Formulation and Characterization of Cubosomes Containing REB for Improvement of Oral Absorption of the Drug in Human Volunteers

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Submitted on: 31-12-2017; Revised on: 05-02-2018; Accepted on: 08-02-2018

ABSTRACT

Objective: To prepare rebamipide (REB) -loaded cubosomal nanoparticles to improve the oral bioavailability of the drug in human volunteers. Methodology: The poorly water soluble drug REB was incorporated into cubosomal nanoparticles by solvent dilution method. The prepared REB-loaded cubosomes were in vitro characterized for particle size, polydispersity index (PDI), zeta potential, drug crystallinity and in vitro drug release. In vivo absorption of REB-loaded cubosomes was compared with drug powder and commercially available tablets Mucosta® (Egypt Otsuka Pharmaceutical Company) in healthy human volunteers. Results: In vitro, REB-loaded cubosomes entrapped about 82.44 % drug and revealed nanometer-size ranged from 303.1 ± 4.9 to 444.9 ± 6 nm. In vitro REB release from cubosomes exhibited a phase of rapid and high release of the drug during the first hour followed by a slower and complete drug release over a period of 3 hrs. The results of bioavailability study in healthy human volunteers showed that cubosomes significantly improved the rate and extent of REB absorption. The mean relative bioavailability of the REB loaded cubosomes compared to the drug powder and commercially available tablets (Mucosta®) was 267.67 % and 166.29 % respectively. Conclusion: The prepared cubosomes significantly improved the In-vivo absorption of REB in comparison with drug powder and commercially available tablets (Mucosta®).

Keywords: Absorption; Bioavailability; Cubosomes; Rebamipide

INTRODUCTION

REB, a 2-(4-chlorobenzoylamino)-3[2(1H)-quinolonin-4-yl] propionic acid (Figure 1), is a potent antiulcer agent that stimulates generation of endogenous prostaglandins in the gastric mucosa, thereby facilitating and accelerating ulcer healing. REB is considered a Biopharmaceutics Classification System (BCS) Class IV according to the US Food and Drug Administration (FDA) due to its low aqueous solubility and poor oral bioavailability. The bioavailability of REB is about 10% in humans.

Improvement of bioavailability of a hydrophobic drug like REB is a major challenge in drug development. Various techniques have been reported to be advantageous for improving solubility and oral bioavailability of REB such as formulation of nanocrystals, solid dispersion, and salt formation techniques.

Cubosomes are discrete, submicron, nanostructured particles of bicontinuous cubic liquid crystalline phase. It has the same microstructure as the parent cubic phase but differ in having larger specific surface area and their dispersions have much lower viscosity in comparison to the bulk cubic phase.

Figure 1: Chemical structure of rebamipide

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Cubosomes have many advantages as drug carriers, such as high drug payloads due to high internal surface area and cubic crystalline structures, relatively simple method of preparation, biodegradability of lipids, capability of encapsulating hydrophilic, hydrophobic and amphiphilic substances, and targeted or controlled release of bioactive agents. Cubosomes have been proposed as an excellent candidate for oral drug delivery system. In most cases, cubosomes as drug delivery system were explored for poorly water-soluble drugs to enhance the oral bioavailability. Sustained release of drug under a controlled manner can also be achieved by using the nanostructure lipid-based liquid crystalline system. In a previous study, it has enhanced bioavailability of simvastatin, a class II drug according to Biopharmaceutics Classification System (BCS), has been achieved by GMO/poloxamer 407 cubic nanoparticles. Taking advantage of their permeation-enhancing effect, on the other hand cubic nanoparticles were evaluated as potential vehicles to improve the oral bioavailability of cyclosporine A, a more challenging BCS IV drug of poor water-solubility and poor permeability and succeeded to achieve 178% improvement in the oral bioavailability.

There were many trials for improvement of REB oral bioavailability like formulation of REB solid dispersion which made an improvement of bioavailability 2.71 times higher than that of the reference product in the ulcer-induced rat model. While in another experiment for preparation of REB lysinate salt no significant difference was observed in bioavailability between REB and REB lysinate. Also there was a trial for formulation of REB nanosuspension, which showed a slight increase in the bioavailability of REB nanosuspension compared with Mucosta® tablet. Accordingly, the objective of this study is to prepare REB-loaded cubosomes and to compare its oral bioavailability with REB powder and REB commercially available tablets (Mucosta®) in healthy human volunteers.

**MATERIAL AND METHODS**

**Material**
REB was a gift sample from Egypt Otsuka Pharmaceutical Company, Glyceryl mono oleate (GMO) was kindly donated from Kerry Group (Ireland), Poloxamer 407 was obtained from Sigma Aldrich Chemie GmbH (Switzerland), deionized water was obtained from Arab Company for Pharmaceutical and Medicinal plants MEPACO-MEDIFOOD (Egypt), Absolute ethyl alcohol was obtained from El-Nasr Pharmaceutical Chemical Company (Egypt), and other chemical were of analytical grade and were used without further purification.

**Methods**

**Construction of HPLC calibration curve**

The HPLC system was consisted of LC20AB solvent delivery system, SIL-20AHT auto-sampler, CBM-20A communication bus, DGU20A3 vacuum degasser, RF20A fluorescence detector (excitation 320 nm; emission 380 nm), and CTO-20A column oven (all of them by Shimadzu Corporation, Japan). The mobile phase used for REB was consisted of acetonitrile: water: acetic acid (30:70:5). REB separation was carried out using a C18 (100 - 4.6 mm, 5mm) analytical column (ACE, Aberdeen, Scotland). Flow rate was set at 0.5mL/min.

Oflaxacin was used as an internal standard. Oflaxacin stock solution was prepared by dissolving 100 μg of Oflaxacin powder in 200 ml acetonitrile and then this solution was vortex mixed until complete dissolution, the concentration of this stock solution is 0.5 μg/ml. For preparation of stock solution 10 mg REB REB powder was dissolved in 200 ml methanol for a final concentration of 50 μg/ml Using methanol, REB stock solution was serial diluted to 10, 5, 2.5, 1, 0.5, 2.5, and 0.1μg/ml. from the dilutions, a calibration curve with different concentration ranges was constructed.

**Preparation of REB loaded cubosomes**
Blank and REB-loaded cubosomal nanoparticle dispersions were prepared through disrupting a cubic gel phase of GMO and water in the presence of poloxamer 407 as a stabilizer by mechanical stirring. The dispersions appeared as uniform opaque white mixtures with no visible signs of aggregates. The final concentration of lipid in the dispersion ranged from 5% w/w (F1 and F2) to 10% w/w (F3 and F4) with respect to the final dispersion weights (100 gm in all formulae). The ratio of GMO to poloxamer 407 in total lipid content was 10:1 w/w in all prepared formulae. The choice of this ratio was based upon observations of Jin et al. who have found that cubosomes of this composition have reasonable physicochemical properties and improved the absorption of a poorly absorbed drug. The compositions of different formulae of REB loaded cubosomes are shown in Table 1.

**Characterization of cubosomes**

**Particle Size and polydispersity index (PDI)**
The measurement of the mean particle size of cubosomes was done using laser diffraction technique on a Zetasizer (Malvern Instruments Ltd. Malvern, UK). Before the measurement, 0.5 ml sample were diluted with deionized water to a volume of 30 ml to obtain a suitable scattering intensity, the size was measured at 25 ºC, each sample of particle size was determined in triplicate and the mean was considered.
Values of PDI give an indication for the homogeneity of the preparation\(^7\).

**Determination of Zeta potential**

The zeta potential of REB loaded cubosomes was measured using Zetasizer Nano (Malvern Instruments, UK). This instrument is laser based multiple angle particle electrophoresis analyzer. It measures the electrophoretic mobility and surface charge. To determine zeta potential, 0.5 ml of the cubosomal nanoparticle was diluted with deionized water to 30 ml, all measurements were done at room temperature (25\(^\circ\)C) and repeated three times \(^8\).

**Entrapment efficiency %**

The entrapment efficiency (EE\%) of the drug was determined by centrifugation method. Briefly, 1 mL of the prepared cubosomes were added to 4 mL deionized water and centrifuged at 15000 rpm for 15 minutes till complete precipitation of the cubosomal nanoparticles. One mL of the clear supernatant was added to 4 mL methanol and vortexed for 5 minutes; 100\(\mu\)L sample of the vortexed solution was placed in clean glass tubes, 20 \(\mu\)L of 36\% hydrochloric acid was added followed by 2 mL of acetonitrile. Solutions were vortex mixed for 1 min, then centrifuged for 20 min at 5000. After centrifugation, supernatant was transferred to clean glass tubes and evaporated to dryness then reconstituted in 200 \(\mu\)L mobile phase and placed in clean injection vials for HPLC analysis\(^5\).

\[
\text{E.E \%} = \frac{W_{\text{initial}} - W_{\text{free}}}{W_{\text{initial}}} \times 100
\]

Where, \(W_{\text{initial}}\) drug is the amount of REB initially used for cubosome preparation and \(W_{\text{free}}\) drug is the amount of the drug not incorporated in to cubosomes and remain in the supernatant.

**Transmission Electron microscope examination**

Morphological examination of cubosomes was carried out using a transmission electron microscope (Jeol Company, Japan) equipped with super twin lens. A droplet of cubosome dispersion was placed on a 200 mesh carbon-coated copper grid, and the excess fluid was removed by an absorbent filter paper. The samples were stained with 1% sodium phosphotungstate solution and were viewed using magnification up to 1,000,000 xs.

**Differential scanning calorimetry**

This test aims to detect any possible change in the physical state of REB when got entrapped in the cubosomes. DSC was carried out for REB loaded cubosomes, blank cubosomes, REB powder, and GMO using a thermal analysis system (DSC-60, Shimadzu, Japan). The samples (5mg) were heated over a temperature range 30 \(^\circ\)C – 350 \(^\circ\)C at a constant rate of 10\(^\circ\)C/min in an aluminum pan under a nitrogen atmosphere. A similar empty pan was used as the reference.

**Infrared Spectroscopy**

IR spectra were obtained using Fourier Transformer Infra-Red (Shimadzu, Japan) for REB loaded cubosomes, blank cubosomes, REB powder, and GMO. Samples were prepared in KBr discs (about 10 mg sample for 100 mg of dry KBr). The IR spectra were obtained in the spectral region 450–4000 cm\(^{-1}\).

**Powder X-ray diffraction**

X-ray diffraction patterns of both blank and REB loaded cubosomes as well as pure REB, and GMO samples were obtained using the X-ray diffractometer. The samples of the prepared cubosomes were separated from fluids using filter paper and left to dry in air for 12 hours. Diffracotgrams were recorded using Cu as tube anode under the following conditions: voltage was 45 kV, the current was 30 mA, steps were 0.02 \(^\circ\) of (\(^2\)Th.), and the counting rate was 0.5 s/step at room temperature. Data were collected from 4 \(^\circ\)C to 40 \(^\circ\)C.

**In vitro release of REB from REB loaded cubosomes**

In vitro release of REB loaded cubosomes was performed using cellulose tube diffusion technique \(^19,20\). Briefly, cellulose tube was soaked in the release media overnight before the experiment. The cellulose tube was filled with an accurately weighed amount of the cubosomes equivalent to 100 mg REB, tightly closed, and was immersed in a receptor compartment containing 900 mL of phosphate buffer (pH 7.4). The release of REB was performed at 37 \(\pm\) 0.5 \(^\circ\)C using the US Pharmacopoeia dissolution apparatus (paddle method) at 100 rpm with 900 mL phosphate buffer as the dissolution medium. Two mL of dissolution media were withdrawn at predetermined time intervals of 15, 30, 45, 60, 75, 90, 105, and 120 min, followed by replacing with an equal volume of fresh media in the same temperature in order to maintain the sink condition. All the dissolution samples were filtered using syringe filter (0.45 \(\mu\)m) prior to the HPLC analysis process. These experiments were performed in triplicates and the mean was considered.

**Study design**

The study was performed to compare the bioavailability of REB loaded cubosomes with REB powder and marketed tablets (Mucosta\(^\circ\)) in human volunteers after oral administration of a single oral dose equivalent to 100 mg of each. Volunteers were fasted overnight (12 hour) and the study performed using a non-blind, two-treatment, two periods, randomized,
crossover design with a washout period of two weeks between each phase. The study was approved by the Helwan University Protection of Human Subjects Committee, and the protocol complies with the declarations of Helsinki and Tokyo for humans. After giving informed written consent, six adult male volunteers were enrolled in this study. First of all, a comprehensive medical examination were performed for the volunteers (including a physical examination, clinical laboratory, vital signs and symptoms, medical history, test for hepatitis B, hepatitis C, syphilis, and HIV, and a drug abuse screening test), also a test for hypersensitivity to REB was performed for all volunteers, and they were not receiving any medication during the study period.

Collection of blood samples

Blood samples (1 ml) were collected directly from each volunteer using I.V. cannula into heparinized tubes at 0, 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, and 12 hours. Collected blood samples were centrifuged at 4000 rpm for 5 minutes, and the plasma was transferred to separate glass tubes to be kept frozen until analysis.

HPLC analysis of REB in plasma

The HPLC system was consisted of a Jasco PU-980 pump, model FP-2020 plus fluorescence detector (λ<sub>ex</sub> 320 nm, λ<sub>em</sub> 380 nm) equipped with L-7200 auto sampler (Hitachi, Tokyo, Japan), a column heater (CH-150, Eldex Lab Inc., CA, USA), and DS-CHROM chromato-integrator (Donam instruments Inc., Seoul, Korea). The chromatographic separation was performed using a Luna C18 analytical column (250mm x 4.6mm I.D., 5 µm, Phenomenex; Torrance, CA), preceded by a guard column (4mm x 3mm I.D.) packed with the same material (Phenomenex; Torrance, CA). Both the analytical and guard columns were kept at 60ºC within a column heater. The mobile phase consisted of acetonitrile–water–acetic acid (30: 70: 5, v/v, pH 2.4) and was delivered at a flow-rate of 1.0 mL/min.

Sample preparation

To 0.5 mL of human plasma in a 2 mL micro tube, 10µL of internal standard solution and 100 µL of 36% hydrochloric acid were added. Then, the plasma was briefly mixed with a vortex mixer and 1 mL of ethyl acetate was added to the sample mixture. The sample solution was vortex-mixed for 5 min, and centrifuged at 5000 g for 1 min. The organic layer was transferred to another tube and evaporated to dryness. The sample residue was re-dissolved with 100 µL of freshly prepared mobile phase, and then 20 µL aliquot was injected onto the HPLC system for analysis.

Construction of the calibration curve

Stock solutions of REB (100µg/ml) and internal standard ofloxacin (10µg/ml) were freshly prepared in methanol and stored at 4ºC. Standard solutions were made by serially diluting the stock solutions in methanol to the required concentrations before use. Calibration curves were constructed with drug free human plasma spiking with stock solutions, which have the final concentrations of REB, 0 (blank), 2, 10, 50, 100, and 500 ng/ml, respectively, in plasma.

RESULTS AND DISCUSSION

Particle size, PDI and Zeta potential

The mean particle sizes of REB loaded cubosomes (Table 2) were in the range of 303.1 ± 4.9 to 444.9 ± 6 nm. Results of particle size distribution

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Table 2. Mean particle size, PDI, Zeta potential and EE % of the prepared cubosomes

<table>
<thead>
<tr>
<th></th>
<th>Mean particle size (nm)</th>
<th>PDI</th>
<th>Zeta potential (mV)</th>
<th>EE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>389.90 ± 8.20</td>
<td>0.334 ± 0.02</td>
<td>-11.4 ± 0.17</td>
<td>88.10 ± 1.26</td>
</tr>
<tr>
<td>F2</td>
<td>303.10 ± 4.90</td>
<td>0.368 ± 0.03</td>
<td>-18.6 ± 0.13</td>
<td>75.80 ± 0.83</td>
</tr>
<tr>
<td>F3</td>
<td>444.90 ± 6.12</td>
<td>0.264 ± 0.04</td>
<td>-13.8 ± 0.26</td>
<td>87.85 ± 0.45</td>
</tr>
<tr>
<td>F4</td>
<td>517.50 ± 6.30</td>
<td>0.358 ± 0.07</td>
<td>-15.0 ± 0.19</td>
<td>78.00 ± 0.85</td>
</tr>
</tbody>
</table>

Figure 3. DSC thermogram of (A) Rebamipide, (B) GMO, (C) Poloxamer 407, (D) blank cubosomes, and (E) rebamipide loaded cubosomes.

Figure 4. IR spectrum of (A) Rebamipide, (B) GMO, (C) Poloxamer 407, (D) blank cubosomes, and (E) rebamipide loaded cubosomes.

Figure 5. Powder X-ray diffraction pattern of (A) Rebamipide, (B) GMO, (C) Poloxamer 407, (D) blank cubosomes, and (E) rebamipide loaded cubosomes.

Figure 6. In-vitro release of rebamipide loaded cubosomes (different formulae) in comparison with rebamipide powder.

showed that incorporation of REB lowered the particle size of the drug loaded cubosomes to be smaller than blank cubosomes. This decrease in particle size differs from the results obtained in the preparation of cubosomes containing 5-flurbiprofen for ocular application[23] where entrapment of the drug led to particle size increase. However, in preparation of amphotericin B cubosomes, entrapment of drug caused no change in the cubosomal particle size[24]. The results of PDI (Table 2) reveal that the particle size
distribution of drug loaded cubosomes shows monodispersity, i.e., narrow range of cubosome particle size distribution\(^2\). Results of zeta potential for different formulae of cubosomes are shown in Table 2 which are in the range $-18.6 \pm 0.13$ mV to $-11.4 \pm 0.17$ mV indicating a good stabilization level of the prepared cubosomal nanoparticles.

**Entrapment efficiency %**

The mean entrapment efficiency of REB is ranged from 75.80 to 88.10 % (Table 2). These results revealed that increasing the concentration of REB led to an increase in the entrapment efficiency, while the concentration of GMO has no effect on the entrapment efficiency.

**Transmission Electron microscope:**

The TEM microphotograph of REB-loaded cubosomes (Figure 2) reveal that the particles are of certain shapes ranged from circular, square or rectangular shapes. The particle size of the photographed cubosomes is in the range of 400 – 500 nm which is in good agreement of the particle size determined by laser diffraction technique.

**Differential scanning calorimetry**

Figure 3 show the DSC thermograms of REB, GMO, poloxamer 407, blank cubosomes and REB loaded cubosomes. DSC thermogram of REB exhibit a sharp characteristic endothermic peak at 306.3 °C, corresponding to its melting point reflecting the crystalline state of the drug. DSC thermogram of GMO show endothermic peak at 37 °C, while that of poloxamer 407 showed an endothermic peak at 57°C. Formulation of blank cubosomes show a significant shift in mixture melting point to about 97.8 °C, while the REB loaded cubosomes do not have a sharp peak indicating the melting point but show a wide peak in the range 108-124.4 °C. These results revealed that the REB when incorporated into the cubosomal nanoparticles may be in the amorphous form, or it may had undergone an interaction with the other ingredients of the formula leading to the disappearance of its characteristic peak at 306.3 °C.

**Infrared Spectroscopy**

The IR analysis was performed to support the results obtained from thermal analysis. The IR spectra for REB (Figure 4) show a characteristic peak for the carbonyl group at 1643 cm\(^{-1}\) and another peak at 3600 cm\(^{-1}\) for the hydroxyl group. The spectra of drug loaded cubosomes showed slight shift and decreased peak intensity of the characteristic OH group of pure drug, while the peak of C=O group was disappeared. This result is in agreement with their DSC thermograms and is attributed to the possible interaction between hydroxyl group of GMO and pure drug carbonyl group. Based on the results of DSC thermograms and IR spectra, it is concluded that GMO showed a remarkable tendency to interact with the pure drug.

**Powder X-ray diffraction**

X-ray diffraction was carried out to confirm the physical state of REB incorporated into cubosomes in comparison to drug-free cubosomes, pure drug, and GMO. It is clear that the diffractogram of the pure REB (Figure 5) exhibit multiple characteristic intensity reflections indicating its crystalline nature. However, these characteristic peaks disappeared in the X-ray diffraction pattern of REB loaded cubosomes. Moreover, the powder X-ray diffraction pattern for the drug loaded cubosomes is without any remarkable difference when compared to the powder X-ray pattern for blank cubosomes. This indicates that the drug was molecularly dispersed or in the amorphous form and confirms previous results from the DSC analysis.

**In vitro drug release from REB loaded cubosomes**

Figure 6 shows the in vitro release profiles of REB-loaded cubosome F1 and F3 in addition to release profile of REB powder. It is found that the drug release was very high during the first hour followed by gradual drug release. The results show approximately the same release pattern for both formulae, but F3 is found to be of higher drug release than F1 with about 1%. According to this data the formula F3 is selected for in the in-vivo studies.

**Construction of standard REB in plasma calibration curve**

The measured peak area ratio of REB was plotted as functions of drug concentration and a linear relationship was obtained with correlation coefficients of about 0.997 over the studied REB concentration range (10 - 500 ng/ml).

**Plasma concentration-time data**

The results of the mean $C_{\text{max}}$, and $\text{AUC}_{0-12}$ after administration of REB powder, F3 and the commercial REB tablets are shown in (Table 3) (Figures 7 – 10). A high improvement in the bioavailability of REB loaded cubosomes is achieved as indicated by significantly higher values of $C_{\text{max}}$ (368.33 ± 4.23 ng/ml) and $\text{AUC}_{0-12}$ (673.89 ± 7.73 ng.hr/ml) compared to both REB powder and Mucosta\textsuperscript{®} tablets (Figure 11).

The mean relative bioavailability of REB-loaded cubosomes, calculated on the basis of $\text{AUC}_{0-12}$, was 267.67 % and 166.36 % compared to plain REB powder, and Mucosta\textsuperscript{®} tablets, respectively. These results indicate that a 167% increase in the oral bioavailability of REB was achieved by REB-loaded cubosomes.
Table 3. Bioavailability parameters of REB in human volunteers (n=6) after administration of a single oral dose (100 mg/kg) of powder REB, Mucosta® tablets and REB-loaded cubosomes (F3)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Plain REB powder</th>
<th>Mucosta® tablets</th>
<th>REB-loaded cubosomes (F3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt; (ng/ml)</td>
<td>157.67 ± 2.73</td>
<td>221 ± 2.53</td>
<td>368.33 ± 4.23</td>
</tr>
<tr>
<td>T&lt;sub&gt;max&lt;/sub&gt; (h)</td>
<td>1.95</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;0-12&lt;/sub&gt; (ng.h/ml)</td>
<td>252.23 ± 4.37</td>
<td>405.26 ± 4.64</td>
<td>673.89 ± 7.73</td>
</tr>
</tbody>
</table>

Each value represents the mean ± standard division of six volunteers
* P<0.05, statistically significant difference based on student t test

Figure 7. Mean plasma concentration of rebamipide after single oral dose of 100 mg rebamipide powder.

Figure 8. Mean plasma concentration of rebamipide after single oral dose of Mucosta® tablet 100 mg.

Figure 9. Mean plasma concentration of rebamipide after single oral dose of rebamipide loaded cubosomes equivalent to 100 mg rebamipide.

Figure 10. Mean plasma rebamipide concentration after administration of single oral dose of (A) rebamipide powder, (B) Mucosta® tablet, and (C) rebamipide loaded cubosomes.
The mean relative bioavailability of REB-loaded cubosomes, calculated on the basis of AUC0-12, was 267.67% and 166.36% compared to plain REB powder, and Mucosta® tablets, respectively. These results indicate that a 167% increase in the oral bioavailability of REB was achieved by REB-loaded cubosomes (F3). This might be due to that, cubosomes as a drug delivery system may enhance the absorption of poorly water soluble drugs through different pathways. The unique liquid-crystalline structure of cubosomes could provide a protection for entrapped drug from degradation in the gastrointestinal tract. Also, the lyotropic property of cubosomes and the secondary nanostructures formed as a result of GMO digestion in gastrointestinal tract, makes it easier for the drug loaded nanoparticles to penetrate the “unstirred aqueous layer” and make closer contact with cell membranes. This close contact in addition to the similarity of lipid bilayer of cubosomes to microstructure of cell membrane, which may promote their uptake by endothelial membranes and facilitate drug absorption.

CONCLUSIONS

In conclusion, the administration of REB loaded cubosomes, formula F3, improved the absorption and bioavailability of the drug. More clinical trials and experimental work are needed for verification and evaluation the use of cubosomes as a drug delivery system for oral lower doses of REB in comparison to the standard oral REB dose.

Conflict of Interest

The authors declare that they don’t have any conflict of interest.