Polymeric colloidal nanocarriers entrapped with *Centella asiatica* extract

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Abstract

Nanoparticle technology plays an important role in loading active ingredients and being able to increase their stability and performance. In this work, *Centella asiatica* extract, a plant with known dermatological and cosmetic applications, was incorporated into colloidal polymer nanocarriers, dispersed in silicophilic medium. Nanocarriers were prepared and characterized for particle size (Dynamic Light Scattering), morphology (Scanning Electron Microscopy) and entrapment efficiency of madecassoside. Physical and chemical stability of the encapsulated extract was monitored for 60 days of storage under specific conditions (25 and 40 °C), quantifying the content of madecassoside by HPLC and the particle size distribution. In vitro safety assays, including halo diffusion, cell viability and cell transformation assay were evaluated for free and encapsulated extracts. Nanocarriers showed an average diameter around 210 nm (Polydispersity Index—PDI 0.2) and the entrapment efficiency of 97.7%. The concentration of madecassoside remained stable over time, indicating that the nanocarriers proposed here were able to protect madecassoside against degradation. In addition, nanocarriers showed non-cytotoxic effects and did not induce cell transformation.

Keywords *Centella asiatica* extract · Nanoparticles · Polymeric nanocarriers · Madecassoside

1 Introduction

Centella asiatica (L.) Urban (*C. asiatica*), also known as Gotu Kola, is a small perennial plant [1] used in traditional medicine in different parts of the world, such as Southeast Asia, India, China and Indonesia. The main constituents responsible for pharmacological activities are the pentacyclic triterpenes (asiatic acid, madecassic acid, asiaticoside, and madecassoside) [2]. *C. asiatica* has significantly drawn the attention of scientific groups in the recent years due to its broad range of applications, such as in skin diseases [3], wound healing [4], anti-aging and skin moisturizing [5], hepatoprotective [6], anticancer, antibacterial, antifungal, anti-inflammatory [3], neuroprotective [7, 8], antioxidant

[9, 10], antidiabetic [11], cognitive function enhancement [12]. The literature has numerous success studies indicating the cosmetic potential of *C. asiatica* in the treatment of wrinkles and photoaging skin [13, 14], cellulite and striae [15], and as a skin moisturizing [4, 16]. The skin aging is a multi-factorial phenomenon induced by intrinsic (chronological aging) and extrinsic factors (like ultraviolet damage, pollution, harsh weather, and cigarette smoke) [17], which the chronological aging leads to a decrease in the number of fibroblasts and, consequently, the synthesis of collagen and elastin also decreases, resulting in skin wrinkles and loss of elasticity [18, 19]. Further, the total hyaluronic acid (HA) level in the epidermis diminishes markedly [20] and the skin becomes drier due to the loss of sebaceous

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glands in the dermis. Sun exposure is the principal cause of extrinsic aging, which UV radiation is absorbed, generating reactive oxygen species (ROS) inducing the synthesis of proteolytic enzymes such as matrix metalloproteinases (MMPs), responsible for the breakdown of the extracellular matrix, resulting in wrinkles [21, 22].

In vitro studies have demonstrated the anti-hyaluronidase and anti-elastase potential of the *C. asiatica* extract, besides the matrix metalloproteinase-1 inhibition (MMP-1) [5]. The *C. asiatica* extract can stimulate collagen synthesis, upregulate aquaporins –3 (AQP3) expression [14], and promote free radical scavenging activity [10, 23]. Besides that, the extract has been shown to be an efficient skin protector against UV radiation [13], comparable with OMC (octyl methoxy cinnamate) in its protection against UVB absorbance, showing its potential as a natural protector against UVB damage [10]. Clinical studies have shown anti-inflammatory, skin hydration and anti-wrinkle potential activities of *C. asiatica* extract [13, 24], showing its great potential of application as a cosmeceutical product.

Nanotechnology combined with cosmeceuticals nowadays is a strategy that can bring many benefits, such as controlled release of the active substances, increased permeation through the skin, greater physical stability, among others [25]. This strategy may be especially important in the case of water-soluble plants extracts, due to its difficulty of permeation through the skin, which naturally has hydrophobic characteristics [26, 27].

Some studies have been carried out to protect *C. asiatica* extract, improving its stability and bioavailability [26, 28]. Yongsirasawad et al. [29] prepared *C. asiatica* extract-loaded Gelatin Nanoparticles (CGNP) by using gelatin one-step desolvation method, achieving an entrapment efficiency around 36% and an antibacterial activity three times higher of the CGNP compared to the crude extract. Moreover, bovine serum albumin (BSA) nanoparticles containing CA extracts, prepared by an adapted desolvation method reached encapsulation efficiency of around 90% and showed the potential of the nanoparticles to protect CA extract against hydrolysis and biotransformation [30].

In addition, CA extract also is also being vehicled in polymeric films for topical application [31]. Electrospun gelatin fiber containing methanolic CA extract cross-linked with glutaraldehyde vapor, resulted in fibers with average diameters between 226 and 232 nm, stable in an aqueous medium and able to promote a controlled release of asiaticoside [32]. In another study, Monton et al. [33] prepared film forming polymeric dispersion (FFPD) containing *C. asiatica* extract using Eudragit® RS 30D and Eudragit® RL 30D as the film forming agents, resulting in a loading efficiency about 100% and achieving formulations able to promote sustained release and accumulation of madecassoside and asiaticoside in the skin layers, with no detection of these

phytoactives in the release medium, indicating the safety of the formulation in not permeating through the dermis. However, most of these studies use organic solvents in their production processes or in the chemical cross-linking agents and several them still use raw materials derived from animals such as gelatin in the preparation of nanostructures, which has been avoided by the cosmetic sector.

In this context, in this work, *C. Asiatica* extract was entrapped in polymeric colloidal nanocarriers (PNC-CA) aiming to improve its stability and control the madecassoside release. The nanocarriers were prepared by using a hydrophilic polymer dissolved in an aqueous phase and emulsified in a silicone phase containing the surfactant, through a process that avoids the use of organic solvents or toxic crosslinking agents. Characterization of the PNC-CA included particles size measurement by photon correlation spectroscopy (PCS); morphology by Scanning Electron Microscopy (SEM); entrapment efficiency of madecassoside; stability of PNC-CA over time and in vitro safety tests.

2 Materials and methods

2.1 Materials

Centella asiatica glycolic extract was purchased from Mapric, São Paulo, Brazil; polydimethylsiloxane fluid DC 200, 100 cP (Focus Química); starch purity gum® 1773 (Ingredion, Canada); - Cyclopentasiloxane SF 1540 silicone (Momentive), sodium chloride (Dinâmica), Madecassoside and Asiaticoside (Sigma Aldrich), DMEM-7777 (Sigma Aldrich). The NCTC-clone L929 mouse connective tissue strain from the American Type Culture Collection [ATCC-(CCL1)] was purchased from the Adolfo Lutz Institute cell bank (Cultcel) and the Balb / c 3 T3 strain was purchased from the Rio de Janeiro cell bank (BCRJ-UFRJ).

2.2 Preparation of polymeric colloidal nanocarrier entrapped with *Centella asiatica* extract

Polymeric colloidal nanocarriers entrapped with *C. asiatica* extract (PNC-CA) was prepared according to the procedure described by Cerize et al. [34]. Briefly, the process was carried out basically in three steps. Step 1: pre-emulsion formation by dispersing the internal phase (*C. asiatica* extract, starch, sodium chloride and water) in the external phase, Polydimethylsiloxane fluid and SF 1540 silicone; Step 2: nanoemulsification of the pre-emulsion formed in Step 1, performed in a high pressure homogenizer at 1000 bar in two cycles; Step 3: extraction of the inner aqueous phase, under controlled pressure (180–210 mmHg) and temperature of 50 °C in order to obtain the PNC-CA.

2.3 Particle size distribution and polydispersity index (PDI)

The particle size distribution and polydispersity index (PDI) were determined based on the analytical technique of Dynamic Light Scattering using the equipment Nanoplus (Particulate Systems). The samples (50 μL) were dispersed in 6 g of fluid silicon SF 1202 containing 2% of emulsifier silform 60-A and homogenized and transferred to the sample cell. The measurements were done in triplicate and the software ZetaSizer Version 6.20 was used to manage the test and treat the data.

2.4 Morphology by scanning electron microscopy

Morphology of PNC-CA was evaluated by Scanning Electron Microscopy (Field Electrons Gum—Scanning Electron microscope—FEG-SEM, model Quanta 3D, FEI). The samples were prepared prior to the analysis: PNC-CA was filtered through a cellulose ester membrane (porosity 0.2 μm) in a vacuum system. The membrane was then washed with cyclohexene to remove the silofilic medium and preserve the nanocarriers. These membranes were then placed in desiccator under vacuum for drying for 24 h. After gold coating (Desc IV, Denton), samples were visualized with the scanning electron microscope (SEM) with an accelerating voltage ranging between 10 and 20 kV.

2.5 Biomarkers content: high performance liquid chromatography (HPLC) analysis

Madecassoside and asiaticoside have been chosen as markers for *C. asiatica* extract. Identification and quantification of madecassoside in the PNC-CA and *C. asiatica* extract were determined by high performance liquid chromatography (HPLC). The methodology used was developed by Institute for Technological Research (IPT), based on the work of Rafamantanana et al. [35]. During the development of the HPLC method, different proportions of mobile phase were tested, as well as the addition of trifluoroacetic acid and methyl tert butyl ether to the aqueous and organic phase, according to previous work of Schaneberg et al [36]. Using the Shim-Pack GIST column, the most efficient method was adapted to work of Rafamantanana et al. [35], and the parameters of Linearity, Limit of Detection (LOD) and Limit of Quantification (LOQ) were considered in order to ensure the quality and reliability of the results.

The quantification of madecassoside was performed by High Performance Liquid Chromatograph (HPLC), model SPD-M20A, UV Vis detector (Shimadzu). The column used was Shim-Pack GIST—Shimadzu (5 μm -C18,

4.6 mm $\times$ 250 mm) and the detection wavelength was set at 206 nm. The mobile phase was a gradient of acetonitrile:water (0.01–15 min., 20:80; 15–25 min., 35:65; 25–30 min., 65:35; 25–40 min., 20:80) and the flow rate was 1.0 mL/min. Prior to injection, samples and standards were filtered through a 0.22 μm membrane syringe filter (PTFE—hydrophilic). The injection volume was 20 μL and it was performed three injections for each standards and samples. The concentrations of madecassoside in the samples were estimated from the standard curve. Asiaticoside was not identified in the extract. The standard curve was prepared from a stock solution of madecassoside (1000 mg L^{-1}), prepared with standard madecassoside (from Sigma Aldrich $\geq 95\%$) using ultrapure water. From this stock solution the calibration curve was prepared, at concentrations of 8; 10; 20; 25; 30; 40 and 50 mg L^{-1} . The linear equation and LOD and LOQ of madecassoside were also determined.

2.5.1 Preparation of PNC-CA samples for madecassoside quantification

In order to eliminate the effect of the matrix in which the nanocarriers were dispersed (silicone), 8 g of the formulation was centrifuged at $15.115 \times g$ for 30 min (25 $^{\circ}\text{C}$). Subsequently, part of the supernatant was removed, and the recovered decanted material was used in the determination of madecassoside content and encapsulation efficiency. Thus, to perform the calculations, a correction factor (FC) was applied:

$$FC = \frac{\text{Mass of decanted material (g)}}{\text{Total sample mass (g)}} \quad (1)$$

where: mass of decanted material is the total mass of product—the mass of supernatant removed after the centrifugation process.

2.5.2 Total madecassoside content (Madeca_{total})

About 0.25 g of the material decanted, obtained as described in Sect. 2.5.1. was added in a 2 mL micro tubes with 1 mL of methanol. The sample was vortexed for 1 min, so that the methanol could break the nanocarriers, releasing the active substance in the medium. This mixture was centrifuged at $2.236 \times g$ for 5 min and supernatant was transferred to another tube. The precipitate was washed twice more with 1 mL of methanol and centrifuged at $8.944 \times g$ and $15.115 \times g$ for 5 min each.

The collected supernatants were concentrated in a vacuum system (40 $^{\circ}\text{C}$) and the concentrated material was resuspended in 1 mL of methanol, filtered through a 0.22 μm hydrophilic PTFE filter and analyzed by HPLC for

madecassoside quantification, according to the method described in Sect. 2.5.

Total concentration of madecassoside in the final product ($[Madeca_{Total}]$ in mg kg^{-1}) was calculated using Eq. (2):

$$[Madeca_{Total}] = \frac{([CE_{madeca}] \times FV)}{W_{sample}} \times FC \quad (2)$$

where: $[CE_{madeca}]$ is the madecassoside concentration obtained by HPLC (in mg L^{-1}); FV is the final volume of the resuspended concentrated material and W_{sample} is the sample weight (mg) in the eppendorf tube.

To obtain the final result of the madecassoside concentration in Eq. (2), it was necessary to multiply by the correction factor (Eq. 1) due to the sample concentration, as mentioned in item Sect. 2.5.1.

2.5.3 Entrapment efficiency

In order to quantify only the madecassoside entrapped into the nanocarrier structure, the precipitate obtained in Sect. 2.5.1 was washed with ethyl acetate and centrifuged again at $8.944 \times g$ for 15 min, followed by removal of the supernatant. The precipitate collected were washed more twice with ethyl acetate and dried at 40°C for 3 h in an oven. Dried material was suspended in water, vortexed and placed in ultrasound bath until total solubilization. The obtained mixture was filtered through a $0.22 \mu\text{m}$ hydrophilic PTFE filter and analyzed by HPLC according to methodology described in Sect. 2.5, thus obtaining only the concentration of madecassoside into the nanostructure.

The entrapment efficiency (EE, %) was calculated according to Eq. (3), dividing the concentration of madecassoside into the nanocarriers $[Madeca_{encapsulated}]$, in mg kg^{-1} (after the correction applied in Eq. 1) by the total concentration of madecassoside $[Madeca_{Total}]$, in mg kg^{-1} .

$$EE(\%) = \left(\frac{[Madeca_{encapsulated}]}{[Madeca_{Total}]} \right) \times 100 \quad (3)$$

2.6 Evaluation of stability of PNC-CA: total madecassoside content and particle size distribution

The stability study of PNC-CA considering the degradation rate of madecassoside in PNC-CA, besides the possible formation of impurities and the particle agglomerations were also evaluated. PNC-CA was stored at 25 and 40°C and the madecassoside content and particle size were evaluated

at days 0, 7, 15, 30 and 60, as described in Sects. 2.5.2 and 2.3, respectively.

2.7 In vitro safety testing

2.7.1 Indirect-contact test: Agar diffusion

This assay was performed according to the methodology described by Rogero et al. [37]. NCTC-929 cells (1×10^5 cells mL^{-1}) were distributed in 6 wells plates and allowed to grow for 48 h in incubator at 37°C , with 5% of CO_2 . After cell confluence, a preparation containing 1.8% of agar in DMEM medium with serum (2 times concentrated and containing 0.01% neutral red) was distributed over the cell monolayer. After solidification, non-toxic filter paper discs were soaked in *C. asiatica* extract solution and PNC-CA and deposited on the cells protected by the agar layer. As negative control it was used filter paper soaked in saline solution and as positive control, latex glove fragment. After 24 h of contact with the cells, the plates were observed macroscopically to verify the presence of a growth inhibition halo around the evaluated material and microscopically to observe the cellular aspects. The images were photo documented with the naked eye and through the microscope (Axiovert 135, Zeiss).

2.7.2 Cell viability

NCTC-929 cells (1×10^5 cells mL^{-1}) were distributed in 96 wells plates ($100 \mu\text{L}/\text{well}$). After 24 h of incubation at 37°C with 5% of CO_2 , the culture medium was replaced by the test materials in different concentrations, diluted in DMEM medium with 5% of Serum Fetal Bovine (SFB). For the empty nanocarriers and PNC-CA the concentration was of 36%, 18%, 9%, 4.5%, 2.3%, 1.1%, 0.6% and 0.3% and for the *C. asiatica* extract the concentration was of 4.32%, 2.16%, 1.08%, 0.54%, 0.27%, 0.135%, 0.0675%, 0.03375%. The concentration of *C. asiatica* extract in the PNC-CA samples is corresponding to the concentrations of the free *C. asiatica* extract in this assay. All cells were incubated for further 24 h under the same conditions as described above. Therefore, cells were washed with phosphate buffer saline (PBS) and stained with neutral red vital dye (NR). This procedure was performed according to the Annex A (Neutral red uptake—NRU—cytotoxicity test) of ISO 10993-5: 2009-10 [38].

The tested material concentrations used here were based on the concentration of the PNC-CA in cosmetic bases, which we considered around 5%.

2.7.3 Cell transformation assay (CTA)

This assay was conducted according to the document Balb/c 3 T3 cell transformation assay (2010) [39]. Balb/c 3 T3 cells (2×10^3 cells mL^{-1}) were cultivated in Petri dishes (diameter = 90 mm) containing 10 mL of DMEM with 10% of SFB (approximately 180 cells/ cm^2) and maintained in incubator at 37 °C, with 5% of CO_2 for 24 h. After that, the medium was removed and test substances were added. Methylcol-antrene reagent (0.1 mg mL^{-1}) was used as positive control of cell transformation. For each material analyzed, 10 mL of culture medium, 1 mL of PNC-CA and 120 μL of *C. asiatica* extract were used. After 72 h, DMEM with tests substances were removed, replaced by fresh DMEM with 10% of SFB and incubated at 37 °C, with 5% of CO_2 for 3 days.

After this time, the medium was removed again, replaced by DMEM with 2% of SFB and incubated at 37 °C, with 5% of CO_2 . This procedure was repeated twice week during 31–32 days. In the sequence, DMEM was removed and cells were fixed and stained with Giemsa 10%. After Giemsa staining, the foci obtained were photographed and interpreted according to the catalog published by Sasaki et al. [40].

The concentrations of madecassoside in the analysis of content, entrapment efficiency and stability study were obtained through triplicate analysis of the samples under study. The results were based on the simple mean and standard deviation of the triplicate mean.

3 Results

3.1 Particle size and polydispersity index (PDI)

The mean particle size of the PNC-CA was 210.5 nm (± 15.7 nm) and the polydispersity index was 0.175 (± 0.020). The Fig. 1 shows the size distribution to the PNC-CA sample.

3.2 Morphology

The Fig. 2 presents the morphology of PNC-CA obtained by Scanning electron microscope (SEM). The image shows particles with size compatible with that obtained by Dynamic light scattering, about 200–300 nm.

Fig. 1 Mean particle size of the PNC-CA

Fig. 2 SEM image of PNC-CA (magnitude of 50,000×)

Fig. 3 HPLC chromatograms. **(a)** Overlapping of all chromatograms; **(b)** Expansion of the chromatograms. Peak 1 and 3 are interfering peaks from matrix of the CA extract; Peak 2 is the madecassoside

3.3 Madecassoside identification and quantification by HPLC

The development of a HPLC method for identification and quantification of madecassoside was performed to assure the reliability of the analyses.

Through a calibration curve, it was obtained linearity in the range of 8–50 mg L⁻¹, with correlation coefficient (R^2) of 0.995. The limit of detection (LOD) and limit of quantification (LOQ) were obtained through linear regression of three calibration curves prepared according to the guidelines of the Brazil's National Health Surveillance Agency [41]. The data obtained were treated statistically with the software Excel 2010 and Action Stat version 3.7. The values of LOD e LOQ obtained were 2.62742 and 7.95369 mg L⁻¹, respectively. The Fig. 3a, b show HPLC overlapped chromatograms of the standard madecassoside (in blue color), madecassoside in *C. asiatica* extract at 12% (in black color) and madecassoside in PNC-CA (in red color). It was eluted

at retention time of approximately 14.2 min. Madecassoside concentration in *C. asiatica* extract and in PNC-CA were 573.8 mg L^{-1} ($\pm 23.4 \text{ mg L}^{-1}$) and 58.86 mg L^{-1} ($\pm 2.21 \text{ mg L}^{-1}$), respectively. The presence of asiaticoside has been investigated but not detected in this extract.

The triterpenoids and saponins present in C.A extract are complex molecules and due to the similarity of the polarity of their structures, their separation is a challenging task [42]. The HPLC technique is widely used for the analysis of these compounds and as a device, in the chromatographic analysis the mobile phase initially has greater polarity, being composed of 80% water and 20% acetonitrile. The addition of ionic and acidic pairs can assist the separation of the RP-C18 chromatographic column. Monton et al. (2018) [43] developed an efficient method of separating madecassoside and asiaticoside using a C18-RP column with a mobile gradient phase of water and acetonitrile adding orthophosphoric acid. In previous studies [36] the chromatographic separation was performed with a column RP-C18, with mobile phase composed of water and acetonitrile with the additives trifluoroacetic acid and methyl tert-butyl ether. In this study, the researchers identified six constituents of the C.A. extract, one of them, asiaticoside B, was described as a structural isomer of madecassoside. As a result in the chromatogram, the madecassoside peak was detected shortly after the asiaticoside B. Rafamantanana et al. (2009) [35] used water and acetonitrile gradient method using an RP-C18 column, with a peak in the chromatogram of the C.A. extract showing which precedes Madecassoside and partially coelutes, being called asiaticoside B. Therefore, the methodology developed in this article also presents a partial coelution of an interfering peak that precedes the peak of interest of the madecassoside marker. In order to check the retention time of the madecassoside marker were done dilutions of C.A. extract which were prepared with the addition of madecassoside standard with known concentration, confirming the retention time of the marker in the chromatographic analysis.

3.4 Entrapment efficiency

The entrapment efficiency of the nanocarriers (EE) was 97.7%, and was determined by the ratio between the concentration of encapsulated madecassoside into the nanocarriers, $[Madeca_{encapsulated}]$, 56.64 mg kg^{-1} and the total concentration of madecassoside in the formulation $Madeca_{Total}$, 58.00 mg kg^{-1} , according to Eq. (2).

3.5 Stability of the CA entrapped in the PNC

3.5.1 Madecassoside content

The stability studies were carried out at 25 and 40 °C, in triplicate for each collected point. The Fig. 4 shows the

Fig. 4 Madecassoside content (mg kg^{-1}) in the PNC-CA, under the storage conditions evaluated (25 and 40 °C) for 60 days

Fig. 5 Average diameter of the nanocarriers during the stability study

variation of the madecassoside concentration during the 60 days evaluated for both condition studied (25 and 40 °C).

The results show that the concentration of madecassoside remains almost constant over time, showing that the nanocarrier was effective in protecting the active in the period of 60 days, even in the extreme conditions of temperature, 40 °C.

3.5.2 Particle size distribution

Figure 5 shows the average diameter of the PNC-CA as a function of time. It is noted that after 15 days there is an

increase in the average size and after that remains constant between 30 and 60 days, for both stored conditions (25 and 40 °C). The increase of particle average size after 15 days of stability evaluation can be associated with the increase of polydispersity index observed in both condition of studies, which varied from 0.111 to 0.264 to the store condition of 25 °C and 0.192 to 0.294 to the store condition of 40 °C, according to the Table 1. The increase of polydispersity index suggests an increase of non-uniformity of the size distribution of particles of colloidal system, which can affect the system stability.

3.6 In vitro safety testing

3.6.1 Indirect test: Agar diffusion

The indirect test—Agar diffusion is based on the presence of a lighter halo around the deposited test material

which indicates cell death and consequently the dispersion of the dye that is retained in the cells. On the contrary, in the absence of halo around the deposited test material and dye dispersion indicate that the cells are live.

The Fig. 6a shows a macroscopic analysis of negative and positive test controls with the absence of growth inhibition halo around the atoxic paper filter and presence of lighter halo around latex glove respectively and the Fig. 6b shows absence of growth inhibition halo for both samples: *C. asiatica* extract and PNC-CA.

Figure 7 shows the optical microscopy in the area around the tests materials. It is observed that for both *C. asiatica* extract and PNC-CA, the cells are morphologically normal indicating non-cytotoxicity of materials as negative control.

Table 1 Polydispersity index of PNC-CA

Storage condition	Time (days)				
	0	07	15	30	60
25 °C	0.163 ± 0.048	0.129 ± 0.104	0.111 ± 0.073	0.264 ± 0.009	0.211 ± 0.058
40 °C	0.163 ± 0.048	0.202 ± 0.115	0.192 ± 0.034	0.294 ± 0.029	0.227 ± 0.026

Fig. 6 Photograph of culture plates from indirect contact cytotoxicity assay. Negative control of cytotoxic effect: non-toxic filter paper (**a**, left well); positive control: piece of latex glove (**a**, right well); Samples of *C. asiatica* extract (**b**, first line) and PNC-CA (**b**, second line)

(a)

(b)

(a)

(b)

(c)

(d)

Fig. 7 Optical microscopy of cells in the area around the test materials. (**a**) Negative control: non-toxic filter paper; (**b**) Positive control of cytotoxic effect: piece of latex glove; (**c**) PNC-CA samples and

(**d**) *C. asiatica* extract. Images from microscope Axiovert 135, Zeiss, magnification (400×)

3.6.2 Cell viability

The cell viability was evaluated after 24 h of contact of cells with the test materials. For the empty nanocarriers and PNC-CA the concentration was of 36%, 18%, 9%, 4.5%, 2.3%, 1.1%, 0.6% and 0.3% and for the *C. asiatica* extract the concentration was of 4.32%, 2.16%, 1.08%, 0.54%, 0.27%, 0.135%, 0.0675%, 0.03375%. The results (Fig. 8) showed that none of the test materials were cytotoxic in the studied concentrations.

Fig. 8 Cell viability (%) after 24 h of cell contact with the test materials

3.6.3 Potential of cell transformation

Aiming to complete the in vitro safety assessment tests, the cell transformation assay—CTA was performed. Figure 9 illustrates cellular aspects of the evaluated conditions. Results showed that both samples *C. asiatica* extract and encapsulated (PNC-CA) did not form type III foci, with characteristics presented by the positive control of cell transformation. Although samples formed foci when compared with the negative control condition, where there are

Fig. 9 Cell Transformation Assay (CTA). (a) Positive control of cell transformation: focus type III—magnification (8x); (b) Negative control of cell transformation magnification (8x); (c) Absence of

focus type III for the cells treated with PNC-CA magnification (20x) and (d) *C. asiatica* extract magnification (20x). The Images were obtained using a Leica MZ8 Stereomicroscope

cells only, and despite the basophilia, there is no piling of the cells, leaving only with focus I aspect, based on the classification proposed by Sasaki et al. [40].

Therefore, the results show that the formulations evaluated do not induce cell transformation in the in vitro assay.

4 Discussion

In this study, PNC-CA were prepared by emulsification process followed by high pressure homogenization, resulting in particles around 210 nm, with PDI of 0.2, indicating a homogeneous particle size distribution. The absence of large particles (low PDI) and the viscosity of silicone (100 cPs) can contribute to the formulation stability, once large particles are prone to aggregation.

The hydrophobic character of the formulation prepared here aims to improve its partition coefficient. Bovine serum albumin (BSA) nanoparticles [44] and transfersomes [45], both loaded with *C. asiatica* extract, and liposomes [27] loading only madecassoside, were prepared aiming to improve partition coefficient and, consequently, bioavailability. However, in these processes, it was used organic solvents, or chemical crosslinking agent or a non-scalable method. The nanocarriers presented in this work have the advantage of being prepared without organic solvents or chemical crosslinking agents, through a robust and scalable process.

The *C. asiatica* extract used in this work was obtained commercially and the biomarker madecassoside was identified and quantified ($0.57 \text{ mg g}^{-1} \pm 0.02 \text{ mg g}^{-1}$). The presence of asiaticoside was investigated but this metabolite was not found. As reported in the literature, *C. asiatica* reach pentacyclic triterpenoid metabolites, like the saponins madecassoside and asiaticoside and their sapogenins (madecassic and asiatic acids), and its composition can vary depending on the plant origin, growth conditions and extraction process [46, 47]. Madecassoside was the highest triterpenoid in the extract used by Hashim (2011) [10], with $3.10 \text{ mg mL}^{-1} \pm 4.58 \text{ mg mL}^{-1}$, while Azaid (2016) [48] used an extract with higher asiaticoside content (7.50 mg mL^{-1}) than madecassoside (0.74 mg mL^{-1}), that is close to the concentration found by us.

The HPLC chromatograms (Fig. 3) showed that madecassoside in *C. asiatica* extract and PNC-CA had the same retention time to the standard madecassoside, confirming the presence of the extract in the nanocarriers.

These triterpenoids, madecassoside and asiaticoside, stimulate Type I collagen secretion by fibroblasts, [49], being able to minimize skin aging process, since the main mechanism is the decrease in collagen levels [50]. Furthermore, madecassoside proved to be an effective inhibitor of hyperpigmentation caused by UVB irradiation,

by suppressing the production prostaglandins (PG) PGE2 and PGF2 α , the main PGs produced by keratinocytes in response to UV irradiation [51].

Entrapment efficiency of *C. asiatica* extract obtained with the preparation process used here was 97.7%, that is higher than the result obtained by Surini et al. [45], that prepared transfersomes containing *C. asiatica* extract by thin layer hydration method and obtained particles around 200 nm and reached 85% of entrapment efficiency. Also the particle obtained in this work was higher than the nanoparticles formed as liposome type spheres by gelatin (115 nm), while the EE was less than 70% [26], and yet higher than the NLCs previously mentioned that reached a maximum of 45% of EE for the glycolic extract of *C. asiatica* with particles size around 110 nm [28].

The protection of pentacyclic triterpenes against degradation provided by the polymeric nanocarriers developed in this work was effective. Madecassoside concentration remained constant over the time, in all stored conditions studied. This protection is crucial once these metabolites can degrade over time and storage conditions. Madecassoside and asiaticoside degraded approximately 20% after 16 weeks under light protected condition [52] and asiatic acid degraded after 6 months under 40 °C [53]. Monton et al. [43] studying the stability of asiaticoside and madecassoside in filming forming polymeric dispersions, using Eudragit® RS 30D and Eudragit® RL 30D as film former, concluded that madecassoside and asiaticoside content kept stable only at 4 °C storage condition, while at 25 and 40 °C, the degradation of triterpenes occur after 90 days of storage. Besides that, entrapment efficiency of *C. asiatica* glycolic extract in NLCs measured by asiaticoside concentration decreased after 30 days of storage at 4 °C from 44.7% to 34% [28].

The mean diameter of the nanocarriers during the stability studies increased between days 15th and 30th for both temperatures, 25 °C and, and then remained stable during the evaluation. These diameters increase observed may have occurred possible due to the aggregation phenomenon of nanoparticles which can be justified by the slightly increase of polydispersity index.

The safety assays shown that *C. asiatica* extract used here has no cytotoxic effects or carcinogenic potential on the studied concentrations. These findings are consistent with literature. Aizad et al. [48] that observed that *C. asiatica* extract was able to stimulate proliferation of human fibroblasts and, on the other hand, inhibited lung cancer cells (A549) growth. Pittella (2009) [54] confirmed the cytotoxic potential of *C. asiatica* against mouse melanoma (B16F1), human breast cancer (MDA MB-231) and rat glioma (C6) cell lines, while no cytotoxic effects were observed in normal hamster kidney (BHK-21) cell lines. The selectivity of action could be related to the differences in

morphology and physiology between tested cell lines, although this is not yet proven [54]. PNC-CA and empty nanocarriers also showed no cytotoxic effects on the studied concentrations.

5 Conclusion

In this work, polymeric colloidal nanocarriers entrapped with *Centella asiatica* extract were prepared for cosmetic application. The particles were prepared by a “clean” process, without the use of organic solvents or chemical cross-linking agents. The extract of *Centella asiatica* used had a high concentration of madecassoside, evaluated by HPLC, with methodology implemented by our group. The high encapsulation efficiency obtained (97.7%) proves the efficiency of the process. The nanocarriers obtained here remained stable under the storage conditions studied and were able to protect madecassoside against degradation. Safety studies have shown that PNC-CAs are safe, non-cytotoxic and do not induce cell transformation.

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Compliance with ethical standards

Conflict of interest The authors certify that they have no conflict of interest in the subject matter of this manuscript.

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