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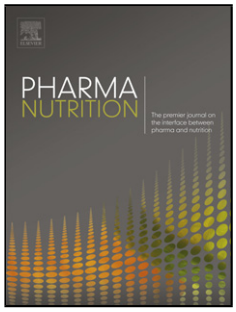

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An *in vitro* approach to study the absorption of a new oral formulation of berberine

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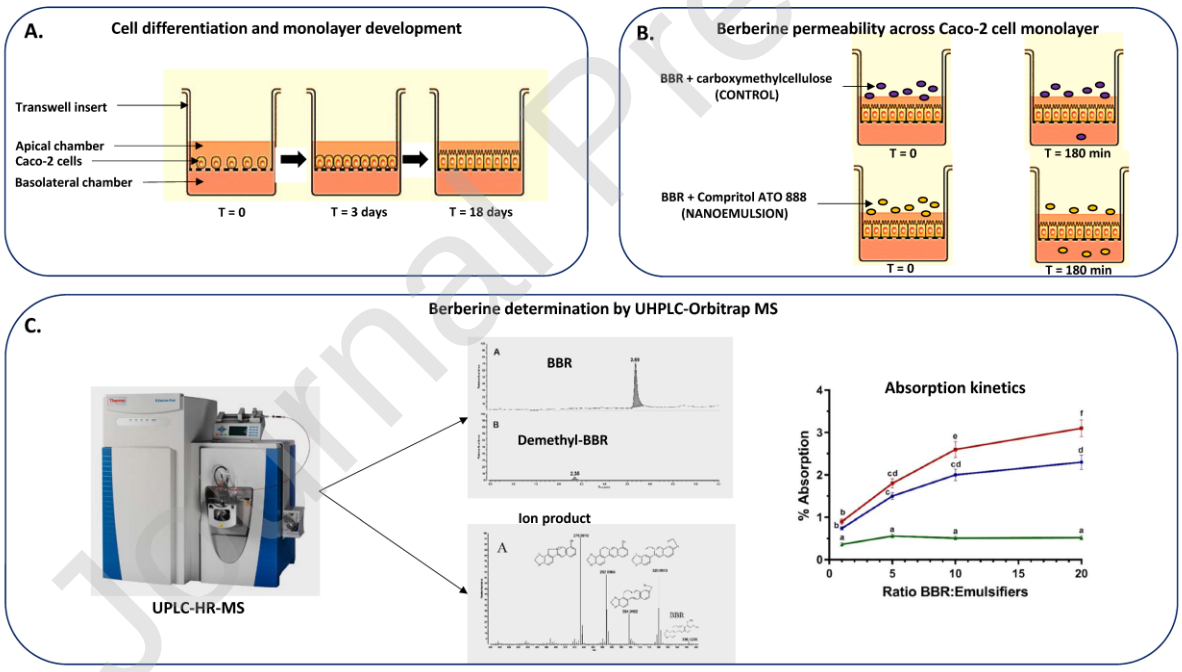
Graphical abstract



Abstract

Background: Berberine (BBR) possesses several biological activities in humans, but the poor water solubility and low oral bioavailability preclude its pharmacological use. To overcome these limitations, several formulations have been prepared including encapsulation, micro- and nano-emulsion. The aim of this study was to develop a nanoemulsion delivery system of BBR and to evaluate its membrane permeability using Caco-2 cell model.
Methods: Nanoemulsions containing different ratios BBR:Compritol ATO 888 (a lipid excipient) were formulated at 25 and 80 °C. The controls consisted of BBR and carboxymethylcellulose. Absorption of BBR nanoemulsions delivery systems was evaluated *in vitro* by using human colon adenocarcinoma cells (Caco-2) Transwell model. The amount of permeated BBR was determined by LC-HR-MS at time zero and every 30 min for 180 min.
Results: Nanoemulsions significantly improved apical-to-basal transport of BBR compared to the control formulation. Kinetics of BBR uptake showed that the maximum amount absorbed was reached after 90-120 min and the percentage of BBR absorbed by Caco-2 cells increased with increasing BBR-to-Compritol ratio (1:20 > 1:10 > 1:5 > 1:1). Moreover, the formulation prepared at 80 °C showed a higher absorption rate (6-fold increment compared to control) than that developed at 25 °C (4.5-fold increment compared to control). Furthermore, demethyl-BBR was detected after 120 min of incubation as partial metabolism of berberine in the intestine.
Conclusions: Overall, in our *in vitro* model, these new nanoemulsions seem to potentially improve the absorption of BBR. However, *in vivo* studies are required in order to demonstrate the bioavailability of BBR from this new formulation.

Keywords: berberine; nanoparticles; Caco-2 cells;*in vitro* absorption; mass spectrometry

1. Introduction

Plants are producers of numerous bioactive molecules (i.e., carotenoids, glucosinolates, polyphenols) with a plethora of biological and functional activities [1-5]. Within bioactives, berberine (BBR), a natural alkaloid isolated from medicinal herbs such as *Coptis chinensis* and *Hydrastis Canadensis*, is traditionally used for the treatment of diarrhea and gastroenteritis [6]. More recently, other biological functions have been attributed to BBR, including hypolipidemic, hypoglycemic, antiarrhythmic, anti-inflammatory, antimicrobial, neuroprotective and antineoplastic activities [7–14]. Regarding the hypolipidemic activity, it should be noted that the mechanism of action of

BBR is different compared to statins. In fact, while statins act by inhibiting the synthesis of endogenous cholesterol and upregulating the low-density lipoprotein (LDL) receptor (LDL-R) in liver and peripheral tissues [15], BBR seems to reduce the amount of circulating cholesterol by increasing the expression and stabilization of LDL-R. Therefore, as a hypolipidemic molecule, BBR reduces cholesterol with a mechanism of action different from that of statins [16].

Despite the potential biological activities in humans and the low toxicity and cost, the therapeutic use of BBR has encountered several challenges. In particular, the main limitations are determined by its poor water solubility and bioavailability, which has been estimated to be less than 1% of the dose ingested [17]. In order to overcome this problem, a possible solution could be the use of a delivery system that can improve its bioavailability. The most common delivery systems consist of the use of polymeric nanoparticles, silica-based nanoparticles, micelles, liposomes, graphene, and lipid nanostructures [18]. Different lipid classes have been utilized as pharmaceutical excipients due to their negligible toxicity [18]. Among them, Compritol 888 ATO has been used in drug encapsulation, as a lubricating agent in the manufacture of oral preparations [19], and as a matrix-forming agent in the preparation of sustained-release tablets [20]. Compritol consists of a mixture of diacyl- (40–60%), monoacyl- (13–21%), and triacyl- (21–35%) glycerols [21], and this particular composition provides high drug entrapment efficiency [22].

Another strategy to enhance the efficiency of transport systems based on nanostructured lipid carriers is to use high process temperature [23]. In fact, it has been shown that temperature has an impact on the particle size and shape of nanosystems, features that can increase the solubility and dissolution rate of encapsulated drugs [24]. In particular, increased temperature leads to increased interfacial tension of the droplets, which may be responsible for the particle size and morphology [25]. In addition, low temperature induces consolidation of droplets in a non-spherical shape and a consequent low diffusion rate [25].

Therefore, the aim of the present study was to design and optimize BBR transport systems based on nanostructured lipid carriers (NLCs) consisting mainly of Compritol and, in lesser amounts, lecithin, developed at different temperatures (25 vs. 80 °C). Moreover, the different formulations were tested *in vitro* to evaluate the kinetics of BBR uptake using Caco-2 cells grown on the Transwell diffusion system as a model of intestinal absorption [26].

2. Materials and Methods

2.1. Materials

Minimum Essential Medium Eagle (MEM; Cat. No. M5650-500mL), penicillin–streptomycin solution (Cat. No. P4333-100mL), MEM Non-Essential Amino Acid Solution (100×) (Cat. No. M7145-100mML), sodium pyruvate (100 mM) (Cat. No. 11360070), Human Caucasian colon adenocarcinoma (Caco-2) cells (Cat. No 09042001-1VL), standards of BBR, methanol, acetonitrile and formic acid were provided by Sigma-Aldrich (St. Louis, MO, USA). Berberis extract at 90% BBR was from Vivatis Pharma (Gallarate, VA, I). Compritol 888 ATO, or glyceryl dibehenate according to the European Pharmacopeia, was supplied by Pharmalabor (Canosa di Puglia, BT, I). Water was obtained from an Arium pro apparatus (Sartorius, Milan, I).Millicell® tissue culture plate well inserts (Cat. No. PIHP01250) were from Merck (Darmstadt, D).

2.2. Preparation of lipid nanostructures containing berberine

Approximately 50, 100, 200, and 500 mg of berberis extract were mixed with 850, 800, 700, and 400 mg of Compritol, respectively, and then 100 mg of lecithin were added to each mixture at room temperature (RT; 25 °C). In this way, the ratio of BBR to emulsifiers was in the range of 1:20–1:1 (w/w). A further hot preparation (80 °C) was made by heating Compritol 5 °C above its melting point, and then adding 100 mg of previously heated lecithin at the same temperature. The mixture was kept in mild agitation and the temperature was fixed to ensure that the lipid material did not solidify. After 10 min, berberis extract was added and the emulsion was obtained by stirring the mixture at 16,000 rpm for 20 min. The emulsion was quickly cooled and reduced to a fine powder. All samples were stored in a refrigerator at 4 °C. The amount of BBR in the formulations was determined as described by Wang [14].The controls consisted of the same amounts of BBR used in the test formulations, while Compritol and lecithin were replaced by carboxymethylcellulose.

2.3. Cell culture

Caco-2 cells were cultured in minimum essential medium (MEM) supplemented with 10% (v/v) fetal bovine serum, antibiotics (50 U per mL penicillin, 50 µg per mL streptomycin), 1% (v/v) of 100× non-essential amino acids, and 1 mM sodium pyruvate. Cell cultures were maintained at 37 °C and 5% (v/v) CO_2 atmosphere. The medium was replaced every

3 days during cell growth and differentiation. For the experiments, cells were used 21 days after reaching confluence to allow for differentiation into intestinal epithelial cells. Cells were used between passages 4 and 10.

2.4. Viability assay

The toxicity of the compounds was tested on Caco-2 cells by Trypan blue exclusion assay using a TC20TM automated cell counter and dual-chamber cell counting slides (BIORAD, Segrate, Milan, I). Caco-2 cells with 80% confluence grade were treated with BBR at different concentrations for 24 h and in the presence of Compritol, lecithin, and cellulose. Subsequently, cells were trypsinized, resuspended, and used for Trypan blue exclusion assay. Three independent experiments were performed in which each condition was tested in triplicate.

2.5. Transepithelial electrical resistance measurement

In order to measure transepithelial electrical resistance (TEER), Caco-2 cells with 80% confluence grade were seeded on Transwell® 24-well permeable media (12 mm, 0.4 μm pore polyester membranes) at a density of approximately 2×10^5 cells per well. Cells were differentiated into polarized monolayers by growing on Transwell inserts for 18–21 d. The volume of medium added to the upper and lower compartments was 0.4 and 0.6 mL, respectively. TEER was measured using a Millicell-ERS Resistance System (Millipore, Bedford, MA, USA) that includes a dual-electrode volt-ohm-meter. TEER was calculated as follows: TEER = (Rm − Ri) × A, where Rm is transmembrane resistance, Ri is intrinsic resistance of cell-free media, and A is the surface area of the membrane in cm^2. Monolayers were used when TEER values were between 350 and 450 Ω cm^2.

2.6. Berberine permeability acrossCaco-2 cell monolayer

In the apical (AP) to basolateral (BP) experiments, 0.5 mL of test solution (70 μM BBR) was added to the AP side of the monolayer at the beginning of the test, and after 30, 60, 90, 120, and 180 min, 25 μL was taken from the basal side. To the different aliquots, 25 μL of methanol was added, the mixture was centrifuged at 6000*g* for 2 min, and the supernatants were stored at –20 °C before LC-HR-MS analysis.

2.7. Berberine determination by UHPLC-Orbitrap MS

The analysis was performed on an Acquity UPLC system (Waters, Milford, MA, USA) coupled with an Orbitrap high-resolution Fourier transform mass spectrometer, Exactive model (Thermo Scientific, Rodano, I), equipped with an HESI-II probe for ESI and a collision cell (HCD). The operative conditions were as follows: spray voltage +4.0 kV, sheath gas flow rate 55 (arbitrary units), auxiliary gas flow rate 20 (arbitrary units), capillary temperature 350 °C, capillary voltage +60 V, tube lens +100 V, skimmer +26 V, and heater temperature 130 °C. A 1.7 µm Kinetex XB C18 column (150 × 2.1 mm, Phenomenex, Torrence, CA, USA) maintained at 45 °C was used for separation. The flow rate was 0.7 mL/min, and the eluents were 0.05% formic acid in water (A) and acetonitrile (B). UPLC separation was achieved by the following linear elution gradient: 5–50% of B for 5 min, then increased to 90% B for 5 min. The acquisition was made in full-scan mode in the range (m/z)+ 100–1000 u, using an isolation window of ±2 ppm. The AGC target, injection time, mass resolution, energy, and gas in the collision cell were 1×10^5, 50 ms, 50 K, 20 V, and N2, respectively. The MS data were processed using Xcalibur software (Thermo Scientific). The peak identity was ascertained by evaluating the accurate mass and the fragments obtained in the collision cell. BBR stock solutions (0.1 mg/mL) were prepared in methanol and stored at –20 °C. Working solutions (n = 5) were prepared in the range of 2–200 ng/mL and stored at 4 °C. Analysis was carried out in duplicate.

2.8. Statistical analysis

Statistical analysis was performed by means of Statistica software (Statsoft Inc., Tulsa, OK, USA). Analysis of variance (ANOVA) was used to assess the effects of the different formulations on BBR uptake in the Caco-2 cell culture model. Post hoc analysis of differences between treatments was assessed by the least significant difference (LSD) test with $p \leq 0.05$ as the level of statistical significance. Data were derived from three independent experiments in which each condition was tested in triplicate. Results were statistically expressed as mean ± standard error of mean.

3. Results

3.1. Effect of BBR on Cell Viability

Table 1 presents the effects of BBR on cellular viability assessed by Trypan blue exclusion assay at all concentrations tested for 24 h and in the presence of Compritol, lecithin, and cellulose solution. The control condition is represented by cells in their normal growth medium without BBR and nanoemulsion formulation. BBR nanoemulsion and unencapsulated BBR did not affect cell viability, which remained higher than 90%.

3.2. Effect of nanostructured lipid carrier (NLC) transport system on BBR intestinal absorption

To assess whether the presence of emulsifiers in formulations containing BBR could improve its absorption, a delivery system consisting of nanostructured lipid carriers was prepared. The effect of different formulations on the absorption of BBR was evaluated by a Caco-2 cell transport model, which comprises a monolayer of cells expressing analogue morphological and functional features of intestinal epithelium such as microvilli and tight junctions [27]. The results reported in Table 2 suggest that fatty acid esters contained in Compritol could significantly improve the transport of BBR. Indeed, formulations containing the tested emulsifiers showed increased BBR uptake compared to controls without lipid carriers. Moreover, the increased uptake determined by emulsifiers was confirmed at all-time points analyzed (30 to 180 min).

In addition, we observed that the absorption of BBR increased in a time-dependent manner for both nanoemulsions and control formulation without emulsifiers. However, the control formulation reached a plateau after approximately 120 min, while the new nanoemulsions containing emulsifiers showed continuous uptake without reaching a plateau until 180 min (Table 2). In addition, the ratio of BBR to emulsifiers had a relevant influence: 50 mg of BBR in the formulation with a ratio of 1:20 led to a higher permeate amount than the control containing 500 mg of BBR (ratio 1:1) without emulsifiers.

Relative to the rate of BBR permeating through Caco-2 cells in the Transwell diffusion system, we found a positive correlation with the BBR to emulsifier ratio. In particular, the highest proportion of BBR uptake was obtained with a ratio of 1:20 (1:20 > 1:10 > 1:5 > 1:1) (Figure 1). In particular, the rate raised from 0.74% (ratio 1:1) to 2.3% (ratio 1:20) for the RT-prepared formulation and from 0.9% (ratio 1:1) to 3.1% (ratio 1:20) for the hot-prepared formulation. The same results cannot be translated to the control preparations, since the rate of BBR permeating through Caco-2 cells remained stable between the different ratios of BBR and cellulose tested, particularly around 0.5%.

Furthermore, we also observed that the formulation prepared at high temperature (80 °C) led to better absorption than the formulation developed at 25 °C for all BBR-to-emulsifier ratios tested (Figure 1). Specifically, the maximum difference in the rate of absorption was 0.8% (hot- vs. RT-prepared solution, 3.1% vs. 2.3%) and it was observed at the highest ratio (1:20).

3.3. Berberine and its metabolites identification by High-Resolution Mass Spectrometry

Figure 2A shows the ions extracted at $(m/z)^{+}$336.1230 u, corresponding to BBR, in a sample obtained after 180 min of incubation with a solution containing BBR:Compritol in a 1:5 ratio. In some basal solutions, obtained after 120 min of incubation with Berberine, the untargeted analysis revealed the presence of a compound with Rt 2.35 min and $(m/z)^{+}$322.1070 u ($C_{19}H_{16}NO_4$), which was 14 kDa less than BBR (Figure 2B). The unknown compound (M1) was then fragmented at different collision energies (30-60 V) and it produced ions with *m/z* 307.0845 u by losing a methyl moiety and the *m/z* 292.0970 by loss of a further hydroxyl group and 278.0015 u by further loss of methyl moiety. Figure 3 reports the fragmentation pattern of BBR and M1. Of note that the fragments with *m/z* 292.0970 and 278.0015 were in common with BBR. Thus, M1 was tentatively identified to be 9-demethyl-BBR (berberrubine) or 10-demethyl-BBR (thalifendine). Table 3 shows the concentrations of demethylated BBR determined in the basal solutions. This compound was not detected in the apical solutions. The BBR metabolite concentration was below the limit of quantification (LOQ) in all control samples (C1-C4), in samples prepared at the lowest ratio BBR:emulsifier (T1: RT and T1: HOT), except after 180 min for the formulation prepared at high temperature. As with BBR, the amount of the metabolite found in basal solutions increased with the increasing BBR-to-emulsifier ratio. In fact, the highest amount of BBR metabolite was detected for the formulation prepared with a ratio of 1:20 (1:20 > 1:10 > 1:5 > 1:1). Moreover, the formulation prepared at high temperature (80 °C) led to a higher amount than the formulation prepared at room temperature for all BBR-to-emulsifier ratios tested (Table 3). No significant difference was highlighted between both samples obtained after 120 and 150 min of incubation and those obtained after 150 and 180 min. On the contrary, samples taken after 120 min resulted significantly different from those obtained after 180 min.

4. Discussion

The aim of the present study was as follows: first, to compare the absorption of BBR from two different formulations (nanostructured lipid carrier vs. carboxymethylcellulose excipient), and second, to evaluate the kinetics of uptake by modifying the temperature for preparing the emulsifier (25 vs. 80 °C) and the ratio of BBR to emulsifier (from 1:1 to 1:20). Absorption was tested by using a Caco-2 cell Transwell model. The results obtained document that the formulations containing emulsifiers increased the rate of BBR absorption compared to the control supplement. The rate of absorption increased with an increased BBR-to-emulsifier ratio.

The absorption of BBR from different formulations has been evaluated in several studies in order to identify new transport systems (such as nano-based carriers) able to enhance its bioavailability [28]. This interest has been mainly attributed to the biological properties of BBR and its potential application in different therapeutic areas, including the prevention and management of lipids and glucose dysmetabolism [29–32]. In fact, it has been recognized that the low lipophilic property of BBR determines its scarce uptake and consequently the biological effect. Thus, the use of a lipid-based complex could represent a valid solution to improve BBR absorption. However, phospholipids could lead to the formation of phytosome, leading to a reduction in the dissolution rate and consequent absorption [33].

Our findings show that an increased BBR-to-Compritol ratio induced an improvement of BBR absorption. These results are probably attributable to the capacity of the surfactant Compritol to act as solid dispersion by enhancing the stability and dissolution rate of BBR, as also documented by Zhang and coworkers [33] in a study in which they processed complex BBR-phospholipids with the carrier TPGS100 and SiO_2. This solid dispersion led to a higher rate of dissolution and stability of the BBR complex. Our results seem to be in accordance with other *in vitro* studies testing the role of different nanoemulsions in the enhancement of BBR intestinal absorption. For example, Deng and colleagues [34] used in their experiments a transport system consisting of Compritol and other excipients such as olive oil, cremophor EL, and d-α-tocopheryl polyethylene glycol 1000 succinate, documenting improved BBR uptake. This nanoemulsion was tested in both Caco-2 cells and RAW 264.7 macrophages, showing improved BBR absorption compared to free BBR. In another study, Kwon and coworkers [35] developed a BBR-loaded mixed micelle formulation by using two surfactants, Pluronic 85 and Tween 80. The authors reported that administration of the BBR-loaded mixed micelle formulation increased BBR solubility and absorption in Caco-2 cells.

Absorption of BBR-loaded nanoemulsions was also assessed in *in vivo* studies. Elsheikh et al. [36] found that their formulation, BBR-loaded cremochylomicrons, enhanced the rate and extent of BBR absorption compared to free BBR in Sprague-Dawley rats. Further, Sun and colleagues [37] assessed the effect of Gelucire 44/14 (composed of polyethylene glycol monoesters and diesters, monoglycerides, diglycerides, and triglycerides) on the transport of BBR using an *in situ* closed-loop method in rats. The authors found that Gelucire 44/14 was able to significantly increase plasma concentration of BBR compared to the control group, and in particular enhanced absorption in the ileum.

Overall, from these studies the important role of the type of excipient in BBR absorption became clear. This effect can be attributed to the capacity of surfactants, used as excipient, to inhibit the P-glycoprotein involved in the efflux of BBR and the CYP2D6 and CYP3A4 in enterocytes, responsible for BBR metabolism [35–37]. Moreover, a mixed micelle formulation consisting of surfactants could increase intestinal absorption by affecting cell integrity, paracellular transport, and macropinocytosis transmembrane mechanisms [38]. Another aim of the study was to evaluate the effect of two temperatures (25 vs. 80 °C), used for the preparation of nanoemulsions, on BBR bioavailability. In our experimental conditions, we found that heating the Compritol, as excipient, at about 80 °C resulted in a further improvement of BBR uptake compared to the same formulation developed at room temperature (25 °C). The contribution of temperature to drug delivery and bioavailability has been evaluated in different studies [23,39-40]. For example, Barthelemy et al., [39] demonstrated that the dissolution rate of coated drug-loaded beads using Compritol increased with the use of high temperature (54 °C). More recently, a review showed that a nanoemulsion based on Compritol had better drug release when prepared under hot conditions compared to the cold analogue formulation. The authors attributed their findings to the positive impact of high temperature on the reduction of vesicular diameter, the entrapment efficiency, and the zeta potential of the nanoemulsions [23]. Furthermore, He et al., [40] evaluated the absorption of silymarin-loaded solid lipid nanoparticles prepared by using cold and hot (85 °C) Compritol. The results showed an increased rate of silymarin release with the hot-prepared formulation, in line with our findings on BBR. Regarding BBR metabolism, it should be noted that we were able to detect the presence, in small amounts, of demethyl-BBR in basal solutions after 120 min of incubation of Caco-2 cells with BBR. Xu and colleagues [41] also detected this metabolite, in addition to other derivatives from phase 2 transformations, in rat plasma after oral ingestion of BBR. Moreover, Liu and coworkers [42] observed the generation of several metabolites in rat enterocyte S9 fractions and intestinal perfusates *in vitro* studies. Among these metabolic derivatives, berberrubine (9-demethyl-BBR), the glucuronide of demethyleneberberine, and jatrorrhizine were the main intestinal metabolic products. A further contribution to BBR metabolism derives from cytochrome P450 enzymes (CYPs) in liver and intestine. In particular, CYP2D6 resulted the primary enzyme involved in the formation of the demethylated and demethylenated products [43]. Thus, our data appear to support the hypothesis that already at the level of enterocytes there is a partial metabolization of BBR to give demethyl-BBR, such as 9-demethyl-BBR (berberrubine) or 10-demethyl-BBR

(thalifendine), a more polar compound than the starting product. Suggesting that, although BBR has a very low bioavailability, its metabolism and the generation of derivatives could be responsible for the biological activities observed *in vivo*.

5. Conclusions

In our experiments, two new formulations prepared at 25 and 80 °C induced a significant increase in BBR absorption compared to the traditional formulation (4.5-fold and 6-fold, respectively). Since we excluded a possible cytotoxicity effect of the emulsifiers, we may assume that the use of Compritol 888 ATO as excipient may represent a useful alternative for the development of transport systems able to deliver and absorb BBR in a more efficient way. However, since the data were obtained *in vitro*, it is highly recommended to substantiate these findings with human studies aimed at evaluating BBR bioavailability.

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References

[1] A. Vaiserman, A. Koliada, A. Zayachkivska, O. Lushchak. Curcumin: a therapeutic potential in ageing-related disorders. PharmaNutrition 14 (2020) 100226.

[2] L.M. Cercato, J.P. Oliveira, M.T. Santana Souza, et al.Effect of flavonoids in preclinical models of experimental obesity. PharmaNutrition 16 (2021) 100260.

[3] T. Bohn, M.L. Bonet,P. Borel, et al. Mechanistic aspects of carotenoid health benefits - Where are we now? Nutr. Res. Rev. (2021) 1-66.

[4] M. Marino, C. Del Bo', D. Martini, M. Porrini, P. Riso. A review of registered clinical trials on dietary (poly)phenols: past efforts and possible future directions. Foods. 9 (2020) 1606.

[5] R. Perez-Gregorio, J. Simal-Gandara.A critical review of bioactive food components, and of their functional mechanisms, biological effects and health outcomes. Curr. Pharm. Des. 23 (2017) 2731-2741.

[6] S. Habtemariam. Berberine and inflammatory bowel disease: A concise review. Pharmacol. Res.113 (2016) 592-599.

[7] M.O. Welcome. Blood brain barrier inflammation and potential therapeutic role of phytochemicals. PharmaNutrition 11 (2020) 100177.

[8] M.O. Welcome. Neuroinflammation in CNS diseases: Molecular mechanisms and the therapeutic potential of plant derived bioactive molecules. PharmaNutrition 11 (2020) 100176

[9] S. Bernardi, C. Del Bo', M. Marino, et al. Polyphenols and intestinal permeability: rationale and future perspectives. J. Agric. FoodChem. 68 (2020) 1816-1829.

[10] M. Pirro, G. Lupattelli, R. Del Giorno, et al. Nutraceutical combination (red yeast rice, berberine and policosanols) improves aortic stiffness in low-moderate risk hypercholesterolemic patients. PharmaNutrition 1 (2013) 73–77.

[11] C.W. Lau, X.Q Yao, Z.Y.Chen, et al. Cardiovascular actions of berberine. Cardiovasc. Drug Rev.19 (2001) 234-44.

[12] A.F. Cicero, A. Baggioni.Berberine and its role in chronic disease. Adv. Exp. Med. Biol.928 (2016) 27-45.

[13] S.H Leng, F.E Lu, L.J. Xu. Therapeutic effects of berberine in impaired glucose tolerance rats and its influence on insulin secretion. ActaPharmacol. Sin.25 (2004) 496–502.

[14] L.Wang, H.Li, S. Wang, et al. Enhancing the antitumor activity of berberine hydrochloride by solid lipid nanoparticle encapsulation. AAPS Pharm. Sci. Tech. 15 (2014) 834-844.

[15] I. Cavallari, A. Delli Veneri,E. Maddaloni,et al. Comparison of lipid-lowering medications and risk for cardiovascular disease in diabetes. Current Diabetes Rep. 18 (2018) 138.

[16] W. Kong,J. Wei, P. Abidi, et al. Berberine is a novel cholesterol-lowering drug working through a unique mechanism distinct from statins. Nat. Med. 10 (2004) 1344–51.

[17] Y.T Liu, H.P Hao, H.G Xie, et al. Extensive intestinal first-pass elimination and predominant hepatic distribution of Berberine explain its low plasma levels in rats. Drug. Metab. Dispos. 38 (2010) 1779-1784.

[18] J. Kumar Patra,G. Das, L.F. Fraceto, et al. Nano based drug delivery systems: recent developments and future prospects. J. Nanobiotech. 16 (2018) 71.

[19] V. Jannin, V. Berard, A. N'Diaye, et al. Comparative study of the lubricant performance of Compritol 888 ATO either used by blending or by hot melt coating. Int. J. Pharm. 262 (2003) 39-45.

[20] S.N. Patere, N.S. Desai, A.S. Jain, et al. Compritol 888 ATO a lipid excipient for sustained release of highly water soluble active: formulation, scale-up and IVIVC Study. Curr. Drug Deliv.10 (2013) 548-556.

[21] M.H. Aburahma, S.M. Badr-Eldin. Compritol 888 ATO: a multifunctional lipid excipient in drug delivery systems and nanopharmaceuticals, Expert Opin. Drug Deliv. 11 (2014) 1865-1883.

[22] K. Manjunath, J.S. Reddy, V. Venkateswarlu. Solid lipid nanoparticles as drug delivery systems. Methods Find. Exp. Clin. Pharmacol. 27 (2005) 127-144

[23] A. Di Costanzo, R. Angelico. Formulation strategies for enhancing the bioavailability of silymarin: The state of the art molecules. 24 (2019) 2155.

[24] J. Siepmann, F. Siepmann. Mathematical modeling of drug dissolution Int. J. Pharm. 453 (2013) 12-24.

[25] Z. Zhang, Z. Shen, J. Wang, et al.Micronization of silybin by the emulsion solvent diffusion method Int. J. Pharm. 376 (2009) 116-122.

[26] A.R. Hilgers, R.A. Conradi, P.S. Burton. Caco-2 cell monolayers as model for drug transport across the intestinal mucosa. Pharm. Res. 7 (1990) 902-10.

[27] Y. Sambuy, I. De Angelis, G. Ranaldi, et al.The Caco-2 cell line as a model of the intestinal barrier: influence of cell and culture-related factors on Caco-2 cell functional characteristics Cell Biol.Toxicol. 21 (2005) 1-26.

[28] M.J. Iqbal, C. Quispe, Z. Javed, et al.Nanotechnology-based strategies for berberine delivery system in cancer treatment: Pulling strings to keep berberine in power front Mol.Biosci. 7 (2021) 624494.

[29] Y. Hu, E.A. Ehli, J. Kittelsrud, et al. Davies. Lipid-lowering effect of berberine in human subjects and rats. Phytomedicine 19 (2012) 861-867.

[30] W. Wei, H. Zhao, A. Wang, et al. A clinical study on the short-term effect of berberine in comparison to metformin on the metabolic characteristics of women with polycystic ovary syndrome. Eur. J.Endocrinol. 166 (2012) 99-105.

[31] Y. Zhang, X. Li, D. Zou, et al. Treatment of type 2 diabetes and dyslipidemia with the natural plant alkaloid Berberine. J.Clin.Endocrinol.Metab. 93(2008) 2559-65.

[32] A. Poli, C.M. Barbagallo, A.F.G. Cicero, et al. Nutraceuticals and functional foods for the control of plasma cholesterol levels. An intersociety position paper.Pharmacol. Res. 134 (2018) 51-60.

[33] Z. Zhang, Y. Chen, J. Deng, et al. Solid dispersion of berberine-phospholipid complex/TPGS 1000/SiO_2: preparation, characterization and in vivo studies. Int. J. Pharm. 465 (2014) 306-316.

[34] J. Deng, Z. Wu, Z. Zhao, et al. Berberine-loaded nanostructured lipid carriers enhance the treatment of ulcerative colitis. Int. J. Nanomedicine 15 (2020) 3937-3951.

[35] M. Kwon, D.Y. Lim, C.H. Lee, et al. Enhanced intestinal absorption and pharmacokinetic modulation of berberine and its metabolites through the inhibition of P-glycoprotein and intestinal metabolism in rats using a berberine mixed micelle formulation. Pharmaceutics 2 (2020) 882.

[36] M.A. Elsheikh, Y.S.R. Elnaggar, D.A. Hamdy, O.Y. Abdallah. Novel cremochylomicrons for improved oral bioavailability of the antineoplastic phytomedicineberberine chloride: Optimization and pharmacokinetics. Int. J. Pharm. 535 (2018) 316-324.

[37] J. Sun, H. Bao, Y. Peng, et al. Improvement of intestinal transport, absorption and anti-diabetic efficacy of berberine by using Gelucire44/14: *In vitro*, *in situ* and *in vivo* studies. Int. J. Pharm. 544 (2018) 46-54.

[38] X. Qu, Y. Zou, C. He, et al. Improved intestinal absorption of paclitaxel by mixed micelles self-assembled from vitamin E succinate-based amphiphilic polymers and their

transcellular transport mechanism and intracellular trafficking routes. Drug Deliv. 25 (2018) 210-225.

[39] P. Barthelemy, J.P. Laforêt, N. Farah, J. Joachim. Compritol 888 ATO: an innovative hot-melt coating agent for prolonged-release drug formulations. Eur. J. Pharm.Biopharm. 47 (1999), 87-90.

[40] J. He, S. Hou, W. Lu,et al. Preparation, pharmacokinetics and body distribution of silymarin-loaded solid lipid nanoparticles after oral administration. J. Biomed. Nanotechnol. 3 (2007) 195–202.

[41] P. Xu, C. Xu, X. Li, et al. Rapid identification of berberine metabolites in rat plasma by UHPLC-Q-TOF-MS. Molecules 24 (2019)1994.

[42] Y. Liu, H. Hao, H. Xie, L. Lai, Q. Wang, C. Liu, G. Wang. Extensive intestinal first-pass elimination and predominant hepatic distribution of berberine explain its low plasma levels in rats. Drug Metab. Dispos. 38 (2010), 1779-84.

[43] Y. Guo, F. Li, X. Ma, X. Cheng, H. Zhou, C.D. Klaassen. CYP2D plays a major role in berberine metabolism in liver of mice and humans. Xenobiotica 41 (2011), 996-1005.

Figure 1. Effect of BBR-to-emulsifier ratio and method of preparing emulsifying solutionon rate of BBR uptake at 180 min. BBR, berberine; RT, room temperature.
Results derived from three independent experiments in which each condition was tested in triplicate. Values with different letters are significantly different ($P < 0.05$). Data are reported as mean ± SEM.

Figure 1

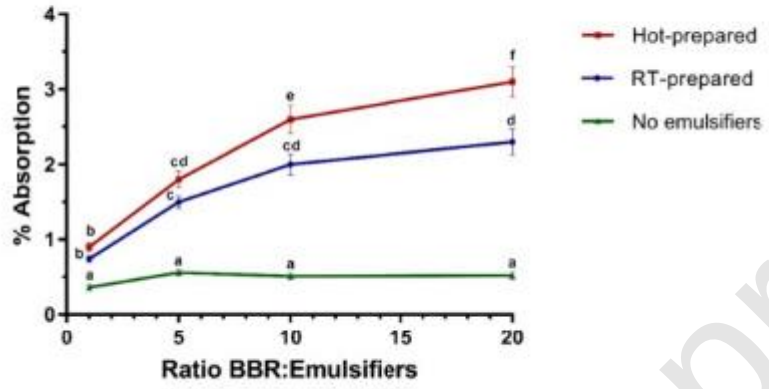

Figure 2. The extracted ion chromatogram of (A) BBR and (B) its metabolite M1 in a basal solution after 180 min incubation with a solution containing BBR:Compritol in a ratio 1:5.

Figure 2

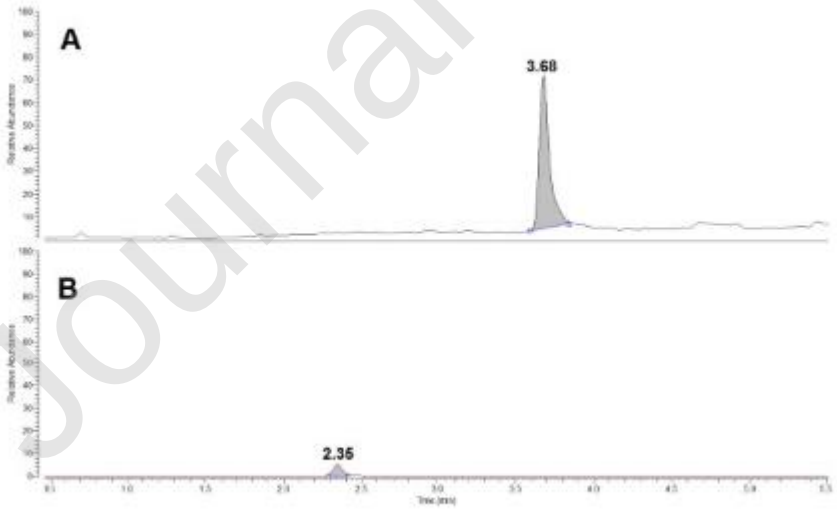

Figure 3. The fragmentation pattern of (A) BBR and (B) its metabolite M1.

Figure 3

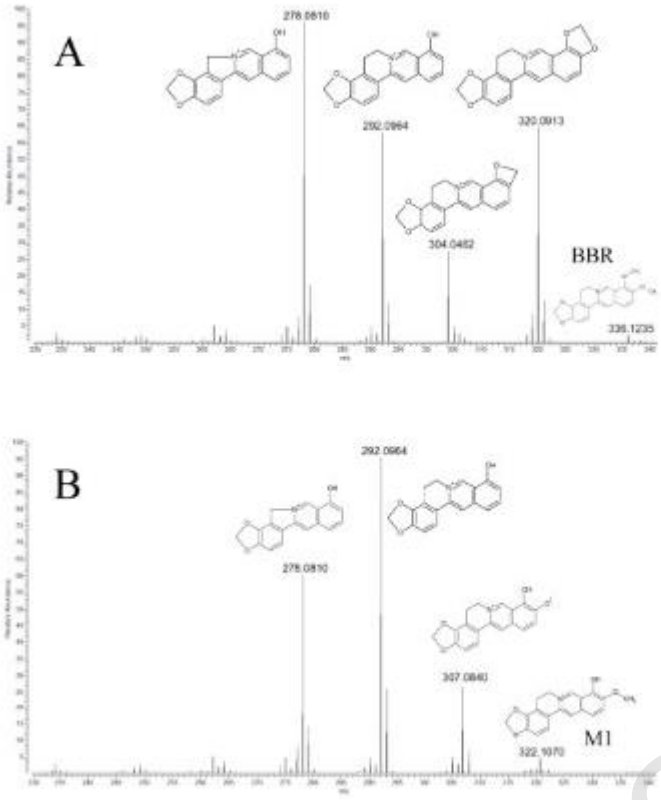

Table 1. Percentage of cell viability following supplementation with BBR evaluated by Trypan blue exclusion assay.

	MEM	C1	C2	C3	C4	Test 1	Test 2	Test 3	Test 4
Viability %	91.9 ± 1.1	92.9 ± 0.9	94.8 ± 0.5	92.3 ± 0.7	92.3 ± 0.7	90.4 ± 0.8	91.7 ± 1.3	93.1 ± 0.7	91.3 ± 0.9

MEM, negative control; C1, BBR 500 mg + cellulose 500 mg; C2, BBR 200 mg + cellulose 800 mg; C3, BBR 100 mg + cellulose 900 mg; C4, BBR 50 mg + cellulose 950 mg; test 1, BBR 500 mg + Compritol 400 mg + lecithin 100 mg; test 2, BBR 200 mg + Compritol 700 mg+ lecithin 100 mg; test 3, BBR 100 mg + Compritol 800 mg + lecithin 100 mg; test 4, BBR 50 mg + Compritol 850 mg + lecithin 100 mg. Results derived from three independent experiments in which each condition was tested in triplicate. Data are reported as mean ± SEM. MEM, minimum essential medium; BBR, berberine.

Table 2. Absorption kinetics of BBR nanoemulsions (T1, T2, T3, and T4) and their controls (C1, C2, C3, and C4) from apical to basal in human Caucasian colon adenocarcinoma cell (Caco-2) model (n = 3). Results derived from three independent experiments in which each condition was tested in triplicate. Data are reported as mean ± SEM.

min	30	60	90	120	150	180
Formulation			µM BBR			
C1	0.00 ± 0.00^{a}	0.07 ± 0.00^{a}	0.13 ± 0.01^{a}	0.20 ± 0.01^{a}	0.21 ± 0.01^{a}	0.24 ± 0.01^{a}
T1 RT	0.04 ± 0.00^{b}	0.14 ± 0.01^{b}	0.28 ± 0.01^{b}	0.47 ± 0.02^{b}	0.46 ± 0.02^{b}	0.51 ± 0.02^{b}
T1 HOT	0.03 ± 0.00^{b}	0.20 ± 0.01^{b}	0.30 ± 0.01^{b}	0.52 ± 0.02^{b}	0.58 ± 0.02^{b}	0.61 ± 0.02^{b}
C2	0.03 ± 0.00^{a}	0.09 ± 0.00^{a}	0.22 ± 0.0^{a}	0.33 ± 0.02^{a}	0.38 ± 0.02^{a}	0.39 ± 0.01^{a}
T2 RT	0.06 ± 0.00^{b}	0.33 ± 0.01^{b}	0.60 ± 0.03^{b}	0.82 ± 0.04^{b}	0.90 ± 0.04^{b}	1.02 ± 0.04^{b}
T2 HOT	0.22 ± 0.01^{c}	0.73 ± 0.03^{c}	0.95 ± 0.04^{c}	1.07 ± 0.04^{b}	1.19 ± 0.05^{b}	1.23 ± 0.05^{b}
C3	0.06 ± 0.00^{a}	0.20 ± 0.01^{a}	0.27 ± 0.01^{a}	0.34 ± 0.01^{a}	0.34 ± 0.01^{a}	0.35 ± 0.02^{a}
T3 RT	0.30 ± 0.01^{b}	0.79 ± 0.03^{b}	0.93 ± 0.04^{b}	1.12 ± 0.04^{b}	1.18 ± 0.04^{b}	1.37 ± 0.06^{b}
T3 HOT	0.30 ± 0.01^{b}	1.13 ± 0.05^{c}	1.26 ± 0.05^{b}	1.42 ± 0.05^{c}	1.67 ± 0.06^{c}	1.78 ± 0.08^{c}
C4	0.05 ± 0.00^{a}	0.22 ± 0.01^{a}	0.30 ± 0.01^{a}	0.34 ± 0.02^{a}	0.34 ± 0.01^{a}	0.36 ± 0.01^{a}
T4 RT	0.58 ± 0.02^{b}	1.01 ± 0.03^{b}	1.12 ± 0.04^{b}	1.31 ± 0.05^{b}	1.42 ± 0.05^{b}	1.57 ± 0.05^{b}
T4 HOT	0.51 ± 0.01^{b}	1.48 ± 0.05^{c}	1.64 ± 0.06^{c}	1.82 ± 0.06^{c}	1.98 ± 0.07^{c}	2.12 ± 0.06^{c}

C, control; T, test; RT, nanoemulsion prepared at room temperature; HOT, nanoemulsion prepared by heating emulsifiers to ~80 °C. C1, BBR 500 mg + cellulose 500 mg; C2, BBR 200 mg + cellulose 800 mg; C3, BBR 100 mg + cellulose 900 mg; C4, BBR 50 mg + cellulose 950 mg; T1, BBR 500 mg + Compritol 400 mg + lecithin 100 mg; T2, BBR 200 mg + Compritol 700 mg+ lecithin 100 mg; T3, BBR 100 mg + Compritol 800 mg + lecithin 100 mg; T4, BBR 50 mg + Compritol 850 mg + lecithin 100 mg.Values with different letters within the same column are significantly different ($P < 0.05$).

Table 3. BBR metabolite concentration in the basal solution after BBR incubation in Caco-2 model (n = 3). Results derived from three independent experiments in which each condition was tested in triplicate. Data are reported as mean ± SEM.

min	30	60	90	120	150	180
Formulation	nM BBR metabolite					
C1	n.d.	n.d.	n.d.	5.1 ± 0.3[a]	5.3 ± 0.3[a]	6.1 ± 0.4[a]
T1 RT	n.d.	n.d.	n.d.	11.7 ± 0.7[a]	11.5 ± 0.6[a]	12.7 ± 0.6[a]
T1 HOT	n.d.	n.d.	n.d.	13.0 ± 0.6[a]	14.0 ± 0.7[a]	15.4 ± 0.7
C2	n.d.	n.d.	n.d.	8.3 ± 0.4[a]	9.5 ± 0.4[a]	9.6 ± 0.4[a]
T2 RT	n.d.	n.d.	n.d.	20.5 ± 0.9	22.6 ± 0.9	25.6 ± 1.1
T2 HOT	n.d.	n.d.	n.d.	26.8 ± 1.1	29.8 ± 1.3	30.7 ± 1.3
C3	n.d.	n.d.	n.d.	8.4 ± 0.4[a]	8.5 ± 0.4[a]	8.7 ± 0.5[a]
T3 RT	n.d.	n.d.	n.d.	28.1 ± 1.2	29.4 ± 1.3	34.2 ± 1.6
T3 HOT	n.d.	n.d.	n.d.	35.6 ± 1.5	41.7 ± 1.7	44.4 ± 1.9
C4	n.d.	n.d.	n.d.	8.5 ± 0.3[a]	8.5 ± 0.3[a]	8.9 ± 0.3[a]
T4 RT	n.d.	n.d.	n.d.	32.9 ± 1.7	35.5 ± 1.9	39.3 ± 2.2
T4 HOT	n.d.	n.d.	n.d.	45.5 ± 1.9	49.4 ± 1.9	52.9 ± 2.5

n.d.: < LOD (5.5 nM, 2 ng/ml). [a]:< LOQ (13.6 nM, 5 ng/ml)

n.d., not detectable; LOD, limit of detection; LOQ, limit of quantification; C, control; T, test; RT, nanoemulsion prepared at room temperature; HOT, nanoemulsion prepared by heating emulsifiers to ~80 °C. C1, BBR 500 mg + cellulose 500 mg; C2, BBR 200 mg + cellulose 800 mg; C3, BBR 100 mg + cellulose 900 mg; C4, BBR 50 mg + cellulose 950 mg; T1, BBR 500 mg + Compritol 400 mg + lecithin 100 mg; T2, BBR 200 mg + Compritol 700 mg+ lecithin 100 mg; T3, BBR 100 mg + Compritol 800 mg + lecithin 100 mg; T4, BBR 50 mg + Compritol 850 mg + lecithin 100 mg.