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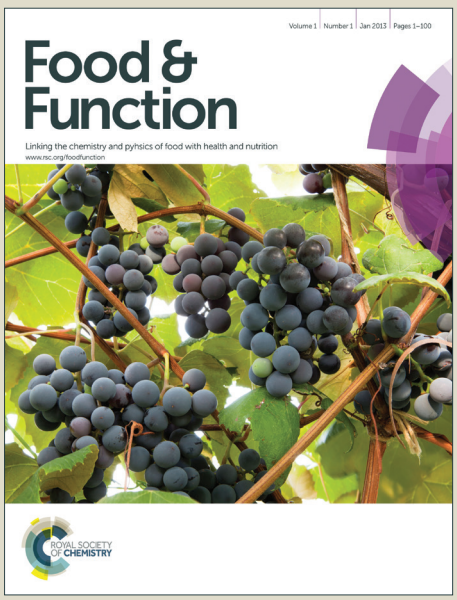

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Phenolic extracts of *Rubus ulmifolius* Schoot flowers: characterization, microencapsulation and incorporation into yogurts as nutraceutical sources

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Abstract

Rubus ulmifolius Schoot (Rosaceae), known as wild blackberry, is a perennial shrub found in wild and cultivated habitats in Europe, Asia and North Africa. Traditionally, it is used for homemade remedies because of its medicinal properties, including antioxidant activity. In the present work, phenolic extracts of *R. ulmifolius* flower buds obtained by decoction and hydroalcoholic extraction, were chemically and biologically characterized. Several phenolic compounds were identified in both decoction and hydroalcoholic extract of flowers, being ellagitannin derivatives the most abundant ones, namely sanguiin H-10 isomer and lambertianin. Additionally, and comparatively with the decoction form, the hydroalcoholic extract presented both higher phenolic content and antioxidant activity. The hydroalcoholic extract was thereafter microencapsulated in an alginate-based matrix and incorporated into a yogurt to achieve antioxidant benefits. In what concerns the performed incorporation tests, the obtained results pointed out that, among the tested samples, the yoghurt containing the microencapsulated extract presented a slightly higher antioxidant activity, and that both forms (free and microencapsulated extract) gave rise to products with higher activity than the control. In conclusion, this study demonstrated the antioxidant potential of *R. ulmifolius* hydroalcoholic extract and the effectiveness of the used microencapsulation technique for its preservation, thus opening new perspectives for the exploitation of these natural phenolic extracts in food applications.

Keywords: antioxidant activity, decoction, microencapsulation, phenolic compounds, *Rubus ulmifolius*

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Introduction

Reactive oxygen and nitrogen species are formed during normal cellular metabolism, but when presented in high concentration they become toxic being this effect related to several chronic diseases such as cancer, cardiovascular and neurodegenerative diseases.[1,2] Exposure to those species from a variety of sources has led the organism to develop defense mechanisms (endogenous defenses) in order to protect the cells against excessive levels of free radicals. Antioxidant defenses can be enzymatic and non-enzymatic. Examples of enzymatic defenses are superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), and glutathione reductase (GSH-R).[2,3] The endogenous non-enzymatic antioxidant defenses include glutathione (GSH), α-tocopherol (vitamin E), ascorbic acid (vitamin C) and lipoic acid.[1,3]

Exogenous antioxidant defenses supplied by diet have gained special interest, namely the use of phenolic compounds from plants. In fact, plants are a natural source of effective bioactive phenolic compounds. Beneficial activities of these compounds include risk reduction of cardiovascular and neurodegenerative diseases, diabetes or osteoporosis. The slower progression of certain cancers is another benefit, enabling plant polyphenols as potential chemopreventive and anti-cancer agent in humans.[4]

Rubus ulmifolius Schoot (Rosaceae), known as wild blackberry, is a perennial shrub found in wild and cultivated habitats in Europe, Asia and North Africa.[5] Traditionally, *R. ulmifolius* is regarded as an interesting medicinal plant and considered to be anticatarrhal, antiseptic, diuretic, anti-inflammatory, antioxidant, astringent, and antispasmodic.[6,7] Decoctions from dry flower buds are used for diarrhea, menstrual pain, menopause disorders, liver diseases, aphtha, gingivitis, hypertension and diabetes.[8]

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The antioxidant properties of methanolic extract from *R. ulmifolius* flowers were previously reported[9] but not for hydroalcoholic extract or for its most used form, decoction. The mentioned extracts could be included in formulations of nutraceuticals or functional foods due to their attractive bioactive properties.

Microencapsulation is a technique that allows bioactive compounds/extracts to be incorporated into a matrix or coating shell in the form of microparticles with diameters ranging from 1 to 1000 micrometers.[10] These microparticles can release their contents along with time by means of different release mechanisms, which are dependent from the used encapsulation materials, production process, final morphology and application. This technology has been used in several fields including pharmaceutical, food and cosmetic. Encapsulation of natural extracts can provide protection against the action of atmospheric agents (light, moisture and heat), ensuring an increase of their stability and thus a control of their bioavailability.[10] There are several documented examples of the application of this technique with natural extracts for production of functional foods. For example, Krishnaiah et al.[11] studied the *Morinda citrifolia* L. fruit extract encapsulation in κ-carrageenan and maltodextrin matrices. This extract is recognized for its antibiotic and antioxidant proprieties due to the presence of high phenolic compounds content. In addition, microcapsules production from cactus pear fruits (*Opuntia ficus-indica*) extracts represents an interesting food additive due to the presence of antioxidants and as a red colorant.[12]

The effective incorporation of microencapsulated natural extracts in foods was performed by Çam et al.[13] and Ezhilarasi et al.[14] by testing the incorporation of microencapsulated *Punica granatum* L. peel and *Garcinia cowa* Roxb. fruit extracts in ice-cream and bread, respectively.

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The present study aimed to characterize the phenolic compounds present in the hydroalcoholic extract and decoction of *R. ulmifolius* flower buds, and to evaluate their antioxidant potential. Furthermore, the hydroalcoholic extract in its lyophilized form was microencapsulated in an alginate matrix by an atomization/coagulation technique. Additionally, an equivalent amount of *R. ulmifolius* hydroalcoholic extract, free and microencapsulated, was added to yogurt samples and its antioxidant activity was evaluated and compared with a control. The results obtained showed the antioxidant potential of *R. ulmifolius* hydroalcoholic extract and the effectiveness of the microencapsulation technique to preserve the antioxidant activity, thus opening new perspectives for the exploitation of these natural phenolic extracts for nutraceutical applications.

Experimental

Plant material

Samples of flower buds from different specimens of *Rubus ulmifollius* Schoot randomly selected were collected in late spring of 2009, in the Natural Park of Montesinho territory, Trás-os-Montes, North-eastern Portugal, considering the Portuguese folk pharmacopoeia, the local medicinal criteria of use and the plants growth patterns. Morphological key characters from the Flora Iberica[15] were used for plant identification. Voucher specimens are deposited in the Herbário da Escola Superior Agrária de Bragança (BRESA). The samples were lyophilized (FreeZone 4.5, Labconco, Kansas, USA), reduced to powder (~20 mesh) and kept in the best conditions for subsequent use.

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116

117 **Standards and reagents**

118 HPLC-grade acetonitrile was obtained from Merck KgaA (Darmstadt, Germany).
119 Formic, acetic were purchased from Prolabo (VWR International, France). Trolox (6-
120 hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), was purchased from Matreya
121 (PA, USA). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar
122 (Ward Hill, MA, USA). Phenolic compound standards were from Extrasynthèse
123 (Genay, France). Alginic acid sodium was obtained from Fluka Chemie, Calcium
124 chloride 2-hydrate were purchased from (Panreac Química S.A.U).
125 All other chemicals were of analytical grade and purchased from chemical suppliers.
126 Water was treated in a Milli-Q water purification system (TGI Pure Water Systems,
127 USA).

128

129 **Extraction procedures for phenolic compounds**

130 An hydroalcoholic extraction was performed using the lyophilized plant material (1 g)
131 stirring with 30 mL of methanol:water (80:20, *v/v*) at 25 ºC at 150 rpm for 1 h and
132 filtered through Whatman No. 4 paper. The residue was then extracted with one
133 additional 30 mL portion of the hydroalcoholic mixture. The combined hydroalcoholic
134 extracts were evaporated at 35 ºC under reduced pressure (rotary evaporator Büchi R-
135 210, Flawil, Switzerland) and then further lyophilized (FreeZone 4.5, Labconco,
136 Kansas, USA).
137 A decoction was also prepared from the lyophilized plant material (1 g), by adding 200
138 mL of distilled water, heating (heating plate, VELP scientific) and boiling for 5 min.

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The mixture was left to stand for 5 min and then filtered under reduced pressure. The obtained decoction was frozen and lyophilized.

The hydroalcoholic extract and lyophilized 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.

Characterization of the extracts in 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.

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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 1700.

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: catechin (y=158.42x-11.38; R^2=0.9999); chlorogenic acid (y=600.27x–763.62; R^2=0.9998); *p*-coumaric acid (y=884.6x+184.49; R^2=0.9999); ellagic acid (y=32.72x+77.8; R^2=0.9999); 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); 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).

Evaluation of *in vitro* antioxidant properties

DPPH radical-scavenging activity

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This methodology was performed using an ELX800 Microplate Reader (Bio-Tek, Bedfordshire, UK). The reaction mixture in each one of the 96-wells consisted of one of the different concentration solutions (30 µL) and methanolic solution (270 µL) containing DPPH radicals (6×10^{-5} mol/L). The mixture was left to stand for 30 min in the dark. The reduction of the DPPH radical was determined by measuring the absorption at 515 nm. The radical scavenging activity (RSA) was calculated as a percentage of DPPH discolouration using the equation: RSA (%) = $[(A_{DPPH}-A_S)/A_{DPPH}] \times 100$, where A_S is the absorbance of the solution when the sample extract has been added at a particular level, and A_{DPPH} is the absorbance of the DPPH solution.[9] The extract concentration providing 50 % of antioxidant activity (EC_{50}) was calculated from the graph of DPPH scavenging activity against extract concentrations. Trolox was used as standard.

Reducing power

This methodology was performed using the Microplate Reader described above. The different concentration solutions (0.5 mL) were mixed with sodium phosphate buffer (200 mmol/L, pH 6.6, 0.5 mL) and potassium ferricyanide (1% w/v, 0.5 mL). The mixture was incubated at 50 ºC for 20 min, and trichloroacetic acid (10% w/v, 0.5 mL) was added. The mixture (0.8 mL) was poured in the 48-wells, as also deionised water (0.8 mL) and ferric chloride (0.1% w/v, 0.16 mL), and the absorbance was measured at 690 nm.[9] The extract concentration providing 0.5 of absorbance (EC_{50}) was calculated from the graph of absorbance at 690 nm against extract concentrations. Trolox was used as standard.

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Inhibition of β-carotene bleaching

A solution of β-carotene was prepared by dissolving β-carotene (2 mg) in chloroform (10 mL). Two millilitres of this solution were pipetted into a round-bottom flask. After the chloroform was removed at 40 ºC under vacuum, linoleic acid (40 mg), Tween 80 emulsifier (400 mg), and distilled water (100 mL) were added to the flask with vigorous shaking. Aliquots (4.8 mL) of this emulsion were transferred into different test tubes containing different concentrations of the samples (0.2 mL). The tubes were shaken and incubated at 50 ºC in a water bath. As soon as the emulsion was added to each tube, the zero time absorbance was measured at 470 nm in a spectrophotometer (AnalytikJena, Jena, Germany). β-Carotene bleaching inhibition was calculated using the following equation: (Abs after 2h of assay/initial Abs) × 100.[9] The extract concentration providing 50% of antioxidant activity (EC_{50}) was calculated from the graph of β-carotene bleaching inhibition against extract concentrations. Trolox was used as standard.

Inhibition of lipid peroxidation using thiobarbituric acid reactive substances (TBARS)

Porcine (*Sus scrofa*) brains were obtained from official slaughtering animals, dissected, and homogenized with a Polytron in ice-cold Tris–HCl buffer (20 mM, pH 7.4) to produce a 1:2 (w/v) brain tissue homogenate which was centrifuged at 3000*g* for 10 min. An aliquot (0.1 mL) of the supernatant was incubated with the different solution concentrations (0.2 mL) in the presence of $FeSO_4$ (10 μM; 0.1 mL) and ascorbic acid (0.1 mM; 0.1 mL) at 37 ºC for 1 h. The reaction was stopped by the addition of trichloroacetic acid (28% w/v, 0.5 mL), followed by thiobarbituric acid (TBA, 2%, w/v, 0.38 mL), and the mixture was then heated at 80 ºC for 20 min. After centrifugation at 3000*g* for 10 min to remove the precipitated protein, the colour intensity of the

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malondialdehyde (MDA)-TBA complex in the supernatant was measured by its absorbance at 532 nm. The inhibition ratio (%) was calculated using the following formula: Inhibition ratio (%) = [(A – B)/A] × 100 %, where A and B were the absorbance of the control and the compound solution, respectively.[9] The extract concentration providing 50% of antioxidant activity (EC_{50}) was calculated from the graph of TBARS formation inhibition against extract concentrations. Trolox was used as standard.

Microencapsulation of the *R. ulmifolius* hydroalcoholic extract

Microspheres containing lyophilized *R. ulmifolius* hydroalcoholic extract were prepared by atomization/coagulation technique. Briefly, sodium alginate was used as the matrix material and $CaCl_2$ aqueous solution as coagulation agent. A hydroalcoholic extract solution was prepared by dissolving 50 mg of the lyophilized extract in 10 mL of distilled water under stirring. Then, this solution was filtered for residues removal and 400 mg of sodium alginate added. The solution was kept under stirring until complete alginate dissolution was achieved. Thereafter the alginate solution containing the extract was atomized using the NISCO Var J30 system (feed rate of 0.3 mL/min and a nitrogen pressure of 0.1 bar) to produce the microspheres. The atomized microspheres were immediately coagulated by contacting with a $CaCl_2$ aqueous solution (250 mL at a concentration of 4% (w/v)), during 4 hours. The resulting microspheres were collected by filtration under reduced pressure and washed twice with distilled water. The obtained microspheres were then lyophilized and stored in dark conditions at 4 ºC.

Microspheres characterization

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Microspheres 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 microspheres after the production and coagulations stages, respectively. It was also possible to infer the presence/absence of extract inside of the microspheres.

The effective extract incorporation into the alginate matrix was inspected by FTIR analysis. For that purpose, spectra of pure alginate, free hydroalcoholic extract of *R. ulmifolius* and the 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} by co-adding 48 scans.

The dry residue (DR) and encapsulation efficiency (EE) were also evaluated. DR was calculated as the ratio between the dry (lyophilized) and the wet microspheres weight (%, w/w). EE evaluation was done through the quantification of the non-encapsulated extract. For that purpose the remaining extract in the coagulation and the in the first washing solution were quantified by HPLC and added. The second washing solution was found to be absent of extract.

The encapsulation efficiency was calculated according to the following expression:

$$EE = [(M_{e\text{-}t}\text{-}M_{e\text{-}ne})/(M_{e\text{-}t})]\times 100$$

In which $M_{e\text{-}t}$ represents the theoretical amount of extract, i.e. the amount of extract used in the microencapsulation process. $M_{e\text{-}ne}$ corresponds to the non-encapsulated extract remaining after encapsulation process (determined by HPLC as previously described). Since the extract corresponds to a complex mixture of several components, the two major compounds derived from the ellagic acid, sanguiin and lambertianin were chosen as the model chemical species to be quantified for EE evaluation purposes.

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284 **Incorporation of free and microencapsulated hydroalcoholic extract of *R.***
285 ***ulmifolius* in a yogurt**

286 The chosen food matrix was a natural yogurt without added sugar and 3.5% (w/w) of
287 fat. The yoghurt (140 g) was placed in a glass container and mixed in order to ensure
288 homogeneity to produce all the samples needed for the assays. Then, this mass was
289 divided into six portions for the preparation of the following samples: two samples of
290 pure yogurt with 25 g each (used as control sample), two samples of yogurt with 25 g
291 each for free extract incorporation (6.25 mg of extract in each one) and two samples of
292 yogurt with 20 g for microencapsulated extract incorporation (40 mg of lyophilized
293 microspheres in each one). The samples were prepared taking into consideration the use
294 of the same extract/yogurt ratio (0.25 mg/g).

295 The antioxidant activity was evaluated at two different sampling times, namely: at
296 initial time (t=0), i.e. immediately after the addition of free or microencapsulate extract,
297 and after 3 days (t=3). The collected samples at t=0 and t=3 were then lyophilized and
298 conditioned for future analysis. The used tests for antioxidant activity evaluation were:
299 DPPH radical scavenging activity and reducing power. The procedures used are
300 described in a previous section (DPPH radical - scavenging activity and Reducing
301 power).

302

303 **Statistical analysis**

304 All the assays were carried out in triplicate and the results are expressed as mean values
305 and standard deviation (SD). The results were analyzed using one-way analysis of

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306 variance (ANOVA) followed by Tukey's HSD Test with α = 0.05, performed with
307 SPSS v. 18.0 program.

308
309

310 Results and discussion

311

312 Phenolic profile of the hydroalcoholic extract and decoction

313 The HPLC phenolic profile of *Rubus ulmifolius*, obtained after hydroalcoholic
314 extraction, and recorded at 280 and 370 nm is shown in **Figure 1**; peak characteristics,
315 identities and quantification are presented in **Table 1**. Twenty-four phenolic compounds
316 were identified in both samples, in which seven were identified as phenolic acid
317 derivatives (di- and caffeolyquinic, *p*-coumaroylquinic, feruloylquinic acids and ellagic
318 acid), eleven as flavonoids (quercetin and kaempferol derivatives and catechin), and six
319 as hydrolyzable tannins (lambertianin, sanguiin and four di-hexahydroxydiphenol
320 (HHDP)-galloyl glucose isomers).
321 Quinic acid derivatives were the main phenolic acids identified, according to their UV
322 (λ_{max} at 314-330 nm) and mass spectra (pseudo molecular ions $[M-H]^-$ at *m/z* 337, 353
323 and 367, all of them yielding a product ion at *m/z* 191, due to the deprotonated quinic
324 acid). Peak 1, the major phenolic acid derivative found, and peak 2 were identified as 3-
325 *O*-caffeoylquinic acid and 3-*p*-coumaroylquinic acid, respectively. Peak 1 yielded
326 deprotonated quinic acid (*m/z* at 191) as base peak and another majority ion
327 corresponding to the hydroxycinnamic acid residue at *m/z* 179 ([caffeic acid-H]$^-$), and
328 peak 2 presented *m/z* 163 ([*p*-coumaric acid-H]$^-$) as base peak, a fragmentation pattern
329 characteristic of the corresponding 3-acylquinic acids according to Clifford et al.[16, 17].
330 Similarly, peak 3 was tentatively identified as 3-*O*-feruloylquinic acid taking into

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account its pseudomolecular ion and fragmentation pattern, yielding a majority ion at *m/z* 193 ([ferulic acid-H]$^-$) as base peak. Peak 19 ([M-H]$^-$ at *m/z* 515) was assigned to 3,5-*O*-dicaffeoylquinic acid based on its elution order and mass spectra characteristics. The 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]$^-$ (~60% base peak).[16,17] Peak 4 presented a UV spectra similar to *p*-coumaric acid, with λ_{max} around 313 nm; the peak area was very small and did not allow obtaining a clear pseudo-molecular ion, although signals at *m/z* 163 ([coumaric acid-H]$^-$) and *m/z* 119 ([coumaric acid-CO_2-H]$^-$) were observed at its retention time, which allowed assigning as a *p*-coumaroyl derivative.

Peak 18 corresponds to ellagic acid and was positively identified according to its retention, mass and UV-vis characteristics by comparison with the commercial standard; this peak was only found in the decoction preparation.

Regarding flavonoids, mainly flavonol derivatives (**Table 1**) were found. Catechin (peak 5), quercetin 3-*O*-rutinoside (peak 13), quercetin 3-*O*-glucoside (peak 15), kaempferol 3-*O*-rutinoside (peak 20) and kaempferol 3-*O*-glucoside (peak 23) were positively identified according to their retention, mass and UV-vis characteristics by comparison with commercial standards. Peaks 14 and 16 presented UV spectra with λ_{max} around 350 nm and an MS^2 product ion at *m/z* 301 indicating that they correspond to quercetin derivatives. According to their pseudo molecular ions [M-H]$^-$ at *m/z* 477 and 463, they were identified as quercetin 3-*O*-glucuronide (peak 14), which was confirmed by comparison with a standard isolated in our laboratory[18], and a quercetin 3-

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355 *O*-hexoside (peak 16). Similar reasoning also allowed assigning peaks 21 and 22 as
356 kaempferol 3-*O*-glucuronide and kaempferol 3-*O*-hexoside, respectively. Peaks 17 and
357 24 should correspond to kaempferol *O*-pentosyl hexoside and kaempferol *O*-
358 acetylhexoside according to their pseudomolecular ions ($[M-H]^-$ at *m/z* 579 and 489,
359 respectively) and MS^2 fragment released at *m/z* 285 (quercetin; $[M-H-132-162]^-$ loss of
360 a pentosyl-hexoside moiety and $[M-H-42-162]^-$, loss of an acetylhexoside moiety,
361 respectively).
362 The remaining detected compounds corresponded to hydrolyzable ellagitannins. Peaks 6
363 and 8 presented the same pseudo molecular ion $[M-H]^-$ at *m/z* 1567, which produced
364 MS^2 fragment ions at *m/z* 1265 (by loss of a hexahydroxydiphenoyl group, HHDP), m/z
365 1103 (loss of HHDP and glucosyl moieties), *m/z* 933 (further loss of a gallate unit), *m/z*
366 631 (loss of an additional HHDP group) and *m/z* 301 (HHDP released after final loss of
367 glucosyl-gallate). The signal detected at *m/z* 783 would correspond to the pseudo
368 molecular doubly charged ion $[M-H]^{-2}$, as established by zoom scan analysis. These
369 characteristics were coherent with the structure of sanguiin H-10 (**Figure 2b**);[19, 20] the
370 observation of two peaks might be due to different configurations in the glucose units,
371 either α- or ß-, as previously observed by Kool et al.[20]. Thus, peaks 6 and 8 were
372 identified as sanguiin H-10 isomers. Peak 7 presented a pseudo molecular ion $[M-H]^{-2}$ at
373 *m/z* 1401 that was doubly charged as showed by zoom scan analysis and its MS^2
374 fragmentation released singly charged product ions at *m/z* 1235, 933, 631 and 301.
375 These characteristics were coherent with the trimeric ellagitannin lambertianin C
376 (**Figure 2c**), composed of three galloyl-bis-HHDP glucose units (molecular mass of
377 2085.8 Da, out of the analyzed *m/z* range), previously described in blackberry fruits[21, 22]
378 and other *Rubus* species.[19, 20, 23] Peaks 9-12 presented a pseudo molecular ion $[M-H]^-$ at

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379 *m/z* 935, releasing MS^2 product ions at *m/z* 633 and 301, likely due to the loss of HHDP
380 and galloyl-glucose moieties, which is consistent with galloyl-bis-HHDP-glucose[21, 24]
381 and allowed their identification as different galloyl-bis-HHDP glucose isomers (**Figure**
382 **2a**).
383 The phenolic profile of both preparations was identical, varying mostly in the
384 concentrations found (**Table 1**). The hydroalcoholic extract presented higher
385 concentration in total phenolic compounds (240.48 mg/g extract) than the decoction,
386 mainly due to the higher concentration in hydrolyzable tannins (203.39 mg/g extract).
387 The most abundant compounds found in both preparations were ellagitannin derivatives,
388 such as a sanguiin H-10 isomer and lambertianin C. These same compounds have also
389 been reported as relevant phenolic compounds in *Rubus* fruits, including blackberries,
390 by other authors.[19-25] Besides ellagitannins, fruits of *Rubus* species are also known to
391 contain some amounts of flavonoids such as quercetin and kaempferol based flavonol
392 conjugates, with the major components being quercetin 3-*O*-glucuronide and quercetin
393 3-*O*-glucoside[26], as well as ellagic acid, which were also found in the herein studied
394 flowers of *R. ulmifolius*. Ellagic acid has been reported to have antiviral activity and
395 provide protection against cancers of the colon, lung, and esophagus, and the health
396 benefits of raspberry consumption have been promoted on the basis of claims of a high
397 ellagic acid and ellagitannin content.[27] To our knowledge, this is the first time that these
398 compounds were identified and quantified in *R. ulmifolius* flower buds.
399
400 ***In vitro* antioxidant properties of the hydroalcoholic extract and decoction**
401 The results obtained in the evaluation of the antioxidant activity of the hydroalcoholic
402 extract and decoction of *R. ulmifolius* are given in **Table 2**. The hydroalcoholic extract

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gave higher antioxidant activity (lower EC_{50} values) in all the *in vitro* assays (EC_{50} values between 34.23 and 1.58 µg/mL) than the decoction preparation (EC_{50} values ranging from 201.72 and 184.21 µg/mL). This is in agreement with the higher phenolic compounds concentration (240.48 mg/g) found in the hydroalcoholic extract in comparison to the decoction preparation (177.44 mg/g).

The methanol extract previously studied by our research group[9] presented slightly higher EC_{50} values ($\leq$ 40 µg/mL) and, therefore, lower antioxidant activity. There are various studies that report the antioxidant activity of fruits of *Rubus* species and one specific[28] that studied the antioxidant activity of *R. ulmifolius* leaves using ABTS radical decolourisation assay. Nevertheless, to our knowledge, there are no reports available on the decoction preparation of the mentioned plant or in its hydroalcoholic extract.

Production of alginate microspheres containing *R. ulmifolius* hydroalcoholic extract

Alginate-based microspheres containing lyophilized *R. ulmifolius* hydroalcoholic extract were prepared by using an atomization/coagulation technique. The produced microspheres were analyzed by MO immediately after the atomization and 4 under coagulating stage (**Figure 3**). Microspheres, in both stages, showed a spherical shape and were perfectly individualized without the presence of agglomerates. Their estimated size was comprised between 79 and 380 µm. In addition, the microspheres presented a lightly homogeneous pink color characteristic of the extract, indicating its incorporation and good distribution inside the microsphere. HPLC analysis of ellagic acid derivatives (sanguiin H-10 and lambertianin C), both in the coagulation and in the first wash

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solutions, showed that these compounds were present in residual concentrations (below the detection limit) or absent. This data allowed an encapsulation efficiency estimation close to 100%.

Microspheres rehydration after lyophilisation

The lyophilized microspheres were rehydrated in distilled water for a period of 24 hours in order to test the initial morphology recovery. **Figure 4** shows the OM analysis of the dried and rehydrated microspheres at magnifications of 40, 100 and 400x. As it can be seen, the final size of the rehydrated microspheres is close to the one of the initial microspheres (before the lyophilization) showing their good rehydration capacity. The water recovery after the 24 hours was 80% of the originally hydrated microspheres (obtained after production).

Fourier Transform Infrared Spectroscopy (FTIR)

The FTIR spectra are shown in **Figure 5** (pure alginate, pure extract and microspheres containing the lyophilized extract). As expected, the microspheres spectrum is dominated by the presence of alginate since a ratio extract/alginate of 100/800 (w/w) was used (see major contributions indicated by the dotted blue line). However, in the microspheres spectrum it is possible to note the contribution from the carbonyl (C=O) and hydroxyl groups (OH) of the extract (indicated by the dashed red lines). The widening of the OHs and C=Os bands can be explained due to the previously stated and represent an evidence of the presence of extract in the microspheres.

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Incorporation of free and microencapsulated hydroalcoholic extract of *R. ulmifolius* in a yogurt

Table 3 shows the obtained results for the antioxidant activity evaluated according to two parameters: DPPH radical - scavenging activity and reducing power. Both forms (microencapsulated and free extract) showed greater activity than the control (EC_{50} lower values for both DPPH radical scavenging activity and reducing power). The results showed that the extract also led to microencapsulated products with better preservation of the antioxidant activity over time (in both tests the EC_{50} values decreased from time 0 to time 3 days).

Figure 6 shows the images of the microspheres incorporated into the yogurt at the initial time (t=0), immediately after the addition of the microencapsulated extract and after 3 days (t=3) at a magnification of 40, 100 and 400x. The image analysis for t=3 days shows that the microspheres preserve their initial morphology, no microspheres disaggregation was noticed. This fact corroborates also the protective effect of the alginate matrix.

Conclusion

In summary, the characterization of *R. ulmifolius* flower buds extracts obtained by decoction and hydroalcoholic extraction revealed the presence of twenty-four phenolic compounds, being ellagitannin derivatives the most abundant ones, namely sanguiin H-10 isomer and lambertianin C. Comparatively with the decoction form, the hydroalcoholic extract presented higher antioxidant activity, which can be correlated with its higher phenolic compounds content. The atomization/coagulation microencapsulation technique was successfully applied to produce microspheres

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containing *R. ulmifolius* hydroalcoholic extract which open new avenues for the exploitation of these phenolic extracts in applications such as the food industry. As a preliminary approach the produced microspheres were incorporated into a natural yogurt indicating that, comparatively with its free form, the microencapsulated one is able to better preserve the extract antioxidant activity along time. In summary, the results demonstrated the potential antioxidant of *R. ulmifolius* hydroalcoholic extract and the efficiency of microencapsulation for its preservation.

Competing interests

The authors declare no competing financial interest.

Acknowledgement

The authors are grateful to Fundação para a Ciência e a Tecnologia (FCT, Portugal) for financial support to CIMO (strategic project PEst-OE/AGR/UI0690/2011) and LSRE (strategic project PEst-C/EQB/LA0020/2011). L. Barros thanks FCT, POPH-QREN and FSE for her researcher contract under "Programa Compromisso com Ciência-2008". The GIP-USAL is financially supported by the Spanish Government through the *Consolider-Ingenio 2010* Programme (FUN-C-FOOD, CSD2007-00063).

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Table 1. Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, identification and quantification (mg/g extract or decoction) of phenolic compounds in *Rubus ulmifolius*. In each row different letters mean significant differences ($p<0.05$).

Peak	Rt (min)	λ_{max} (nm)	Molecular ion $[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Hydroalcoholic	Decoction
1	5.2	326	353	191(100),179(67),173(6),135(55)	3-*O*-Caffeoylquinic acid	17.83 ± 0.55[a]	13.69 ± 0.63[b]
2	6.9	310	337	191(18),173(6),163(100),119(53)	3-*p*-Coumaroylquinic acid	1.45 ± 0.00[a]	1.18 ± 0.12[b]
3	8.0	326	367	193(100),191(5),173(9),134(35)	3-*O*-Feruloylquinic acid	0.82 ± 0.04[a]	0.62 ± 0.02[b]
4	9.1	313	-	163(8),119(100)	*p*-coumaroly derivative	0.14 ± 0.00[b]	0.70 ± 0.03[a]
5	10.5	278	289	245(93),203(72),137(42)	Catechin	2.83 ± 0.10[a]	2.29 ± 0.37[b]
6	12.1	240	1567	1265(5),1235(4),1103(5),933(27),783(37),631(100),301(11)	Sanguiin H-10 isomer	2.44 ± 0.08[b]	14.43 ± 1.19[a]
7	14.1	242	$[1401]^{2-}$	1235(5),933(11),631(20),301(10)	Lambertianin C	56.73 ± 0.89[a]	25.67 ± 2.69[b]
8	14.9	244	1567	1265(5),1235(12),1103(4),933(100),783(10),631(86),301(4)	Sanguiin H-10 isomer	133.44 ± 2.64[a]	83.81 ± 1.10[b]
9	16.3	256	935	633(21),301(51)	Galloyl-bis-HHDP-glucose isomer	4.50 ± 0.04[b]	5.46 ± 0.05[a]
10	17.1	256	935	633(8),301(24)	Galloyl-bis-HHDP-glucose isomer	4.28 ± 0.04[a]	4.49 ± 0.02[a]
11	17.9	256	935	633(12),301(14)	Galloyl-bis-HHDP-glucose isomer	0.56 ± 0.07[a]	0.37 ± 0.06b
12	18.6	256	935	633(11),301(15)	Galloyl-bis-HHDP-glucose isomer	1.43 ± 0.10[b]	3.63 ± 0.20[a]
13	18.9	354	609	301(100)	Quercetin 3-*O*-rutinoside	0.48 ± 0.03[a]	0.48 ± 0.05[a]
14	19.6	354	477	301(100)	Quercetin 3-*O*-glucuronide	4.52 ± 0.12[a]	4.33 ± 0.10[a]
15	19.9	354	463	301(100)	Quercetin 3-*O*-glucoside	2.54 ± 0.03[a]	2.05 ± 0.27[a]
16	20.2	348	463	301(100)	Quercetin *O*-hexoside	1.34 ± 0.08	nd
17	20.9	348	579	285(100)	Kaempferol *O*-pentosyl hexoside	1.15 ± 0.11[a]	1.11 ± 0.05[a]
18	21.0	251/363	301	284(5),229(6),185(3)	Ellagic acid	nd	5.69 ± 0.28
19	21.9	328	515	353(100),191(90),179(60),173(2),135(27)	3,5-Di-*O*-caffeoylquinic acid	1.04 ± 0.04[b]	1.34 ± 0.16[a]
20	22.3	354	593	285(100)	Kaempferol 3-*O*-rutinoside	0.62 ± 0.11[b]	0.76 ± 0.03[a]
21	22.6	350	447	285(100)	Kaempferol *O*-hexoside	0.51 ± 0.06[a]	0.49 ± 0.01[a]
22	23.5	347	461	285 (100)	Kaempfero *O*-glucuronide	0.99 ± 0.07[a]	1.03 ± 0.06[a]
23	23.8	347	447	285(100)	Kaempferol 3-*O*-glucoside	0.71 ± 0.01[a]	0.74 ± 0.06[a]
24	26.3	354	489	285(100)	Kaempferol acetylhexoside	0.10 ± 0.01[a]	0.10 ± 0.01[a]
					Total phenolic acid derivatives	21.28 ± 0.59[b]	23.21 ± 0.96[a]
					Total hydrolyzable tannins	203.39 ± 3.23[a]	137.85 ± 2.59[b]
					Total flavonoids	14.45 ± 0.44[a]	13.38 ± 0.05[b]

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Table 2. Antioxidant activity of *Rubus ulmifolius* hydroalcoholic extract and decoction (mean ± SD).

Antioxidant activity	Hydroalcoholic	Decoction
DPPH scavenging activity (EC_{50}, μg/mL)	34.23 ± 2.75[a]	184.21 ± 21.40[b]
Reducing power (EC_{50}, μg/mL)	29.27 ± 0.80[a]	191.23 ± 0.58[b]
β-carotene bleaching inhibition (EC_{50}, μg/mL)	3.90 ± 0.46[a]	197.04 ± 4.81[b]
TBARS inhibition (EC_{50}, μg/mL)	1.58 ± 0.07[a]	201.72 ± 3.67[b]

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

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Table 3. Antioxidant activity of yogurt enriched with *Rubus ulmifolius* extract and microencapsulated (mean ± SD).

	Control yogurt		Yogurt with incorporated extract		Yogurt with microencapsulated extract	
	0 days	3 days	0 days	3 days	0 days	3 days
DPPH scavenging activity	91.19 ± 1.24	146.17 ± 5.16	49.34 ± 0.49	49.88 ± 2.31	90.71 ± 3.84	84.15 ± 1.71
Reducing power	2.86 ± 0.01	13.52 ± 0.66	16.34 ± 0.07	2.11 ± 0.12	15.68 ± 0.08	1.42 ± 0.04

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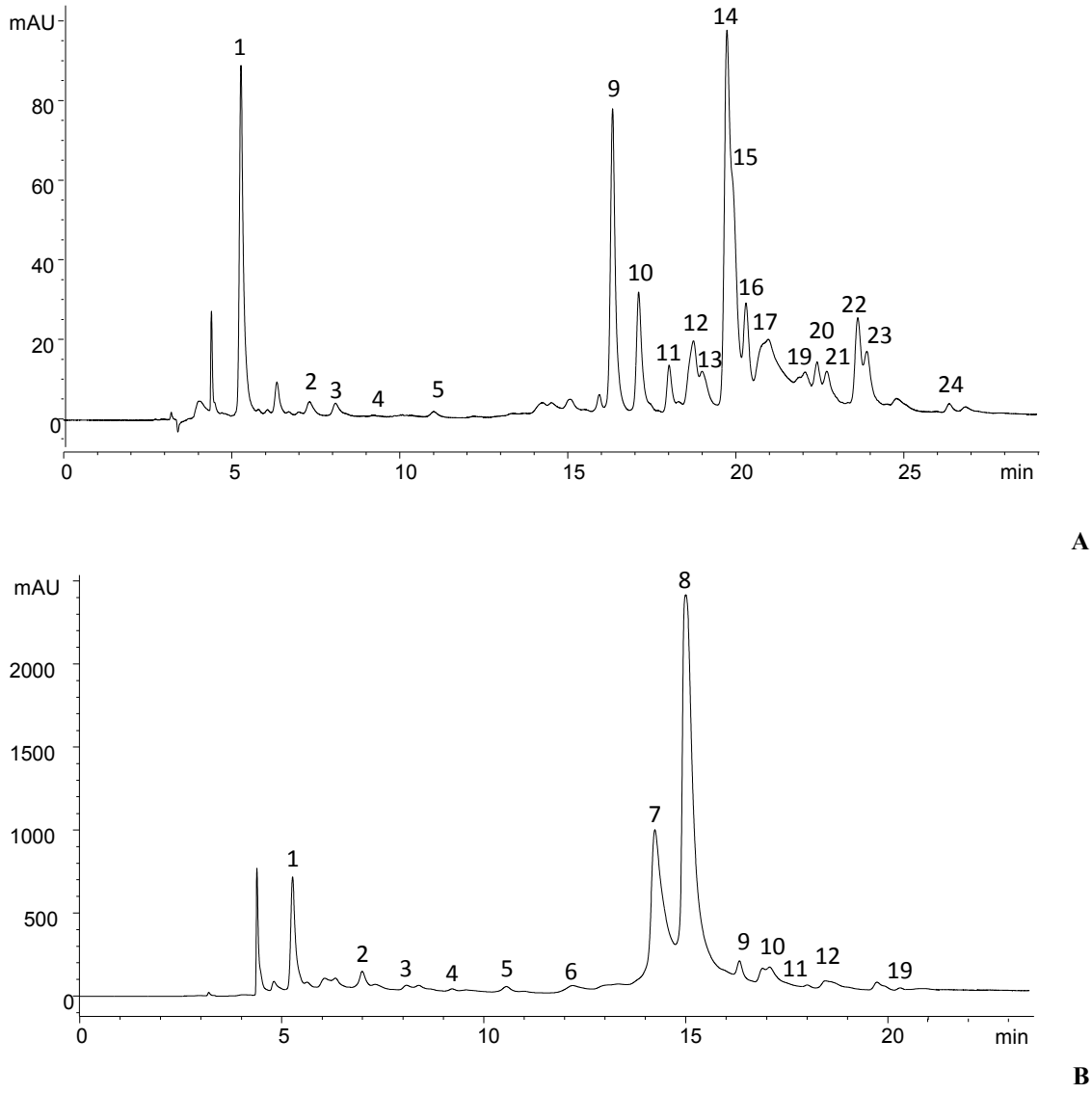

Figure 1. HPLC phenolic profile of *Rubus ulmifolius* hydroachoolic extract, obtained at 370 nm (A) and 280 nm (B).

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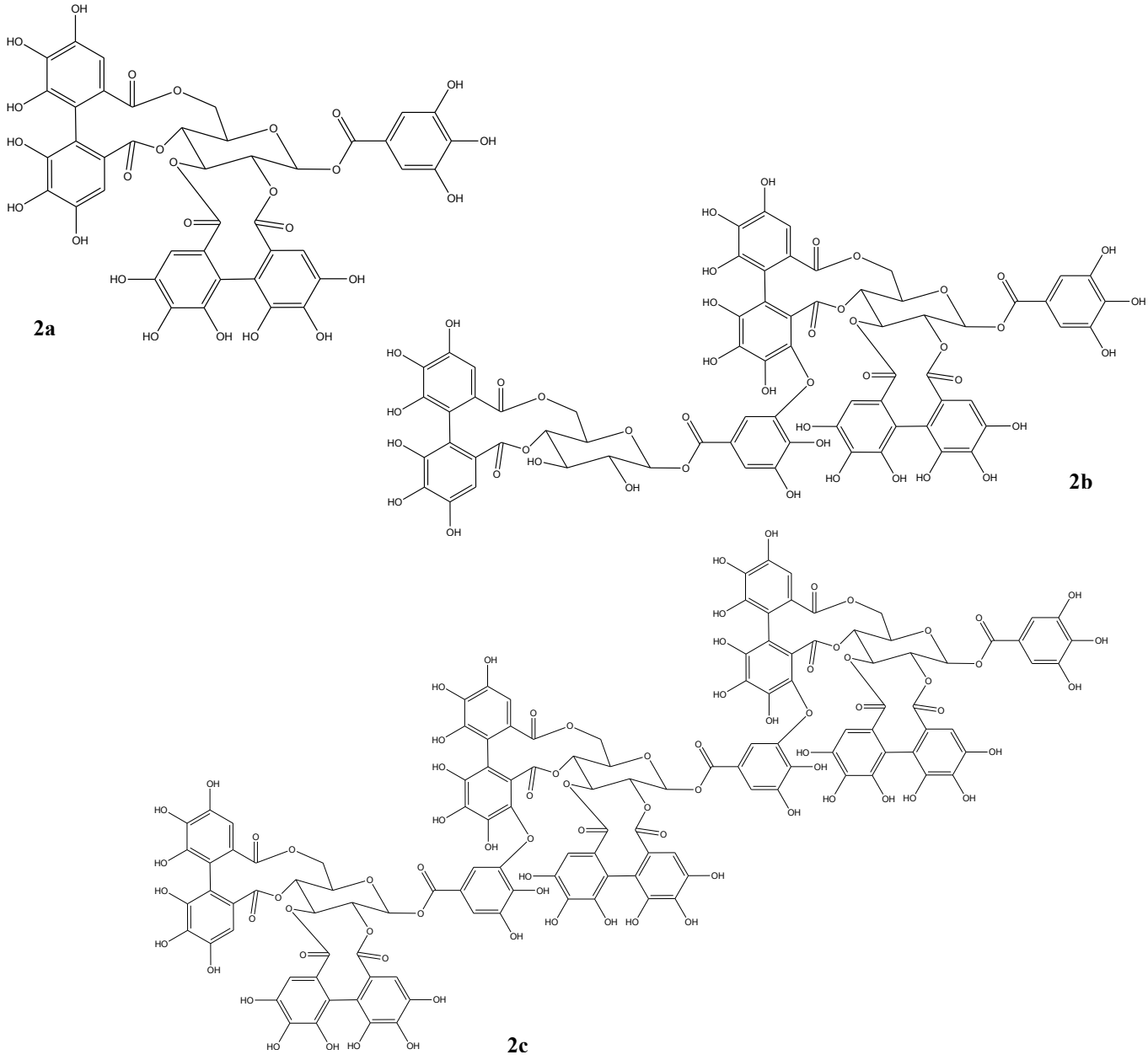

Figure 2. Structures of ellagitannins found in *Rubus ulmifolius*, formed by dimers (sanguiin H-10) (**2b**) and trimers (lambertianin C) (**2c**) of galloyl-bis-HHDP glucose (**2a**).

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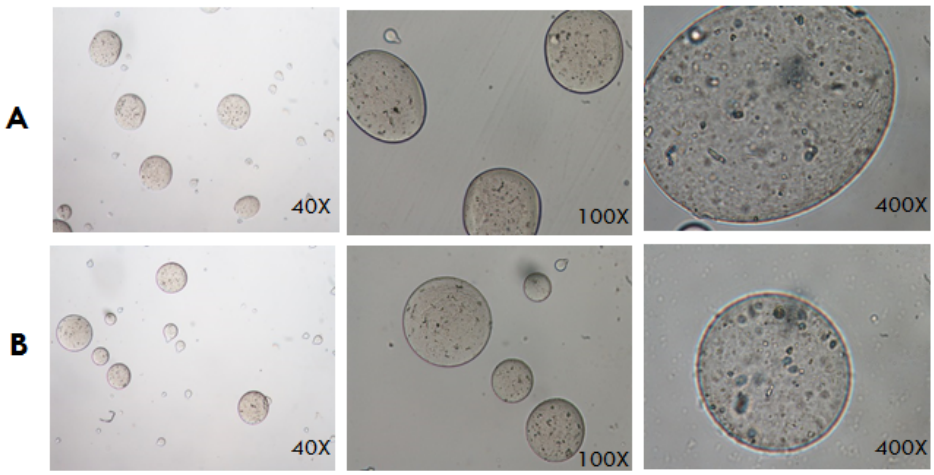

Figure 3. OM analysis with magnification of 40, 100, 400X of: the microspheres immediately after atomization (A), and after 4 hours coagulation period under stirring at 400 rpm (B).

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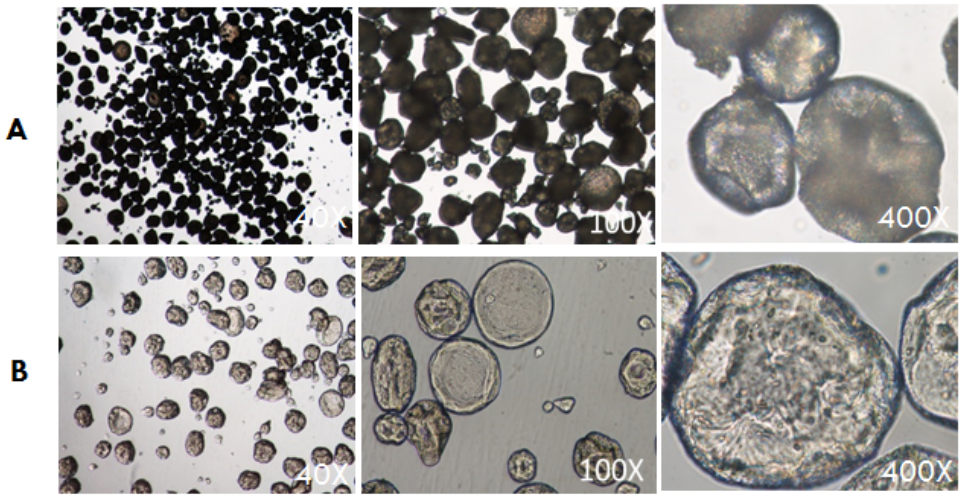

Figure 4. Microspheres morphology analysis by OM under magnifications of 40, 100 and 400X. (A) lyophilized microspheres, (B) microspheres after 24 hours of rehydrating.

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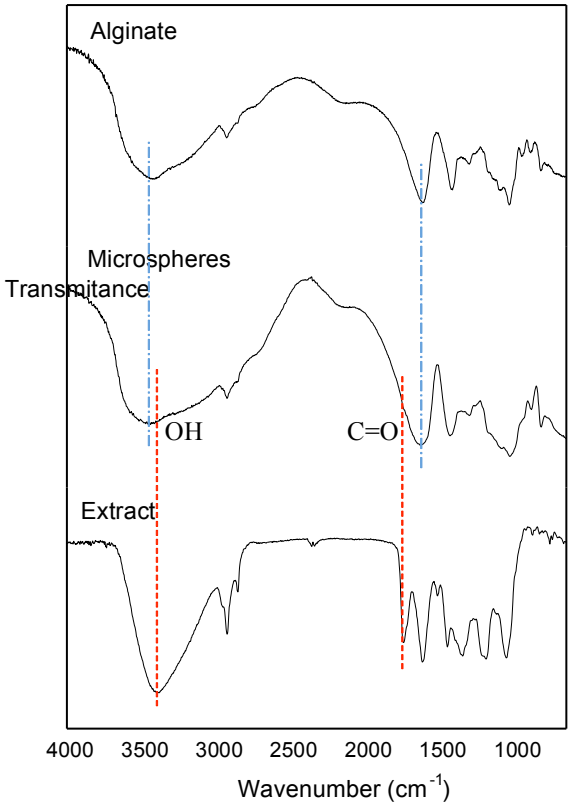

Figure 5. FTIR spectra of alginate, lyophilized extract and produced microspheres.

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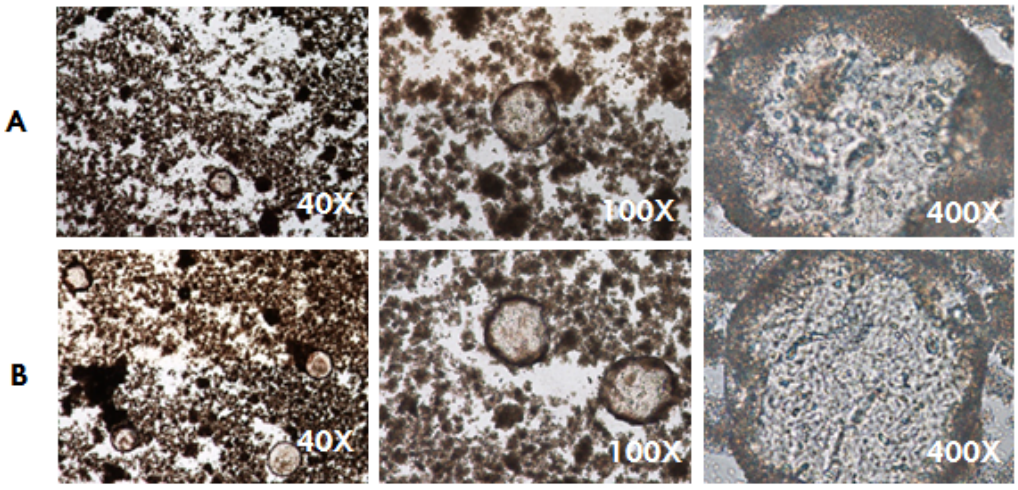

Figure 6. Microspheres incorporation in a natural yogurt analysis by OM under magnifications of 40, 100 and 400X. (A) microspheres at the initial time (t0), (B) microspheres after three days (t3). The white arrow puts in evidence the incorporated microspheres structures.

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TOC graphic

Phenolic extracts of *Rubus ulmifolius* Schoot flowers: characterization, microencapsulation and incorporation into yogurts as nutraceutical sources

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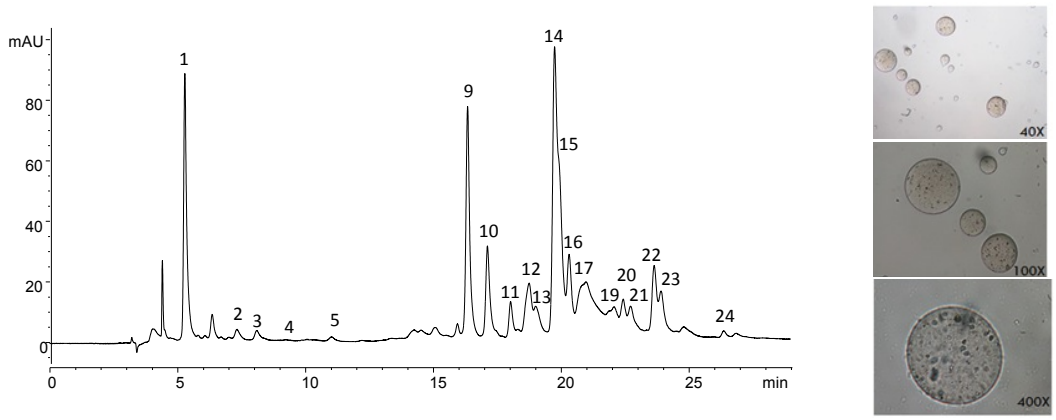

Microencapsulation techniques were used to incorporate enriched phenolic extracts into dairy products.

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Graphical Abstract

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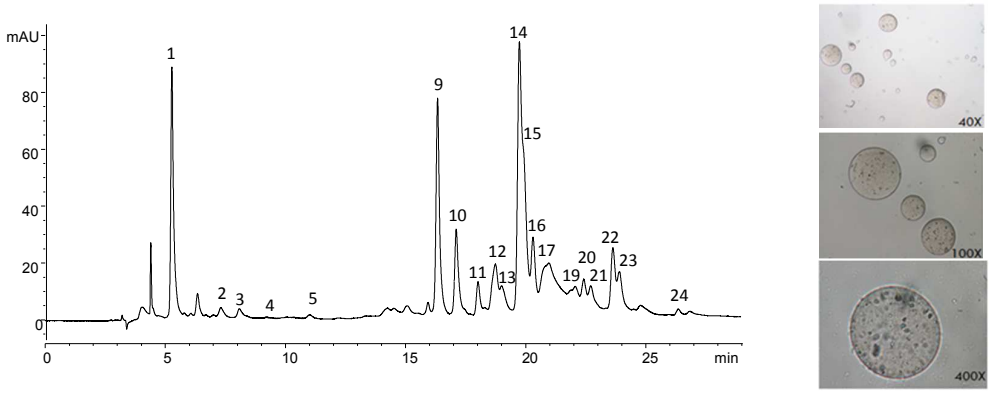

Microencapsulation techniques were used to incorporate enriched phenolic extracts into dairy products.

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