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Research Article

Nanoencapsulation of the Aranto (*Kalanchoe daigremontiana*) Aquoethanolic Extract by Nanospray Dryer and its Selective Effect on Breast Cancer Cell Line

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ABSTRACT
The Aranto (*Kalanchoe daigremontiana*) is a succulent and xerophytic plant that contains glycosides, flavonoids and lipids; these last ones include triterpenoids and bufadienolides that have cytotoxic activity against various cancer cell lines. In this investigation applied to anticancer therapy, it was obtained an aquoethanolic extract from *K. daigremontiana*, and nanoencapsulated in Poly (D, L-lactide-*co*-glycolide) (PLGA), by the nanospray drying technique. *In vitro* experiments showed that the nanocapsules (NC) containing the aquoethanolic extract from *K. daigremontiana* had a better cytotoxic effect (IC_{50} 48.53 µg/ml), compared to the non-encapsulated aquoethanolic extract (IC_{50} 61.29 µg/ml) in the MDA-MB-231 metastatic breast cancer cell line. On the other hand, when comparing the cytotoxic effect in the breast non-cancerous cell line MCF 10A we found no cytotoxic effect of the NC containing the aquoethanolic extract of *K. daigremontiana* with concentrations of the extract equal or lower than 200 µg/ml, whereas the aquoethanolic extract which was not encapsulated had a positive cytotoxic effect (IC_{50} 100.2 µg/ml). These results showed an important selectivity of the cytotoxicity of the NC with the aquoethanolic extract to the cancerous cells.

Keywords Breast cancer, *Kalanchoe daigremontiana*, Poly (D,L-lactide-*co*-glycolide), Cytotoxicity, Nanospray Dryer, nanocapsules

INTRODUCTION

The use of nanoparticles is very important for nanomedicine as drug delivery systems, because the drugs can be controllably and sustainably released. Nanomedicines have a subcellular size, and biocompatibility with tissue and cells[1]. Investigations by Anand et al.[2] showed that the use of nanostructured drugs minimizes the degradation of the active compounds, increases its permanence inside the targeted organ and reduces their toxicity. Therefore, the particle size can play an important role[3], since large particles can potentially induce a permanent damage to the cell membrane, while small particles can pass through the membrane and damage within the cell. The shape also plays an important role, because non-spherical particles might have a different biological response compared with spherical nanoparticles[4,5]. Respecting the polymers used in the preparation of nanoparticles, the high molecular weight generally leads to nanoparticle systems with low polydispersity index[6]. Furthermore, Siqueira et al.[7] when using cellulose (polymer of high molecular weight which has 3000 glucose units), obtained favorable results observing nanoparticles with almost spherical morphology, associated with a form factor of 0.78 ± 0.06. For nanomedicine, the molecular recognition has become very important; in this phenomenon an antibody, a fragment of DNA or other molecule, binds or hybridizes to its target; that is why the "nanometer" scale is often crucial for the ability of a nano-bio system to bind with high specificity the analyte of interest[8].

Employing a nanosystem containing the drugs, that could cross the cell membrane and reach the specific intracellular target, may allow an efficient therapeutic response for antineoplastic drugs, mainly because they have their targets within the tumor cells[9]. Recent studies[10] showed that the nanoparticles, prepared with polymers conjugated to the drugs, accumulate passively in the tumors, they contain larger pores than the normal tissue (100-1000 nm in diameter). This agglomeration occurs even in the absence of ligands; this passive mechanism has been called

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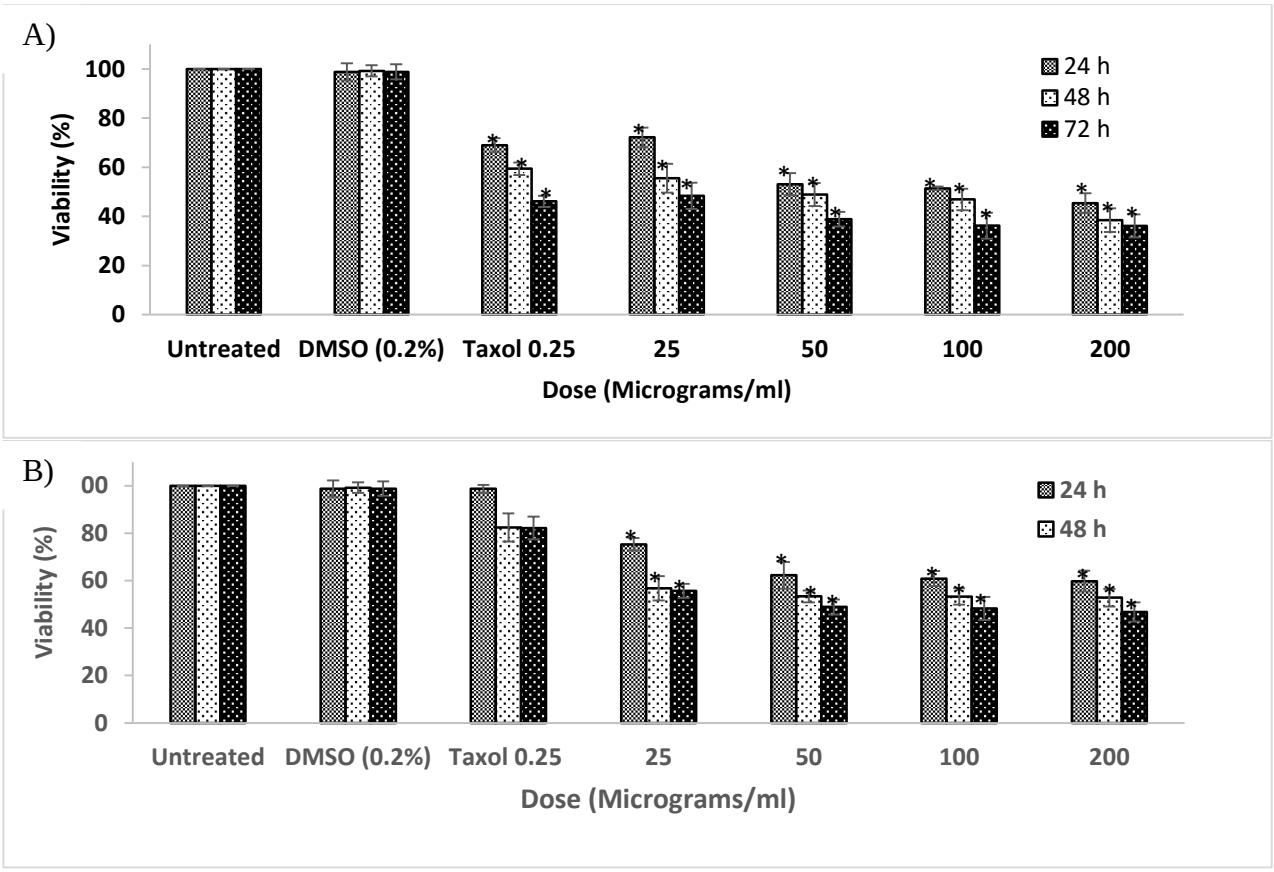

Figure 1: Effect of the aquoethanolic extract of K. daigremontiana in A) tumor cell line MDA-MB-231, B) normal cell line MCF10A. (* p <0.05) compared to untreated cells

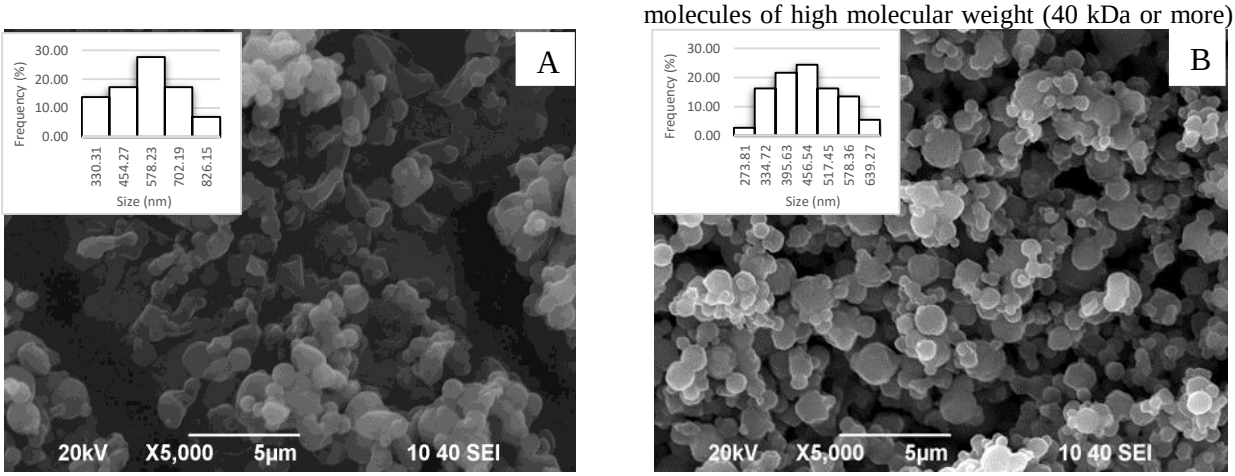

Figure 2: Nanocapsules of PLGA obtained by nanospray dryer A) without extract and B) with aquoethanolic K. daigremontiana extract.

"Enhance of permeability and retention" (EPR)[11]. This phenomenon explained by Maheda,[12] is based on the theory that tumor cells, because of its rapid growth, induce the production of new blood vessels that are poorly organized, these blood vessels present surface aberrations, thus allowing the passage of macromolecules and nanoparticles to the tumor tissue and afterwards leading to a selective accumulation in the interstitial space. Whereby it has been specified that, in the field of oncology, molecules of high molecular weight (40 kDa or more) as well as pharmaceutical vehicles with a nanometer size, are able to accumulate themselves in various pathological tissues such as solid tumors[9].

Usually, cytostatic and cytotoxic drugs, radiation and surgery, are used to fight against cancer. It is also common to use, in advanced pathologies, alternatives that seek the acceleration of the cell apoptosis process, like ethnomedicine[13,14]. Such is the case of various species of the genus of *Kalanchoe*, that have been studied, mainly

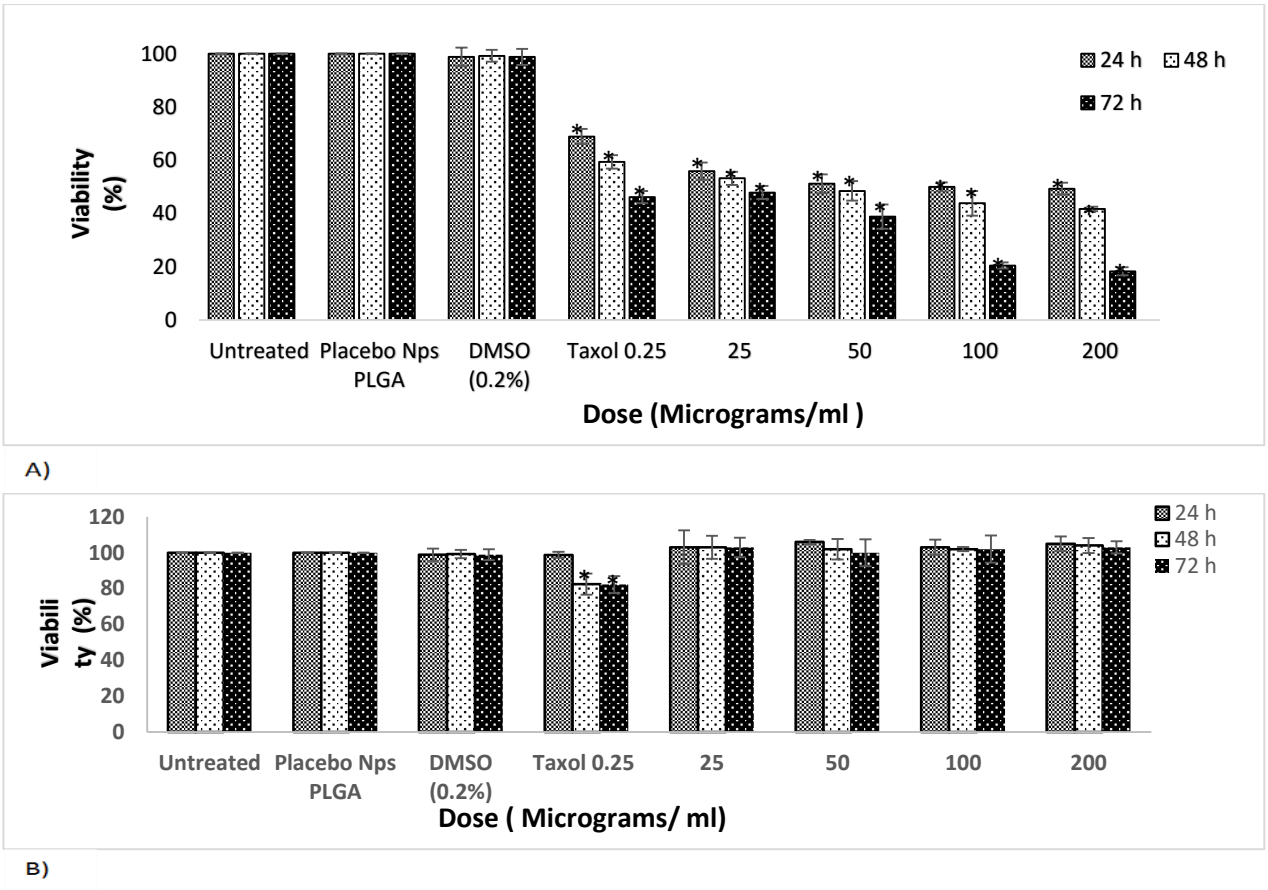

Figure 3: Effect of PLGA nanoparticles of aquoethanolic K. daigremontiana extract A) tumor cell line MDA-MB-231, B) Normal MCF10A cell line. (* $p < 0.05$) compared to untreated cells.

focusing on their antitumor activity, testing different cell lines such as KB cells, human lung carcinoma cells A 549, ileocecal colorectal adenocarcinoma cells HCT-8, human nasopharyngeal carcinoma and intramuscular carcinosarcoma cells, reporting inhibitory activity[15,16]. Nowadays, *in vitro* cytotoxicity of some species of *Kalanchoe* (*K. pinnata, K. daigremontiana* and *K. flammea*) have been evaluated in uterine cervical and prostate cancer cell lines with moderate cytotoxic results[17]. Preliminary evaluations, by our group, showed that the aquoethanolic extract of *K. daigremontiana* induces cytotoxicity in the human mammary gland adenocarcinoma cell line MDA-MB-231. Due to these results, and the needs related to cancer therapy, it was proposed the evaluation of the effect of the free and nanoencapsulated herbal medicine in the viability of MDA-MB-231 cells and compare its behavior with the normal breast cells MCF 10A.

MATERIALS AND METHODS

Preparation of the aquoethanolic extract of K. daigremontiana.

Foremost, about 18 kg of the plant were chosen and collected from the Hidalgo state, in the municipality of Ixmiquilpan, Mexico. Subsequently, this was sanitized with a solution of sodium hypochlorite (NaClO, 1%), and then dried in a vacuum oven at 30 °C to dryness. The dried material was ground to a granulometry of 80 mesh in a hammer mill (Mikro pulverizer 2TH model, USA). The sieved material was stored in polyethylene containers for later uses. The extraction was carried out by maceration; for that, 10g of the dry sample of *K. daigremontiana* were added to 200 ml of the aquoethanolic solution (33.3% ethanol in deionized water, 18μΩ cm^{-1}, Easypure, EU), the mixture was stirrer at room temperature during 24 h. Then, the extract was centrifuged (SIGMA Laborzentrifugen, Model: 2-16: Germany) at 3000 rpm for 5 min. The supernatant solution was separated and the residues were washed consecutively, four times, until the total extract was obtained. The supernatant was concentrated in a rotavapor (rotary evaporator, Yamato, model RE500) at 40 rpm in a water bath at 35 °C and concentrated to dryness in a vacuum oven (Thelco Precision, Model 29) at 30 °C, 15 lb. The extract was stored for later uses.

Preparation of PLGA nanoparticles

Polymeric nanoparticles were prepared with PLGA (Poly(D,L-lactide-co-glycolide), 75:25), mol wt 66,000-107,000 (Sigma Aldrich), directly by the drying method, using a nano spray dryer[18]. A 0.5% w/v solution was prepared (PLGA as polymer and the dry extract of *K. daigremontiana*, in a ratio of 50% w/v total solids) employing acetone as solvent. The solution was magnetic stirred at 300 rpm, constantly, and feed to the Nano spray dryer equipment (Model B-90, BÜCHI Labortechnik AG, Flawil, Switzerland). The drying process was develop at 120 °C, 35 mbar.

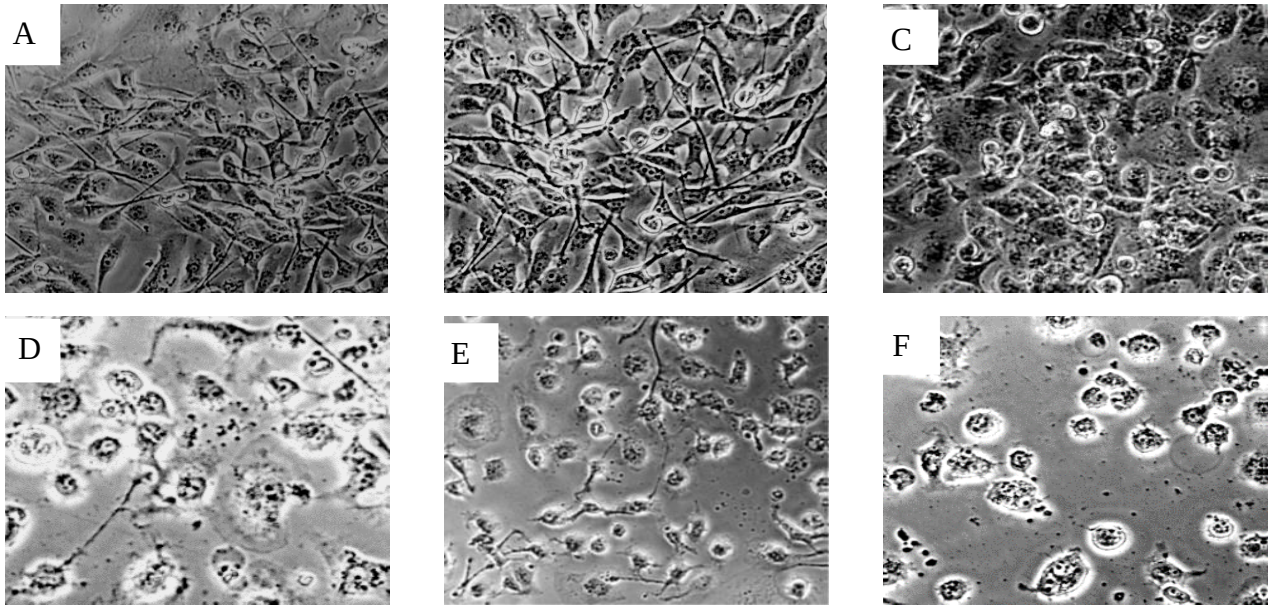

Figure 4: Micrographs of cell line MDA-MB-231 (20X) 24h treatment time. A) Untreated cells B) cells with DMSO C) cells with Taxol, D to F) cells with aquoethanolic K. daigremontiana extract at doses 25, 50, and 100 µg/ml respectively.

Particle characterization: size, zeta potential and morphology by SEM

The size and morphology of the nanoparticles, in dry, were determined by scanning electron microscopy (SEM, JSM-6390LV, JEOL, Japan), the samples were placed in holders attached to graphite tape, and then were covered with a thin layer of silver by a vacuum sputtering process (Desk IV, Denton vacuum, USA). The micrographs were performed at 5000 and 10000 magnifications. The hydrodynamic diameter of the nanoparticles and their polydispersity index (PdI) were measured by dynamic light scattering (Zetasizer Nano Zs, Malvern Instruments, Ltd., Malvern, UK) at 25 °C, whereas the surface charge (Zeta potencial, mV) was measured by electrophoretic mobility and analyzed in folded capillary cells with the same instrument and experimental conditions.

Cell line and culture conditions

The human mammary adenocarcinoma cell line MDA-MB-231 was used and cultured in DMEM (Dulbecco's Modified Eagle's Medium, Gibco) supplemented with 5% fetal bovine serum (Invitrogen). Whereas the non-cancerous human breast epithelial cell line MCF 10A was cultured in DMEM-F12 (Gibco), supplemented with 10% fetal bovine serum (Invitrogen), EGF (100 mg/ml, Sigma), insulin (100 mg/ml, Sigma) and hydrocortisone (1 mg/ml, Sigma). Both cell lines were incubated at 37 °C, in an atmosphere of 5% CO_2 and constant humidity[19].

In vitro cytotoxicity testing

For the evaluation of the antitumor activity of the nanoparticles formulated with the aquoethanolic extract of *K. daigremontiana*, the tumor cell line MDA-MB-231 and the normal cell line MCF 10A were used. The cells were harvested, centrifuged and resuspended in medium, DMEM and DMEM-F12, respectively. Then, a cell account was made for sowing 7000 cells per well, in 96-well microplates, and then incubated at 37 °C for 24 h, 5% of CO_2 and constant humidity. Three negative controls were used, 1) cells without treatment, 2) cells treated with 0.2% DMSO and 3) PLGA nanoparticles without aquoethanolic extract; on the other hand, 0.25 µg/ml taxol was used as positive control. Different concentrations of the aquoethanolic extract free and incorporated into the NC were tested (25, 50, 100, 200 µg/ml) at 24, 48 and 72 h. After this time, 20 µl of the 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT, Sigma) solution (5 mg/ ml) were added to each well. Then, the plates were incubated for 1.5 h at 37 °C in darkness. After incubation, the supernatant was discarded and 100 µl of DMSO were added to each well. The analysis was carried out in an ELISA reader (Labsystem Multiskan MS) at 570 nm. The calculations of the viability percentages were performed by eq. 1[19].

$$Viability\ (\%) = \frac{(OD\ Problem)}{(OD\ Control)} X\ 100 \quad \text{Eq. 1}$$

Further, a nonlinear regression between percentage survival and the log concentration was developed to calculate the IC_{50} value by the 5.0 Graph Pad Prims software. The cytotoxicity was determined by MTT assays by triplicate.

RESULTS AND DISCUSSION

In vitro assays of cell viability

The aquoethanolic extract of *K. daigremontiana* observed in Fig. 1A indicate that has a lethal effect on the MDA-MB-231 cells in a dose- and time-dependent way. Therefore, a significant decay in cell viability started even though with the lowest dose. For the dose of 25 µg/ml, there were observed reductions on the cell viability of approximately 30% for 24 h and 50% after 72 h of treatment. When the cells were treated with a dose of 100 µg/ml and evaluated 24 h later, the viability decreased to 51% and to 36% after 72 h of treatment. The treatment with

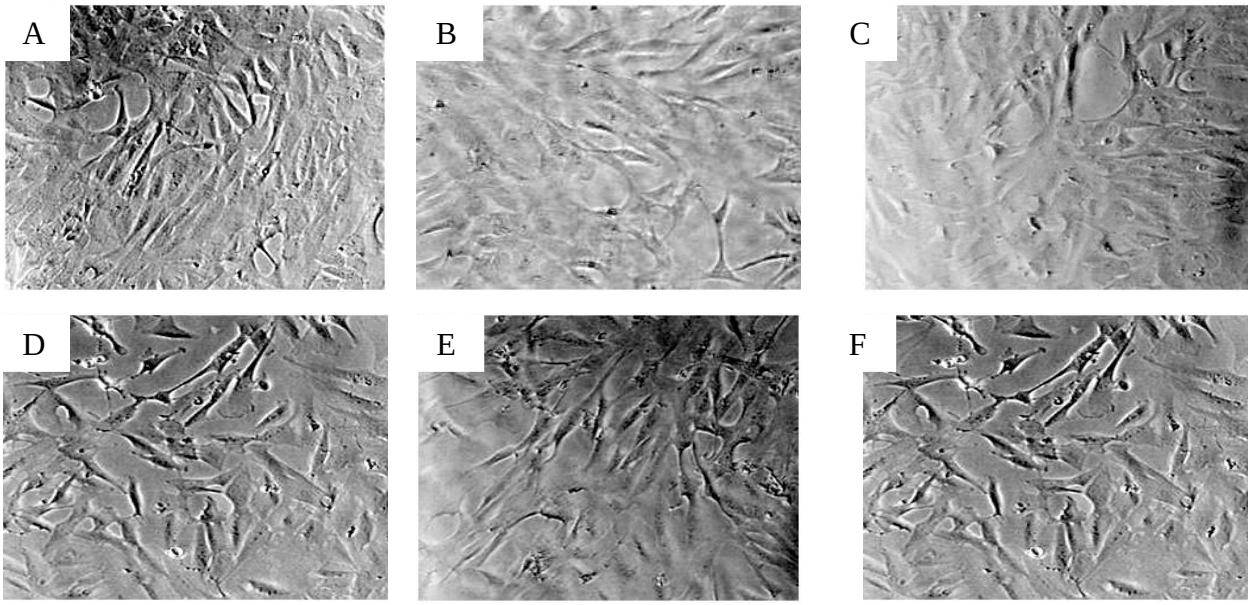

Figure 5: Micrographs of normal cell line MCF10A (20X), treatment time 24 h. A) Untreated cells B) control cells with PLGA; C to F) cells with nanocapsules of PLGA aquoethanolic K. daigremontiana extract at doses 25, 50, 100, and 200 µg/ml respectively

a dose of 200 µg/ml, showed a cell viability about 45% and up to 36% at 72h.

Evaluations in the normal breast cell line MCF 10A with the aquoethanolic extract of *K. daigremontiana* (Fig. 1B) also showed a cytotoxic effect, producing a significant decline in cell viability, with a similar behavior to the tumor cell line MDA-MB -231, although the values of the decrease in viability were lower for the different treatments. For a 25 µg/ml dose, the cell death was about 25% at 24 h and about 50% at 72 h. With the 100 µg/ml dose the cell viability decreased to 61% and 48% after 24 h and 72 h, respectively and, with the dose of 200 µg/ml, the viability decreased to 60% at 24 h and decreased to 47% at 72 h.

These results indicate that the aquoethanolic extract of *K. daigremontiana* has a positive cytotoxic effect in both breast cell lines, the tumorigenic and normal ones, having no selectivity in their cytotoxic action; however, the normal cells were slightly less sensible to the treatment.

Preparation and characterization of nanocapsules

Nanocapsules of PLGA were formed with the dry aquoethanolic extract of the *K. daigremontiana* dissolved in acetone, by using a nano spray dryer; they were characterized in terms of their morphology, size, SEM microstructure and surface charge by ξ potential. Nanocapsules without the *K. daigremontiana* extract were also prepared as a control, see Fig. 2.

The PLGA particles size distribution, without containing Aranto extract was of 552.5 ± 154.71 nm evaluated by SEM (Fig. 2A) and for the distribution of PLGA nanoparticles formulated with aquoethanolic *K. daigremontiana* extract was of 453.36 ± 95.18 nm, both presenting a shape close to spherical (Fig. 2B), obtaining a 90% of encapsulation. It is well known that opsonization is influenced by the size and surface characteristics of the particles, when the particles are very small, <200 nm in diameter they are quickly eliminated from our system[10]. Therefore the optimal size range of the nanoparticles, to keep them from being eliminated and ensure penetration through the pores of tumor cells, should be of 200-800 nm diameter. On the other hand, the hydrodynamic diameter of the NC without the extract was of 197.13 + 4.56 nm, whereas the NC containing the extract showed diameters of 267.66 + 6.32 nm. These differences observed in the samples are explained because: 1) the dry samples allow the aggregation of the NC making difficult the correct measurement of the samples; so then, the best technique to determine the size of the NC was DLS and, 2) the hydrodynamic diameter values observed in the NC without the extract and the NC containing the extract, are explained due to the inclusion of the extract within the NC body, what increases the size of the particles.

These NC containing the extract where developed as an alternative to study the future cytotoxic effect of the aquoethanolic extract *K. daigremontiana* in "live" models and to target nanocapsules to tumor cells, based on the passive diffusion principle[11].

We used PLGA due to the absence of toxicity in its degradation products, and because of its potential adjustable velocity of degradation[20,21]. The biodegradation of PLGA is developed with a very slow speed and thus, do not affect the normal cell functions[18]. Investigations had reported the preparation of PLGA nanoparticles with Taxol (paclitaxel) for the treatment of brain cancer[22], colon cancer[23], leukemia and cervical cancer cells[24]. All of them concluding that the use of nanoencapsulated drugs induce a better cytotoxic effect in the treatment of various cancer cell lines. There were not observed changes in the zeta potential values of the NC when they were evaluated without the extract (- 13.76 $\pm$ 0.55 mV, in PBS and -0.09 + 0.17 in DMEM) and with the extract (-14.73 $\pm$ 1.81 mV,

in PBS and -0.10 ± 0.04 in DMEM). Both formulations showed negative zeta potential values.

Cell viability tests with the nanoencapsulated extract

The release of the active agent from the polymeric matrix of the PLGA nanoparticles is a complex process, which is attributed to two phenomena: the diffusion of the drug due to the physicochemical properties of the phytomedicine and the degradation of the PLGA, what decreases the molecular weight of the polymer, allowing the drug to travel easier across the matrix[25]. The results in Fig. 3A show that for the tumor cell line (MDA-MB-231), by the performing of cytotoxicity tests with untreated cells, cells treated with DMSO and cells treated with nanocapsules of PLGA (without extract), no cell death was observed. In contrast, the cells exposed to PLGA nanocapsules containing the *K. daigremontiana* extract showed a significant decrease in their viability, even for the lowest dose (25 µg/ml), in which a cell death of 34% was observed over a period of 24 h, whereas 52% cells were unviable at 72 h. When cells were treated with a dose of 100 µg/ml, the cell viability was decreased by 50% at 24h and after 72h a cell death of 80% was observed, similar results were found when the cells were treated at a dose of 200 µg/ml. In the assays developed to evaluate the toxicity of the NC loaded with the *K. daigremontiana* aquoethanolic extract in the normal cell line (MCF 10A, see Fig. 3B) it was observed that the untreated cells, DMSO-treated cells and cells treated with NC without the extract (placebo) did not showed affected cell viability values. On the other side, the administration of 0.25 µg/ml of taxol decreased by 20% the cell viability at 48 h and 72 h of treatment. However, the NC loaded with the aquoethanolic extract at different concentrations (25, 75, 100 and 200 µg/ml) did not cause cell death in the normal cells as it did in the tumor cell line.

This effect is very important, because it evidences selectivity in the cytotoxicity of the nanoencapsulated *K. daigremontiana* aquoethanolic extract, induced mainly by the nanoencapsulation process and not by the components. The NC containing the extract shows *in vitro* a selectivity that differs from most of the drugs in clinical use, that do not discriminate cancer cells from normal cells[11].

IC_{50}Values

The NC containing the *K. daigremontiana* extract had a better cytotoxic effect (IC_{50} 48.53 µg/ml) than the aquoethanolic extract administered un-encapsulated (IC_{50} 61.29 µg/ml) in the MDA-MB-231 cells. For the MCF 10A cells, the un-encapsulated extract had a positive cytotoxic effect (IC_{50} 100.2 µg/ml), whereas the nanoencapsulated *K. daigremontiana* extract did not induce cell death to these cells. Investigations developed by other authors in these vegetal species have shown IC_{50} values dependent on the cell line[26].

Effect of the aquoethanolic extract on cell morphology

The results of cell viability at 24 h were corroborated by the cell micrographs shown in Fig. 4 and 5. Fig. 4 shows that the MDA-MB-231 control cells: A) untreated cells and B) cells with DMSO have an elongated and well-defined shape and are confluent. When cells were treated with taxol (Fig. 4 C), it was observed a change in the morphology, resulting in a rounded (spherical) shape, a reduction in the cellular size, a cell membrane deformation and greater separations between the cells. Interestingly, when tumor cells were exposed to the *K. daigremontiana* aquoethanolic extract (25, 50, and 100 µg/ml, Fig. 4 D, E and F, respectively) their morphology also changed, probably due to the induction of an apoptotic process, deduced by the morphological events such as nuclear fragmentation and condensation, separation of cytoplasmic organelles and deformations in the cell membrane. These modifications are intensified with increased concentrations of the extract. This result corroborates the findings of the viability assays. The same behavior was observed by[14], when the *Coptidis rhizome* aqueous extract was administered to the MDA-MB-231 cell line.

When the cancer cells MDA-MB-231 were treated with the *K. daigremontiana* aquoethanolic extract nanoencapsulated in PLGA, they showed a similar behavior to the treatment with the un-encapsulated extract. The NC with the extract produced a cell death, probably by apoptosis, with the morphological changes listed previously. Surprisingly, the cell death increased when the NC were used, compared with the un-encapsulated extract at the same concentration and time of treatment (see Fig. 4). The normal cells MCF 10A, when adding *K. daigremontiana* aquoethanolic extract nanoencapsulated with PLGA in doses of 25, 50, 100 and 200 µg/ml (Fig. 5), did not present changes in their morphology and showed the same development as the controls. This means: they had an elongated shape, were confluent, and did not present apparent modifications in cell membranes and inside them.

The results generated by the treatment with the *K. daigremontiana* extract nanoencapsulated have a very important contribution and could be attributed to the small size of the capsules that can be introduced in tumor cells and not in the normal breast cells, due to the difference in surface morphology of the cells. This size of NC would help the penetration of nanoparticles into the cell and thereby carry out the combined process of erosion and diffusion of the polymeric matrix, releasing the active ingredient (*K. daigremontiana*) of the PLGA nanocapsules within, causing the cell death.

CONCLUSIONS

The *K. daigremontiana* aquoethanolic extract nanoencapsulated with PLGA was obtained by the spray drying method. The treatment with the NC containing the *K. daigremontiana* aquoethanolic extract had performed better than the crude extract, in the cellular breast cancer cell line MDA-MB-231. The nanoencapsulated extract showed a selective cytotoxic effect, avoiding cell death of normal cells (MCF 10A) and causing the death of tumor cells, this contribution is very important for the chemotherapy.

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