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α-amyrin-loaded nanocapsules produce selective cytotoxic activity in leukemic cells

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ABSTRACT

Introduction: Amyrins are triterpenes that have attractive pharmacological potential; however, their low water solubility and erratic stomach absorption hinders their use as a drug. The aim of this paper was to develop a novel α-amyrin-loaded nanocapsule for intestinal delivery and evaluate, preliminarily, its cytotoxic ability against leukemic cells.
Material and methods: Five nanocapsule formulations were designed by the solvent displacement-evaporation method. Poly-ε-caprolactone, Eudragit® E100, and Kollicoat® Mae 100 P were used as film-former materials. Particle size, polydispersity index (PdI), zeta potential, and the pH of all formulations were measured. The cytotoxic potential of the nanocapsules was evaluated in vitro using different leukemic lineages
Results: Nanocapsules coated with Kollicoat® Mae 100 P presented the smallest particle size (130 nm), the lowest zeta-potential (−38 mV), and the narrowest size distribution (PdI = 0.100). The entrapment efficiency was 65.47%, while the loading capacity was 2.40%. Nanocapsules release 100% of α-amyrin in 40 min (pH 7.4), by using a possible mechanism of swelling-diffusion. The formulation showed excellent on-shelf physicochemical stability during one year. Additionally, nanocapsules produced a selective cytotoxic effect on a human leukemia lineage Kasumi-1, an acute myeloid leukemia cell line, and produced cell death by apoptosis
Conclusion: α-amyrin-loaded nanocapsules appear to be a promising nanoformulation that could be used against leukemia.

1. Introduction

Plant-derived products from the Amazon Rainforest are among the main natural resources of Brazil. Species like *Carapa guianensis*, *Copaifera* spp., *Astrocaryum murumuru*, *Pterodon emarginatus*, and *Protium* spp. have enormous pharmacological potential that as yet has only been partially exploited [1](Ferreira et al., 2017). Research into the development of pharmaceutical products using plant-derived substances from the Amazonia contributes to scientific, economic, and social development in Brazil.

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Protium species are widespread in the Amazonia [2]. These species produce a light brown oleoresin popularly known as "*breu branco*", which is commercialized in informal markets in Brazil, and has modest economic importance. Oleoresin is used for reducing joint inflammations [3] and to treat infected wounds and skin ulcers [2]. The oleoresin infusion is used for cleaning infected wounds, diabetic foot, and as a remedy for reducing high levels of blood glucose [2]. Despite the popular utility of Protium *spp*. oleoresin, studies for validating the use of this plant-derived product are scarce.

The oleoresin from Protium *spp* contains flavonoid and phenolic compounds [4], and approximately 45% of the isomers α- and β-amyrin [2,5]. Amyrins are triterpenes that have a gastro-protective effect [4,5], and anti-inflammatory, and hypoglycemic activity [3,5,6]. As such, the presence of flavonoid and phenolic compounds explains the ethnobotanical use in wound and ulcer healing.

The triterpenes α and β-amyrin have shown cytotoxicity against some cancer cell lines such as HL-60, promyelocytic leukemia, SF-295, glioblastoma, HCT-8, colon cancer, MDAMB-435, and melanoma [7]. Amyrins have also been evaluated against other cancer cell lines, including glioblastoma, breast and colorectal adenocarcinoma, liver and lung carcinoma, pancreatic adenocarcinoma, prostate and renal carcinoma [8]. In all cases, both triterpenes showed a weak and selective antitumor effect mediated by apoptosis [7,8]. For these reasons, the oleoresin of Protium *spp*. represents not only a valuable extractivist product, but a source of natural compounds with great pharmacological potential, which justifies the growing interest of the scientific community in these compounds.

However, the low water solubility of amyrins hinders their use, *in natura*, as active ingredients in pharmaceuticals. Alpha-amyrin has an erratic absorption when administered by the oral route, and a long half-life and a low clearance, which causes toxicity in rats [9]. In order to overcome these problems, strategies, such as salt formation, complexation and solid dispersion, have been used to enhance the amyrins' bioavailability [1,4]. One approach to overcome their solubility problem and the erratic oral absorption could be the syntheses of polymeric nanocapsules as carrier systems for amyrins, however, this solution has not yet been reported. Nonetheless, Rodrigues et al. [10] prepared an α- and β-amyrin-loaded nanoemulsion for a possible intravenous administration, but the pharmacological effect was never evaluated (or at least was never reported). On the other hand, Fernandes et al. [11] prepared a nanoemulsion using a vegetal extract rich in amyrins, intended as an insecticide. To the best of our knowledge, there is no nanocapsule formulation containing α-amyrin.

Nanotechnology is an innovative science that allows us to design pharmaceuticals with new and better performance [12]. Nanocapsules, nanospheres and nanoemulsions are nanoparticles that allow us to enhance the bioavailability of drugs with low water solubility and, at the same time, increase the drugs' potency and efficacy. Among the nanoparticle systems, nanocapsules have shown potential as carriers of lipophilic drugs. The polymeric shell of nanocapsules protects the drug from the degradative effects of factors such as light, oxygen, and the acidic environment of the stomach [13,14]. In addition, the use of surfactants in the formulation of nanocapsules (as plasticizers and/or as stabilizers) also improves the water solubility of lipophilic drugs [15]. Thus, the aim of this paper was to develop a novel polymeric α-amyrin-loaded nanocapsule (ANC) for intestinal delivery and evaluate its cytotoxic effect against leukemic cell lines.

2. Material and methods

2.1. Chemicals

All the ingredients used for the preparation of the nanocapsules were of pharmaceutical quality (Sigma, USA). Kollicoat® Mae 100 P was kindly donated by BASF-SP (Brazil). Poly-ε-caprolactone (PCL) was purchased from Sigma (USA), and Eudragit® E100 was purchased from Evonik (Brazil). Chemicals and buffer solutions were purchased from Alphatec (Brazil). Alpha-amyrin with 98% purity was kindly provided by the Phytochemistry Laboratory, Faculty of Pharmaceutical Sciences, Federal University of Amazonas, Brazil.

2.2. Preparation of the nanocapsule

The ANC was prepared by interfacial polymer deposition following the solvent displacement method [12]. Preparation of the *organic phase*: in a magnetic stirrer at 400 rpm (Fisatom, Brazil), an acetone solution of monolauryl sorbitan ester (Span 20) and isopropyl palmitate was prepared. Afterwards, the polymer (previously dissolved in 5 mL of ethanol 96%) was added and stirring was maintained for 20 min. The *aqueous phase* was composed of water and polyoxyethylene (20) sorbitan monooleate (Tween 80), and stirring was maintained at 400 rpm for 20 min, at room temperature. The organic phase was added to the aqueous phase (≈1 mL/min) using a burette, and the mixture was stirred for an additional 15 min. Subsequently, the mixture was homogenized (Ultra-turrax®, IKA, Switzerland) at 10 krpm for 5 min. To form the ANC, the solvent was eliminated in a vacuum rotary evaporator (IKA, Switzerland) at 50 °C.

To select the best polymer for preparation of the nanocapsules, five formulations (Table 1) were prepared using Poly-ε-caprolactone, Eudragit® E100, and Kollicoat® Mae 100 P.

2.3. Droplet size and morphology

The particle size and polydispersity index were measured by PCS (photon correlation spectroscopy) using a Zetasizer Nano (Malvern, UK), at a wavelength of 633 nm, 173° scatter angle, and 25 °C [13] (Rodriguez et al., 2017). Before the measurements, the nanocapsule solution was filtered using a Millipore® membrane (0.45 mm). Measurements were made in triplicate, and the mean ± standard deviation was recorded.

The morphology of the nanoparticles was evaluated using scanning electron microscopy (TESCAN, Czech Republic). The assay was performed with a scattering electron detector, at 15 kV, a sample distance of 8.0 mm, and 15.6 Kx magnification. Samples were metalized with a thin gold layer, using BAL TEC equipment (Czech Republic).

2.4. Zeta potential and conductivity

Zeta potential and conductivity were measured in a particle size analyzer (Zetasizer, Malvern, UK), using a disposable polycarbonate zeta cell. Measurements of electrophoretic mobility were automatically converted to zeta potential using the Smoluchowsky approximation. Measurements were made at 25 °C, using a voltage of 150 V [13]. Each sample was measured in triplicate, and the mean ± standard deviation was recorded.

Table 1
Experimental design for preparation of the nanocapsule.

Organic phase	F1	F2	F3	F4	F5
Isopropil palmitate (mg)	500	500	500	500	500
α-amyrin (mg)	50	50	50	50	50
Span 20 (mg)	–	–	500	–	500
Poly-ε-caprolactone (mg)	350	–	–	–	–
Eudragit® E100 (mg)	–	350	350	–	–
Kollicoat® Mae 100 P (mg)	–	–	–	350	350
Ethanol 96% (mL)	–	–	–	5	5
Acetone (mL)	15	15	15	15	15
Aqueous phase					
Tween 80 (mg)	500	500	500	500	500
Water (MiliQ®) (mL)	50	50	50	50	50

2.5. Encapsulation efficiency and loading capacity

An aliquot of 400 μL of the nanocapsule suspension was placed in a 1 mL ultrafiltration tube (MS®, USA), which was then centrifuged at 13 Krpm for 30 min, at 5 °C. In a 2 mL Eppendorf tube, we placed 200 μL of the supernatant plus 12 μL of ethyl acetate, and 96 μL of acetonitrile. The sample was filtered using a Millipore® membrane (45 μm) and 20 μL was immediately injected into the HPLC system. The assay was performed in a Phenomenex Luna 5 μm C18 column (25 × 5 cm) using the acetonitrile: water (60:40 v:v) mixture as the mobile phase. The runtime was 15 min, at a flow rate of 0.8 mL/min. The signal detection was made at 200 nm. The amount of α-amyrin was determined by the calibration curve method, using α-amyrin as standard (Sigma, USA). The HPLC method was validated (data not shown) to evaluate the purity of the α-amyrin, to determine the entrapment efficiency, and to evaluate the ANC loading capacity.

The entrapped efficiency (EE) was calculated as:

$$EE\ (\%) = 2 \text{ x } (50 - ACL) \quad (1)$$

Where: 50, is the mass of α-amyrin (mg) used for preparation of the nanocapsules, and ACL is the mass of α-amyrin quantified in the supernatant liquid (mg).

To determine the loading capacity, 250 mL of nanosuspension was lyophilized (Terroni, Brazil) and the content of α-amyrin in a weighted mass (50 mg) of the lyophilized nanocapsules was calculated.

$$Loading\ capacity\ (\%) = (AC / W) \text{ x } 100 \quad (2)$$

Where: AC is the α-amyrin content in mg, and W is the mass of lyophilized nanocapsules used for analysis (50 mg).

2.6. Release profile

The release profile was assessed, in vitro, in a paddle dissolution apparatus (Nova Etica, Brazil) at 37 ± 1 °C using 500 mL of PBS buffer (pH 7.4) as the dissolution medium. An aliquot of 0.25 g of lyophilized nanocapsules was placed in the dissolution medium and stirred at 75 rpm for 60 min. Aliquots of 3 mL were removed every 5 min and replaced with fresh PBS solution. One mL of the sample was used for measuring the particle size and polydispersity index. The other two milliliters were centrifuged at 14 Krpm for 25 min, at 4 °C. One mL of the supernatant liquid was filtered through a Millipore® membrane (0.45 μm), and 20 μL of filtrated supernatant liquid was directly injected into the HPLC system (as described in Section 2.5). The assay was performed in triplicate.

2.7. Effect of pH

The effect of pH on ANC particle size and ζ-potential was determined using an MPT-2 titrator (Malvern, UK) coupled to a particle size analyzer (Zetasizer). Sodium hydroxide (0.1 mol/L) and hydrochloric acid (0.1 mol/L) were used as titrants. The instrument was calibrated using buffer solutions (pH 4, pH 7, pH 10; Alphatec, Brazil). Measurements were performed in triplicate at 25 °C.

2.8. Thermal effect

The effect of temperature on the particle size and polydispersity index of the nanocapsules was evaluated. The nanocapsule suspension was heated from 20° to 70°C, at intervals of 5 °C. The sample was maintained for 5 min at each temperature before the measurement. Particle size and polydispersity index were measured in triplicate, and results were expressed as the mean ± standard deviation.

2.9. Stability

Samples of the nanocapsules of 50 mL were kept in amber flasks for a year and a half at 25 ± 2 °C, and 65% relative humidity. The particle size, polydispersity index, ζ-potential, and pH were measured on days 0, 7, 15, 30, 60, 90, 180, 360, and 540. All the measurements were made in triplicate, and results were expressed as the mean ± standard deviation.

2.10. Cytotoxic activity

Human peripheral blood mononuclear cells (PBMC) from healthy donors were collected after informed consent was obtained from the patients. Separation of mononuclear cells was performed by gradient centrifugation methods using Ficoll Histopaque-1077 (1.077 g/cm^3) (Sigma-Aldrich, Germany) following the manufacturer's instructions, at 400 g for 30 min. The use of human samples was approved by the Ethics Committee at the Federal University of São Paulo, under authorization number 35853720.2.0000.0021.

The Jurkat (human acute T lymphocytic leukemia), Molt (human acute T lymphocytic leukemia) and Kasumi-1 (human acute myeloid leukemia) cell lines were cultivated in RPMI 1640 medium (Merk, Germany). K562 (human chronic myeloid leukemia), and C1498 (mouse acute myeloid leukemia) were cultivated in DMEM medium (Merk, Germany). The medium was supplemented with 10% fetal bovine serum (Cultilab, Brazil), 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified atmosphere at 37 °C, under 5% CO_2. Cytotoxic activity on PBMC cells and leukemia lineages (10^5 cells/mL) was assayed in 96-well microplates. The cytotoxic effect of ANC was compared with the effect of blank nanocapsules ($_b$ANC) prepared under the same conditions as described in 2.2, but without α-amyrin, though at the same concentrations (10 and 50 μg/mL).

After 48 h of incubation with the tested substances, the medium was removed and 100 μL of 10% resazurin solution was added. After 5 h, the fluorescence was read at 530 nm (excitation) and 590 nm (emission) using a microplate reader (FlexStation 3, Molecular Devices, Germany). Each experiment was performed in triplicate. The mean values of the cellular metabolism of all groups were compared using one-way ANOVA, followed by Tukey's HSD (Honestly Significant Difference) test, with significance at $p < 0.05$.

2.11. Annexin V/7-AAD assay

Cells were plated (10^5 cells/mL) and stimulated with the test substance for 24 h. After this period, the cells were washed and resuspended in the buffer solution (0.01 M HEPES, pH = 7.4, 0.14 M NaCl, and 2.5 mM $CaCl_2$). The suspension of cells was labeled with annexin V-fluorescein isothiocyanate (FITC) and 7-AAD (7-amino-actinomycin D) (Becton Dickinson, USA) according to the manufacturer's instructions. The cells were incubated in the dark for 20 min at room temperature and, subsequently, 10,000 events per sample were collected in a flow cytometer (Accuri C6, Becton Dickinson, USA). The data analysis was performed using the FlowJo software, version 10.7 (Becton Dickinson, USA).

2.12. Cleaved Caspase-3

Cells (Kasumi-1) were plated (10^5 cells/mL) and stimulated with an ANC solution at 1.40 μg/mL (EC_{50} concentration) for 24 h. After the treatment, the cells were fixed with 2% paraformaldehyde for 30 min and then permeabilized with 0.01% saponin in PBS for 20 min at room temperature. Subsequently, the cells were incubated for one hour with cleaved caspase-3 antibody FITC conjugate (Becton Dickinson, USA) at room temperature. Subsequently, 10,000 events per sample were collected in a flow cytometer (Accuri C6, Becton Dickinson, USA). The data analysis was performed using the FlowJo software, version 10.7 (Becton Dickinson, USA). Geometric mean (GM) of fluorescence

intensity was used to quantify the cleaved caspase-3.

2.13. Statistical analysis

All data were analyzed using the Stat Graphic Centurion v.XV.1 program (StatEase, CO, USA). Analysis of variance (ANOVA) followed by Tukey's HSD test were performed to evaluate statistical differences among the means of various groups. A statistically significant difference was considered at $p < 0.05$.

3. Results

The α-amyrin used for the preparation of the nanocapsules exhibited 98.0% purity. The analytical procedure was previously validated (data not shown). The HPLC chromatogram showed a single peak at a retention time of 5.186 min (Fig. 1). The figure also presents the calibration curve used for the quantitation (area = 53001*C – 655991; R^2 = 0.9974).

3.1. Preparation and characterization of nanocapsules

Table 2 shows the particle size, polydispersity index, ζ-potential, and the conductivity of the five formulations (F1-F5). After the preparation (24 h), all formulations appear as homogeneous liquids with a pale and translucent aspect. F1 showed a high polydispersity index, a bimodal particle size distribution, with a low value of ζ-potential. After 48 h, F1 presented phase separation with solid particles of α-amyrin at the bottom (assessed by FTIR). Thus, the F1 formulation was discarded. Formulations F2 and F3, 24 h after the preparation, showed polydispersity indexes greater than 0.308, with a bimodal particle size distribution, and ζ-potential less than −17 mV (Table 2). Contrarily, 24 h after the preparation, F4 showed a monomodal size distribution, ζ-potential −33.56 mV, and a conductivity higher than 0.200 $\mu\Omega/cm^{-1}$. After the same period, F5 showed a yellowish pale translucent aspect, with a small particle size (130 nm) and a low polydispersity index (0.100), and a high ζ-potential of −38.30 mV (Table 2).

After 48 h, formulations **F2** and **F3** presented phase separation. One phase was completely clear at the bottom (aqueous phase), and the other one had a white foamy aspect on the surface. **F4** showed a milky appearance, with signs of coalescence. However, after 48 h, **F5** presented the same yellowish pale, and translucent aspect observed the day before, with practically no variation in its properties (Table 2). Thus, only **F5** was analyzed further.

Fig. 2 shows the particle size distribution (**A**), ζ-potential (**B**), and the morphology of nanoparticles (**C**) of **F5**, prepared with Kollicoat® Mae100P as the film-former polymer, 48 h after the preparation. The encapsulation efficiency of the process was 65.47%, while the loading capacity was 2.40%. Nanocapsules presented a spherical morphology with a tendency to agglomerate (Fig. 2**C**).

3.2. Release profile

The release profile of the α-amyrin-loaded nanocapsules was performed in PBS buffer (pH 7.4) as the dissolution medium (Fig. 3). The percentage of α-amyrin released, the particle size and polydispersity index were measured every five minutes. After 30 min, the nanocapsules had released 90% of the amyrin content. By this time, the particle size had increased to 1100 nm, 8-fold the particle size at time 0 (131 nm), with a polydispersity index of 1.000 (complete dispersal system). The increase in particle size was correlated with the α-amyrin released from the nanocapsules' core, and showed a strong relationship between them ($R^2 = 0.9720$, $F = 279.30$, $p = 0.0001$).

3.3. Effect of pH

The effect of pH on particle size and ζ-potential is presented in Fig. 4. ζ-potential remained between −0.10 mV at pH1 and −4.5 mV at pH 5. From pH 5 upwards, ζ-potential decreased to −22 mV at pH7, increasing up to −16 mV at pH 9. The particle size increased from 118 nm at pH 1–473 nm at pH 5. From pH 5 upwards, the size was practically constant up to pH 9. Polydispersity index (in parenthesis) also increased to 0.586 at pH 9.

3.4. Thermal effect

Fig. 5 presents the effect of temperature (from 10° to 70 °C) on nanocapsule' particle size and polydispersity index. The size presented practically no changes, and remained between 99 and 103 nm in the range of temperatures. In contrast, the polydispersity index diminished from 0.120 at 10 °C to 0.070 at 60 °C.

3.5. Stability study

Table 3 shows the particle size, polydispersity index, and ζ-potential of ANC, measured on days 0, 7, 15, 30, 60, 90, 180, 360 and 540 after the preparation.

The size diminished from 128 nm at time 0–122 nm after 540 days (Table 3). The width of the size distribution was narrower over time, with a polydispersity index of 0.095 18 months after the preparation,. In a similar manner, ζ-potential diminished (increasing in moduli) to reach −45.23 mV after 360 days, and contributed to the stabilization of the system. The pH remained practically constant over time (values between 5.11 and 5.13).

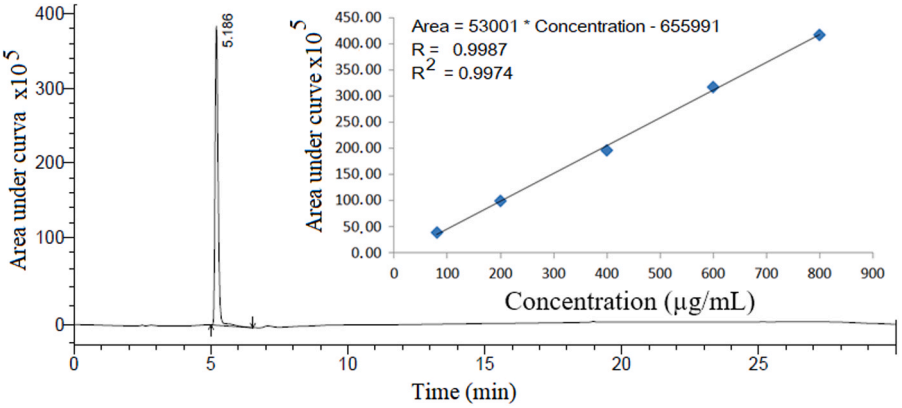

Fig. 1. HPLC chromatogram for quantitating α-amyrin with the statistical parameters of the calibration curve.

Table 2
Main properties of the α-amyrin-loaded nanocapsule formulations prepared for selecting the film-former polymer.

Test	Time (h)	Particle size (nm)	Polydispersion index	ζ-potential (mV)	Conductivity (μΩ /cm^{-1})
F1	24	143.49 ± 46.81	0.371 ± 0.041	-16.27 ± 9.33	0.296 ± 0.095
F2	24	364.35 ± 55.52	0.327 ± 0.080	-5.17 ± 1.67	0.208 ± 0.015
F3	24	321.30 ± 15.54	0.308 ± 0.056	-16.97 ± 4.18	0.261 ± 0.007
F4	24	405.20 ± 36.11	0.426 ± 0.057	-33.56 ± 20.70	0.290 ± 0.139
F5	24	129.75 ± 0.77	0.100 ± 0.055	-38.30 ± 0.70	0.153 ± 0.003
	48	130.18 ± 1.28	0.101 ± 0.052	-38.65 ± 1.22	0.160 ± 0.018

n = 3.

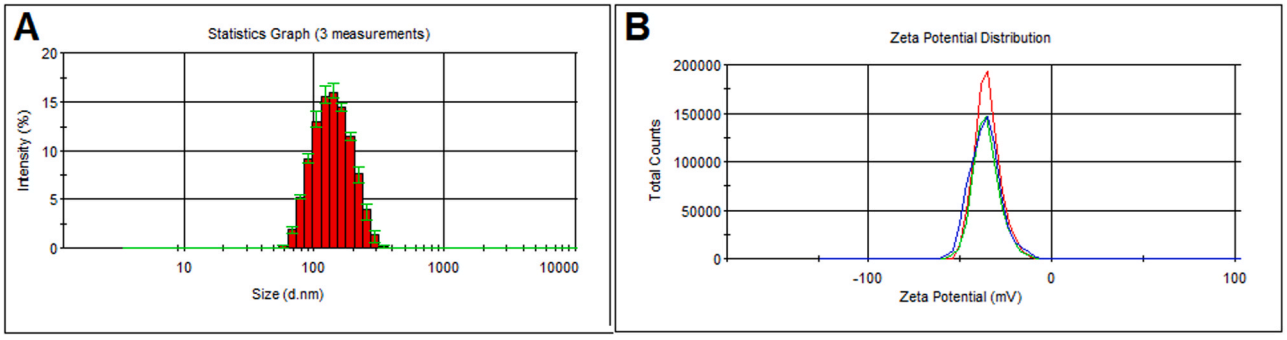

Fig. 2. Particle size distribution (A), ζ-potential (B), and morphology of the **F5** formulation evaluated by SEM, 48 h after the preparation. Particle size: 130.15 nm, polydispersity index 0.101, and ζ-potential −38.65.

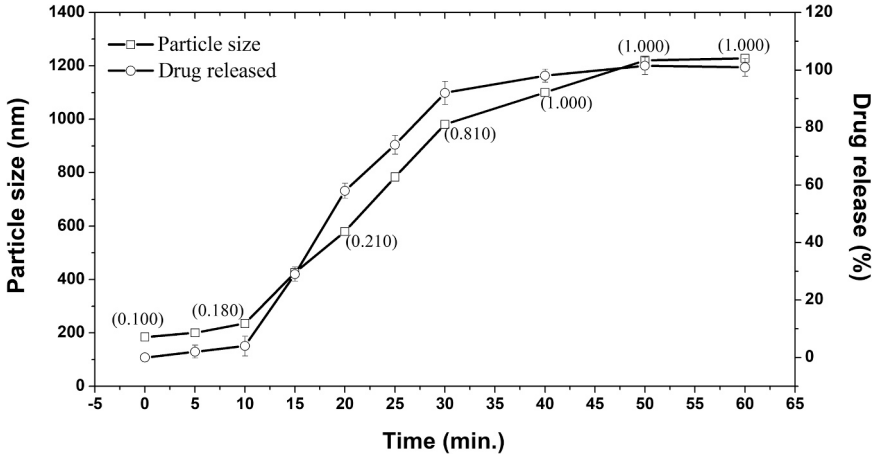

Fig. 3. Release profile of the α-amyrin-loaded nanocapsules in PBS buffer (pH 7.4). The graph also presents the effect of the time on the particle size of the nanocapsules and the polydispersity index (in parenthesis) in the dissolution medium.

3.6. ANC cytotoxic effect

Cytotoxicity of the ANC with 10 and 50 μg/mL was assessed in leukemic cell lineages (Fig. 6). Blank nanocapsules with 10 and 50 μg/mL did not reduce the metabolic activity of myeloid leukemias, such as K562, Kasumi-1 and C-1498 (mouse acute myeloid leukemia), however, levels of 50 μg/mL reduced the cell metabolism of Jurkat (leukemic T-cell lymphoblast) and Molt4 (human acute T cell leukemia) to below 20%. The cell metabolism of Jurkat, Molt 4 cells, and K562 (human chronic myeloid leukemia) was reduced when treated with ANC with 50 μg/mL. In addition, ANC at 10 μg/mL reduced the metabolic activity of Kasumi-1 cells and C1498 cells to six and five percent, respectively, though these lineages were not affected by the blank nanocapsules at any concentration (Fig. 6). Thus, the cell lineages Kasumi-1 and C1498 were selected to perform cytotoxicity curves for 24 h.

The cells were incubated with nanocapsule solutions (10 and 50 μg/mL) for 48 h. Blank nanocapsule solutions ($_b$ANC), at the same concentration (10 and 50 μg/mL), were used as the negative controls. In each case, the unstimulated cell culture was used as control. The data were expressed as the means ± SEM of three independent experiments, and were performed in duplicate. Different letters in a column represent statistically significant difference by the ANOVA test, followed by Tukey's HSD test at $p < 0.05$. ***a***, denotes a non-significant statistical difference when compared to the control; ***b***, ***c***, and ***d*** denote statistically significant difference when compared to the control; the same letters in columns denote that the means are statistically equal.

The cytotoxicity of the ANCs was evaluated against Kasumi-1 and C1498 cell lines (Fig. 7, Table 4). The nanocapsules exhibited potent cytotoxicity against both cell lineages. Nonetheless, the cytotoxicity was more potent against Kasumi-1 cells (EC_{50} of 1.40 ± 0.09 μg/mL) than against the C1498 cells (EC_{50} of 9.00 ± 0.20 μg/mL). The ANCs did not produce cell death in normal human peripheral blood mononuclear cells (PBMC), which were used as the non-tumoral cell control.

EC_{50} is the concentration of a drug that gives a half-maximal

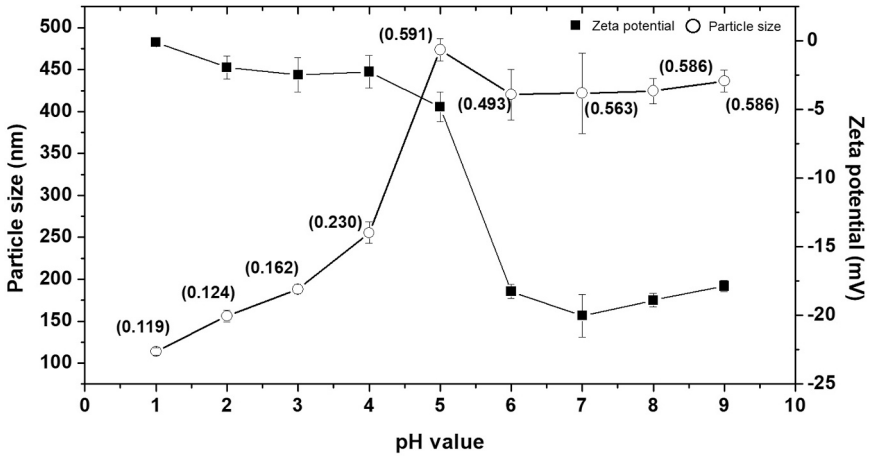

Fig. 4. Effect of pH on particle size and ζ-potential of the α-amyrin-loaded nanocapsules.

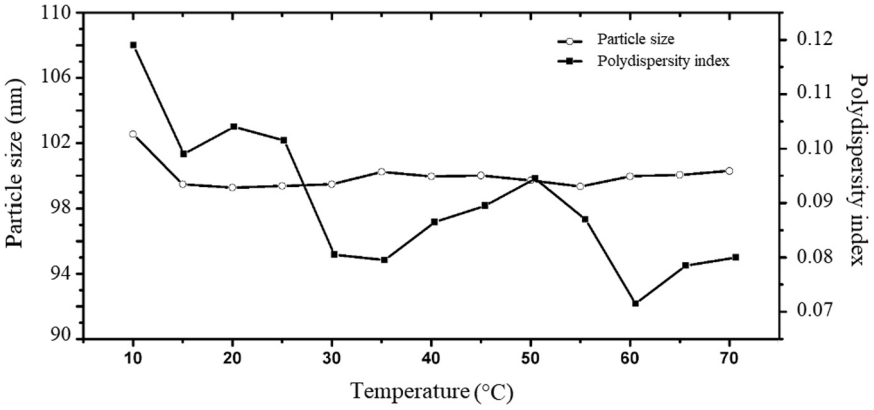

Fig. 5. Effect of temperature (from 10° to 70 °C) on the particle size and polydispersity index of α-amyrin-loaded nanocapsules.

Table 3
Particle size, polydispersity index, ζ-potential, and pH of the nanocapsule formulation according to the elapsed time.

Time (days)	Particle size (nm)	Polydispersity index	ζ-potential (mV)	pH
0	128.80 ± 1.89	0.107 ± 0.011	-35.63 ± 0.55	5.12 ± 0.08
7	129.20 ± 0.40	0.108 ± 0.014	-34.56 ± 0.55	5.11 ± 0.05
15	127.38 ± 1.62	0.081 ± 0.017	-32.96 ± 1.06	5.11 ± 0.07
30	128.60 ± 1.68	0.128 ± 0.013	-35.93 ± 1. 90	5.12 ± 0.08
60	129.38 ± 1.22	0.128 ± 0.013	-38,60 ± 1.01	5.12 ± 0.09
90	123.36 ± 0.32	0.111 ± 0.015	-36.70 ± 1.57	5.11 ± 0.05
180	122.70 ± 0.55	0.094 ± 0.017	-43.20 ± 0.35	5.12 ± 0.07
360	121.00 ± 0.15	0.090 ± 0.007	-43.55 ± 0.41	5.13 ± 0.12
540	122.00 ± 0.14	0.095 ± 0.005	-45.23 ± 0.41	5.11 ± 0.18

n = 5.

response. E_{max} is the maximal effect at high drug concentrations. Different letters in a column indicates statistically significant differences at $p < 0.05$ (n = 5).

The mechanism of cell death induced by the α-amyrin nanocapsules in Kasumi-1 cells was assessed. The cell culture was treated with a nanocapsule solution at the EC_{50} (1.40 μg/mL). The annexin V and 7-ADD assay confirmed the cell death of the Kasumi-1 cells by apoptosis (Fig. 8A and B), but with lower efficacy than shown in the resazurin assay (Fig. 6). Cell death by apoptosis was confirmed via the quantification of cleaved Caspase-3 (Fig. 8C).

4. Discussion

The use of herbal products to treat chronic diseases has increased dramatically in the last years. Botanical-based products are used for complementing the pharmacotherapy of numerous diseases such as cancer, type II diabetes, and others [16]. However, the purity, efficacy and safety of substances of natural origin to be used as drugs must be rigorously evaluated. The α-amyrin used in this study exhibited the same purity as that available on the market (98%), and was of HPLC/GC grade (https://pubchem.ncbi.nlm.nih.gov/substance/329757908). The analytical procedure used for quantitating the purity of the α-amyrin was validated and can be used successfully for quality purposes and for studying the chemical stability of this active ingredient.

Nanocapsules are widely used as carriers of poorly soluble drugs to improve stability, and/or bioavailability [17,18]. To select the coating material for the α-amyrin nanocapsules, three different polymers were used, namely PCL, Eudragit® E100, and Kollicoat® Mae 100 P. Nanocapsules prepared with PCL (**F1**) initially showed a low particle size (143 nm) and a slightly higher polydispersity index (0.371), with a bimodal size distribution (Table 2). After 48 h, **F1** showed phase separation with solid particles at the bottom. PCL is a biocompatible material that usually produces good coating layers [19](Pohlmann et al., 2017). However, in the **F1** formulation, it seems that PCL formed an incomplete and/or weak coating, which allowed the release of α-amyrin from the core, thus destabilizing the system, and producing the precipitation.

Formulations **F2** and **F3** (Eudragit® E100-coated) showed a phase separation different to **F1**. In both cases, the aqueous phase (at the bottom) was clear. Eudragit® E100 is based on 2-(dimethylamine) ethyl

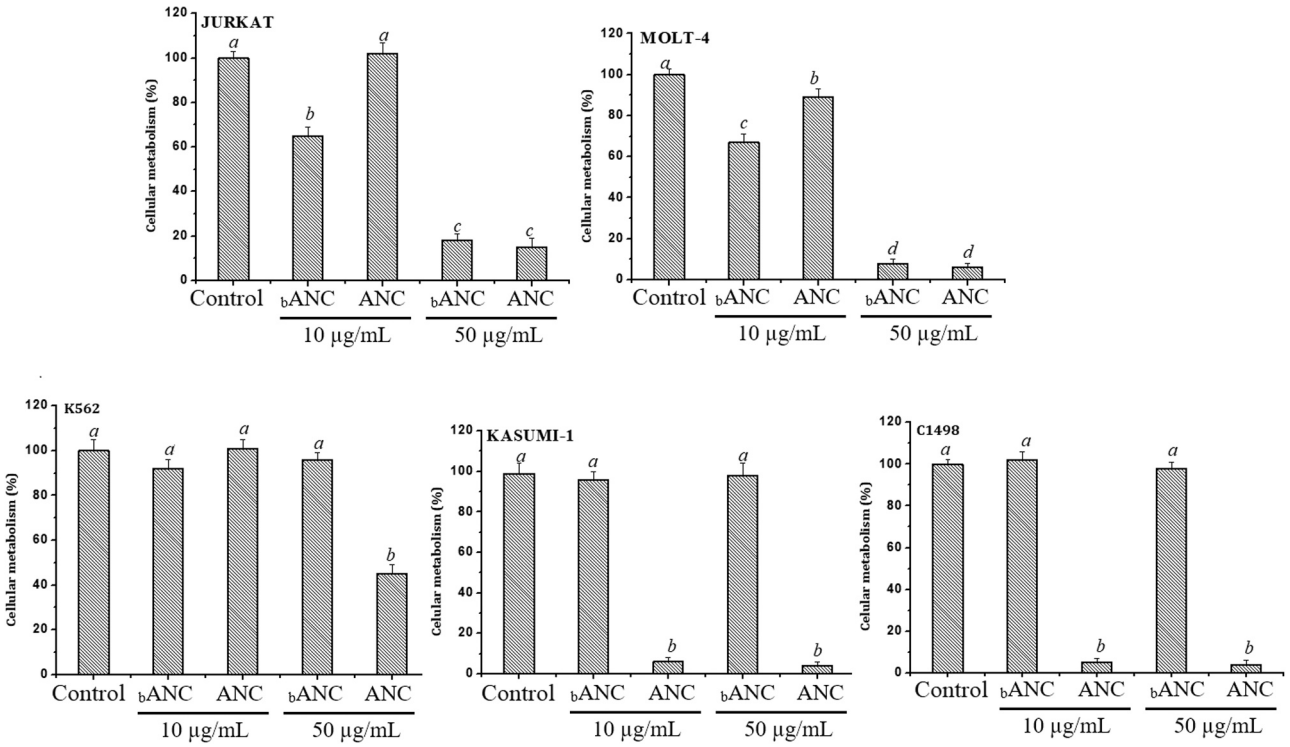

Fig. 6. Selection of leukemic lineage susceptive to α-amyrin-loaded nanocapsules (ANC).

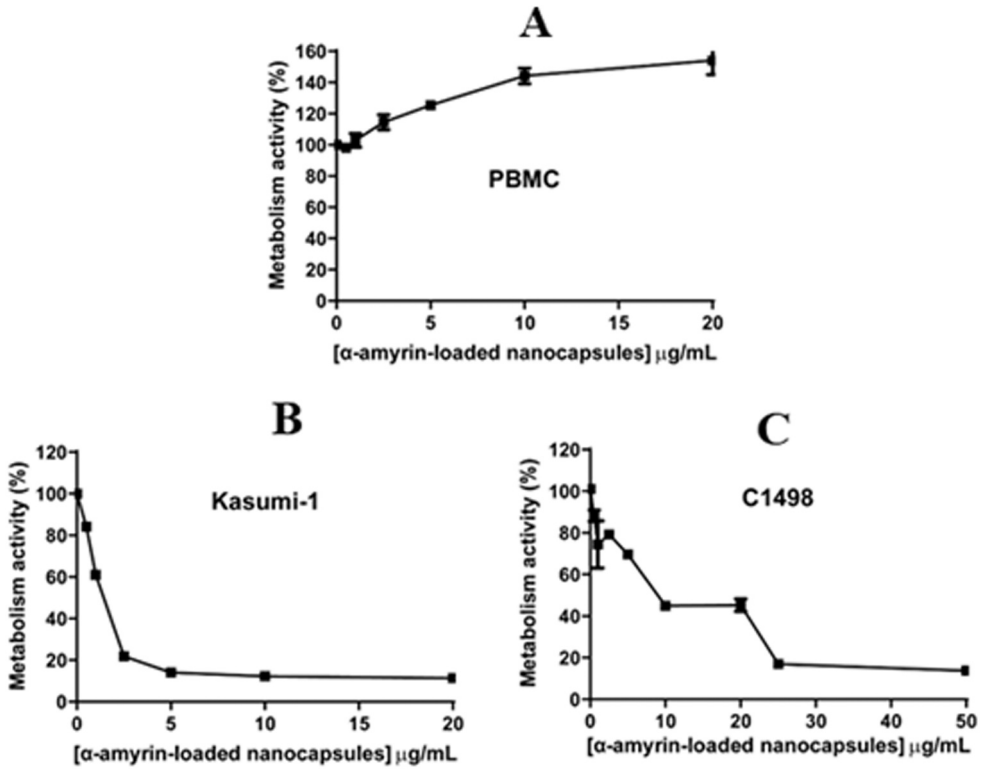

Fig. 7. Cytotoxicity curves of α-amyrin-loaded nanocapsules against leukemic cells: (A) human peripheral blood mononuclear cells (PBMC, used as normal cell control); (B) Kasumi-1; and (C) C1498. The cells were incubated with ANC solutions for 24 h. The results were expressed as the means ± SEM of the three independent experiments and were performed in duplicate.

methacrylate, methyl methacrylate, and n-butyl methacrylate [20], with relatively high plasticity [21]. It is possible that the presence of Tween 80 produced a weak layer that broke up and released the α-amyrin contained in the core.

Nanocapsules prepared with Kollicoat® Mae 100 P (**F4** and **F5**) exhibited different behavior. **F4** (405 nm) initially showed a

Table 4
Effects of α-amyrin-loaded nanocapsules on leukemia cellular lineages.

Cell lineage	EC_{50} (μg/mL)	E_{max} (μg/mL)
C1498	9.00 ± 0.20[a]	89.00 ± 0.50[a]
Kasumi-1	1.40 ± 0.09[b]	87.00 ± 0.30[b]
t-test, p-value	9.12, 0.0001	4.89, 0.0080

monomodal size distribution, but with a large dispersion of sizes (polydispersity index of 0.426). This formulation became turbid after 48 h, with a milky aspect, which could be a result of particle aggregation, and which is a clear sign of instability [13,18]. Contrarily, after 48 h of the preparation, **F5** still had the same turbid aspect, a low particle size (129 nm) with a narrow size distribution (polydispersity index 0.100), and a highly modular value of ζ-potential (−35.63 mV, Fig. 2). It seems that the presence of Span 20 in the organic phase in **F5**, in combination with Tween 80 (in the aqueous phase) allows us to obtain a perfect dispersion, something that was not accomplished in **F4** due to the absence of Span 20 in the organic phase. Kollicoat® Mae 100 P is composed of 97% methacrylic acid-ethyl methacrylate (1:1), 2.3% Tween 80%, and 0.7% sodium dodecyl sulfate [22]. This polymer is partially neutralized with NaOH to facilitate water dispersion; however, it produces elastic and resistant layers around the lipid core, especially when used with non-ionic surfactants [13,14]. According to these results, formulation **F5** was selected for characterization, stability studies, and evaluation of its biological activity.

The SEM analysis of formulation **F5** showed particles with a spherical shape, and a size greater than that measured by PCS. This probably occurs due to the sample preparation process. The presence of surfactants on the nanoparticle's surface helps the aggregation process during the metallization as a result of water evaporation, which results in agglomerates with a rounded shape, as observed in Fig. 5C.

The drug-loaded contents can affect the pharmacological effect of the nanocapsules, the release profile, and the stability of the nanocapsules [22]. The high entrapment efficiency is one of the most important characteristics of nanocapsules prepared with Kollicoat® Mae 100 P as a film former, with values above 90% [13,14]. In this study, a good encapsulation efficiency (65.47%) was obtained, which agrees with results reported for other nanoparticles prepared with Kollicoat® Mae 100 P [13,14]. Loading capacity is another important parameter of the nanoparticle's preparation process. This is the amount of drug available per unit weight of the nanocapsules. The ANCs showed a loading capacity of 2.40%, which represents 240 mg/g of lyophilized nanocapsules. This value was used to calculate the concentrations of all the nanocapsule solutions used for evaluating the biological activity. To our knowledge, this is the first report on the preparation of polymeric α-amyrin-loaded nanocapsules.

The physical properties of the nanoparticles, such as particle size, ζ-potential, and the pharmacokinetic ones, such as the release profile, can be affected by external factors like pH and temperature, among others [17]. The effect of pH and temperature on ANC particle size and ζ-potential was evaluated. Changes in pH from 1 to 5 had a trivial effect on ζ-potential, which maintained values between −0.10 and −4.50 mV. From pH 5 upwards, ζ-potential decreased (increased the module) with few variations beyond pH 7. Probably, the increase of OH-ions in solution, due to the addition of NaOH, helps to form an anionic layer around the nanocapsules, and contributes to produce the negative ζ-potential. This result agrees with Rodriguez et al. [13], who prepared loratadine-loaded nanocapsules using Kollicoat® Mae 100 P as a film-former polymer. In their study, the ζ-potential showed the same behavior observed in the α-amyrin-loaded nanocapsules.

Similarly, the particle size increased from 118 nm at pH 1–473 nm at pH 5 and showed a positive correlation. At pHs from 6 to 9, the particle size increased to 430 nm, but with a noticeable increase in the polydispersity index. This means that at pHs above 5.5, the particles are unstable, which is probably due to aggregation and disaggregation

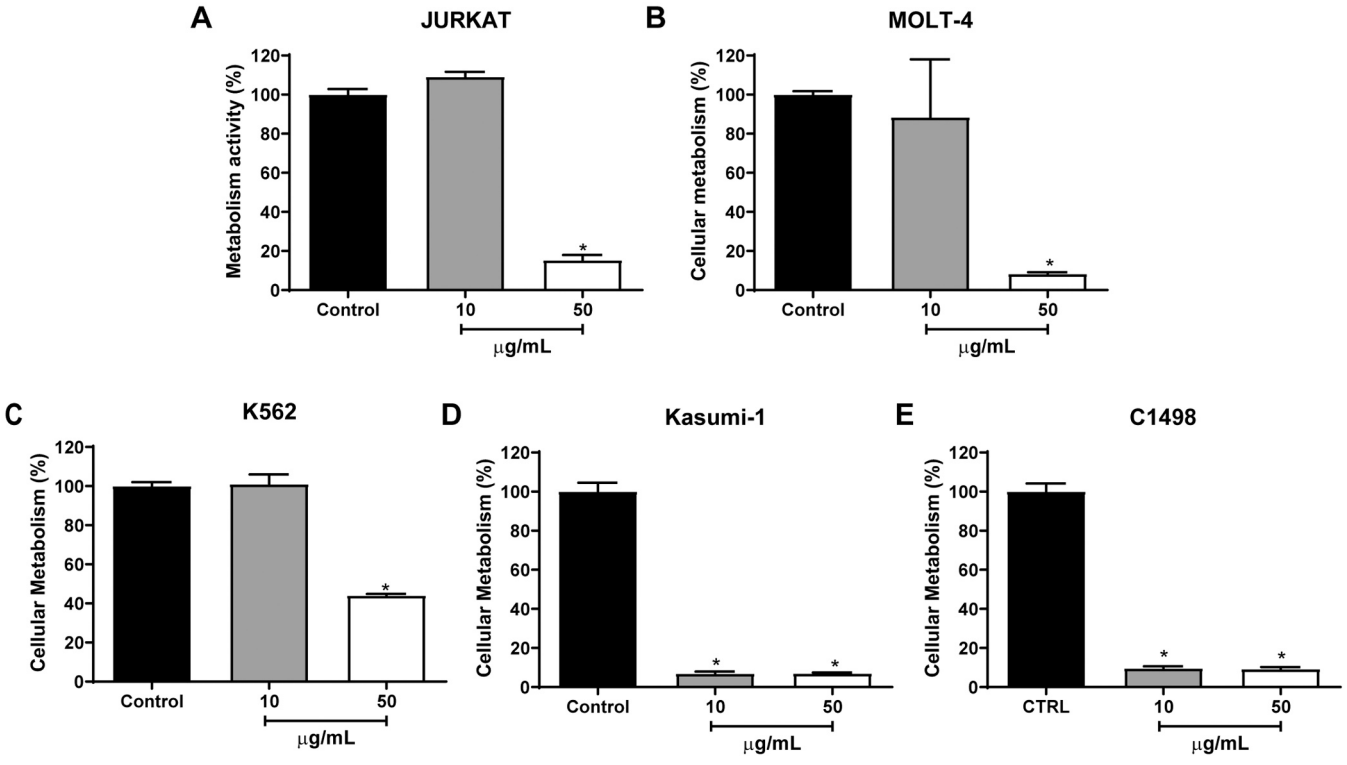

Fig. 8. α-amyrin loaded nanocapsules (ANCs) promote cell death by apoptosis in Kasumi-1 lineage. Cells (10^5/mL) were stimulated with the ANCs at the EC_{50} (1.4 μg/mL) for 24 h. (A) typical counterplots of the cell death profile analyzed by annexin V-FITC/7-AAD; (B) mean of annexin V/7-AAD assay; (C) quantitation of cleaved Caspase-3. These results are represented as mean ± SEM of three independent experiments and were performed in duplicate. Unstimulated cells were used as control. The * denotes statistically significant differences when compared to the control at $p < 0.05$.

processes and/or the increase in the volume of the particle [13]. Similar behavior was reported in other nanoparticles prepared with Kollicoat® Mae 100 P [13,14]. Methacrylic acid-copolymers permit the preparation of resistant nanocapsules, and keep the size and volume practically intact at pH < 5. However, at pHs above 5.5, the particle size increases to break up the shell and releases the content. The same behavior was observed in nanoparticles prepared with the extract of *Cassia grandis* [14], and in loratadine-loaded nanocapsules [12]. In both systems, the coating layer was produced using Kollicoat® Mae 100 P, combined with Tween 80 as the plasticizer. This result suggests that α-amyrin-loaded nanocapsules can remain intact in the acidic environment of the stomach (pH 1.5–2.5).

In the pharmaceutical industry, some operations and processes promote heat and mass transfer, which affect the excipient's and the drug's stability. The effect of temperature on the nanocapsule's main properties was evaluated. The particle size remains between 99 and 103 nm at temperatures between 10 and 70 °C. The size distribution was narrower as the temperature increased, and showed a polydispersity index of 0.120 at 10 °C, and 0.070 at 70 °C. This behavior suggests excellent thermal stability from 10° to 70 °C, which is crucial for further scaling-up of processes. This result agrees with those reported by Rodriguez et al. [13] and Lafourcade et al. [14]. Kollicoat® Mae 100 P is a mixture of methacrylic acid-ethyl acrylate (1:1), Tween 80 (2.7%) and sodium dodecyl sulfate (0.7%). This polymer forms an elastic coating that is capable of expanding the volume up to 8-fold without breaking up [24], which could explain the good thermal stability of α-amyrin-loaded nanocapsules.

Pharmaceuticals need to preserve their physical, chemical, pharmacological and biopharmaceutical stability throughout their lifetime. Stability implies maintaining (within certain limits) the product properties unaltered under standard storage or certain stress conditions [25]. Stability studies allow us to evaluate the effects of time and/or external factors (e.g., temperature, light, humidity, oxygen absorption) on the properties of the formulation. Additionally, stability studies allow us to define the best form of packaging and storage of the final product [24]. In this study, the stability of α-amyrin-loaded nanocapsules was evaluated during a year and a half, under shelf conditions, when kept in amber flasks, at 25 ± 2 °C, and under normal conditions of humidity and illumination (Table 3).

After 18 months, the particle size of the nanocapsules was 122 nm, with a low polydispersity index (0.095). On the other hand, ζ-potential was practically constant during the first three months though it diminished to −45.23 mV after 18 months. The high modular value of the ζ-potential contributes to system stabilization [18]. The excellent stability observed is probably associated with the dynamic equilibrium reached by the nanocapsule system. The last statement can be validated by the narrowing base of the size distribution (lowest polydispersity index) because of the reduction in size and a high and stable zeta potential during the period of the study [13,14,18]. In addition, the use of non-ionic surfactants (Tween 80, Span 20) impairs the particle aggregation in the solution due to a steric effect, which enhances the system stabilization [26]. The pH behavior during the period (practically constant) also reflects the excellent stability of the α-amyrin-loaded nanocapsules.

To produce the pharmacological effect, the encapsulated drug needs to be released from the core of the nanocapsules. There are various mechanisms of drug release, e.g., diffusion, degradation, swelling followed by diffusion, and others. In this study, a possible correlation between the α-amyrin release and the increase of the ANC's particle size was evaluated. Kollicoat® Mae 100 P produces polymeric shells that are capable of increasing their volume if heated or when immersed in liquids that have a pH above 5.5 [13]. The release of α-amyrin showed a linear correlation with the increase in particle size ($R^2 = 0.9720$). After 30 min, 91% of α-amyrin was released, and the particle size had increased to 1000 nm, showing it to be a polydisperse system (polydispersity index = 0.810). One hundred percent of the α-amyrin was released after 50 min. Despite α-amyrin-loaded nanocapsules having short time-release at pH 7.4, this could be sufficient to guarantee adequate release and absorption. This test suggests that the release of α-amyrin may occur through a swelling-diffusion mechanism, where the core-shell swells creating openings that become bigger as the size increase, thus allowing the release of the α-amyrin. However, other tests must be conducted to confirm the release mechanism with greater precision.

The treatment of cancer is one of the challenges of modern science. Every year, many plant-derived compounds are evaluated in the search for new substances to treat all kinds of cancer. However, the number of substances with some usefulness to treat cancer are few. Nanoencapsulated α-amyrin reduced the cellular metabolism of Kasumi-1 cells by 94%, and that of C1498 cells by 95%. (Fig. 6). Thus, these cell lines were selected to construct the cytotoxicity curves and estimate the nanocapsules' EC_{50} and E_{max} values [27].

The potency (EC_{50}) and efficacy (E_{max}) are two parameters estimated from the cytotoxicity curve. EC_{50} is the drug concentration that produces 50% of its maximum response. E_{max} is the highest response produced by the drug. In other words, at a concentration higher than E_{max}, the drug will not present any additional effect [27]. The α-amyrin-loaded nanocapsules produced a notable cytotoxic effect against both cell lineages (Kasumi-1 and C1498, Table 4). However, the cytotoxicity was more potent against Kasumi-1 cells (EC_{50} 1.40 μg/mL) than the C1498 cells (EC_{50} of 9.00 μg/mL). In terms of efficacy, the nanocapsules showed similar efficacy (but were statistically different, $p < 0.05$) as antileukemic agents against Kasumi-1 and C1498 cells, and exhibited an E_{max} of 87.00 μg/mL and 89.00 μg/mL, respectively.

The mechanism of cell death induced by ANCs in Kasumi-1 cells (acute myeloid leukemia) was evaluated at the ANCs' EC_{50} concentration (1.40 μg/mL). The annexin V and 7-ADD assays showed that approximately 20% of the cell population was going through an early apoptosis stage after 24 h of treatment (Fig. 8A and B). Cellular death by apoptosis was confirmed by the quantitation of cleaved Caspase-3 (Fig. 8C). Therefore, it is expected that an increasing number of cells undergo the next phases of apoptosis between 24 h and 48 h of treatment. This result agrees with Barros et al. [7] and Mishra et al. [8], who found that amyrin esters triggered cellular death by apoptosis in human leukemia cells (HL-60) and human breast cancer cells (MCF-7). This is also the case with pentacyclic triterpenes like α and β-amyrin, which exert beneficial effects in metabolic disorders [5]. Not surprisingly, α-amyrin-loaded nanocapsules did not cause the death of human peripheral blood mononuclear cells (PBMC) (Fig. 7A), which suggests a selective cytotoxicity against leukemic cells (Fig. 7). Other authors have found evidence of the selectivity of α and β-amyrin against HL-60 tumor cells [7]. Nonetheless, further studies are needed to better understand and take advantage of this apparent selectivity.

5. Conclusions

One of the main problems when working with natural extracts is the low yield of the active compounds, which makes further synthesis necessary in order to increase not only the amount but also the purity of the active compound. On the other hand, molecules of natural origin frequently need to be chemically transformed to increase their potency and efficacy. The α-amyrin-loaded nanocapsules developed herein showed excellent potency and efficacy as antileukemic agents without presenting a cytotoxic effect on normal cells, which suggests a selective cytotoxicity against leukemic cells. However, other studies must be conducted to ensure their safe use.

Conflict of interest statement

The authors declare no competing interests.

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