Exploring the antioxidant potential of *Helichrysum stoechas* (L.) Moench phenolic compounds for cosmetic applications: chemical characterization, microencapsulation and incorporation into a moisturizer

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Abstract

The present work explores the antioxidant potential of *Helichrysum stoechas* (L.) Moench phenolic compounds for cosmetic applications involving the following steps: chemical characterization, microencapsulation and incorporation into a moisturizer. Eighteen different phenolic compounds were identified in flowering aerial parts (decoction and hydroalcoholic extract), being 3,5-*O*-dicaffeoylquinic acid and myricetin *O*-acetylhexoside the most abundant phenolic acid and flavonoid, respectively. Comparatively to the decoction form, the hydroalcoholic extract presented both higher antioxidant activity and higher phenolic content, being its lyophilized form chosen to proceed with microencapsulation studies. Double emulsion/evaporation microencapsulation technique was applied to produce polycaprolactone based microspheres containing *H. stoechas* hydroalcoholic extract, which were then successfully incorporated into a moisturizer. The results obtained demonstrated the antioxidant potential of *H. stoechas* hydroalcoholic extract and the viability of its microencapsulation, thus opening new perspectives for the exploitation of these natural phenolic extracts in applications such as the cosmetic industry.

Keywords: Helichrysum stoechas, phenolic compounds, decoction, antioxidant activity, microencapsulation, cosmetics

1. Introduction

Natural matrices represent a rich source of biologically active compounds and are an example of molecular diversity, with recognized potential for the development of cosmetics or cosmeceuticals. Bioactive properties of various plants are connected with the presence of phenolic compounds, especially flavonoids (Mishra et al., 2008). The biological, pharmacological and medicinal properties of this group of compounds have been extensively reviewed (Marchand, 2002) and related to their antioxidant properties by preventing UV induced oxygen free radical generation and lipid peroxidation (Kaur and Saraf, 2011).

Cosmetic and cosmeceutical preparations from herbal origin are most popular among consumers, as these agents are typically nontoxic and possess strong antioxidant activity. Since oxidative stress is one of the major mechanisms for skin aging and dermatological disorders, phytochemicals such as phenolic compounds could be useful for treating or preventing those conditions (Singh and Agarwal, 2009).

Ethnobotanical surveys conducted in Northeastern Portugal have highlighted the medicinal use of decoctions of *Helichrysum stoechas* (L.) Moench (shrubby everlasting; port. douradinha) for cold, bronchitis and fever (Carvalho, 2010). Moreover, the antioxidant potential of extracts of this plant (Carini et al., 2001; Albayrak et al., 2010; Barros et al., 2010) could also support the development of cosmetic/cosmeceutical products, similarly to some documented examples dealing with the encapsulation of various plant extracts and isolated compounds. For example, Harris et al. (2011) studied the *Ilex paraguariensis* extract encapsulation in microspheres and nanoparticles of chitosan. This extract presents a high content of phenolic compounds with several pharmacological properties including anti-aging activity. In addition, the cutaneous

absorption of green tea extract compounds was studied, both in free and microencapsulated forms (Wisuitiprot et al., 2011). In what concerns the encapsulation of isolated compounds for cosmetic applications, rosmarinic acid (Kim et al., 2010) and quercetin (Sacalia and Mezzena, 2009) were studied using polycaprolactone and lipidic matrices, respectively.

Although antioxidant properties of *H. stoechas* methanol extract (Carini et al., 2001; Albayrak et al., 2010; Barros et al., 2010) and phenolic compounds (Lavault and Richomme, 2004) have been already investigated, there are no reports neither on the hydroalcoholic extract nor on its most used form, decoction. Moreover, to the best of our knowledge, microencapsulation of such extracts has never been studied.

This work aimed to characterize phenolic compounds of the hydroalcoholic extract and decoction of *H. stoechas* flowering aerial parts, and evaluate their antioxidant potential. Furthermore, the microencapsulation of the lyophilized hydroalcoholic extract was tested by means of water-in-oil-in-water (w/o/w) solvent evaporation technique, a widely used method in pharmaceutical and cosmetic applications (Li at al., 2008, Koo et al., 2008, Giri et al., 2012).

2. Materials and methods

2.1. Plant material

Helichrysum stoechas (L.) Moench (Asteraceae) is a perennial species growing to 0.5 m, in dry, rocky and sandy habitats of the Natural Park of Montesinho territory, Trás-os-Montes, North-eastern Portugal. The inflorescences and leafy flowering stems of about 15 cm long were randomly gathered from several specimens in 2009 late spring and

early summer, considering the Portuguese folk pharmacopoeia descriptors, the local medicinal criteria of use and the species growth patterns.

Morphological key characters from the Nova Flora de Portugal (Franco, 1994) were used for plant identification. Nomenclature follows The Plant List 2013 (http://www.theplantlist.org/). Voucher specimens are kept in the Escola Superior Agrária de Bragança Herbarium (BRESA). The plant material was lyophilized (FreeZone 4.5, Labconco, Kansas, USA), reduced to powder (~20 mesh) and kept in the best conditions for subsequent use.

2.2. Standards and Reagents

HPLC-grade acetonitrile was obtained from Merck KgaA (Darmstadt, Germany). Formic and acetic acids were purchased from Prolabo (VWR International, France). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was purchased from Matreya (Pleasant Gap, PA, USA). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). Phenolic compound standards were from Extrasynthèse (Genay, France). All other chemicals were of analytical grade and purchased from chemical suppliers. Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

Polycaprolactone diol (Mw 3000) (PCL) was supplied by Solvay Caprolactones (Solvay Interox Ltd, United Kingdom) and Tergitol 61E was supplied by Dow Surfactants (The Dow Chemical Company, USA). Methylene Chloride (DCM) was purchased from Sigma-Aldrich (USA) and Polyvinylalcohol (PVA, trade name Celvol 840) was supplied by Celanese Chemicals (USA).

2.3. Extraction procedures for phenolic compounds

A hydroalcoholic extraction was performed using the lyophilized plant material (1 g) stirring with 30 mL of methanol:water (80:20, *v/v*) at 25 ºC at 150 rpm for 1 h and filtered through Whatman No. 4 paper. The residue was then extracted with one additional 30 mL portion of the hydroalcoholic mixture. The combined extracts were evaporated at 35 ºC under reduced pressure (rotary evaporator Büchi R-210, Flawil, Switzerland) and then further lyophilized (FreeZone 4.5, Labconco, Kansas, USA)

A decoction was also prepared from the lyophilized plant material (1 g), by adding 200 mL of distilled water, heating (heating plate, VELP scientific) and boiling for 5 min. The mixture was left to stand for 5 min and then filtered under reduced pressure. The obtained decoction was frozen and lyophilized.

The lyophilized hydroalcoholic extract and decoction were re-dissolved in methanol:water (80:20, *v/v*) and water, respectively (final concentration 2.5 mg/mL), for phenolic compounds determination and antioxidant activity evaluation. The final solutions were further diluted to different concentrations to be submitted to distinct *in vitro* assays.

2.4. Characterization of phenolic compounds

The extracts were analysed using a Hewlett-Packard 1100 chromatograph (Agilent Technologies) with a quaternary pump and a diode array detector (DAD) coupled to an HP Chem Station (rev. A.05.04) data-processing station. A Waters Spherisorb S3 ODS-2 C_{18}, 3 μm (4.6 mm × 150 mm) column thermostatted at 35 °C was used. The solvents used were: (A) 0.1% formic acid in water, (B) acetonitrile. The elution gradient established was isocratic 15% for 5 min, 15% B to 20% B over 5 min, 20-25% B over

10 min, 25-35% B over 10 min, 35-50% for 10 min, and re-equilibration of the column, using a flow rate of 0.5 mL/min. Double online detection was carried out in the DAD using 280 nm and 370 nm as preferred wavelengths and in a mass spectrometer (MS) connected to HPLC system via the DAD cell outlet.

MS detection was performed in an API 3200 Qtrap (Applied Biosystems, Darmstadt, Germany) equipped with an ESI source and a triple quadrupole-ion trap mass analyzer that was controlled by the Analyst 5.1 software. Zero grade air served as the nebulizer gas (30 psi) and turbo gas for solvent drying (400 ºC, 40 psi). Nitrogen served as the curtain (20 psi) and collision gas (medium). The quadrupols were set at unit resolution. The ion spray voltage was set at -4500V in the negative mode. The MS detector was programmed for recording in two consecutive modes: Enhanced MS (EMS) and enhanced product ion (EPI) analysis. EMS was employed to show full scan spectra, so as to obtain an overview of all of the ions in sample. Settings used were: declustering potential (DP) -450 V, entrance potential (EP) -6 V, collision energy (CE) -10V. EPI mode was performed in order to obtain the fragmentation pattern of the parent ion(s) in the previous scan using the following parameters: DP -50 V, EP -6 V, CE -25V, and collision energy spread (CES) 0 V. Spectra were recorded in negative ion mode between *m*/*z* 100 and 1500.

The phenolic compounds were characterized according to their UV and mass spectra and retention times compared with standards when available. For the quantitative analysis of phenolic compounds, a 5-level calibration curve was obtained by injection of known concentrations (2.5-100 μg/mL) of different standard compounds: caffeic acid ($y=617.91x–691.51$; $R^2=0.9991$); chlorogenic acid ($y=600.27x–763.62$; $R^2=0.9998$); *p*-coumaric acid ($y=884.6x+184.49$; $R^2=0.9999$); isorhamnetin 3-*O*-glucoside

(y=262.31x–9.8958; R^2=1.000); isorhamnetin 3-*O*-rutinoside (y=327.42x+313.78; R^2=0.9991); ferulic acid (y=505.97x-64.578; R^2=0.9999); kaempferol 3-*O*-glucoside (y=190.75x–36.158; R^2=1.000); kaempferol 3-*O*-rutinoside (y=175.02x–43.877; R^2=0.9999); myricetin (y=741.41x-221.6; R^2=0.999); quercetin 3-*O*-glucoside (y=316.48x–2.9142; R^2=1.000); quercetin 3-*O*-rutinoside (y=222.79x–243.11; R^2=0.9998). The results were expressed in mg per 100 g of dry weight (dw).

2.5. Evaluation of in vitro antioxidant properties

DPPH radical-scavenging activity was evaluated by using an ELX800 microplate reader (Bio-Tek Instruments, Inc; Winooski, VT, USA), and calculated as a percentage of DPPH discolouration using the formula: $[(A_{DPPH}-A_S)/A_{DPPH}] \times 100$, where A_S is the absorbance of the solution containing the sample at 515 nm, and A_{DPPH} is the absorbance of the DPPH solution. Reducing power was evaluated by the capacity to convert Fe^{3+} to Fe^{2+}, measuring the absorbance at 690 nm in the microplate reader mentioned above. Inhibition of β-carotene bleaching was evaluated though the β-carotene/linoleate assay; the neutralization of linoleate free radicals avoids β-carotene bleaching, which is measured by the formula: β-carotene absorbance after 2h of assay/initial absorbance) × 100. Lipid peroxidation inhibition in porcine (*Sus scrofa*) brain homogenates was evaluated by the decreasing in thiobarbituric acid reactive substances (TBARS); the colour intensity of the malondialdehyde-thiobarbituric acid (MDA-TBA) was measured by its absorbance at 532 nm; the inhibition ratio (%) was calculated using the following formula: [(A - B)/A] × 100%, where A and B were the absorbance of the control and the sample solution, respectively (Barros et al., 2010). The results were expressed in EC_{50} values (sample concentration providing 50% of

antioxidant activity or 0.5 of absorbance in the reducing power assay). Trolox was used as standard.

2.6. Microencapsulation of the hydroalcoholic extract

Microspheres incorporating lyophilized *H. stoechas* hydroalcoholic extract were prepared by double emulsion/evaporation technique, following the general procedure adapted from Giri at al. (2012). Briefly, PCL was used as the matrix material, Tergitol 61E (HLB 3) as the surfactant for the preparation of the primary w/o (water-in-oil) emulsion, and DCM as the oil phase to solubilize PCL. PVA was the protective colloid for the stabilization of the secondary o/w (oil-in-water) emulsion. For the preparation of the primary emulsion, an aqueous solution was prepared by dissolving 70 mg of the lyophilized extract in 4 mL of distilled water under stirring, then filtered and added with 1.5% (w/w) of Tergitol 61E. The oil phase was prepared by dissolving 800 mg of PCL in 20 mL of DMC. The ratio extract/polymer was 70/800 (w/w), which is a value in accordance with the ones typically used in literature for microencapsulation purposes. The w/o emulsion was prepared by adding the aforementioned aqueous solution to the oil phase followed by homogenization at 11000 rpm during 2 minutes using a Cat Unidrive X 1000 homogenizer. Finally, the w/o/w emulsion was prepared through the addition of the previously prepared w/o emulsion to 200 mL of a PVA aqueous solution (1%, w/v) under continuous stirring (400 rpm) during 10 minutes. Microspheres consolidation was achieved by DCM evaporation using a Buchi Rotovapor R-114 in two steps: 2 hours at 40 ºC and atmospheric pressure followed by one hour under reduced pressure (600 mbar). The resulting microspheres were firstly collected by

sedimentation, washed twice with distilled water and thereafter recovered through filtration. The microspheres were then lyophilized and stored in dark conditions at 4 ºC.

2.7. Microspheres characterization

Microspheres in solution form were analyzed by optical microscopy (OM) using a Nikon Eclipse 50i microscope equipped with a Nikon Digital Sight camera and NIS Elements software for data acquisition. OM analysis was applied to access size and morphology of the primary and secondary emulsions, as well as of the consolidated microspheres. Evidence of successful extract microencapsulation was inspected by FTIR analysis. For that purpose, spectra of PCL, hydroalcoholic extract and corresponding microspheres were collected on a FTIR Bomen (model MB 104) by preparing KBr pellets at a sample concentration of 1% (w/w). Spectra were recorded at a resolution of 4 cm^{-1} between 650 and 4000 cm^{-1} and by co-adding 48 scans.

2.8. Microspheres incorporation into a base moisturizer

Lyophilized microspheres were incorporated in a base formulation prepared by an o/w technique. The water phase (WP) comprises 15 mL of water (no thickener was used), whereas the oil phase (OP) accounts with 2.0 mL of almond oil added with 0.5 g of Olivem 1000 (emulsifier), 0.2 g of Olivem 300 (emollient and co-emulsifier) and 0.25 g of Olivax (moisturizer agent). Olivem and Olivax are trademarks of BT Company (Milan, Italy). The WP and OP were heated separately to 70-75 ºC until homogenization. Thereafter they were left to cool to 40 ºC before adding 100 mg of microspheres to the oil phase. Finally the WP was mixed with OP and vigorously stirred together. For control purposes, a moisturizer sample without added microspheres was

also prepared. Samples with and without microspheres were analyzed by OM and compared.

2.9. Statistical analysis

All the assays were carried out in triplicate and the results are expressed as mean values and standard deviation (SD). The results were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's HSD Test with $\alpha = 0.05$, performed with SPSS v. 18.0 program.

3. Results and discussion

3.1. Phenolic profile of the hydroalcoholic extract and decoction

The HPLC phenolic profile of a *Helichrysum stoechas* sample, obtained after hydroalcoholic extraction, and recorded at 280 and 370 nm is shown in **Figure 1**; peak characteristics and tentative identities are presented in **Table 1**. Eighteen compounds were detected, nine of which were phenolic acid derivatives. Among them, six compounds (peaks 1, 3, 9, 11, 13 and 16) were caffeoylquinic acid derivatives identified according to their UV spectra and pseudomolecular ions. Peak 1 ($[M-H]^-$ at *m/z* 353) was identified as 3-*O*-caffeoylquinic acid, yielding the base peak at *m/z* 191 and the ion at *m/z* 179 with an intensity >73% base peak, characteristic of 3-acylchlorogenic acids as reported by Clifford et al. (2003 and 2005). Peak 3 was identified as 5-*O*-caffeoylquinic acid by comparison of its UV spectrum (λ_{max} 326 nm) and retention time with a commercial standard. Peaks 4 and 5 were tentatively identified as 5-*O*-coumaroylquinic and 5-*O*-feruloylquinic acids, respectively, taking into account their

pseudomolecular ions, fragmentation patterns and relative intensities similar to 5-*O*-caffeoylquinic acid.

Peaks 9, 11 and 13 ([M-H]$^-$ at *m/z* 515) corresponded to dicaffeoylquinic acids and were assigned to 3,5-*O*- (peaks 9 and 11) and 4,5-*O*- dicaffeoylquinic acids (peak 13) based on their elution order and fragmentation patterns (Clifford et al, 2003; Clifford et al, 2005). The fragmentation pattern of peaks 9 and 11 was similar to the one previously reported by Clifford et al. (2005) for 3,5-*O*-dicaffeoylquinic acid. MS^2 base peak was at *m/z* 353, produced by the loss of one of the caffeoyl moieties ([M-H-caffeoyl]$^-$), and subsequent fragmentation of this ion yielded the same fragments as 5-caffeoylquinic acid at *m/z* 191, 179 and 135, although in this case with a comparatively more intense signal at *m/z* 179 ([caffeic acid-H]$^-$, ~70% base peak). These peaks (9 and 11) were identified as *cis* and *trans* 3,5-*O*-dicaffeoylquinic acid, respectively, based on the elution order described in a previous study (Barros et al., 2012). Peak 13 was assigned to 4,5-*O*-dicaffeoylquinic acid since its fragmentation was identical to that previously reported by Clifford et al. (2005). In this case, the signal at *m/z* 335 was barely detectable (<3 % of base peak) and the intense signal at *m/z* 173 is characteristic for an isomer substituted at position 4, which indicated loss of the caffeoyl moiety at position 5.

Peak 16 presented a pseudomolecular ion at *m/z* 529 that yielded fragments at *m/z* 367 ([*O*-feruloylquinic acid-H]$^-$), 353 ([caffeoylquinic acid-H]$^-$, and 179 and 193, corresponding to deprotonated caffeic acid and deprotonated ferulic acid, respectively, which allowed its identification as a caffeoyl-*O*-feruloylquinic acid.

Peak 2 was identified as protocatechuic acid by comparison of its UV spectrum (λ_{max} 260, sh296 nm) and retention time with a commercial standard.

The remaining phenolic compounds corresponded to flavonol derivatives (**Table 1**). Myricetin 3-*O*-glucoside (peak 6) and quercetin 3-*O*-glucoside (peak 8) were positively identified according to their retention, mass and UV-vis characteristics by comparison with commercial standards. Pseudomolecular ($[M-H]^-$ at *m/z* 463) and product (*m/z* at 301, quercetin) ions of peak 14 allowed its identification as a quercetin hexoside. This peak showed λ_{max} at higher wavelength (370 nm) than quercetin 3-*O*-glucoside (344 nm) and similar to quercetin aglycone. According to Mabry et al. (1970), the introduction of a glycoside on the hydroxyls at positions 7, 3' or 4' should not have effect on maximal wavelength or the spectrum shape in relation to the aglycone. Thus, peak 14 was tentatively assigned as quercetin 7-*O*-hexoside.

Peaks 10 and 12 ($[M-H]^-$ at *m/z* 505) should correspond to quercetin *O*-acetylhexosides according to their pseudomolecular ion and MS^2 fragment released at *m/z* 301 (quercetin; $[M-H-42-162]^-$, loss of an acetylhexoside moiety). Similarly, peaks 7, 15 and 17 were assigned as acetylhexosides of myricetin, isorhamnetin and kaempferol, respectively, based on their pseudomolecular and product ions. The bathochromic displacement in λ_{max} observed in peak 7 (366 nm) in relation to myricetin 3-*O*-glucoside (peak 6; 354 nm) might allow assigning it as myricetin 7-*O*-acetylhexoside.

Finally, peak 18 presented a pseudomolecular ion $[M-H]^-$ at *m/z* 609, releasing two MS^2 fragments ions at *m/z* 463 ([M-H-146, loss of a *p*-coumaroyl moiety]$^-$) and an *m/z* 301 ([M-H-162, loss of a hexosyl moiety]$^-$), although the loss of 146 mu could also correspond to a rhamnosyl residue, the maximum at 315 nm in the UV spectrum of the peak pointed to a coumaroyl substituent. Thus, the compound would correspond to a quercetin 3-*O*-[*p*-coumaroyl]-glucoside. Lavault and Richomme (2004), identified a similar compound, quercetin 3-*O*-[6-trans-*p*-coumaroyl]-*β*-D-glucoside

(helichrysoside), presenting the same MS and UV spectra, therefore peak 18 was assigned to this compound.

trans 3,5-*O*-Dicaffeoylquinic acid was the most abundant phenolic acid (21.76 and 9.54 mg/g of extract and decoction, respectively) (**Table 2**), whereas myricetin 7-*O*-acetylhexoside was the most abundant flavonoid found (21.76 and 9.54 mg/g of extract and decoction, respectively). The hydroalcoholic extract presented higher concentration in phenolic compounds (135.61 mg/g of extract) than the decoction sample (60.37 mg/g of decoction). Protocatechuic acid was only found in decoction preparation (**Table 2**), which could be formed as a degradation product of quercetin, due to the high temperature applied in this preparation.

There are some similarities in the phenolic profile identified in *H. stoechas* flowers from Portugal and samples from Italy (Carini et al., 2001) and France (Lavault and Richomme, 2004). A sample from Turkey (Albayrak et al., 2010) presented a very different profile, being chlorogenic acid the only compound found in common. Lavault and Richomme (2004) identified eighteen phenolic compounds (α-pyrones, phloroglucinols, phenolic acids, flavonoids, and coumarin), being identified four phenolic acids and flavonoids; quercetin-3-O-*β*-D-glucoside, helichrysoside (quercetin 3-*O*-[6-trans-*p*-coumaroyl]-*β*-D-glucoside), quercetagetin 7-*O*-*β*-D-glucoside, 5-caffeoylquinic acid and 3,5-dicaffeoylquinic acid were also identified in the sample studied herein. Carini et al. (2001) identified five phenolic acids and seven flavonoids; the phenolic acids profile was similar to the Portuguese sample, whereas for flavonoids some differences were found. Those authors identified flavones, such as apigenin and naringenin derivatives. Lavault and Richomme (2004) and Carini et al. (2001) did not

present quantification data on these compounds. Albayrak et al. (2010) were the only authors that presented quantification data, in which the major component present was chlorogenic acid, followed by apigenin 7-glucoside. The quantities found by those authors were lower than the ones obtained herein.

3.2. In vitro antioxidant properties

The results obtained in the evaluation of the antioxidant activity of the hydroalcoholic extract and decoction of *H. stoechas* are given in **Table 3**. The hydroalcoholic extract gave higher antioxidant activity (lower EC_{50} values) in all the *in vitro* assays (EC_{50} values between 79.84 and 36.62 µg/mL) than the decoction preparation (EC_{50} values ranging from 435.20 to 147.03 µg/mL). This is in agreement with the higher phenolic compounds concentration (135.61 mg/g) found in the hydroalcoholic extract in comparison to the decoction preparation (60.37 mg/g). Regarding radical scavenging activity using DPPH assay, the hydroalcoholic extract showed lower activity than methanol extracts of *H. stoechas* from Turkey (EC_{50} value 7.95 µg/mL, Albayrak et al., 2010). The methanol extract previously studied by our research group (Barros et al., 2010) presented higher EC_{50} values (≤ 520 µg/mL) and, therefore, lower antioxidant activity. In our knowledge, no reports are available on the decoction preparation of the studied plant.

3.3. Microspheres preparation and incorporation in moisturizers

PCL based microspheres incorporating lyophilized *H. stoechas* hydroalcoholic extract were prepared by double emulsion/evaporation technique. The encapsulation process was monitored by OM analysis by cheeking several key points of the process. **Figure**

2A shows the prepared w/o emulsion evidencing the individualized spherical aqueous droplets with an estimated size of 10 µm dispersed in the organic medium (DCM containing PCL). **Figure 2B** and **Figure 2C** show the final w/o/w emulsion right after preparation and after 10 minutes under stirring at 400 rpm, respectively. The obtained embryonic microspheres are spherical in shape and it can be noticed the presence of droplets in its interior (water droplets containing the extract). Additionally, as expected, a size reduction was observed as a consequence of stirring, being the final size of the embryonic microspheres estimated to be comprised between 40 and 170 µm.

Figure 3 shows the microspheres consolidation process. During this stage, the size of the obtained microspheres decreased accompanying DCM evaporation. As it can be noticed in **Figure 3A** (micrograph after one hour evaporation at 40 ºC and atmospheric pressure) consolidation was negligible, becoming evident only after two hours under evaporation in the same conditions (**Figure 3B**). To ensure total DCM removal, and consequently total microspheres consolidation, evaporation proceeded under reduced pressure during one additional hour (**Figure 3C**). Final size of the obtained microspheres was estimated to be comprised between 30 and 100 µm.

FTIR analysis of PCL, lyophilized extract and corresponding microspheres is shown in **Figure 4**. As expected microspheres spectrum was dominated by the presence of PCL (a ratio extract/PCL of 70/800 (mg/mg) was used). Nevertheless, comparatively to the PCL spectrum, in the microspheres both OH and C=O stretching bands become more prominent compared to the CH stretching zone. This could be explained by an effective extract incorporation, containing compounds rich in OH and C=O moieties.

The obtained lyophilized microspheres were easily dispersed in the oil phase, contrary to the aqueous phase, where they remain agglomerated. For this reason, the oil phase

was chosen as the more adequate vehicle to proceed with microspheres incorporation during the moisturizer preparation (an oil-in-water emulsion process). **Figure 5** presents the images of the produced base moisturizer **(Figure 5A)**, as well as of the moisturizer added with the lyophilized microspheres under 100X **(Figure 5B)** and 400X **(Figure 5C)** magnification. As it can be observed, microspheres are clearly visible in the moisturizer matrix as individualized particles.

4. Conclusions

Eighteen different phenolic compounds were identified, being 3,5-*O*-dicaffeoylquinic acid the most abundant phenolic acid and myricetin *O*-acetylhexoside the most abundant flavonoid. Comparatively with the decoction form, the hydroalcoholic extract presented the highest antioxidant activity, which can be correlated with its higher phenolic compounds content.

The double emulsion/evaporation microencapsulation technique was successfully applied to produce microspheres containing *H. stoechas* hydroalcoholic extract, which opens new avenues for the exploitation of these phenolic extracts in applications such as the cosmetic industry. As a preliminary approach microspheres were incorporated into a moisturizer. The envisaged methodology comprises its addition to the oil phase guarantying a good dispersion, i.e. the absence of agglomerates.

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References

Albayrak, S., Aksoy, A., Sagdic, O., Hamzaoglu, E. 2010. Compositions, antioxidant and antimicrobial activities of *Helichrysum* (Asteraceae) species collected from Turkey. Food Chem. 119, 114-122.

Barros, L., Dueñas, M., Carvalho, A.M., Ferreira, I.C.F.R., Santos-Buelga C. 2012. Characterization of phenolic compounds in flowers of wild medicinal plants from Northeastern Portugal. Food Chem. Toxicol. 50, 1576-1582.

Barros, L., Oliveira, O., Carvalho, A. M., Ferreira, I.C.F.R. 2010. *In vitro* antioxidant properties and characterization in nutrientes and phytochemicals of six medicinal plants from the Portuguese folk medicine. Ind. Crops Prod. 32, 572-579.

Carini, M., Aldini, G., Furlanetto, S., Stefani, R., Facino, R.M. 2001. LC coupled to ion-trap MS for the rapid screening and detection of polyphenol antioxidants from *Helichrysum stoechas*. J Pharm. Biomed. Anal. 24, 517–526.

Carvalho A.M. 2010. Plantas y sabiduría popular del Parque Natural de Montesinho. Un estudio etnobotánico en Portugal. Madrid: CSIC, Biblioteca de Ciencias.

Clifford, M.N., Johnston, K.L., Knight, S., Kuhnert, N.A. 2003. A hierarchical scheme for LC-MSn identification of chlorogenic acids. J. Agric. Food Chem. 51, 2900-2911.

Clifford, M.N., Knight, S., Kuhnert, N.A. 2005. Discriminating between the six isomers of dicaffeoylquinic acid by LC-MSn. J. Agric. Food Chem. 53, 3821-3832.

Franco, João do Amaral. 1984. Nova Flora de Portugal. Continente e Açores-Clethraceae (Compositae). Vol. III. Lisboa.

Giri, T.K., Choudhary, C., Ajazuddin, A.A., Badwaik, H., Tripathi, D.K. 2012. Prospects of pharmaceuticals and biopharmaceuticals loaded microparticles prepared by double emulsion technique for controlled delivery. Saudi Pharm. J. 21, 125-141.

Harris, R., Lecumberri, E., Mateos-Aparicio, I., Mengíbar M., Heras A. 2011. Chitosan nanoparticles and microspheres for encapsulation of natural antioxidants extracted from *Ilex paraguariensis*. Carbohydr. Polym. 83, 803-806.

Kaur, C.D., Saraf, S. 2011. Development of photoprotective creams with antioxidant polyphenolic herbal extracts. Res. J. Med. Plant 6, 83-91.

Kim, H.J., Kim, T.H., Kang, K.C., Pyo, H.B., Jeong, H.H. 2010. Microencapsulation of rosmarinic acid using polycaprolactone and various surfactants. Int. J. Cosmet. Sci. 32, 185-191.

Koo, B.M., Jung, J.E., Han, J.H., Kim, J.W., Han, S.H., Chung, D.J., Suh, K.D. 2008. Encapsulation and stabilization of photo-sensitive antioxidants by using polymer microcapsules with controlled phase heterogeneity. Macromol. Rapid Commun. 29, 498-502.

Lavault, M., Richomme, P. 2004. Constituents of *Helichrysum stoechas* variety olonnense. Chem. Natural Comp. 40, 118-121.

Li, M., Rouaud, O., Poncelet, D. 2008. Microencapsulation by solvent evaporation: State of the art for process engineering approaches. Int. J. Pharm. 363, 26-39.

Marchand, L.L. 2002. Cancer preventive effects of flavonoids--a review. Biomed. Pharmacothe. 56, 296-301.

Mabry, T.J., Markham, K.R., Thomas, M.B. 1970. The Systematic Identification of Flavonoids. New York: Springer-Verlag Publication.

Mishra, K.P., Ganju, L., Sairam, M., Banerjee, P.K., Sawhney, R.C. 2008. A review of high throughput technology for the screening of natural products. Biomed. Pharmacother. 62, 94-98.

Sacalia, S., Mezzena, M. 2009. Incorporation of quercetin in lipid microparticles: Effect on photo and Chemical stability. J. Pharm. Biomed. Anal. 49, 90-94.

Singh, R.P., Agarwal R. 2009. Cosmeceuticals and Silibinin. Clinical Dermatology, 27, 479-484.

Wisuitiprot, W., Somsiri, A., Ingkaninan, K., Waranuch, N. 2011. *In vitro* human skin permeation and cutaneous metabolism of catechins from green tea extract and green tea extract-loaded chitosan microparticles. Int. J. Cosmet. Sci. 33, 572-579.

Table 1. Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, tentative identification of phenolic acids and flavonoids in *Helichrysum stoechas*

P1eak	Rt (min)	λ_{max} (nm)	Molecular ion $[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification
1	5.26	326	353	191(100),179(73),173(7),135(55)	3-*O*-Caffeoylquinic acid
2	6.14	260,sh296	163	109(100)	Protocatechuic acid
3	8.03	326	353	191(100),179(17),173(17),135(9)	5-*O*-Caffeoylquinic acid
4	13.09	310	337	191(100),173(7),163(9),119(3)	5-*p*-Coumaroylquinic acid
5	14.81	330	367	191(100),173(10),134(7)	5-*O*-Feruloylquinic acid
6	16.23	354	479	317(100)	Myricetin 3-*O*-glucoside
7	19.67	366	521	317(100)	Myricetin 7-*O*-acetylhexoside
8	20.64	344	463	301(100)	Quercetin 3-*O*-glucoside
9	21.78	326	515	353(100),335(13),203(3),191(73),179(69),173(22),161(5),135(22)	*cis* 3,5-*O*-Dicaffeoylquinic acid
10	22.19	354	505	301(100)	Quercetin *O*-acetylhexoside
11	22.22	328	515	353(100),335(5),191(97),179(79),173(9),161(5),135(37)	*trans* 3,5-*O*-Dicaffeoylquinic acid
12	24.33	358	505	301(100)	Quercetin *O*-acetylhexoside
13	24.91	330	515	353(97),335(3),299(4),255(5),203(16),191(30),179(75),173(100),161(4),135(27)	4,5-*O*-Dicaffeoylquinic acid
14	27.21	370	463	301(100)	Quercetin 7-*O*-hexoside
15	27.91	354	519	315(100)	Isorhamnetin *O*-acetylhexoside
16	28.31	326	529	367(96),353(8),335(6),193(100),191(15),179(6),173(22),134(4)	Caffeoyl-*O*-feruloylquinic acid
17	29.41	366	489	327(57),285(100)	Kaempferol *O*-acetylhexoside
18	31.77	315,sh360	609	463(70),301(40)	Quercetin 3-*O*-[*p*-coumaroyl]-glucoside

Table 2. Phenolic compounds quantification (mg/g of extract or decoction) in hydroalcoholic extract and decoction of *Helichrysum stoechas* (mean ± SD).

Phenolic compounds	**Hydroalcoholic extract**	**Decoction**
3-*O*-Caffeoylquinic acid	0.68 ± 0.01	0.99 ± 0.22
Protocatechuic acid	nd	1.14 ± 0.05
5-*O*-Caffeoylquinic acid	21.94 ± 0.28	12.12 ± 0.12
5-*p*-Coumaroylquinic acid	0.29 ± 0.29	0.26 ± 0.03
5-*O*-Feruloylquinic acid	0.55 ± 0.55	0.52 ± 0.06
Myricetin 3-*O*-glucoside	9.37 ± 0.08	3.08 ± 0.19
Myricetin 7-*O*-acetylhexoside	**21.76 ± 0.52**	**9.54 ± 0.05**
Quecetin 3-*O*-glucoside	9.28 ± 0.43	3.80 ± 0.19
cis 3,5-*O*-Dicaffeoylquinic acid	2.00 ± 0.01	0.79 ± 0.08
Quercetin *O*-acetylhexoside	2.63 ± 0.36	1.36 ± 0.06
trans 3,5-*O*-Dicaffeoylquinic acid	**43.31 ± 0.15**	**15.65 ± 0.11**
Quercetin 7-*O*-acetylhexoside	4.49 ± 0.16	2.18 ± 0.01
4,5-*O*-Dicaffeoylquinic acid	4.94 ± 0.24	2.97 ± 0.11
Quercetin 7-*O*-hexoside	4.93 ± 0.27	1.73 ± 0.05
Isorhamnetin *O*-acetylhexoside	0.97 ± 0.04	0.35 ± 0.01
Caffeoyl-*O*-feruloylquinic acid	0.45 ± 0.02	0.20 ± 0.02
Kaempferol *O*-acetylhexoside	4.61 ± 0.10	2.16 ± 0.02
Quercetin 3-*O*-[*p*-coumaroyl]-glucoside	3.43 ± 0.11	1.51 ± 0.09
Total phenolic acids	74.16 ± 0.44[a]	34.64 ± 016[b]
Total flavonoids	61.45 ± 1.67[a]	25.72 ± 0.02[b]
Total phenolic compounds	135.61 ± 2.10[a]	60.37 ± 0.14[b]

In each row different letters mean significant differences ($p<0.05$).

Table 3. Antioxidant activity of *Helichrysum stoechas* hydroalcoholic extract and decoction (mean ± SD).

Antioxidant activity	Hydroalcoholic extract	Decoction
DPPH scavenging activity (EC_{50}, µg/mL)	36.62 ± 0.39^{b}	435.20 ± 2.66^{a}
Reducing power (EC_{50}, µg/mL)	75.14 ± 3.77^{b}	187.57 ± 1.98^{a}
β-carotene bleaching inhibition (EC_{50}, µg/mL)	79.84 ± 1.45^{b}	171.08 ± 1.71^{a}
TBARS inhibition (EC_{50}, µg/mL)	51.62 ± 1.27^{b}	147.03 ± 7.03^{a}

In each row different letters mean significant differences ($p<0.05$).

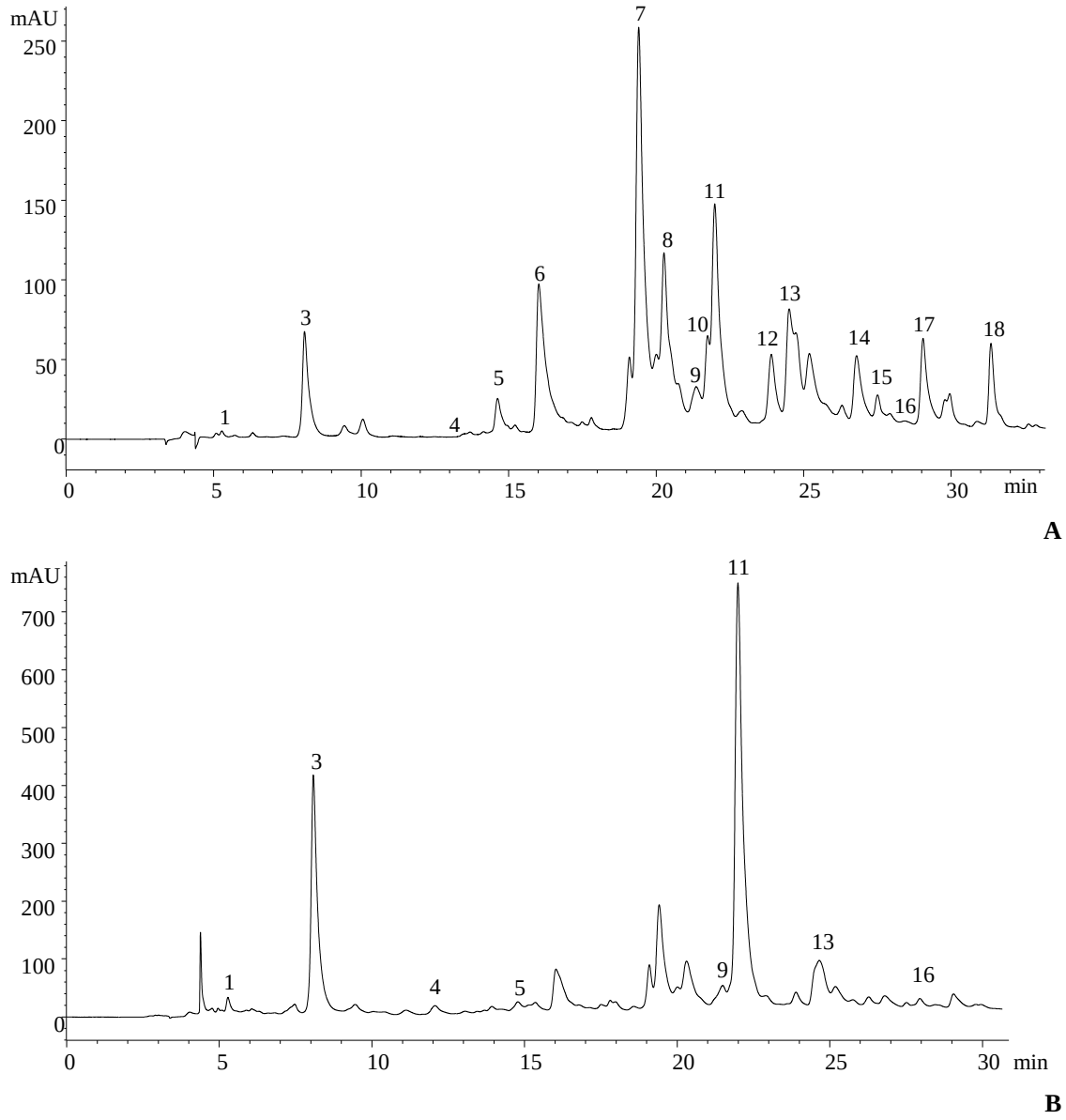

Figure 1. HPLC phenolic profile of *Helichrysum stoechas* hydroalcoholic extract recorded at 370 nm (A) and 280 nm (B).

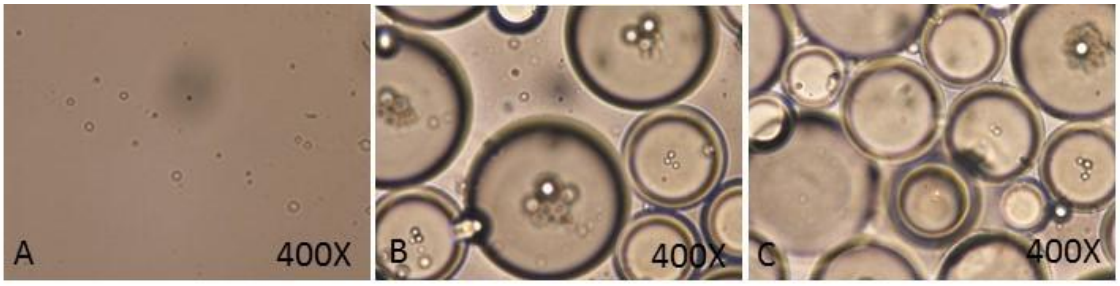

Figure 2. Optical microscopy analysis with magnification of 400X of: the w/o emulsion (A), w/o/w emulsion immediately after its preparation (B) and after 10 minutes under stirring at 400 rpm (C).

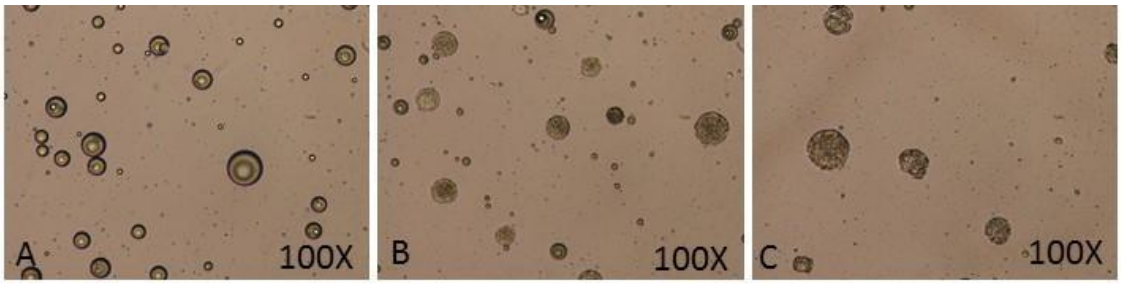

Figure 3. Microspheres morphology evolution during the solvent evaporation process (magnification of 100X): (A) after one hour under evaporation at 40 ºC and atmospheric pressure, (B) after two hours under evaporation at 40 ºC and atmospheric pressure, and (C) after one additional hour at 40 ºC under reduced pressure.

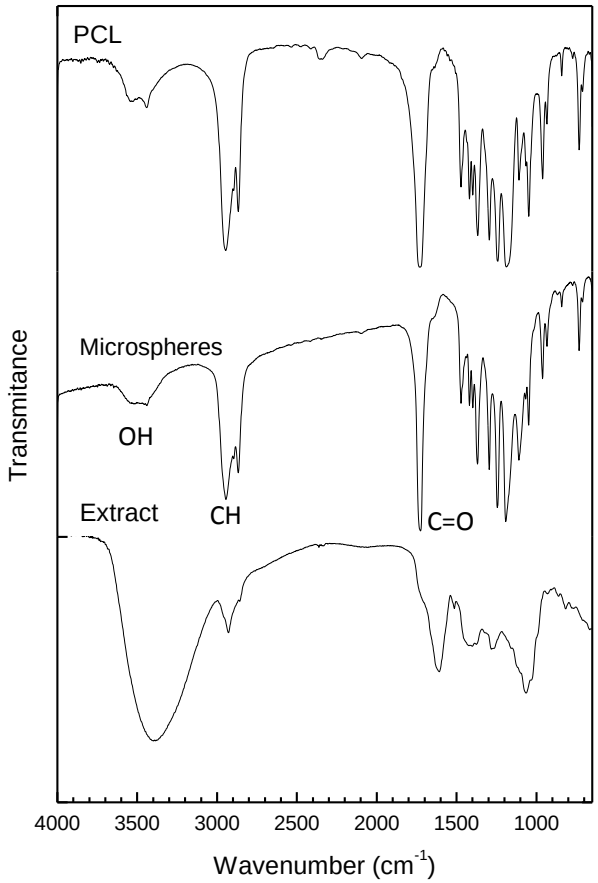

Figure 4. FTIR spectra of polycaprolactone (PCL), lyophilized extract and produced microspheres.

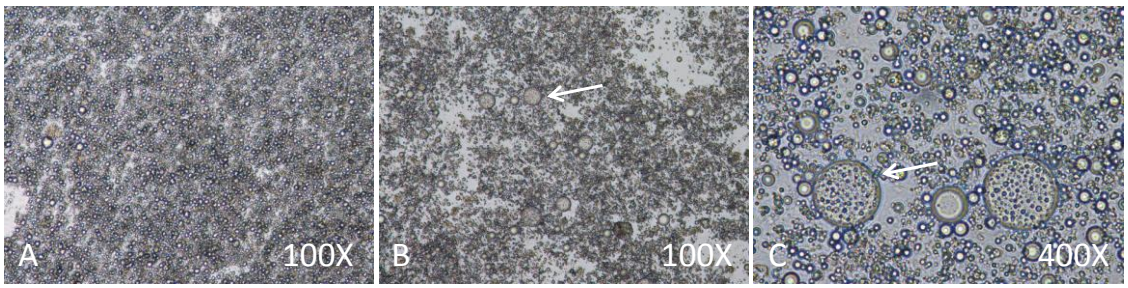

Figure 5. Microspheres incorporation in a moisturizer. (A) Base moisturizer, (B) moisturizer with incorporated microspheres under 100X magnification, and (C) moisturizer with incorporated microspheres under 400X magnification. The blank arrow puts in evidence the incorporated microspheres structures.