Research Article

Green Extraction of Rosa canina L. and Prunus spinosa L. by NaDES and their Encapsulation in Chitosan Nanoparticles for Cosmetic Industry

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Highlights

1. Rosehips and blackthorns are rich in polyphenols for antioxidant activity
2. Rosehips and blackthorns were extracted by an ethanolic solution or a NaDES mixture
3. NaDES mixture showed a better extraction of polyphenols than ethanolic solution
4. NaDES extracts were encapsulated into different chitosan nanoparticles
5. Chitosan nanoparticles cross-linked with pentasodium tripolyphosphate solid are suitable for topical use

Abstract

The cosmetic industries increasingly value innovative natural ingredients as markers of sustainability. This work investigated the preparation of extracts from rosehips and blackthorns using natural deep eutectic solvents (NaDES) and their encapsulation in chitosan nanoparticles for cosmetic applications. The phenolic composition of the extracts, obtained either using ethanolic solution or NaDES, was evaluated through phytochemical assays. Chitosan nanoparticles were prepared using various crosslinking agents and polymer concentrations, followed by characterization of their physicochemical properties. Cell viability studies were conducted on the human dermal fibroblast cell line WS1. The NaDES extracts exhibited promising results, showing a total phenolic content of 35.26±2.41 and 8.65±0.42 mg GAE/g FW and antioxidant activity by DPPH assay of 92.67±0.74 and 86.06±1.88 % for rosehip and blackthorn, respectively. The optimized nanoparticles formulation was achieved using pentasodium tripolyphosphate solid as the crosslinking agent and a chitosan concentration of 2 mg/mL. Nanoparticle characterization revealed that they were small in size with entrapment efficiency of 63.43±0.54 and 72.64±0.68% for rosehips and blackthorns extract, respectively. Furthermore, the chosen formulations were stable for 8 weeks and exhibited good cell viability. In conclusion, chitosan nanoparticles loaded with NaDES extract of rosehip and blackthorn could be a novel and green antioxidant formulation for cosmetic application.

Keywords: *Rosa canina* L. extract; *Prunus spinosa* L. extract; natural deep eutectic solvent (NaDES); antioxidant activity; chitosan nanoparticles; cosmetic ingredients.

1. Introduction

Nowadays, the worries about climate change urge a new approach to production and consumption behavior. Cultivation of wild edible plants and green processing methods could improve goods production's sustainability (Chemat et al., 2019; Karapatzak et al., 2023; Luo et al., 2022). Among wild edible plants, it is worth noting *Rosa canina* L. and *Prunus spinosa* L., plants from the Rosaceae family, that are spontaneously growing on hills and low mountains in Europe, Asia, and North Africa (Magiera et al., 2022; Popović-Djordjević et al., 2023). These plants can often be observed growing together, typically as roadside species, and they were used as a fence to protect the cultivated lands from livestock and other intruders. Their spiny shrubs, highly resistant to harsh environmental conditions, are strategically planted to create biophysical barriers or for ornamental purposes, increasing the preservation of endemic species and biodiversity (Di Martino et al., 2020). Moreover, *R. canina* L. and *P. spinosa* L. could be domesticated for crop production of fruits known for their healthy properties due to polyphenols, vitamins, and other bioactive compounds. For this reason, they are used in the food, pharmaceutical, and cosmetic industries (Kayahan et al., 2023; Negrean et al., 2023).

Polyphenols combined with vitamins are the main bioactive compounds responsible for the beneficial properties of these berries. In particular, the fruits of *R. canina* L., known as rosehips, contain various phenolic compounds such as gallic and ellagic acids, catechins, quercetin, and anthocyanins (Alves et al., 2022; Jovanović et al., 2023; Stănilă et al., 2015). The blue fruits of *P. spinosa* L. named blackthorns contain more than 400 polyphenols, in particular cumaric and caffeic acids, catechins, rutin, quercetin, myricetin, condensed proanthocyanidins, anthocyanins such as cyanidin and peonidin (Coppari et al., 2021; Cosmulescu et al., 2017; De Luca et al., 2023a). It has been demonstrated that polyphenols could act as a scavenger for free radicals, preserving the anatomic-physiological asset of tissues and preventing damage (Magiera et al., 2022).

In recovering polyphenols from vegetable matrices, cosmetic industries are looking for sustainable extraction processes that could avoid pollutant solvents, reducing energy and water consumption and waste production. Traditional organic solvents, namely methanol, ethanol, acetone, and ethyl acetates, have several disadvantages, such as toxicity, volatility, non-degradability, and

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flammability. They are also costly, and their use in extraction poses potential dangers to human health and the environment (Pai et al., 2022; Rodríguez-Martínez et al., 2022).

A new sustainable strategy to substitute organic solvents in the extraction processes is the use of natural deep eutectic solvents (NaDES), which are designable solvents formed by mixing two or more natural, inexpensive, biodegradable components. NaDES are emerging as green and sustainable solvents for efficiently extracting bioactive compounds such as phenolic acids, flavonoids, and other polyphenols from plant materials. They are a mixture of hydrogen-bond donors (HBDs) and hydrogen-bond acceptors (HBAs) at an appropriate molar ratio to form a eutectic mixture (Rashid et al., 2023; Salazar-Bermeo et al., 2023). Moreover, NaDES extracts, prepared with safe constituents, are becoming attractive as they could be directly added as ingredients for the final products, avoiding other pollutant processes such as evaporation or concentration processes, reducing energy consumption, and limiting water addition (Cannavacciuolo et al., 2022; Mohd Fuad et al., 2021).

Natural extract properties could be better preserved and adapted to skin application through nanosystems encapsulation (Pai et al., 2022). Chitosan nanoparticles represent an effective and sustainable tool for the encapsulation of polyphenols. Chitosan (CS), [poly(β-(1/4)-2-amino-2-deoxy-D-glucose)], is a natural cationic polysaccharide derived from chitin. CS is a suitable material for developing drug delivery systems as nanoparticles due to its gel-forming ability. The ionotropic gelation is the most crucial technique for ionic crosslinking of CS (Algharib et al., 2022; Aranaz et al., 2021).

This work aims to prepare a NADES extract of rosehips and blackthorns rich in polyphenols as a more sustainable alternative to conventional ethanolic extraction. The physicochemical properties of the extracts mainly related to the phenolic composition were analyzed. Then, the extracts were encapsulated in CS nanoparticles using different crosslinking agents and polymer concentrations. In each step of the production method, the pollutant aspects were considered, and the more eco-friendly methods were chosen to improve the entire sustainable production process concerning the selection of natural raw materials, green extraction of bioactive compounds, and the water-saving nanoencapsulation technique.

2. Materials and Methods

2.1. Materials

Rosehips and blackthorns were collected in November 2022 from wild plants in Montefalcone nel Sannio (CB, Molise, Italy), GPS coordinates 41°51'29.6"N 14°36'55.2"E. After collection, the samples were stored at -18±2°C until use. *P. spinosa* L. and *R. canina* L. were identified by Prof. M. Mandrone (University of Bologna). Voucher samples were deposited in the herbarium of Bologna University Botanical Garden with the codes BOLO0602029 and BOLO060203, respectively. Low molecular CS(Mw 150 kDa, viscosity 20–300 cP, T = 20 C, 1% in 1% acetic acid; deacetylation degree 95%) and pentasodium tripolyphosphate (TPP; MW 368 Da) was purchased from Sigma-Aldrich (Milan, Italy). Folin-Ciocalteu reagent was sourced from Titolchimica (Pontecchio Polesine, Italy). Dulbecco's modified Eagle medium supplemented with 4.5g/L D-glucose was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA) as well as bromuro di 3-(4,5-dimetiltiazol-2-il) -2,5-difeniltetrazolio (MTT) reagent. Fetal bovine serum (FBS), trypsin /EDTA (5g porcine trypsin and 2g EDTA), L-glutamine, penicillin, and streptomycin were purchased from Euroclone S.p.A. (Milan, Italy). WS1 human fibroblasts were sourced from American Type Culture Collection ATCC, (Manassas, VA, USA). All other chemicals were purchased from Sigma-Aldrich (Milan, Italy). Phosphate buffer at pH 5.5 (PBS) was composed of 1.5 g/L Na_2HPO_4 × 12 H_2O, 13.61 g/L KH_2PO_4 and 2.66 g/L NaCl. Ultrapure water (18.2 MΩ cm) was obtained with a MilliQ apparatus by Millipore (Milford, MA, USA).

2.2. Preparation and characterization of NaDES

A mixture of NaDES was selected based on studies to extract phenolic compounds using the heating methodology as a simple and fast method, saving energy consumption (Jamaleddine et al., 2022; Kaoui et al., 2022; Rodríguez-Martínez et al., 2022). Moreover, the mixture was selected considering its final utilization as an ingredient of a cosmetic formulation. It was composed of lactic acid, sodium acetate, and water. Lactic acid was included in the NaDES mixture as it improves the

169 solubilization of CS and is an ordinary pH regulator in cosmetics. Sodium acetate increases the
170 encapsulation efficiency of CS nanoparticles (Ngo et al., 2016). The 35% of water was added as
171 the optimal percentage to reduce the viscosity and the density of NaDES, which is beneficial to the
172 mass transport from plant matrices to solutions. Concerning the preparation, lactic acid (HBD) was
173 mixed with sodium acetate (HBA) in proper molar ratios (3:1) with 35% water content along with
174 heating at 80ºC by a magnetic stirrer for 30 minutes. The mixture was stored in a flask covered
175 with parafilm to avoid water absorption. The pH was measured by a digital pHmeter (Crison
176 Instruments, S.A. Barcelona, Spain). The density was calculated from the ratio weight/volume of 10
177 mL of the mixture. The viscosity was assessed by a rotational viscometer (Fungilab, Barcelona)
178 using spindle number 8 at 100 rpm. All measurements were performed at room temperature in
179 triplicate.
180
181 *2.3. Green extraction of rosehips and blackthorns*
182 A hydroalcoholic solution (50% EtOH/ 50% water *v/v*) or the prepared NaDES were used as
183 solvents for polyphenols extraction. Rosehip seeds were removed, and blackthorn stones were
184 accurately separated from the pulp using a thin knife. The pulp fruits were then finely chopped and
185 homogenized. 1.0 g of each type of fruit pulp was mixed with 10mL of the hydroalcoholic solution
186 or NaDES and sonicated for 90 min at room temperature. Then, the mixtures were centrifuged at
187 5000 rpm for 10 minutes. The supernatants were collected and filtered by a Buchner funnel with
188 filter paper. The final volume of extracts was measured using a cylinder before storage at 4.0±1.0
189 °C in the dark until use.
190
191 *2.4. Total Phenolic Content (TPC), Total Flavonoid Content (TFC), Antioxidant Activity (AA) of*
192 *extracts*
193 The extracts' Total Phenolic Content (TPC) was determined using the Folin–Ciocalteu reagent
194 (Singleton et al., 1999). Previously, 1mL of each extract was diluted (1:20 *v/v*). Then, 0.2 mL of
195 extract solution was added to 1.00 mL of 1:10 diluted Folin–Ciocalteu's phenol reagent, followed by
196 adding 0.8 mL of sodium carbonate solution (7.5% *w/v*). After 30 min in the dark at 40.0±1.0°C, the

absorbance at 750 nm was measured spectrophotometrically (UV-Vis 1601 spectrophotometer, Shimadzu, Milan, Italy). Distilled water was used as a blank. TPC was estimated from a standard curve of gallic acid (R^2 = 0.999), and results were expressed as gallic acid equivalent in mg/mL extract (mg GAE/mL extract).

The Total Flavonoid Content (TFC) was determined using the pharmacopeial method with minor modifications (Polumackanycz et al., 2020). Briefly, 1 mL of 2% (*w/v*) $AlCl_3$ was added to 1 mL of the extract solution. After incubation at room temperature for 30 min in the dark, the absorbance was measured at 430 nm. The TFC was estimated from a standard curve of quercetin (R^2 = 0.999) and was expressed as mg of quercetin equivalent/mL extract (mg QE/mL of extract).

The Antioxidant Activity (AA) was determined by the 2,2-di-phenyl-1-picrylhydrazyl radical (DPPH, Sigma Aldrich) reduction assay as described by Brand-Williams *et al*. with minor modification (Brand-Williams et al., 1995). Briefly, the solutions of extract and ascorbic acid (used as standard antioxidant compounds) were mixed with a solution of DPPH (0.1 mM in methanol) at room temperature. The mixtures were kept in the dark for 30 min, and the absorbance was measured at 517 nm. Methanol was used as a blank solution, and DPPH solution was the control. Results are expressed as a percentage of inhibition of the DPPH radical according to the following equation: Inhibition % = $[(A_0\text{-}A)/A_0]$ where A_0 was the absorbance of DPPH control and A was the absorbance of the sample with DPPH.

The pH value of extract solutions was determined by using a digital pH-meter (Crison Instruments, S.A. Barcelona).

All measurements were performed in triplicate, and average values were calculated.

2.5. HPLC analysis of Low molecular weight phenolics

The presence of low molecular weight phenolic acid, flavanols, flavonols, and anthocyanins in *R. canina* and *P. spinosa* extracts was investigated according to (Castro Marin and Chinnici, 2020) using a HPLC instrument equipped with a quaternary gradient pump Jasco PU-2089, an autosampler Jasco AS-2057 Plus Intelligent Sampler and two detectors: A Jasco UV/Vis MD-910 PDA detector and a Jasco FP-2020 Plus Fluorescence detector. The column was a C18 Poroshell

120 (Agilent Technologies), 2.7 µm (4.6 x 150 mm), operating at 35° C with a flow of 0.8 mL/min. Elution solvents were 2% acetic acid in HPLC- grade water (Eluent A) and 2% acetic acid in HPLC-grade acetonitrile (Eluent B). Gradient elution was as follows: from 98% to 95% A in 10 min, 95% to 90% A in 7 min, 90 to 82% A in 6 min, 82% to 80% A in 3 min, 80% to70% A in 3 min, 70% to 50% A in 3 min, 50% to 0% A in 4 min and finally to 98% A in 1 min. Extracts (1.0 g of each type of fruit pulp was mixed with 10mL of each solvent, NADES, or 50% ethanol/water) were diluted 1:1 with HPLC eluent A, filtered with a PDVF syringe filter (Merck, Darmstadt, Germany) before injection. Identification was accomplished by using pure standards (when available) or taking into account the UV/VIS spectrum of each eluted peak. For each compound, semi-quantification was expressed as % peak area with respect to the total peak area within the pertaining chemical family detected at a specific wavelength (e.g., 308 nm for coumaric acid and derivatives, 324 nm for caffeic acid and derivatives, 365 nm for quercetin, its derivatives and all the flavonols, 520nm for anthocyanins). Flavanols were detected by fluorescence with λex 280nm and λem 345nm. All the analyses were carried out in triplicate.

2.6. Preparation of CS nanoparticles: influence of crosslinking agents, CS concentration, and preparation method.

CS nanoparticles were prepared using the ionic gelation method. Different types of crosslinking agents namely, tripolyphosphate (TPPaq), phytic acid (PAaq), or sodium alginate (SAaq) were solubilized in water. Firstly, the extracts were diluted (1:25 v/v), and CS was added to the extract solution (25 mL) and stirred at 200 rpm for 24 hours at room temperature, obtaining the cationic phase. Then, the pH of the CS solutions was measured (pHmeter, MicroPH CRISON 2000, Carpi, Italy) and adjusted at pH 5.0 using NaOH 2.5% w/v. Then, the crosslinking agents were added in proper molar ratios (1:5) to the prepared CS solution with a constant stirring of 200 rpm at room temperature until the appearance of a homogenous opalescence. Moreover, a simplified method to save water and improve sustainability was tested; we modified the crosslinking agent addition into the CS solution, comparing the TPPaq (solubilized in water) with TPPs (poured as a solid salt). Moreover, different concentrations of chitosan ranging from 2 to 5 mg/mL were tested to optimize

253 the formulation. Three replicates were prepared for each formulation, and samples were stored at
254 room temperature until use.
255
256 *2.7. Characterization of CS nanoparticles*
257 Particle sizes and polydispersity index (PDI) of nanoparticles were measured by photon correlation
258 spectroscopy (PCS) using a Brookhaven 90-PLUS (New York, USA) with a He–Ne laser beam at a
259 wavelength of 532 nm (scattering of angle 90°) To carry out the measurements nanoparticle
260 suspensions were dispersed in ultrapure water with a dilution of 1:16 (v/v), selected to ensure that
261 the average count rate remained between 100 and 500 kilo counts per second. The measurements
262 were performed at room temperature with five runs for each determination.
263 Zeta-potential measurements were taken at 25°C using a Malvern Zetasizer 3000 HS (Malvern
264 Instruments Ltd., Malvern, UK). Nanoparticle suspensions were diluted in ultrapure water (1:16
265 v/v). Both the particle size and the zeta-potential measurements were run in triplicate.
266 Entrapment efficiency (EE) was determined by the ultracentrifugation method. Nanoparticle
267 suspensions were centrifuged (Microspin 12, Highspeed Mini-centrifuge, Biosan, Riga, Latvia) at
268 14.000 rpm for 30 min at 25.0±1.0 ◦C. After the centrifugation, 0.2mL of the supernatants were
269 withdrawn, and the TPC was assessed, as reported in paragraph 2.4. The EE was calculated using
270 the following equation: $EE\% = TPC_{extract} - TPC_{surnatant} / TPC_{extract} \times 100$. The experiments were carried
271 out in triplicate.
272
273 *2.8. Stability studies*
274 Nanoparticle stability was assessed by measuring the size and the PDI over 8 weeks of storage in
275 the dark at room temperature. Aliquots of suspension were diluted in water (1:16 *v/v*) at the
276 scheduled time (0,2,4,6,8 weeks), and the size and PDI of nanoparticles were determined using
277 PCS. Three replicates for each sample were assessed.
278
279 *2.9. In vitro release studies*

The *in vitro* release of polyphenols was determined using a Franz-type static glass diffusion cell (15 mm jacketed cell with a flat-ground joint and clear glass with a 12 mL receptor volume; diffusion surface area = 1.77 cm^2) equipped with a V6A Stirrer (PermeGearInc., Hellertown, PA, USA). A cellulose membrane (MF-Millipore cellulose nitrate 0.22 µm, Sartorius Stedim, Biotech GmbH, Germania) was placed between the receptor and the donor compartments, and 12 mL of pH 5.5 phosphate buffer was used as the receptor medium. For comparison, 1mL of each nanoparticle suspension or the pure extract solutions was loaded in the donor compartment. The systems were kept at 32.0±1.0 °C under magnetic stirring (100 rpm/min). Aliquots (0.2 mL) of the solution in the receptor compartment were withdrawn at a predetermined time (1, 2, 3, 4, 5, and 6 hours), and the release medium was refilled with the same volume. The release profile of polyphenols from nanoparticle suspensions compared to the pure extracts was determined by assessing the TPC as reported in paragraph 2.4. The experiments were performed in triplicate.

2.10. Cell viability

Cellular metabolic activity, as an indicator of cell viability and cytotoxicity, was measured by MTT assay on a human dermal fibroblast cell line WS1. Cells were grown in DMEM high glucose, supplemented with 10% FBS, 2 mM L-Glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin, at 37°C in a 5% CO_2/95% air humidified atmosphere. The biocompatibility of the formulations obtained was measured after 24 hours of treatment on a 96-well plate (Corning®, Corning, NY, USA). Specifically, 7000 cells per well were seeded and left to adhere overnight. Subsequently, the formulations prepared at different concentrations of CS (2 and 3mg/mL) were filtered (0.45 µm) and added to the cultured cells at different concentrations ranging from 1.25 to 10%. After 24 hours of treatment, 10µL of MTT solution (5 mg/mL stock solution) were added to each well, and the plate was incubated at 37°C 5% CO_2/humidified air. After 4 h, the cell culture medium was removed, and the formazan crystals were solubilized by adding 100µL/well of 2-propanol. The absorbance at 570 nm was measured and recorded with a multimode plate reader EnSpire® (PerkinElmer, Waltham, USA). The sample absorbance at 690 nm was used as a

reference wavelength for correction. Solutions without CS and TPP were used as a control. All the experiments were performed in four replicates.

2.11. Statistical Analysis

All the experiments were performed in triplicate, and the results were expressed as mean ± standard deviation (SD). Significant differences between average values were assessed through analysis of variance (one- and two-way ANOVA) using the software GraphPad Prism V6(GraphPad, San Diego, CA, USA). Differences were deemed significant for *p < 0.05, **p< 0.01, ***p<0.001.

3. Results and Discussion

3.1. Green extraction of rosehips and blackthorns and phytochemical characterization

The extraction of bioactive compounds has been demonstrated to be better and more efficient using green extraction technologies. For this reason, we have compared the traditional hydroalcoholic solvents with an innovative and sustainable NaDES mixture. Hence, the pulp fruits of rosehips and blackthorns were extracted by ethanol/water (50:50, *v/v*) or NaDES. The NaDES was a clear and stable solution. Its pH was 3.76±0.02, the viscosity 50.0±8.2 cP, and the density 1.173±0.04 g/mL.

After the extraction and the centrifugation, the extract solutions were analysed and TPC, TFC and AA were reported in Table 1.

Table 1: TPC (mg GAE/g FW), TFC (mg QE/g FW), and AA% of rosehip extract (RC) and blackthorn extract (PS) obtained by the hydroalcoholic solution or NaDES*

Sample	TPC (mg GAE/g FW)	TFC (mg QE/g FW)	AA %
RC NaDES	35.26 ± 2.41	0.42 ± 0.18	92.67 ± 0.74
RC 50% EtOH	29.60 ± 3.56	0.32 ± 0.06	91.90 ± 1.67
PS NaDES	8.65 ± 0.42	0.55 ± 0.13	86.06 ± 1.88

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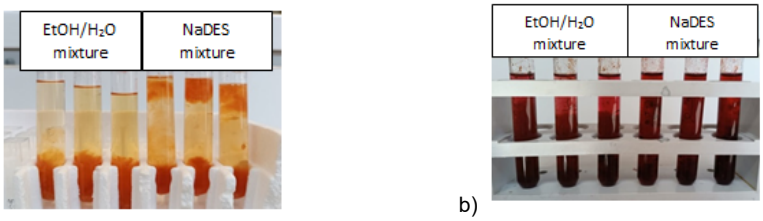
PS 50% EtOH	5.38 ± 0.46	0.53 ± 0.06	84.42 ± 0.40

*RC 50% EtOH-rosehip extract by ethanol solution; PS 50% EtOH-blackthorn extract by ethanol solution; RC NaDES-rosehip extract by NaDES; PS NaDES-blackthorn extract by NaDES.FW-fresh weight of pulp. Values are expressed as mean ± SD (n=3).

The final volume of NaDES extracts (8.5±0.2 mL and 9.5±0.1mL for RC and PS, respectively) are lower than hydroalcoholic extracts (9.0±0.1 mL and 9.8±0.1 mL for RC and PS, respectively). This could be attributed to the NaDES viscosity, which decreases the separation efficiency between solution and matrix. Concerning the different values between the two types of fruits, the final extract volume of RC is lower than PS, probably due to the different composition of fruit pulps. As shown in Figure 1, it is worth that noting the higher swelling of the RC matrix that makes the separation by centrifugation and filtration more difficult due to the higher amount of extract held by the matrix.



a) b)

Figure 1: Mixtures of hydroalcoholic solution and NaDES with rosehip pulp (a) and blackthorn pulp (b) before centrifugation.

The phytochemical characterization was performed to analyze the main phenolic compounds in the extracts, focusing on their antioxidant activity that could reduce oxidative damage and exert cosmetic benefits. For this reason, the TPC, TFC, and AA values of hydroalcoholic and NaDES extract of RC and PS were determined, and the results are reported in Table 1.

Considering the TPC determination results, RC and PS NaDES have a higher value (35.26 ± 2.41 and 8.65 ± 0.42 mg GAE/g FW, respectively) than the hydroalcoholic extracts (29.60 ± 3.56 and

5.38 ± 0.46 mg GAE/g FW) suggesting that the NaDES mixture could be more effective in recovering phenolics compounds. This ability of NaDES is attributed to the hydrogen bonding interactions between hydroxyl and carboxylic groups of NADES molecules and hydroxyl groups naturally present in phenolic compounds (Kaoui et al., 2022).

Concerning the TFC values, the NaDES solvent demonstrated to be effective in extraction of flavonoids from rosehips pulp (0.42 ± 0.18 mg QE/g FW for NaDES and 0.32 ± 0.06 mg QE/g FW for hydroalcoholic solution) and from blackthorn pulp (0.55 ± 0.13 mg QE/g FW for NaDES and 0.53 ± 0.06 mg QE/g FW for hydroalcoholic solution). According to the literature, flavonoids highly contribute to the antioxidant activity of rosehips and blackthorn extracts (Peña et al., 2023; Pinacho et al., 2015).

The reduction of DPPH could measure the AA, which is related to the antiaging efficacy of a cosmetic formulation. As can be observed in Table 1, all extracts exert antioxidant activity, and the AA values are similar for NaDES and the ethanolic extracts (92.67±0.74 and 91.90±1.67 % for RC; 86.06 ± 1.88 and 84.42±0.40 % for PS). Focusing on the differences between the two types of fruits, the AA of RC is higher than PS; this result agrees with the literature that identifies rosehip as one of the most antioxidant fruits due to its considerable amount of antioxidant compounds (Cosmulescu et al., 2017).

3.2. HPLC analysis of low molecular weight phenolics

The content in monomeric or dimeric phenolics of the extracts was evaluated using an HPLC-DAD approach to elucidate the presence of distinct compounds and better discuss the extraction efficiencies exerted by the NaDES and hydroalcoholic solvents. For this characterization and for both the plant extracts, identification was carried out by matching the UV spectrum and retention time of unknown compounds with that of pure standards. These compounds were hence quantified using calibration curves built with the respective standard, and their amounts in the extracts are reported in Table 2. In the chromatograms also appeared a number of peaks whose identification was only tentatively assigned to a putative phenolic class based on their UV spectrum features

(Taniguchi et al., 2023) and the elution order on similar C18 columns (Figures S1 and S2). No quantification was carried out for these latter compounds,.

According to previous reports (Fascella et al., 2019; Guimarães et al., 2013), Rosehip extracts appeared to be mainly composed of flavanols, ellagic acid, and flavonols (Table 2 and Figure S1).

Table 2: Identified phenolic compounds and their amount (µg/g FW) in rosehip (RC) and blackthorn (PS) extracted by NaDES mixture in comparison with 50% ethanol (EtOH). N.d.: Not detected. [1] Fluorimetric detection.

Compound	Detection (nm)	**RC-NaDES**	**RC-ETOH**
(+)-catechin	FLD[1]	45.12 ± 2.24	18.83 ± 0.33
Procyanidin B_2	FLD	43.35 ± 1.80	9.24 ± 0.12
(-)-epicatechin	FLD	4.90 ± 0.62	2.40 ± 0.09
Sum Flavanols		*93.37* ± 4.53	*30.47* ± 0.86
Ellagic acid	365	6.50 ± 0.31	3.46 ± 0.31
Sum EA derivatives		*6.50* ± 0.31	*3.46* ± 0.31
Quercetin-3-*O*-glucoside	365	7.23 ± 0.12	4.75 ± 0.21
Quercetin	365	1.41 ± 0.10	n.d.
Sum Flavonols		8.74 ± 0.26	4.75 ± 0.21
Total phenolics		**108.92 ± 4.63**	**38.68 ± 2.87**

Compound	Detection (nm)	**PS-NaDES**	**PS-ETOH**
3-Caffeoylquinic acid	324	170.30 ± 10.32	97.21 ± 10.24
5-Caffeoylquinic acid	324	33.31 ± 2.54	4.15 ± 0.71
Caffeic acid	324	61.55 ± 4.28	25.5 ± 0.63
Sum Caffeic derivatives		265.16 ± 18.44	126.86 ± 10.64
Rutin	365	24.6 ± 2.12	19.37 ± 0.94
Quercetin-3-*O*-glucoside	365	8.28 ± 0.34	5.94 ± 0.29
Quercetin	365	3.59 ± 0.54	1.88 ± 0.20
Sum Flavonols		36.47 ± 3.98	27.19 ± 3.41
Total phenolics		**301.64 ± 14.41**	**154.05 ± 13.24**

In particular, (+)-catechin and procyanidin B2 were quantified to be the main low molecular phenolics, followed by quercetin-3-*O*-glucoside and ellagic acid (Table 2). Rosehip extract chromatograms also showed a large hump at rT comprised between 15 min and 28 min (Figure S1). Based on UV and fluorometric spectra, it was tentatively elucidated to be due to oligomeric

395 procyanidins with polymerization degree >5, which in reversed-phase chromatography are known
396 to elute unresolved (Karonen et al., 2004).
397 The qualitative composition in distinct phenolics of rosehip extracts largely corresponded to what
398 was already reported in the bibliography for those Mediterranean wild fruits (Fascella et al., 2019;
399 Guimarães et al., 2013). In addition, the amount and the relative proportion of those compounds
400 were found to be in the same order of magnitude as other reports based on HPLC-DAD analysis
401 (Guimaraes et al., 2013; Fecka, 2009)
402 Phenolic acids and flavonols were the major constituents of blackthorn extracts (Table 2). The
403 main low molecular weight phenolic compound was 3-caffeoylquinic acid (neochlorogenic acid),
404 followed by caffeic acid. Flavonols were another class of compounds, most of which were
405 quercetin glycosides such as rutin or quercetin-3-*O*-glucoside. Their amounts were in accordance
406 with previous reports on the phenolic composition of this species (Guimaraes et al., 2013; De Luca
407 et al., 2023; Popovic et al., 2020). Apart from those depicted in Figure S2, two additional
408 chromatographic peaks (not shown) having UV/VIS spectra with maximum absorbance at 520-525
409 nm were tentatively identified as anthocyanins. In this respect, other researchers reported the
410 presence of 4 main anthocyanins in blackthorn extracts, unanimously identified as glucosides or
411 rutinosides of cyanidin and petunidin, respectively (De Luca et al., 2023; Popović-Djordjević et al.,
412 2023).
413 As far as the extraction method is concerned, it is clearly evident from Table 2 that in rosehip,
414 NaDES extracts were richer than hydroalcoholic extracts, as is also apparent by comparing the
415 respective chromatograms (Figure S3 and S4). The relative proportion of each phenolic class
416 seemed to be influenced by the two extraction methods, particularly for ellagic acids, and flavonols,
417 which were poorly extracted with 50% ETOH (Figure S4). Chromatogram comparison also
418 suggests the better extraction efficiency of NaDES for oligomeric procyanidins (see the height of
419 the broad peak eluting between rT 15.00 min and 28.00 min in Figure S3). For blackthorn, the
420 greater overall efficiency of NaDES extraction keeps being clear, but it seems to be restricted
421 almost completely to the phenolic acids fraction, while for flavonols, 50% ETOH and NaDES

demonstrated to be qualitatively and quantitatively more comparable (Table 2 and Figures S5 and S6).

3.3. Preparation and characterization of CS nanoparticles

Currently, increasing attention in cosmetic fields is focused on natural sources of raw materials and eco-friendly processes of production. For this reason, in this study, NaDES extracts of RC and PS were chosen as a natural source of bioactive cosmetic compounds. To facilitate their skin application, CS nanoparticles were prepared for some valuable properties: protection of encapsulated extracts, a sustained release of bioactive compounds that could facilitate their skin absorption, and other properties such as mucoadhesive and antibacterial activity (Aranaz et al., 2021). The chitosan polymer chain has amino groups that are positively charged and could interact with the negative charge of crosslinking agents forming nanoparticles (Raza et al., 2020). In this work, ionic gelation was chosen as a simple and fast method for nanoparticle preparation. Different anions and different concentrations of CS, were tested to select the more promising formulation for cosmetic and pharmaceutical use.

3.2.1. Influence of the different crosslinking agents

Different aqueous solutions of tripolyphosphate (TPPaq), phytic acid (PAaq), or sodium alginate (SAaq) were used in the same molar ratio (5:1) to crosslink the CS (2mg/mL) and form nanoparticles. Their sizes, PDI, ζ-potential, and EE%, were measured 24h after preparation to complete the ionic gelation process.

Table 3: Effect of different crosslinking agents (TPPaq, PAaq, and SAaq) on sizes (nm), PDI, ζ-potential (mV), and EE% of CS nanoparticles of NaDES rosehip extract (RC-CS) after 24 hours from preparation*

Sample	Size (nm)	PDI	ζ-potential (mV)	EE%
RC-CS-TPPaq	185.33 ± 12.01	0.190 ± 0.045	+ 27.49 ± 0.56	49.92 ± 0.80
RC-CS-PAaq	389.2 ± 14.35	0.308 ± 0.007	+ 23.70 ± 1.31	41.35 ± 1.71

RC-CS-SAaq	480.7 ± 30.35	0.293 ± 0.002	+ 24.41 ± 0.97	48.04 ± 0.25

*Chitosan (2mg/mL) nanoparticles of rosehip extract crosslinked by TPPaq (RC-CS-TTPaq) or Phytic acid (RC-CS-PAaq) or Sodium alginate (RC-CS-SAaq). Values are expressed as mean ± SD (n=3)

As reported in Table 3, the TPPaq favors the formation of smaller nanoparticles (185.33 ± 12.01nm) than PAaq, and SAaq, having a size of 389.2 ± 14.35 and 480.7 ± 30.35 nm, respectively. It has been reported that nanoparticles smaller than 300 nm favor skin penetration; for this reason, the TPPaq forms CS nanoparticles more suitable for topical use. Concerning the PDI, TPPaq leads to the lowest value of PDI (0.190 ± 0.045), indicating that the system is the most homogeneous among all formulations. ζ-potential indicates the surface electric potential of particles in solution. Generally, the ζ-potential values above +30 mV or below −30 mV are considered a suitable values for the vesicles' stability. The ζ-potential values of prepared CS nanoparticles are all positive due to the positive charge of the polymer. However, slight differences observed can be attributed to the different types of crosslinking agents. In particular, in the case of TPPaq, the value is higher than PAaq, and SAaq, and it could predict the higher stability of the system. Finally, the EE% values of TPPaq (49.92 ± 0.80%) indicate that this anion leads to a better encapsulation of polyphenols. For this reason, the TTPaq was selected as the best crosslinking agent for the following experiments.

3.3.2. Influence of preparation method using TPP solid or TPP solution

TPP is the most utilized crosslinking agent for CS nanoparticle preparation. Ordinarily, it is used after solubilization in water; the higher the concentration of TPP, the higher the nanoparticle sizes. It has also been demonstrated that the concentration of salts in a CS solution plays a role in determining the final characteristics of nanoparticles (Sreekumar et al., 2018). Recently, water–saving or waterless processes have become more and more attractive in improving manufacturing sustainability (Aguiar et al., 2022). For this reason, in this study, we compared TPPaq as a solution in water or TPPs poured as a solid salt to assess the final characteristic of CS 2mg/mL nanoparticles.

Table 4: Size (nm), PDI, ζ-potential and EE% of CS nanoparticles of rosehips and blackthorns extract by NaDES formed with TPPs or TPPaq after 24 hours from preparation *

Sample	Size (nm)	PDI	Zeta Potential (mV)	EE%
RC-CS-TPPs	237.60 ± 12.96	0.190 ± 0.043	22.66 ± 2.15	63.43 ± 0.54
RC-CS-TPPaq	185.33 ± 12.01	0.190 ± 0.045	27.49 ± 0.56	49.92 ± 0.80
PS-CS-TPPs	172.95 ± 0.85	0.210 ± 0.005	20.31 ± 0.97	72.64 ± 0.68
PS-CS-TPPaq	152.35 ± 1.75	0.240 ± 0.003	16.90 ± 5.46	62.53 ± 4.13

*Chitosan nanoparticles containing RC NaDES crosslinked by TTPs (RC-CS-TPPs) or TTPaq (RC-CS-TPPaq) and PS NaDES crosslinked by TTPs (PS-CS-TPPs) or TTPaq (PS-CS-TTPaq). *Values are expressed as mean ± SD (n=3)

Concerning the size, the CS nanoparticles obtained by TPPaq are smaller than those of TPPs , as shown in Table 4. However, for all samples, the size remains less than 300 nm, making them suitable for topical use (Raszewska-Famielec and Flieger, 2022).

A PDI value less than 0.3 indicates that the system is homogeneous (Hassane Hamadou et al., 2022); as shown in Table 5, the physical state of TPP does not significantly affect the value of PDI, which remains in all cases less than 0.3.

According to the literature, the value of zeta potential helps predict the system's stability if its value is at least ± 30mV (Mahmood et al., 2019). In our study, all samples have ζ-potential values lower than + 30mV; for this reason, the stability of the different formulations was also assessed by monitoring the size and PDI of nanoparticles over 8 weeks. The results are reported in Figure 2.

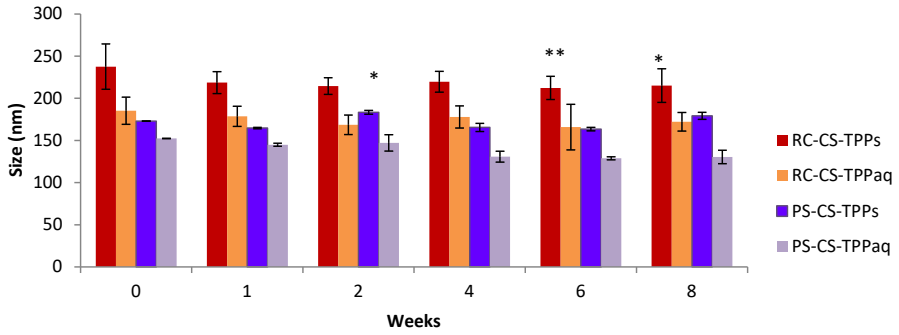


Figure 2: Size of CS nanoparticles loaded with rosehip extract or blackthorn extract during 8 weeks of storage at 25.0 ± 2.0 °C.

Figure 2 shows that all formulations maintain similar sizes over 8 weeks, and nanoparticles obtained by TPP solid have good stability.

Finally, the value of EE is significantly higher for the TPPs than the TPPaq, indicating that this method can favor the encapsulation of polyphenol in CS nanoparticles.

To sum up, TPPs leads to CS nanoparticles with optimal sizes for topical use, good homogeneity, stability over 8 weeks, and higher entrapment efficiency. Moreover, it could represent a more sustainable option for saving water and reducing the total preparation volume. For these reasons, we selected this method of adding TPP for the following studies.

3.3.3. Influence of CS concentration

According to the literature, the higher CS concentration, the higher the size of nanoparticles and the variation of other parameters such as PDI and EE% (Sreekumar et al., 2018). In this study, different concentrations of CS ranging from 2 to 5 mg/mL were tested to optimize the formulation. TPPs was used as crosslinking agent, and size, PDI, and EE% were measured, and the results are reported in Figure 3.

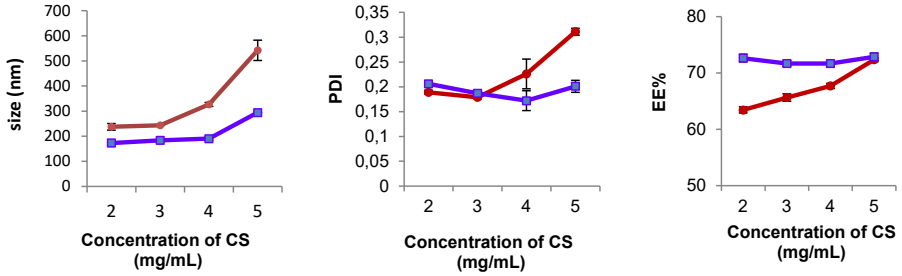
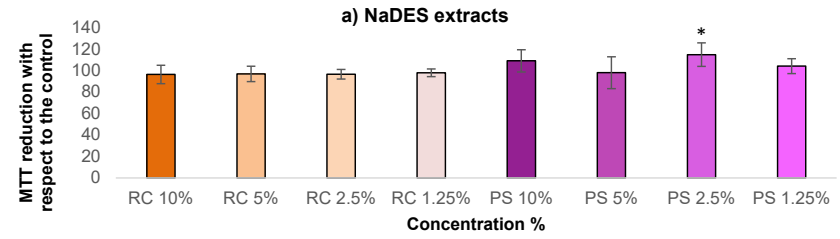


Figure 3: Size (nm), PDI, and EE%, of CS (2,3,4,5 mg/mL) nanoparticles of rosehip extract (RC-CS-TPPs) (red line) and blackthorn extract (PS-CS-TPPs) (violet line) after 24 hours from preparation.

512 Figure 3 shows that the size of RC-CS-TPPs containing 4 and 5mg/mL of CS are 326.85 ± 8.15
513 and 542.5 ± 40.6 nm, respectively ($p<0.001$). Otherwise, a lower size increase related to the CS
514 concentration can be observed for PS-CS-TPPs reaching the value of 294.3 ± 1.60 nm at 5 mg/mL
515 ($p<0.001$). The different trends in the size increase of nanoparticles could be attributed to their
516 chemical composition. PS extracts contain a high amount of anthocyanins, and, as reported in the
517 literature, increasing CS concentration determines a low increase in nanoparticle size due to the
518 higher crosslinking between CS and anthocyanins (Wang et al., 2021).
519 A similar trend is followed for the PDI for the two types of loaded extract. In particular, the
520 polydispersion of RC-CS-TPPs 5mg/mL is 0.311±0.007, indicating a lowering in the system
521 homogeneity with the increasing concentration of polymer.
522 Considering the EE% for RC-CS-TPPs, it tends to increase with CS concentration almost linearly
523 until 5mg/mL ($p<0.001$); instead, it appears to remain similar for all concentrations of PS-CS-TPPs,
524 confirming that the extract compounds could have different interactions with CS chain.
525 Considering these results, it can be concluded that the composition of the extract heavily
526 influences the final characteristic of nanoparticles. Focusing on an extract preparation for skin
527 application, we select 2 and 3 mg/mL of CS for sizes smaller than 300nm, narrow PDI, and good
528 entrapment efficiency as the more promising formulations for topical use.
529
530 *3.4. Cell viability studies*
531 MTT assay was used to assess the biocompatibility of CS formulations on WS1 fibroblasts, the
532 primary cells of connective tissue. Different concentrations of formulations ranging from 1.25 to
533 10% (*v/v*) were tested. As shown in Figure 4, none of the treatments had a detrimental effect on
534 WS1 cells, including the higher concentration, and it allows the treatment dose to be adjusted
535 according to different needs, even at high concentrations. Moreover, both loaded formulations
536 show a positive modulation in the cell viability compared to the unloaded chitosan nanoparticles.
537 This positive modulation effect could be better investigated in further studies with extended times.
538



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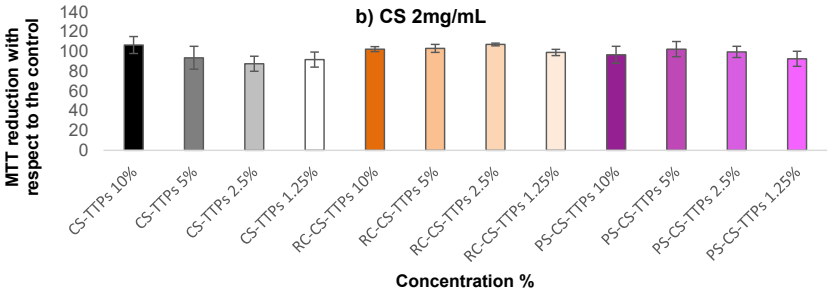

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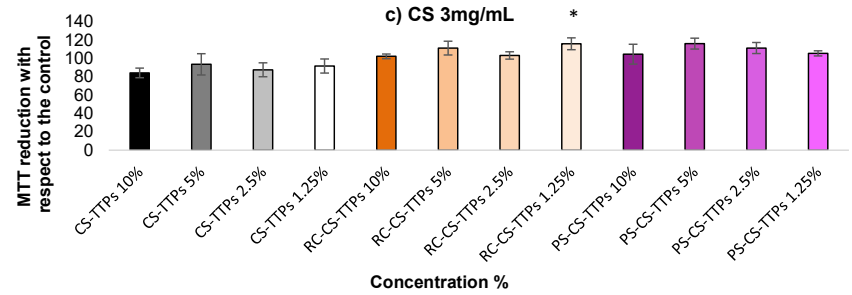

541

542 Figure 4: MTT assays on WS1 cells treated for 24 hours with increasing concentrations of a) RC
543 NaDES extract and PS NaDES extract. b) CS-TPPs, RC-CH-TPPs, PS-CS-TPPs – 2 mg/mL; c)
544 CS-TPPs, RC-CS-TPPs, PS-CS-TPPs – 3 mg/mL. Effects on cell viability were tested in
545 comparison with control cells. (*p<0.05)

546

547 *3.5. In vitro* release studies

548 The *in vitro* release studies describe the TPC released from CS nanoparticles over time and could
549 be helpful in predicting the skin absorption of bioactive compounds.

Figures 5a and 5b show the *in vitro* release profiles of polyphenols from RC-CS-TPPs and formulation PS-CS-TPPs with 2 and 3mg/mL of CS over a period of 6 hours.

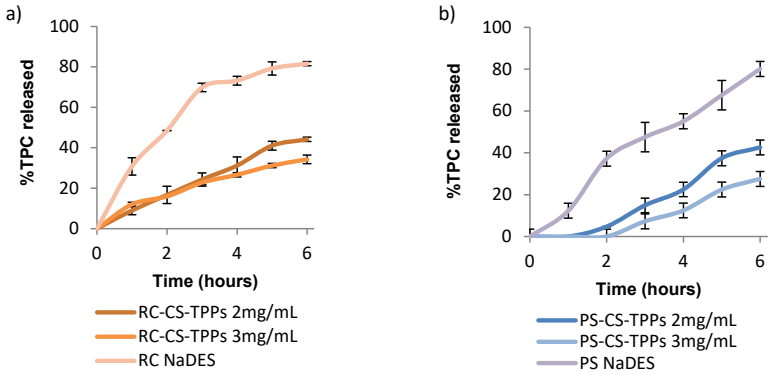

Fig. 5: *In vitro* release profiles of TPC in PBS: EtOH (7:3 *v/v*) from a) RC-CS-TPPs 2mg/mL, RC-CS-TPPs 3mg/mL and RC NaDES; b) PS-CS-TPPs 2mg/mL, PS-CS-TPPs 3mg/mL and PS NaDES.

As expected, all encapsulated formulations presented a lower TPC released over time than the non-encapsulated extract. In fact, the amount of TPC released from NaDES extracts in 6 hours was higher than 80% both for rosehip and blackthorn. For the encapsulated extract, the higher the amount of CS, the slower the polyphenols release ($p<0.01$ after 4 h). This effect is probably due to the entrapment of polyphenols in the nanoparticles and the higher bulkiness of the polymer, which limits the diffusion of the bioactive compounds. A prolonged release of polyphenols through extract encapsulation could be profitable in favor of their skin absorption.

4. Conclusions

The green extraction methods of bioactive compounds from vegetable matrix is a starting point for more sustainable pharmaceutical and cosmetic production. Initially, a mixture of NaDES suitable for polyphenols recovery was compared to an ethanolic solution to extract rosehip and blackthorn pulp. Phytochemical investigation of extracts revealed that the NaDES mixture is more effective

than the ethanolic solution in extracting phenolic compounds from the matrix. These bioactive compounds developed a valuable antioxidant activity for cosmetic applications. Moreover, it represents a more green extraction process, and the extract could be directly implemented in nanoparticle preparation, avoiding evaporation and other preservation or preparation processes, saving energy, and reducing water consumption. CS nanoparticles loaded with rosehip and blackthorn extracts prepared by a NADES mixture were successfully obtained.

Different crosslinking agents were tested, and CS/TPPs at a molar ratio of 5:1 were found to be the best option for obtaining nanoparticles with suitable characteristics for topical use. Results demonstrated that TPP solid salt is effective in obtaining CS nanoparticles with nanometric sizes, avoiding water consumption. Subsequently, different concentrations of CS ranging from 2 to 5 mg/mL were tested to optimize the formulation. The results showed that the CS 2mg/mL formulations lead to rosehip and blackthorn nanoparticles with small sizes, narrow PDI, highest zeta potentials, and good EE%. Moreover, the CS 2mg/mL formulations demonstrated stability for eight weeks, and they are safe for cells and guarantee a slow TPC release profile.

In conclusion, RC-CS-TPPs 2mg/mL and PS-CS-TPPs 2mg/mL prepared with NaDES extracts represent a new environmentally friendly option for cosmetic industries looking for more sustainable natural ingredients.

CRediT authorship contributions Statement

Conceptualization, T.C., V.S.; methodology, V.S., T.C., M.R., M.M., C.C., F.C.; validation, T.C., V.S., M.M., C.C. M.R., F.C.; investigation, V.S., M.R., M.M., I.C., C.C., F.C., T.C.; data curation, T.C., V.S., M.R. and C.C.; writing—original draft preparation, T.C., V.S., M.R., C.C., F.C., J.M.; writing—review and editing, T.C, V.S., C.C., M.M., J.M., A.A., B.L., and F.B.; supervision and project administration T.C.

All authors have read and agreed to the published version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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