Preparation and optimization of berberine phospholipid complexes using QbD approach and *in vivo* evaluation for anti-inflammatory, analgesic and antipyretic activity

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ABSTRACT: Berberine (BER) is a benzylisoquinoline alkaloid found in many plants. It has high water solubilitybut its bioavailability is low in oral administration due to the first-pass effect in the liver and intestine. Phytosomesare are prepared by complexing herbal active ingredients and phospholipids (PLs). The aim of this study is to prepare BER-phospholipidcomplexes (BPCs) using the reverse phase evaporation method. In the preparation of the formulations, the effects of BER:PL ratio (w/w), reaction temperature (°C) and reaction time (h) on the specifications of the complexes such as particle size (PS), zeta potential (ZP) and encapsulation efficiency (EE%) were investigated. The distribution of PS of obtained complexes was ranging between 339 -1259 nm and their ZP were within -4,27- -5,15 mV. The drug EE% was relatively high. Quality by Design (QbD) methods have been used in the formulation design and selection of the optimum formulation. BPCs anti-inflammatory, analgesic and antipyretic activities are evaluated by using in vivo methods.Carrageenan-, Prostaglandin E2 (PGE$_2$)- and serotonin-induced hind paw edema, acetic acid-induced increase in capillary permeability and subcutaneous air-pouch models for the anti-inflammatory activity, inhibition of p-benzoquinone-induced abdominal constriction and hot plate test for the analgesic activity, Freund's complete adjuvant-induced pyrexia model for the antipyretic activity were used in mice and rats. Results showed that BPCs showed potent anti-inflammatory, analgesic and antipyretic activities at the dose of 209 mg/kg.

KEYWORDS: Berberine;phytosomes; analgesic; anti-inflammatory; qbd approach

1. INTRODUCTION

Phytocomponents are generally well-soluble structures in waterbut they do not have a sufficient hydrophilic-lipophilic balance for an ideal bioavailability [1]. At this point, drug delivery systems were required to obtain maximum efficacy from phytocomponents and to carry these components to the effect site [2].

In the last quarter-century, researchers have been working on a special formulation called phytosomes for phytocomponents and herbal extracts. Phytosomes are also defined as PL-based complex systems. They are prepared to increase the oral bioavailability of phytocomponents and their extracts [3]. The complexation of plant extracts or water-soluble phytocomponents and PLs significantly increases the bioavailability of the substances by increasing penetration through lipoidal biological membranes [4-7]. These complex compounds are obtained by complexing products of natural origin with natural PLs such as soy lecithin and egg yolk lecithin in a suitable solvent [8]. The chemical structure of the complexes formed are similar to the cell membrane and they are defined as phyto-lipid delivery systems [9]. The choice of phytocomponents depends on its structure and its pharmacokinetic profile. It is preferable to prepare phytosome formulations of phytocomponents that cannot pass through the intestinal membranes by passive diffusion, as they usually

contain long side chains and numerous ring structures [10]. Water-soluble phytocomponents (generally polyphenolics) can be converted to phyto-lipid delivery systems known as phytosomes [11].

BER is isolated from various plant species, especially *Berberis* L. (barberry) which also grows in the worldwide. BER is poorly soluble in oil and has a high molecular weight. It is accepted that BER has low oral bioavailability due to its properties that make it difficult to pass through the intestinal wall, such as the first-pass effect in the intestine and the liver, showing self-aggregation that causes a diminish in the solubility of the gastrointestinal tract and low permeability from the intestinal membranes [12]. The improvement of the bioavailability of hydrophilic drugs such as BER can be achieved by the phytosome complexing technique, which increases lipid-rich membrane penetration. In addition, phyto-PL complexes can help reduce their dosage and increase their duration of action, thanks to their extended drug release [13]. It was therefore considered to be a suitable candidate for preparing phyto-PL complex [14]. In recent years, antihypertensive [15], hypoglycemic [16], anticancer [17], antidepressant [18] and hepatoprotective [19] effects of BER have been studied by various researchers. The anti-inflammatory activity of BER has been demonstrated by different researchers by using different models [20,21]. BER was found to act by inhibiting elastolytic activity of elastase enzyme, which plays a role in the development of inflammatory illness [21].

The implementation of the QbD opinion results in a better product, process and interrelation understanding [22]. As stated by the ICH Q8 (R2), QbD application in the pharmaceutical field begins with defining quality target product profile (QTTP) which describes the quality, safety, and efficacy characteristics of the product [23]. In terms of product quality, a Critical Quality Attribute (CQA) is a chemical, biological, physical, or microbiological characteristic or property or that must be kept in a proper limit range. Critical process parameters (CPPs) are parameters that have an influence on a CQA and thus must be controlled or monitored to guarantee the process produces the desirable quality [24]. Optimization is a mathematical method of looking for the most advantageous solution to a problem. Mathematical optimization techniques must be used to identify the optimum formulation properties and/or the process variables to achieve the desired product properties [25]. With the aid of statistical or artificial intelligence computer software there is a chance to obtain detailed information on the impact of the process and formulation parameters to critical quality attributes with optimum formulation or process conditions [26].

In this study, an experimental design study was conducted to follow the alterations in the outputs of the formulations and to evaluate them using Modde Pro 12.1 (Sartorius Stedim Data Analytics AB) and INForm ANN (Intellygensys, UK) statistical computer software. Then optimum formulation was used to evaluate the anti-inflammatory activity of the BPCs by using in vivo methods. Carrageenan-, PGE_2- and serotonin-induced hind paw edema [27], acetic acid-induced increase in capillary permeability [28] and subcutaneous air-pouch [29] models for the anti-inflammatory activity were used in mice and rats. p-benzoquinone-induced abdominal constriction test [30] and hot plate test [31] were used for the analgesic activity. At the last step of this study; the antipyretic activity of the BPCs in comparison with BER by using in vivo methods was evaluated. For this reason, Freund's complete adjuvant-induced pyrexia model [32] was used.

2. RESULTS

2.1. Development of HPLC method

BER solution was prepared in water at a concentration of 100 μg/mL. The clean and sharp peak of BER was obtained at 346 nm in 3,85 minutes. Regression analyses were performed. Linearity and range, specificity, precision (repeatability and intermediate precision), limit of detection (LOD) and limit of quantification (LOQ) were evaluated as analytical validation parameters. Acceptance criteria for these parameters were set according to official FDA guidelines [33]. Measured validation parameters were within acceptance limits (relative standard deviation < 2%).

2.2. Preparation and physical characterization of BPCs

In the formation of BPCs, the phytoactive component combines with the polar part of the PL with the help of hydrogen bonds or covalent bonds. Phosphatidylcholine is an amphiphilic molecule consisting of hydrophilic choline and lipophilic phosphatidyl groups; the polar choline part combines with the bioactive molecule through hydrogen bonds, while the lipophilic and nonpolar phosphatidyl group surrounds the complex of the choline-bioactive molecule. The formulations were prepared using reverse-phase evaporation method. 27 different formulations, offered by statistical software, were prepared by changing the parameters of BER:PL ratio (w/w), reaction temperature (°C) and reaction time (h). While preparing formulations, it was

seen that reaction temperature is a very important parameter, especially in complex formation. In the prepared formulations, ZP, polydispersity index (PDI), EE% and PS analyzes were performed (Table 1).

Table 1. Characteristics of different BPCs formulations

Formulation	ZP (mV) ± S.D.	PS (nm) ± S.D.	PDI ± S.D.	EE%± S.D.
F1	-4.27 ± 0.08	462.8 ± 27.8	0.32 ± 0.003	40.80± 3.21
F2	-4.27 ± 0.26	580.7 ± 32.4	0.30 ± 0.011	38.78± 1.23
F3	-4.27 ± 0.24	572.4 ± 22.7	0.26 ± 0.005	32.84± 0.85
F4	-4.28 ± 0.07	541.5 ± 24.5	0.08 ± 0.006	48.01± 2.52
F5	-4.27 ± 0.16	632.5 ± 45.6	1.00 ± 0.012	38.52± 1.78
F6	-4.27 ± 0.17	794.1 ± 67.1	1.00 ± 0.015	40.98± 4.56
F7	-4.27 ± 0.21	575.8 ± 22.3	0.84 ± 0.010	55.45± 1.90
F8	-5.15 ± 0.12	555.8 ± 25.6	0.12 ± 0.005	50.30± 2.05
F9	-5.03 ± 0.14	794.6 ± 77.9	1.00 ±0.012	46.46± 4.98
F10	-4.58 ± 0.05	751.8 ± 34.8	0.13 ± 0.002	40.33± 1.63
F11	-5.15 ± 0.10	667.9 ± 42.3	0.32 ± 0.009	40.51± 7.32
F12	-4.27 ± 0.17	846.9 ± 80.2	0.28 ± 0.008	40.45± 4.92
F13	-4.27 ± 0.19	827.0 ± 54.6	0.09 ± 0.003	44.06± 2. 19
F14	-4.28 ± 0.05	765.2 ± 37.8	0.31 ±0.013	46.87± 1.94
F15	-4.27 ± 0.07	844.6 ± 98.5	0.07 ± 0.002	45.27±3.84
F16	-4.63 ± 0.23	587.1 ± 3.49	0.38 ± 0.009	43.49± 6.57
F17	-5.15 ± 0.12	339.2 ± 19.5	0.28 ± 0.010	48.03± 3.76
F18	-4.27 ± 0.35	794.9 ± 63.2	0.92 ± 0.011	64.54± 5.46
F19	-4.28 ± 0.55	987.6 ± 32.4	0.35 ± 0.007	38.00± 4.72
F20	-4.27 ± 0.14	968.4 ± 11.6	0.33 ± 0.008	54.79± 5.49
F21	-4.28 ± 0.22	1258.7 ± 98.3	0.12 ± 0.004	40.36± 1.74
F22	-4.27 ± 0.34	826.6 ± 26.06	0.10 ± 0.011	45.59± 2.38
F23	-4.27 ± 0.60	1203.6 ± 54.6	1.00 ± 0.010	39.68± 4.09
F24	-5.15 ± 0.30	1082.1 ± 100.5	0.11 ± 0.09	38.63± 2.10
F25	-4.28 ± 0.08	632.4 ± 0.71	0.50 ± 0.011	51.31± 1.09
F26	-4.27 ± 0.21	1009 ± 45.6	1.00 ± 0.013	46.76± 3.45
F27	-5.03 ± 0.15	1064.5 ±94.3	0.634 ± 0.137	52.98± 6.79

It is observed that BER and PL reacting in a ratio of 1:7 usually achieved better EE% than other conditions; however, PDI was also observed to increase due to increased rates of use of substances. In formulations prepared by reverse-phase evaporation method, EE% was found to vary between 32.8% and 64.54%. The ZP is the repel or draw force value measurement between the particles. The ZP of the BPCs were between -4,27- -5,15 mV. Evaluated formulation parameters did not significantly affect ZP.

2.3. Determining the ideal formulation with QbD approach

The outcomes of the experiments were evaluated by the software. As a result, an optimum formulation was offered by the software. After the evaluation of the reverse phase evaporation method using the software, the formulation found is named with FModd code. In the formulation obtained from the software, BER: PL ratio (w/w) was 1: 7, the reaction temperature was 60 °C and the reaction time was 1 hour. The results of the formulation which gave the closest results according to the software and the optimum results obtained from the software. The results of modeling software were obtained as -4.48 mV, 843nm, 0.368 and 50.11% for ZP, PS, PDI and EE% respectively. The closest results according to the software were found with formulation F25 (Table 1).

2.4. In vivo studies

Carrageenan-induced hind paw edema in mice is a biphasic event. The early phase (90-180 min.) of the inflammation is due to the release of histamine, serotonin and similar substances. The later phase (270-360 min) is associated with the activation of kinin-like substances, i.e. prostaglandins, proteases and lysosome [34]. As shown in Table 2, the BER and the BPCs did not show any anti-inflammatory activity in the carrageenan-induced hind paw edema model at the dose of 104.5 mg/kg. However, BER and BPCswas found to have anti-inflammatory effectsprostaglandin E_2 at the dose of 209 mg/kg, which is support this hypothesisin 14.9-21.8 % and 19.0-30.2 %, respectively.

Table 2. Effect of the test materials on carrageenan-induced paw edema model in mice

Material	Dose (mg/kg)	Swelling thickness (x10^{-2}mm) ±S.E.M. (inhibition %)			
		90 min	180 min	270 min	360 min
Control		42.5±4.1	58.6±3.7	59.4±3.9	51.5±3.4
BER	104.5	44.6±3.1	47.9±3.5 (18.3)	51.2±3.1 (13.8)	58.8±3.5 (5.2)
	209	35.3±4.6 (16.9)	45.8±3.6 (21.8)*	47.0±3.2 (20.9)	43.8±3.0 (14.9)
BPCs	104.5	37.1±4.4 (12.7)	51.3±4.1 (12.5)	51.2±4.4 (13.8)	49.7±3.9 (3.5)
	209	31.4±3.7 (26.1)*	40.9±3.7 (30.2)**	46.8±4.0 (21.2)	41.7±4.2 (19.0)
Indomethacin	10	31.2±2.8 (26.6)*	40.1±2.5 (31.6)**	35.6±2.9 (40.1)***	29.3±2.4 (43.1)***

*:$p<0.05$. **:$p<0.01$. ***:$p<0.001$ significant from the control. S.E.M.: standard error mean; n=7 (seven animals were used in each group).

In particular, the identification of the inhibitory effect on various enzyme systems and mediators in the clarification of the mechanism of action of the compounds provides important explanatory information [29]. The prostaglandins occurring by the cyclooxygenase pathway are the most significant inflammatory mediators in the body [35]. Nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin show their effects by inhibiting the synthesis of prostaglandins. For this purpose, the PGE_2-induced hind paw edema model was performed in mice to determine the effect of BER and BPCs on prostaglandins. As shown in Table 3, it was determined that BER and complex formulation did not show any anti-inflammatory effect at the relevant doses in the PGE_2-induced hind paw edema model.

In the anti-inflammatory activity assay based on the inhibition of acetic acid-induced capillary permeability increase, the BER and BPCs was determined to have a dose-dependent anti-inflammatory effect. Although at the dose of 104.5 mg/kg, BER and BPCs had a low anti-inflammatory effect, at 209 mg/kg dose of BER 21.2 % and BER and BPCs had an anti-inflammatory effect of 31.9 % (Figure 1).

As shown in Table 3, BER and BPCs were determined to have a dose-dependent anti-inflammatory effect in the serotonin-induced hind paw edema model. The anti-inflammatory effect of BER and BPCs was found to be low at the 104.5 mg/kg dose in this model. On the other hand, BER (7.7-29.4% inhibition) and BPCs (13.2-33.7% inhibition) were found to have anti-inflammatory effects in at 209 mg/kg dose.

The effects of BER and BPCs on the subcutaneous air-pouch method which has a strong anti-inflammatory effect on acute models were investigated. In the local inflammation by Freund's Complete Adjuvant injected into the air pouch formed on the back of the mice, the BER and BPCs was determined to have an anti-inflammatory effect at the dose of 209 mg/kg with 28.2 % inhibition (Figure 2).
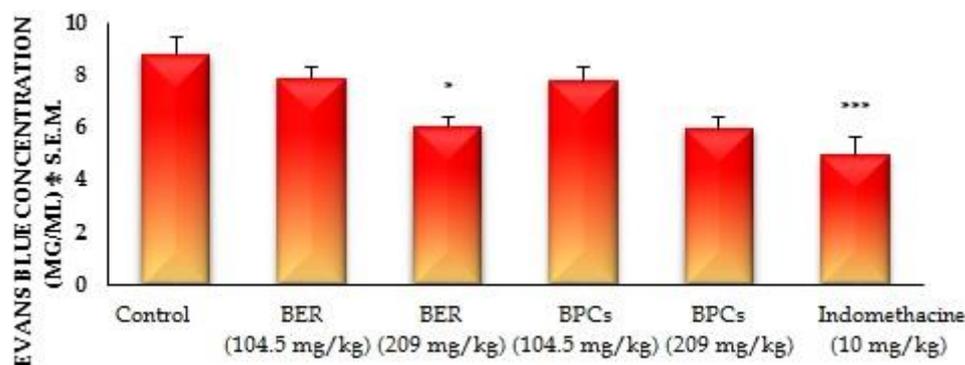
BER is known to possess antipyretic effect and this was also demonstrated in our previous study using FCA-induced subacute pyrexia model in rats [32]. Anti-inflammatory drugs that inhibit prostaglandin biosynthesis are capable of suppressing fever [36]. In the present study, in order to determine the inhibitory effect of the BER and BPCs on fever, a subacute model was set using killed mycobacterial toxin (FCA) in mice. Since the the BER and BPCs administered, doses (104.5 mg/kg and 209 mg/kg) were preferred to avoid toxicity, especially deaths due to gastric bleeding in multiple-dose regimen.

Table 3. Effect of the test materials on PGE_2-induced and serotonin-induced paw edema in mice

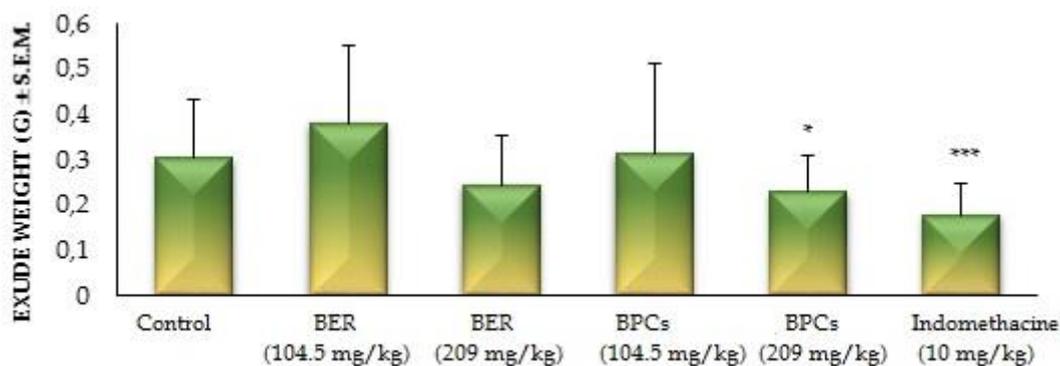| Material	Dose (mg/kg)	Swelling thickness ($\times 10^{-2}$mm) ±S.E.M. (inhibition %)					
		0 min	15 min	30 min	45 min	60 min	75 min
Control	-	1.6±0.6	13.1±1.2	19.5±1.4	20.8±1.9	18.1±1.4	10.3±0.9
BER	104.5	1.5±0.7	15.4±1.5	19.9±1.7	18.3±1.1 (12.0)	17.3±1.1 (4.4)	9.8±0.7 (4.9)
	209	1.6±0.8	14.3±1.1	18.2±1.1 (6.7)	17.6±1.3 (15.4)	17.0±1.1 (6.1)	8.9±0.5 (13.6)
BPCs	104.5	1.7±0.5	13.9±1.4	19.8±1.7	21.5±1.4	19.2±0.8	10.6±0.5
	209	1.5±0.9	11.2±1.3 (14.5)	16.2±1.0 (16.9)	17.5±1.3 (15.9)	15.1±0.8 (16.6)	8.9±0.8 (13.6)
Indomethacin	10	1.6±0.5	10.4±0.7 (20.6)	14.9±1.0 (23.6)	14.7±1.1 (29.3)**	13.2±0.9 (27.1)*	7.2±0.8 (30.1)**

Material	Dose (mg/kg)	Swelling thickness ($\times 10^{-2}$mm) ±S.E.M. (inhibition %)					
		0 min	6 min	12 min	18 min	24 min	30 min
Control		3.5±0.4	9.1±0.6	12.3±0.5	16.9±1.2	21.4±1.3	23.5±1.4
BER	104.5	3.4±0.3	8.3±0.5 (8.8)	10.9±0.4 (11.4)	16.5±0.9 (2.4)	18.2±0.5 (14.9)	20.5±1.1 (12.8)
	209	3.6±0.5	8.4±0.5 (7.7)	10.1±0.6 (17.9)	13.4±0.6 (20.7)	15.1±0.8 (29.4)**	17.4±0.5 (25.9)*
BPCs	104.5	3.3±0.4	9.3±0.5	10.9±0.8 (11.4)	17.1±0.7	17.8±0.6 (16.8)	20.2±1.3 (14.0)
	209	3.5±0.4	7.9±0.3 (13.2)	8.6±0.9 (27.6)*	11.2±0.5 (33.7)*	15.4±0.7 (28.0)*	17.8±0.7 (24.3)
Indomethacin	10	3.4±0.5	7.5±0.7 (17.6)	8.1±0.7 (34.1)**	10.8±0.5 (36.1)**	13.9±0.5 (35.0)**	16.5±0.8 (29.8)**

*:p<0.05. **:p<0.01. ***:p<0.001 significant from the control, S.E.M.: standard error mean; n=7 (seven animals were used in each group).

*:p<0.05. **:p<0.01. ***:p<0.001 significant from the control, S.E.M.: standard error mean; n=7 (seven animals were used in each group).
Figure 1. Effect of the test materials on acetic acid-induced capillary permeability increase model in mice

*:p<0.05. **:p<0.01. ***:p<0.001 significant from the control, S.E.M.: standard error mean; n=7 (seven animals were used in each group).
Figure 2. Effects of the test materials on subcutaneous air-pouch method

Increased body temperature and pain are the most prominent among the symptoms observed in response to inflammation in the body [37]. It is also ideal to have antipyretic and analgesic effects for anti-inflammatory compounds. In order to evaluate the analgesic activity, BER and BPCs was studied against p-benzoquinone induced writhings in mice. As shown in Figure 3, BER and BPCs did not show any analgesic effect at 104.5 mg/kg dose in the p-benzoquinone-induced pain test. On the other hand, it was determined that the BPCs had shown 30.7 % analgesic activity at the dosage of 209 mg/kg.
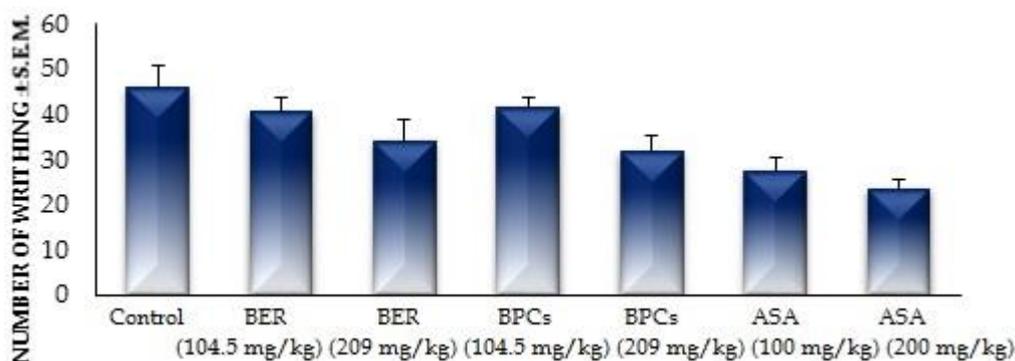
Another activity method for determining the analgesic effect was used the hot plate test in mice. The hot plate test is usually a test for compounds acting on opioid receptors. According to the results of this test, there is no significant analgesic activity of the BER and BPCs at the dose of 104.5 mg/kg. However, the BPCs showed an analgesic effect with 28.2 % at 209 mg/kg. The results suggest that BER and BPCs had no analgesic effect on opioid receptors when used at a dose of 104.5 mg/kg (Figure 4).

The antipyretic effect of BER and BPCswas evaluated by observing the variance of heat among the treated and untreated hind paws by using a digital thermometer every other day in Freund's Complete Adjuvant-induced-treated rats. As shown in Table 4 BER and BPCs possessed a notable antipyretic activity. In addition, physical changes (body weight, behavior) were not observed in the mice at the end of the experiments.

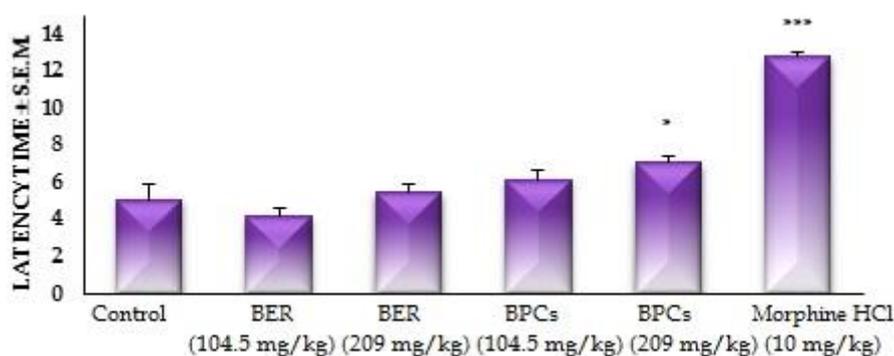**Table 4.** Antipyretic effect of the test materials on FCA-induced fever in rats

Days	Material (Dose)					
	Mean change in fever between two paws ± S.E.M.					
	Control	BER (104.5 mg/kg)	BER (209 mg/kg)	BPCs (104.5 mg/kg)	BPCs (209 mg/kg)	Indomethacin (10 mg/kg)
3	38.3 ± 0.11	39.1 ± 0.14	38.8 ± 0.16	38.5 ± 0.09	38.4 ± 0.11	38.7 ± 0.09
4	39.6 ± 0.10	39.7 ± 0.15	39.7 ± 0.18	$.9 \pm 0.12$	39.6 ± 0.12	39.5 ± 0.11
6	40.1 ± 0.13	40.2 ± 0.24	$39.1 \pm 0.14*$	40.1 ± 0.19	39.4 ± 0.11	$39.1 \pm 0.08*$
8	39.9 ± 0.17	40.3 ± 0.19	$39.5 \pm 0.17*$	40.7 ± 0.13	$39.1 \pm 0.12*$	$39.3 \pm 0.14*$
10	40.3 ± 0.20	40.7 ± 0.25	$39.8 \pm 0.11*$	40.1 ± 0.15	$39.1 \pm 0.17*$	$39.0 \pm 0.10**$
12	38.9 ± 0.15	40.5 ± 0.21	39.0 ± 0.14	39.2 ± 0.17	$38.4 \pm 0.11*$	$38.5 \pm 0.08**$
14	38.8 ± 0.19	39.9 ± 0.22	39.1 ± 0.12	39.0 ± 0.19	$38.3 \pm 0.15*$	$38.1 \pm 0.08**$

* p<0.05. **p<0.01. *** p<0.001 significant from the control, S.E.M.: standard error mean; n=7 (seven animals were used in each group).

*:p<0.05. **:p<0.01. ***:p<0.001 significant from the control, S.E.M.: standard error mean; n=7 (seven animals were used in each group).

Figure 3. Effect of the test materials against p-benzoquinone-induced writhing in mice

*:p<0.05. **:p<0.01. ***:p<0.001 significant from the control, S.E.M.: standard error mean; n=7 (seven animals were used in each group).

Figure 4. Effect of the test materials on latency of pain in mice exposed to the hot plate

3. DISCUSSION

BPCs were produced by reverse-phase evaporation method characterized by the reaction temperature, reaction time, and BER:PL ratio. The ratio of BER and PL are one of the important parameters in terms of the efficiency of the complexation process and the stability of the complex. The reaction time and reaction temperature were also seen as critical formulation parameters affecting the complex formation. The experimental area was designed with a statistical software using these parameters and the software offered us 27 different formulations; these formulations were prepared. In the formulations prepared, studies such as determination of EE%, ZP and PS for physical characterization were carried out. In order to obtain the optimum formulation among these, all results were entered into the optimisation software and the product features that we gave the FModd code were recommended to us by the software. Among the formulations we prepared, it was seen that F25 was the most compatible formula for FModd and was chosen as the optimum formula. This formulation has a relatively high loading capacity and a low PS. Formula F25 was examined under an optical microscope and vesicular structures were observed.

QbD approach in formulation design has an important role in the optimal formulation and process condition selection and experimental design in terms of time-saving and convenience. Risk-based determination of critical attributes and parameters to evaluate them with statistical software like the MODDE Pro software that finds the ideal formulation among formulations prepared according to the proper experimental design gave important benefits.

In the analytical validation studies carried out to evaluate the specificity parameter, a peak of a PL, which is used as an excipient in BPCs, was observed at 346 nm near the BER. Chromatograms were overlaying to see if the peaks made interference and it was seen that they made a small interference. In order to evaluate whether this attempt is meaningful, R (selectivity) value was calculated. In chromatography, it is necessary to have R=1 to accept that two peaks are separated from each other in quantitative terms. And while this separation value means that the two bands overlap each other by 2%, in the case of R= 1.5, this overlap falls

to 1%, and the two peaks are completely separated from each other[38]. The R (selectivity) value calculated by using retention times and peak widths of the substances was 1.616. This value shows us that the peaks are completely separated from each other. There is no interference between BER and the PL used in BPCs formulation. As a result, a specific, precise, accurate HPLC method has been developed and fully validated according to the FDA guidelines for the quantitative determination of BER from BPCs.

BER is an active substance with high hydrophilic properties; therefore, it is very difficult for it to pass through biological barriers in the body. Therefore, BPCs was prepared and in vivo anti-inflammatory, analgesic and antipyretic activities were determined. Previous studies [32] have shown that BER has anti-inflammatory activity at a dose of 209 mg/kg. 104.5 mg/kg and 209 mg/kg doses were used in our studies to compare the efficacy of pure BER and BPCs. As far as we know, the complexation of water-soluble BER and PL significantly increases the bioavailability of substances by increasing penetration through lipoidal biological membranes. In this study, due to the high transfer rate of BPCs through cell membranes, an increase in oral bioavailability was expected. Therefore, it was hoped that the analgesic, antipyretic activity and anti-inflammatory effect would be enhanced with the low-dose BPCs. However, no analgesic efficacy was found when the dose was 104.5 mg/kg. It was also observed that the anti-inflammatory effect was significantly reduced. When the antipyretic effect was evaluated, this activity was seen at both the 209 mg/kg dose and the 104.5 mg/kg dose.

4. CONCLUSION

We successfully prepared BPCs by a simple method as an effective drug delivery system. BPCs have small particle size and narrow particle size distribution. Due to this characteristic; they have the capacity as a suitable candidate for phytoconstituents delivery systems. QbD approach in formulation design guided us in choosing an optimal formulation. The antipyretic activity was detected in BPCs both at a dose of 104 mg/kg and at a dose of 209 mg/kg. However, the analgesic and anti-inflammatory effects were only seen at the high dose of 209 mg/kg.Furtherstudieswillfocus on its oral bioavailability in ordertobetterobservetheadvantage of theBPCs.

5. MATERIALS AND METHODS

5.1. Materials

BER chloride form ($C_{20}H_{18}ClNO_4$, MW 371,81 g/mol) was obtained from Sigma-Aldrich (Germany). L-α-Phosphatidylcholine from egg yolk were purchased from Sigma- Aldrich (Germany). Analytical HPLC-grade acetonitrile was purchased from Carla Erba Reagents. Freund's Complete Adjuvant (FCA) was obtained from Sigma (USA). *P*-benzoquinone were purchased from Merck. All other chemicals and reagents were purchased from Sigma-Aldrich (Germany) unless otherwise noted.

5.2. Preparation of BPCs

The reverse-phase evaporation method was used to prepare BPCs. The amounts ofegg yolk PL calculated according to different ratios, were dissolved in 10 mL ethanol. A solution of BER at concentration of 2 mg/ml in water was prepared and added to the PL solution. Then, this mixture was mixed on a magnetic stirrer (Wisestir SMHS3, Daihan Scientific, Korea) in different reaction temperatures (40, 50, 60 °C), BER:PL ratio (w/w) (1:3, 1:5, 1:7) and reaction times (1, 2, 3 hours) at 400 rpm to create w/o type emulsion. Ethanol was removed using a rotary evaporator (Buchi 200, BÜCHI Labortechnik AG, Switzerland) to obtain an aqueous dispersion containing BPCs.After the formulation was prepared, it was centrifuged (Sigma 3-30 KS, Germany) and the unloaded active ingredient was separated. The complexes were dispersed in water, then the BPCs suspension was frozen at -20 °C and lyophilized (Christ Gamma 2-16 LSCPlus, Germany) for 48 hours. The obtained samples were stored under vacuum. In the study, different formulations were prepared as shown in Table 5and evaluated by ZP, PDI, EE%, and PS analysis.

5.3. Development and validation of the quantification method

High-performance liquid chromatography (HPLC) was used for the assay of BER in the BPCs. The wavelength was set at 346 nm throughout the analysis (Agilent 1260 Infinity II, Santa Clara, USA). BER in water, serial concentrations (1-20 µg/mL) were prepared by diluting from 100 µg/mL stock solution. The analyzes were carried out at 30 °C and samples were injected into the system as 10 µL. A calibration equation

was calculated. The developed quantification method was validated with analytical validation parameters (precision, the limit of detection, the limit of quantification, specificity).

5.4. Physical characterization of BPCs

5.4.1. Encapsulation efficiency

The indirect method was used to calculate the EE% of BPCs. BPCs suspension was centrifuged at 8 °C, 20000 rpm, for 10 minutes and the supernatant was taken and this process was repeated 3 times. After centrifugation of the complexes, the quantity of unloaded BER in the supernatant was defined by HPLC (n=3). The EE% was calculated through Equation 1.

$$EE\% = \frac{\text{Total BER weight} - \text{Unloaded BER weight}}{\text{Total BER weight}} \times 100 \qquad (Eq.1)$$

Table 5. Formulations prepared by reverse-phase evaporation method

Formulation	Reaction time (h)	BER:PL ratio (w/w)	Reaction temperature (°C)
F1	1		
F2	2	1:3	
F3	3		
F4	1		
F5	2	1:5	40
F6	3		
F7	1		
F8	2	1:7	
F9	3		
F10	1		
F11	2	1:3	
F12	3		
F13	1		
F14	2	1:5	50
F15	3		
F16	1		
F17	2	1:7	
F18	3		
F19	1		
F20	2	1:3	
F21	3		
F22	1		
F23	2	1:5	60
F24	3		
F25	1		
F26	2	1:7	
F27	3		

5.4.2. Zeta potential

ZP of complexes was measured to determine the surface charge of the complexes on the zeta sizer (NanoSeries, Nano-ZS, Malvern Instruments, UK) (n=3). A disposable cuvette was used for ZP evaluation.

5.4.3. Particle size and polydispersity analysis

PS and PDI of BPCs were determined by dynamic light scattering (DLS) method using zeta sizer (NanoSeries, Nano-ZS, Malvern Instruments, UK). Analyses were conducted at 25°C. Disposable cuvettes were used for the measurement and 5 measurements were done for each sample.

5.5. Determining the ideal formula with QbD approach

In this study, CQA were determined as ZP (mV), PS (nm), PDI and EE% respectively. And, the relationship between these critical attributes and critical formulation and process parameters established as BER:PL ratio (w/w), reaction temperature (°C), reaction time (h) were investigated and the pharmaceutically acceptable BPCs formulation were achieved using QbD approach.

The MODDE Pro (Sartorius Stedim Data Analytics) software was used to design the experiments and determine the ideal formulation. MODDE is a software used to create and evaluate statistical experimental designs. It supports models such as Full factorial, Fractional factorial, General subset designs, Rechtschaffner L-designs, Onion, Plackett Burman, D-Optimal designs, Reduced combinatorial designs, which can be selected based on the number of factors for the scanning phase. After the scan, the quadratic polynomial (model) experiment design is usually examined to understand how factors react in more detail, make estimates, find optimization, or find a field of study. Within the software, Three-level full factorial, central composite (CCC, CCO, and CCF), Box Behnken, Onion and D-Optimal etc. designs are used for RSM reviews [39].

According to the determined formulation properties, the software offered 27 different experiments with full factorial design. The experimental results obtained from the reverse-phase evaporation method were entered into the software and then an optimum formulation was offered by the software. The experimental data were fitted by using the Partial least squares regression (PLS) method in the statistical module from Modde 11 Pro software. Moreover, the validity of the experimental design was checked by the analysis of variance (ANOVA) test [40].

5.6. In vivo studies

5.6.1. Experimental Animals

The in vivo experiments were conducted on male mice of BALB/c strain (25–30 g) and Wistar albino rats (180-200 g). All animals were provided from Gazi University Animal Experiments Laboratory (Ankara, Turkey) and reserved in 12 h light/dark cycle at room conditions with free admission to laboratory food and water tap ad libitum for 3 days before the pharmacological experiments. For BER and BPCs experimental group, seven mice were used. All the studies were performed conferring to the international rules regarding animal experiments and biodiversity rights (Gazi University Animal Experiments Local Ethics Committee: G.Ü.ET-15.011).

5.6.2. Preparation of test samples for bioassay

In our previous anti-inflammatory activity study, BER was found effective anti-inflammatory, analgesic, and antipyretic effects at 209 mg/kg dose [41]. So, 104.5 mg/kg and 209 mg/kg doses of BER and BPCs were tested due to the high anti-inflammatory effect of BER at 209 mg/kg dose in this study. All of the test materials were given in 104.5 mg/kg and 209 mg/kg doses after suspending in 0.5% sodium carboxymethylcellulose (CMC) suspension in distilled water and administered by gastric gavage to mice and rats. The control group animals received the same experimental treatment as those of the test groups except the drug treatment was replaced with applicable volumes of dosing vehicle. Indomethacin (10 mg/kg and 0.5 mg/ear) in 0.5% CMC was used as reference drug.

5.6.3. Anti-inflammatory activity models

Carrageenan-induced hind paw edema model: Test samples and the reference drug indomethacin (10 mg/kg) were managed to groups of mice and after an hour, carrageenan suspension in a saline solution was injected into the subplantar tissues of the right hind paws of mice to form an edema. Saline solution was injected into the subplantar tissues of their left hind paws as a control. Volumes of the paws were measured using a gaugecalipers (Ozaki Co., Tokyo, Japan) at 90-min intervals, and any differences among the two hind paws were noted as the levels of edema. The results achieved from test samples and control group animals were statistically assessed [27,32].

PGE$_2$-induced hind paw edema model: Test samples and the reference drug indomethacin (10 mg/kg) were managed to groups of mice and after an hour, PGE$_2$ suspension in a Tyrode solution was injected into the subplantar tissues of the right hind paws of mice to form an edema. Tyrode solution was injected into the subplantar tissues of their left hind paws as a control. Volumes of the paws were measured using a gauge caliper (Ozaki Co., Tokyo, Japan) at 15-min intervals, and any differences among the two hind paws were noted as the levels of edema. The results achieved from test samples and control group animals were statistically assessed [27,32].

Serotonin- induced hind paw edema model: Test samples and the reference drug indomethacin (10 mg/kg) were managed to groups of mice and after an hour, serotonin in 0.5 μg/5 μLTyrode's solution was injected into the subplantar tissues of the right hind paws of mice to form an edema. Tyrode solution was injected into the subplantar tissues of their left hind paws as a control. Volumes of the paws were measured using a gauge calipers (Ozaki Co., Tokyo, Japan) at 6-min intervals, and any differences among the two hind paws were noted as the levels of edema. The results achieved from test samples and control group animals were statistically assessed [27,32].

Acetic acid-induced increase in capillary permeability: Thirty minutes after orally managing test samples and indomethacin (10 mg/kg) to different groups of mice, 4% Evans Blue in a saline solution was injected into the marginal tail vein of each mouse. 10 min later, 0.5% acetic acid solution was injected intraperitoneally. 20 min later, mice were euthanized by cervical dislocation, and the peritoneum were opened. Contents of peritoneum were transferred to 10-mL volumetric flasks with 0.1 N NaOH solution and washed with distilled water. After adding distilled water to the volumetric flasks to confirm that the volume was 10 mL, the solution's absorbance was measured at 590 nm wavelength by Beckman DU Spectrophotometer [28,29].

Subcutaneous air-pouch method: Three milliliters of air was injected subcutaneously on the dorsum of each mouse under light anesthesia to make an ellipsoid or oval-shaped air pouch. After twenty-four hours, 1 mL of Freund's Complete Adjuvant (FCA) was injected into the air pouch as a test injection [35]. Test samples, as well as vehicle for control, were given per osonce daily for 5 next days. Animals were then sacrificed with high dose anesthesia and the pouch was carefully excised and the whole granulation tissue inside the pouch was collected with a Pasteur pipette into a vial and weighed.

5.6.4. Analgesic activity

***p*-benzoquinone-induced abdominal constriction test:** Sixty minutes after orally administering test samples and the reference drug ASA (100 mg/kg) to different groups of mice, 2.5 % (w/v) p-benzoquinone solutiondissolved in distilled water and was injected intraperitoneally at a dose of 0.1 mL/10 g. 5 min later, the writes response of each animal was counted for 15 min. The inhibition in writing reflex was statistically evaluated [30,41].

Hot plate test: Animals were placed on a hot plate apparatus (Ugo Basile, Comerio, Italy) that was thermostatically preserved at 55±1 °C. Animals, non-treated (CMC 0.5 % suspension, p.o.) or treated with the test samples at 100 mg/kg, orally, were placed on glass funnels in the heated surface and the time among placing the animals and the beginning of licking paws or jumping were noted as latency of response. As the reference drug, Morphine HCl was used at 10 mg/kg dose [31].

5.6.5. Antipyretic activity

Freund's complete adjuvant-induced pyrexia model: Inorder toconclude the antipyretic activity, rats treated with FCA (Freund's complete adjuvant) [32]. The temperature on the surface of the right and left hind paws of each rat was measured each other day with a clinical contact digital thermometer (Prima long) and the difference between these two values was compared with that of control group and results were appraised statistically.

5.6.6. Statistical analysis of data

Animal experiments data was expressed as the mean standard error (±S.E.M). Statistical variancesamong the control and treated groups were assessed by ANOVA and Students-Newman-Keulsposthoc tests. $p < 0.05$ was considered to be significant [* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$].

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