellar solution was filtered through a 0.22µm nylon filter.

2-3. Characterization of Ber-loaded micelles:

The characterization study was conducted at the Research Institute for Biotechnology and Bioengineering, Isfahan University of Technology, Isfahan, Iran.

2-3-1. Assessment of the drug loading content and the retention efficiency:

The Ber encapsulated within polymeric micelles had to be extracted first with 100% ethanol, and then the concentrations were measured using an UV-vis spectrophotometer set to 350 nm. Standard solutions of Ber dissolved in 100% ethanol at concentrations ranging from 1 to 10 mg/mL were used to calibrate the content determination method. Freshly prepared mBer was assessed to determine the initial drug loading capacity and retention efficiency of the micelle. The data was recorded as four replicates. The retention efficiency (RE) and the drug loading content (DL) were determined using the following formulas (180):

$$RE (\%) = \frac{\text{mass of drug loaded in micelles}}{\text{mass of drug initially added}} \times 100$$

$$DL (\%) = \frac{\text{mass of the drug loaded in micelles}}{\text{mass of the feeding polymers and drug}} \times 100$$

2-3-2. Determination of micelle zeta potential, particle size, and polydispersity index:

They were assessed based on a method referred to as dynamic light scattering (DLS). The formulations of the tested samples were initially

diluted with deionized water in order to achieve a final concentration of F127 at 200 $\mu\text{g/mL}$, and the pH was 6.8. The examined samples were thereafter positioned inside the electrophoretic cell of the Zetasizer-3000 instrument. The equilibration of the instrument was done at room temperature for 2 min, then the He-Ne laser was fixed at 90° and adjusted at 633nm (204). The mean values of the zeta potential, which is a parameter that assesses the electrochemical equilibrium at the interface between a particle and a liquid, were estimated. It allows for the evaluation of nanoparticles' surface charge and predicts the physical stability of particles in dispersion systems and their tendency to interact with other molecules as it measures the magnitude of electrostatic repulsion or attraction between particles (205). The hydrodynamic size of the particles and the polydispersity index (PDI) were also measured. All the measurements were conducted on four replicates.

2-3-3. Morphological analysis of the created micelle by transmission electron microscopy(TEM):

TEM was employed to look into the formulation's morphology. The micelles were imaged using a JEM-100CX electron microscope (203).

2-3-4. Fourier transform infrared spectroscopy:

To assess the state of Ber inside polymeric micelles. The infrared spectrograms were obtained by employing the Nicolet 6700 FT-IR spectrometer, utilizing the KBr technique, within the spectral range of $4000\text{--}400\text{ cm}^{-1}$. FTIR spectra of Ber, mBer, and the corresponding void micelles were acquired at ambient temperature (203).

2-3.5. X-ray diffraction:

Diffraction patterns of Ber, mBer, and corresponding empty blank micelles were recorded using an X-ray diffractometer (XRD). The conditions of the analysis were set as follows: voltage 40 kV, current 30 mA, and diffraction patterns were collected with 2θ at an angle range of $5\text{--}70^\circ$ (206).

2-4. Assessment of in vitro release of Ber from prepared micelles:

The evaluation of Ber release from loaded micelles was conducted in pH 7.4 conditions in order to simulate physiological circumstances. A predetermined quantity of mBer was evenly distributed into a 4 mL solution of phosphate-buffered saline (PBS). Subsequently, a volume of 2 mL of mBer was introduced into a dialysis cassette possessing a molecular weight of 14,000 Da to ensure that both free Ber and the free polymer were diffused out of the cassette. Next, both ends of the bag were sealed and then immersed in 50 mL of PBS (pH 7.4). The samples were maintained at 37 °C and shaken at 100 rpm. At the appropriate intervals (0, 0.5, 1, 2, 3, 6, 10, 12, 24, and 48 h), a volume of 100 µL was taken out of the release medium. Subsequently, an equivalent volume of fresh medium was introduced into the release medium. UV-visible spectrophotometry was employed to determine the concentration of Ber in each sample

2-5. Evaluation of micelles' toxicity via in vitro hemolysis assay:

A hemolysis test was performed on empty micelles, free Ber, and mBer samples. The micelles' toxicity was measured based on the principle of hemoglobin release from erythrocytes, as previously reported (199,207). The defibrinated blood of a rabbit was diluted tenfold with PBS and subsequently, the sample was subjected to centrifugation at a speed of 2,000 (rpm) for a duration of 15 minutes. The supernatant was removed, and the precipitate was rinsed thrice using a PBS. Subsequently, it was centrifuged for 15 minutes at a speed of 2,000 (rpm). To achieve a concentration of 2% (v/v percent) in the blood cell mixture, 50 microliters of the samples to be tested were mixed with 500 microliters of blood cells. The resulting suspensions were then incubated at a temperature of 37°C for a duration of 3 hours, simulating a physiologically appropriate dilution setting for intravenous injection (with a concentration of 1µM of Ber). As a negative control for this experiment, red blood cells suspended in

physiological saline (0.9% sodium chloride) indicated no hemolysis, but erythrocytes suspended in deionized water produced 100% hemolysis and were considered a positive control. The following equation was used to calculate the hemolysis percentage (199):

$$\text{Hemolysis percentage} = \frac{[\text{Test sample absorbance} - \text{Negative control absorbance}]}{[\text{Positive control absorbance} - \text{Negative control absorbance}]} * 100.$$

2-6. Tissue culture: In flat bottom 96-well cell tissue culture plates, H9c2 rat embryonic cardiomyoblast cell line (ATCC® CRL-1446™), and SKOV-3 Human Caucasian Ovarian Adenocarcinoma cell line (ATCC® HTB-77™) were cultured separately in Dulbecco's modified eagle medium (DMEM) which was supplemented with 10% fetal bovine serum (FBS), 1% of penicillin 10,000 international unit and 10 mg streptomycin, and 25 µg amphotericin B. For maintenance, 1 mM sodium pyruvate and 2 mM glutamine were also added to culture media. The cultured cells were incubated at 37°C in an air atmosphere with 5% CO₂ for 24 h after being seeded into each well at a density of 5.0×10^3 cells per well and the cellular attachment to the well bottom was achieved. The workplace is the Iraqi Center for Cancer Research and Medical Genetics.

2-7. Cell culture viability assay, and IC₅₀ calculation

2-7-1. Experimental groups: Both types of cell lines were subdivided into seven treated groups according to the received treatment. Experimental groups were treated separately with 1 µL of Dimethyl sulfoxide (DMSO) and contained various serial concentration dilutions of either: free Ber, Berberine-loaded Pluronic F127 Micelles (mBer), Dox, Bre-Dox, or mBer-Dox combination, at a constant molar ratio of 1:1, and void nanomicelles. Another set of cells that serve as the solvent control were treated only with PBS containing 0.5% DMSO. All the wells had a 0.5% final concentration

of DMSO. Concentrations of Ber or mBer used were varied from 25 – 1600 μ M and 2.5-40 μ M in H9c2 cells and SKOV-3 cells respectively.

Dox concentration varied from 0.125 – 8 μ M and 0.0125-0.6 μ M in H9C2 cells and SKOV-3 cells respectively. Ber-Dox and mBer-Dox concentrations were ranged from 0.125 – 8 μ M and 0.0125– 0.6 μ M in H9c2 cells and SKOV-3 cells respectively, and for void micelles were ranged from 2.5 -1600 μ M in both cell lines. After the treatment, the plates were incubated for another 48 hours, and the MTT assay was performed to determine the level of cell viability.

2-7-2. MTT assay: The Colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test was used to evaluate cells viability based on their metabolic activity. Normal viable cells have NAD(P)H-dependent cellular oxidoreductase enzymes, mainly located in the mitochondria and other organelles such as endoplasmic reticulum, which can reduce a yellow tetrazolium salt into insoluble purple crystal compound called formazan. The resultant formazan crystals can be solubilized by adding DMSO, resulting in a colored soluble solution that can be quantified by measuring the absorbance using a microplate reader. The number of viable cells is proportional to the rate of tetrazolium reduction (208).

The assay was performed by adding 10 μ l of the MTT reagent of 5 mg/mL to each well containing cultured cells and the cells were incubated further under the same conditions for 4 h. Next, the supernatant will be removed using multichannel pipette followed by adding 150 μ L of DMSO and 25 μ l of glycine to each well with well mixing to lyse the cells and ensure complete solubilization of the formazan crystals. Using a microplate (ELISA) reader, the 492 nm absorbance of tested cells and control cells was used to assess the relative proportion of inhibited cells, assuming

100% survival from the vehicle-only control. The percentage inhibition of treated cells was estimated using the following formula:

$$\text{Percentage of Inhibited Cells} = 100 - (\text{Treated Cells Absorbance} / \text{untreated Cells Absorbance}) \times 100$$

The half-maximal inhibitory concentration (IC₅₀), which shows the dose required to produce a 50% inhibition of cellular viability, was calculated for each drug alone or in combination. The results of the experiment were produced by plotting the measured absorbance after various treatments against the different concentrations of the test substances using the linear regression analysis. The quadruplicate was performed for all concentrations in all treated groups.

2-7-3. Combination index (CI) calculation: At the same concentrations used in the experimental groups for the calculation of the inhibitory concentrations and IC₅₀ for Ber, Dox, Ber-Dox, and mBer-Dox utilizing the MTT test, fractions of affected cells (fa) vs. combination index (CI) graphs for Ber and Dox in combination in two different cell lines, H9c2 and SKOV-3, were also constructed utilizing CI analysis (209,210). CalcuSyn software (version 2, New jersey, USA) was used to analyze the CI equation algorithms. The CI can be estimated for different fractions of affected cells (Fa). The benefit of showing CI versus Fa is that it displays the interaction between Dox and Ber in combination at all affected cell levels (1 to 99%).

We got the CI equation for the two drugs as follows:

$$CI = (Ber1/BerX) + (Dox1/DoxX)$$

where Ber1 and Dox1 stand for concentrations of Ber and Dox alone, respectively, to exert a certain effect. BerX and DoxX display the concentrations of Ber and Dox respectively in the combination that has the same effect.

When the CI is 1, there is additive interaction; when it is <1 , there is synergy; and when it is >1 , there is antagonism (209).

We used the same principle to figure out how Dox and mBer interact in the two different cell lines by calculating the CI value and comparing it to how Dox and free Ber interact.

For each H9c2 and SKOV-3 cell line, we generated a plot of mean CI versus fa data that is representative of four replicates.

2-8. Evaluation of the cardioprotective effect of mBer on H9c2 cells treated with 1 μ M Dox:

2-8-1. Experimental groups:

H9c2 cells were subdivided into seven treated groups based on the received treatment, and each group was treated separately with 1 μ L of Dimethylsulfoxide (DMSO) at a final concentration of 0.5% in each well. In this study, the experimental groups included the control group, Ber-treated group, berberine-loaded pluronic F127 micelles (mBer)-treated group, Dox-treated group, Ber-Dox combination, and mBer-Dox combination-treated groups at 1:1 molar ratio, as well as the void F127 micelles-treated group (Em). All groups received treatment and were subsequently incubated for 48 hours. The treatment with the 1 μ M concentration of Ber or mBer and the 1 μ M cardiotoxic concentration of Dox was followed as a treatment regime for the next set of experiments in this study.

2-8-2. Evaluation the effect of mBer on the viability of H9c2 cells treated with Dox:

In order to evaluate the preventive efficacy of Ber or mBer against DIC, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay was utilized to determine the viability of cells., which was performed in quadruplicate. MTT assay works on the basis that only living cells, owing to their active metabolism, can transform MTT into purple

formazan, which has the highest absorbance at 492 nm. The quantity of the formed formazan is quantified spectrophotometrically, and it is directly correlated with cell viability (208). Briefly, the assay was conducted by adding 10 µL of a 5 mg/ml MTT reagent to each well containing cultured cells, followed by a 4 hours' incubation under similar conditions. After removing the supernatant with a multichannel pipette, the cells were lysed, and formazan crystals were fully solubilized by adding 150 µl of DMSO and 25 µl of glycine to each well with good mixing. A microplate (ELISA) reader was used to compare the absorbance at 492 nm between test and control cells in order to determine the percentage of cells that were inhibited, assuming that control cells treated with vehicle alone would have a survival rate of 100%. The following formulas were used to estimate treated cells' percentage inhibition:

Percentage of Inhibited Cells = 100 - (Absorbance of Treated Cells / Absorbance of untreated Cells) x 100

Percentage of Cell viability = 100 - Percentage of Inhibited Cells

2-8-3. Estimation of cardiomyocytes toxicity:

2-8-3-1. Measurement of lactate dehydrogenase (LDH):

The leakage of LDH from the H9c2 cells was used to determine the cytotoxicity. After the cells were treated and incubated for 48 hours, the culture media was collected, the levels of LDH in a cell culture medium were measured with the use of a LDH activity colorimetric assay kit purchased from Thermo Fischer Scientific, USA (Cat. NO.: C20300). In accordance with the guidelines provided by the manufacturer, the reaction mixture was distributed evenly among the wells in the plate, which was then incubated for 0.5 h at 25 °C followed by adding 50 µL of stop solution, an ultraviolet spectrophotometer was used to measure the absorbance at both 490 nm and 680 nm after the addition of stop solution. The determination of LDH activity involved subtracting the value of

absorbance measured at 680 nm (background signal) from the value of absorbance at 490 nm.

2-8-3-2. Determination of creatine kinase myocardial band(CK-MB):

CK-MB levels were calculated in the culture medium used to grow the cells utilizing ELISA Kit specific for this enzyme in rats purchased from Elabscience, China (Cat. NO.: E-EL-R1327). The samples were centrifuged for 20 minutes at 1000g at 4°C, and the supernatant was collected for analysis. According to the manufacturer's guidelines, samples from the culture media or standards were added to the kit's micro ELISA plate, which had already been coated with specific CK-MB antibody. The plate was then incubated at 37°C with biotinylated antibody specific to this antibody and avidin-horseradish peroxidase (HRP) were added successively to the plate and incubated at 37°C. After washing the unconjugated components, the substrate solution was added to each well. Only in wells containing rat CK-MB, a biotinylated detection antibody, and an Avidin-HRP conjugate was the reaction started, and the fluid color in the well turned blue. Next, the reaction became stopped by the addition of the stop solution. At absorption of 450 nm, the optical density which is corresponding to the concentration of CK-MB was determined spectrophotometrically. By comparing the absorbance of the samples to the standard curve (Figure 2-1), it was possible to compute the concentration of Rat CKMB in the test samples.

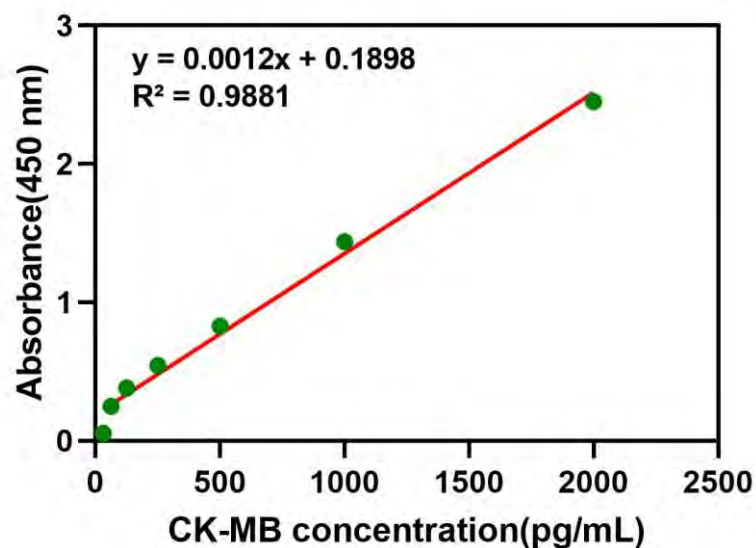


Figure 2-1: The standard curve of CK-MB

2-8-3-3. Determination of Cardiac Troponin I (cTn-I) release:

cTn-I was estimated in the cell culture supernatant utilizing ELISA Kit specific for this enzyme in rats purchased from Elabscience, China (Cat. NO.: E-EL-R1253). The cell culture supernatant samples were centrifuged for 20 minutes at 1000g at 2-8°C. The wells were filled with 100μL of standard or sample and then incubated at 37 °C for 90 minutes. After discarding the liquid, each well received 100μL of Biotinylated detection antibody working solution and was incubated at 37 °C for 60 minutes. After aspiration, the plate was rinsed three times. The fluid was aspirated and the plate rinsed five times after the addition of 100μL of HRP conjugate working solution and incubation at 37°C for 30 minutes. Following the addition of 90μL of Substrate Reagent and 15 minutes of incubation at 37 °C, 50μL of stop solution was added. The plate was quickly read at 450 nm so that the results could be calculated.

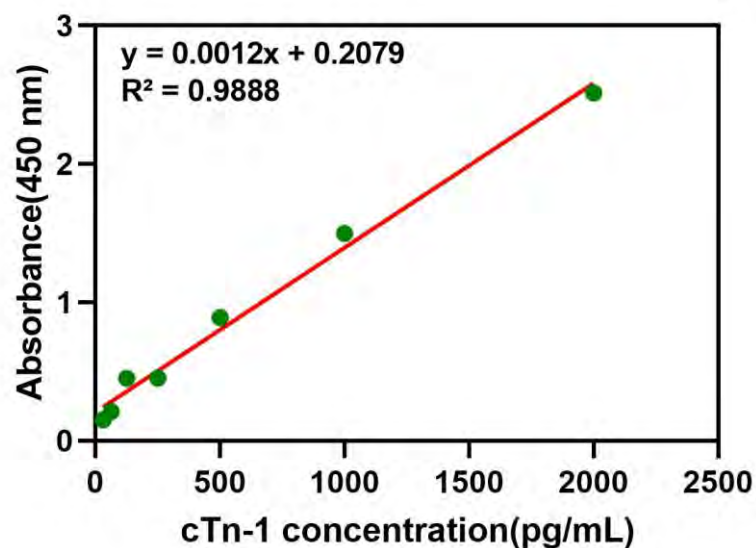


Figure 2-2: The standard curve of cTn-I

2-8-4. Evaluation of oxidative stress status

2-8-4-1. Measurement of malondialdehyde (MDA) level:

MDA levels were used as indicative of lipid peroxidation. MDA concentration (nmol/mg protein) was determined colorimetrically based on its reaction with thiobarbituric acid using the MDA Content Assay Kit (Sunlong Biotech Co., China, Cat. No.: AK0662-50T-48S) and measuring absorbance at 532 nm (211).

2-8-4-2. Assessment of Reduced glutathione (GSH) level:

GSH is an important antioxidant involved in the elimination of toxic compounds, such as free radicals. Using the GSH Assay Kit (Sigma-Aldrich Co., USA, Cat. No.: MAK364), the GSH concentration was determined colorimetrically via an enzymatic cycling approach in the presence of GSH and a chromophore. Reducing the chromophore produces a stable product that can be measured at 450 nm to determine the GSH quantity in the sample (212).

2-8-4-3. Evaluation of catalase (CAT) activity:

CAT is a ubiquitous enzyme that acts as the primary catalyst for the degradation of hydrogen peroxide (H₂O₂). At a wavelength of 240 nm, H₂O₂ displays a prominent absorption peak. Due to the decomposition of water and oxygen facilitated by CAT, the absorbance of the reagent at 240 nm is observed to decrease. CAT activity was quantified in accordance with the manufacturer's instructions (Sunlong Biotech Co., China, Cat.No. AK0580) (213).

2-8-4-4. Determination of Superoxide dismutase (SOD) activity:

SOD is a crucial antioxidant enzyme that transforms the superoxide radical into oxygen molecules and hydrogen peroxide. In accordance with the manufacturer's guidelines (Sunlong Biotech Co., China, Cat. No.: AK0584), the SOD activity was measured using spectrophotometry, and the absorbance was at 560 nm (214).

2-8-5. Measurement of some pro-inflammatory cytokines levels:

The level of some inflammatory cytokines including interleukin-1 β (IL-1 β) and interleukin-6 (IL-6) was determined in cells culture supernatant using specific ELISA kits purchased from Beyotime Institute of Biotechnology, China (Cat. NO.: PI303 and. PI328 for IL-1 β and IL-6 respectively). The samples were centrifuged for 20 minutes at 1000g and 4°C to obtain the supernatant from the cell cultures. Quantitative measurement of the desired interleukins was performed by reading absorbance at 450 nm in a microplate reader after the ELISA stages had been processed per the manufacturer's guidelines. The signal detected is proportional to concentration of targeted interleukins in the samples.

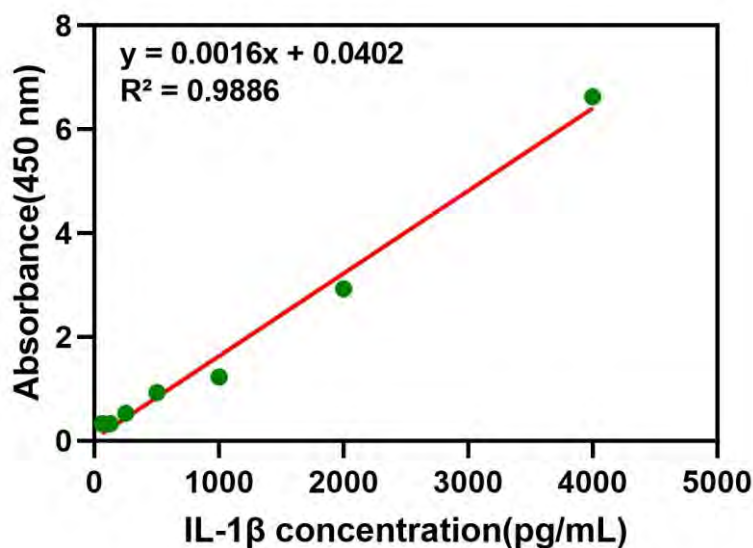


Figure 2-3: The standard curve of IL-1β

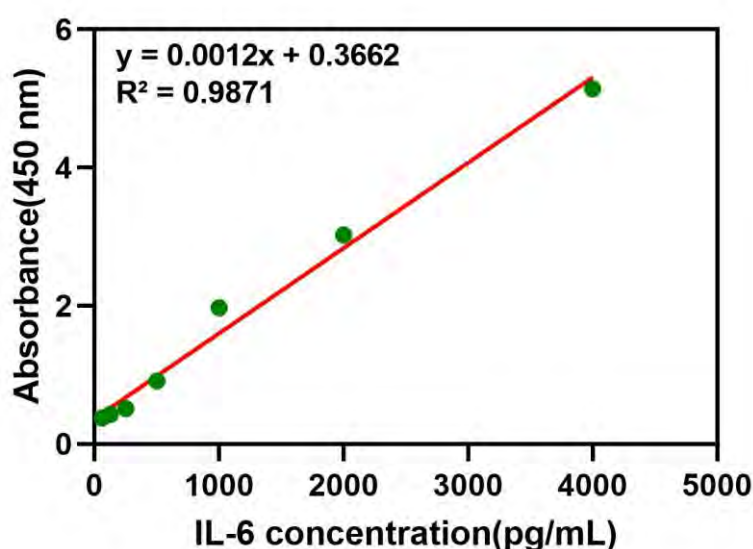


Figure 2-4: The standard curve of IL-6

2-8-6. Assessment of Caspase-3/7 activity:

H9c2 cells were seeded in opaque 96-well cell culture plates and treated in the same manner as in the viability analysis test in cultured cells. Based on the manufacturer's instructions. After 48 hours' post-treatment, 100 μ L of the Caspase-Glo[®] 3/7 reagent (Promega, USA, Cat. No.: G8090) was applied to each well at a volume ratio of 1:1. Next, the samples were left at

25°C for 3 hours before the luminescence was measured using a GloMax® microplate reader to estimate the caspase 3/7 activity. The workplace is the Research Institute for Biotechnology and Bioengineering, Isfahan University of Technology, Isfahan, Iran.

2-9. Statistical analysis:

The present study employed a one-way analysis of variance (ANOVA) to analyze the data, which were assumed to follow a normal distribution. Subsequently, the least significant differences (LSD) test was conducted as a post hoc analysis to ascertain any statistically significant differences. The statistical software SPSS package, specifically version 29, was utilized for the analysis. A significance level of $P < 0.05$ was employed to determine statistical significance (215).

Chapter Three

Results and Discussion

3-1. Drug loading and retention efficiency:

Ber was intended to be encapsulated within a pluronic F127 micelle to enhance its delivery. The results indicated that Ber was successfully loaded into Pluronic F127 micelles. The feeding weight of Ber can influence the drug loading (DL) and retention efficiency (RE) of micelles. Consequently, for maximum encapsulation efficiency of Ber, we investigated the effects of varying Ber feeding weights on loading efficiency, while other variables, such as temperature, pH, and solvent type, remained constant during the creation of micelles. As the amount of Ber utilized in the preparation rose from 1 mg to 10 mg, the DL increased from 2.2 ± 0.24 % to 5.3 ± 0.25 % (Figure 3-1), but the RE of the micelles considerably dropped as a result of this change (Figure 3-2).

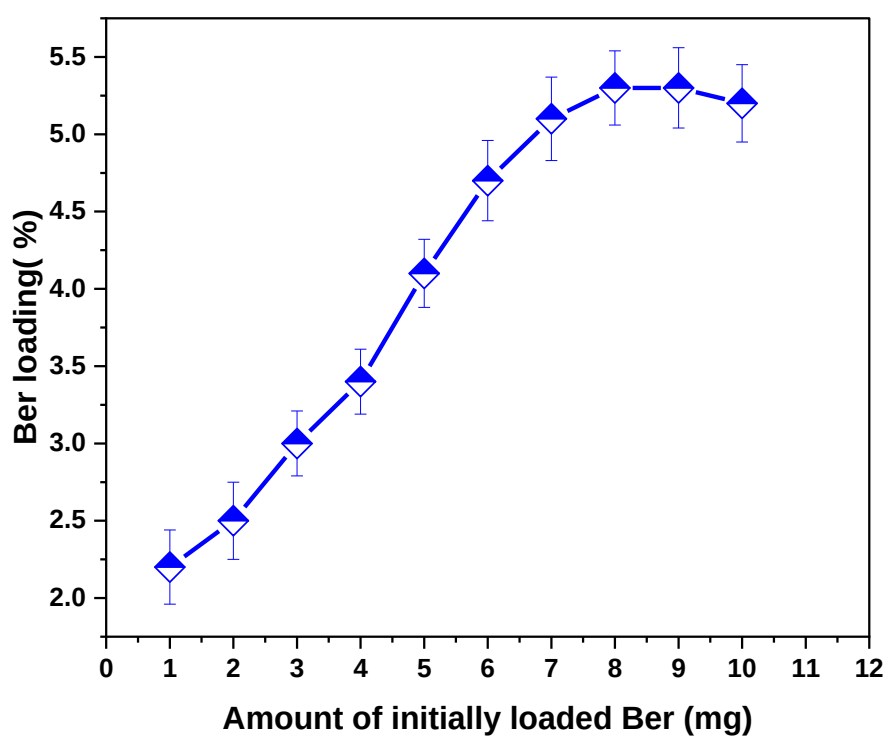


Figure 3-1: The relationship between the amount of initially loaded Ber and the loading capacity (%) of the created mBer. Data presented as mean $\pm$ SD; n = 4.

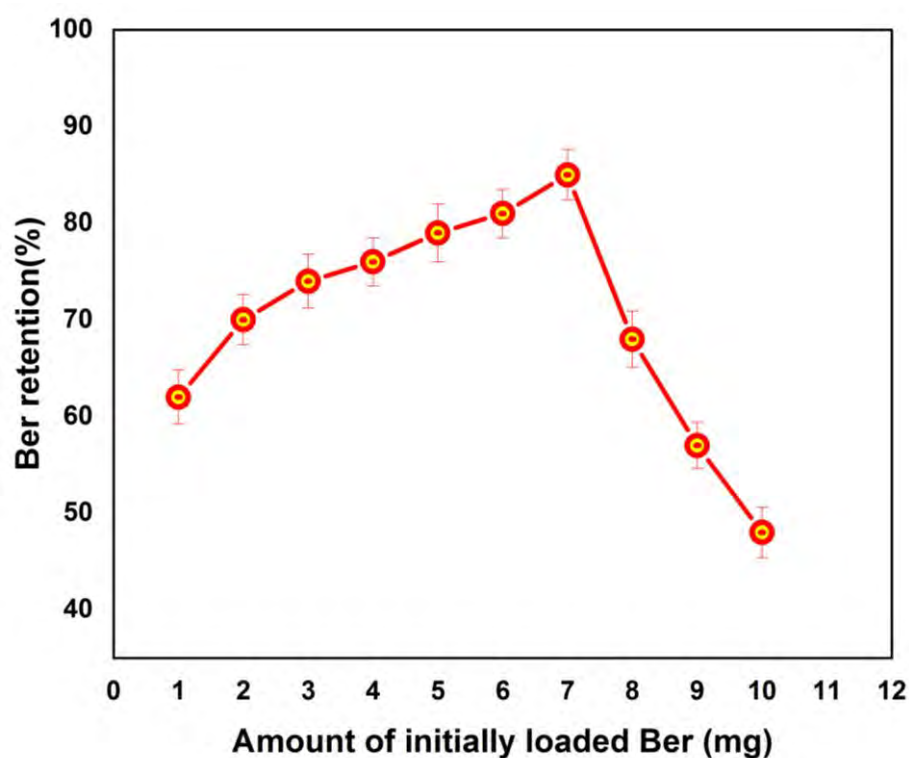


Figure 3-2: Effect of different amounts of loaded Ber on the retention capacity (%) of the created mBer. Data presented as mean $\pm$ SD; n = 4.

To top it all off, when the feeding Ber weight was 7 mg, the RE achieved its maximum value of $85 \pm 2.6\%$ (Figure 3-2). Micelle formulations exhibited an increase in DL but a reduction in RE in response to increasing Ber feeding (Table 1). The concentration of 7 mg of Ber was chosen as a suitable amount for making mBer in subsequent experiments.

Table 3-1: The retention efficiency and loading capacity percentages of mBer. The values indicate the mean $\pm$ SD; n = 4.

Weight of feeding Ber(mg)	Loading efficiency(%)	Retention efficiency(%)
1	2.2$\pm$ 0.24	62$\pm$2.8
2	2.5 $\pm$ 0.25	70$\pm$2.6
3	3.0$\pm$0.21	74$\pm$2.8
4	3.4$\pm$0.21	76$\pm$2.5
5	4.1$\pm$0.22	79$\pm$3.0
6	4.7$\pm$0.26	81$\pm$2.5
7	5.1$\pm$0.27	85$\pm$2.6
8	5.3$\pm$0.24	68$\pm$2.9
9	5.3$\pm$0.26	57$\pm$2.4
10	5.2$\pm$0.25	48$\pm$2.6

The thin film hydration technique was used to create mBer in this study. According to the findings of this study, the observed pattern of increasing DL but decreasing RE with increasing Ber addition during micelle formulations may be due to the saturation of the hydrophobic micelle core with Ber during micelle production. This finding is consistent with what was believed to occur when creating a different type of polymeric micelle, where the core was so saturated with drug molecules that precipitation of these molecules could occur on the exterior of the micelle (216).

3-2. Size and Zeta potential of micelles under hydrodynamic conditions:

DLS was used to measure the mean hydrodynamic particle size, size distribution, and zeta potential of the created micelles. The hydrodynamic size of the produced mBer was around 82.2 $\pm$ 10.3 nm. The mBer size distribution closely resembles a normal distribution, as depicted in figure 3-3. This result

suggests that the predicted formation of monodisperse nanoscale micelles has occurred. The PDI value in the created mBer was 0.176 ± 0.029 , indicating that the created micelles had a relatively confined size distribution. The zeta potential of a micelle dispersion system is a crucial characteristic for predicting its physical storage stability as well as measuring particle aggregation tendency. Following what is depicted in figure 3-4, the micelles that had been loaded with Ber had a zeta potential of -47.4 ± 0.19 mV.

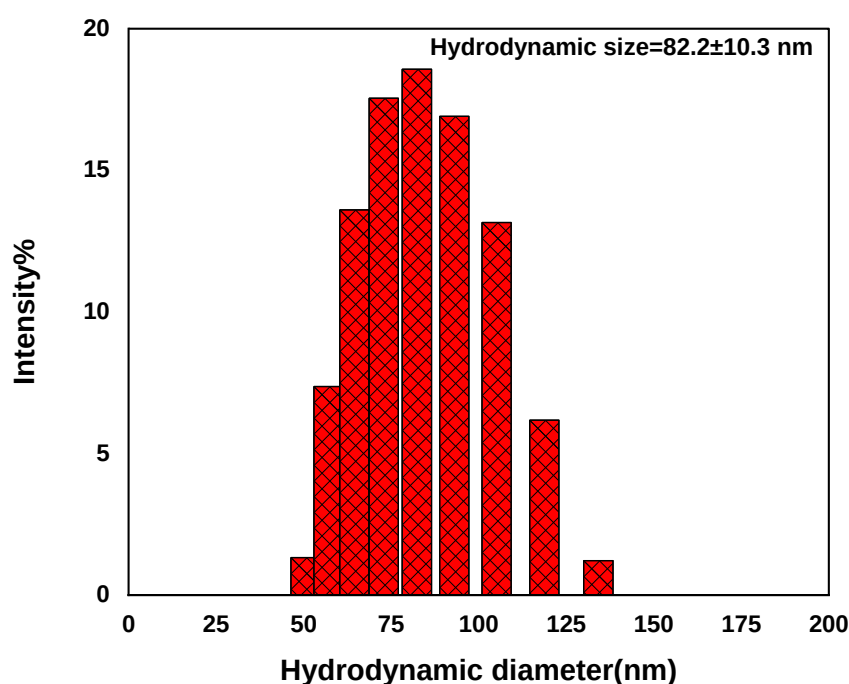


Figure 3-3: Hydrodynamic particle size (nm) and distribution of mBer as measured by DLS.

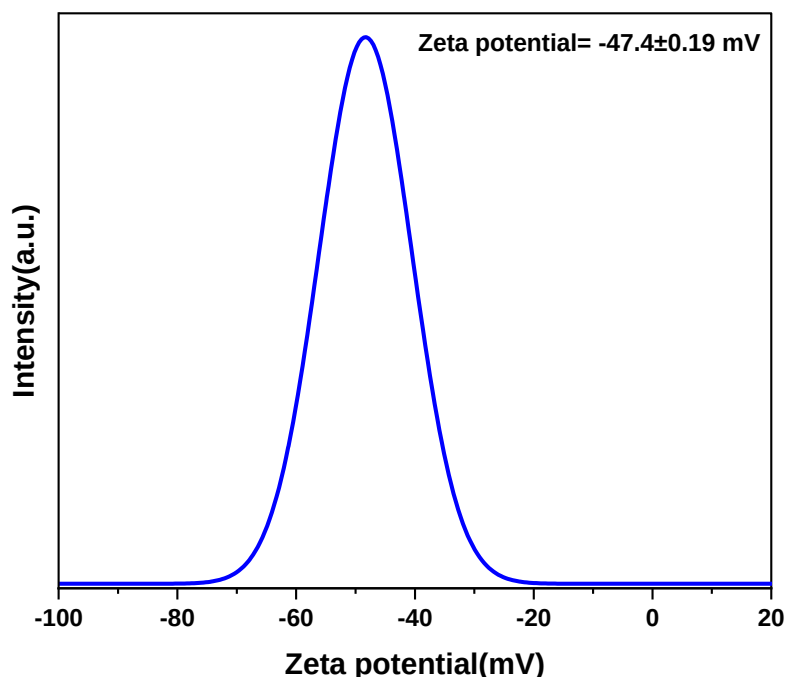


Figure 3-4: Zeta potential (mV) of the mBer. Data presented as mean $\pm$ SD. n = 4.

A dimensionless number, PDI, expresses the particle size distribution in the system under study. The closer the polydispersity value approaches zero, the more uniform the particle population. (217). The created micelles in this study had PDI values of less than 0.3, indicating that the measured micelles possessed a narrow size distribution (218). The zeta potential is a commonly employed metric for assessing and forecasting the physical stability of particle dispersion systems. A zeta potential exceeding $|20|$ mV generates electrostatic repulsive energy that impedes particle aggregation and growth (219). As evidenced by the measured zeta potential value, the analysis conducted in this study indicates a low probability of aggregation and particle growth for the created mBer.

3-3. Analysis of morphological characteristics with TEM:

The mBer solution visually appeared transparent and yellowish (figure 3-5a). TEM results showed the formed micelles loaded with Ber have a moderately uniform particle size with an average of 10.26 ± 0.13 nm (Figure 3-

5b). Our results exhibited a polydispersed, spherical morphology of mBer similar to grains and Upon closer inspection of the TEM image, it became clear that these micelles had both bright and dark regions (Figures 3-5c and 3-5d).

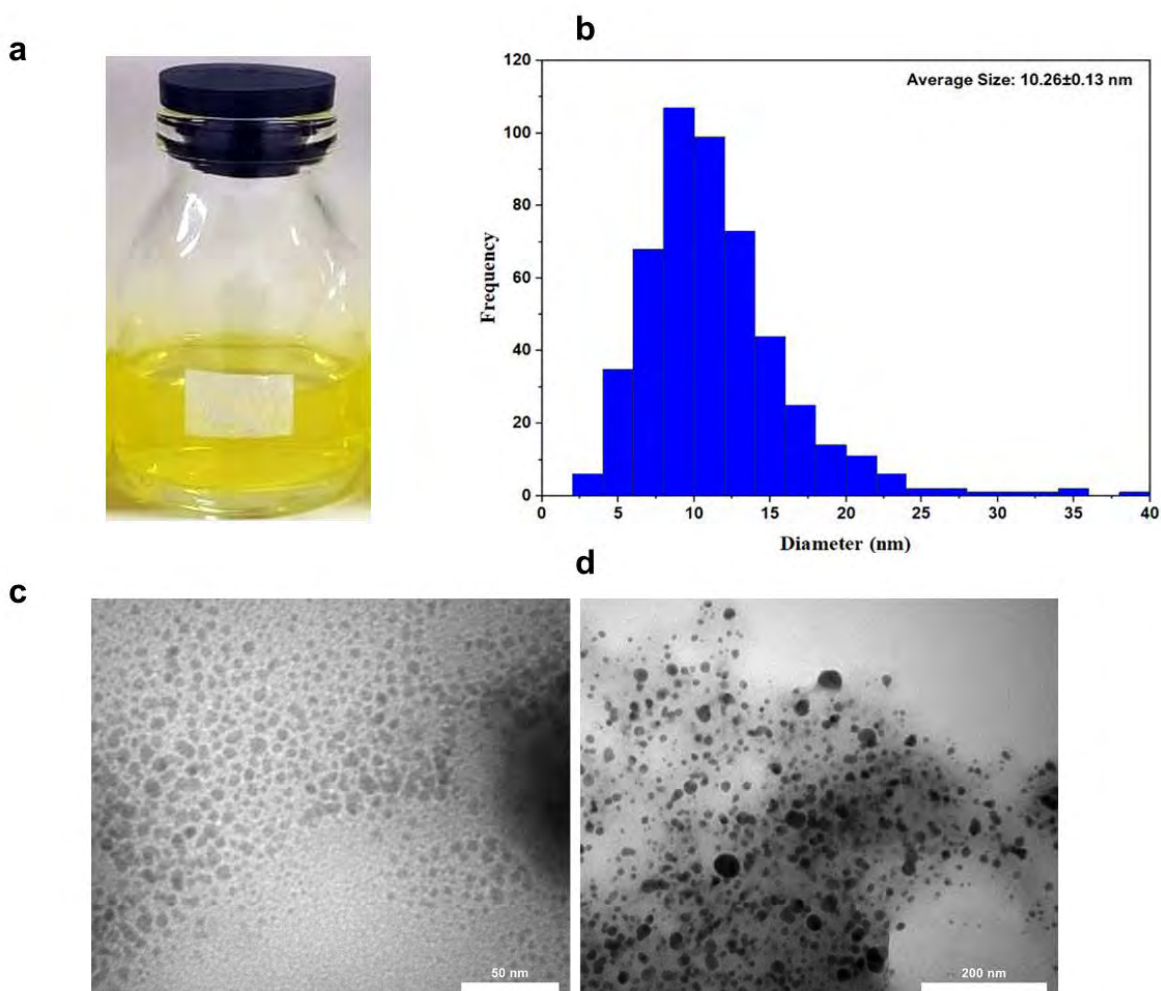


Figure 3-5: Morphological Characteristics of mBer: **a)** photographic images of mBer; **b)** particle size distribution of mBer measured by TEM; **c)** TEM image of the mBer formulation (bar = 200 nm); and **d)** TEM micrograph of the mBer formulation (bar = 50 nm).

The majority of the pluronic polymer micelles created in this study were found to have spherical morphological characteristics, which is in accordance with the previous reports (220,221). TEM images of the produced micelles displayed bright and dark regions. The PEO block of the pluronic copolymers

may be responsible for the light regions, while the Ber-loaded hydrophobic interior of the PPO chains may be responsible for the dark regions. The average particle size of the mBer we made, as measured by DLS or TEM, shows clear differences between the two methods, but it can be used to show that the micelles we made were small enough to pass through the cell membranes. In light of the literature review to date, it is evident that the optimal nanoparticle size is approximately less than 100 nm. At this size, the particle could pass through cellular barriers such as the blood-brain barrier, where smaller particles are believed to penetrate deeper into tissue layers (222). The micelle size is inversely proportional to the interface area; therefore, small micelles increase the contact area with the body's targets, delivering a sufficient quantity of loaded drug due to the high ratio of surface area that comes into contact with nanoparticles (223). Also, the small size of nanoparticles prevents quick clearing via the lymphatic system, where it has been demonstrated that particulates 200 nm or larger tend to activate the lymphatic system and are quickly removed from circulation (224,225).

It is important to note that there is a difference in the nanoparticle size reported from DLS and TEM images. Size estimation by TEM, which is a number-based observation, requires the examination of a sample in its dry state, whereas size estimation by DLS, which is an intensity-based observation, permits the examination of a sample in its solvated state, where solvent molecules will surround a micelle. DLS measures the hydrodynamic size. Under the effect of Brownian motion, the hydrodynamic diameter gives information about the core of a micelle, any material around it, and the molecules of the solvent that interact with a micelle in different ways that don't involve covalent bonds. Using TEM to estimate the size of a nanoparticle is based on an estimation of the size of the compact particle without the presence

of a hydration layer. Consequently, the hydrodynamic diameter measured by DLS remains relatively greater than the TEM-estimated size (226).

3-4. FTIR analysis:

The FTIR spectra of mBer were compared to those of blank micelles made of Pluronic F127 and free Ber. The results of these comparisons are presented in figure 3-6. In the FTIR spectrum, pure Ber can be shown to exhibit a variety of distinct absorption bands. The characteristic absorption band is typically located at 1506 cm^{-1} . The blank F127 micelles sample exhibits distinctive absorption bands at 2889 cm^{-1} , and 1010 cm^{-1} . The absorption bands of Ber-loaded micelles were quite comparable to those of blank micelles, with the exception of the Ber-specific peaks. On the basis of these findings, it is possible to draw the conclusion that Ber was successfully localized and contained within the hydrophobic core of the micellar particle. Interestingly, the micelles that had been loaded with Ber did not show any additional peaks or variations in the location of the characteristic peak. This demonstrates that no new chemical compound was created throughout the procedure. Micelles loaded with Ber did not display any new peaks or shifts in the typical peak location (Figure 3-6). This is evidence that no new chemical compound was produced.

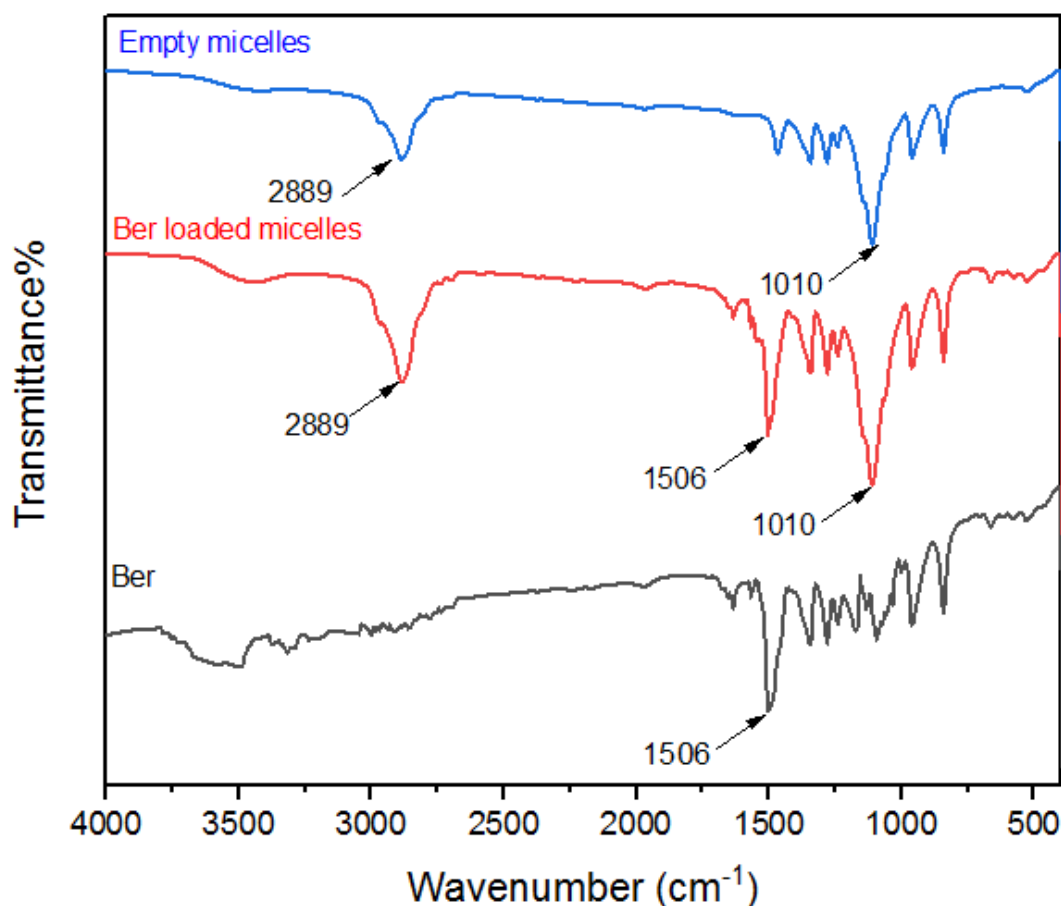


Figure 3-6: FTIR spectra of Ber, empty pluronic F127 micelles, and pluronic F127 micelles loaded with Ber.

The significant absorption peak at 2889 cm⁻¹ that has been seen in the FTIR spectra of pluronic F127 has been prominently linked to C–H stretching vibrations and 1010 cm⁻¹ has linked to C–C stretching vibration (227). The characteristic peak at 1506 cm⁻¹ in Ber has been attributed to furyl group C=C bond vibrations (228,229). The resultant mBer spectra show all of the unique absorption bands of the polymer and the Ber, albeit with slightly less prominent peaks than those seen in the spectra of the pure components. During the process of mBer synthesis, there was no discernible peak shift, which suggests that no new chemical bonds were formed and that there were only some physical interactions that took place between the Ber and the polymer.

3-5. X-Ray diffraction (XRD) analysis:

Ber, pluronic F127, and mBer were examined using XRD in order to confirm the physical existence of Ber within the synthesized micelles. The results obtained from the XRD spectra are depicted in figure 3-7. Ber showed two sharp peaks with maximum intensities at 16.3° and 25.3° , while pluronic F127 showed two sharp peaks at 19.1° and 23.3° . In addition to this, the diffraction patterns of Ber and Ber-loaded micelles were completely different from one another.

To be more specific, the distinct Ber peaks that were previously observed in the diffraction patterns of the free Ber are nowhere to be detected in the diffraction pattern of the Ber-loaded micelles, revealing that Ber had been encapsulated within the polymeric micelles in an amorphous form and that there was relatively no free Ber present on the surface of the micelles.

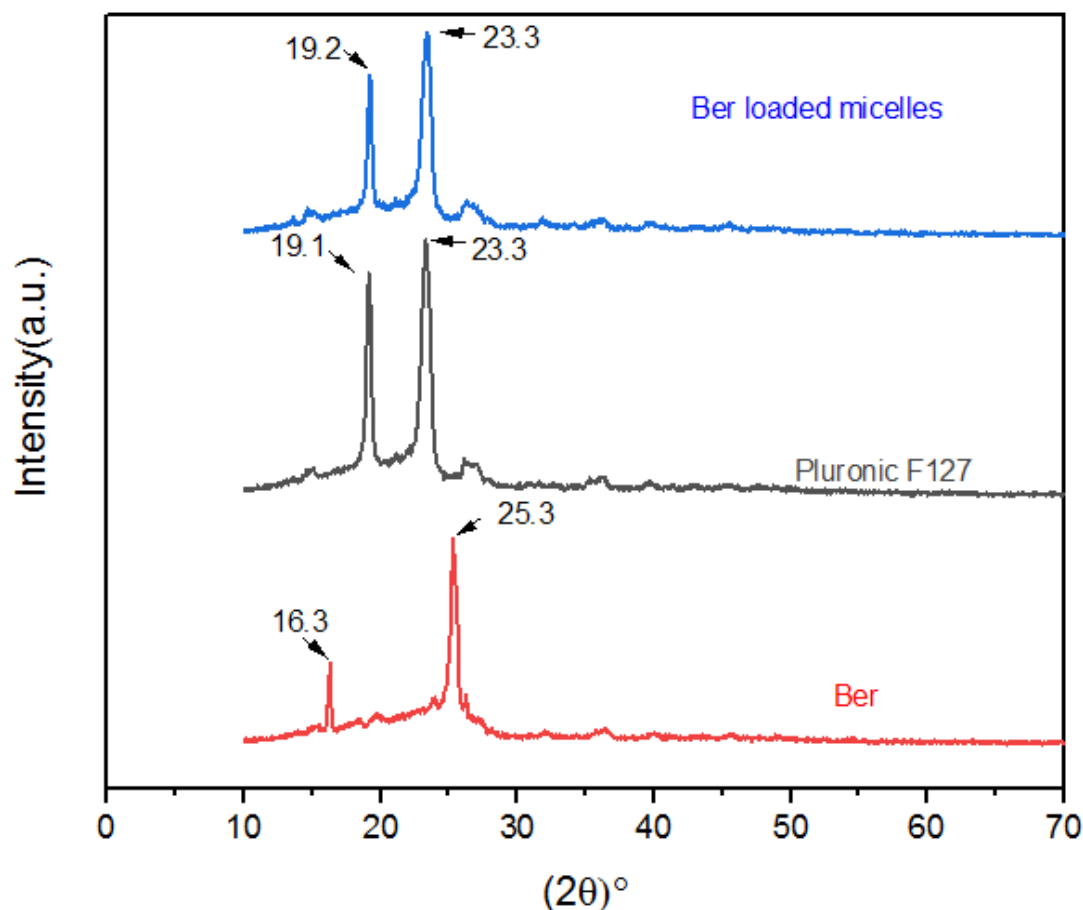


Figure 3-7: XRD diffractogram patterns for Ber, empty pluronic F127 micelles, and Ber-loaded pluronic F127 micelles.

The two sharp peaks that Ber exhibited in XRD spectra in this study are consistent with what has been previously documented about the XRD peaks of Ber (203,230). The results of the current XRD characterization study showed that micellar formulations have broken up the crystalline structure of Ber, leaving behind amorphous particles in the micellar core compartment, and this is consistent with reports that Ber molecules were transformed from their crystalline state into an amorphous one (203,206). Our study shows that the polymer and Ber work well together, and it suggests that pluronic F127 is a good carrier for loading Ber. Less crystalline and amorphous substances have larger free energies and more solubility capacity than their crystalline counterparts (231). Thus, physical modification of Ber may increase its solubility and dissolution rates compared to crystalline Ber. Thus, a micellar

formulation may improve Ber's solubility and release by modifying its crystalline nature.

3-6. Ber release from micelles in Vitro:

At a pH of 7.4, the behavior of mBer's release was studied via in vitro testing. This was done to mimic the release that happens in the normal physiological environment of the body. Figure 3-8 depicts the shift that occurs in the Ber release rate from both the free stock solution of Ber and mBer throughout the course of time. Ber was released from polymeric micelles at a much slower rate than it was from the free solution. After 3 h, it was determined that just 25% of the Ber had been released from the mBer, whereas around 50% of the Ber had been found to have been released from the PBS solution over the same time period. After 12 h, almost 95% of the Ber had been released from the free stock solution, but about 65% of the Ber that had been incorporated into the micelles was still there. At a pH of 7.4 and after 24 h, the release of the Ber from the free solution was nearly complete, and the amount that was released reached over 98%, but only about half of the loaded amount of Ber in mBer was released.

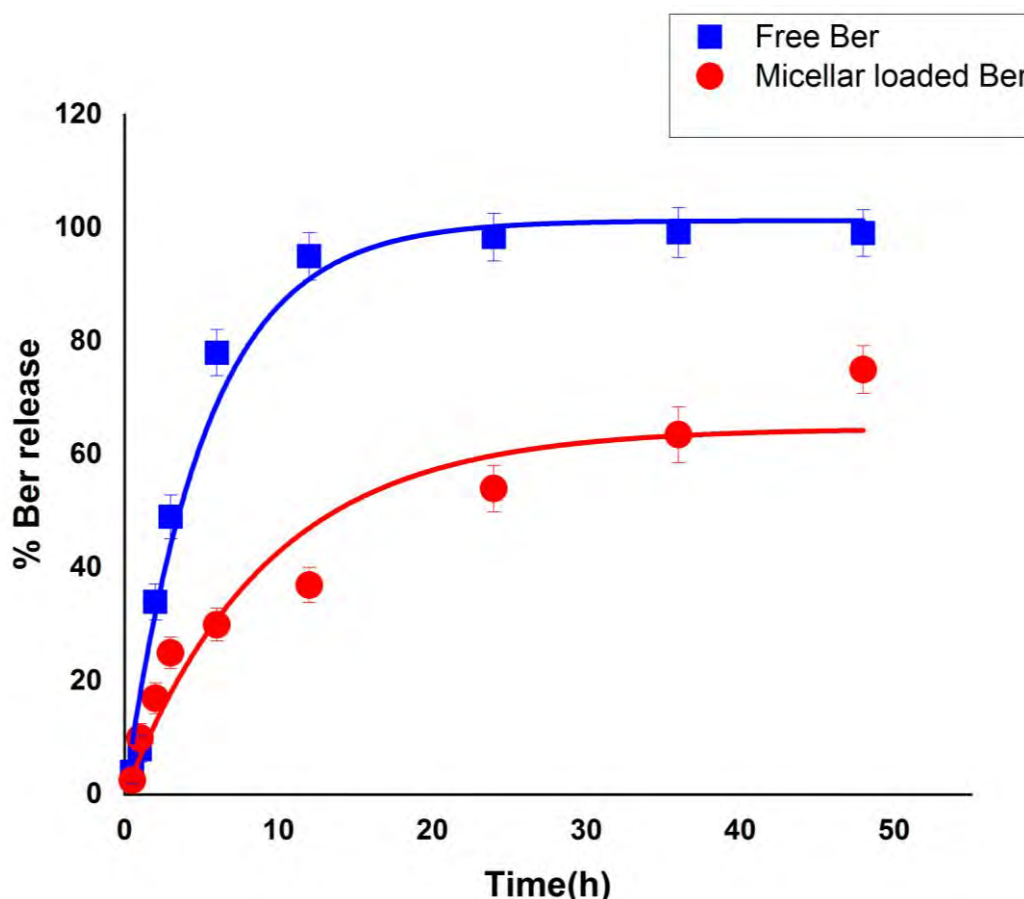


Figure 3-8: Ber release from Ber stock solution and mBer over 48 h at pH 7.4. Data depicted as mean Ber released (%)±SD; n = 4.

On the basis of in vitro release tests, it would suggest that the micelles have the capacity to release Ber from the produced micelles in both burst-release and sustained-release behaviors. Ber incorporation close to the micellar surface or Ber molecules already existing in the microchannels of micelles are likely responsible for the initial burst release of Ber from the micelle particles (232,233). The sustained-release behavior may result from the hydrophobic Ber being stably incorporated into the micelle core and slowly diffusing out of the micelles (234). However, we predict that increased blood volume dilution, dynamic clearance processes, and plasma protein binding will accelerate micelle dissociation in vivo. Previous research has demonstrated that the hydrophobic core and kinetic stability of F127 micelles may lengthen the circulating times of enclosed molecules (235,236). Since in vitro release

kinetics do not necessarily correspond to in vivo circulating times, it is reasonable to infer that mBer will circulate in the body for a longer duration than free Ber.

3-7. Toxicity evaluation using in vitro hemolysis:

A standard hemolysis assay was used to check the compatibility of a micelle formulation made for Ber with red blood cells. Free Ber in solution increased hemolysis insignificantly ($P>0.05$) more than the negative control (cells were suspended in normal saline), an empty micelle vehicle, and Ber-loaded micelles, as shown in figure 3-9. Over all, compared to the positive control group (cells were incubated with distilled water), the Ber-loaded micelles did not cause any obvious hemolysis. The micelles produced were clearly very compatible with red blood cells, both on their own and when loaded with Ber.

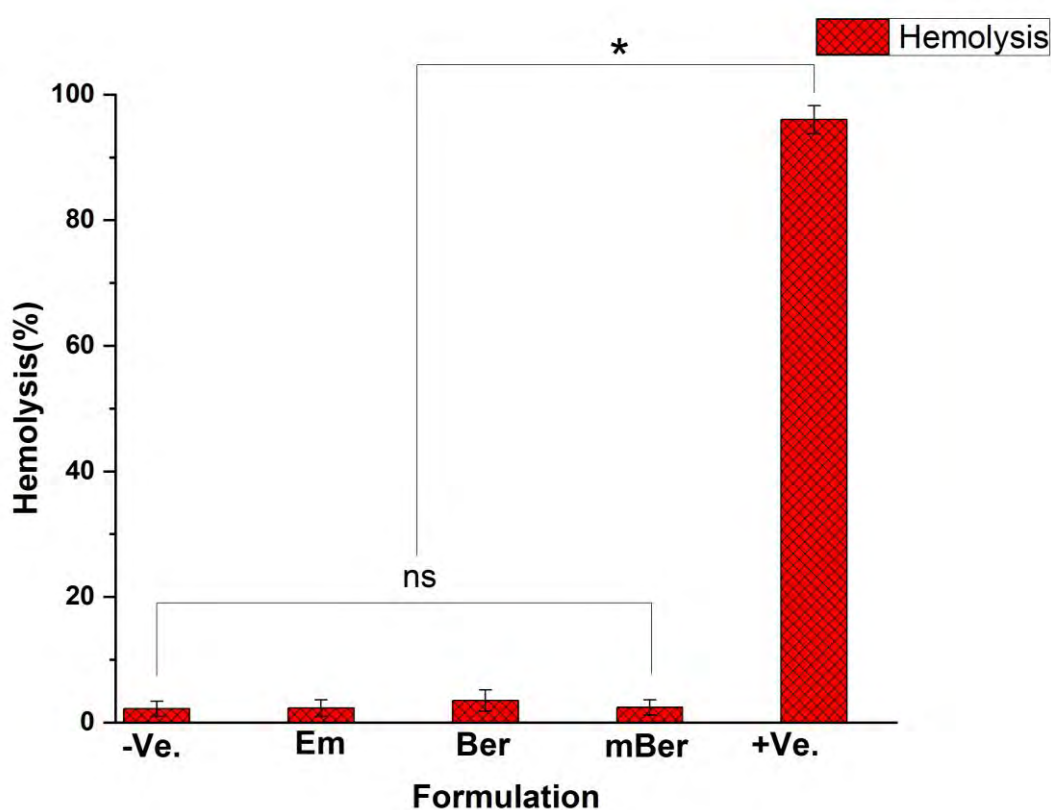


Figure 3-9: Hemolysis tests of the created mBer. After incubation of red blood cells with different samples include physiological saline as a negative control (-Ve.), empty micelles (Em), free Ber (Ber), micellar-loaded berberine (mBer), and distilled water as a positive control (+Ve.). Data presented as mean $\pm$ SD; n = 4. * indicated a statistically significant difference ($P < 0.05$).

Hemolysis is one of the key indicators of both acute toxicity and compatibility issues with new foreign materials. The net surface charge of the particle and the chemical composition of the used materials are the main factors affecting the incidence of hemolysis (237,238). Blood cells rapidly adsorb to some nanoparticles to produce a protein corona that affects their interaction with blood components (239). Numerous studies have linked nanoparticle-induced coagulopathy to cardiovascular disease. Nanoformulation can disrupt the coagulation system and alter hemostatic equilibrium, causing life-threatening disorders such as deep vein thrombosis and intravascular coagulopathy (240). Thus, researchers should carefully study the interactions between newly created nanoparticles and blood cells. This will improve nanoformulation hemocompatibility, simplify clinical studies, and speed up the market introduction of new nanoformulation-based products.

Both on their own and after being loaded with Ber, the micelles that were created as an outcome of this experiment showed a high level of compatibility with red blood cells. The results of this test suggest that the generated mBer can be safely administered via intravenous injection.

3-8. mBer reduces DOX-mediated cytotoxicity in H9C2 cells:

On rat's H9c2 cell culture, the potential inhibitory effect of different serial concentration dilutions of free Ber, mBer, Dox, Bre-Dox, or mBer-Dox combination, and empty F127 micelles was tested and compared with the control group of the cell line treated only with PBS containing 0.5% DMSO, which is considered the vehicle control group. In the MTT experiment, cell

viability was expressed as a percentage of inhibition of treated cells relative to untreated control. Treatment of H9c2 cells with free Ber or mBer did not affect the viability of cells except after relatively high concentrations for both Ber forms (Figure 3-10). As shown in figure 3-11, the inhibitory effect of Dox on the viability of H9c2 cells increases as Dox concentration increases. Starting at 2.85% inhibition at the lowest utilized concentration 0.125 μ M and increasing to 89% inhibition at the highest tested concentration 8 μ M. Combination of Ber and Dox considerably mitigated the cytotoxicity of Dox on H9c2 cells (Figure 3-11). The IC₅₀ values of free and micellar-loaded Ber in H9c2 cells after 48 h of incubation were 3565.08 $\pm$ 37 μ M and 3439.46 $\pm$ 39 μ M respectively (Figure 3-12), indicating that both Ber and mBer are particularly nontoxic to cultured cardiomyocytes (H9c2 cells). Empty polymeric micelles alone exhibited no cytotoxic to H9c2 cultured cells and that is why it was not shown on the figure 3-10.

Following 48 hours of incubation, the 50% growth inhibition concentration (IC₅₀) of Dox in H9c2 cells was 4,42 μ M (Figure 3-12). The increase in the IC₅₀ of Ber-Dox when compared to the IC₅₀ of Dox (Figure 3-12) demonstrated that Ber had cardioprotective properties. When the micellar form of Ber is used with Dox, the cardioprotective effect of Ber against Dox in H9c2 cells is clearly improved. When combined with Dox, mBer is more effective and potent at protecting cultured cardiac cells than free Ber (Figure 3-11), as evidenced by the fact that the IC₅₀ of the combination of mBer and Dox was significantly ($P < 0.05$) higher than that of Ber-Dox in combination (Figure 3-12).

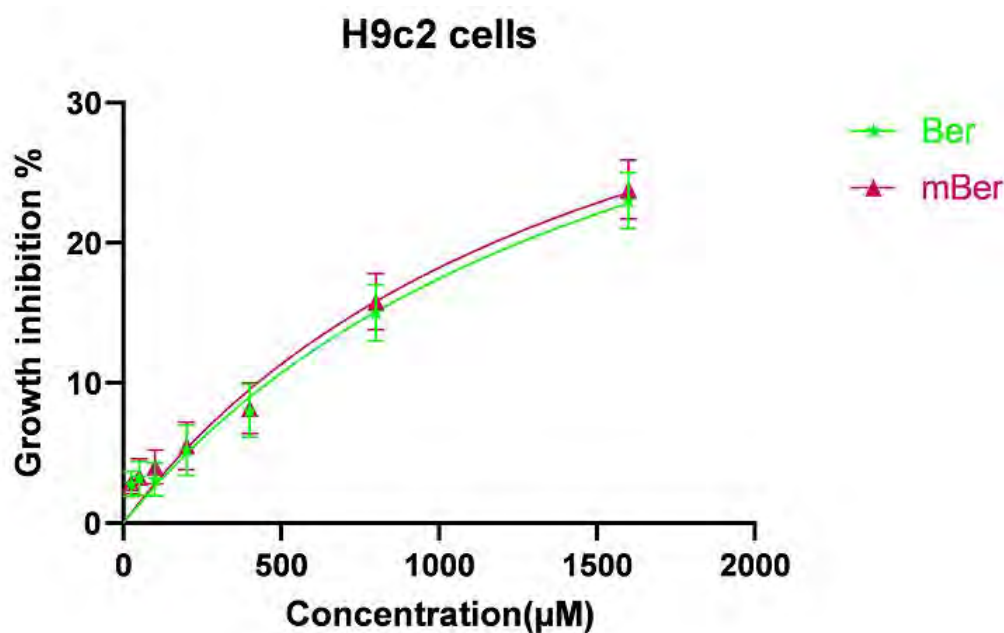


Figure 3-10: The growth inhibition percentage of free Ber and mBer in H9C2 cell line. Data presented as mean $\pm$ SD(μ M); n = 4.

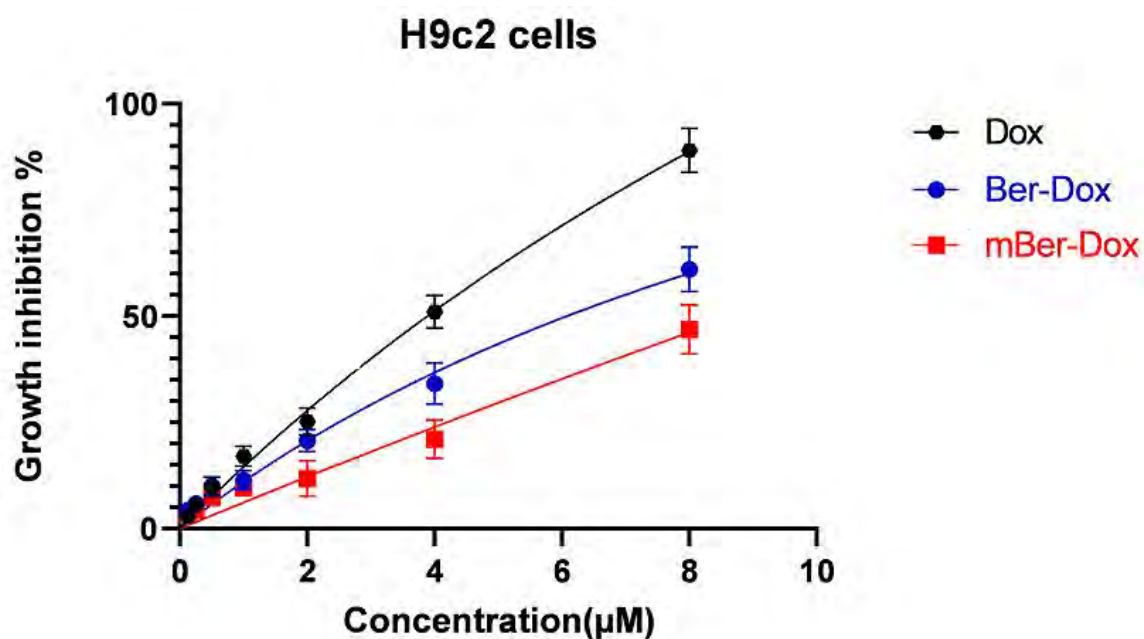


Figure 3-11: A comparison of growth-inhibition percentages of Dox, Ber-Dox, and mBer-DoX in H9C2 cell line. Data presented as mean $\pm$ SD(μ M); n = 4.

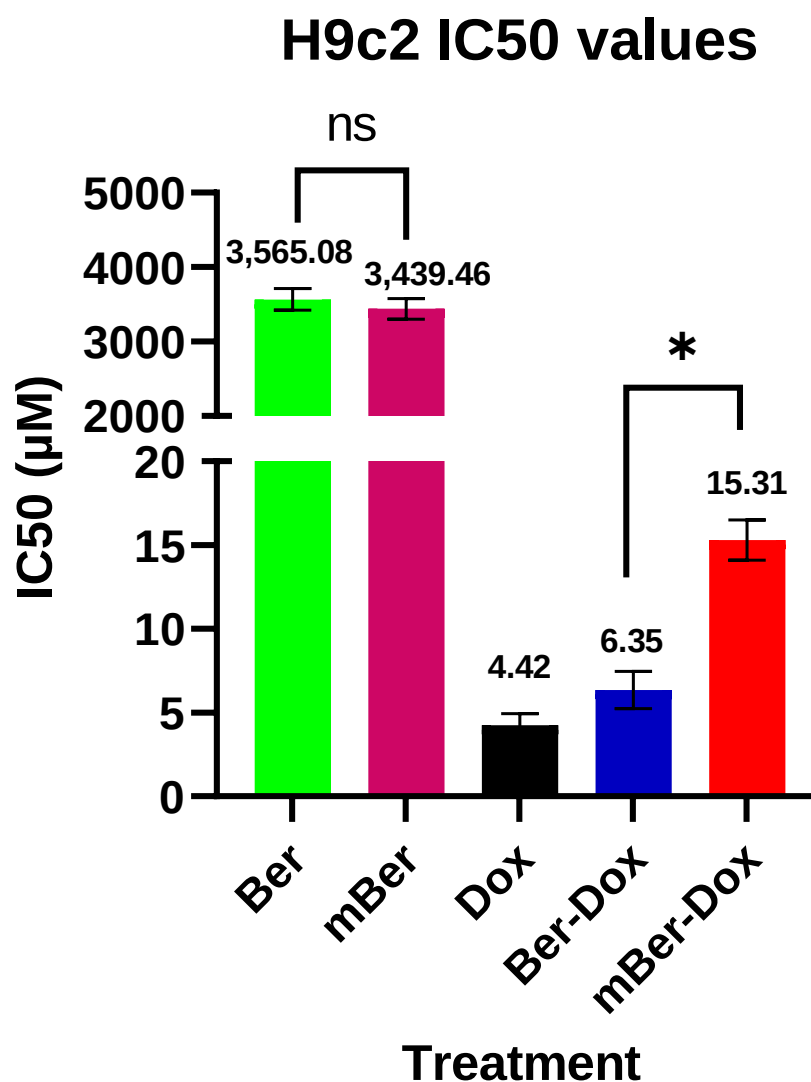


Figure 3-12: The IC 50 values of Dox,Ber-Dox, and mBer-DoX in H9C2 cell lines. Data presented as mean± SD(μM); n = 4. ns: no significant difference $p>0.05$.* denoted to significant differences $p<0.05$.

In this experiment, the MTT test was used to find out how effective Ber and its micellar form are at protecting the cardiomyocytes in vitro. We found that Ber has a cardioprotective effect. Interestingly, the polymeric nanoformulation of Ber greatly outperformed the activity of the free Ber, as evidenced by the experimental results of this work, which demonstrated that Ber loaded micelles treatment with increasing concentrations provided a significant protection against Dox. This significant improvement in the cardioprotective activity of the polymeric nanoformulation of Ber over the free

form of Ber, as measured by the IC₅₀ value on H9c2 cells, can be attributed to the advantages of micelles in enhancing the permeability of Ber and the cellular retention effect, which permits the release and availability of Ber nanoparticles readily in the treated cells.

3-9. Antagonistic action of mBer and Dox in H9c2 cells:

The CI was used to figure out how Ber and Dox affect H9c2 cells together. The plots of CI vs. all fractions affected (Fa) for Ber-Dox and mBer-Dox are shown in figure 3-13. Values of CI that are less than 1 indicate synergy, while values of CI that are greater than 1 indicate antagonism. Based on the data, both the Ber-Dox and mBer-Dox combinations were antagonistic (CI > 1) in H9c2 cells (Figure 3-13), but the mBer-Dox combination was more antagonistic than the Ber-Dox combination. These data further support Ber's antagonistic effect against DIC, and the cardioprotective effect of Ber is enhanced when it is loaded in the F127 micelle.

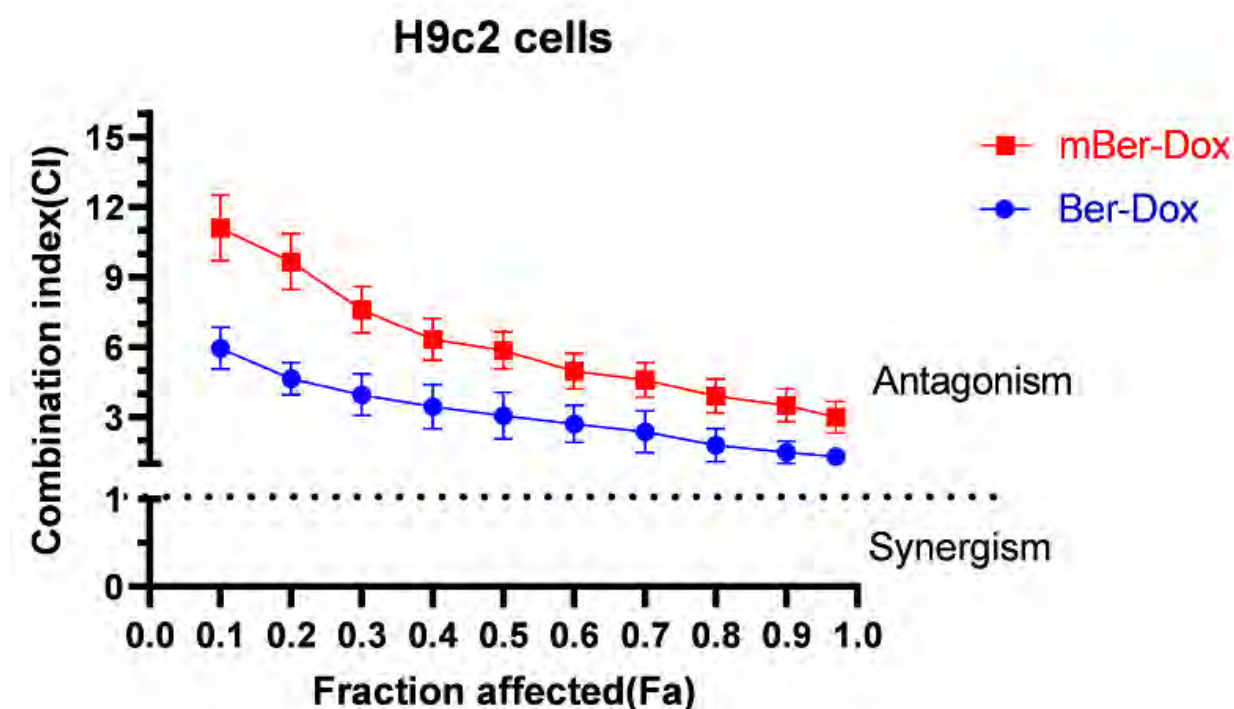


Figure 3-13: CI vs Fa plots for the Ber-Dox and mBer-Dox combinations in H9c2 cells. CI values of 1, 1, and > 1 correspondingly imply synergy, additivity, and antagonism. Data presented as mean CI $\pm$ SD.(n = 4).

Ber's antagonistic effect against Dox in H9c2 cells may be a result of this compound's free radical scavenging activities and/or interference with cell signaling pathways, which leads to the attenuation of Dox-induced damage. It was reported that Ber exhibits cardio-protective properties via multiple mechanisms, such as minimizing free radical injury, and preventing mitochondrial dysregulation (241). In a similar way, Ber protects cultured cardiomyocytes from Dox toxicity by lowering SIRT1-induced p66shc expression, which reduces the damage caused by Dox(140).

3-10. Chemosensetizing effect of mBer in SKOV-3 cells:

To assess the possible inhibitory effects of various concentrations of free Ber, mBer, Dox, Ber-Dox, or mBer-Dox combination, and void F127 micelles on human SKOV-3 cells, the MTT assay was performed, and the results were also represented as a percentage of inhibition relative to the untreated control. The results demonstrate that both Dox and Ber reduced cell viability in the SKOV-3 cell line in a concentration-dependent manner (Figure 3-14 and 3-15).

Treatment of SKOV-3 cells with free Ber exhibited dose-dependently low cytotoxicity. mBer augments the cytotoxicity of Ber in comparison to its free form (Figure 3-14). Micelles devoid of Ber had no effect on SKOV-3 cells. Dox's cytotoxicity against SKOV-3 cells is clearly increased when combined with Ber. Whereas there is a shift in the potency and efficacy of the cytotoxicity against SKOV-3 cells when mBer was used in combination with Dox compared to the situation when Ber and Dox were used in combination (Figure 3-15). Also, contrary to what was seen in H9c2 cells, when SKOV-3 cells were treated with both Ber and Dox, the IC₅₀ was significantly ($p < 0.05$) lower than when Dox was used alone. This further suggests that Ber makes

cancer cells more sensitive to chemotherapy when it is combined with Dox. Intriguingly, the micelle form of Ber enhances Ber's chemosensitizing effect on SKOV-3 cells, as indicated by a further significant ($p < 0.05$) drop in IC₅₀ value (Figure 3-16).

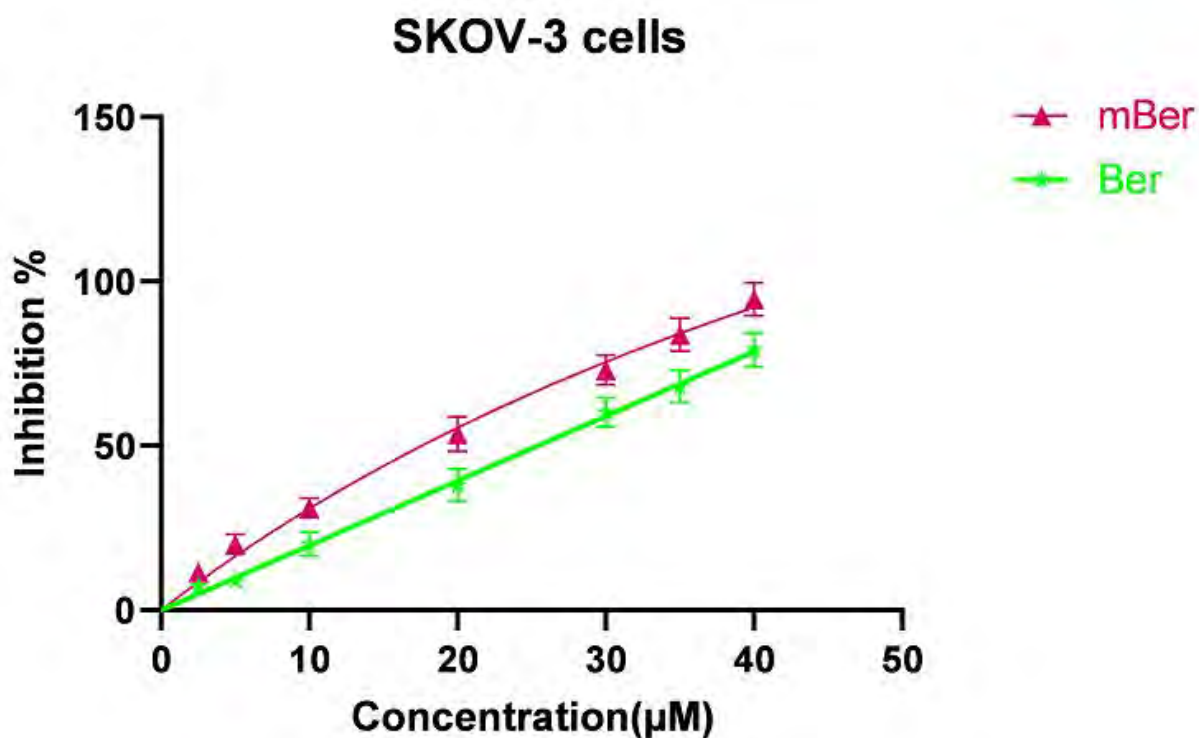


Figure 3-14: A comparison of the growth inhibition percentages of free Ber and mBer in SKOV-3 cells.

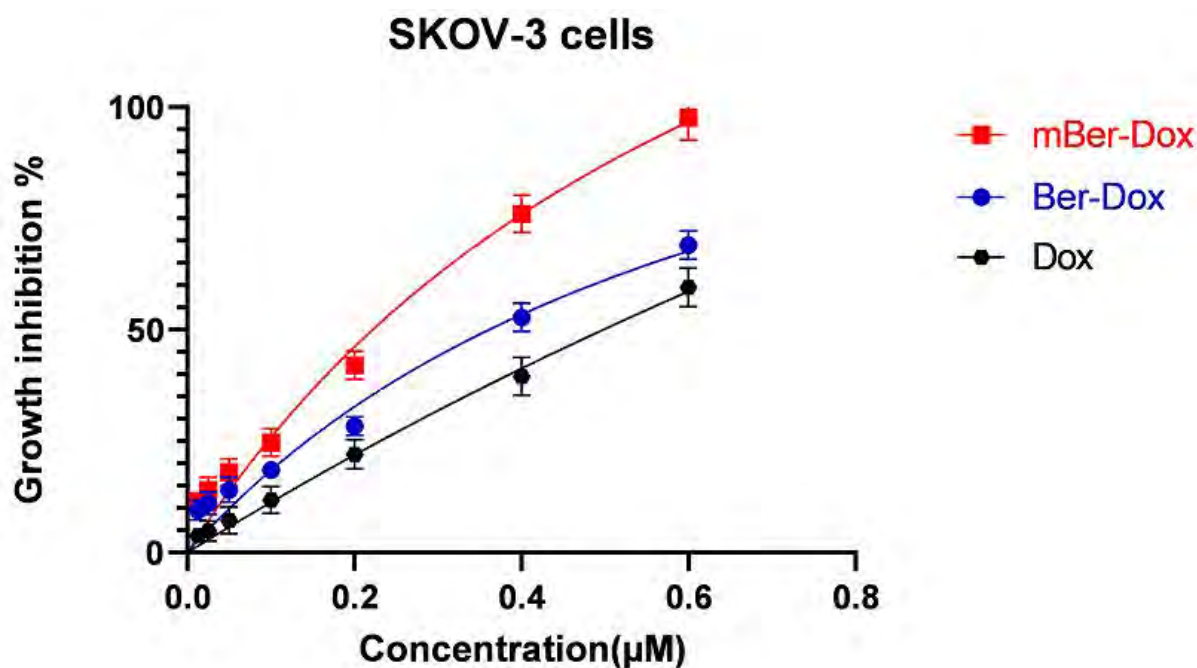


Figure 3-15: Growth-inhibition percentages of Dox, Ber-Dox, and mBer-DoX in SKOV-3 cell line. Data presented as mean $\pm$ SD(μ M); n = 4.

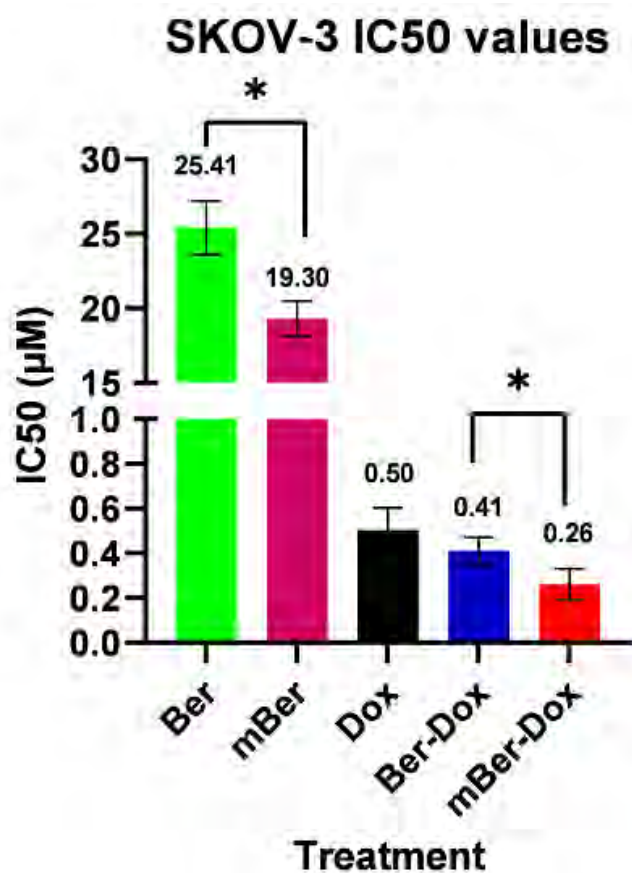


Figure 3-16: The IC₅₀ values of Dox, Ber-Dox, and mBer-DoX in SKOV-3 cell lines. Data presented as mean $\pm$ SD(μ M); n = 4. * denoted to significant differences p<0.05.

With contrast to the findings observed with H9c2 cells, we found that free Ber has an inhibitory effect on SKOV-3 cells. The anticancer properties of Ber have been documented and have been attributed to its capacity to remove free radicals, induce apoptosis and cell cycle arrest, decrease angiogenesis and inflammation, and regulate the activity of several signaling pathways (242). The inhibitory effect of Ber on SKOV-3 cells was enhanced when a nanoformulation of Ber was utilized. Intriguingly, we also confirmed that Ber and Dox work well together in SKOV-3 cell line. This suggests that Ber makes SKOV-3 cells more sensitive to chemotherapy when it is combined with a highly toxic drug like Dox. The CI index corroborated the IC₅₀ value, demonstrating that the synergistic anticancer activity of mBer and Dox was improved. One possible explanation is that F127 micelles improve Ber's permeability, delivery, and retention inside the cell. Ber, in particular, is rapidly effluxed from cells because it is a substrate for P. glycoprotein drug efflux transporter(103) .

Inhibition of the P-glycoprotein by Pluronic F127 micelles has been reported (243). These findings suggest that F127 micelles contribute to enhanced cellular retention of Ber. One way that both Dox and Ber act as anti-cancer agents is by inducing apoptosis (244,245).

3-11. Synergistic action of Ber and Dox in SKOV-3 cells:

In SKOV-3 cells, both combined treatment showed antagonistic effect ($CI < 1$) only at relatively low concentrations (low fraction effected) which is less relevant to clinical therapy, but substantially showed synergistic effect as their concentrations increase. The effect of Ber-Dox combination was found to be synergistic after a Fa of 0.25, whereas the mBer-Dox co-delivery showed synergistic effect earlier after a fa of 0.15. Notably, after those points, which are more relevant to clinical therapy, it was revealed that they both have an antagonistic cytotoxic effect. In general, the mBer-Dox combination

demonstrates superior cytotoxic synergy in SKOV-3 cells than the Ber-Dox combination (Figure 3-17).

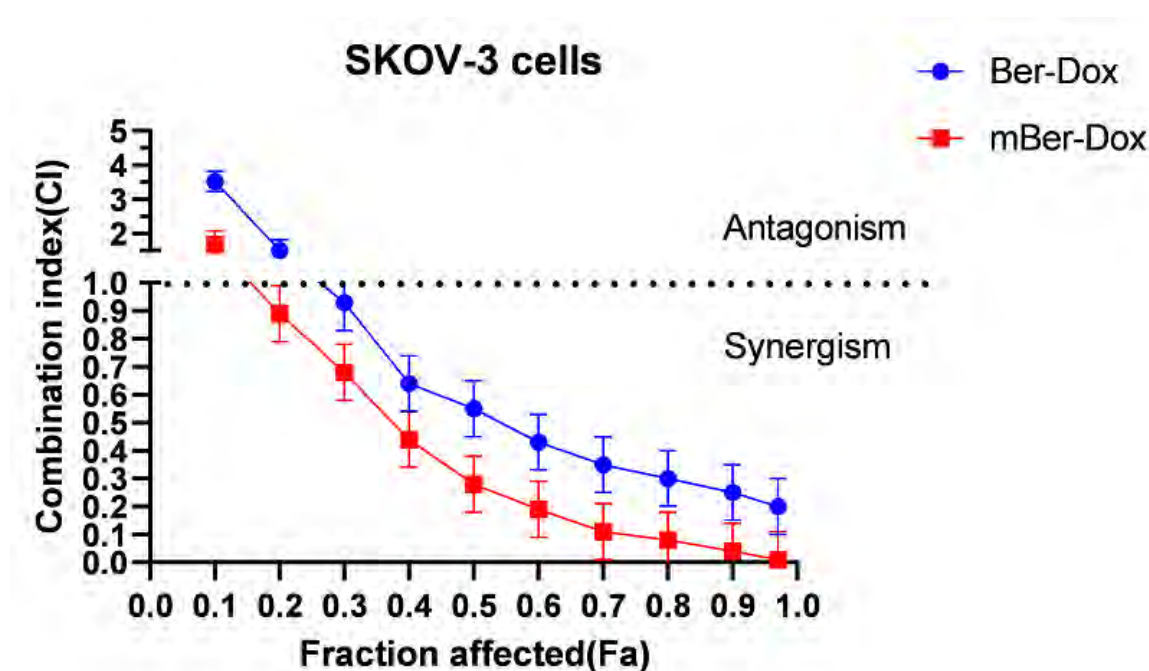


Figure 3-17: Ber-Dox and mBer-Dox CI values in SKOV-3. Where values below 1, at 1, and above 1 denote synergistic, additive, and antagonistic effects, respectively. Data presented as mean CI $\pm$ SD. (n = 4).

Based on the idea of "independent similar action", which is a situation in which the combined effect of two drugs is equivalent to the greater of their individual effects (246), this seems to be the case. Thus, the combination of Dox and Ber may have a synergistic anti-cancer effect. We showed that Ber, a naturally occurring phytochemical molecule, can protect cultured neonatal rat cardiomyocytes from Dox's toxic effects and make a cultured human breast cancer cell line more sensitive to the drug's effects. In agreement with our findings, it has been reported that Ber significantly reduced the apoptosis in H9c2 cardiomyocytes induced by hypoxia (247). Conversely, a number of studies have indicated that Ber promotes apoptosis in cancer cell lines in a concentration-dependent way (248). According to the findings of the present

study, it can be concluded that the effect of Ber on apoptosis differs depending on the type of cell employed and the concentration used.

3-12. The effect of mBer on the viability of H9c2 cells treated with Dox 1 μ M:

Cell viability after treatment with empty micelles, Ber, or mBer (98 ± 3.6 , 95 ± 3.7 , and $94.7 \pm 3.6\%$ respectively) was not statistically ($p > 0.05$) different from the control, demonstrating that neither polymeric micelles nor Ber in its free or micellar form per se produce cytotoxicity in H9c2 cells. Treatment of H9c2 cells with 1 μ M concentration of Dox, resulted in significantly ($p < 0.05$) lower viability ($52.00 \pm 2.50\%$) (Figure 3-18). Compared to the control cells. Dox-induced cell death was significantly ($p < 0.05$) mitigated by pretreatment with free Ber, which increased cell viability to $72.7 \pm 3.0\%$. Our evaluation of the protective effects of mBer against DIC in H9c2 cells revealed that the cell viability was significantly ($p < 0.05$) greater with mBer ($84.2 \pm 3.10\%$) compared to Ber alone (Figure 3-18).

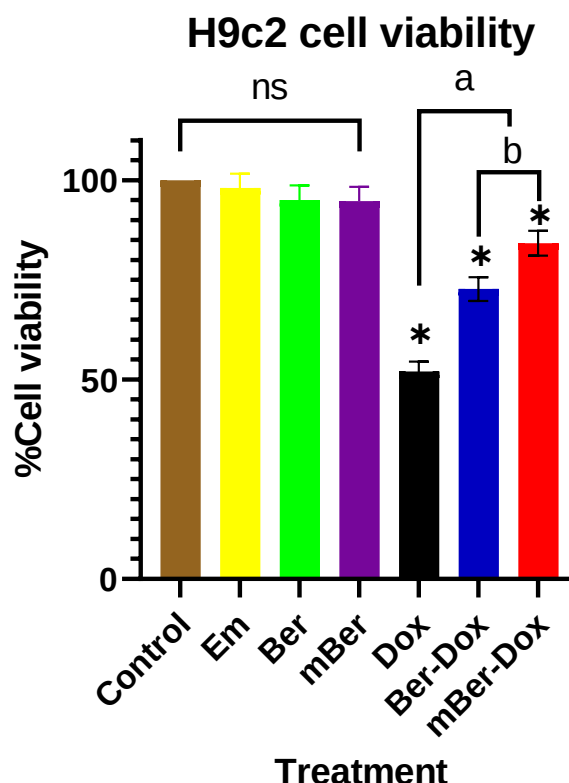


Figure 3-18: H9c2 cell viability using MTT reduction assay. The data are presented in the form of mean value $\pm$ SD derived from four independent replicate. ns: not statistically significant ($p > 0.05$) compared to control, * significantly different ($p < 0.05$) compared to control, a: significantly different ($p < 0.05$) compared to Dox and b: significantly different ($p < 0.05$) compared to Ber-Dox. Em: empty blank micelles; Ber: berberine; mBer: berberine-loaded pluronic F127 micelles; Dox: doxorubicin.

We used a concentration of 1 μ M of Dox in this study because this concentration is representative of what is seen in human plasma after therapeutic doses of Dox have been delivered (249). In clinical practice, after Ber was given to patients, its maximum concentration in the plasma could reach 1 μ M (250).

In accordance with this, we find that Ber was not cytotoxic and did not display clear cellular toxicity in cultured cardiomyocytes. Therefore, in the present investigation, we looked at how Ber affected Dox-induced cultured cardiomyocyte damage at doses of 1 μ M, and we found Ber

reduced DIC when co-delivered with Dox. Interestingly, Ber did not induce significant cytotoxic effects on cultured cardiomyocytes when used alone. We have found that the F127 micelle is a useful carrier for Ber, substantially enhancing its efficacy against DIC while maintaining its nontoxic effect on cultured cardiomyocytes.

3-13. mBer attenuate DIC by reduction of LDH, CK-MB, and cTn-I release:

LDH release was utilized as an indicator of cardiomyocyte damage. The control group exhibited the lowest cytotoxicity at 63 ± 8.3 U/L, while the Dox group exhibited the highest at 317 ± 17.4 U/L. LDH leakages from cells treated with void micelles, free Ber, and mBer were 66 ± 8.9 , 68 ± 9.3 , and 60.5 ± 10.3 U/L, respectively, which were not significantly ($p > 0.05$) different from the control, indicating that these compounds do not cause cytotoxicity in H9c2 cells. Treatment with either free Ber or mBer in combination with Dox significantly ($p < 0.05$) reduced Dox injury to cultured cardiomyocytes, as evidenced by the reduced release of LDH to 265 ± 16.1 and 210 ± 15.2 U/L, respectively, compared to Dox-treated cells. mBer was significantly ($p < 0.05$) more effective than free Ber in this regard (Figure 3-19a).

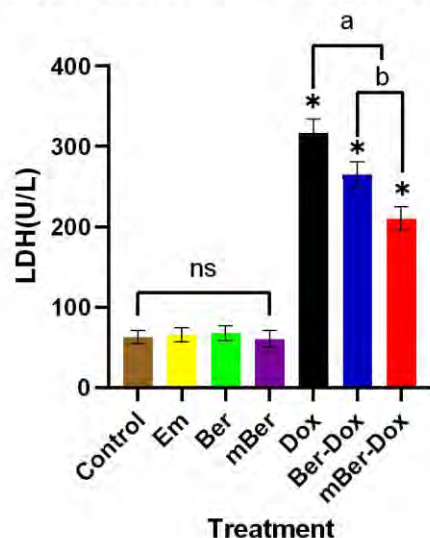
To learn more about how Ber and mBer affect DIC, the leakage level of CK-MB, which is another marker for cardiotoxicity, was measured. Compared to the control group (0.61 ± 0.16 pg/ml), Dox significantly ($p < 0.05$) increased CK-MB release (2.82 ± 0.25 pg/ml). The effects of Dox were relatively countered by combining it with Ber or mBer, which significantly ($p < 0.05$) decreased CK-MB release to 2.11 ± 0.22 and 1.52 ± 0.22 respectively (figure 3-19b).

Once again, mBer significantly ($p < 0.05$) reduced CK-MB release better than free Ber. As was indicated before, Em, Ber, and mBer did not

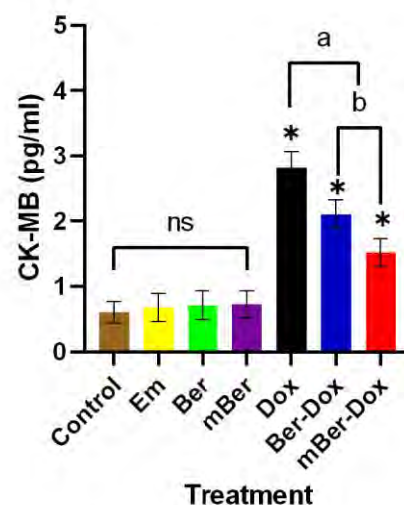
induce significant leakage of CK-MB, which indicated that they were not cytotoxic to H9c2 cells (Figure 3-19b).

Treatment with Em, Ber, and mBer did not significantly ($p > 0.05$) alter cTn-1 release levels from H9c2 cells compared to the control cells 23 ± 2.1 pg/ml. cTn-1 level was significantly ($p < 0.05$) elevated in Dox-treated cells 66 ± 4.2 pg/ml compared to the control group. The combination of Ber and Dox significantly ($p < 0.05$) decreased cTn-1 release to 55.4 ± 3.6 pg/ml. Moreover, compared to Ber-Dox-treated cells, cTn-1 level was significantly ($p < 0.05$) reduced in mBer-Dox-treated cells (45.67 ± 3.2 pg/ml), indicating enhanced therapeutic potential of Ber when loaded in to pluronic micelles (Figure 3-19c).

a- LDH levels released from H9c2 cells



b- CK-MB levels released from H9c2 cells



c- cTn-1 leakage from H9c2 cells

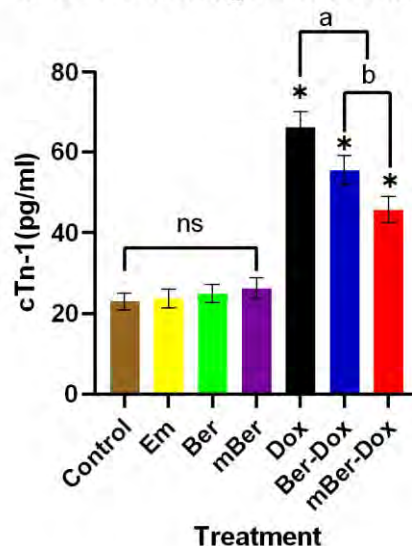


Figure 3-19: The effects of mBer on Dox-induced cardiomyocyte toxicity markers Leakage in H9c2 cells. Cardiotoxicity parameters included a: release of lactate dehydrogenase (LDH), b: Creatine kinase myocardial band (CK-MB) leakage, and c: Cardiac Troponin I (cTn-I) release. The data are presented as mean value $\pm$ standard deviation based on four independent replicates. ns: not statistically significant ($p > 0.05$) versus the control, * statistically significant ($p < 0.05$) versus the control, b: statistically significant ($p < 0.05$) versus Dox, and c: statistically significant ($p < 0.05$) versus Ber-Dox. Em: void blank micelles; Ber: berberine; mBer: pluronic F127 micelles loaded with berberine; Dox: doxorubicin.

LDH, CK-MB, and cTn-I are all well-recognized surrogate markers for cardiotoxicity, with their elevations serving as quantitative indicators of cellular membrane integrity loss (251). LDH is a stable enzyme found in all cell types; upon plasma membrane disruption, it is rapidly released into the cell culture medium and is regarded as a hallmark of cellular damage (252). Creatine Kinase (CK) is a crucial enzyme in the production and utilization of cellular energy. The CK-MB (most prominent in cardiac muscle) is a dimer of two polypeptide chains consisting of M and B subunits which release after cardiomyocytes injury; other kinds of CK are detected in various tissues (253). Troponin (Tn) is a family of regulatory proteins that play a role in skeletal and cardiac muscle contraction. cTn-I molecules are composed of amino acid sequences that are unique to cardiac tissue, making it a highly specific method for detecting cardiac injury. cTn-I follows a similar release pattern to that of CK-MB, however CK-MB levels recover after a relatively shorter time, the concentration of cTn-I remains elevated for an extended duration, thereby offering an extended period during which cardiac injury can be detected (254). The levels of LDH, CK-MB, and cTnI in Dox-treated H9c2 cells are much higher than in untreated cells, as predicted. This is consistent with previous research indicating that Dox compromises the integrity of myocardial cell membranes (255).

Interestingly, simultaneous treatment with free Ber or mBer reduced LDH, CK-MB, and cTn-I levels in Dox-treated H9c2 cells. Ber's ability to protect H9c2 cells from Dox-induced injury and to reduce the release of cardiac LDH, CK-MB, and cTn-I might be due to Ber's antioxidant properties and/or interaction with cell signaling pathways. Ber's protective antioxidant capabilities have been demonstrated both here and in earlier studies (256).

3-14. mBer mitigates the oxidative stress induced by Dox:

Afterward, the amounts of variables linked to oxidative stress were assessed. MDA production was dramatically increased by Dox treatment in the H9c2 cells (8.6 ± 0.61 nmol/mg), whereas Ber co-treatment significantly ($p < 0.05$) reduced the Dox-induced increase in MDA level. Compared to Ber-Dox-treated cells, mBer-Dox-treated cells demonstrated a significant reduction ($p < 0.05$) in MDA levels. The levels of MDA in Em-treated cells, Ber-treated cells, and mBer-treated cells are not significantly different from those in control-treated cells (Figure 3-20a).

Also, oxidative stress was measured by looking at GSH content, a key factor in ROS production, in H9c2 cells. GSH level significantly ($p < 0.05$) went down to 17.26 ± 1.8 nmol/mg protein in Dox-treated cells after 48 hours of incubation relative to control group (40 ± 2.31 nmol/mg protein), which was expected. In contrast, the GSH level of cells that were treated with both Dox and Ber was significantly ($p < 0.05$) lower than the GSH level of cardiomyocytes that were only treated with Dox. This demonstrates how Ber can lessen the oxidative stress that Dox causes in H9c2 cells. Comparing cells treated with Ber-Dox to those treated with mBer-Dox, the level of GSH was significantly ($p < 0.05$) greater in those who received mBer-Dox, indicating further improvement in Ber delivery. The GSH level did not significantly ($p > 0.05$) differ between the negative control and EM cells, nor between the Ber and mBer treated cells (Figure 3-20b).

CAT, in contrast to MDA, showed the opposite pattern. CAT is an important antioxidant defense for cells. Figure 3-19c shows that 48 hours of Dox treatment caused a decline in cellular CAT activity in H9c2 cells (2.73 ± 0.41 μ mol/min/mg protein). The cellular CAT activity in H9c2 cells was reduced 2.5-fold relative to control cells. Ber in combination with Dox attenuated Dox-induced CAT activity reduction. In terms of CAT activity

enhancement when co-treated with Dox, mBer significantly ($p < 0.05$) outperformed free Ber. The treatment of H9c2 cells with Em. Ber, mber did not result in any significant ($p > 0.05$) alteration in the activity of cellular CAT (Figure 3-20c).

Moreover, oxidative stress was measured by looking at SOD activity, another key factor against oxidative stress, in H9c2 cells. The SOD activity in H9c2 cells treated with Dox was 104 ± 7.8 U/mg which is exhibited a significant ($p < 0.05$) decrease compared to control cells following an incubation period of 48 hours, which was expected. In contrast, the activity of SOD in the cells that were treated with both Dox and Ber (139 ± 8.1 U/mg) was significantly ($p < 0.05$) higher than the SOD activity in the cells that were only treated with Dox. This demonstrates how Ber can lessen the oxidative stress that Dox causes in H9c2 cells. Comparing cells treated with Ber-Dox to those treated with mBer-Dox, the activity of SOD was significantly ($p < 0.05$) higher in those treated with mBer-Dox (169 ± 8.9 U/mg), indicating further improvement in Ber delivery. The SOD activity did not differ significantly ($p > 0.05$) between the negative control and void micelles treated cells, nor between the Ber and mBer treated cells (Figure 3-20d).

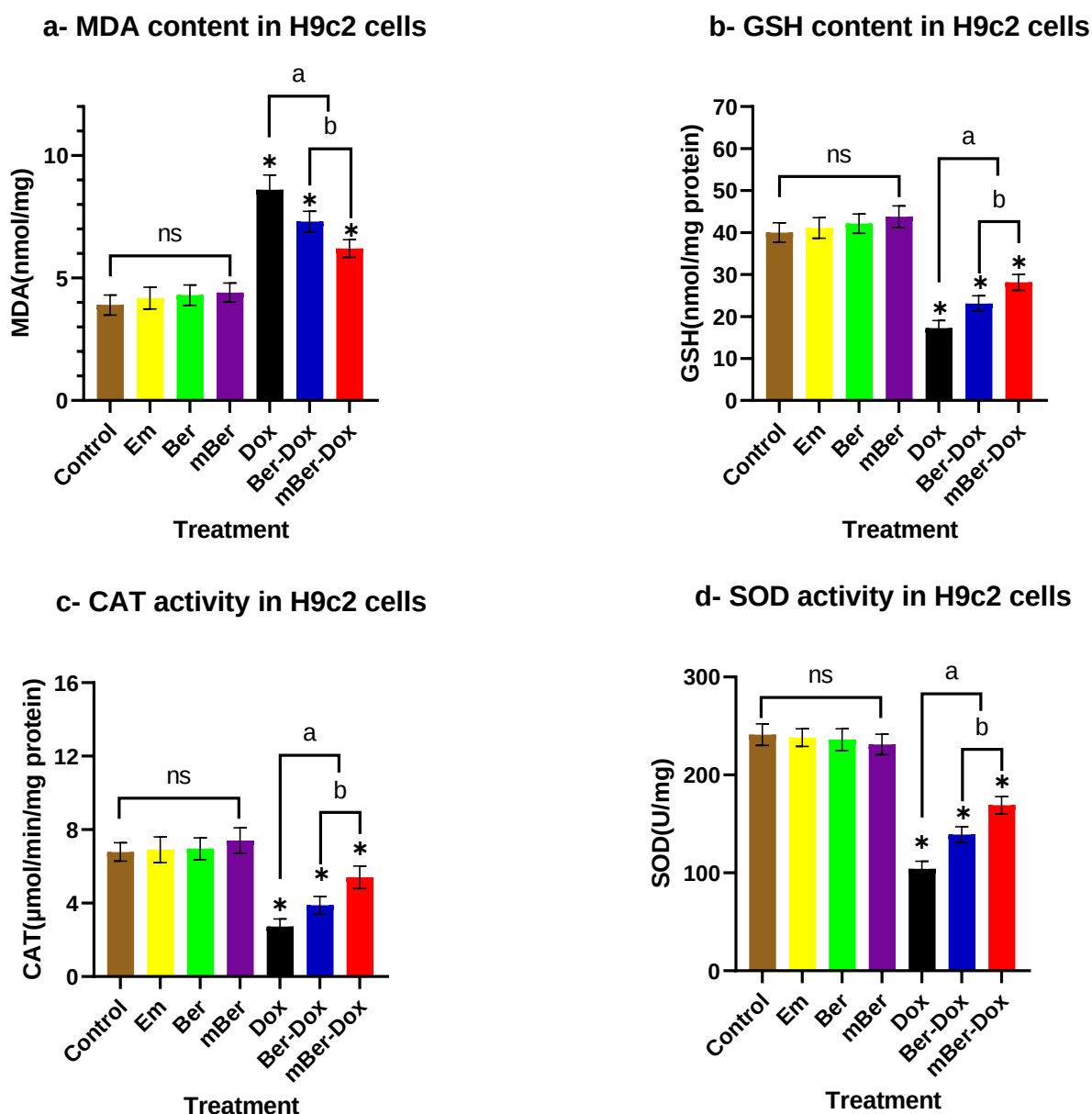


Figure 3-20: mBer suppresses Dox-induced oxidative stress. The oxidative stress parameters in H9c2 cells were: a: Lipid peroxidation indicated by malondialdehyde (MDA) level; b: Reduced glutathione (GSH) level; c: Catalase (CAT) activity; and d: Superoxide dismutase (SOD) (GPx) activity. Based on quadruplicate, the data are shown as mean $\pm$ standard deviation. ns: not statistically significant ($p > 0.05$) compared to the control; *: statistically significant ($p < 0.05$) compared to the control; a: statistically significant ($p < 0.05$) compared to Dox; and b: statistically significant ($p < 0.05$) compared to Ber-Dox. Em stands for empty micelles, Ber for berberine, mBer for pluronic F127 micelles containing berberine, and Dox for doxorubicin.

Although a multifaceted and complex process involved in DIC, oxidative damage and antioxidant deficiency in cardiomyocytes have been extensively implicated as a major cause of DIC (257). Dox exhibits a pronounced affinity towards iron, and the creation of iron-Dox complexes induces a significant imbalance in the redox status and unrestrained ROS production which ultimately induces oxidative damage (258). Cardiomyocytes are more susceptible to reactive oxygen species-induced oxidative injury due to their relatively lower levels of antioxidant enzymes (259). Oxidative stress has been found to have negative consequences by many ways such as inducing lipid peroxidation (260). In the current investigation, MDA level was assessed as a biomarker for lipid peroxidation. The administration of Dox resulted in a notable elevation of MDA levels in Dox-treated cells compared to the control group, which is in line with previous studies (261).

Cellular membranes that are abundant in peroxidation-prone lipids, such as in cardiac cells, are the primary targets of free radical damage mediated by Dox. The aforementioned radical-induced impairment results in the formation of ongoing and hazardous aldehydes, namely MDA. The aldehydes possess the ability to undergo diffusion within the cellular environment and may even traverse the plasma membrane. They can subsequently initiate an attack on macromolecular targets located at a considerable distance from their site of origin, thereby functioning as secondary cytotoxic messengers (241).

Additionally, our findings validated that Dox exhibited a significant reduction in the antioxidant content and performance of CAT and SOD. The findings presented herein are consistent with previous studies (256). The significant increase in MDA level and the decrease in the activity of the antioxidant enzymes CAT and SOD after Dox treatment as compared to

the control group, which is presented here, unequivocally point to the presence of severe oxidative stress. The treatment of cells with Ber-Dox or mBer-Dox resulted in a reduction of MDA levels in comparison to the group treated with Dox, with a more pronounced effect observed in the mBer-Dox treated group. This is in line with what has been previously published, which states that Ber significantly decreased cardiac MDA in rats that had been treated with Dox (241). This evidence from the current data suggests that the robust antioxidant potential of Ber is in opposition to Dox-induced oxidative stress. Also, the treatment with Ber or mBer resulted in a significant enhancement of the antioxidative activities of GSH, CAT, and SOD against Dox, which is consistent with prior investigations (256). Ber is a type of metal chelator that has the ability to chelate transition metals, including iron which is particularly significant as iron has been shown to contribute to the initiation of ROS (262).

The cardioprotective properties of Ber may be attributed to its antioxidant potential, which entails the elimination of free radicals and the binding of iron through chelation, ultimately resulting in a decrease in the generation of ROS. Importantly, an enhancement in antioxidant activity is evident in H9c2 cells receiving mBer-Dox treatment groups in comparison to the Ber-Dox-treated group. The findings suggest that the incorporation of Ber within the micelles enhances the antioxidant capacity of this phytochemical.

3-15. mBer alleviates cardiac cytokines secretion in Dox-treated H9c2 cells:

To determine whether Ber in its free or micellar form could protect cardiomyocytes from Dox-induced injury, the levels of inflammatory cytokines IL-1 β and IL-6 were measured in the culture media of H9c2 cells. After 48 hours of exposure to 1 μ M Dox, the levels of IL-1 β and IL-6

in the culture medium of Dox-treated cardiomyocytes were 273.42 ± 20.2 pg/ml and 138.2 ± 8.2 pg/ml respectively which were significantly ($p < 0.05$) higher than those of control cells (63 ± 10.9 and 26.71 ± 4.2 pg/ml for IL-1 β and IL-6 respectively) (Figure 3-21a). A rise in cytokines levels caused by Dox was shown to be significantly ($p < 0.05$) lessened by combining treatment with either Ber or mBer. However, combination treatment with mBer resulted in an additional significant reduction ($p < 0.05$) in cytokines (142 ± 15.9 pg/ml IL-1 β and 79.2 ± 5.9 pg/ml IL-6) than did combined treatment with Ber (196 ± 14.8 and 97.3 ± 6.3 pg/ml for IL-1 β and IL-6 respectively). Again, Em, Ber, and mBer do not have any significant ($p > 0.05$) effect on the level of cytokines that are released into the culture medium of cardiomyocytes (Figure 3-21b).

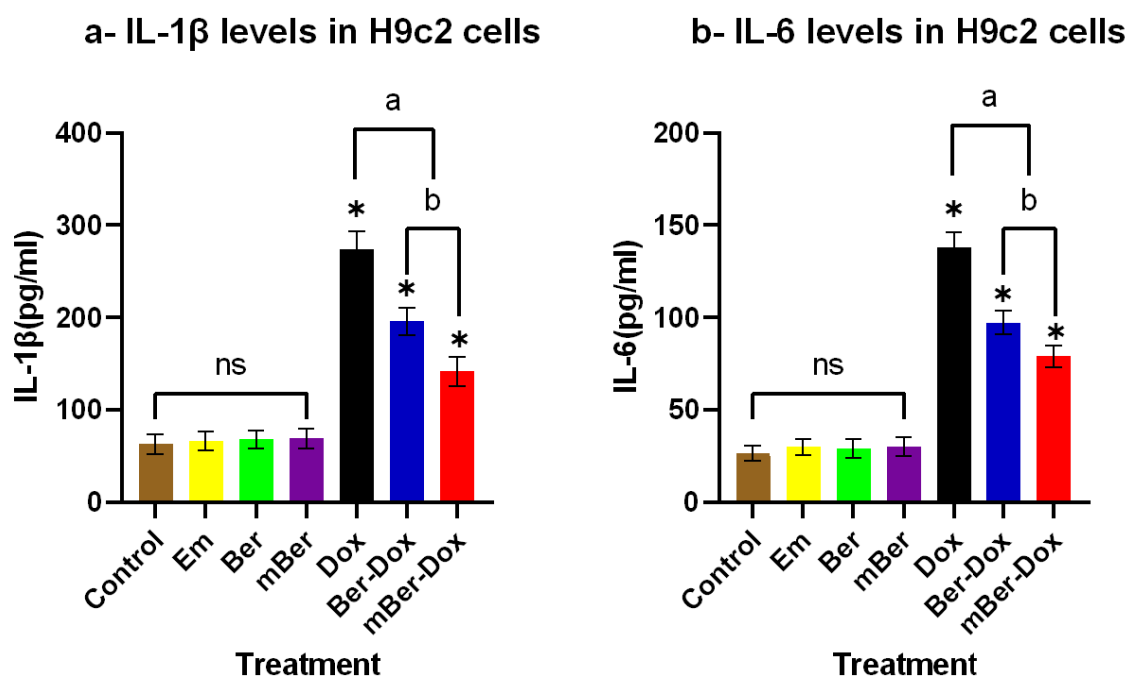


Figure 3-21: The effect of mBer on Dox-induced proinflammatory cytokine elevation in H9c2 cells. The studied inflammatory markers included a: interleukin-1 β (IL-1 β) and b: interleukin-6 (IL-6). Data indicate Four biological replicates' mean $\pm$ SD. ns: not statistically significant compared to the control ($p > 0.05$); *: statistically significant compared to the control; a: compared to Dox; and b: compared to Ber-Dox ($p < 0.05$).

Notably, the expression of inflammatory cytokines is directly induced by oxidative stress, which further escalates inflammatory processes (263). Numerous studies support the idea that targeting inflammation and oxidative stress is an effective therapeutic approach to reduce DIC. There is mounting evidence linking pro-inflammatory cytokines to the onset of numerous cardiac disorders. Among these cytokines are interleukins, which play crucial roles in the onset and progression of a number of cardiac diseases, including coronary atherosclerosis and congestive heart failure (258). Dox is capable of causing significant cardiac toxicity that is associated with a significant inflammatory response, as reported here. Nuclear factor-kappa B becomes active quickly following Dox injury in the cardiomyocytes, where it stimulates the synthesis of multiple pro-inflammatory cytokines, including IL 1 β , TNF- α , and IL-6 (264). These cytokines further worsen inflammatory damage by coordinating a pro-inflammatory milieu and recruiting leukocyte infiltration into the myocardium. Therefore, prior studies have shown that reducing cardiomyocyte mortality and cardiac dysfunction after Dox administered is possible via the regulation of cardiac inflammation (265). We found that the co-delivery of Ber in free or micellar form with Dox resulted in a significant reduction of IL-6 and IL-1 β levels induced by Dox treatment. The anti-inflammatory properties of Ber in cardiac cells have been previously linked to its ability to impede the phosphoinositide 3-kinase (PI3K)/AKT signaling pathway. This, in turn, leads to a decrease in the release of several pro-inflammatory cytokines, including IL-6, IL-1 β , and tumor necrosis factor TNF- α , in cardiac myocytes (266). The findings indicate that the treatment with Ber-Dox in comparison to Dox alone resulted in a decrease in the levels of IL-6 and IL-1 β , suggesting that Ber could have the ability to mitigate the inflammatory response triggered by

Dox in cardiomyocytes, and this ability was improved when Ber was loaded into pluronic micelles.

3-16. mBer protects H9c2 cells from Dox-induced cell apoptosis:

Figure 3-22 depicts the caspase 3/7 activity of H9c2 cells in the presence of different treatments. Dox-treated cells exhibited fivefold more caspase 3/7 activity than untreated control cells. Ber-Dox or mBer-Dox significantly ($p < 0.05$) reduced caspase 3/7 activity in H9c2 cells compared to Dox treatment alone while maintaining apoptotic activity beyond control level. Notably, there is a significant ($p < 0.05$) reduction in caspase 3/7 activity in cells treated with mBer-Dox as compared to those treated with Ber-Dox. Em, Ber, and mBer have no discernible effect on the activity of Caspase 3/7 in H9c2 cells (Figure 3-22).

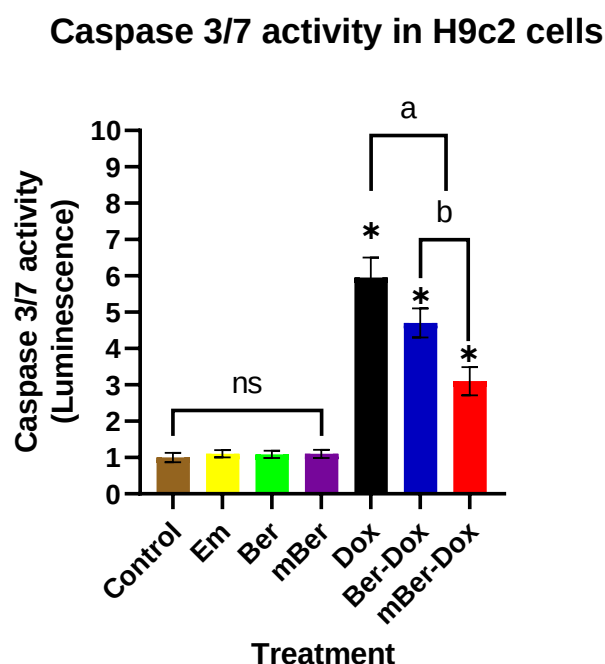


Figure 3-22: mBer inhibits Caspase 3/7 activity in Dox-treated H9c2 cells. The presented data illustrates the mean $\pm$ SD from Four distinct biological replicates. The notation "ns" denotes no statistically significant difference when compared to the control group, while "*", "a", and "b" signify significant differences in relation to the control group, Dox, and Ber-Dox, respectively ($p < 0.05$).

Various anticancer agents, such as Dox, can induce apoptosis through the activation of caspases 3/7, implying either an intrinsic or extrinsic pathway for ultimate cell death. Therefore, evaluating caspase 3/7 may provide insights into ultimate cell death regardless of the specific apoptotic mechanism being used (267). Thus, the utilization of caspase3/7 as a biomarker to assess apoptosis-initiated cell death could offer a close approximation of the effect of Ber on the Dox-induced cardiomyocytes apoptosis.

Following treatment with Dox, a substantial increase in caspase 3/7 activity was seen in H9c2 cells compared to that observed in the control cells and this is consistent with prior reports about Dox in cultured cardiomyocytes (268).

Reduced caspase 3/7 activity was detected in H9c2 cells in culture as a result of the concurrent treatment of Ber with Dox, irrespective of the existence or absence of micelles. It is possible that the antagonistic interaction between Ber and Dox in H9c2 cells in terms of apoptosis is caused by a reduction in both the activity of caspase 3/7 and reactive oxygen species in cardiomyocytes induced by Ber treatment. This is corroborated by previous reports that Ber treatment reduced apoptotic and oxidative stress in response to ischemia injury by lowering caspase-3 activity and MDA levels (269). However, mBer co-delivery did not completely inhibit Dox-induced caspase 3/7 activation and return to normal levels (untreated), indicating that DIC is not completely mitigated. A possible rationale for this occurrence is that Dox-induced caspase 3/7 activation may involve other mechanisms that might not be affected by Ber treatment at the current concentration.

H9c2 cells are likely to undergo a complex and multifaceted process of cellular apoptosis upon exposure to Dox. This is supported by previous

research indicating that caspase-12 activation plays a role in mediating Dox-induced cardiomyocyte apoptosis (270).

Chapter Four

Conclusions and Recommendations

Conclusions:

1-Using the thin-film hydration approach, we were able to create spherical Ber-loaded F127 micelles with high entrapment efficiency that possess desirable physicochemical features.

2- Ber's in vitro release behavior from the micelles demonstrated both fast and sustained release properties.

3- mBer were shown to be relatively non-toxic and biocompatible via in vitro hemolysis testing.

4-The utilization of mBer presents a more suitable option compared to conventional free Ber in mitigating the elevated levels of oxidative stress, inflammation, and apoptosis-inducing factors in cardiomyocytes exposed to Dox treatment.

5- Our findings indicated that polymeric F127 micelles are a relatively simple strategy that could serve as a promising nanocarrier for enhancing Ber delivery.

6- In cultured cardiomyocyte cells, the cardioprotective effect of mBer against Dox is greater than the cardioprotective effect of free Ber. This is due to its enhanced effectiveness against DIC.

7- The combination of mBer and Dox could be a novel approach that offers the potential for a promising and effective agent for the treatment of tumors.

Recommendations:

- 1-To confirm the chemosensitizing and cardioprotective effects of mBer, further research is required to validate these findings through in vivo studies and in clinical settings.
- 2- The findings of our study offer a logical approach for developing more phytochemical adjunctive therapies with Dox that have the potential to be efficacious for patients undergoing chemotherapy.
- 3- Evaluation of the chemosensitizing effect of the newly developed mBer on several types of cancer cell lines, encompassing not only an ovarian adenocarcinoma cell line but additionally others.
- 4- Assessment of the cytoprotective efficacy of the created mBer on several other cell types, such as hepatocytes and renal cells, among others.
- 5- Explore how the mBer-Dox combination treatment affects cellular metabolomics and gene expression profiling in cardiomyocytes and several cancer cell lines.

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الخلاصة

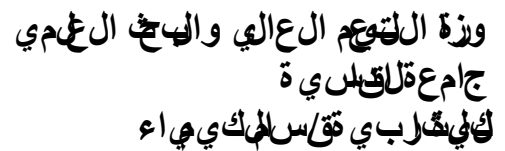
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تتبعن توغر رية تارث احيوي ئيلاي ئيى او بتوك و افصالي الذي لى و عالا ولى 27 (بغاغ ييىة
(الذي لى و غم كمش (ح عالا 11 غم ضرر عدي غرو و غل بلعومش. اعل عبت روض ع غي عت غل بلعومش اى عيىة غرو ع
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اطروحہ مقدمہ الی
 مجلسینفالتربیہ -جامعہ القدس
 وہی جزء منقطلباتیل درجہ التتوراتوعلفی الکی واء

بأشرف
أ. د انوار جلیل بٹھان