

Lipid Nanocarriers for Oral Delivery of *Serenoa repens* CO₂ Extract: A Study of Microemulsion and Self-Microemulsifying Drug Delivery Systems

Authors

Clizia Guccione, Maria Camilla Bergonzi, Khaled M. Awada, Vieri Piazzini, Anna Rita Bilia

Affiliation

Department of Chemistry, University of Florence,
Sesto Fiorentino (Florence), Italy

Key words

Serenoa repens CO₂ extract, Arecaceae, microemulsions, self-microemulsifying drug delivery systems, oral absorption, PAMPA

received November 29, 2017

revised March 5, 2018

accepted March 6, 2018


Bibliography

DOI <https://doi.org/10.1055/a-0589-0474>

Published online March 15, 2018 | *Planta Med* 2018; 84: 736–742 © Georg Thieme Verlag KG Stuttgart · New York | ISSN 0032-0943

Correspondence

Prof. Dr. Anna Rita Bilia
Department of Chemistry, University of Florence
Via Ugo Schiff 6, 50019 Sesto Fiorentino (FI), Italy
Phone: + 39 05 54 57 37 08
ar.bilia@unifi.it

 Supporting information available online at
<http://www.thieme-connect.de/products>

ABSTRACT

The aim of this study was the development and characterization of lipid nanocarriers using food grade components for oral delivery of *Serenoa repens* CO₂ extract, namely microemulsions (MEs) and self-microemulsifying drug delivery systems (SMEDDSs) to improve the oral absorption. A commercial blend (CB) containing 320 of *S. repens* CO₂ extract plus the aqueous soluble extracts of nettle root and pineapple stem was formulated in two MEs and two SMEDDSs. The optimized ME loaded with the CB (CBM2) had a very low content of water (only 17.3%). The drug delivery systems were characterized by dynamic light scattering, transmission electron microscopy, and high-performance liquid chromatography (HPLC) with a diode-array detector analyses in order to evaluate the size, the homogeneity, the morphology, and the encapsulation efficiency. β -carotene was selected as marker for the quantitative HPLC analysis. Additionally, physical and chemical stabilities were acceptable during 3 wk at 4°C. Stability of these nanocarriers in simulated stomach and intestinal conditions was proved. Finally, the improvement of oral absorption of *S. repens* was studied *in vitro* using parallel artificial membrane permeability assay. An enhancement of oral permeation was found in both CBM2 and CBS2 nanoformulations comparing with the CB and *S. repens* CO₂ extract. The best performance was obtained by the CBM2 nanoformulation (~ 17%) predicting a 30–70% passive oral human absorption *in vivo*.

Introduction

Saw palmetto (*Serenoa repens* [W.Bartram] Small [Arecaceae]; synonym: *Sabal serrulata* [Michx.] Schult.f.) is a small palm tree, native to southeastern North America, particularly Florida [1]. Since the 1990s, saw palmetto has been one of the 10 top-selling herbal medicines in the United States, with a worldwide turnover of about \$700 million per year [2].

In Europe, numerous preparations containing saw palmetto extracts are marketed as herbal medicinal products or food supplements and they are widely used to treat discomforts related to prostatic hyperplasia [3–5]. Saw palmetto berry herbal drug and its extracts obtained by suitable extracting procedures using hydroalcoholic mixtures, supercritical carbon dioxide, or n-hexane

are reported in United States and European pharmacopoeias in dedicated monographs [6, 7].

Recently, the European Medicine Agency has indicated that preparations based on the hexane soft extract (DER 7–11:1; 320 mg daily) can be designated as well-established herbal medicinal products and can be used for the symptomatic treatment of benign prostatic hyperplasia [4].

Moreover, the effectiveness of a special botanical product (Prostamev Plus) based on saw palmetto (320 mg) plus pineapple stem extract (25 mg) and plus nettle root extract (120 mg) has been recently compared to saw palmetto alone in reducing the symptoms of inflammatory prostatitis (bacterial and nonbacterial ones). Prostamev Plus represents a unique combination of botanicals because bromelain present in the pineapple stem extract is

ABBREVIATIONS

BCS	Biopharmaceutics Classification System
CB	commercial blend
CBM1	microemulsion n. 1 loaded with the commercial blend
CBM2	microemulsion n. 2 loaded with the commercial blend
CBS1	self-microemulsifying drug delivery system n. 1 loaded with the commercial blend
CBS2	self-microemulsifying drug delivery system n. 2 loaded with the commercial blend
DER	drug/extract ratio
DLS	dynamic light scattering
HCOOH	formic acid
ME	microemulsion
PAMPA	parallel artificial membrane permeability assay
PDI	polydispersity index
PVDF	Durapore® polyvinylidene fluoride
SGM	simulated gastric medium
SMEDDS	self-microemulsifying drug delivery system
SIM	simulated intestinal medium
TEM	transmission electron microscopy

particularly active in localized inflammation in the presence of edema, while nettle extract is particularly effective in the symptomatic treatment of micturition disorders [8]. Hence, the clinical study confirmed the advantage of using a cotreatment that associates nettle and pineapple to saw palmetto because of the improvement of uroflowmetry values and in the scores of the International Index of Erectile Function-5 and National Institute of Health-Chronic Symptom Index questionnaires. The study reported no side effects in addition to those provided for the antibiotic therapy alone. In conclusion, the groups treated with Prostamev Plus showed an improvement of all the investigated parameters compared to the groups treated with saw palmetto alone, with or without associated antibiotic therapy [9].

Prostamev Plus is marketed in the form of soft gelatin capsules for oral administration containing a coarse suspension due to the mixture of lipid and water-soluble constituents, namely saw palmetto CO₂ extract plus nettle root extract and pineapple stem extract. For instance, the lipid saw palmetto CO₂ extract has a similar behavior of the low soluble drugs, which have high intra- and inter-subject variability and lack of dose proportionality because the absorption rate from the gastrointestinal lumen is controlled by the dissolution. Various approaches can be used to improve the dissolution rate of these drugs and consequently to optimize their bioavailability after oral administration. Among them are MEs, which are optically isotropic and thermodynamically stable liquid preparations with an average structure size below 300 nm. They consist of the oil phase, water, and surfactants and are especially suitable for plant extracts and complex mixtures. Preconcentrates of MEs represent alternative flexible formulations. These are homogeneous liquids, which contain oil, surfactants, and the drug

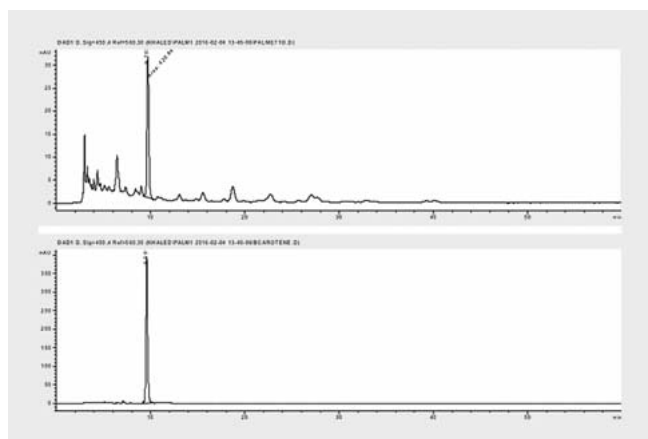
without aqueous phase. They are also known as SMEDDSs. When a SMEDDS is in contact with water or gastrointestinal fluid, it spontaneously forms a ME with gentle agitation. The digestive motility of the stomach and the intestine can provide the agitation necessary for self-emulsification. SMEDDSs form transparent MEs with a droplet size of less than 300 nm and are generally preferred over the regular MEs for oral preparations because SMEDDSs can decrease the volume and it is easy to fill in the soft gelatin capsules.

The aim of the present study is the development of innovative formulations based on the complex mixture of lipophilic and hydrophilic constituents, namely from saw palmetto CO₂ extract plus nettle root extract and pineapple stem extract. Based on the solubility studies of the different extracts, two MEs and two SMEDDSs were developed and fully characterized by DLS, TEM, and HPLC-DAD in order to evaluate the size, the homogeneity, the morphology, and the loading capacity and encapsulation efficiency. The chemical and physical stabilities were also investigated including the stability in simulated gastric fluid, followed by simulated intestinal fluid stability, avoiding precipitation or increase in the globule size as this may affect the product performance *in vivo*. Therefore, the components used in the system should have high solubilization capacity for the drug, ensuring the solubilization of the drug in the resultant dispersion. The *in vitro* permeation studies were assessed by PAMPA to determine the suitability of the developed nanoformulations and compare the permeation of the conventional coarse suspension loaded in soft gelatin capsules.

Results and Discussion

The study started with the investigation of aqueous solubility of the extracts of the CB. Both nettle (*Urtica urens* L. [Urticaceae])/ *Urtica dioica* L. [Urticaceae]) dried extract (70% EtOH) root and pineapple (*Ananas comosus* L. [Bromeliaceae]) stem extract (1850 GDU/g bromelain) were highly soluble in water according to the BCS classification. The doses of nettle (120 mg) and pineapple (25 mg) extracts were soluble in less than 250 mL water. By contrast, saw palmetto CO₂ extract (320 mg) resulted very poor soluble in 250 mL water [10].

The successive step was the selection of a representative marker among the constituents of saw palmetto extract for the HPLC-DAD analysis, being a GC instrument not suitable for the settled assays in order to develop and characterize the innovative drug delivery systems. The saw palmetto extract was solubilized in a mixture of MeOH/dichloromethane (1:1) and it was analyzed using different columns and solvent mixtures. The presence of β -carotene was established on the basis of the characteristic UV spectrum and on the value of the retention time by comparison with β -carotene standard. Accordingly, β -carotene was selected as a suitable marker of both saw palmetto commercial extract and the CB because no interferences were found with the other constituents of the preparation. An HPLC-DAD method previously developed and validated in our lab for the analysis of goji berry and its extracts [11] was used for the quantification of β -carotene, which resulted ~ 0.18 mg/mL of extract. The chromatographic profile of the commercial extract at 450 nm is reported in



► **Fig. 1** Chromatographic profile of the *Serenoa repens* extract (top) and chromatogram of β -carotene standard reference (bottom) at 450 nm.

► **Fig. 1.** Accordingly, the HPLC-DAD method was used to evaluate the solubility of the saw palmetto extract in various oils and surfactants to select the appropriate components of the MEs and SMEDDSs (► **Table 1**). Soy oil was selected as the oily phase, while Tween 80 and Kolliphor EL were selected as surfactants.

Accordingly, two MEs were obtained using a water titration method. Each sample was visually checked after equilibrium and determined as being a clear ME, emulsion, or gel. Their composition is reported in ► **Table 2**. The extracts of CB were added to the lipophilic phase and the corresponding loaded MEs (CBM1 and CBM2) were obtained using deionized water added drop by drop, under gentle agitation, to the oily mixture. CBM1 and CBM2 composition is reported in ► **Table 2**. CBM2 (**Fig. 1S**, Supporting Information) had a lower content of water when compared with CBM1 (30.2 vs. 17.3%). A pseudoternary diagram of CBM2 is available as Supporting Information

Two SMEDDSs loaded with the CB were also developed, namely CBS1 and CBS2. Their composition is reported in ► **Table 2**.

All developed preparations (M1, M2, CBM1, CBM2, CBS1, and CBS2) were characterized by DLS in terms of size, polydispersity and ζ -potential. All the formulations were homogeneous systems, with a narrow size distribution and low values of the PDI and mean diameter (► **Table 3**). Among the loaded systems, CBM2 and CBS2 were the most appropriate. Analysis by TEM of MEs confirmed the size of the internal phases. In ► **Fig. 2**, TEM analysis of CBM2 is reported, confirming the presence of droplets with a size of ~ 200 nm. TEM analysis of CBS1 and CBS2 were carried out using distilled water after dilution 1:50 v/v. In ► **Fig. 3**, TEM analysis of CBS2 after dilution is reported confirming the presence of droplets with a size of not more than 200 nm.

The optimized nanoformulations were stored away from light at 4°C for 21 d in order to assess their physical stability by monitoring the size of the dispersed phase by DLS. CBS2 was quite stable: no phase separation occurred and the size of the droplets remained nearly constant, as reported in ► **Fig. 4A**. In the case of ME CBM2, no phase separation occurred during storage; however, the size of the droplets decreased slightly at day 14 to return to

► **Table 1** Solubility of β -carotene from saw palmetto extract in various oils, surfactants, and solvents. Data displayed as the mean \pm SD (n = 3). Measurements were from three independent experiments.

Excipient/solvent	β -carotene solubility (mg/mL)	Percentage (%) of solubilised saw palmetto extract
Almond oil	0.095 \pm 0.008	52.78
Soybean oil	0.116 \pm 0.012	64.44
Vitamin E	0.101 \pm 0.015	56.11
Sunflower oil	0.096 \pm 0.010	56.33
Oleic acid	0.101 \pm 0.014	56.11
<i>C. sativa</i> seed oil	0.111 \pm 0.011	61.67
<i>B. officinalis</i> seed oil	0.123 \pm 0.013	68.33
Labrafil	0.094 \pm 0.007	52.22
Capryol 90	0.118 \pm 0.012	65.56
Triacetin	0.020 \pm 0.001	11.11
<i>A. spinosa</i> kerne oil	0.103 \pm 0.011	57.22
Tween 80	0.121 \pm 0.012	67.22
Tween 20	0.109 \pm 0.010	60.56
Transcutol HP	0.087 \pm 0.008	48.33
Kolliphor EL	0.122 \pm 0.013	67.78
Dicloromethane:MeOH 1:1	0.180 \pm 0.010	100.56
Water	Not detected	–

the original values at day 21. This behavior could be due to the fact that MEs are thermodynamically stable and form spontaneously (or with very low energy input) under the right conditions, but they are highly dynamic systems and, as such, undergo continuous and spontaneous fluctuations that consist of phase inversion and changes in droplet size [12].

TEM analysis confirmed the data obtained by DLS analyses: sizes ranged between 200 and 250 nm for extract loaded nano-carriers. In addition, chemical stability was obtained by quantifying the residual amount of β -carotene by using the HPLC-DAD method reported in the experimental part. As reported in ► **Fig. 4B**, the concentration of β -carotene did not decrease significantly during the whole period of the test. After 3 wk the residual β -carotene was ~ 89% in the CBM2 and ~ 95% in the CBS2.

To further evaluate the appropriateness of the developed nanosystems for oral use, intragastric stability was tested in SGM (pH 2) in the presence of pepsin for 2 h, followed by treatment with SIM (pH 7) in the presence of the pancreatin-lipase-bile extract mixture for 2 h. Samples were collected and analyzed by DLS analysis to check their physical stability. The analyses confirmed the physical stability of the systems in terms of size and homogeneity. CBM2 was stable and no aggregation or degradation phenomena occurred. Sizes of the dispersed phase in SGM were 114.4 \pm 1.5 nm, while in SIM were 109.0 \pm 2.5 nm at the end of the tests and were comparable with those found before

► **Table 2** Composition of the optimised MEs and SMEDDSs.

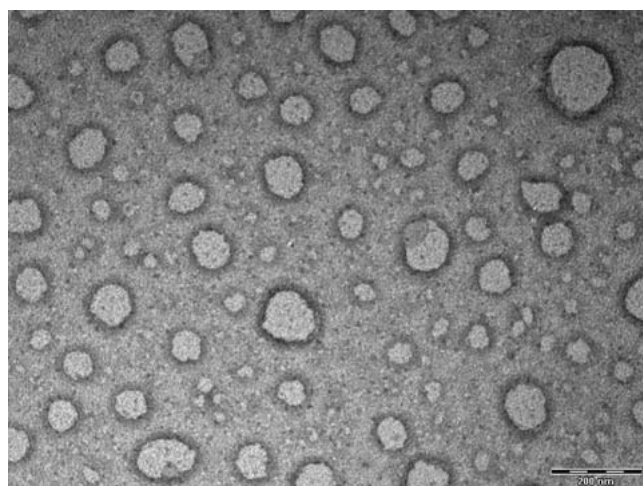
Nanocarrier	Soy oil (g)	Kolliphor EL (g)	Tween 80 (g)	Water (g)	Saw palmetto extract (g)	Nettle extract (g)	Pineapple extract (g)
M1	0.2	–	1.8	4.9	–	–	–
M2	0.2	1.8	–	5.0	–	–	–
CBM1	0.055	–	0.50	2.25	3.20	1.20	0.25
CBM2	0.15	1.33	–	1.29	3.20	1.20	0.25
CBS1	0.28	–	2.52	–	3.20	1.20	0.25
CBS2	0.28	2.52	–	–	3.20	1.20	0.25

► **Table 3** DLS characterization of MEs and SMEDDSs in term of size (nm), polydispersity, and ζ -potential (mV). Data displayed as the mean \pm SD (n = 3). Measurements were from three independent experiments.

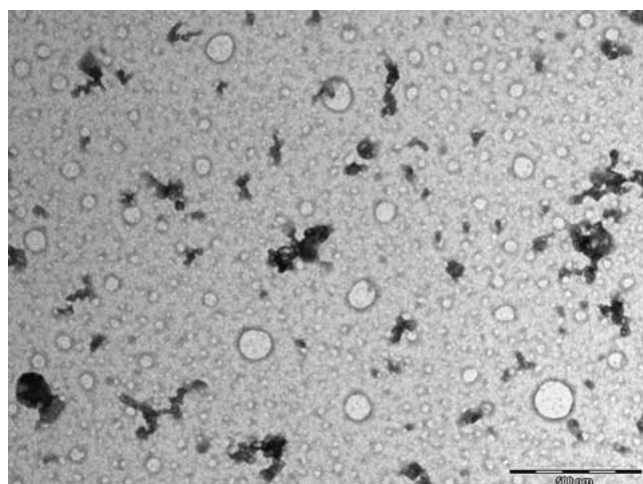
Nano-carrier	Size (nm)	Polydispersity	ζ -potential (mV)
M1	15.8 \pm 2.1	0.22 \pm 0.09	– 43.50 \pm 2.33
M2	18.5 \pm 4.0	0.18 \pm 0.11	– 18.6 \pm 3.12
CBM1	243.6 \pm 11.4	0.31 \pm 0.19	– 35.2 \pm 6.31
CBM2	220.1 \pm 9.6	0.30 \pm 0.26	– 16.7 \pm 4.11
CBS1	399.7 \pm 18.4	0.41 \pm 0.10	– 20.3 \pm 1.55
CBS2	239.0 \pm 13.4	0.28 \pm 0.14	– 25.9 \pm 2.56

the tests. In addition, the CBS2 was stable after the tests in SGM (173 \pm 3.5 nm) and SIM (144.6 \pm 2.7 nm).

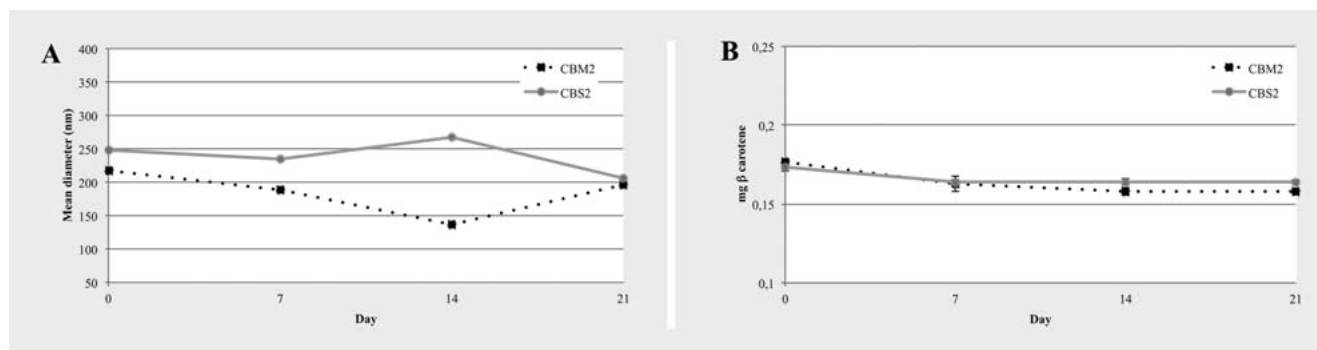
Finally, in order to predict the oral permeability of saw palmetto extract, the PAMPA test was utilized. Recently, PAMPA has gathered considerable interest in pharmaceutical research as a helpful complement and in many cases an alternative test to the Caco-2 assay [13–16]. The experiments were carried out measuring the ability of the saturated aqueous solution of saw palmetto extract, of the saturated aqueous solution of the CB, of CBM2 and CBS2, to diffuse from the donor to acceptor compartment, through a membrane, in order to evaluate the influence of the formulation on the permeability of saw palmetto. Both formulations displayed an appreciated permeation of β -carotene in comparison with the extract and the CB, used as control (► **Table 4**). After 2 h of PAMPA test, permeated β -carotene was under the limit of detection (LOD = 0.87 μ g) for all samples, with the exception of CBS2, which displayed 2% of permeated β -carotene. After 4 h, the permeation increased for both the nanoformulations, while it was detected a permeation for the extract and the CB. After 6 h the best formulation for the oral delivery was the CBM2, with 17% of permeated β -carotene. This value is quite significant because according to the literature [17] for low soluble drugs a PAMPA flux in the range from 5 to 25% corresponds to the 3–70% passive oral human absorption *in vivo*. Finally, a required recovery more than 80% for an acceptable *in vitro* prediction was obtained for almost all the samples (► **Table 4**).



► **Fig. 2** TEM image of nanoformulation CBM2.



► **Fig. 3** TEM image of nanoformulation CBS2 after dilution with water (1:50 v/v).



► **Fig. 4** Physical stability (A) and chemical stability (B) of CBM2 and CBS2 within 3 wk. Data are the mean \pm SD ($n = 3$). Measurements were from three independent experiments.

Accordingly, to the increase of passive diffusion, an enhancement of the oral bioavailability is expected. ME and SMEDD manufacturing and scale-up is simple and do not require specialized equipment and can represent a good alternative to the traditional formulations. Additional imperative advantages of MEs over conventional formulations are almost 100% entrapment of drug, with a high stability over time and high versatility for both polar and lipophilic constituents, and potentially excellent vectors for various routes of administration.

Materials and Methods

Materials

The following products were supplied by Farmaceutica MEV: saw palmetto berry supercritical CO₂ extract (DER 8.0–14.3:1, containing not less than 70.0% and not more than 95.0% of fatty acids and not less than 0.2% and not more than 0.5% of sterols, calculated on an anhydrous basis, lot n. 15/01604); nettle (*U. urens/U. dioica*) root dried extract (70% v/v EtOH, DER 12–16:1, containing 0.82% β -sitosterol, lot n. 15/01509); pineapple (*A. comosus*) stem powder (bromelain was 1850 GDU/g, lot n. 15/00941). Authentic samples of these extracts are maintained at the Department of Chemistry “Ugo Schiff” under the following voucher specimens: 20–2016 (saw palmetto extract), 21–2016 (nettle extract), 22–2016 (pineapple extract).

β -carotene (purity $\geq 98\%$, UV) was from Extrasynthese. Soybean oil, almond oil, tween 20, tween 80, DL- α -tocopherol acetate (vitamin E, purity $\geq 96\%$, HPLC), kolliphor EL, and triacetin were purchased from Sigma-Aldrich; oleic acid was from Farmitalia, Carlo Erba Spa; transcutool HP, capryol 90, and labrafil were from Gattefossé; sunflower oil was from Coop; and *Cannabis sativa* L. (Cannabaceae) seed oil, *Borago officinalis* L. (Boraginaceae) seed oil, and *Argania spinosa* (L.) Skeels (Sapotaceae) kernel oil were purchased from Galeno. EtOH and MeOH analytical reagent, acetone, and MeOH HPLC grade and HCOOH ($\geq 98\%$) were purchased from Sigma-Aldrich. Water was purified by a Milli-Q_{plus} system from Millipore. Phosphotungstic acid was from Electron Microscopy Sciences. Cholesterol, lecithin, dichloromethane, DMSO, 1,7-pctadiene ($\geq 98\%$), PBS bioperformance certified, lipase from por-

► **Table 4** Quantity (%) of β -carotene permeated in the PAMPA test. Data are the mean \pm SD ($n = 3$). Measurements were from three independent experiments.

Sample	Incubation time	% permeated β -carotene	% of recovery
CBM2	2 h	nd	–
	4 h	2.0 \pm 0.1%	99%
	6 h	17.4 \pm 2.1%	99%
CBS2	2 h	2.2 \pm 0.3%	87%
	4 h	4.2 \pm 0.4%	83%
	6 h	7.1 \pm 0.3%	78%
CB	2 h	nd	–
	4 h	nd	–
	6 h	1.3 \pm 0.2%	86.5%
Saw palmetto extract	2 h	nd	–
	4 h	nd	–
	6 h	3.1 \pm 0.4%	98%

nd: not detected

cine pancreas, pepsin from porcine gastric mucose, bile salts, HCl were purchased from Sigma-Aldrich.

Methods

HPLC-DAD assays

For qualitative and quantitative analysis, an HP 1100 L instrument with a diode array detector and managed by a HP 9000 workstation (Agilent Technologies) was used. Data were processed with HP ChemStation software (Agilent). Separation was performed at 24°C on Luna RP C18 (250 \times 4.6 mm), 5 μ m particle size (Phenomenex). The mobile phase consisted of an isocratic mixture of Acetone/MeOH 55:45 v/v. The flow rate was 1 mL/min and the total run time was 20 min, with a post time of 3 min. The sample injected volume was 10 μ L. The UV spectra were recorded between 200 and 600 nm. Chromatographic profiles were registered at 254, 350, 430, and 450 nm. The identification of β -caro-

tene was performed by comparing the retention time and the UV spectra of the peaks in the samples with those of authentic reference samples.

Calibration curve: Standard solutions were freshly prepared by a serial dilutions of stock solution of β -carotene in acetone/MeOH 1:1 to obtain a range of concentration between 0.002 and 0.352 $\mu\text{g/mL}$.

Quantitative determination of β -carotene: External standard method was applied to quantify β -carotene using a regression curve and to increase confidence in our data. Samples were analyzed in triplicate. Measurements were performed at 450 nm, the maximum absorbance of β -carotene. Results were expressed as the mean \pm standard deviation (SD) of three separate experiments.

Preparation of samples for HPLC-DAD analysis: Commercial saw palmetto extract and the CB were solubilized in MeOH:dichloromethane 1:1, ultrasonicated for 5 min and centrifuged for 4 min at 14 000 rpm prior to injection in HPLC. Aqueous solubility was determined by dissolving commercial extract in deionized water at room temperature until saturation. The residue was eliminated by centrifugation and the solution was analyzed by HPLC.

Solubility studies

To find out an appropriate excipient to develop MEs and SMEDDSs, the solubility of saw palmetto extract and the CB in different oils and surfactant was determinate as follows. An excess amount of SM extract was added to 5 mL of each selected excipient (almond oil, soybean oil, tocopherol acetate, sunflower oil, *C. sativa* seed oil, *B. officinalis* seed oil, *A. spinosa* kernel oil, triacetin, oleic acid, labrafil, capryol 90, transcitol HP, kolliphor EL, tween 20, tween 80, and water). Each mixture was shaken at 25 °C for 24 h and then was centrifuged at 14 000 rpm for 10 min. The concentration of the components of the extract was determined by HPLC-DAD after dilution with MeOH/dichloromethane (1:1). The analyses were from three independent experiments.

Preparation of MEs, CBM1, and CBM2

Pseudo-ternary phase diagrams were constructed using Chemix School version 3.60 software to obtain the concentration range of all components in which they form ME. The pseudo-ternary phase diagrams were constructed using the water titration method. Each oil-surfactant mixture was diluted at 50 °C under vigorous stirring dropwise with water. After equilibrium, each sample was visually checked and the phase boundary was determined by observing the changes in the sample appearance from turbid to transparent or from transparent to turbid and by evaluating if ME, emulsion, or gel was present. Optimized preparations are reported in ► **Table 2**.

CBM1 and CBM2 were prepared by dissolving the CB into the oil-surfactant mixture, by adding the required quantity of water, and stirring for 24 h to form a clear and transparent dispersion (► **Table 2**).

Preparation of SMEDDSs, CBS1, and CBS2

Empty SMEDDSs were prepared under magnetic stirring with weighted quantity of oil and surfactant for 24 h at room temperature (► **Table 2**). The mixture was diluted up 100 times with Milli-

Q water and mixed by vortex until a transparent preparation was obtained. CBS1 and CBS2 were prepared by dissolving the CB in the mixture of oil and surfactant at room temperature for 24 h. After a 100-fold dilution with Milli-Q water, this mixture was mixed by vortex until a transparent preparation was obtained. The resulting nanoformulations were stored at 4 °C.

Self-emulsification and precipitation assessment evaluation of SMEDDSs

Self-Emulsification time and precipitation assessment evaluation of the self-emulsifying properties of SMEDDSs formulations were performed by visual assessment as previously reported [17]. In brief, different compositions were categorized on speed of emulsification, clarity, and apparent stability of the resultant emulsion. Visual assessment was performed by dropwise addition of 1 mL of SMEDDS into 250 mL of distilled water. This was done in a glass beaker at room temperature, and the contents were gently stirred magnetically at \sim 100 rpm. Precipitation was evaluated by visual inspection of the resultant emulsion after 24 h. The nanoformulations were then categorized as clear (transparent or transparent with bluish tinge), nonclear (turbid), stable (no precipitation at the end of 24 h), or unstable (showing precipitation within 24 h).

Robustness to dilution for SMEDDS

Robustness to dilution was studied by diluting SMEDDS to 50, 150, and 250 times with distilled water and phosphate buffer with gastric and intestinal pH. The diluted SMEDDSs were stored for 24 h and observed for any signs of phase separation, precipitation, or coalescence. A confirmation of the robustness to dilution of CBS2 at acid pH was obtained by TEM analysis.

Characterization of MEs and SMEDDSs in terms of particle size, polydispersity index, and ζ -potential

Particle size of the developed formulations was measured by a DLS, Zetasizer Nano series ZS90 (Malvern Instruments) equipped with a JDS Uniphase 22 mW He-Ne laser operating at 632.8 nm, an optical fiber-based detector, a digital LV/LSE-5003 correlator, and a temperature controller (Julabo water bath) set at 25 °C. Time correlation functions were analyzed to obtain the hydrodynamic diameter of the particles and the particle size distribution (PDI) using the ALV-60X0 software V.3.X provided by Malvern. Autocorrelation functions were analyzed by distribution, fitting a multiple exponential to the correlation function to obtain particle size distributions. In particular, polydispersity values were calculated for each peak as peak width/mean diameter. Scattering was measured in an optical quality 4 mL borosilicate cell at a 90-degree angle, diluting the samples from 5 to 30-fold in Milli-Q water. ζ -potentials of the nanocarriers were measured using a Malvern Instruments Zetasizer Nano series ZS90. For all samples, an average of three measurements at stationary level was taken. The temperature was kept constant at 25 °C by a Haake temperature controller. The ζ -potential was calculated from the electrophoretic mobility, μE , using the Henry correction to Smoluchowski's equation. The analyses were from three independent experiments.

Morphological analysis of MEs and SMEDDSs

Nanocarrier's dispersions were analyzed in terms of morphology and mean diameter by transmission electron microscope (TEM, Jeol Jem 1010) and by scanning electron microscope (SEM, Phenom G2 ProX, Phenom-World, Alfatext). Ten microliters of ME or SMEDDS dispersion diluted 10 times was applied to a carbon film-covered copper grid. Most of the dispersion was blotted from the grid with filter paper to form a thin film specimen, which was stained with a phosphotungstic acid solution 1% w/v in sterile water. The samples were dried for 3 min and then were examined under a JEOL 1010 electron microscope at an accelerating voltage of 64 kV.

Stability studies of MEs and SMEDDSs

In order to evaluate the stability of the different formulations, the samples were put into sealed glass vials and stored at 4 °C for 21 d. Their chemical and physical stabilities were studied by monitoring the occurrence of phase separation, dispersed phase size, and drug content at predetermined intervals (1 wk) by DLS and HPLC/DAD analyses. Furthermore, to mimic physiological dilution process after oral administration, the samples were diluted 10, 20, and 30-fold with Milli-Q water. The dilutions were followed by gentle vortexing for 2 min at room temperature. DLS analyses confirmed the physical stability of the systems in terms of size and homogeneity. The analyses were from three independent experiments.

In vitro stability of MEs and SMEDDSs in the presence of SIM and SGM

The intragastric stability was tested in SGM as described previously [15]. Briefly, 5 mL of formulation were suspended in 5 mL SGM (0.32% w/v pepsin, 2 g of sodium chloride, and 7 mL HCl dissolved in 1 L Milli-Q water and pH adjusted to 1.8 using 1 M HCl) and incubated in a water bath at 37 °C under shaking speed of 100 strokes/min. After 2 h, sample was collected to analyze the size and PDI. After digestion in simulated stomach condition, the same sample was subjected to digestion under simulated intestinal condition containing intestinal enzyme complex (lipase 0.4 mg/mL, bile salts 0.7 mg/mL, and pancreatin 0.5 mg/mL) and calcium chloride solution 750 mM at pH 7.0, 37 °C under shaking speed of 100 strokes/min. After 2 h digestion in SIM, the sample was collected and its physical stability was checked by DLS analysis. The analyses were from three independent experiments.

In vitro PAMPA

The PAMPA assay was used to predict passive absorption. The assay is carried out in a 96-well, MultiScreen-IP PAMPA (Millipore Corporation) filter plate. The ability of compounds to diffuse from a donor compartment, through a PVDF membrane filter pretreated with a lipid-containing organic solvent, into an acceptor compartment was evaluated. Ten microliters of lecithin (10 g/L) and cholesterol (8 g/L) in 1,7-octadiene solution were added to the filter of each well. Immediately after the application of the artificial membrane, 150 µL of drug containing donor solutions (saw palmetto extract, blend of extracts, CBS2 and CBM2 at the concentration) were added to each well of the donor plate. Three hundred microliters of buffer (PBS, pH 2) was added to each well

of the acceptor plate. The acceptor plate was then placed into the donor plate, ensuring that the underside of the membrane was in contact with buffer. The plate was covered and incubated at room temperature under shaking for 6 h and permeation was evaluated at 0.5, 2, 4, and 6 h.

At the end of the experiment, the samples from the donor and receptor plate were analyzed for β -carotene concentration by HPLC-DAD. The experiment was performed in triplicate and mean of three samples was used in the data analysis. Permeability of the compound across the compartments and the recovery of the experiments were calculated using the following formula:

% β carotene permeated =

$$\frac{\text{mg acceptor compartment}}{\text{mg donor compartment} + \text{mg acceptor compartment}} \times 100$$

$$\text{Recovery \%} = \frac{\text{mg donor compartment} + \text{mg acceptor compartment}}{\text{mg } \beta \text{ carotene in the preparation}}$$

Supporting Information

A pseudoternary diagram of CBM2 is available as Supporting Information.

Acknowledgements

Financial support was granted from Farmaceutica MEV, which also provided the extracts as gifts. Dott.ssa M. Cristina Salvatici Centro di Microscopia Elettronica "Laura Bonzi", ICCOM, and Consiglio Nazionale delle Ricerche (CNR) is gratefully acknowledged.

Conflict of Interest

The authors declare no conflict of interest.

References

- [1] Bennett BC, Hicklin J. Uses of saw palmetto (*Serenoa repens*, Arecaceae) in Florida. *Econ Bot* 1998; 52: 381–393
- [2] Blumenthal M. The ABC Clinical Guide to Herbs. New York, NY: Thieme; 2003
- [3] Scientific Foundation for Herbal Medicinal Products. ESCOP Monographs. *Serenoae Repentis Fructus (Sabal Fructus)*. Saw Palmetto Fruit. Stuttgart: Thieme; 2003
- [4] European Medicines Agency. EMA/HMPC/280079/2013. Committee on Herbal Medicinal Products (HMPC) European Union Herbal Monograph on *Serenoa repens* (W. Bartram) Small, Fructus. London, UK: EMA; 2015
- [5] World Health Organisation. WHO Monographs on Selected Medicinal Plants – Volume 2. *Fructus Serenoae Repentis*. Geneva, Switzerland: WHO; 2004: 285–299
- [6] Saw Palmetto Extract. United States Pharmacopeia and National Formulary (USP 40-NF 35). Rockville, MD: United States Pharmacopeial Convention; 2017
- [7] Saw Palmetto Extract. European Pharmacopoeia. 9th ed. Strasburg, Germany: Council of Europe; 2017
- [8] European Scientific Committee on Phytotherapy. Scientific Foundation for Herbal Medicinal Products. ESCOP Monographs. *Urticae Radix*. Nettle Root. Stuttgart: Thieme; 2003

- [9] Marzano R, Dinelli N, Ales V, Bertozzi MA. Effectiveness on urinary symptoms and erectile function of Prostamev Plus® vs. only extract *Serenoa repens*. Arch Ital Urol Androl 2015; 87: 25–27
- [10] Bilia AR, Piazzini V, Guccione C, Risaliti L, Asprea M, Capecchi G, Bergonzi MC. Improving on nature: the role of nanomedicine in the development of clinical natural drugs. Planta Med 2017; 83: 366–381
- [11] Karioti A, Bergonzi MC, Vincieri FF, Bilia AR. Validated method for the analysis of goji berry, a rich source of zeaxanthin dipalmitate. J Agric Food Chem 2014; 62: 12529–12535
- [12] Heuschkel S, Goebel A, Neubert RH. Microemulsions – modern colloidal carrier for dermal and transdermal drug delivery. J Pharm Sci 2008; 97: 603–631
- [13] Piazzini V, Rosseti C, Bigagli E, Luceri C, Bilia AR, Bergonzi MC. Prediction of permeation and cellular transport of *Silybum marianum* extract formulated in a nanoemulsion by using PAMPA and Caco-2 cell models. Planta Med 2017; 83: 1184–1193
- [14] Piazzini V, Monteforte E, Luceri C, Bigagli E, Bilia AR, Bergonzi MC. Nanoemulsion for improving solubility and permeability of *Vitex agnus-castus* extract: formulation and *in vitro* evaluation using PAMPA and Caco-2 approaches. Drug Deliv 2017; 24: 380–390
- [15] Righeschi C, Bergonzi MC, Isacchi B, Bazzicalupi C, Gratteri P, Bilia AR. Enhanced curcumin permeability by SLN formulation: the PAMPA approach. LWT-Food Sci Technol 2016; 66: 475–483
- [16] Bergonzi MC, Hamdouch R, Mazzacupa F, Isacchi B, Bilia AR. Optimization, characterization and *in vitro* evaluation of curcumin microemulsions. LWT-Food Sci Technol 2014; 59: 148–155
- [17] Kansy M, Senner F, Gubernator K. Physicochemical high throughput screening: parallel artificial membrane permeation assay in the description of passive absorption processes. J Med Chem 1998; 41: 1007–1010