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Self-microemulsifying system of an ethanolic extract of *Heliopsis longipes* root for enhanced solubility and release of affinin

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Abstract

Self-microemulsifying or self-nanoemulsifying drug delivery systems (SMEDDS/SNEDDS) are well known to improve the dissolution and increase the oral bioavailability of hydrophobic drugs, including herbal extracts. Organic extracts of *Heliopsis longipes* root and affinin, its main component, induce a vasodilator effect; however, they are poorly water soluble and therefore are difficult to administer and dose by the oral route. This research aimed to develop, through pseudo-ternary phase diagrams, a self-microemulsifying system prepared from an ethanolic extract of *H. longipes* root (HL-SMDS). In addition, the optimized lipid-based formulation was characterized and its in vitro gastrointestinal simulated dissolution was determined. The formulation composed of Transcutol, 55% (solubilizer); Tween80/PG, 10% (surfactant/co-solvent); Labrasol, 35% (surfactant); and the herbal extract was selected as optimal and identified as a SMEDDS, since when coming into contact with water, it forms a micro-emulsion with droplet sizes less than 100 nm. The stability tests showed that HL-SMDS remained stable over time under extreme conditions. Furthermore, the amount of affinin released from HL-SMDS at pH 1 and 6.8 was higher than that of the ethanolic extract from *H. longipes* root. These results indicate that HL-SMDS is a novel alternative to improve the aqueous solubility and therefore the oral bioavailability of the ethanolic extract of *H. longipes* root.

Keywords Self-microemulsifying drug delivery systems (SMEDDS), *Heliopsis longipes*, Affinin, Spilanthol, Herbal medicines

Introduction

Medicinal plants have been considered over the years as the origin or starting point of the development of medicines, since they have contributed to the discovery of new bioactive compounds and the obtaining of herbal medicines. In addition, plants used in traditional medical systems are more accessible to the population, specifically those with fewer resources and poor medical services (Acosta-Recalde et al. 2018; World Health Organization (WHO) 2019). However, many of the compounds or extracts obtained from medicinal plants are poorly water-soluble, which hinders their subsequent formulation in pharmaceutical forms that would allow their administration, dosage, and bioavailability (Rohini et al. 2021; Chairuk et al. 2020).

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The oral bioavailability of a drug depends on its solubility and dissolution rate in the gastrointestinal (GI) fluid. Therefore, these parameters are important factors to consider when developing drug delivery systems. In order to enhance the solubility and dissolution rate of poorly water-soluble drugs (BCS II and IV class), new formulation strategies have been proposed to improve the bioavailability of these molecules, including micronization or nanosizing, salt formation, complexation with cyclodextrins, co-solvent and surfactant-based solubilization, amorphous solid dispersions, and lipid formulations (micro- and nano-emulsions, and other lipid-based nanostructures) (Porat and Dahan 2018; Alshamrani et al. 2022; Pouton 2006).

Self-emulsifying drug delivery systems (SEDDS) are lipid-based formulations (LBFs) that emulsify (o/w) in aqueous media and generate nano-droplets, when subjected to a gentle agitation (Pouton 1997; Anton and Vandamme 2011), simulating what happens with the peristaltic movements of the gastrointestinal tract (Pouton 1997; Shah and Agrawal 2020; Oliveira and Bruschi 2022). Ideally, SEDDS are composed of isotropic mixtures of natural or synthetic oils with lipophilic surfactants (HLB: 8–12) and hydrophobic co-solvents/co-surfactants (Pouton 2006; Pouton and Porter 2008). On the other hand, the use of hydrophilic surfactants (HLB > 12), hydrophilic co-solvents, and co-surfactants would allow obtaining self-microemulsifying drug delivery systems (SMEDDS) and self-nanoemulsifying drug delivery systems (SNEDDS). The advantages of SEDDS, SMEDDS, and SNEDDS have increased their use in the last decades as an alternative to solubilize and improve the dissolution rate and bioavailability of hydrophobic drugs (Pavoni et al. 2020; Tan et al. 2021).

The composition and properties of SEDDS, SMEDDS, and SNEDDS are summarized in the Lipid Formulation Classification System (LFCS), which categorizes lipid formulations into four groups (types I, II, IIIA, IIIB, and IV), classified according to their formulation components, hydrophobicity, dispersibility, and digestability (Pouton and Porter 2008; Niederquell and Kuentz 2013; Williams et al. 2014).

Currently, some pharmaceutical journals describe SEDDS as nano-emulsions or micro-emulsions without any distinction, which generates confusion among the scientific community, despite the fact that both systems have different physical and physicochemical properties (Pavoni et al. 2020; McClements 2012). Micro-emulsions (MEs) are isotropic dispersions that are spontaneously formed when oils, surfactants, and optionally one or more co-solvents come into contact with water. They exist in a thermodynamic equilibrium that depends on the composition, temperature, and atmospheric pressure (Anton and Vandamme 2011; Pavoni et al. 2020; McClements 2012).

As previously mentioned, one of the main challenges in the formulation of new drugs is their low solubility in water. In fact, it has been estimated that around 80% of all new drug candidates are poorly soluble in water (Acharya et al. 2011; Quirino-Barreda et al. 2017; Shanley 2018), which is the case of the organic extracts obtained from *Heliopsis longipes* root and affinin. This species, endemic to Mexico, is employed in Mexican Traditional Medicine for the treatment of some illnesses, including toothache, gingival disease, and muscle pain (Déciga-Campos et al. 2012; Mata et al. 2019; Cariño-Cortés et al. 2010; Cilia-López et al. 2010). The major components found in *H. longipes* root are alkamides, mainly (2E, 6Z, 8E) -N-isobutyl-2,6,8-decatrienamide also known as affinin or spilanthol (Willig et al. 2019). Both the extracts prepared from *H. longipes* root, as well as affinin in its pure state, have shown different biological and pharmacological activities (Althaus et al. 2017; Barbosa et al. 2016; Rosa-Lugo et al. 2017). In addition, our research group demonstrated that affinin, and the dichloromethane and ethanolic extracts from *H. longipes* root induce a concentration-dependent vasodilator effect (Castro-Ruiz et al. 2017). Therefore, we hypothesized that this plant's roots could be used to develop a useful herbal medicine to treat high blood pressure.

Considering that the organic extracts obtained from *H. longipes* root are lipophilic, it was necessary to find suitable formulations that would allow their administration in animal or human models and for developing herbal medicines. Thus, the present work aimed to develop, through pseudo-ternary phase diagrams, a self-microemulsifying system prepared from an ethanolic extract of *H. longipes* root. The optimized lipid-based formulation was characterized, and its in vitro gastrointestinal simulated dissolution was assessed.

Materials and methods

Materials

Reagents and solvents (absolute ethanol, acetonitrile, acetic acid, hydrochloric acid, monobasic potassium phosphate, sodium hydroxide, carboxymethylcellulose) used in the present investigation (A.R. and HPLC grade) were obtained from JT Baker® (Phillipsburg, NJ, USA). Double-distilled water was used throughout the work. Lyontec® Chemicals, S. de R. L. de C.V. (Mexico), donated the lipid excipients (Transcutol® HP, Labrafil®, and Labrasol®) from Gattefossé® (USA) which used to obtain the SEDDS formulations, and Tween® 80, Propylene glycol®, and affinin standards were acquired from ChromaDex® Standards (USA).

Preparation of the ethanolic extract from *H. longipes* root

One kilogram of the dried and ground root was subjected to an extraction process by maceration with absolute ethanol (1:10 plant material: solvent) at room temperature for 1 week. Thereafter, the plant material was filtered and this process was repeated twice using a new solvent. The filtrates were combined, and the solvent was removed with a rotary evaporator (Heidolph® VV 2000, Germany).

Validation of the analytical method for detecting and quantifying affinin

The validation of the analytical method used to determine and quantify affinin was carried out based on the criteria established by the Validation Guide of Analytical Methods edited by the National College of Pharmaceutical Chemical Biologists of Mexico, AC (CNQFB 2012). The acceptance criteria for suitability, linearity, accuracy, repeatability, limits of detection, and quantification agree with what was previously reported by other authors (CNQFB 2012; Bae et al. 2010; Raposo and Ibelli-Bianco 2020).

Validation of the analysis system

Accuracy The system's accuracy was determined by analyzing 6 replicates of the same affinin solution (45 µg/mL). The areas of the peaks were obtained, as well as the average ($\bar{y}$), the standard deviation, and the coefficient of variation (CV).

Suitability A suitable solution was prepared (2 mg/mL of the extract was maintained for 7 days at 40°C). Thereafter, a solution at a concentration of 45 µg/ml was prepared and injected 5 times. Considering the area under the curve of the 5 injections, the CV was calculated. The capacity factor (K'), resolution (R), tailing factor (T), and the number of theoretical plates (N) were determined for each injection.

Linearity To determine the linearity of the system, 6 replicates of the calibration curve (15, 30, 45, 60, 75, 90 µg/mL) were injected. Subsequently, the coefficient of determination (r^2) and the confidence interval for the slope (CI (β_1)) were obtained.

Validation of the analysis method

Accuracy and repeatability Six spiked samples of affinin, whose concentrations fell within the calibration curve (15, 20, 25, 30, 45, and 50 µg/mL), were prepared. Then, the difference in the areas was calculated to determine the quantity of affinin that was recovered. A graph of the amount added vs. the amount recovered was constructed, the recovery percentage was calculated, and the CV and IC (µ) were determined.

Linearity To evaluate the linearity of the method, 3 different concentrations of affinin were prepared and added to an extract solution. Three levels were selected as follows: 15, 30, and 45 µg/mL; each level was evaluated in triplicate. A graph of the amount added vs. the amount recovered was constructed. Subsequently, r^2, IC (β_1), IC (β_0), and the recovery percentage were calculated, and from these values, the CV and IC (µ) were determined.

Intermediate precision A sample of the analyte (60 µg/mL) was prepared, 3 injections were performed by two different analysts on two different days and the CV was determined.

Detection and quantification limits Three different concentrations of affinin, 15, 30, and 45 µg/mL were prepared, and the analytical response was measured.

Affinin quantification

Affinin quantification was carried out on a WATERS® 600E HPLC, with a diode array detector (WATERS 2998), with a ZORBAX® ECLIPSE XDB-C8 column (4.5×150 mm, 5-µm particle size and 120 Å pore size); an isocratic system was used, with a mobile phase of acetonitrile: 1% acetic acid in water, in a 1:1 ratio, with a flow of 1 mL/min. The injection volume was 20 µL, and the analysis time was 9 min.

To determine the affinin content, a calibration curve was obtained using an affinin standard in a range of 15 to 90 µg/mL (15, 30, 45, 60, and 90 µg/mL). To prepare the sample, 2 mg of the ethanolic extract was weighed, dissolved in 1 mL of absolute HPLC-grade ethanol, sonicated for 15 min, and filtered through nylon membranes with a 0.45-µm pore size. Subsequently, an aliquot of 100 µL was taken and completed to 1 mL with mobile phase; the sample was injected in triplicate.

Characterization of the quantified ethanolic extract of *H. longipes* root

Organoleptic properties

The organoleptic properties of the ethanolic extract obtained from *H. longipes* root, such as color, smell, and taste, were evaluated.

Spectrophotometric determination of color The spectrophotometric indicators of color (SIC) of the ethanolic extract of *H. longipes* root and the self-emulsifying samples were determined through a portable

spectrophotometer (Konica Minolta® CM-600d, Japan) using an illuminant D65, a viewing angle of 10°, and the CIELab space for obtaining L* (lightness), Hue h°, and C* (saturation) values. The samples were analyzed in triplicate, and the standard error of the mean (SEM) was calculated.

Determination of the refractive index (RI)

The refractive index (RI) of the ethanolic extract of *H. longipes* root was determined by dissolving 400 mg of extract in 1 mL of absolute ethanol and subsequently measured with a digital refractometer (HI96801, HANNA® instruments) at 25 °C; zero calibration was performed with distilled water, the sample was measured in triplicate, and the SEM was calculated.

Determination of water solubility at 25 ℃ and 37 ℃

To carry out this test, 50 mg of the extract was taken and added to 100 mL of distilled water in a container with a lid. Subsequently, they were kept under agitation and controlled temperature for 24 h. Next, 1 mL of the sample was taken and filtered, and the concentration of affinin was determined by HPLC; after 48 h, the procedure was repeated. This assay was performed in triplicate for each analysis temperature (OECD 1995).

Microbiological analysis

For the microbiological analysis, 5 g of the ethanolic extract obtained from *H. longipes* root was sent to the Clinical Services and Molecular Diagnostics Unit of the Faculty of Chemistry of the Autonomous University of Querétaro. The tests considered in the Mexican Herbal Pharmacopoeia (FHEUM 3.0) were carried out: aerobic mesophilic bacteria, fungi, yeasts, *Escherichia coli*, and *Salmonella* spp. The results were analyzed according to the limits established in the Mexican pharmacopoeia for plant material or products containing extracts with or without additives and materials for internal use (FHEUM 2022). The determinations were made according to the Official Mexican Standards (NOM) (NOM-092-SSA1-1994 1995; NOM-111-SSA1-1994 1995; NOM-210-SSA1-2014 2015).

Obtaining a self-microemulsifying system

Analyses of solubility of the ethanolic extract of *H. longipes* root in water and in different excipients were carried out. Four different systems composed of three excipients were proposed (Table 1), and thereafter, ten formulations were prepared for each system (Table 2) to construct pseudo-ternary phase diagrams (Morales Florido 2018). Each system's ability to dissolve the extract was evaluated by observing the absence of sediment, turbidity, or opalescence.

Table 1 Composition of the four SEDDS proposed to find the optimal system to dissolve the ethanolic extract from *H. longipes* root

System	Excipient 1	Excipient 2	Excipient 3
A	Transcutol® HP	Labrasol®	Labrafil®
B	Transcutol® HP	Tween® 80	Labrafil®
C	Transcutol® HP	Propylene glycol (PG)	Tween® 80
D	Transcutol® HP	Tween 80®/PG (1:1)	Labrasol®

Table 2 Formulations prepared for each of the A, B, C, and D systems

Formulation	Excipient 1	Excipient 2	Excipient 3
1	0.80	0.10	0.10
2	0.65	0.25	0.10
3	0.65	0.10	0.25
4	0.50	0.35	0.15
5	0.50	0.35	0.25
6	0.50	0.15	0.35
7	0.25	0.50	0.25
8	0.25	0.25	0.50
9	0.10	0.80	0.10
10	0.10	0.10	0.8

Thereafter, water was added to each one of the formulations proposed and the resulting systems were visually observed. The presence of a transparent liquid was indicative of a micro-emulsion.

Selecting the optimal self-microemulsifying system

For obtaining the optimal SMEDDS containing the ethanolic extract of *H. longipes* root (HL-SMDS), a simplex design of increased lattice was carried out, using the system that fulfilled optimal physical properties regarding appearance, particle size, and solubilizing capacity of the extract.

Characterization of the HL-SMDS

Determination of organoleptic properties

The organoleptic properties, such as color, smell, and taste of the HL-SMDS, were determined, considering that changes in these characteristics might indicate instability. Color determination was performed as described in Spectrophotometric determination of color section .

Determination of droplet size and polydispersity index

The mean droplet size and the corresponding polydispersity index (PDI) were determined by dynamic light scattering using a Litesizer® 500 (AntonPaar® Instruments, Mexico) at 25℃. Samples were analyzed in triplicate, the

data were reported using the DTS® (nano) software, and the results were reported as the mean and SEM of at least ten measurements on each sample.

Determination of the Zeta potential

For measurement of the Z potential, a Zetameter Zetaprobe 300 (Malvern® Panalytical, Ltd., Mexico) was used at 25℃. The samples were analyzed in triplicate. The results were expressed as mean and SEM of at least ten sample measurements (Morales Florido 2018).

Determination of morphology

The morphology was observed using a scanning electron microscope (Hitachi®, model SU8230, cold cathode field emission), STEM mode, and an acceleration voltage of 30 kV and W.D 8 mm, and the sample was prepared on a copper grid for TEM. HL-SMDS (50 µL, 7 mg/mL) was diluted in water (1:20 dilution), and a sample drop was placed on the copper grid.

Determination of transmittance

The transmittance was determined using a UV/VIS spectrophotometer (Model: VE-5100UV, Scientific Company Vela Quin S.A. de C.V.), measurements were made in triplicate at 650 nm, and the SEM was calculated.

Determination of the refractive index

The refractive index was determined using a digital refractometer (HANNA® HI96801, Instruments) at 25℃. The zero calibration was performed with distilled water. The sample was measured in triplicate, and the SEM was calculated.

Determination of pH

The pH was determined using a pH meter (Navi® pH meter F-51, HORIBA®, Ltd., Kyoto, Japan). The electrode of the pH meter was immersed in the sample, and the pH was recorded after reaching equilibrium. The samples were analyzed in triplicate, and the SEM was calculated.

Physical and chemical stability at different pH values

To determine the physical and chemical stability of the HL-SMDS, two media that simulate its passage through the gastrointestinal tract (GIT) were prepared. For this, a 0.1 N hydrochloric acid solution was obtained, starting from 8.5 mL of 37% fuming hydrochloric acid, and it was completed to 1 L with deionized water; finally, the pH was adjusted to 1. To obtain the simulated intestinal medium, 6.8 g of monobasic potassium phosphate was dissolved in 250 mL of deionized water, then 77 mL of 0.2 N sodium hydroxide was added, and it was completed with deionized water to 1 L. Subsequently, the pH was adjusted to 6.8 with sodium hydroxide.

To carry out the test, 3 mL of HL-SMDS was taken and placed in 30 mL of the solution, stirred at 200 rpm, and a constant temperature of 25℃. Samples were taken at 0.5, 1, 2, and 4 h and filtered, and the affinin content was quantified by HPLC. The droplet size was measured using the same analysis conditions at each time through a Litesizer 500 (Litesizer 500, Anton Paar Instruments, Austria) (Morales Florido 2018).

Accelerated stability study

Stability studies of HL-SMDS were performed. Since the HL-SMDS is considered a simile of a drug for new health registration, the conditions established in NOM-073-SSA1-2015 (stability of drugs, medications, and herbal remedies) were selected.

HL-SMDS was kept for 6 months in a stability chamber (Lumistell® stability chamber, model ITH-75, Mexico) at a temperature of 40℃ ± 2℃ and 75% ± 5% relative humidity (R.H.). Measurements were made at 0, 3, and 6 months, and the parameters measured were color, smell, pH, weight loss, and identity (initial and final) (NOM-073-SSA1 2015).

Affinin release kinetics

For this study, 3 mL of HL-SMDS and/or ethanolic extract of *H. longipes* root dissolved in carboxymethylcellulose was taken and placed inside bags made with a 12 to 14 kDa cellulose membrane (Spectra/Por®), which were sealed on both sides and tied to the dissolver stem (Hanson Research® SR8 Plus Dissolution test station), which was kept at 37℃, with an agitation of 50 rpm.

Five hundred milliliters of simulated acid or intestinal medium was added to the dissolving vessels; after the system was assembled, 3 mL samples were taken without medium replacement at 5 min, 10 min, 15 min, and 30 min and 1, 2, 3, 4, and 8 h. The affinin content in each sample was quantified by HPLC, with the method previously described (Morales Florido 2018).

Statistical analysis

Data were reported as the standard error of the mean ± SEM, analyzed using a one-way ANOVA test, and differences between them were assessed using Tukey's test. The differences between the means were considered significant when $p < 0.05$. Data analysis was performed with the GraphPad Prism 9 program.

Results and discussion

Validation of the analytical method

Validation of the analytical method is a fundamental element in quality systems, since it provides evidence that testing laboratories meet the requirements for a specific analytical determination, which guarantees

quality, reliability, reproducibility, and safety of the results (Raposo and Ibelli-Bianco 2020).

According to our results, both the system and the method for affinin quantification met the criteria established in the Validation Guide of Analytical Methods edited by the National College of Pharmaceutical Chemical Biologists of Mexico, AC. The results are listed in Table 3.

Affinin quantification

The previously validated method was used for affinin quantification in the ethanolic extract of *H. longipes* root. A calibration curve was obtained using 6 concentrations of the affinin standard, with $r^2 > 0.98$.

The ethanolic extract batch of *H. longipes* root used in this study had an average affinin content of 342 ± 0.3 µg/mg of extract. This result may vary depending on the plant material used to obtain the extract, due to various factors, such as the harvesting time and area, as well as the growth and maturity stage of the plant (Li et al. December 2019; Mahajan et al. 2020).

Characterization of the quantified ethanolic extract

The ethanolic extract from *H. longipes* root exhibited a refractive index of 1.41 ± 0.002 and an aromatic odor. The color of the extract, evaluated using CIE L*a*b color space, had values of L* = 25.3 (low lightness), Hue h° = 37.1° (orange-red color area), and chroma C* = 4.2 (low saturation). In addition, its consistency was viscous, its flavor was intense and unpleasant, and it produced a tingling sensation that lasted several minutes.

Regarding the miscibility test, we found that the system was already in equilibrium after 48 h, since the affinin concentration did not vary by more than 15%, at either of the two test temperatures, according to what is established in the corresponding OECD guide (OECD 1995). Approximately 80 µg of affinin/mg of extract was solubilized in water, representing 23% of dissolved affinin, considering the results obtained when this compound was quantified in the extract (342 µg of affinin/mg of extract). The low aqueous solubility of affinin, our chemical marker, evidenced the need to apply new solubilization techniques.

Medicinal plants used as raw materials usually have a large number of aerobic and anaerobic bacteria and filamentous fungi from the soil. The most common microorganisms found in pharmaceutical products are *Klebsiella* spp., *Escherichia coli*, *Salmonella* spp., and *Pseudomonas aeruginosa*, among others (FHEUM 2022; Eissa 2017; Myemba et al. 2022). Therefore, in this work, a microbiological analysis of the ethanolic extract of *H. longipes* root was carried out. The results obtained were within the limits established in the FHEUM 3.0 for plant material or products containing extracts with or without additives and materials for internal use (Table 4).

Obtaining the self-microemulsifying system

The four systems (A, B, C, D) described in the methodology section were prepared using the excipient mixtures shown in Table 1. The excipients were perfectly mixed in all formulations, and no turbidity was observed. However, when adding water, A and B formed emulsions with a milky-white color. So we continued working with C

Table 3 Parameters evaluated for the validation of the analytical method used in quantifying affinin

Parameters	Results	Criteria of acceptance[a]
System accuracy	CV = 1.2%	CV ≤ 1.5%
System suitability	CV = 0.8%; *K* = 4.5; *R* = 3.2; *T* = 0.9; *N* = 6529	CV ≤ 2%, K' > 2; *R* > 2; *T* < 2; *N* > 2000 (each injection)
System linearity	$r^2 = 0.99$ IC(β_1) = 128,382–14,1873	$r^2 \geq 0.98$ IC(β_1) must not include zero
Accuracy and repeatability of the method	CV = 1.3% IC(µ) = 99.7–103.6% Recovery average = 101.6%	CV of the recovery percentage < 2% IC(µ) must include 100% or that the arithmetic average of the recovery percentage is included in the interval: 98–102%
Method Linearity	$r^2 = 0.99$ IC(β_1) = 0.7–1.2 IC(β_0) = -5.7–10.9 $CV_{y/x}$ = 1.4%	$r^2 > 0.98$ IC(β_1) must include the unit IC(β_0) must include zero $CV_{y/x}$ of the recovery percentage < 2%
Intermediate precision of the method	CV = 1.2%	CV ≤ 2%
Detection and quantification limits	LD = 1.7 µg/mL LC = 5.3 µg/mL $r^2 = 0.99$ IC(β_1) = 17,7366–91,883	$r^2 \geq 0.98$ IC(β_1) must not include zero

[a] CNQFB, 2012

Table 4 Results of the microbiological analysis of the ethanolic extract obtained from *H. longipes* root

Microorganism	Quantified *H. longipes* root ethanolic extract	Acceptance criteria[b]	
		Plant material or products containing extracts with or without additives	Materials for internal use
Aerobic bacteria	< 10 UFC/g	Maximum 5×1^5 UFC/g	Maximum 1^5 UFC/g
Mushrooms	< 10 UFC/g	Maximum 5×1^4 UFC/g	Maximum 1^3 UFC/g
Yeasts	< 10 UFC/g	Maximum 5×1^4 UFC/g	Maximum 1^3 UFC/g
Escherichia coli	< 3 MPN/g [a]	None	None
Salmonella spp (1 g)	None	None	None

[a] MPN most probable number

[b] FHEUM, 2022

Table 5 Formulations prepared with the excipients of system D obtained from an augmented lattice simplex experimental design

Formulation D	Transcutol®	Tween® 80/ PG(1:1)	Labrasol®	Droplet size (nm)
1	0.60	0.10	0.30	69 ± 2.5
2	0.57	0.12	0.31	78 ± 4.4
3[a]	0.55	0.10	0.35	82 ± 2.6
4	0.55	0.15	0.30	94 ± 8.0
5	0.54	0.13	0.33	73 ± 4.5
6	0.52	0.12	0.36	76 ± 13.1
7	0.52	0.16	0.32	85 ± 7.8
8	0.50	0.10	0.40	78 ± 4.7
9	0.50	0.15	0.35	97 ± 5.6
10	0.50	0.20	0.30	94 ± 9.0

[a] HL-SMDS optimal formultion

and D, which showed characteristics of micro-emulsions since they were transparent solutions. This possibility was later confirmed when measuring their droplet size, which was between 50 and 200 nm.

During the development of drug delivery systems, including herbal medicines, one of the main limitations of intragastric administration to preclinical animal models is that substances must have low viscosity due to the difficulty of passing through the intragastric cannula. System C had high viscosity, since it contained a high proportion of Tween® 80. So we decided to continue working with system D, which was a low-viscosity micro-emulsion.

Optimization of system D was carried out using an augmented lattice simplex design, selecting 10 new formulations (Table 5) whose droplet size, in the absence of extract, was between 50 and 200 nm, thus corroborating that system D was a micro-emulsion. Then, 30 mg of the quantified ethanolic extract of *H. longipes* root was added to each of the 10 formulations, considering that in previous studies our research group observed that this amount of the ethanolic extract of *H. longipes* root, allows a dose of 150 mg of affinin/kg body weight in rats. It is important to mention that this dose of affinin elicits significant pharmacological effects when orally administered. The particle size was determined by adding 700 µL of water, simulating what would happen in the rat stomach, considering that gastrointestinal fluids are aqueous, presenting droplet size values below 100 nm (with < 100 nm but > 50-nm droplet size) (Fig. 1).

Figure 1 shows contour and three-dimensional response surface plots of the ten D-system formulations loaded with 30 mg of the extract of *H. longipes* root. These plots were employed to visualize the relationship between the droplet size and the proportions of the excipients used. In the case of this lipid system, it was observed that as the percentage of Transcutol® and Labrasol® increases, the droplet size decreases, in such a way that the use of Transcutol® and Labrasol®, in intermediate percentages, and a small proportion of Tween® 80/PG allows obtaining smaller droplet size.

The formulation consisting of Transcutol®, 55%; Tween®80/PG, 10%; Labrasol®, 35%; and 30 mg of the ethanolic extract of *H. longipes* root (HL-SMDS) was selected as optimal, considering its viscosity, the solubility of the extract, and the average droplet size (82 ± 2.6 nm). HL-SMDS corresponds to a IIIB system of the LBF classification of Pouton (2006), characterized by containing water-soluble components with low oil content (Pouton 2006).

Characterization of the self-microemulsifying system HL-SMDS

The color of HL-SMDS, which was evaluated using the CIE L*a*b color space, had values of L* = 34.6 (low luminosity), Hue h° = 65° (yellow–red color area), and a chroma C* = 19.1 (low saturation). The HL-SMDS retained the original flavor of the extract and the tingling

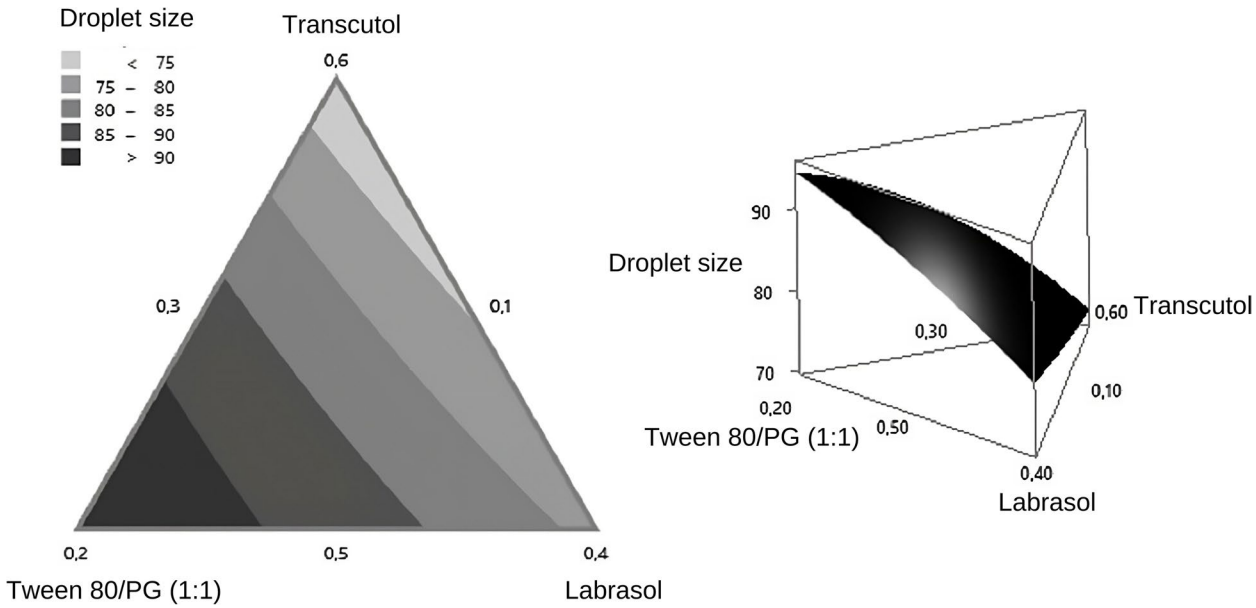

Fig. 1 Contour and three-dimensional response surface plots of the ten D-system formulations loaded with the extract of *H. longipes* root

sensation with an additional slight oil taste. Its odor was characteristic of the extract and the excipients.

The droplet size of HL-SMDS was 82 ± 0.8 nm with a PDI value of 0.3 ± 0.6 and a zeta potential of − 1.3 ± 0.0. These values were in the range described for SMEDDS (Feeney et al. 2016; Wang et al. 2020). The morphology of HL-SMDS was observed by STEM, and Fig. 2 shows the presence of spherical droplets that did not aggregate. Furthermore, the droplet size of HL-SMDS obtained from the STEM images was approximately 80 nm, similar to that measured by dynamic light scattering.

The refractive index of HL-SMDS was 1.44 ± 0.002, and when adding water (700 µL) to form the micro-emulsion, the refractive index was 1.40 ± 0.002. The pH of HL-SMDS was 6.9 ± 0.0, and its transmittance was 80 ± 0.3%.

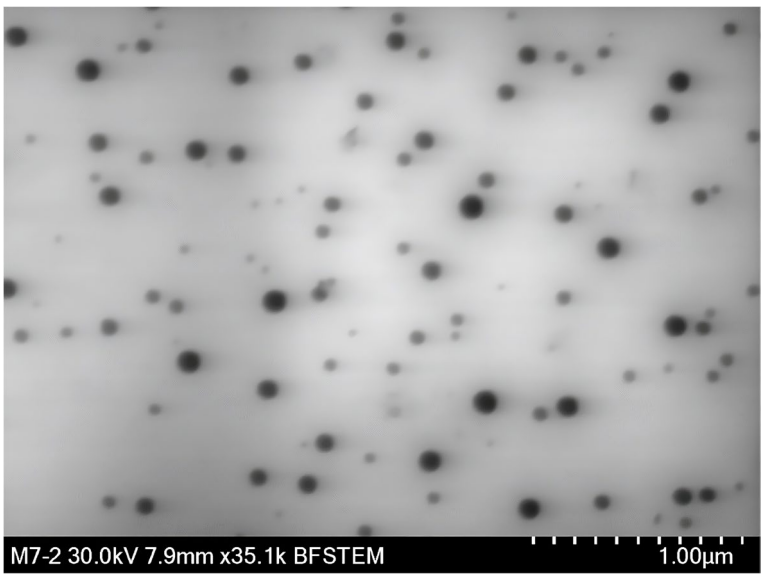

Fig. 2 STEM image of the micro-emulsion from HL-SMDS after 20-fold dilution with distilled water (1:20 dilution). The scale bar represents 1.00 µm

Stability at different pH values

Characterization of the chemical and physical stability of SMEDDS and SNEDDS is important, especially when administered orally (Feeney et al. 2016; Wang et al. 2020). Therefore, it was relevant to verify the stability of HL-SMDS at different pHs, simulating its passage through the gastrointestinal system, by determining both the chemical stability of the active principle (affinin) and the droplet size when HL-SMDS came into contact with water (Chaudhari and Akamanchi 2019).

To carry out the stability determinations, HL-SMDS was diluted in simulated gastric (HCL solution of pH 1) and intestinal (NaOH solution of pH 6.8) fluids, and samples of both solutions were taken at different times. However, as shown in Fig. 3A, the pH did not have a significant effect on affinin (the active principle and chemical marker) concentration over time, thus showing the chemical stability of this compound.

As shown in Fig. 3B, the droplet size values of HL-SMDS in both simulated gastrointestinal media increased as time passed. This physical instability behavior might be attributed to the Ostwald ripening phenomenon, which is explained according to the Laplace equation, where the internal pressure of droplets differs from the pressure of the surrounding liquid in a magnitude that is directly proportional to the interfacial tension of the drop and inversely proportional to its radius. Consequently, when two oil drops of different sizes come into contact in emulsions, micro- or nano-emulsions, the bigger drops "devour" smaller drops (the fluid moves from higher to lower pressure) (Liu and Hu 2020; Park et al. 2020).

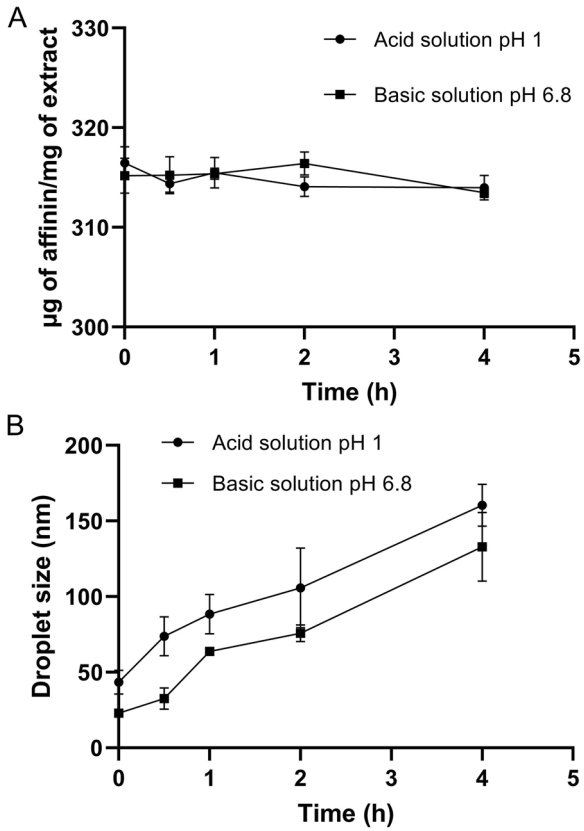

Fig. 3 **A** Concentration of affinin in the ethanolic extract of *H. longipes* root. **B** Droplet size of HLSMDS. Both **A** and **B**, in two solutions at different pH values through time, at a constant temperature of 25 °C and stirring at 200 rpm. Data expressed as mean ± SEM ($n = 3$)

It is important to mention that according to our results, the droplet size of the lipid system did not exceed 160 nm during the 4 h that the stability test lasted. This means that during this period of time, the system remained as a micro-emulsion. In a previous study, the pharmacokinetic parameters of affinin were determined in rats and its calculated half-life was 1.13 h. It was also found that 4 h after oral administration of this compound, more than 70% had been eliminated from the rat serum, and after 6 h, no affinin concentration was found at all (Veryser et al. 2016). These findings indicate that the greatest absorption of affinin occurs in a period of time less than 4 h, a period of time in which our system remains as a micro-emulsion. Thus, the fact that the droplet size of HL-SMDS increases as a function of time has no significance in the bioavailability of the main active principle contained in the ethanolic extract of *H. longipes* root.

Accelerated stability study

According to the NOM, the stability studies of drugs, medicines, and herbal remedies represent the scientific evidence supporting their useful life period. In Mexico, the climatic zone recognized to carry out these stability studies is climatic zone II (subtropical, with possible high humidity), according to the classification of the World Health Organization indicated in point 12.23 of this standard (NOM-073-SSA1 2015).

In this work, the temperature was kept at 40℃ ± 2 ℃ and a relative humidity of 75% ± 5%. The results obtained from this analysis (Table 6) showed that the three replicates of HL-SMDS remained stable during all the time the test lasted. The concentration of affinin did not vary, and the weight loss did not exceed 5%, despite the fact that HL-SMDS was not in the final container, as suggested by the NOM. Neither the color nor the odor of HL-SMDS changed, and the pH remained stable. Specifically, the color of HL-SMDS had values of $L^* = 37.4$ (low luminosity), Hue $h° = 74.2°$ (yellow–red color area), and chroma $C^* = 23.8$ (low saturation). It is evident that long-term studies must be carried out on HL-SMDS, considering its final dosage form.

Table 6 Stability measurements of HL-SMDS

Time (months)	Color	Odor	pH	Weightloss (%)	Quantification (µg affinin/mg of extract)
0	Yellow–red color area	Aromatic	6.9 ± 1	-	254.3 ± 2
3	Yellow–red color area	Aromatic	7.0 ± 2	0	255.5 ± 1
6	Yellow–red color area	Aromatic	6.9 ± 0	2.1	254.6 ± 3

Affinin release kinetics

The release profile of HL-SMDS and the *H. longipes* ethanolic extract in different pH aqueous media are presented in Fig. 4. The affinin release from HL-SMDS, in both media, was higher than that of the ethanolic extract.

In the pH 1 medium, the cumulative dissolution rate of affinin contained in HL-SMDS reached more than 50% in approximately 15 min. By contrast, the dissolution rate of affinin contained in the ethanolic extract only reached 16%. Regarding the dissolution rate of affinin in the basic solution (pH 6.8), approximately 40% of affinin contained in HL-SMDS was released after 8 h. However, the dissolution rate of affinin contained in the ethanolic extract only reached 20%, evidencing that in both times (15 min and 8 h) and for both media (pH 1 and 6.8), and the dissolution of affinin contained in the extract was half of that obtained for HL-SMDS.

HL-SMDS represents a self-microemulsifying system that, in addition to increasing the solubility and release of affinin, is easy to prepare. Moreover, the chromatographic method that was developed and validated to quantify affinin, which is the chemical marker, is simple and fast. However, one of the disadvantages of this lipid system is the fact that the droplet size tends to increase once it comes into contact with water. Although according to pharmacokinetic studies carried out previously with affinin, during the period of time in which this compound is absorbed (less than 4 h), the lipid system remains as a microemulsion, and it is necessary to carry out in vivo pharmacokinetic and pharmacological studies to verify if indeed, HL-SMDS exerts the desired pharmacological effect and the bioavailability of affinin, the main active compound of *H. longipes* root, is improved.

Conclusions

In order to increase the aqueous solubility and favor the bioavailability of an ethanolic extract of *H. longipes* root, a self-microemulsifying system was developed, using pseudo-ternary phase diagrams. The compositions of the optimal lipid system were Transcutol, 55%; Tween80/PG, 10%; and Labrasol, 35%. The lipid system loaded with the *H. longipes* ethanolic extract (HL-SMDS) consisted of mostly spherical droplets with size of less than 100 nm. HL-SMDS significantly enhanced the solubility of the ethanolic extract of *H. longipes* root and remained stable over time under extreme conditions (40℃ ± 2℃ and

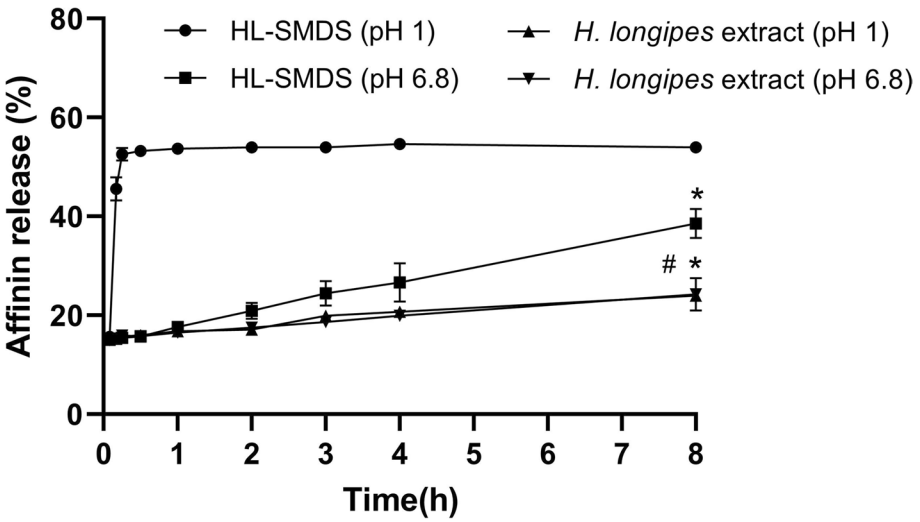

Fig. 4 Release profile of affinin from HL-SMDS and the ethanolic extract of *H. longipes* root in two solutions at different pH through time, at a constant temperature of 37 °C and stirring at 50 rpm. Data expressed as mean ± SEM ($n = 3$), *$p < 0.05$ vs. HL-SMDS (pH 1), #$p < 0.05$ HL-SMDS (pH 6.8) vs. *H. longipes* extract (pH 1 and 6.8)

75% ± 5% relative humidity). Moreover, it significantly enhanced the release of affinin, and the main bioactive compound contained in *H. longipes* root. The results derived from the present investigation indicate that HL-SMDS constitutes a promising lipid-based formulation that would allow the administration and improve the bioavailability of the ethanolic extract of *H. longipes* root, which could used for the development of a useful herbal medicine for the treatment of cardiovascular diseases.

Abbreviations

ANOVA	Analysis of variance
BCS	Biopharmaceutical Classification System
CIE	International Commission on Illumination
CV	Coefficient of variation
FHEUM	Herbal Pharmacopoeia of the United Mexican States
GI	Gastrointestinal
HCl	Hydrochloric acid
HLB	Hydrophilic-lipophilic balance
HL-SMDS	SMEDDS containing the ethanolic extract of *H. longipes* root/optimal formulation
HPLC	High-Performance Liquid Chromatography
IC	Confidence interval
LBFs	Lipid-based formulations
LFCS	Lipid Formulation Classification System
MEs	Micro-emulsions
MPN	Most probable number
NaOH	Sodium hydroxide
NOM	Official Mexican Standard
OECD	Organization for Economic Cooperation and Development
PDI	Polydispersity index
PG	Propylene glycol
RH	Relative humidity
RI	Refractive index
SEDDS	Self-emulsifying drug delivery systems
SEM	Standard error of the mean
SIC	Spectrophotometric indicators of color
SMEDDS	Self-microemulsifying drug delivery systems
SNEDDS	Self-nanoemulsifying drug delivery systems
STEM	Scanning transmission electron microscopy
TEM	Transmission electron microscopy
UFC	Colony-forming unit
UV/VIS	Ultraviolet-visible

Supplementary Information

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Additional file 1. Raw data validation.

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Institutional review board statement

This study was approved by the Bioethics Committee of the Faculty of Chemistry of the Autonomous University of Querétaro, (trade number: CBQ20/116).

Authors' contributions

Conceptualization, D.M-M, A.R-M, and C.T.Q-B; methodology, D.M-M, F.J.L-V, E.M-L, and M.E; validation, C.I-A; formal analysis, D.M-M and E.M-L; investigation, D.M-M, A.R-M, and C.T.Q-B; resources, A.R-M and C.T.Q-B; writing—original draft preparation, D.M-M; writing—review and editing, AR-M, C.T.Q-B, and C.I-A; visualization, D.M-M.; supervision, D.M-M, A.R-M, C.T.Q-B, F.J.L-V, and M.E.; project administration, A.R-M; funding acquisition, D.M-M and A.R-M. All authors have read and agreed to the published version of the manuscript.

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Availability of data and materials

The data that support the findings of this study are available from the corresponding author [A.R-M], upon reasonable request.

Declarations

Competing interests

The authors declare that they have no competing interests.

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