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OPERATING AND COMPOSITIONAL VARIABLES FOR PREPARATION OF BETULINIC ACID NANOEMULSIONS

VARIABLES DE OPERACIÓN Y COMPOSICIÓN PARA LA PREPARACIÓN DE NANOEMULSIONES DE ÁCIDO BETULÍNICO

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Abstract

Betulinic acid is a triterpene with remarkable biological activities, including anticancer and highlights the anti -HIV activity. In recent years, nanoemulsions have been used as delivery systems to improve the bioavailability of lipophilic bioactive compounds. In this work response surface methodology (RSM) by a central composite design was employed to evaluate the effects of emulsification power, ultrasonication time, emulsifier concentration in the characteristics of betulinic acid nanoemulsions. Optimal conditions for nanoemulsions formation were obtained and different emulsifiers and oils as the dispersed phase were studied. The effect of pH on globule size and zeta potential of betulinic acid nanoemulsions was studied. The statistical model used for globule size effectively fitted the experimental data with a R^2 of 0.94. Operating conditions (power and emulsification time) and the quadratic terms, had significant effects on globule size. The optimal conditions to prepare nanoemulsions with an mean diameter of 64 nm were using 15% emulsifier (tween 60), and an ultrasonication power of 20 W during 220 s. Nanoemulsions prepared with medium chain oil as the dispersed phase had the lowest mean globule size; however, after storage time, nanoemulsions with the mixture medium chain oil:olive oil, the globule size remained unaltered. The pH variation changed the globule surface charge, values from -30 mV were obtained, and after storage time there was no significant change in the zeta potential.

Keywords: betulinic acid, nanoemulsion, ultrasonication, globule size, delivery system.

Resumen

El ácido betulínico es un triterpeno con actividades biológicas notables; entre ellas se destacan la anticancerígena y anti-VIH. En los últimos años, las nanoemulsiones se ha utilizado como sistemas de suministro para mejorar la biodisponibilidad de compuestos bioactivos lipofílicos. En este trabajo se empleó la metodología de superficie de respuesta (RSM) mediante un diseño compuesto central para evaluar los efectos de la potencia de emulsificación, el tiempo de tratamiento con ultrasonido, y la concentración de emulsificante en las características de las nanoemulsiones con ácido betulínico. Se obtuvieron condiciones óptimas de formación de las nanoemulsiones y se probaron diferentes emulsificantes y aceites como fase dispersa. Se estudió el efecto del pH en el tamaño de glóbulo y el potencial zeta de las nanoemulsiones. El modelo estadístico utilizado para el tamaño de glóbulo se ajustó con eficacia a los datos experimentales con un R^2 de 0.94. Las condiciones de operación (potencia y tiempo de emulsificación), así como sus términos cuadráticos, tuvieron efecto significativo en el tamaño de glóbulo obtenido. Las condiciones óptimas para la formación de nanoemulsiones con un diámetro medio de 64 nm fueron 15% de emulsionante (tween 60), y una potencia de ultrasonicación de 20W durante 220s. Las nanoemulsiones con el aceite de cadena media como fase dispersa tuvieron el menor tamaño de glóbulo, sin embargo, después del almacenamiento, en las nanoemulsiones con la mezcla de aceite de cadena media: aceite de oliva, el tamaño de globulo permaneció sin cambios. La variación del pH, cambió la carga superficial de los glóbulos, se obtuvieron valores de -30 mV, y después del tiempo de almacenamiento no hubo cambio significativo en el potencial zeta.

Palabras clave: ácido betulínico, nanoemulsión, ultrasonicación, tamaño de glóbulo, sistema de suministro.

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1 Introduction

Nanotechnology has been applied in many scientific areas offering the possibility to improve many systems or products when they are at nano-scale. Nanoemulsions, as conventional emulsions, are colloidal dispersions of two immiscible liquids stabilized by a thin layer of emulsifier; they are thermodynamically unstable systems with a spontaneous tendency to separate. Because of their small globule size, nanoemulsions can remain stable against sedimentation or creaming, and by employing the appropriate emulsifier, nanoemulsions can be protected against flocculation and coalescence. The main destabilization mechanism of nanoemulsions is Ostwald ripening (molecular diffusion of the disperse phase from small droplets to bigger ones) which is dependent on nanoemulsion polydispersity and the difference in solubility between small and large droplets (Tadros *et al*., 2004; Solans *et al*., 2005; Sharma *et al*., 2010). Nanoemulsions can be prepared by using the chemical potential of the components (low-energy emulsification), or by means of a mechanical device (high-energy emulsification). High-energy methods are widely used for preparation of nanoemulsions, including high-pressure homogenizers, microfluidizers, and ultrasonication devices, which can reduce the globule size at nanometric scale. However, it is necessary to evaluate the possible variables during the formation of nanoemulsions, such as emulsifier type and concentration, oil ratio, applied pressure and number of cycles during microfluidization and homogenization, amplitude and ultrasonication time (Silva *et al*., 2012, Solans *et al*., 2005, Donsi *et al*., 2012).

Nanoemulsions are transparent or translucent, which makes them attractive for their use in pharmaceutical, food, cosmetic products, chemical and agrochemical industries; and have been employed for the delivery of active compounds such as drugs, pesticides or personal care products. The main fields of application of nanoemulsions are in health care and pharmaceuticals and can be developed in different types of formulations, such as liquids, creams, foams and sprays (Solans and Solé, 2012; Lee *et al*., 2011, Maali and Hamed Mosavian, 2012). In the pharmaceutical field, nanoemulsions have been applied as carriers of lipophilic compounds, mainly drugs, and are used as drug delivery systems for parenteral (intravenously, intramuscularly, or subcutaneously), topical, ocular, nasal, and the most common, oral administration.

Nanoemulsions have been developed for the solubilization of lipophilic drugs, mainly for use in cancer and tumor therapy, reducing side effects of chemotherapy or increasing the therapeutic efficacy of lipophilic anticancer drugs such as dacarbazin, paclitaxel and tamoxifen (Ganta and Amiji, 2009; Tagne *et al*., 2008; Kabanov *et al*., 2002; Constantinides *et al*., 2004; Tiwari and Amiji 2006). Nanoemulsions have also been used as carriers for antiretroviral drugs to treat acquired immunodeficiency syndrome (Vyas *et al*., 2008; Shah and Amiji 2006), antihypertensive drugs (Shafiq *et al*., 2007), antiprotozoan drugs with schistosomicidal activity (Araujo, *et al*., 2007; Singh and Vingkar, 2008; Santos-Magalhães and Mosqueira, 2010), topically administered drugs (aspirin and lidocaine) and for intravenous delivery of anticonvulsant drugs (Subramanian *et al*., 2008; Sadurni *et al*., 2005; Kelman *et al*., 2007). The globule size in nanoemulsions allows more superficial area, greater transport and exposure of the active compound, reduce the amounts necessary to be used in nanoemulsion formulation, and finally increase solubility, absorption and bioavailability (Sozer and Kokini, 2009, Gutiérrez *et al*., 2008).

Nanoemulsions are more commonly prepared as oil-in-water (O/W) than water-in-oil (W/O) emulsions; O/W nanoemulsions have been used as carriers for the solubilization of hydrophobic substances such as vitamins, carotenes, phytosterols, polyphenols, oils and fatty acids (ω-3 and ω-6), among others (Chen *et al*., 2006; Chaparro-Mercado *et al*., 2012). Many liphophilic compounds can prevent or reduce the risk of some illnesses and are of great interest in the market of functional ingredients and food industry where nanotecnology has also been applied for their transporting (Chen *et al*., 2006). Nanoemulsions allow solubilization of functional hydrophobic substances and represent carrier systems for these beneficial compounds in food industry. Nanoemulsions protect against environmental conditions during processing, storage and the further use of the food, and at the same time do not affect food quality, enhancing bioavalability and delivery to a specific site in the body (Weiss *et al*., 2008; El Kinawy *et al*., 2012). Food aplications for nanoemulsions are in the beginnings and research should be pursued, to apply nanoemulsions in a food matrix it is necessary to previously study the factors affecting the formation and stability of nanoemulsiones and foods, their

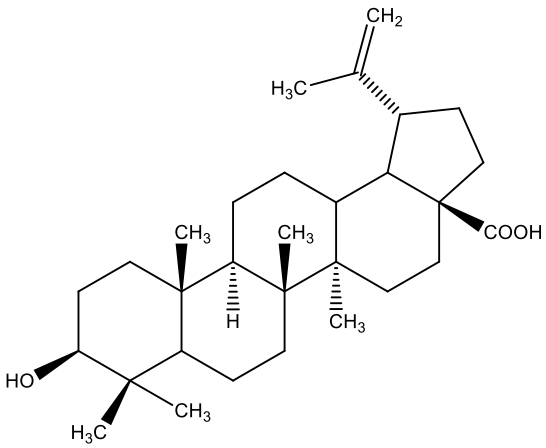

Fig. 1. Chemical structure of betulinic acid.

effects on health, and finally attain the public acceptance for the implementation of these nanosystems in the food industry (Chau *et al*., 2007; Dowling, 2004).

Betulinic acid (Fig. 1) is a pentacyclic triterpene of natural origin, which has anticarcinogenic, anti-HIV, antimalarial and anti-inflammatory activities. Compared with clinically used drugs, betulinic acid has shown selective cytotoxic activity and is capable of inducing apoptosis in a variety of cancer cell lines such as melanoma, ovarian, cervix and lung carcinomas among others (Fulda, 2008; Yogeeswari and Sriram, 2005; Alakurtti *et al*., 2006; Aiken *et al*., 2005; Zuco *et al*., 2002; Pisha *et al*., 1995). As a result of their potential antitumor activity, betulinic acid has been tested *in vivo* and *in vitro* on melanoma cells, achieving a complete inhibition of tumor growth without causing toxicity. Studies by Fulda and Debatin (2005) show the betulinic acid effect combined with anticancer drugs such as Taxol, cisplatin, doxorubicin, actinomicin D, in order to induce apoptosis and their combination can induce apoptosis in cancer cells by different routes and prevent resistance by cancer cells.

Betulinic acid has been administered topically *in vivo* (Ciurlea *et al*., 2010), intraperitoneal (Zuco *et al*., 2002; Jaguer *et al*., 2008; Cheng *et al*., 2003; Sawada *et al*., 2004) and orally (De Melo *et al*., 2009; Akowuah and Zhari, 2008; Chintharlapalli *et al*., 2011). The liphophilic character of betulinic acid causes low absorption in the gastrointestinal tract and poor bioavalability; nanoemulsions as lipid carrier systems can be the alternative to improve betulinic acid solubility and absorption. There are very few studies using betulinic acid nanoemulsions; in these works the preparation has been through the use of microfluidizers and are only focused on the effects associated with administration (antitumor and antiangiogenic activities), so nanoemulsions were not characterized and their stability evaluated (Ciurlea *et al*., 2010; Dehelean *et al*., 2011). Experimental design and surface response, are methodologies used to determine the optimal values of the variables involved in the formation of nanoemulsions stabilized by food grade emulsifiers and biopolymers, which ensure high stability and can be employed for the protection and controlled release of bioactive compounds (Yuan *et al*., 2008; Li and Chiang, 2012; Chaparro-Mercado *et al*., 2012). To the best of our knowledge, there are no reports on the preparation of nanoemulsions of betulinic acid by ultrasonic treatment nor on the evaluation of the influence of emulsification conditions on the characteristics of betulinic acid nanoemulsions and their stability during storage. The objective of this work was to prepare nanoemulsions containing betulinic acid by ultrasonic emulsification. Response surface methodology was employed to find the optimal conditions of betulinic acid nanoemulsion formation. Conditions in the ultrasound equipment and composition were studied related to globule size, distribution and stability.

2 Materials and methods

2.1 Chemicals and reagents

Betulinic acid was obtained from Indofine Chemicals (Hillsborough, NJ). Emulsifiers polyoxythylene sorbitan monolaurate (Tween 20), polyoxyethylene sorbitan monomyristate (Tween 40), polyoxyethylene sorbitan monostearate (Tween 60) and polyoxyethylene sorbitan monooleate (Tween 80) were purchased from Mallinckrodt (Mallinckrodt Baker, Mexico City). Lecithin, composed of 95 % soy phosphatidylcholine was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Mill-Q water was used to carry out all the experiments. Medium chain triglyceride oil of pharmaceutical grade was provided by Now Foods (Bloomindale, IL) and extra-virgin olive oil was obtained from the local market. Other chemicals used in this work were analytical grade or better.

2.2 Betulinic acid nanoemulsion formation

Emulsions were prepared using betulinic acid and medium chain oil as the dispersed phase and Milli-Q water and tween as the continuous phase. The

dispersed phase was set to a content of 5% w in the final nanoemulsion. Betulinic acid was first dissolved in medium chain oil in a proportion of 0.5 mg/g of emulsion, then the dispersed phase was added to the continuous phase and this mixture was pre-emulsified using a rotor-stator homogenizer Ultraturax (T25 digital, IKA) at 5000 rpm for 5 min. The formed coarse emulsion was passed through a S-450D Branson digital sonifier (Emerson Electric Co., St. Louis, MO) at determined power amplitude and time, according to an experimental design. Betulinic acid nanoemulsions prepared under the optimal conditions obtained from the results of the experimental design were changed in composition: different emulsifiers at the same concentration were used (Tween 20, 40, 60, 80 and lecithin) and the oily phase was varied from medium chain oil, olive oil and a 1:1 mixture of both. After ultrasonication, emulsions were collected and analyzed. The effect of pH was studied for the best conditions obtained, pH values in nanoemulsions were adjusted using a pH meter (Thermo Orion 5-star) to 2.5, 5, 7.5 and 10, employing NaOH or HCl (1M). Nanoemulsions adjusted to different pH values were stored at 5 °C for 60 days and monitored. In all treatments, nanoemulsions were prepared in duplicate.

2.3 *Experimental design*

Response surface methodology (RSM) was employed to evaluate the effect of the independent variables (ultrasonication power, ultrasonication time, and emulsifier concentration), on the globule size and distribution (PDI) of the betulinic acid nanoemulsions. The experiments were designed according to the central composite design (CCD). Twenty treatments were carried out randomly in order to study the main, quadratic and the interaction effects of the independent variables on the response variables. The design with the uncoded independent variables used is listed in Table 1. A second-order polynomial equation was used to predict particle size and distribution of the betulinic acid nanoemulsions as a function of the independent variables (Eq. 1).

$$y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad (1)$$

In this equation, y represents globule size or globule size distribution, X_1 is the ultrasonication power amplitude, X_2 is the ultrasonication time and X_3 is the emulsifier concentration (tween 20). An analysis of variance was conducted to determine the statistical significance of factors; variables were considered significant when the p-value became $p < 0.05$. Estimation of goodness of fit for the experimental data was measured by the regression coefficient (R^2). The optimization tool of the statistical software was used with the data of the CCD to optimize the variables in betulinic acid nanoemulsion formation, leading to the smallest globule size and size distribution; these variables were used to prepare betulinic acid nanoemulsions at optimal conditions. The experimental design matrix, data analysis and optimization procedure were performed using the Minitab v.16 statistical software (Minitab Inc., State College, PA).

Table 1. Treatments in the central composite experimental design

Treatment	Power amplitude (W)	Time (s)	Emulsifier (%w)
1	28.11	108.65	7.03
2	51.89	108.65	7.03
3	28.11	251.35	7.03
4	51.89	251.35	7.03
5	28.11	108.65	12.97
6	51.89	108.65	12.97
7	28.11	251.35	12.97
8	51.89	251.35	12.97
9	20	180	10
10	60	180	10
11	40	60	10
12	40	300	10
13	40	180	5
14	40	180	15
15	40	180	10
16	40	180	10
17	40	180	10
18	40	180	10
19	40	180	10
20	40	180	10

2.4 *Characterization of globule size and distribution*

Globule size was determined by dynamic light scattering using a Zetasizer Nano-ZS90 (Malvern Instruments Ltd, Worcestershire, UK), samples (5 μL) were diluted in distilled and deionized Milli-Q water (1000 μL). Globule size of betulinic acid nanoemulsions was described by the cumulants mean

(z-average) diameter. Globule size distribution was given by the polidispersity index (PDI) that measures the width of size distribution, ranging from 0 to 1.0.

2.5 Zeta potential

The surface charge of the globules (zeta potential) was measured using a Zetasizer Nano-ZS90. Samples were prepared diluting 25 μL of the nanoemulsion with 2000 μL of Milli-Q water. Measurements were carried out at 25 °C.

2.6 Stability studies

Betulinic acid nanoemulsions formed at the optimal conditions obtained from CCD, were stored at 5 °C for 60 days. Nanoemulsion characterization (globule size, size distribution and zeta potential) was monitored during this time.

2.7 Statistical analysis

The experimental design, surface response of the fitted polynomial equations and comparison of means by Tukey's test ($p < 0.05$) were carried out using the Minitab v.16 statistical software (Minitab Inc. State College, PA).

3 Results and discussion

3.1 Experimental design

The experimental data for globule size and distribution obtained were used to calculate the coefficients that fit the equations to predict the values for globule size and distribution. Experimental and predicted values obtained are given in table 2. Predicted values were very close to experimental data. The R^2 values for globule size and distribution were 0.941 and 0.84, respectively; suggesting that for globule size the model provided a good fit to the experimental results, and described the influence of the evaluated variables. In all treatments the mean globule size obtained were below 200 nm; however, all the PDI of size distributions were greater than 0.2.

Table 2. Experimental and predicted values for response variables

Treatment	Mean globule size (nm)		PDI	
	Experimental	Predicted	Experimental	Predicted
1	156.7	159.1	0.220	0.244
2	149.8	153.3	0.258	0.232
3	137.8	137.6	0.270	0.258
4	134.8	138.9	0.279	0.286
5	127.8	130.6	0.253	0.243
6	106.5	113.6	0.280	0.288
7	89.8	93.1	0.304	0.325
8	78.7	83.2	0.440	0.411
9	134.8	133.2	0.268	0.252
10	128.1	120.0	0.293	0.314
11	158.7	152.7	0.228	0.228
12	112.8	112.8	0.339	0.343
13	156.6	154.2	0.240	0.241
14	90.6	83.4	0.341	0.345
15	112.4	112.9	0.301	0.304
16	114.3	112.9	0.309	0.304
17	111.5	112.9	0.305	0.304
18	123.3	112.9	0.289	0.304
19	102.3	112.9	0.363	0.304
20	112.2	112.9	0.300	0.304

Table 3. Regression coefficients and analysis of variance of the regression coefficients

Variable	Mean globule size			PDI		
	Regression coefficient	F- Value	P- value	Regression coefficient	F- Value	P- value
β_0	297.543			0.27782		
Linear effects						
β_1	-2.649	5.950	0.035	-0.00045	-0.121	0.906
β_2	-0.526	9.820	0.011	-0.00035	-0.610	0.556
β_3	-5.194	1.430	0.259	-0.01187	-0.790	0.448
Quadratic effects						
β_{11}	0.034	11.010	0.008	-0.00005	-1.473	0.172
β_{22}	0.001	18.940	0.001	0.00000	-1.270	0.233
β_{33}	0.234	2.010	0.186	-0.00043	-0.759	0.465
Interaction effects						
β_{12}	0.002	0.810	0.390	0.00001	1.487	0.168
β_{13}	-0.080	2.070	0.181	0.00041	2.128	0.059
β_{23}	-0.019	4.140	0.069	0.00008	2.542	0.029

The regression coefficients for the response surface equation and the F- and *p*-values are depicted in table 3. The analysis of variance shows the significance of the variables evaluated: globule size was affected by ultrasonication conditions, mainly by the time; these results fitted by the polynomial equation, suggest that ultrasonication variables had a negative single and positive quadratic effects. Emulsifier concentration did not show a significant effect on globule size, hence, by using a proper arrangement of conditions in the ultrasound device, it would be possible to obtain betulinic acid nanoemulsions with mean globule size smaller than 100 nm. In the analysis of variance for globule size distribution the interaction of ultrasonication time-emulsifier concentration had a significant negative effect and the other terms were non-significant.

The effect of the variables on the globule size are shown in the response surface (figure 2); surface responses were generated by varying two of the independent variables and, the other remained at the central point. In figure 2a the effect of ultrasonication time and power amplitude on globule size are depicted, when the emulsifying time increased the mean globule size was reduced, however, from 20 W to 40 W of power amplitude, after 250 s, no further decrease in mean globule size was observed. Under these conditions mean globule sizes below 100 nm could not be obtained; globule sizes of 123, 106 and 116 nm were the lowest obtained for 20, 40 and 60 W, respectively. Similar results were reported by Tang and co-workers (2013), who used 50 - 70 % of amplitude, and obtained an equilibrium in emulsion droplet diameter after 60 s. When the emulsifier concentration available to cover the globule is constant, more emulsifying time can form new smaller globules; however, the emulsifier may not completely cover the globules, and coalescence occurs, so no further reduction in the globule size is obtained. For 60 W of power amplitude, sonication times longer than 250 s produce mean globule sizes up to 121 nm at 300 s; the increase in globule size is reported as over-processing (Jafari *et al*., 2006, 2007; Tadros *et al*., 2004; Kentish *et al*., 2008). During over-processing, the higher power amplitude (60 W) caused an increase in the energy and shear applied to disrupt the globules; consequently, the new globules formed are subjected to higher rates of collisions and re-coalescence occurs; therefore, combinations of high power amplitude and time, did not necessarily cause further reductions in globule size, and are unfavorable under specific circumstances. Figure 2b was generated varying the ultrasonication power amplitude and emulsifier concentration, and maintaining the emulsification time at 180 s. When emulsifier concentration was set at 5% and the power amplitude in the ultrasonifier device

was 40 W, a reduction of mean globule size was observed, then, increasing the power amplitude to 60 W caused overprocessing. This may happen if not enough emulsifier is available to cover the globule rapidly and completely. Additionally, increased power amplitude, produced more shear forces to be applied to globule disruption, and higher collision rates between the newly formed globules occurs, leading to a net increase in globule size. Therefore, over-processing is controlled by emulsifier adsorption, concentration, and globule collisions (as a result of increased energy input during emulsification). Working at 40% of power amplitude and at emulsifier concentrations greater than 12%, globule size values under 100 nm were obtained; similarly, these globule sizes were also obtained using 15% emulsifier and a power amplitude greater than 26%. During emulsification a dynamic process occurs, first the break up of globules by the energy applied, then the emulsifier adsorption forming a membrane around the entire surface of the globule, and finally the globule collisions, wherein the emulsifier should protect the globule from coalescence; then, the use of appropriate combinations of energy applied and emulsifier concentration during globule disruptions can provide satisfactory results in the formation of nanoemulsions. Figure 2c shows the emulsifier concentration and emulsification time combinations at 40 W of power amplitude. In this figure the smallest globule size (68 nm) was obtained. Working from 210 to 300 s of emulsification time and at emulsifier concentrations above 11 %, it was possible to attain globule sizes smaller than 100 nm. At the highest emulsifier concentration (15%), together with an increase in the emulsification time beyond 275 s, it was possible to obtain small globule sizes (less than 70 nm). Emulsifiers reduce the interfacial tension, and thus, decrease the energy necessary to disrupt the globule. At emulsifier concentrations of 15%, less energy is needed to be applied to achieve globule disruption. In addition, longer emulsification times implicate more energy applied, resulting in a decrease in globule size (68 nm). During this study emulsifier concentrations above 11 % and diferent combination of power amplitude and emulsification time made possible to obtain globules sizes smaller than 100 nm.

The CCD was used to find the optimum values for the independent variables; however, in some cases the values of the independent variables considered optimal for a response may not be similar to another response.

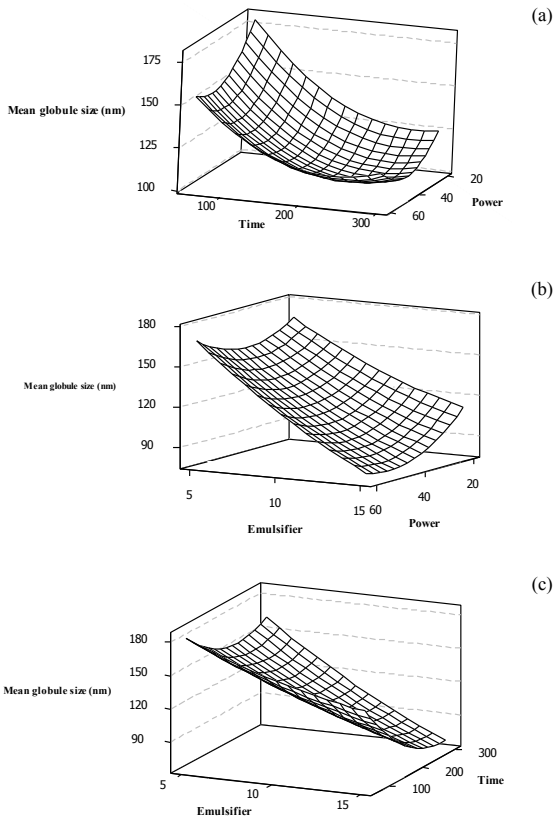

Fig. 2. Response surface plots for globule size of betulinic acid nanoemulsions as a function of emulsifier concentration, power and ultrasonication time.

In this work three sets of optimal conditions were found in order to minimize the mean globule size and size distribution (PDI). The first optimization set was intended to minimize the mean globule size without taking into account the PDI; the second set was to minimize the PDI without taking into account the globule size; and finally the last set of optimization conditions was intended to minimize both the globule size and PDI. Table 4 shows the optimal conditions obtained for each set and the predicted as well as the corresponding experimental values obtained. The difference between the predicted globule size value and the experimental data in Set 1 was 4.5 %, which demonstrated that the model properly fit the experimental data, and it can be used as a tool for globule size prediction. In Set 2, it was difficult to obtain a good fit for PDI from the experimental data, such that the R^2 value for the distribution was 0.84; then the model is not properly fitted to the experimental data.

Table 4. Predicted and experimental values at optimal conditions for betulinic acid nanoemulsion formation.

Treatment	Variable			Predicted value		Experimental value	
	Power amplitude (W)	Time (s)	Emulsifier (%w)	Mean globule size (nm)	PDI	Mean globule size (nm)	PDI
Set 1	47.5	283	15	66.3	-	69.3 ± 2.5^a	0.427 ± 0.017^a
Set 2	60	60	5	-	0.1548	165.4 ± 2.1^b	0.227 ± 0.007^b
Set 3	20	220	15	101	0.276	84.7 ± 2.3^c	0.285 ± 0.007^c

Each value represents the mean ± S.D. (n = 4).

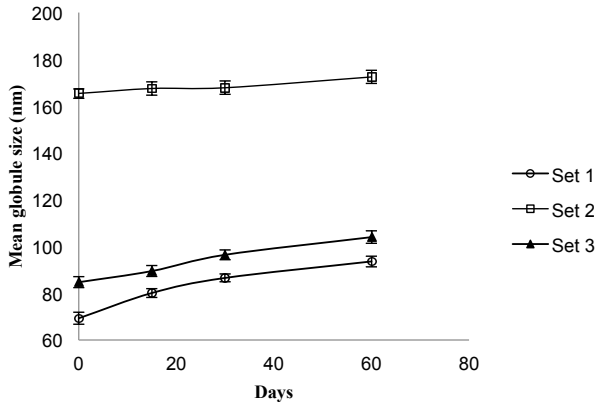

Fig. 3. Mean globule size obtained during storage time.

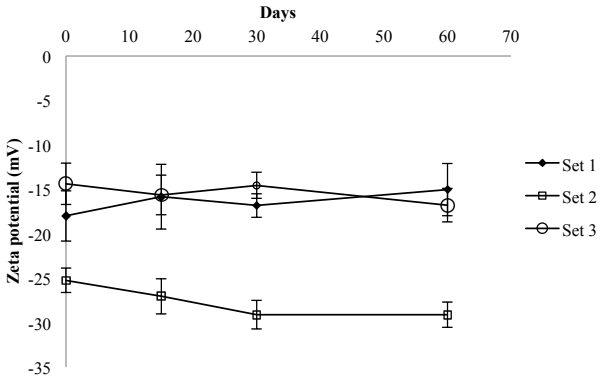

Fig. 4. Nanoemulsion surface charge during storage time.

Similar results were obtained in Set 3 where the PDI is included too, affecting the prediction of the globule size values.

3.2 Storage stability

Samples prepared at optimal conditions were stored for 60 days, and characterization was made during this time. The increase in mean globule size with time is depicted in figure 3; the set 2 was the most stable nanoemulsion with an increase of only 4 %; however, the mean globule size obtained was greater than 150 nm. The PDI in sets 1, 2 and 3 after 60 days were 0.168, 0.165 and 0.171, respectively, with no significant differences between them ($p < 0.05$). The mean globule size obtained in set 3 was below 100 nm and after storage time, it had an increase in mean globule size of 22 %. The surface charge was monitored during storage and these data are depicted in figure 4. The use of non-ionic emulsifiers to stabilize betulinic acid nanoemulsions apparently did not contribute to the surface charge of the globule, however the charge in nanoemulsions can be probably attributed to the oils, which may contain high concentrations of electrolytes and therefore increased the negative surface charge in emulsions (Hsu and Nacu, 2003); also an increase in zeta potential after sonication has been reported (Stalidis *et al*., 1990). By analyzing the surface charge of betulinic acid nanoemulsions in the set 2, the initial high negative surface charge (-25 mV) could have caused the inter-globules repulsion and thus provided greater stability, although set 2 had the lowest content of emulsifier (5 %). In sets 1 and 3 where the emulsifier was 15 %, the surface charge was -18 and -14.4, respectively, which did not favor electrostatic stability and measurable globule coalescence occurred.

3.3 Effect of oil type on betulinic acid nanoemulsions characteristics

Previous studies have revealed that the bioavailability of some lipophilic compounds is related to the length of the fatty acid chain used as a dispersed phase in an emulsion (Clark *et al*., 2000; Ahmed *et al*., 2012); also, oil properties such as solubility, viscosity and interfacial tension can change the globule size of the

emulsion (Ahmed *et al*., 2012). Two oils (medium chain and olive oil) and a 1:1 mixture of them were used as dispersed phase to prepare betulinic acid nanoemulsions and the effect of different oils on the globule size of the nanoemulsions was evaluated. The dispersed phase was set at 5% in the nanoemulsions and the emulsification conditions were according to set 3. The medium chain oil was composed by caprilic acid (56 %) and capric acid (44%); olive oil was composed mainly by oleic acid (75 %), and the mixture used was composed by caprilic acid (35 %), capric acid (26%) and oleic acid (30%). The nanoemulsion characteristics obtained according to the type of oil used are included in table 5. Betulinic acid nanoemulsions with medium chain oil as dispersed phase had the lowest mean globule size (85 nm); using the mixture of long and medium chain oils the mean globule sizes in the nanoemulsions were of 90 nm; with the use of the mixture of oils it is possible to take advantage of the beneficial effects associated with these oils. There was no significant difference ($p < 0.05$) in PDI from nanoemulsions in which medium chain oil or the mixture were used as dispersed phase. In nanoemulsions with olive oil, the PDI was smaller than 0.2, which is indicative of good stability (Klang and Valenta, 2011).

Stability studies were carried out during 60 days of storage, the oil used as a dispersed phase affected the stability; these results are shown in Fig. 5. Betulinic acid nanoemulsions prepared with medium chain oil, olive oil and the mixture had mean globule sizes of 104, 107.4 and 90.4 nm, respectively after storage. During storage the mean globule size in nanoemulsions with the mixture and olive oil remained unaltered. Nanoemulsions with medium chain oil after 30 and 60 days had a raise in globule size of 14 and 23%, respectively, and a reduction in the PDI from 0.285 to 0.175 after 60 days of storage. Ostwald ripening is the main mechanism for instability in nanoemulsiones with sizes below to 500 nm, in which the globule size increases and PDI decreases; this has already been reported in previous studies where short chain oil was used as dispersed phase (Klang and Valenta, 2011; Ahmed *et al*., 2012, Lee *et al*., 2011). Ostwald ripening is a process of gradual growth of the larger disperse phase globules at the expense of small droplets by means of the molecular diffusion through the continuous phase. This destabilization mechanism can be explained by the difference in chemical potential between small and large droplets, where the solubility of the dispersed phase in the continuous phase is a result of the globule size. The smaller the droplet, the higher the solubility of emulsified oil. Oswald ripening is strongly influenced by the solubility and diffusion coefficient of the disperse phase. A way to prevent Ostwald ripening rate in emulsions is the use of less polar oils to decrease the disperse phase solubility. The diffusion coefficient largely depends on the molecular weight; then higher molecular weight oil reduces Ostwald ripening rate. Nanoemulsions using long chain triglycerides (with greater molecular weight) in the disperse phase are more stable that those containing short chains, and the employment of a mixture of short and long chains has also been reported to improve the stability (Ahmed *et al*., 2012). Therefore, the lack of change in the mean globule size in the nanoemulsions prepared with olive oil as the dispersed phase, could be attributed to the presence of long chain fatty acids with low water solubility and high molecular weight, that allows a reduction of this instability phenomenon. Nanoemulsions with the mixture of oils as dispersed phase had globule sizes greater than the obtained with the nanoemulsions prepared with medium chain oil. However, they had the smallest mean globule size after storage. This supports using of a mixture of oils as a convenient option for improved stability.

Table 5. Characteristics of nanoemulsions prepared with different disperse phases.

Oil	Mean globule size (nm)	PDI
MCT	84.7 ± 2.3^a	0.285 ± 0.007^a
Olive:MCT	90.1 ± 2.7^b	0.276 ± 0.037^a
Olive	108.2 ± 2.9^c	0.196 ± 0.022^b

Each value represents the mean ± S.D. (n = 4).

Table 6. Mean globule size characteristics obtained using different emulsifiers

Emulsifier	Mean globule size (nm)	PDI
Tween 20	84.7 ± 2.3^a	0.285 ± 0.01^a
Tween 40	65.4 ± 0.3^b	0.206 ± 0.01^b
Tween 60	63.9 ± 0.4^b	0.398 ± 0.10^c
Tween 80	$1469. \pm 1.7^c$	0.100 ± 0.02^d
Lecithin	263.6 ± 2.7^d	0.237 ± 0.01^e

Each value represents the mean ± S.D. (n = 4).

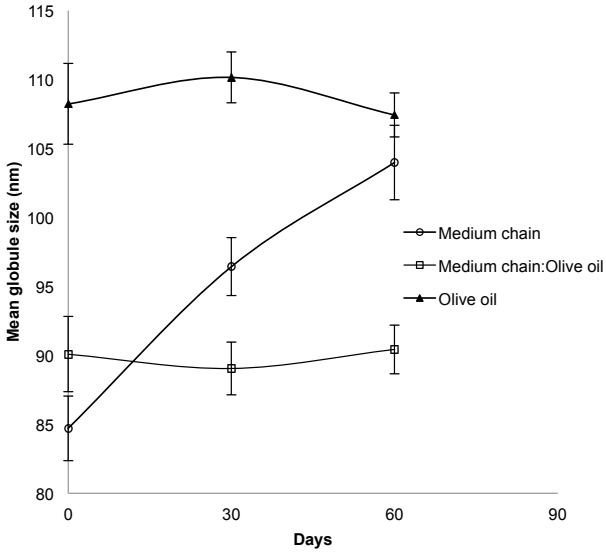

Fig. 5. Nanoemulsion mean globule size using different oils during storage time.

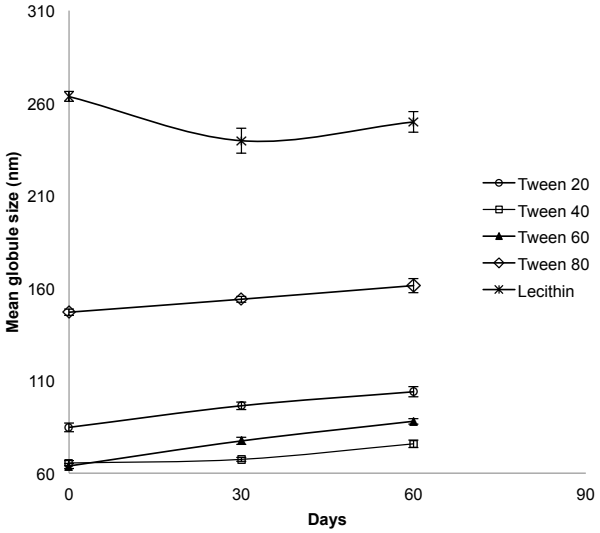

Fig. 6. Nanoemulsion mean globule size using different emulsifiers.

3.4 Effect of the type of emulsifier on globule size of betulinic acid nanoemulsions

Different emulsifiers were used in the preparation of betulinic acid nanoemulsions and their effect on mean globule size was evaluated. The emulsifier concentration was set at 15 %, according to the optimal conditions in set 3, and medium chain oil was used as dispersed phase; results obtained are shown in Table 6. The mean globule size obtained using different emulsifiers ranged between 64 and 147 nm; the mean globule size in betulinic acid nanoemulsions using Tween 80 was the largest; however, the distribution was the most uniform, having a PDI value of 0.1. Betulinic acid nanoemulsions with mean globule sizes of 64 nm were obtained when tween 60 was used as emulsifier. In nanoemulsions prepared with lecithin it was not possible to obtain globule sizes below 200 nm. A more thorough study is necessary to minimize globule size using lecithin as sole emulsifier. The results obtained could be attributed to the differences in the hydrophilic-lipophilic balance (HLB) values of the emulsifiers, type of emulsifier (ionic or non-ionic) and the provided stabilization mode (steric, electrostatic, etc), and authors have reported the effect of molecular structure in emulsifiers such as Tween (Chaparro-Mercado *et al*., 2012; Wang *et al*., 2009). Stability studies are depicted in Fig. 6; initially betulinic acid nanoemulsions prepared with Tween 40 and Tween 60 as emulsifiers had the lowest globule size (65 and 64 nm, respectively). However, nanoemulsions had mean globule sizes of 76 and 88 nm, respectively after storage; being the nanoemulsion made with Tween 40, the lowest in mean globule

size after 60 days. Betulinic acid nanoemulsions made with Tween 60 initially had 64 nm of mean globule size; however, these nanoemulsions had an increase in globule size of 38% after storage. Nanoemulsions made with tween 80 had mean globule sizes up to 100 nm and after 60 days they only showed an increase of 9.8%. In regard to their storage stability, nanoemulsions made with Tween 40, had a mean globule size below 100 nm and a smaller increase in mean globule size (15%) after storage.

3.5 Effect of pH in nanoemulsion characteristics and stability

The pH can exert a decisive effect on nanoemulsions; variations at different levels of pH values cause a change in the surface charge of the globules and therefore in their stability during storage. An increase in the surface charge of the globules favors the electrostatic repulsion and decreases flocculation and further breakup of the nanoemulsion. The surface charge in the globule can be measured by laser Doppler electrophoresis and is reported as zeta potential. The pH from betulinic acid nanoemulsions was varied to values that can be found in different systems such as foods and drugs (pH 2.5, 5, 7.5 and 10). Figure 7 shows the effect of pH change on the zeta potential of the nanoemulsions. The change in pH caused an increase in the negative globule surface charge and values from -16 mV to -30 mV were obtained. Similar reports about the relationship of zeta potential with pH values have already been reported by other authors (Lee *et al*., 2011; Qian *et al*., 2012; Tang *et al*., 2013). These changes in surface charge have been attributed to the preferential adsorption of OH^- groups at the interface, so that an increase in pH toward alkaline values increases the negative surface charge (Hsu and Nacu, 2003). After storage, and at all tested pH values, no significant differences ($p < 0.05$) in the zeta potential of the nanoemulsions compared to the initial charge were found. The pH variations had no effect on mean globule size of the nanoemulsions, which increased significantly from 8 to 10% in all nanoemulsions prepared at different pH values after storage (figure 8). Reports in which the change in pH ionized the active compound, show that tends to move towards the surface of the globule, where it competes with the emulsifier in the interface and thereby increases the droplet coalescence and consequently their globule size (Tang *et al*., 2013).

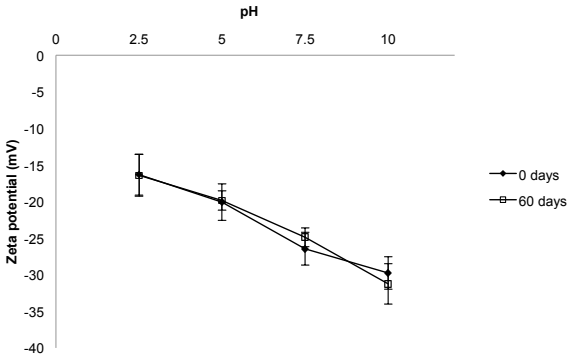

Fig. 7. Zeta potential for nanoemulsions at different pH values.

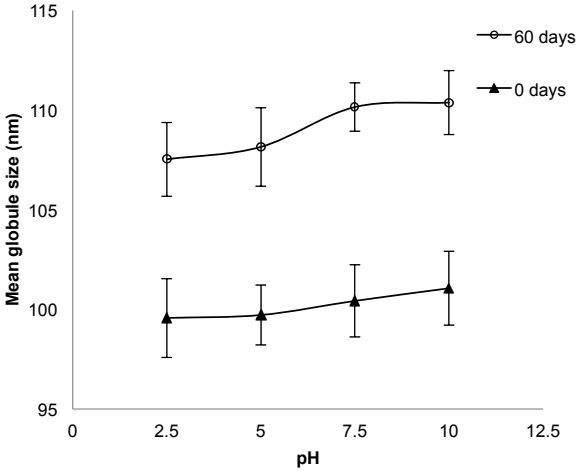

Fig. 8. Nanoemulsion mean globule size at different pH values.

In this study, the presence of betulinic acid lowered the pH due to the acidic nature of the compound, when the variation of pH was performed in the dispersed phase, the size of globule remained constant, indicating that betulinic acid is still part of hydrophobic globule core, and does not interact at the interface. The change in zeta potential, could be attributed to the adsorption of OH^- groups by the oil, but when globule is covered with a non-ionic emulsifier, that offers steric stabilization against globule collisions. Thus, for nanoemulsions with zeta potential values of ca. -14 mV, which is lower than the reported levels that provide good electrostatic stability against coalescence of the droplets (-30 mV), their globules had similar stability as nanoemulsions with zeta potential values greater than -30 mV (Zhao *et al*., 2010). In systems where nanoemulsion globules are covered by a non-ionic emulsifier, the variation in the pH may not have an effect on surface charge because

the emulsifier employed does not ionize. This type of emulsifier provides steric stabilization to the globule, so that a change in the surrounding ions does not alter its emulsifying capacity; however, the change in the surface charge of the globule could possibly have an effect on the oxidative stability during storage. No change occurs on globule size with the variation of pH values found in food, however, it is necessary to study the application of nanoemulsions in different food matrices.

Conclusions

In this research, the designed model fitted to experimental values for mean globule size for betulinic acid nanoemulsions and it could efficiently predict results. The central composite experimental design made it possible to find the optimal values of the independent variables for betulinic acid nanoemulsion formation to produce mean globule sizes below 70 nm. The type of oil used had an effect on the mean globule size of betulinic acid nanoemulsions as well as on their storage stability. The characteristics of the dispersed phase are important variables to consider in the formation and stability of nanoemulsions. Due to the nonionic nature of the emulsifiers used, variations in pH values caused no changes in the globule size of the nanoemulsions. We propose that these conditions could be applied in various food matrices at different pH values. Our data show the combination of compositional and operational variables for betulinic acid nanoemulsions formation in which mean globule sizes near 64 nm can be obtained.

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References

Ahmed, K., Li, Y., McClements, D.J., and Xiao, H. (2012). Nanoemulsion- and emulsion-based delivery systems for curcumin: Encapsulation and release properties. *Food Chemistry 132*, 799-807.

Aiken, C. and Chen, C.H. (2005). Betulinic acid derivatives as HIV-1 antivirals. *Trends in Molecular Medicine 11*, 31-36.

Akowuah, G.A. and Zhari, I. (2008). Determination of betulinic acid from Orthosiphon stamineus leaf extract in rat plasma. *Pharmazie 63*, 788-790.

Alakurtti, S., Makela T., Koskimies, S. and Kauhaluoma, Y.J. (2006). Pharmacological properties of the ubiquitous natural product betulin. *European Journal of Pharmaceutical Sciences 29*, 1-13.

Araujo, S.C., Mattos, A.C.A., Teixeira, H.F., Coelho, P.M.Z., Nelson, D.L. and Oliveira, M.C. (2007). Improvement of *in vitro* efficacy of novel schistomicidal drug by incorporation into nanoemulsions. *International Journal of Pharmaceutics 337*, 307-315.

Chaparro-Mercado, M.C., García-Ochoa, F., Hernández-Sánchez, H., Alamilla-Beltrán, L., Quintanilla-Carvajal, M.X., Cornejo-Mazón, M., Pedroza-Islas, R. and Gutierrez-López, G.F. (2012). Design of an interstitial structure for a grape seed oil emulsion by design of experiments and surface response. *Revista Mexicana de Ingeniería Química 11*, 11-21.

Chau, C.F., Wu S.H. y Yen, G.C. (2007). The development of regulations for food nanotechnology. *Trends in Food Science & Technology 18*, 269-280

Chen, H., Weiss, J. and Shahidi, F. (2006). Nanotechnology in nutraceuticals and functional foods. *Food Technology 60*, 30-6.

Chintharlapalli, S. Papineni, S. Lei, P., Pathi S. and Safe, S. (2011). Betulinic acid inhibits colon cancer cell and tumor growth and induces proteasome-dependent and -independent downregulation of specificity proteins (Sp) transcription factors. *BioMed Central Cancer 11*, 371.

Ciurlea, S.A., Dehelean, C.A., Ionescu, D., Berko, S., Csanyi, E., Hadaruga, D.I., Ganta, S., and Amiji, M.M. (2010). A comparative study regarding melanoma activity of Betulinic acid on topical ointment vs. systemic nanoemulsion delivery systems. *Journal of Agroalimentary Processes and Technologies 16*, 420-426.

Clark, R.M., Yao, L., She, L. and Furr, H.C. (2000). A Comparison of Lycopene and Astaxanthin Absorption from Corn Oil and Olive Oil Emulsions. *Lipids 35*, 803-806.

Constantinides, P.P., Tustian, A. and Kessler, D.R. (2004). Tocol emulsions for drug solubilization and parenteral delivery. *Advanced Drug Delivery Reviews 56*, 1243- 1255.

Dehelean, C.A., Feflea, S., Ganta, S. and Amiji, M. (2011). Anti-angiogenic effects of betulinic acid administered in nanoemulsion formulation using chorioallantoic membrane assay. *Journal of Biomedical Nanotechnology 7*, 317-24.

De Melo, C.L., Queiroz, M.G.R., Arruda, A.C., Rodriguez, A.M., De Sousa, D.F., Almeida, J.G., Pessoa, O.D., Silveira, E.R., Menezes, D.B., Melo, T.S., Santos, F.A. and Rao, V.S. (2009). Betulinic Acid, a Natural Pentacyclic Triterpenoid, Prevents Abdominal Fat Accumulation in Mice Fed a High-Fat Diet. *Journal of Agricultural and Food Chemistry 57*, 8776-8781.

Donsì, F., Sessa, M., and Ferrari, G. (2012). Effect of Emulsifier Type and Disruption Chamber Geometry on the Fabrication of Food Nanoemulsions by High Pressure Homogenization. *Industrial & Engineering Chemistry Research 51*, 7606-7618.

Dowling, A.P. (2004). Development of nanotechnologies. *Materials Today 7*, 30-35

El Kinawy, O. S., Petersen, S. and Ulrich, J. (2012). Technological Aspects of Nanoemulsion Formation of Low-Fat Foods Enriched with Vitamin E by High-Pressure Homogenization. *Chemical Engineering & Technology 35*, 937-940

Fulda, S. y Debatin, K.M. (2005). Sensitization for Anticancer Drug-Induced Apoptosis by Betulinic Acid. *Neoplasia 7*, 162-170.

Fulda, S. (2008). Betulinic acid for cancer treatment and prevention. *International Journal of Molecular Sciences 9*, 1096-1107.

Ganta, S. and Amiji, M. (2009). Coadministration of Paclitaxel and Curcumin in Nanoemulsion Formulations To Overcome Multidrug Resistance in Tumor Cells. *Molecular Pharmaceutics 6*, 928-939.

Gutiérrez, J.M., González, C., Maestro, A., Solè, I., Pey, C.M., and Nolla, J. (2008). Nano-emulsions: New applications and optimization of their preparation. *Current Opinion in Colloid and Interface Science 13*, 245-251.

Hsu, J.P. and Nacu, A. (2003). Behavior of soybean oil-in-water emulsion stabilized by nonionic surfactant. *Journal of Colloid and Interface Science 259*, 374-381.

Jafari, S.M., He, Y., & Bhandari, B. (2006). Nano-emulsion production by sonication and microfluidization - A comparison. *International Journal of Food Properties 9*, 475-485.

Jafari, S.M., He, Y. and Bhandari, B. (2007). Production of sub-micron emulsions by ultrasound and microfluidization techniques. *Journal of Food Engineering 82*, 478-488.

Jäger, S., Laszczyk, M.N. and Scheffler, A. (2008). A Preliminary Pharmacokinetic Study of Betulin, the Main Pentacyclic Triterpene from Extract of Outer Bark of Birch (*Betulae alba* cortex). *Molecules 13*, 3224-3235.

Kabanov A.V., Batrakova E.V. and Alakhov V.Y. (2002) Pluronic block copolymers as novel polymer therapeutics for drug and gene delivery. *Journal of Controlled Release 82*, 189-212.

Kelmann, R.G., Kuminek, G., Teixeira, H.F. and Koester, L.S. (2007). Carbamazepine parenteral nanoemulsions prepared by spontaneous emulsification process. *International Journal of Pharmaceutics 342*, 231-239.

Kentish, S., Wooster, T.J., Ashokkumar, M., Balachandran, S., Mawson, R., and Simons, L., (2008). The use of ultrasonics for nanoemulsion preparation. *Innovative Food Science & Emerging Technologies 9*, 170-175.

Klang, V. y Valenta, C. 2011. Lecithin-based nanoemulsions. *Journal of Drug Delivery Science and Technology 21*, 55-76.

Lee, S.J., Choi, S.J., Li, Y., Decker, E.A. and McClements, D.J. (2011). Protein-Stabilized Nanoemulsions and Emulsions: Comparison of Physicochemical Stability, Lipid Oxidation, and Lipase Digestibility. *Journal of Agricultural and Food Chemistry 59*, 415-427.

Li, P.H. y Chiang, B.H. (2012). Process optimization and stability of D-limonene-in-water nanoemulsions prepared by ultrasonic emulsification using response surface methodology. *Ultrasonics Sonochemistry 19*, 192-197.

Maali, A. and Hamed-Mosavian, M.T. (2013). Preparation and Application of Nanoemulsions in the Last Decade (2000-2010). *Journal of Dispersion Science and Technology 34*, 92-105.

Pisha, E., Chai, H., Lee, I.S., Chagwedera, T.E., Farnsworth, N.R., Cordell, G.A., Beecher, C.W.W., Fong, H.H.S., Kinghorn, A.D., Brown, D.M., Wani, M.C., Wall, M.E., Hieken, T.J., Gupta, T.K. and Pezzuto, J.M. (1995). Discovery of betulinic acid as a selective inhibitor of human melanoma that functions by induction of apoptosis. *Nature Medicine 1*, 1046 - 1051.

Qian, C., Decker, E.A., Xiao, H. and McClements, D.J. (2012). Physical and chemical stability of β-carotene-enriched nanoemulsions: Influence of pH, ionic strength, temperature, and emulsifier type. *Food Chemistry 132*, 1221-1229.

Sadurní, N., Solans, C., Azemar, N. and García-Celma, M.J. (2005). Studies on the formation of O/W nano-emulsions, by low-energy emulsification methods, suitable for pharmaceutical applications. *European Journal of Pharmaceutical Sciences 26*, 438-445.

Santos-Magalhaes, N.S. and Mosqueira, V.C. (2010). Nanotechnology applied to the treatment of malaria. *Advanced Drug Delivery Reviews 62*, 560-575.

Sawada, N., Kataoka, K., Kondo, K., Arimochi, H., Fujino, H., Takahashi, Y., Miyoshi, T., Kuwahara, T., Monden, Y. and Ohnishi, Y. (2004). Betulinic acid augments the inhibitory effects of vincristine on growth and lung metastasis of B16F10 melanoma cells in mice. *British Journal of Cancer 90*, 1672-1678.

Shafik, A., El Sibai, O., Shafik, A.A., and Shafik, I.A. (2007). Mechanism of gastric emptying through the pyloric sphincter: A human study. *Medical Science Monitor 13*, CR24-CR29.

Shah, L.K. and Amiji, M.M. (2006). Intracellular delivery of Saquinavir in biodegradable polymeric nanoparticles for HIV/AIDS. *Pharmaceutical Research 23*, 2638-2645.

Sharma, N., Bansal, M., Sharad, V., Sharma, P.K. and Kulkarni, G.T. (2010). Nanoemulsion: A new concept of delivery system. *Chronicles of Young Scientists 1*, 2-6.

Silva, H.D., Cerqueira M.A., and Vicente, A.A. (2012). Nanoemulsions for Food Applications: Development and Characterization. *Food and Bioprocess Technology 5*, 854-867.

Singh K. and Vingkar S. (2008). Formulation, antimalarial activity and biodistribution of oral lipid nanoemulsion of primaquine. *International Journal of Pharmaceutics 347*, 136-143.

Solans, C., Izquierdo, P., Nolla, J., Azemar, N. and Garcia, C.M.J. (2005). Nano-emulsions. *Current Opinion in Colloid and Interface Science 10*, 102-110.

Solans, C. and Solé, I. (2012). Nano-emulsions: Formation by low-energy methods. *Current Opinion in Colloid and Interface Science 17*, 246-254.

Sozer, N. and Kokini, J.L. (2009). Nanotechnology and its applications in the food sector. *Trends in Biotechnology 27*, 82-89.

Stalidis, G., Avranas, A., and Jannakoudakis, D. (1990). Interfacial properties and stability of oil-in-water emulsions stabilized with binary mixtures of surfactants. *Journal of Colloid and Interface Science 35*, 313-324.

Subramanian, B., Kuo, F., Ada, E., Kotyla, T., Wilson, T., Yoganathan, S. and Nicolosi, R. (2008). Enhancement of anti-inflammatory property of aspirin in mice by a nano-emulsion preparation. *International Immunopharmacology 8*, 1533.

Tadros, T., Izquierdo, P. Esquena, J. and Solans, C. (2004). Formation and stability of nanoemulsions. *Advances in Colloid and Interface Science 108-109*, 303-318.

Tagne J.B., Kakumanu S. and Nicolosi R.J. (2008). Nanoemulsion preparations of the anticancer drug dacarbazine significantly increase its efficacy in a xenograft mouse melanoma model. *Molecular Pharmaceutics 5*, 1055-63.

Tang, S.Y., Shridharan, P. and Sivakumar, M. (2013). Impact of process parameters in the generation of novel aspirin nanoemulsions -Comparative studies between ultrasound cavitation and microfluidizer. *Ultrasonics Sonochemistry 20*, 485-497.

Tiwari, S.B. and Amiji, M.M. (2006). Improved oral delivery of paclitaxel following administration in nanoemulsion formulations. *Journal of Nanoscience and Nanotechnology 6*, 3215-3221.

Vyas, T.K., Shahiwala, A. and Amiji, M.M. (2008). Improved oral bioavailability and brain transport of Saquinavir upon administration in novel nanoemulsion formulations. *The International Journal of Pharmaceutics 347*, 93-101.

Wang, L., Dong. J., Chen, J., Eastoe, J. and Li, X. (2009). Design and optimization of a new selfnanoemulsifying drug delivery system. *Journal of Colloid and Interface Science 330*, 443-448.

Yogeeswari, P. and Sriram, D. (2005). Betulinic acid and its derivatives: a review on their biological properties. *Current Medicinal Chemistry 12*, 657-666.

Yuan, Y., Gao, Y., Mao, L., Zhao, J. (2008). Optimization of conditions for the preparation of β-carotene nanoemulsions using response surface methodology. *Food Chemistry 107*, 1300-1306

Zhao, Y., Wang, C.G., Chowb, A.H.L., Ren, K., Gong, T., Zhang, Z.R. (2010). Selfnanoemulsifying drug delivery system (SNEDDS) for oral delivery of zedoary essential oil: Formulation and bioavailability studies. *Int. J. Pharm. 383*, 170-177.

Zuco, V., Supino, R., Righetti, S.C., Cleris, L., Marchesi, E., Gambacorti, P., and Formelli, F. (2002). Selective cytotoxicity of betulinic acid on tumor cell lines, but not on normal cells. *Cancer Letters 175*, 17-25.