

Self-Nanoemulsifying Drug Delivery System (SNEDDS) Using Lipophilic Extract of *Viscum album* subsp. *austriacum* (Wiesb.) Vollm

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Background and Purpose: Natural products are potential sources of anticancer components. Among various species, the lipophilic extract of the *Viscum album* subsp. *austriacum* (Wiesb.) Vollm. (VALE) has shown promising therapeutic potential. The present work aimed to qualify the plant source and characterize the extract's chemical profile. In addition, a self-nanoemulsifying drug delivery system (SNEDDS) containing VALE (SNEDDS-VALE) was developed.

Methods: *V. album* subsp. *austriacum* histochemistry was performed, and the chemical profile of VALE was analyzed by GC-MS. After the SNEEDS-VALE development, its morphology was visualized by transmission electron microscopy (TEM), while its stability was evaluated by the average droplet size, polydispersity index (PDI) and pH. Lastly, SNEDDS-VALE chemical stability was evaluated by LC-DAD-MS.

Results: The histochemical analysis showed the presence of lipophilic compounds in the leaves and stems. The major compound in the VALE was oleanolic acid, followed by lupeol acetate and ursolic acid. SNEDDS was composed of medium chain triglyceride and Kolliphor[®] RH 40 (PEG-40 hydrogenated castor oil). A homogeneous, isotropic and stable nanoemulsion was obtained, with an average size of 36.87 ± 1.04 nm and PDI of 0.14 ± 0.02 , for 14 weeks.

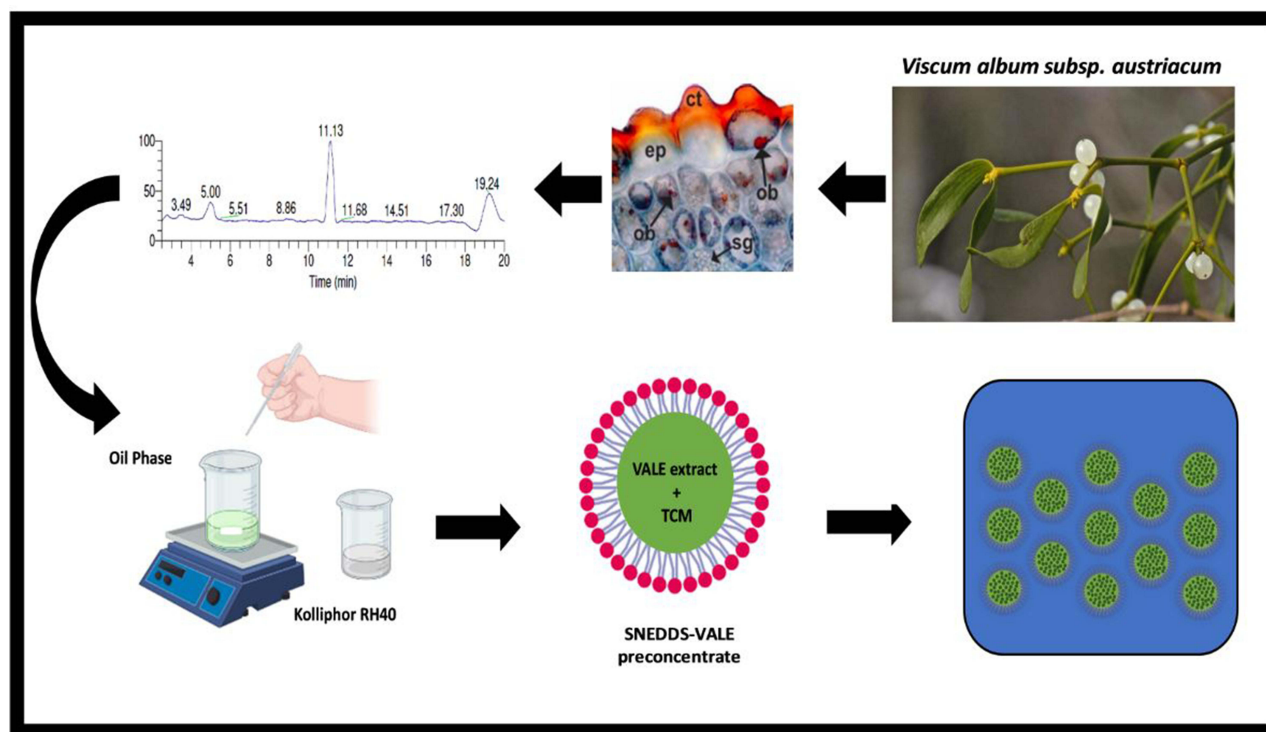
Conclusion: This is the first histochemistry analysis of *V. album* subsp. *austriacum* growing on *Pinus sylvestris* L. which provided detailed information regarding its lipophilic compounds. A homogeneous, isotropic and stable SNEDDS-VALE was obtained to improve the low water solubility of VALE. Further, in vitro and in vivo experiments should be performed, in order to evaluate the antitumoral potential of SNEDDS-VALE.

Keywords: *Viscum album* subsp. *austriacum*, mistletoe, lipophilic extract, oleanolic acid, SNEDDS

Introduction

Among several parasitic plants, the European mistletoe (*Viscum album* L.) is one of the most studied.^{1,2} This species has many distinct host trees, which leads to different chemical metabolic profiles,³⁻⁵ and biological activities.⁶ Medicines

Graphical Abstract



from the *V. album* have been used worldwide in complementary cancer therapy since 1917, mainly in central Europe.⁷ The commercial products are generally prepared by aqueous extractions that are rich in lectins and viscotoxins.^{8,9} These aqueous-soluble compounds are capable of inducing cytotoxic effects on tumor cell lines in addition to induce immunological response.^{8,10–12} However, a lipophilic extract from the *V. album* obtained by ultra-critical CO₂ extraction (VALE) has also presented an interesting therapeutic potential.^{13–16}

The main active components of VALE are considered to be the triterpenes betulinic acid (BA), oleanolic acid (OA), and ursolic acid (UA).¹³ These pentacyclic triterpenes have interesting activities in different biological conditions, considering their lipophilic behavior.¹⁷ The antiproliferative effect of the extract and its fractions (BA, OA, UA) were observed, in addition to their ability to promote apoptotic cell death in MOLT4, K562 and U932 leukemia cells.¹³ In the literature, the use of oleanolic acid resulted cytotoxic activity against A549 and H460 human non-small cell lung cancer cells and promoted autophagy, dependent of ROS, in HepG2 and SMC7721 hepatoma cell lines by suppression of the PI3K/Akt1/mTOR signaling pathway.^{18,19} The therapeutic application of betulinic acid in the treatment of different cancers has also been described, including hepatoma, prostate, pancreatic, breast and leukemia cancer cell lines.²⁰ Moreover, UA isolated from *Oldenlandia diffusa* inhibited the proliferation of doxorubicin-resistant human hepatoma cell line (R-HepG2) through apoptosis and loss of mitochondrial membrane potential.²¹ Considering the antitumor potential of VALE observed in vitro, clinical case studies were subsequently carried out, which demonstrated complete remission and strong improvement with topical treatment of an ointment with 10% VALE in basal cell carcinoma, actinic keratosis and squamous cell carcinoma.^{16,22}

Despite the potential anticancer effects of VALE, it is challenging to deliver its active compounds topically or via the gastrointestinal tract using conventional pharmaceutical preparations. These challenges are mainly due to poor solubilization of the extract related to its lipophilic characteristics.¹⁷ In this sense, nanotechnology has been used to solve some of these barriers.²³ The use of lipid-based nanocarriers, such as self-nanoemulsifying drug delivery systems (SNEDDS), constitutes an outstanding formulation approach for enhancing aqueous solubility.^{24–26}

SNEDDS are isotropic mixtures of oil and surfactants that form a self-nanoemulsifying system in situ when exposed to an aqueous environment, producing an oil-in-water nanoemulsion with a droplet size of 20–200 nm.²⁷ In this context, the development of a SNEDDS containing VALE is a promising alternative for the delivery of its active substances. It allows overcoming the limitations related to the administration of the extract, increasing its solubility and improving its oral absorption and bioavailability. Furthermore, other advantages of this system are its high stability and physical and chemical protection of the encapsulated active ingredients, favoring the therapeutic efficacy of VALE.

The first aim of this study was to qualify the plant source and to provide the chemical quality control of the herbal extract. The subsequent aim was to use the characterized extract in the development of a suitable SNEDDS formulation to enhance the solubility and stability of VALE.

Materials and Methods

Reagents and Materials

Viscum album subsp. *austriacum* (Wiesb.) Vollm. was collected from *Pinus sylvestris* L., during the winter season, on a cultivated natural site that belongs to the Society for Cancer Research, Switzerland. The samples of plants were identified by Dr. Marcelo Guerra Santes (Universidade Estadual do Rio de Janeiro-UERJ), and a voucher (C.H.Quaresma 18.329) was provided by the Herbarium of the Faculdade de Formação de Professores, UERJ, Brazil. *V. album* lipophilic extract (VALE) was donated by Hiscia Institute, Switzerland. The extract was obtained by supercritical CO₂ extraction method, from green organs (leaves and stems) of *Viscum album* subsp. *austriacum*. PEG-40 hydrogenated castor oil (Kolliphor[®] RH40), sorbitan monooleate (Span 80[®]), polysorbate 80 (TWEEN 80[®]), Pyridine, N,O-bis-(trimethylsilyl) trifluoroacetamide (BSTFA), trimethylchlorosilane (TMCS) and the commercial standards of stigmaterol, β -sitosterol, lupeol, ursolic acid, oleanolic acid, betulinic acid and saturated alkanes C7-C30 were purchased, (Sigma-Aldrich, St. Louis, MO, USA/São Paulo, Brazil) as well as medium chain triglycerides (MCT) (Farmácia Quintessência, Rio de Janeiro, Brazil), isopropyl myristate (Vetec Química Fina LTDA, Rio de Janeiro, Brazil), grape seed oil, sunflower oil, sesame oil, sweet almond oil, sorbitol and mineral oil (Ferquima Indústria e Comércio LTDA, Vargem Grande Paulista, São Paulo, Brazil).

Histochemical Analysis

Microchemical analyses of *Viscum album* subsp. *austriacum* harvested in the winter were performed using specific reagents and stains to detect, within cells and tissues, the distribution and accumulation of lipophilic compounds.^{28,29} Free-hand cross-sections of fresh material (leaves and stems) were exposed to Sudan III, which stains lipophilic compounds in red, and Sudan black, which stains lipophilic compounds in black.²⁹ Besides, Nile blue sulfate was used to detect neutral fats in red-to-pink color and fatty acids in blue color.³⁰ The microscopic procedures were conducted in the Laboratory of Pharmacognosy at the State University of Ponta Grossa (UEPG, Brazil).

VALE Extract Qualitative Analysis by TLC

Thin-layer chromatography (TLC) analyses were achieved by silica gel 60 F254 (250 μ m thickness, SiliCycle, Quebec, Canada) using n-hexane and ethyl acetate (7:3 v/v) as mobile phase. For the sample solution, 20 mg VALE extract was dissolved in 20 mL of n-propanol (10 mg/mL). The oleanolic acid standard solution was prepared by dissolving 10 mg of the compound in 5 mL of n-propanol (2 mg/mL). For the standard solution of betulinic acid and ursolic acid, 5 mg of each standard were weighed and dissolved together in 10 mL of n-propanol (1 mg/mL). A 10 μ L spot was done for each standard solution and the VALE solution sample and the plate was left to run through a 10 cm path inside a proper vat containing the mobile phase mentioned above. At the end, the plate was left to dry under ambient conditions before the revelation. The detections were done by spraying anisaldehyde-sulphuric acid reagent. After being heated for 10 minutes at 100 °C, the plates were observed under ultraviolet light at 254, 365 nm and white light.³¹ Spots were identified by R_f-values and color compared to the standard oleanolic acid.

Phytochemical Characterization of VALE by GC-MS

VALE and the analytical standards were derivatized by silylation,³² converting hydroxyl and carboxyl groups in the extract molecules into trimethylsilyl (TMS). Cholesterol was added in a concentration of 200 $\mu\text{g}\cdot\text{mL}^{-1}$ as an internal standard. 125 μL of pyridine, 125 μL of N,O-bis-(trimethylsilyl) trifluoroacetamide (BSTFA), and 25 μL trimethylchlorosilane (TMCS) were added to 2 mg of VALE. Then the tube was heated (70°C) for 30 minutes. Subsequently, the derivatized sample was analyzed by gas chromatography-mass spectrometry (GC-MS). For quantitative analysis, β -sitosterol, stigmasterol, lupeol, oleanolic acid, betulinic acid, β -amyryn acetate, lupeol acetate, and ursolic acid calibration curves were prepared with the following concentrations: 10, 25, 50, 100, 150 and 200 $\mu\text{g}\cdot\text{mL}^{-1}$. Sample analysis was performed on a gas chromatograph (ThermoFisher Scientific, Trace 1300 Series GC) coupled to a triple-quadrupole mass spectrometer (ThermoFisher Scientific, TSQ8000) as follows: 1 μL of the sample was injected (ThermoFisher Scientific, Triplus RSH) using a split ratio of 1:200 and injector temperature at 250°C. The column oven temperature was programmed to start at 80°C, followed by a temperature gradient of 20°C/min up to 180°C, held for 5 min, which was then followed by a temperature gradient of 15°C/min up to 285°C, held for 12 min, totaling 29 min. A solvent delay time of 5.5 min was used to avoid a large solvent peak at the start of the run. A nonpolar HP-5 column (Agilent Technologies; 50m x 0.200 mm x 0.33 μm) was used to separate the compounds. Helium was used as carrier gas at a flow rate of 1.2 mL/min, and the transfer line was maintained at 290°C. The electron ionization voltage was 70 eV, ion source was maintained at 250°C, and the quantitation of compounds was performed by selected reaction monitoring (SRM) acquisition mode as described in [Table S1](#). Tracefinder Forensics v4.1 (Thermo Scientific) software was used for the identification and quantification of metabolites.

SNEDDS Pre-Formulation Study

Solubility Studies

As VALE has low solubility in water due to its chemical composition, the solubility study of this extract was evaluated at different concentrations (1 to 20%), by sequential weight addition of the following pharmaceutical oils in triplicate: Medium-chain triglyceride oil (MCT), Isopropyl myristate, Mineral, Sorbitol, Grape seed, Sunflower, Sesame and Sweet almond. Considering the nature of the extract, 0.1 g of it was added to a 5 mL glass stoppered vial and increasing parts of the solvent were added and homogenized by magnetic stirring, at room temperature for 15 min. Afterward, the vials containing the oil phase were left at 25°C for 24 h to reach equilibrium. Visual observation was done using the reference table “Description and relative solubility of USP and NF articles”, a qualitative parameter to determine the approximate solubility following the United States Pharmacopeia.^{33,34}

Surfactant Selection

A preliminary hydrophilic-lipophilic-balance (HLB) scan test was performed, in which the chosen oil and a pair of surfactants or *Smix* (Tween 80[®]/Span 80[®]) with a known HLB value were used in a fixed proportion (1:3). The HLB range investigated was from 8 to 15, where oil phase and *Smix* were heated at 40 \pm 1.0°C and the continuous phase (water) was heated at 70 \pm 2.0°C. The water phase (80.0% w/w) was slowly added into the oil phase (5% MCT /15% *Smix* w/w) under a magnetic agitation speed of 500 rpm. Emulsions were kept under mixing for 1 h, until reaching room temperature, 25 \pm 2.0°C.³⁵ The HLB number of the surfactant mixture was calculated using the weight fraction of the corresponding surfactants, as can be seen in the following equation:

$$\text{HLB}(\text{mixture}) = \text{HLB A} \times \%A + \text{HLB B} \times \%B \rightarrow A\% + B\% = 100\% \text{ Smix}$$

Ternary Phase Diagram Construction

A ternary phase diagram (TPD) was constructed using the aqueous titration method to identify the right proportions of oil, surfactant and water contents necessary to develop spontaneous nanoemulsion drug delivery systems.³⁶ The selected oil phase containing VALE and the surfactant were added in flasks at fixed ratios (9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, and 1:9) while predetermined and increasing proportions of water (variable component, 8 to 88%) were added to each flask. The aqueous phase titration comprises 99 formulations in which oil/surfactant ratio (OSR) range from 1:9 to 9:1. After each titration, samples were homogenized at 25 \pm 1.0 °C in a stirring plate and kept for 24 h to reach equilibrium. The

formulation macroscopic aspects were then observed according to the variation in their viscosity and stability (presence of creaming, coalescence and/or phase separation). Such features were evaluated by visual inspection and classified as NE (nanoemulsion), PS (phase separation), or MA (macroemulsion). The results were plotted using OriginPro software (version 8.0), and the different regions generated were identified in the triplot graph. From the spontaneous nanoemulsification area (NE), an optimal formulation composition was selected to solubilize the VALE in order to create the self-nanoemulsifying drug delivery system (SNEDDS).^{24,36}

SNEDDS Preparation

BLANK-SNEDDS (without VALE) and VALE-SNEDDS were prepared using the self-nanoemulsification method (low energy), through a two-step addition of the components.²⁷ For the SNEDDS-VALE preparation, 150 mg of VALE was solubilized in 29% wt of MCT under constant magnetic stirring at 200 rpm for 30 min. The oil phase was then kept for 24 h protected from light to reach equilibrium. Then, 68 wt % of PEG-40 hydrogenated castor oil was added to the oil phase, followed by further agitation at 200 rpm for 1 h. The theoretical amount of VALE was 29 mg/g, and the SNEDDS was carried out at 25°C.

Droplet Size, Polydispersity and pH Determination

Droplet size and SNEDDS polydispersity index were carried out by the dynamic light scattering (DLS) method (Zetasizer Nano S90, Malvern Panalytical, Spectris Company, Egham, UK). The samples were diluted 1:100 v/v in distilled water and analyzed at room temperature (25°C). All measurements were made in triplicate and data were represented as average size in nanometers and PdI (mean \pm SD).³⁷ For pH determination, the pre-concentrated samples were diluted 1:10 v/v and verified using a pH meter at 25°C (Digimed pHmeter, São Paulo, Brazil).³⁸

Transmission Electron Microscopy Analysis

The morphology of SNEDDS-VALE and SNEDDS-BLANK-were evaluated by transmission electron microscope (TEM), at 80 K and at 80-kV magnification (Nano Imaging Lab facilities, Basel University, Swiss Nanoscience Institute, Switzerland). Both samples (10 μ L) were diluted 1:10 with double distilled water and taken in a Formvar-coated grid, following negative stain with 10 μ L of uranyl acetate 2.0% (w/v), for 1 min. After 1 min, the excess was dried using filter paper and the grids were left to dry at room temperature (25 \pm 1°C) before being submitted to analysis by TEM (CM100, Philips).³⁹

Physical Stability Study

The VALE-SNEDDS was evaluated for its physical stability in triplicate by three different methodologies (a-c). At the end of each procedure, the samples were visually evaluated for instability presence regarding creaming, cracking or phase separation. a) Heating and cooling cycle: the samples were submitted to six cycles, at 4°C and 40°C, at minimum of 48 h of storage, in each temperature per cycle. In the absence of any of the phenomena described above, the samples would proceed to the centrifugation tests; b) Centrifugation: SNEDDS-VALE samples were centrifuged at two consecutive cycle parameters: 5,000 rpm for 30 min and 10,000 rpm for 30 min. The samples were submitted to the freeze-thaw cycle when instability absence was detected; c) Freezing and thawing cycle: six cycles at 25°C and -20°C were applied, in a minimum of 48 h of storage, in each temperature per cycle. The last stability test consisted of the droplet size and polydispersity index measurements of all samples by DLS technique.⁴⁰⁻⁴²

Accelerated Stability Study

A short-term stability assessment was performed by preparing a new formulation batch in the same conditions as described in the SNEDDS preparation section. The stability was studied by dynamic light scattering (Zetasizer Nano ZS90). The SNEDDS-VALE was stored in glass vials and submitted to different temperatures (25 \pm 5°C and 40 \pm 5°C) for 14 weeks. At predetermined temperatures, droplet size and polydispersity index (PdI) were measured immediately and after 1, 3, 6 and 14 weeks. The formulation was diluted 100-fold with distilled water, and the measurements were taken in triplicate to evaluate the stability over a higher period of time.^{34,43,44}

Robustness to Dilution

Under physiological conditions, the SNEDDS emulsification process occurs spontaneously and gradually.⁴⁵ The dilution robustness test aims to verify the performance of the SNEDDS preconcentrate in the formation of a stable nanoemulsion, under biological mimetic conditions (pH variations and different dilutions). For the robustness dilution test, SNEDDS-VALE was subjected to the following dilutions, ie, 1:50, 1:100, and 1:500, in three different solvents media (distilled water; hydrochloric acid buffer at pH 1.2; phosphate buffer at pH 6.8). The droplet size and PDI parameters were evaluated by the DLS method.^{46,47}

Oleanolic Acid Content by HPLC-DAD-MS

The samples SNEDDS-VALE, SNEDDS-BLANK and VALE free extract were dissolved in n-propanol at 10 mg/mL (in relation to the VALE extract) and the oleanolic acid chemical standard in the same solvent at 2 mg/mL. The chromatographic profile and the quantitative HPLC analysis were performed using a UHPLC-MS (Dionex Ultimate 300 coupled Thermo LCQ Fleet Ion Trap mass spectrometer, Thermo Fisher, San Jose, CA, USA) equipped with a quaternary pump, a column oven, and a DAD detector. Analyses were performed on a reverse-phase C18 column (250 mm × 4.6 mm × 5.0 μm; Kromasil, Akzo Nobel) equipped with a precolumn (3 × 10 mm). The flow rate was 1.0 mL/min and an isocratic condition of acetonitrile (ACN) in 0.1% aqueous formic acid (85:15) in 15 min was used with some adjustments.⁴⁸ The quantification of oleanolic acid was performed at 210 nm and its concentration was calculated by a calibration curve obtained via linear regression (Excel[®] software). Data acquisition and processing were carried out shortly after (Xcalibur[®] software). The oleanolic acid quantification of the pre-concentrated samples was performed after 24 h and 12 months of storage at 25°C.

Results

Histochemistry Aspects of *Viscum album* subsp. *austriacum*

Viscum album subsp. *austriacum* (Figure 1a and b) is a hemiparasite growing on the branches of conifer trees such as *Pinus sylvestris*. Its growth is dichasium with several articulated, glabrous and green stems (Figure 1c), green to yellowish-green leaves (Figure 1d) and globular, whitish and translucent berries (Figure 1e).⁴⁹ The characterization of chemical compounds found in plant tissues can be facilitated by light microscopy-based microchemical investigations carried out using certain reagents and stains.^{50–53} Besides, the use of dyes allows us to localize the distribution and accumulation of secondary metabolites, inside certain cells and tissues, such as lipophilic substances.⁵⁴ For this purpose, *Viscum album* subsp. *austriacum* leaves and stems were subjected to histochemical tests to highlight the compartmentalization sites of lipophilic compounds. The samples of vegetative organs analyzed reacted positively with Sudan III and Sudan black, showing lipophilic compounds in cuticles of the leaves [lamina (Figure 2a) and petiole (Figure 2b)] and stems (Figure 2c). Besides, oil bodies are found in the lamina epidermis of the leaves (Figure 2a), ground parenchyma of the petiole (Figure 2b), in the epidermis of the stems (Figure 2c), mesophyll of the leaves (Figure 2d), and phloem and xylem parenchyma cells of the leaves (Figure 2e–j). The same structure is spread in all tissues such as cortical, vascular and pith of the stems (Figure 2k–m). These oil bodies (Figure 2g, j and m) also stain with Nile blue, indicating the presence of neutral fat in these structures.

VALE Extract Qualitative Analysis by TLC

Typical triterpenes compounds at white light, after revelation with anisaldehyde-sulphuric acid reagent, were observed for the VALE using TLC (Supplementary Material, Figure S1). VALE extract showed different blue-violet and red-violet color bands, which are characteristics for triterpenes.³¹ VALE exhibited one main blue-violet spot with Rf-value similar to the oleanolic acid standard (Rf 0.57) and other zones of blue-violet and red-violet color with less intensity. These are probably attributed to other pentacyclic triterpenes markers, once it was localized next to ursolic and betulinic acid standards (Rf 0.66 and Rf 0.73, respectively).

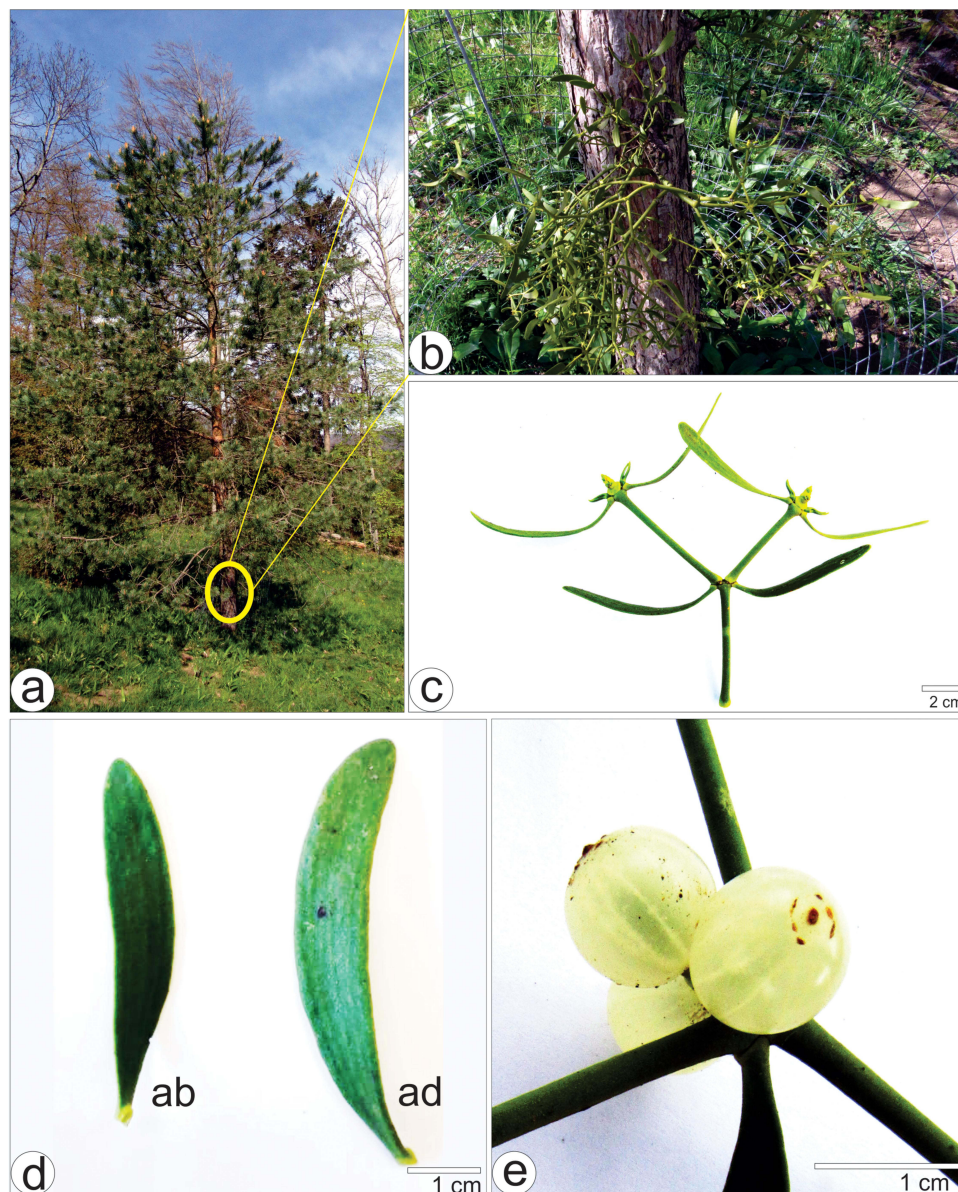


Figure 1 Morphology of *Viscum album* subsp. *austriacum*. Plants growing on branches of host *Pinus sylvestris* (a and b); Several shoots originate from the same node and stems with opposite leaves (c); Leaves on adaxial (ad) and abaxial (ab) side (d); Berries (e).

Phytochemical Characterization of VALE

The GC-MS analysis showed a chromatogram composed of eight main peaks (Figure 3) that were identified according to the MS/MS fragmentation and chemical standards. The confirmation ions used for *Viscum album* lipophilic extract (VALE) were analyzed by selected reaction monitoring mode (Supplementary Material, Table S1). Considering these main compounds present in the VALE, six of them were terpenes (lupeol, oleanolic acid, betulinic acid, β -amyrin acetate, lupeol acetate, and ursolic acid) and 2 phytosterols (β -sitosterol and stigmasterol). Each compound was quantified using a calibration curve and data is shown in Table 1. The silylation process improved the ionization of β -sitosterol, stigmasterol, lupeol, oleanolic acid, betulinic acid, and ursolic acid. The major compound in the VALE was the oleanolic acid, with 59.1 ± 5.77 mg/g followed by lupeol acetate (33.9 ± 2.59 mg/g) and ursolic acid (31.5 ± 2.74 mg/g).

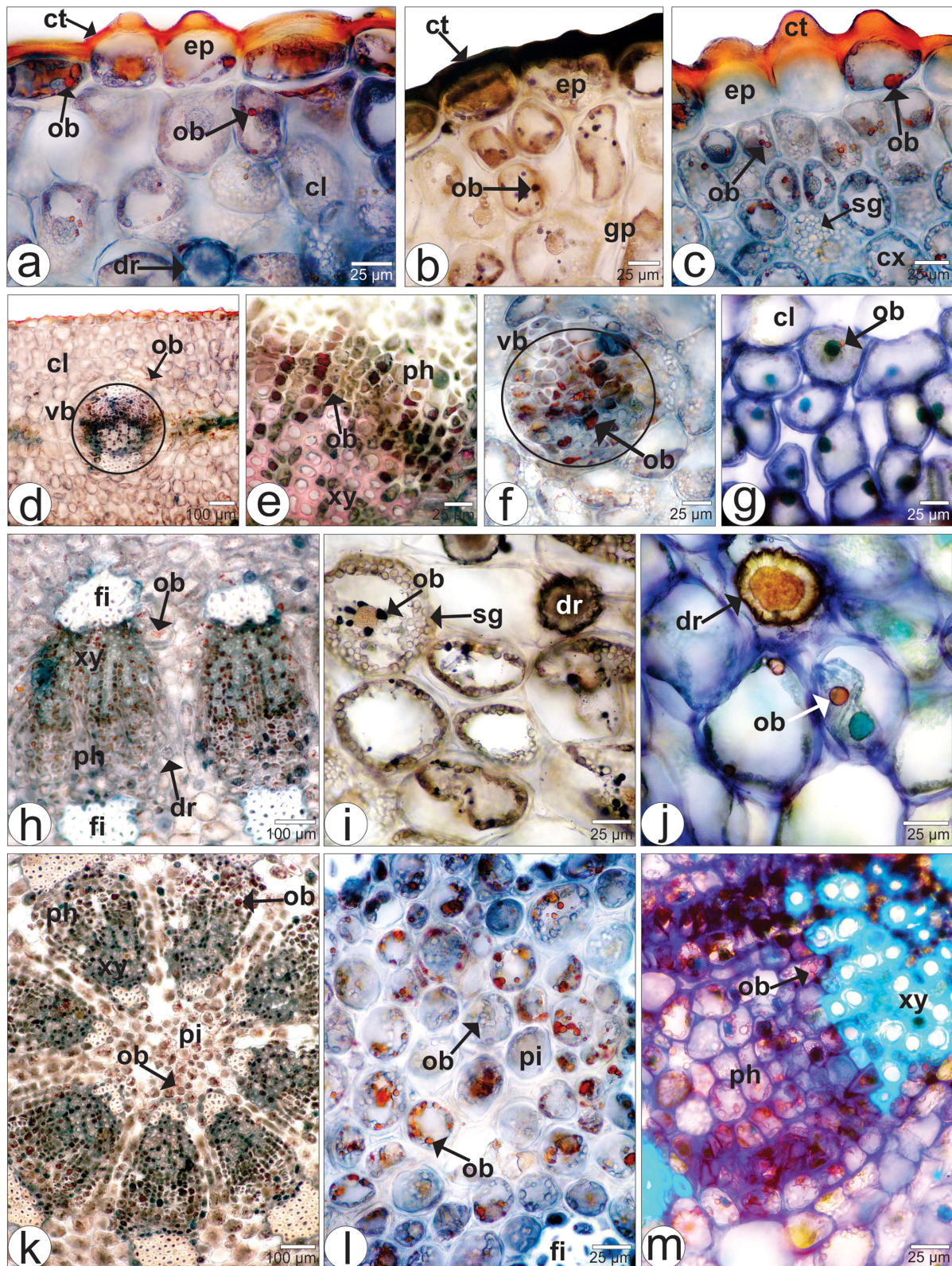


Figure 2 Histochemical tests of *Viscum album* subsp. *austriacum* in cross-section. Lamina of the leaf with Sudan III (a); petiole with Sudan Black (b); stem with Sudan III (c); midrib of the leaf with Sudan III (d) and Sudan black (e); Lamina of the leaf with Sudan III (f) and Nile blue (g); petiole with Sudan III (h), Sudan black (i) and Nile Blue (j); Stem with Sudan III (k and l) and Nile Blue (m).

Abbreviations: cl, chlorenchyma; ct, cuticle; cx, cortex; dr, druse; ep, epidermis; fi, fibers; gp, ground parenchyma; ob, oil bodies; ph, phloem; pi, pith; vb, vascular bundle; xy, xylem.

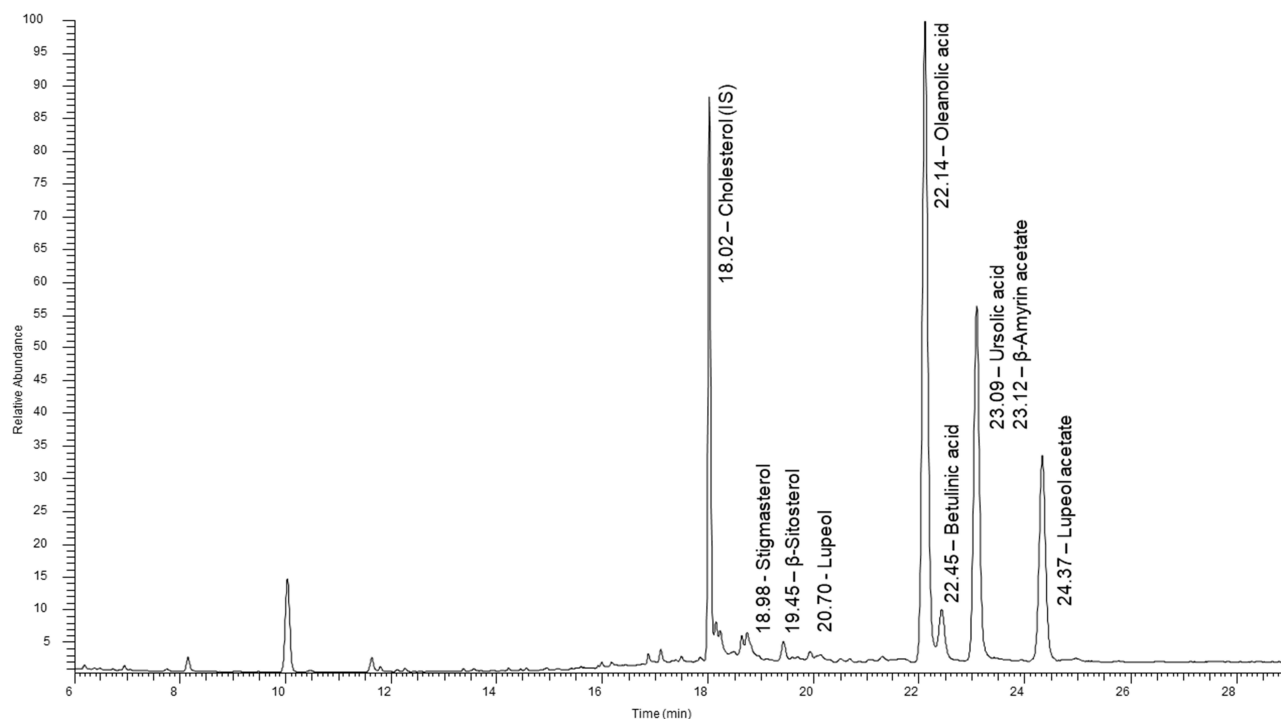


Figure 3 GC-MS chromatogram profile of VALE derivatized by silylation method. The identified phytocompounds and their respective retention times were stigmasterol (18.98); β -sitosterol (19.45); lupeol (20.7); oleanolic acid (22.14); betulinic acid (22.45); ursolic acid (23.09); β - amyryn acetate (23.12) and lupeol acetate (24.37).

SNEDDS-VALE Pre-Formulation Study

Among the pharmaceutical oils tested, only the medium chain triglycerides (MCT) and isopropyl myristate were able to solubilize VALE, as shown in [Table 2](#). The range of VALE solubility in both solvents has an estimated concentration between 33 and 100 mg of VALE per g of oil (33–100 mg/g). Oils with moderate chain length, such as MCT, efficiently generate nanoemulsifying systems.⁵⁵ Their lipophilicity and concentration used in the formulation are important parameters to get the nanoemulsion with a proper range of droplet size (20–200 nm).⁵⁶

The HLB study of MCT varied from 8 to 15 (ideal for o/w emulsions) and showed the presence of translucent (HLB = 13) and slightly bluish formulations (HLB 14 and 15), respectively ([Supplementary Material, Table S2](#) and [Figure S2](#)). The results corroborate the HLB presented in the literature for MCT regarding an o/w system, which may differ depending on the origin of the oil, since the composition of fatty acids in MCT presents a range of variation between caprylic acid (50 to 65%) and capric acid (30 to 45%).⁵⁷

Table 1 Quantitative Analysis of Identified Compounds in the VALE Extract

Retention Time (Min)	Compounds	Content (mg/g)
18.98	Stigmasterol TMS	0.10 \pm 0.01
19.45	β -Sitosterol TMS	1.03 \pm 0.12
20.70	Lupeol TMS	0.17 \pm 0.04
22.14	Oleanolic acid 2TMS	59.1 \pm 5.77
22.45	Betulinic acid 2TMS	3.59 \pm 0.17
23.09	Ursolic acid 2TMS	31.5 \pm 2.74
23.12	β -Amyrin acetate	30.6 \pm 5.83
24.37	Lupeol acetate	33.9 \pm 2.59

Abbreviation: TMS, Trimethylsilyl.

Table 2 VALE Apparent Solubility in Different Pharmaceutical Oils

Oils	Solubility*
Medium-chain triglycerides (MCT)	Soluble
Isopropyl myristate	Soluble
Mineral	Slightly soluble to insoluble
Sorbitol	Slightly soluble to insoluble
Grape seed	Slightly soluble to insoluble
Sunflower	Slightly soluble to insoluble
Sesame	Slightly soluble to insoluble
Sweet almond	Slightly soluble to insoluble

Notes: *Parts of solvent required for 1 part of solute: Freely soluble: from 1 to 10; Soluble: from 10 to 30; Sparingly soluble: from 30 to 100; Slightly soluble: from 100 to 1000; Insoluble: greater than or equal to 10,000.

Based on the HLB study, PEG-40 hydrogenated castor oil presented a theoretically required HLB value close to the oil of choice (14–16). Furthermore, this surfactant demonstrated compatibility with other components of the formulation (extract enriched in triterpenes and MTC) and is known for its emulsification efficiency in the development of nanoemulsions containing at least one of those components.^{55,58,59} Thus, MCT and PEG-40 hydrogenated castor oil were chosen as the oil phase and the emulsifying agent respectively.

A ternary phase diagram was constructed in order to identify the nanoemulsifying region and the right proportion of oily phase, surfactant and water (Figure 4). In the TPD study, all oil/surfactant ratios (OSR) were prepared containing the

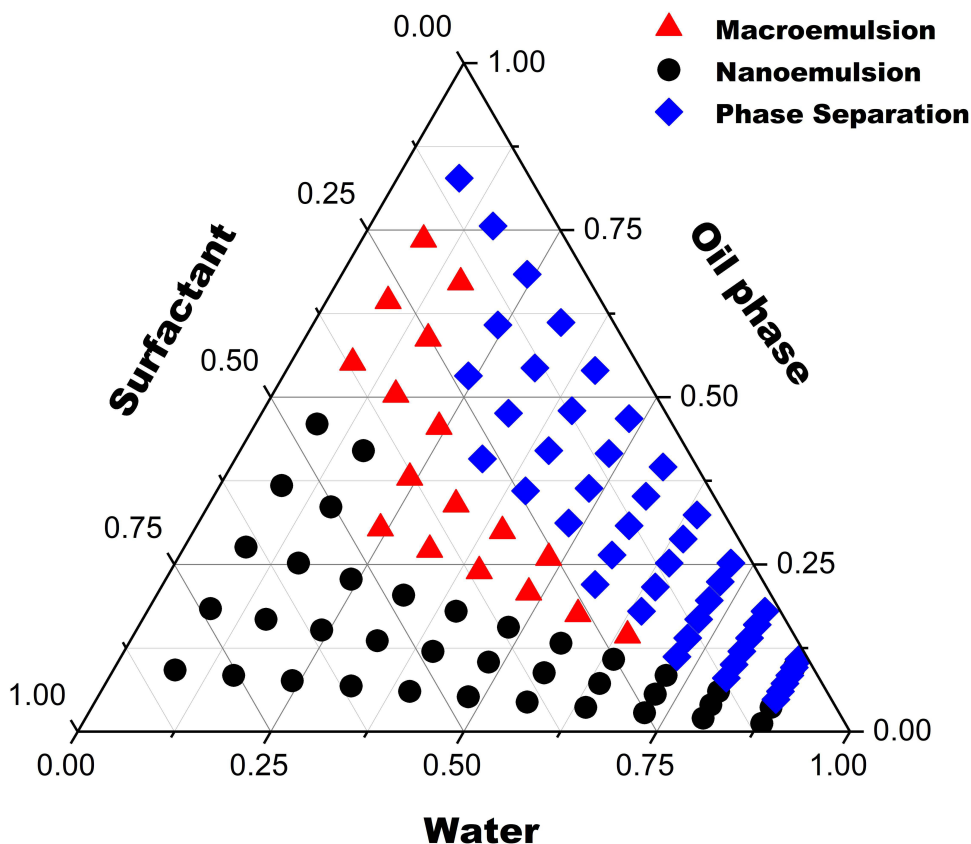


Figure 4 The ternary phase diagram depicting the nanoemulsion (black circle), macroemulsion (red triangle) and phase separation (blue square) regions in a system composed of oil phase (MCT and VALE), Surfactant (PEG-40 hydrogenated castor oil) and water.

herbal constituent since its presence directly influenced the formation of the self-emulsification region.²⁷ Among the 99 generated formulations, a percentage of 32% were macroscopically classified as nanosystems in the TPD (Figure 4).

The OSR of 1:9 and 2:8 generated stable nanosystems, despite the higher amount of water. However, at lower water concentrations ($\leq 56\%$) a transparent gel-like system with high viscosity was observed for both proportions (1:9 and 2:8), which is probably due to the initial swelling of surfactant chains by water addition, promoting a phase equilibrium consistent with a w/o nanoemulsion system. Additionally, the amount of oil present at those proportions was not enough to solubilize a considerable fraction of lipophilic extract, attributing the major solubilization function of the extract to the surfactant. The 3:7 OSR also originated stable translucent nanoemulsions independent of the water addition and with a lower surfactant concentration. The other proportions of oil/surfactant (4:6; 5:5; 6:4; 7:3; 8:2; 9:1) presented an initial w/o nanoemulsion regions (4:6 and 5:5) that were progressively altering its phase behavior for macroemulsions at surfactant concentrations $<42\%$. Below this percentage, the occurrence of physical destabilization resulted in the formation of turbid macro-emulsified systems containing larger droplet size, that are mostly governed by the Ostwald ripening phenomenon and led to phase break under increase in volumetric alteration.⁶⁰ After the phase behavior composition study, the optimum formulation considering the nanoemulsion region in the phase diagram presented the proportions of oil phase and surfactant corresponding to 32% and 68%, respectively. Regarding the oily phase, 9% of its total is represented by the presence of the lipophilic extract of VALE (2.9% in total formulation).

Droplet Size, Polydispersity, pH Determination of SNEDDS-VALE

Mean droplet size and PDI values of the SNEDDS-VALE obtained by DLS are shown in Figure 5A and B. After diluting samples (1:100 v/v in distilled water), mean droplet size and PDI were 37.00 ± 1.04 nm and 0.12 ± 0.10 , respectively. The samples presented a narrow size distribution in function of volume and intensity parameters. Transmission electron microscopy (TEM) highlights the morphological aspects of the SNEDDS-VALE, such as: droplets with uniform spherical shape homogeneously dispersed, with nanometric sizes varying from 30 to 50 nm (Figure 5C and D). The particle size observed by TEM is in accordance with dynamic light scattering (DLS) in which concerns the size distribution and

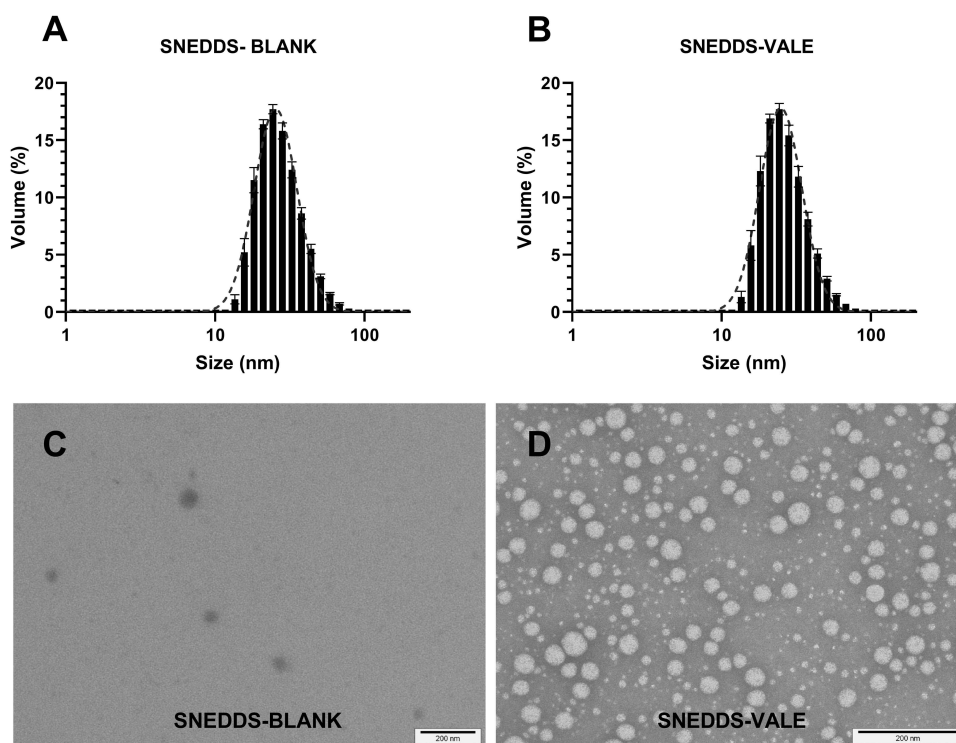


Figure 5 Particle size distribution graphs by volume distribution (A and B) and representative images of transmission electron microscopy (C and D). (A and C) correspond to the blank formulation, without VALE. (B and D) correspond to the formulation loaded with VALE. Scale-bar = 200 nm; sample dilution 1:10.

Table 3 Physical Stability Results of SNEDDS-VALE: Droplet Size (D.nm), Pdl and pH. Results are Expressed as Mean \pm SD (n = 3)

Sample	Heating-Cooling	Centrifugation	Freeze-Thaw	Size (d.nm) \pm SD	Pdl \pm SD	pH \pm SD
1	Approved	Approved	Approved	35.94 \pm 0.22	0.11 \pm 0.01	5.02 \pm 0.02
2	Approved	Approved	Approved			
3	Approved	Approved	Approved			

Note: Sample: SNEDDS-VALE, 3 independent batches.

average size of the droplets of SNEDDS-VALE. Besides, no variations were observed in relation to the pH of the SNEDDS-VALE comparing the 24 h time value (5.0 ± 0.01) with the final pH verified at the end of the physical stability test (5.03 ± 0.02). Together, these data indicate a high stability of the self-nanoemulsifying system.

Physical Stability Study

In order to check the stability of the formulation and classify it as a nanoemulsifying system, SNEDDS-VALE was exposed to different stages of stress, centrifugation study, heating and cooling cycle, and freezing and thawing cycle, as described before. The results obtained are shown in Table 3. At the end of each stress process, instability phenomena such as phase separation, turbidity, creaming, or cracking were not observed. Additionally, SNEDDS-VALE presented small droplet size and Pdl values consistent with monodisperse systems and nanoemulsion parameters.⁶¹ The DLS results (mean droplet size, Pdl) and pH were obtained in triplicate (sample 1, 2 and 3) using three different assays (Table 3).

Accelerated Stability Studies

It was shown that SNEDDS-VALE was able to keep its hydrodynamic diameter and Pdl parameters after 14 weeks of storage at 25°C and 40°C (75 \pm 5% RH) (Figure 6). There was no phase separation, presence of precipitates or color alteration during the stability assay.

Robustness to Dilution

The SNEDDS-VALE chosen through the phase diagram proved to be stable during the physical stability test and, therefore, it was considered eligible for the dilution robustness test. The dispersibility studies of the SNEDDS-VALE in the presence of distilled water, HCl buffer (pH 1.2), and phosphate buffer (pH 6.8) after 24 h are presented in Table 4. No significant differences were observed in relation to the average size as a function of the variation of the pH value. Furthermore, the formulation maintained its physical (size and Pdl) and visual (monodisperse and isotropic) characteristics even after 24 h in the tested media, without signs of instability phenomena such as flocculation or phase separation.

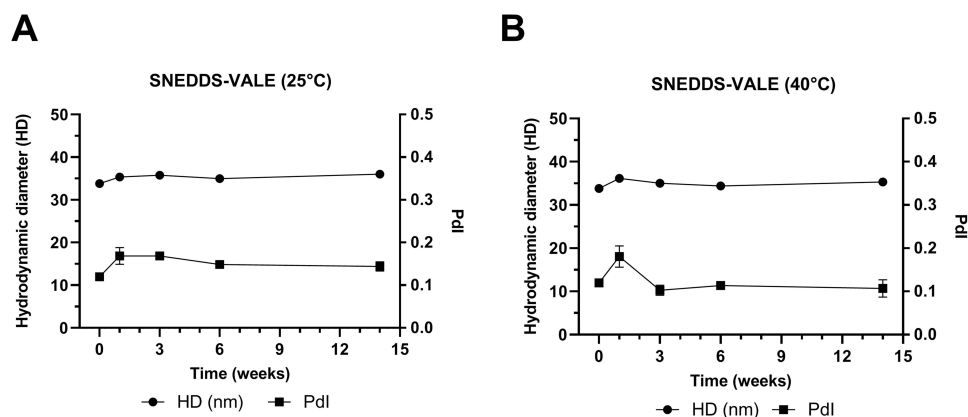


Figure 6 Average hydrodynamic diameter (HD.nm) and polydispersity index (Pdl) of SNEDDS-VALE submitted to accelerated stability assay at temperatures of 25 °C (A) and 40 °C (B) for 14 weeks.

Table 4 Dilution Study of SNEDDS-VALE Formulation in Distilled Water, HCL Buffer (pH 1.2), and Phosphate Buffer (pH 6.8) After 24 h. Results are Expressed as Diameter Size (D.nm) and Polydispersity Index (Pdl) (Mean \pm SD) (n = 3)

Dilution	Distilled Water		pH 6.8		pH 1.2	
	Size (d.nm)	Pdl	Size (d.nm)	Pdl	Size (d.nm)	Pdl
1:50	38.47 \pm 0.19	0.21 \pm 0.02	41.78 \pm 0.20	0.21 \pm 0.01	41.08 \pm 0.52	0.22 \pm 0.01
1:100	38.56 \pm 0.27	0.21 \pm 0.01	41.44 \pm 0.62	0.19 \pm 0.02	40.93 \pm 0.48	0.22 \pm 0.01
1:500	39.17 \pm 0.36	0.19 \pm 0.02	41.93 \pm 0.88	0.20 \pm 0.02	40.56 \pm 0.37	0.19 \pm 0.02

Chemical Stability of the Oleanolic Acid Content in VALE Extract and SNEDDS-VALE

UHPLC-DAD-MS analysis of the VALE used to produce the SNEDDS formulation revealed a major metabolite at the same retention time (11.1 min) of the oleanolic acid reference compound (Figure 7). The molecular mass (455 amu according to ESI-MS) and blue-violet band on TLC (Figure S1, Supplementary Material) after staining with vanillin sulphuric acid reagent suggested to be triterpene. The major peak (11.1 min) of VALE was increased after its fortification with the oleanolic acid reference compound (*data not shown*).

The quantification of oleanolic acid, the chemical VALE marker, was done by a UV detector. The calibration curve of the oleanolic acid was prepared and generated the following linear regression, $y = 3,000,000x - 843,201$, with R^2 of 0.9996. In this analysis, the oleanolic content in VALE was 11.7%.

The chromatograms of VALE and SNEDDS-VALE showed the presence of a peak in the same retention time of the oleanolic acid (11.1 min) by HPLC analysis (Figure 7). It was also possible to see that the SNEDDS-BLANK did not present this peak demonstrating no influence of the SNEDDS excipients in VALE chemical compounds. Besides, the SNEDDS-VALE presented a content of 120.64 ± 0.36 mg OA/ g VALE after 24 h preparation, and 116.6 ± 3.28 mg OA/ g VALE when stored at

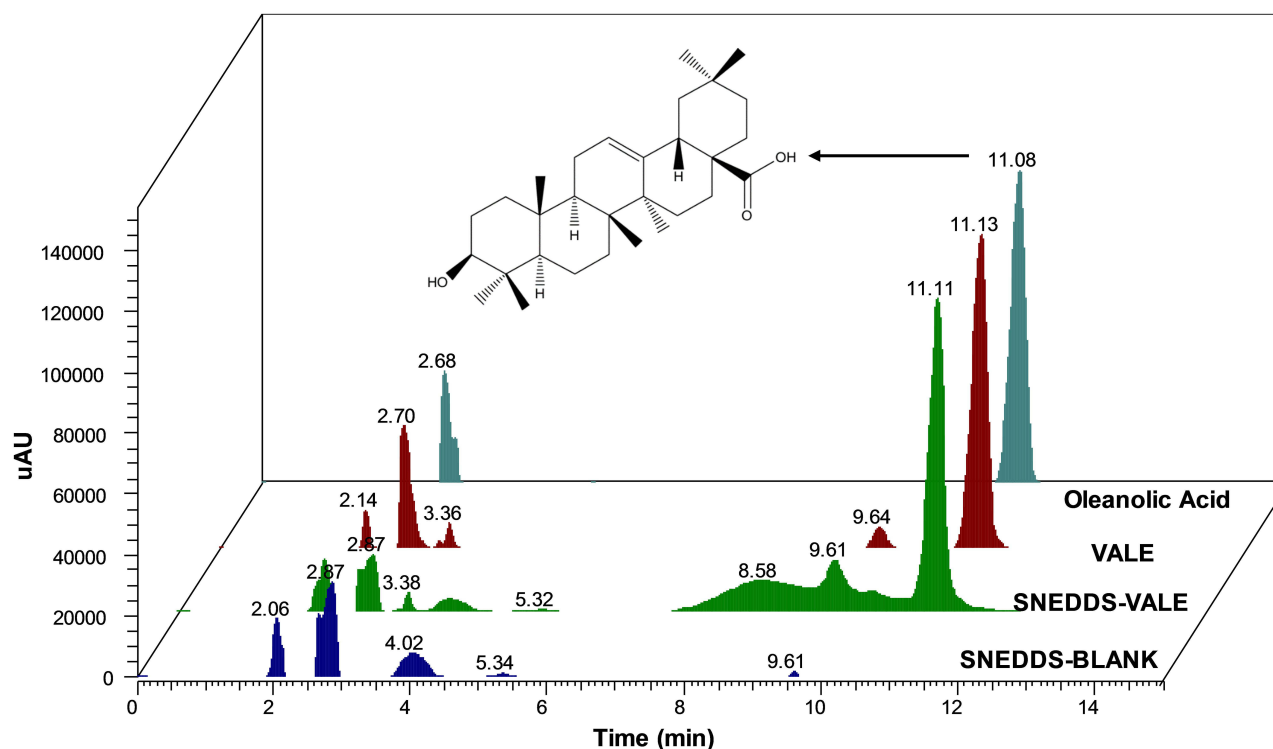


Figure 7 Chromatograms of the SNEDDS-BLANK (blue), SNEDDS-VALE (green), VALE extract (red) and oleanolic acid standard (light blue/green) by HPLC-DAD-MS analysis. Peaks (Retention time of 11.1 min) were assigned to the reference compound.

room temperature for 365 days. It means there was no statistical difference of this majority compound over time (*t*-test, $p < 0.05$).

Discussion

Histochemical analysis of the *Viscum album* subsp. *austriacum* was used as the first evaluation of the starting plant material for VALE production. Optical microscopy using standard lipophilic reagents was performed for the first time in this subspecies and showed the localization of lipophilic compounds in plant organs, (leaves and stems) describing the tissues in which they are present. Thus, the identification of the lipophilic content in the leaves and stems of this subspecies led to the choice of these organs as plant raw material to obtain the VALE extract. The identification through histochemical analysis of tissues and organs in which lipophilic content is present may also be used as a quality parameter for plant raw materials associated with other microscopic analysis (morpho-anatomy) of the same subspecies.^{54,62,63} It may help confirm the subspecies identity and investigate the plant parts (organs) that will be used to acquire the extract since these factors directly influence the lipophilic constituents of the VALE. Almeida et al⁴⁹ also found oily bodies scattered in the organs of *V. album* subsp. *album* using the same dyes, corroborating the results above, since this method allows the comparison between different plant organs and subspecies.⁶⁴ Studies involving the anatomy, micromorphology and histochemistry of *Viscum album* are still limited in the literature, and their continuation will allow a more precise identification of this species and, perhaps, a future differentiation regarding its subspecies.^{49,65,66}

VALE is a complex mixture whose components present a high hydrophobic profile.¹³ The chemical characterization study identified OA as the major compound in the VALE (59.1 ± 5.77 mg/g), as well as the presence of the other pentacyclic triterpenes as betulinic and ursolic acid.

OA is well known in the literature for its therapeutic potential, in which evidence from in vitro and in vivo studies points to its anticancer, antibacterial, antidiabetic, anti-inflammatory, antioxidant, immunoregulatory, hepatoprotective and anti-atherosclerotic effects, through a complex targeting of cell signaling pathways.^{67–74} The other pentacyclic triterpenes present in VALE share most of the pharmacological properties of OA, as they have great structural similarity. However, the particularities of each molecule may influence the intensity of the effect evaluated, resulting in greater or lesser bioactivity.^{17,75,76}

The presence of the above-mentioned pentacyclic triterpenes has been already described by Urech et al¹³ in the *Viscum album*. Moreover, according to Kuonen et al¹⁵ and Estko et al,¹⁴ oleanolic acid is the main compound of VALE, corroborating our results. The phytochemical characterization by GC-MS showed for the first time the presence of the phytosterols, β -sitosterol and stigmasterol in the VALE. Furthermore, the technique allowed us to quantify and confirm the identity of other terpenes in VALE composition, such as lupeol, lupeol acetate and β -amyrin acetate, reinforcing the lipophilic character of this extract. Some of these components, especially OA, are well known for their low water solubility and permeability, being classified by the Biopharmaceutics Classification System (BCS) as IV category.^{77,78} Thus, the formulation of the VALE in a nanocarrier system, such as SNEDDS, would overcome its lipophilic pharmaceutical limitations.^{58,79,80}

In our pre-formulation study, only the medium chain triglycerides (MCT) and isopropyl myristate were able to solubilize VALE at a 1:20 proportion. Among the advantages of using MCT in a SNEDDS is its ease of absorption and digestion, favoring the lymphatic transport of the digested MCT product together with endogenous lipoproteins.^{81,82} Besides, since MCT is synthetically originated from a natural source and presents extensive use in pharmaceutical technology, including the development of drug delivery systems containing pentacyclic triterpenes,^{55,83,84} it was chosen as the oil phase for the development of present SNEDDS.

PEG-40 hydrogenated castor oil is a biocompatible and nonionic surfactant known by presenting an HLB between 14 and 16. The presence of a large number of ethylene oxide groups gives a strong emulsification ability when compared to other agents.⁸⁵ In the SNEDDS formulation process, the surfactant affinity for the oil phase and its HLB value are important parameters to be considered, since it influences the nanoemulsification process and stability, especially when using a low-energy input method to obtain a nanosystem.^{27,86} Based on our study, PEG-40 hydrogenated castor oil presented an HLB value close to the oil of choice. Furthermore, this surfactant is frequently used in the literature in association with MCT or extracts rich in pentacyclic triterpenes in the development of lipid nanosystems due to its

emulsification efficiency and high compatibility with these components.^{55,58,59} These factors guided its choice as an emulsifying agent for the development of SNEDDS-VALE.

In the next step, after oil phase and surfactant selection, the proportion of each constituent in the SNEDDS was defined following the TPD. In SNEDDS systems, the minimum concentration of surfactant capable of generating stable nanoemulsions with low droplet sizes (20–200 nm) and polydispersity values (<0.3) are desirable.²⁷ High surfactant concentrations may promote *in vivo* precipitation phenomena, affecting the bioavailability.⁴⁵ In this sense, the ideal proportion selected by the TPD (OSR 3:7) used the lowest concentration of surfactant capable of generating a macroscopic stable nanoemulsion system containing an oil phase amount that could completely solubilize the VALE extract.

Although SNEDDS-VALE may contain a high amount of PEG-40 hydrogenated castor oil according to some authors, the percentage in which this excipient is present in the formulation allows it to act in a dual role, being the nanoemulsifying agent and also an extract solubilizer, facilitating the complete incorporation of it into the system. Furthermore, similar proportions of PEG-40 hydrogenated castor oil close to the used in SNEDDS-VALE were observed in studies involving the development of SNEDDS and self-microemulsifying systems (SMEDDS) even with the addition of cosolvents in its composition.^{85,87,88} PEG-40 hydrogenated castor oil also presents less toxicity compared to other surfactants of the same class, being considered safe and well tolerated for the nanosystem purpose since it is widely used in nanoemulsified delivery systems containing anticancer agents.^{27,89,90}

The study of the TPD allowed the classification of formulations based on their macroscopic appearance in order to determine the theoretical limit of each component. However, this technique may be linked to observational errors since the time required for the system to reach equilibrium, especially near to the phase limits, could be longer than the time predetermined for the assay (24 h). In order to identify and exclude the presence of undesirable metastable systems in the nanoemulsion region, the physical stability study was performed and no alterations in pH, PDI and droplet size parameters were observed at the end of the assay.⁹¹

A system's kinetic stability is one of the main features of a nanoemulsion. Due to their very small droplet size, its kinetics destabilization is very slow and solely governed by the Ostwald ripening process.⁶⁰ However, unlike conventional nanoemulsions, SNEDDS goes through the process of nanoemulsification in the body, once in contact with biological fluids it should not be influenced by factors such as sample dilution or temperature variations.⁹² To ensure a satisfactory performance *in situ*, SNEDDS must be evaluated using a methodology that mimics the variables present in the physiological environment.⁹³ It is known that the presence of electrolytes can directly influence the stability of formulations due to their ability to interact with the components of the formulation, especially the surfactant.^{27,94} Besides, the nanoemulsification process remains under the influence of factors such as the volume of biological fluids and gastrointestinal motility.⁹⁵ Our results indicate that the formulation maintained its colloidal stability when nanoemulsified in the USP gastrointestinal mimetic buffers, pH 1.2 and 6.8.⁴⁰ In addition, the characterization showed a nanometric droplet size and a monodisperse system coherent with other works for the oral administration route. The comparison between the blank and loaded formulation was made to determine whether the presence of VALE would affect the process of obtaining nanoemulsions in terms of average droplet size and distribution. The absence of significant changes in these parameters indicates that the presence of VALE, at the concentration used in the present study, did not interfere with the interaction among the nanoemulsion ingredients, neither with the interfacial film generation. Besides, the high stability to this system could be attributed to the nanometric size of the droplets generated.^{96,97} As expected, the spherical morphology visualized by TEM confirmed nanodroplets in a uniform distribution as previously presented by DLS results. Considering the character of a self-nanoemulsifying drug delivery system, particle size and its homogeneity is very critical, once it will impact factors such as drug release profile, cellular uptake and biodistribution.⁹⁸

In addition, to evaluate the chemical stability of SNEDDS-VALE, the content of VALE majority marker was done by HPLC-UV (Figure 5) and the analytical method demonstrated to be specific for the quantification of oleanolic acid in SNEDDS-VALE. In this analysis, the oleanolic content in the VALE was 11.7% and similar results were also described by other authors that found a content ranging from 9.3% to 10% of oleanolic acid in the same extract.^{14,15} Chemical standardization of an herbal product is important for quality control and to assure its biological activities.^{2,3,6} The

constant quality of the herbal material and in-process controls during the production procedure in each and every step leads to the desired batch-to-batch consistency.⁹⁹

The accelerated stability conducted at 25°C and 40°C for fourteen weeks showed no alterations in the droplet size and PDI values highlighting the stability of the SNEDDS-VALE under these conditions. Furthermore, at the end of one year, the oleanolic acid content was approximately 100% of the start point indicating no significant losses of the chemical marker in the formulation. Wang et al,¹⁰⁰ using an accelerated stability study, showed that the content of free oleanolic acid (control) in a solid dispersion formulation did not suffer changes or deterioration after 6 months of evaluation. However, Xi et al⁸⁰ demonstrated that oleanolic acid was not chemically stable after 15 days at 60°C in a SNEDDS presenting a reduction of 10%, emphasizing its thermal instability and the need to avoid storage of oleanolic acid formulations at high temperatures. According to our data and the above-mentioned authors, the oleanolic acid seems to be stable at room temperature in both nano and oral classical formulations. In short, VALE delivery through a nanosystem, may be promising in terms of improving the oral bioavailability and chemical stability of its phytochemicals when compared to classical systems.¹⁰¹

Conclusion

This is the first histochemical analysis of leaves and stems of *V. album* subsp. *austriacum* growing in *Pinus sylvestris*. The features found in this study help the species identification and support the quality control of plant material. A homogeneous, isotropic and stable SNEDDS-VALE was obtained to improve the low water solubility of this lipophilic extract encouraging its biological uses. The primary lipophilic components of VALE, namely oleanolic acid, present promising therapeutic potential and further in vivo experiments should be done, to evaluate the anticancer potential of the developed SNEDDS-VALE.

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Disclosure

The authors report no conflicts of interest in this work.

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