Baccharis dracunculifolia extract-loaded chitosan nanoparticles: development, physicochemical characterization and cytotoxicity evaluation

Extrato de *Baccharis dracunculifolia* encapsulado em nanopartículas de quitosana: desenvolvimento, caracterização físico-química e avaliação da citotoxicidade

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Thalita Dias de Oliveira
Farmacêutica pela Universidade Federal de Juiz de Fora; Faculdade de Farmácia da Universidade Federal de Juiz de Fora
Rua: José Lourenço Kelmer s/n, Bairro São Pedro, CEP: 36036-900,Juiz de Fora/MG
thalita.diasoliveira@gmail.com

Lorena Rodrigues Riani
Mestre em Ciências Farmacêuticas pela Universidade Federal de Juiz de Fora; Faculdade de Farmácia da Universidade Federal de Juiz de Fora
Rua: José Lourenço Kelmer s/n, Bairro São Pedro, CEP: 36036-900,Juiz de Fora/MG
lorena_riani@yahoo.com.br

Mirsiane Pascoal Costa
Farmacêutica pela Universidade Federal de Juiz de Fora; Faculdade de Farmácia da Universidade Federal de Juiz de Fora
Rua: José Lourenço Kelmer s/n, Bairro São Pedro, CEP: 36036-900,Juiz de Fora/MG
mirsih.pc@gmail.com

Rodrigo Luiz Fabri
Doutor em Ciências Biológicas pela Universidade Federal de Juiz de Fora; Instituto de Ciências Biológicas, Departamento de Bioquímica, Universidade Federal de Juiz de Fora
Rua: José Lourenço Kelmer s/n, Bairro São Pedro, CEP: 36036-900,Juiz de Fora/MG
rodrigolfabri@yahoo.com.br

Jorge Willian Leandro Nascimento
Doutor em Fármaco e Medicamentos pela Universidade de São Paulo; Instituto de Ciências Biológicas, Departamento de Farmacologia, Universidade Federal de Juiz de Fora
Rua: José Lourenço Kelmer s/n, Bairro São Pedro, CEP: 36036-900,Juiz de Fora/MG
jorge.willian@ufjf.edu.br

Frederico Pittella Silva
Doutor em Bioengenharia, com ênfase em Ciências Farmacêuticas pela The University of Tokyo, Japão; Faculdade de Farmácia da Universidade Federal de Juiz de Fora, r. José Lourenço Kelmer s/n, Bairro São Pedro, CEP: 36036-900,Juiz de Fora/MG
frederico.pittella@ufjf.edu.br

Ademar Alves da Silva Filho
Doutor em Ciências Farmacêuticas (Fármaco e Medicamentos) pela Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo; Faculdade de Farmácia da Universidade Federal de Juiz de Fora, r. José Lourenço Kelmer s/n, Bairro São Pedro, CEP: 36036-900,Juiz de Fora/MG
ademar.alves@ufjf.edu.br

Guilherme Diniz Tavares
Doutor em Fármaco e Medicamentos pela Universidade de São Paulo; Faculdade de Farmácia da Universidade Federal de Juiz de Fora, r. José Lourenço Kelmer s/n, Bairro São Pedro, CEP: 36036-900,Juiz de Fora/MG
guilherme.tavares@farmacia.ufjf.br

RESUMO
Nesse estudo investigamos a adequabilidade da encapsulação do extrato em acetato de etila do ápice foliar de *Baccharis dracunculifolia*(BdAA) (Asteraceae), uma planta medicinal brasileira com promissoras aplicações farmacêuticas, em nanopartículas de quitosana (BdAA-Qui). As nanopartículas foram desenvolvidas pelo método de gelificação iônica e caracterizadas em relação ao diâmetro hidrodinâmico médio (DHm), índice de polidispersividade (IP), potencial zeta (PZ), eficiência de encapsulação (EE), com quantificação do Artepillin C, marcador químico do extrato, por CLAE-DAD, morfologia (por microscopia eletrônica de transmissão – MET) e citotoxicidade em linhagem celular de fibroblastos (L929). As BdAA-Qui apresentaram DHm superior às nanopartículas controle (sem o extrato). Esse pode ser considerado o primeiro indício da ocorrência da encapsulação dos constituintes do extrato na matriz polimérica das nanopartículas. Além disso, o IP obtido apresentou valor próximo a de sistemas monodispersos. Essa característica foi comprovada pelas imagens de MET. O valor de PZ positivo (20,4±0,85 mV) é característico da protonação da molécula de quitosana e pode implicar em estabilidade satisfatória das nanopartículas desenvolvidas. Ainda, esse valor foi semelhante àquele obtido para as nanopartículas controle, o que pode sugerir que os constituintes do extrato estejam no interior da nanopartícula e não adsorvidos em sua superfície. Em relação à EE, encontramos um valor elevado (cerca de 85%). Esse dado pode ser devido à interação eletrostática entre a quitosana e o Artepillin C (pka 4.65), o qual encontra-se prioritariamente sob a forma ionizada no pH padronizado para o preparo das nanopartículas (4.7). Por fim, a formulação desenvolvida não alterou a viabilidade das células durante o período de exposição (24 ou 48h). Esse dado preliminar é particularmente importante para garantir a segurança de uso do extrato encapsulado em nanopartículas de quitosana. Pelo exposto, o carreador escolhido mostrou-se adequado para encapsular o extrato BdAA visando futuras aplicações farmacêuticas.

Palavras-chave: *Baccharis dracunculifolia,* ápice foliar, Artepillin C, nanopartículas de quitosana

ABSTRACT
In this study, we investigated the suitability of encapsulating the ethyl acetate extract from the leaf bud of *Baccharis dracunculifolia* (BdAA), a Brazilian medicinal plant with promising pharmaceutical applications, in chitosan nanoparticles (BdAA-Ch). The nanoparticles were developed by the ionic gelation method and characterized concerning

the mean hydrodynamic diameter (Z-average), polydispersity index (IP), zeta potential (ZP), morphology (by transmission electron microscopy – TEM), encapsulation efficiency (EE), with quantification of Artepillin C, the chemical marker of extract, by HPLC-DAD, and cytotoxicity in a fibroblast cell line (L929). BdAA-Ch nanoparticles presented Z-average superior to control nanoparticles (without the extract). This finding can be considered the first indication of the extract encapsulation in the nanoparticles. Furthermore, the obtained IP presented a value close to that of monodisperse systems. TEM images have proved this feature. The positive PZ value (20.4±0.85 mV) is characteristic of the chitosan's protonation and may imply satisfactory stability of the developed nanoparticles. Furthermore, this value was similar to that obtained for the control nanoparticles, suggesting that the extract's constituents are inside the nanoparticle and not adsorbed on its surface. Concerning EE, we found a high value (around 85%). This data may be due to the electrostatic interaction between chitosan and Artepillin C (pka 4.65), which is primarily in the ionized form at the standardized pH to prepare nanoparticles (4.7). Finally, the developed formulation did not change cell viability during the exposure period (24 or 48h). This preliminary data is particularly important to guarantee the safety of using the extract encapsulated in chitosan nanoparticles. For these reasons, chitosan proved to be suitable for encapsulating the BdAA extract aiming for future pharmaceutical applications.

Keywords: *Baccharis dracunculifolia,* leaf bud, Artepillin C, chitosan nanoparticles

1 INTRODUCTION

Baccharis dracunculifolia DC (Asteraceae), commonly known as “alecrim do campo” or “vassorinha”, is a native medicinal plant of South America,found mainly in Brazil (Rodrigues et al., 2020). Previous studies with extracts of *B. dracunculifolia* aerial parts have identified many chemical compounds, such as phenylpropanoids, flavonoids, benzofurans, furanoditerpenes, and triterpenes (Da Silva Filho et al, 2004; Da Silva Filho, 2012), as well as promising antioxidant (Veiga et al., 2017), antimicrobial (Da Silva Filho et al., 2008), anti-inflammatory (Santos et al., 2010), anti-parasitic (Da Silva Filho et al., 2009), and anti-ulcer (Lemos et al, 2007) properties. Among compounds, Artepillin C is the main substance found in the leaf buds of *B. dracunculifolia* (Park et al., 2004) and has demonstrated many important biological activities, including antioxidant, anti-inflammatory, and anticancer (Shahinozzaman et al., 2020).

These findings demonstrate the great potential for using these plant extracts in the pharmaceutical field. In this context, it is essential to design a suitable formulation for the delivery of this bioactive compounds to guarantee its stability, safety, and efficacy. In this sense, nanostructured formulations have been extensively investigated, with particular attention to polymeric nanoparticles (Moritz et al., 2015; Tavares et al., 2020). Several biocompatible, biodegradable, and non-toxic polymers can be used for the preparation of

nanoparticles. From this perspective, chitosan (Ch) has been widely investigated (Zhao et al., 2018; Rizeq et al., 2019).

Ch is a biodegradable, hydrophilic, low toxicity, and biocompatible aminopolysaccharide obtained from the alkaline deacetylation of chitin, a polymer found in crustacean shells, insect cuticles, and the cell walls of some fungi (Franco et al., 2020; Du et al., 2014). Due to its mucoadhesive characteristics and its antioxidant, healing, antimicrobial, and anti-inflammatory actions, and since its use in humans is approved by different regulatory agencies, this polymer has been studied for the preparation of different drug delivery systems, especially nanoparticles (Desai et al., 2016; Quiñones et al., 2018). Ch nanoparticles have been evaluated for administration by different routes, such as oral, topical, parenteral, ophthalmic, and pulmonary (Mohammed et al., 2017), and are capable of encapsulating different active substances, for instance, synthetic drugs, proteins, nucleic acids and plant extracts (Servat-Medina et al., 2015; Ahmed et al., 2016; Nguyen et al., 2019).

Therefore, the goal of this study was to develop and characterize *Baccharis dracunculifolia* leaf buds´ extract-loaded chitosan nanoparticles aiming to establish the suitability of this carrier for future pharmaceutical applications of the extract.

2 MATERIAL AND METHODS

2.1 MATERIAL

Ethyl acetate, acetic acid, and DMSO were purchased from Synth (São Paulo, SP, Brazil). Chitosan, sodium tripolyphosphate, DMEM, fetal bovine serum, antibiotic solution (penicillin/streptomycin), and 3-(4,5-Dimethyl- 2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were acquired from Sigma-Aldrich (Saint Louis, MO, USA).

3 METHODS

3.1 ETHYL ACETATE EXTRACT OF THE LEAF BUDS FROM *B. dracunculifolia*

The study was developed following the Brazilian Federal Law (n° 13.123/2015) on Access to Genetic Heritage, registered under nº AE32DB3. Aerial parts (leaf buds) of *B. dracunculifolia* were collected on the campus of the Federal University of Juiz de Fora - UFJF (21°46'34.8"S 43°21' 52.4"W) in October 2016. The plant material was previously authenticated by P.L. Viana (Federal University of Minas Gerais), and a voucher specimen (CESJ 47482) was deposited at the Leopoldo Krieger Herbarium in the

Institute of Biological Sciences (UFJF).

Leaf buds (7.0 g) were dried, powdered, and extracted, by maceration, using ethyl acetate as solvent. After, solvent was removed under vacuum (Rotary evaporator R-210, Buchi, Switzerland) at 40°C to produce the crude ethyl acetate extract of the *B. dracunculifolia* leaf buds (BdAA) (5.25 g).

3.2 NANOPARTICLE PREPARATION

Ch nanoparticles (unloaded nanoparticles; n=3) were developed by the ionic gelation method (Calvo et al., 1997) with few modifications. First, chitosan (1.75 mg/mL) was solubilized in a 1% (v/v) acetic acid solution under magnetic stirring for 30min, with subsequent pH adjustment to 4.7 with a dilute aminomethyl propanol solution. Afterward, the crosslinking agent sodium tripolyphosphate (TPP - 1 mg/mL, pH 9) was slowly dropped, under constant agitation in a magnetic stirrer (1250 rpm; 1h), to obtain the nanoparticles. The chitosan:TPP ratio was kept at 5:2. To incorporate the extract to the nanoparticles (BdAA-Ch nanoparticles; n=3), ethanolic solutions of the extract at concentration of 50% (concerning the concentration of Ch) were added to the Ch solution and the agitation was kept for 30 min. Finally, TPP was dropped as described above.

3 NANOPARTICLES CHARACTERIZATION

3.1 SIZE AND POLYDISPERSION MEASUREMENTS

The mean particle size (as the hydrodynamic diameter; Z-average) and polydispersity index (PdI) were measured by Dynamic Light Scattering (DLS) using a Zetasizer Nano series (Malvern Instruments, Worcestershire, United Kingdom). Each analysis was performed at 25 °C, with a detection angle of 90°. The results were expressed as the mean hydrodynamic diameter, the standard deviation of the size distribution, and the PdI (n = 3).

3.2 ZETA POTENTIAL DETERMINATION

The zeta potential (ZP) values were obtained by electrophoretic mobility (Zetasizer Nanoseries, Malvern Instruments, Worcestershire, United Kingdom). For the determination, the samples were directly placed in an electrophoretic cell. The analyses were performed in triplicate.

3.3 MORPHOLOGICAL EVALUATION

The morphological characteristic of nanoparticles was assessed by transmission electron microscopy (MET) using the JEM-1011 equipment (Jeol LTD., Tokyo, Japan) operated at an accelerating voltage of 80kV. For analysis, an aliquot of both formulations (control and BdaAA-Ch nanoparticles) were placed on an amorphous carbon-coated ParlodionR 200 mesh (CF200-Ni, SEM) nickel grid and then dried for 24h at room temperature. Finally, the bright field option was selected for morphology analysis up to 50,000x magnification.

3.4 DETERMINATION OF ENCAPSULATION EFFICIENCY (EE)

EE was indirectly determined, using the ultrafiltration/centrifugation method (Wallace et al., 2012), by quantifying the free extract present in the supernatant considering its chemical marker, Artepillin C. Artepillin C was isolated according to previous report (Riani et al., 2021). The BdAA-Ch nanoparticles were diluted in ultrapure water, and aliquots (n=3) were transferred to Amicon ultrafiltration units (MWCO 10,000, Millipore, Germany) and centrifuged at 4.000 rpm for 30 minutes. The samples were analyzed by HPLC with a Diode Array Detector and an autosampler, using the methodology proposed by Nobushi et al. (2012). Equation 1 was used to calculate the EE.

$$EE\ (\%) = \frac{T_e - S_e}{T_e} \times 100 \quad \text{(Equation 1)}$$

Where T_e is the total amount of Artepillin C in the extract, and T_s is the amount of Artepillin C quantified in the supernatant.

4 CYTOTOXICITY EVALUATION

L929 cells were seeded in 96-well plates at a density of $5x10^3$ cells/well with DMEM containing fetal bovine serum (10%) and antibiotic solution (penicillin/streptomycin; 1%). Then, the plates were incubated overnight at 37°C in 5% CO_2. Ch nanoparticles and BdAA-Ch nanoparticles were added to the wells (n=5) in the following concentrations: 0.5; 5.0; 50, and 100 µg/mL. Cells with culture medium were considered negative control. The plates were incubated under the conditions described for 24 or 48h. After this time, 3-(4,5-Dimethyl- 2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was added, and the plates were incubated for 4h. The precipitate formed was dissolved with DMSO, and their absorbance was measured on a spectrophotometer (Spectramax 190, USA) at 540 nm. Negative control was considered as 100% viable cells.

Data were expressed as means ± standard deviations. The statistical analysis was performed by Student's t-test for paired samples. The level of *p-value* < 0.05 was considered significant.

5 RESULTS AND DISCUSSION

Several biological activities have been attributed to *B. dracunculifolia* extracts, such as antioxidant and anti-inflammatory (Santos et al., 2010; Veiga et al., 2017). Also, the scientific and commercial interests of *B. dracunculifolia* has grown in the last years, since this plant has been described as the major botanical source of the Brazilian green propolis (Brandenburg et al., 2020; Rodrigues et al., 2020; Park et al., 2004).

Considering this high pharmaceutical and commercial potentials, it is essential to develop formulations aimed at the proper administration of these extracts and guarantee their safety and efficacy attributes. In this sense, nanoparticulate formulations present advantages compared to conventional formulations, such as release control, increased bioavailability, and reduced toxicity of the encapsulated substance (Patra et al., 2018). Thus, we investigated the suitability of chitosan nanoparticles as a carrier for the BdAA, a promising extract for pharmaceutical applications (Munari et al., 2010). The results for Z-average, PdI, and ZP are shown in Table 1.

Table 1 -Z-average, PdI and ZP of nanoparticles*

	Ch	**BdAA-Ch**
Z-average (nm)	178.8±2.81	274.3±3.8
PdI	0.3±0.007	0.39±0.02
ZP (mV)	19.3±1.73	20.4±0.85

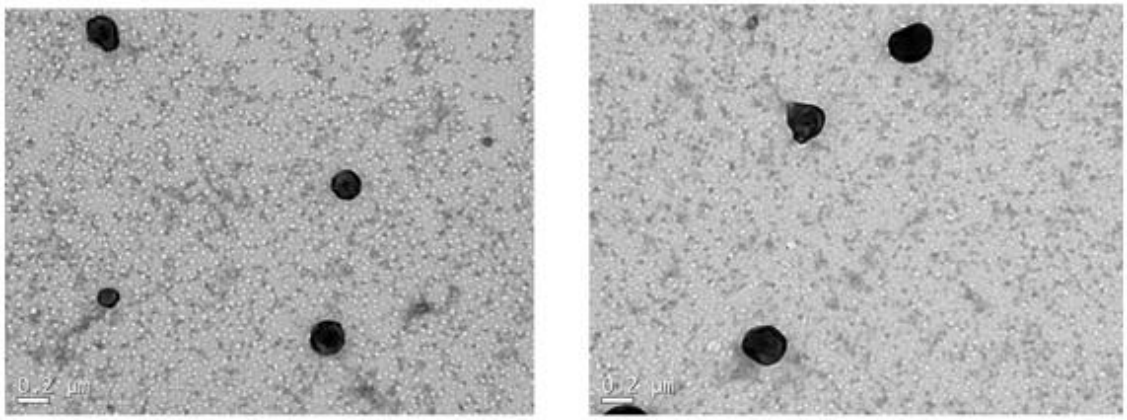
*Values are expressed as mean ± sd (n = 3).

Ch nanoparticles had characteristics similar to those found in other scientific studies (Cafaggi et al., 2007; Miladi et al., 2015; Picchi et al., 2021). In this sense, it should be noted that the obtained PdI value (0,3±0,007) reflects the achievement of a monodisperse system, which is crucial for maintaining the physical stability of a nanocarrier (Tavares et al., 2020) and may indicate that the methodology used was effective for the preparation of Ch nanoparticles. Furthermore, the ZP value (19,3±1,73 mV) is due to the ionization of the chitosan molecule in an acidic environment and can contribute to physical stability due to the repulsion between particles (da Silva et al., 2020). For BdAA-Ch nanoparticles, we noticed an increase in Z-average, which may be due to the encapsulation of the extract in the polymer matrix. Furthermore, a more polydisperse system was obtained, probably due to the heterogeneous incorporation of

the extract´s constituents in nanoparticles. Also, ZP showed no significant change, suggesting that the substances were incorporated more internally into the nanoparticles and not adsorbed on their surface.

TEM images (Figure 1) revealed that the particles have a spherical shape and monodisperse behavior, corroborating the DLS results. Furthermore, a slight increase in the size of BdAA-Ch nanoparticles could be observed compared to Ch nanoparticles. This observation may be an additional indication of the occurrence of encapsulation of the constituents of the extract.

Figure 1. TEM images of (a) Ch nanoparticles and (b) BdAA-Ch nanoparticles. Bars = 200 nm.

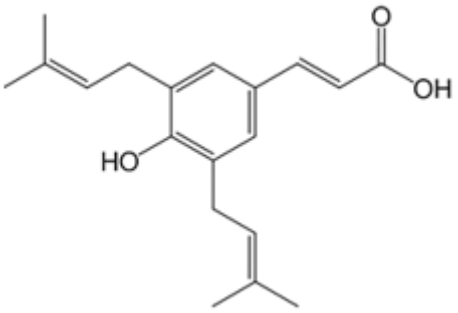


(a) (b)

The concentration of Artepillin C in the extract of *B. dracunculifolia* was equal to 1.198 µg/mL (equivalent to 1.59% of the BdAA extract). Thus, after quantifying this compound in the supernatant of the nanoparticles, we found an EE equal to 84.97± 5.6% (1.018 µg/mL of Artepillin C). We consider that the high encapsulation of Artepillin C is an important result, as this compound is one of the main substances found in the leaf bud of *B. dracunculifolia* and has promising biological activities (Shahinozzaman et al., 2020; Beserra et al., 2020).

The incorporation of substances into chitosan-based nanoparticles is mainly governed by the electrostatic interaction of these positive particles with anionic bioactive (Mudhakir et al., 2014). In this sense, Artepillin C (Figure 2) is a molecule derived from cinnamic acid, which has a carboxylic group in its chemical structure.

Figure 2 - Chemical structure of Artepillin C.



This group is subject to ionization as a function of the pH of the medium and, therefore, the molecule presents a predominance of negative charge at pH values above its pka (4.65) (Camuri et al., 2018). Thus, since the pH value for the preparation of chitosan nanoparticles was standardized at 4.7, this compound is ionized. This fact may justify its high encapsulation in the polymeric matrix of nanoparticles.

Cell viability results of Ch nanoparticles show that there was no significant difference between the different concentrations of nanoparticles and the control group, regardless of exposure time ($p > 0.05$) (Figure 3). This data may corroborate the chitosan's biocompatibility and low toxicity proportions (Rodrigues et al., 2012). Similarly, BdAA-Ch nanoparticles did not reduce cell viability compared to the control group ($p > 0.05$), even after 48 hours of exposure (Figure 3). This result indicates that the encapsulation in chitosan nanoparticles can contribute to the safe use of BdAA extract.

Figure 3 - Cytotoxicity evaluation by MTT assay of Ch nanoparticles in L929 cells (results are expressed as mean ± s.d.; n = 5). (a) after 24 hours of exposure. (b) after 48 hours of exposure.

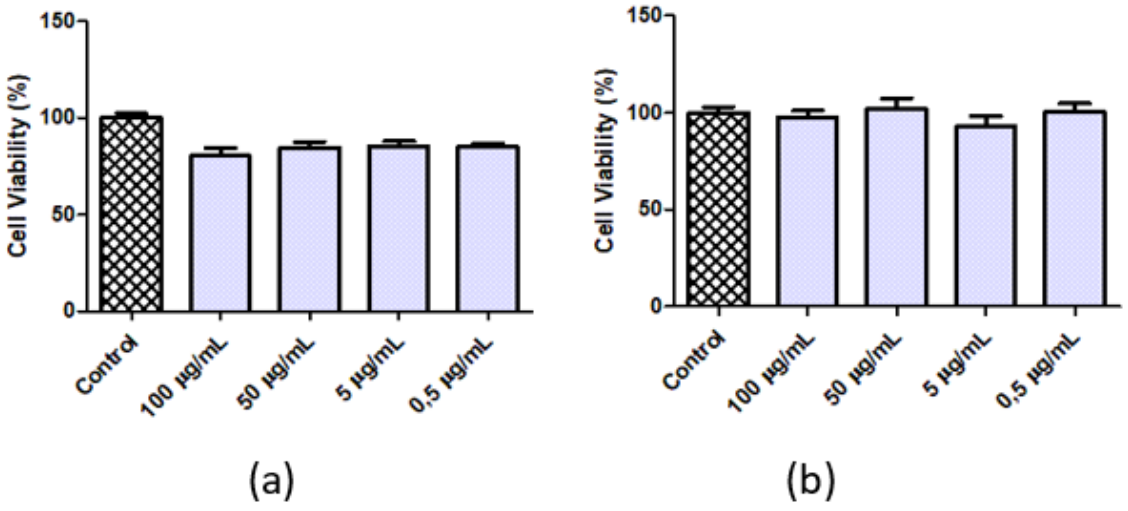
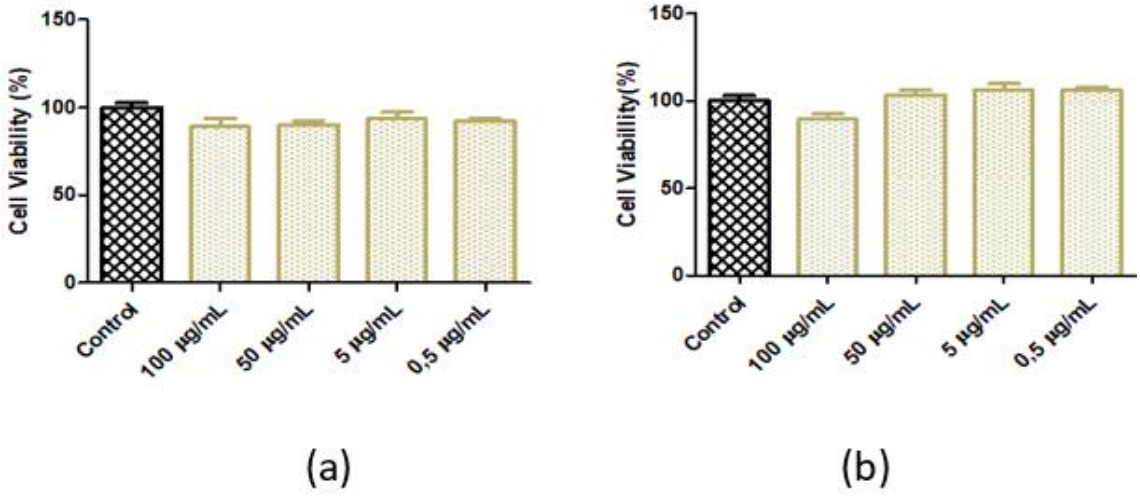


Figure 4 - Cytotoxicity evaluation by MTT assay of BdAA-Ch nanoparticles in L929 cell (results are expressed as mean ± s.d.; n = 5). (a) after 24 hours of exposure. (b) after 48 hours of exposure.



6 CONCLUSION

Our study resulted in the successful development of chitosan nanoparticles aiming at the delivery of BdAA extract. Ch-BdAA nanoparticles presented suitable physicochemical characteristics based on size, PdI, and ZP evaluation. By the TEM images, we proved the obtainment of nanometric particles and with a monodisperse profile. The encapsulation of the extract resulted in high incorporation of Artepillin C, its chemical marker. In cell viability tests, the developed nanoparticles proved to be safe, which demonstrates the biocompatibility of chitosan and validates this choice for the extract encapsulation.

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