MICROENCAPSULATED AND NANOENCAPSULATED FLAVONOIDS AGAINST GLIOBLASTOMA CELLS

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ABSTRACT: The applications of microcapsules include the controlled release of drugs, protection against rust or protection of substances against interactions with the environment. Biodegradable microcapsules are typically used to increase the therapeutic value of water-soluble or –insoluble medicinal drugs, increasing bioavailability, solubility and retention time. Flavonoids are a class of metabolites with many biological properties. In the present study we prepared microcapsules and nanocapsules of the flavonoids apigenin, 7-apiosyl scutellarein and quercetin to be tested against different glioblastoma (GBM) cell lines. Nanocapsules of 7-apiosyl-scutellarein reduced the viability of U87 and GBM 95 cell lines. Microcapsules of apigenin and quercetin reduced cell viability and number of cells in culture in all GBM cell lines tested. Flavonoids and microcapsules were not cytotoxic to astrocytes. This is the first report of 7-apyosil-scutellarein nanocapsules and their assays against gliomas.
KEYWORDS: Flavonoids, microcapsules, nanocapsules, NMR Solid analysis, glioblastoma.

1 | INTRODUCTION

Flavonoids are a large family of natural products that can be found in nearly all plant species. At present, flavonoids comprise more than 6500 natural compounds. They play an important role in the interaction of plants with the environment, being responsible for the color of flowers, fruits and sometimes of leaves, and also have a protective function against the harmful effects of ultraviolet radiation (1). Flavonoids play an important role in protecting plants against invasion by microorganisms. This function involves not only constituting derivatives, but also those that are accumulated as phytoalexins in response to this attack (2). Their distribution varies in different organs, tissues and growth stages and is influenced by environmental factors (1). The study of flavonoids is becoming increasingly interesting because of their important metabolic roles in plants and animals and their potent and diversified biological activities (3).

Malignant gliomas are primary brain tumors representing about 78% of all malignant tumors of the central nervous system (4). Glioblastoma (GBM) is one of the most aggressive human cancers. Despite current advances in multimodality therapies such as surgery, radiotherapy and chemotherapy, the outcome for patients with high grade gliomas remains fatal (5). Previous *in vitro* studies have demonstrated the potential inhibition of glioma cell growth by various flavonoids, leading to cell death as a sign of loss of viability. Quercetin acted in a dose- and time-dependent manner causing apoptosis through oxidative stress (6-8). Clinical studies of quercetin have been limited by its extreme water insolubility requiring dissolution in dimethylsulfoxide which is not biologically inert, with cases of neurological toxicity as well as cardiovascular and respiratory problems having been reported (9). Apigenin inhibited cell proliferation and reduced TGF-β, a multifunctional cytokine that stimulates migration and tissue invasion by glioma cells (10).

Microencapsulated products are widely used in the food, pharmaceutical and cosmetic industries. The particles obtained are called microcapsules or microspheres

according to their internal structure, i.e., core-shell-like or matrix, respectively. Microparticles may contain a solid, liquid or gaseous active substance ranging in size between about 1 micron and 1 millimeter. Particles of smaller size, from 1 nanometer to 1 micrometer, are called nanoparticles, nanocapsules and nanospheres and can also be distinguished according to their internal structure (11). Encapsulated drugs can have better solubility, with improved biological barrier crossing properties and better controlled release kinetics, leading to substantial clinical advantages including dose reduction, prevention of side effects and improvement of bioavailability within the targeted tumor cell (12-13). Resveratrol-loaded lipid-core nanocapsules and quercetin nanoliposomes induced C6 glioma cell death (14-16). Many polymers can be used for encapsulation through different techniques. Polylactic acid and its glycolic acid copolymers (poly(lactic-co-glycolic acid, PLGA) are widely employed for the preparation of sustained-release preparations and PLGA microparticles have been successfully employed against glioma cells (17). In the present study we prepared microcapsules of the flavonoids apigenin, 7-apiosyl scutellarein and quercetin for testing against different lines of glioblastoma cells.

2 I EXPERIMENTAL

2.1 General experimental procedures.

Quercetin, apigenin, PLGA and poly vinyl alcohol (PVA) were obtained from Sigma. 7-Apyosil scutellarein was isolated from *Lantana trifolia* as described below. HCl, acetonitrile, methanol, and acetone were purchased from Vetec and used without further purification. ^{1}H (400 MHz), DEPT-Q (125 MHz), COSY, HSQC, and HMBC spectra were acquired at room temperature on a Bruker Topspin 400 NMR spectrometer (Rheinstetten, Germany) with tetramethylsilane as an internal standard. ESIMS was performed using an Esquire 3000 plus Bruker Daltonics spectrometer with a nebulizer: 27 Psi, Drygas: 7 L/min., Temp: 300°C, HV: 4000V. UV-vis absorption was measured with a Shimadzu, model UV-mini 1240-reader. Centribio centrifuge 80-2b. Hitachi TM3000 - Tabletop Microscope. Ultrasonic Processor, Hielscher, Germany. Malvern, Zetasizer ZS90 nanoseries; Malvern Instruments, UK. Column chromatography (CC) was performed on Sephadex LH-20 (Amersham Biosiences). Thin-layer chromatography (TLC) was performed on silica gel plates 60 PF254 (Merck) analyzed with a UV lamp (254 and 366 nm). All CC fractionations were based on TLC analyses. Cells were photographed with a Nikon Eclipse TE300 microscope and quantified with Image J software.Aabsorbance of the MTT assay was measured at 570 nm with a Victor 3 Perkin Elmer counter.

2.2 Flavonoid isolation

Leaves from *Lantana trifolia* were colleted in Paracambi, Rio de Janeiro, Brazil in

2013 when the plants were in bloom. About 0.5 kg of dried leaves were pulverizied and exhaustively extracted with ethyl acetate. The extract was then concentrated in a rotary evaporator and applied to a Sephadex colum with metanol. About 30 fractions of 150 mL were collected. The final fractions (25-30) contained the isolated flavonoid (**1**) identified by comparison of their spectroscopic data, especially NMR, with literature data (18).

2.3 Microcapsule preparation:

Apigenin (**5**), 7-apyosil scutellarein (**1**) and quercetin (**6**) microcapsules were prepared by a single emulsion evaporation method as described in (19). Briefly 40 mg from **1** and 160 mg PLGA were dissolved in 4 mL dichloromethane and the flavonoids apigenin and quercetin were dissolved in 4 mL acetonitrile. Next, these solutions were added separately dropwise to a 20 mL aqueous solution of 3% PVA (3%) and sonicated for 20 minutes using a Biovera Cristofoli sonicator (42 KHz). The solvent was evaporated under magnetic stirring for 12 h at room temperature and flavonoid microparticles were isolated by 60 min. centrifugation at 4000 rpm and then lyophilized. After centrifugation, the supernatant was recovered and assayed for unentrapped drug. The sediment was washed using the same amount of distilled water as of the supernatant and again centrifuged at 4000 rpm for 30 min. The washing process was repeated 3 times. All washes were collected, lyophilized and assayed for unentrapped drug. The microcapsules of apigenin (**2**), 7-apyosil scutellarein (**3**) and quercetin (**4**) were then obtained.

2.4 Scanning electron microscopy:

The external morphology of the microcapsules was analyzed with a Hitachi TM3000 - Tabletop Microscope. The microcapsules were fixed on a brass stub using double-sided adhesive tape and then observed under high vacuum conditions.

2.5 Particle size analisys and zeta potential

The average particle size and polydispersity index (PDI) of the microcapsules were analyzed with a laser particle size analyzer (Malvern, Zetasizer ZS90 nanoseries; Malvern Instruments, UK) using distilled water as a dispersant. The samples (20 mg in 100 mL distilled water) were stirred in an ultrasound bath (Ultrassonic Processor, Hilscher, Germany) for 12 min at 100% amplitude and 0.5 cycles per minute to form emulsions. The samples were then diluted 1:200 (v/v) and equilibrated in the instrument for 10 seconds. Data were collected using at least 100 measurements and processed using the Smoluchowski equation. Measurements were made in triplicate and the results are shown as mean ± standard error.

2.6 Flavonoid quantification in the microcapsules and nanocapsules

Samples of **2-4** loaded microcapsules and nanocapsules were dispersed in 80% ethanol (3mL) and stirred for 5 min, followed by centrifugation at 4000 rpm for 10 min for extraction of apigenin, 7-apyosil scutellarein and quercetin. The supernatant was reacted with 2% $AlCl_3$ in ethanol for 30 min at room temperature and absorbance was determined spectrophotometrically at 420 nm (20, 21). The amount of flavonoids present in the microcapsules was defined by comparison to a standard solution of quercetin. Microcapsules **2-4** with ethanol were used as control.

2.7 NMR Solid analisys

The cross-polarization magic angle spinning (CP/MAS) ^{13}C NMR spectrum (Figure 2) of the solid samples was obtained using a Bruker spectrometer Ultra Shield NMR at 400 MHz, cross-polarization pulse sequence (cp.av), 30 °C, a 100.61-MHz measurement frequency (22), with a contact time of 2 ms, 8 kHz spinning rate and 2048 scans.

2.8 GBM cell lines

The U87 cell line was purchased from ATCC® (U87MG - ATCC® HTB-14TM). The GBM 95 and GBM 02 cell lines were established in our laboratory as previously described (23). GBM cell lines were cultivated in Dulbecco's modified Eagle's medium-F12 (DMEM-F12) supplemented with 10% fetal bovine serum (FBS) and maintained at 37°C in a CO_2 incubator.

2.9 Human astrocyte cell culture

A primary culture of human astrocyte cells was prepared as previously described (24). Cells were cultivated in DMEM F-12 with 10% FBS and maintained at 37°C in a CO_2 incubator.

2.10 MTT assay

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT – Sigma) was used to analyze the mitochondrial activity of viable cells after treatment with flavonoids. Cells were plated with DMEM F-12 and 10% FBS and cultivated for 24 h before treatment. 7-Apiosyl-scutellarein (**1**), a microcapsule of apigenin (**2**), a nanocapsule of 7-apiosyl-scutellarein (**3**), a microcapsule of quercetin (**4**), apigenin (**5**) and quercetin (**6**) were dissolved in DMSO. Next they were diluted with DMEM-F12 supplemented with 10% FBS in order to treat cells with concentrations of 10, 50 and 75 µM. After 24 and 48 h, MTT was added to each well at a final concentration of 0.5 mg/ml. After 2 h of incubation, the

formazan reaction product was dissolved with DMSO and absorbance was measured at 570 nm (Victor 3 Perkin Elmer).

2.11 Hoechst staining

Hoechst staining (Invitrogen) was performed in order to quantify the number of cells in each treatment condition. Cells were plated with DMEM F-12 and 10% FBS and cultivated for 24 h before treatment with the encapsulated flavonoids apigenin and quercetin (50 and 75 µM). After 48 h, cells were fixed with 4% paraformaldehyde and stained with Hoechst. Each condition was photographed with a Nikon Eclipse TE300 microscope and quantified with Image J software.

2.12 Statistical analysis

Data were analyzed statistically by one-way ANOVA and the Tukey post-test, with the level of significance set at * $p<0.05$; ** $p<001$ and *** $p<0.001$. All analyses were carried out using the GraphPad Prism software.

3 I RESULTS AND DISCUSSION

3.1 Flavonoid quantification, scanning electron microscopy, particle size analisys and zeta potential

7-Apyosil scutellarein (**1,** Fig. 1) was isolated from *Lantana trifolia* as described in the experimental section. The quantification of **2, 3** and **4** detected 83% apigenin (Fig. 1), 85.5% 7-apyosil-scutellarein and 53% quercetin (Fig.1) in the formulation, indicating that the method was not efficient for quercetin. Some literature data (25) describe a direct correlation between increasing PLGA concentration and particle size as well as encapsulation efficiency for quercetin nanoparticles. For in vitro experiments, the concentrations were calculated considering loss during the process. Scanning microscopy analysis of the capsules revealed the presence of different microcapsule and nanocapsule sizes. Microcapsules were detected in 7-apiosyl scutellarein (Fig. 2). The figure shows that the capsules form aggregated particles that cannot be separated in order to obtain images, probably because of the lyophilization process.

The microcapsules were evaluated for particle size, polydispersity index, zeta potential, electrophoretic mobility and conductivity and the results are presented in Table 1. Comparison with literature data showed that the present data disagree with previous results that have reported smaller quercetin loaded PLGA (≈400 nm) (26). Molecular weight, the concentrations of polymer and of encapsulated active are factors that can affect particle size (27, 28). Pool et al. have suggested that the molecular weigth of PLGA can influence

the viscosity of the internal phase, leading to a decrease in net shear stress producing larger particles. The molecular weight of PLGA used in the present study varied between 30 and 60 kDa, a fact that could explain the variation in particle size. The electrical charge measured by the zeta potential of free and loaded flavonoid was negative, indicating the presence of ionized carboxyl groups in the PLGA matrix (29). The difference between the values of apigenin- and quercetin-loaded microparticles indicates that the presence of each specific flavonoid alters the electrical charge of the polymer particles, as described by (26) for quercetin and catechin nanoparticles. By this analysis it can be seen that the technique was most effective for (**3),** a nanocapsule with a particle size of 485.2 nm,.

Microcapsules and nanocapsules	particle size (d.nm)	polydispersity index	Z potential (mV)	mobility (umcm/Vs)	conductivity (mS/cm)
apigenin (**2**)	1775	0.346	-17.2	-1.344	0.00281
7-apiosyl scutellarein (**3**)	485.2	0.777	-26.0	-2.342	0.005200
quercetin (**4**)	1220	0.837	-11.9	-1.108	0.00653

Table 1: Particle size, polydispersity index, zeta potential, electrophoretic mobility and conductivity of microcapsules and nanocapsules.

3.2 NMR Solid analisys

The microcapsules and nanocapsules were then additionally characterized by ^{13}C solid NMR (solid-state NMR) (30). ^{13}C NMR spectroscopic data of flavonoids and PLGA isolated in a liquid state were compared to solid state microcapsules and nanocapsules (Fig.3) in order to determine the patterns of the signals assigned to the flavonoids that are repeated at a lower intensity in solid state. This permitted us to deduce that not all flavonoid subjected to the encapsulation process was effectively retained in the microcapsules, as shown by drug quantification.

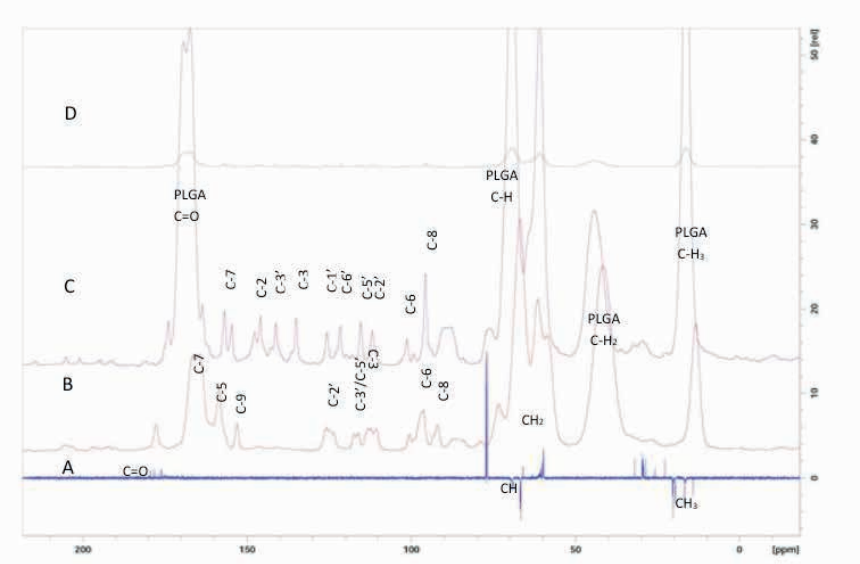

Fig-3: Comparison of NMR liquid analisys (^{13}C-DEPTQ), PGLA (A) and solid analisys microcapsules: (B) apigenin (2), (C) quercetin (4) and nanocapsules of 7-apiosyl scutellarein (D;3)

3.3 GBM viability analysis: Nanoencapsulated 7-apiosyl-scutellarein against GBM

To investigate if 7-apiosyl-scutellarein (**1**) and the 7-apiosyl-scutellarein nanocapsule (**3**) had a cytotoxic effect against glioblastoma cell lines, we treated U87, GBM 95 and GBM 02 cells with 10, 50 and 75 μM of these compounds for 24 and 48 h. The treatment with 7-apiosyl-scutellarein (**1**) had no significant effect on GBM cell viability. However, the 7-apiosyl-scutellarein nanocapsule (**3**) showed cytotoxicity against U87 and GBM 95 after 48 h of treatment. In U87, 50 μM 7-apiosyl-scutellarein nanocapsules (**3**) reduced viability by 42% compared to control (Fig 4B). In GBM 95, treatment with 75 μM 7-apiosyl-scutellarein nanocapsules (**3**) decreased cell viability by 46% (Fig. 4F). 7-Apiosyl- scutellarein (**1**) and 7-apiosyl-scutellarein nanocapsules (**3**) were not cytotoxic to human astrocyte cells (Fig. 4G and H).

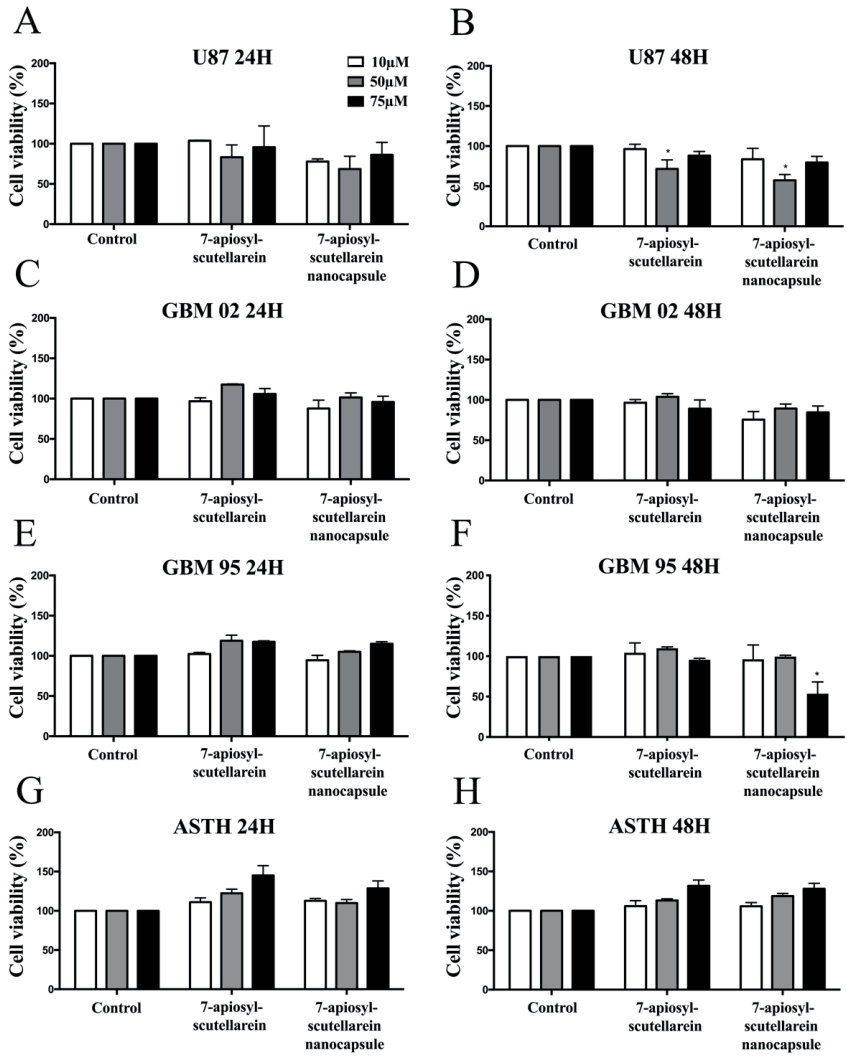

Fig-4: Graphs show the percentage of viable cells after treatment with 10 μM, 50 μM, and 75 μM of 7-apiosyl scutellarein (**1**), and nanocapsule 7-apiosyl scutellarein (**3**) in glioblastoma cell lines U87 (A and B), GBM 95 (C and D) and GBM 02 (E and F) and human astrocyte cell culture (ASTH - G and H) for 24 and 48 h determined by MTT assay. The values (A-H) were normalized by the control (DMSO) and represent the mean and standard error of three independent experiments carried out in triplicate. * $p<0.05$; ** $p<0.01$ *** $p<0.001$.

3.4 GBM analysis: microencapsulated apigenin and quercetin against GBM

Apigenin (**5**) and quercetin (**6**) have antitumor potential against glioblastomas (31, 32). To test if these encapsulated flavonoids would still be cytotoxic to glioblastoma cell lines, we performed an MTT assay treating U87, GBM 95, and GBM 02 cell lines with 10, 50 and 75 μM of the flavonoids and the encapsulated flavonoids for 24 and 48 h. Under these

conditions, apigenin (**5**) did not show a significant effect on GBM cell viability. However, the 48 h treatment with apigenin microcapsules (**2**) decreased the viability of GBM 95, GBM 02, and U87 cell lines (Fig. 5A-F). In GBM 02 and GBM 95, the 48 h treatment with both quercetin (**6**) and quercetin microcapsules (**4**) showed a cytotoxic effect (Fig. 5A-F). In U87 cells, however, the 48 h treatment with 75 μM of the quercetin microcapsule (**4**) reduced cell vibility by 50.5%, while quercetin (**6**) showed no cytotoxic effect (Fig. 5B). None of them was cytotoxic to a human astrocyte culture (Fig. 5G and H).

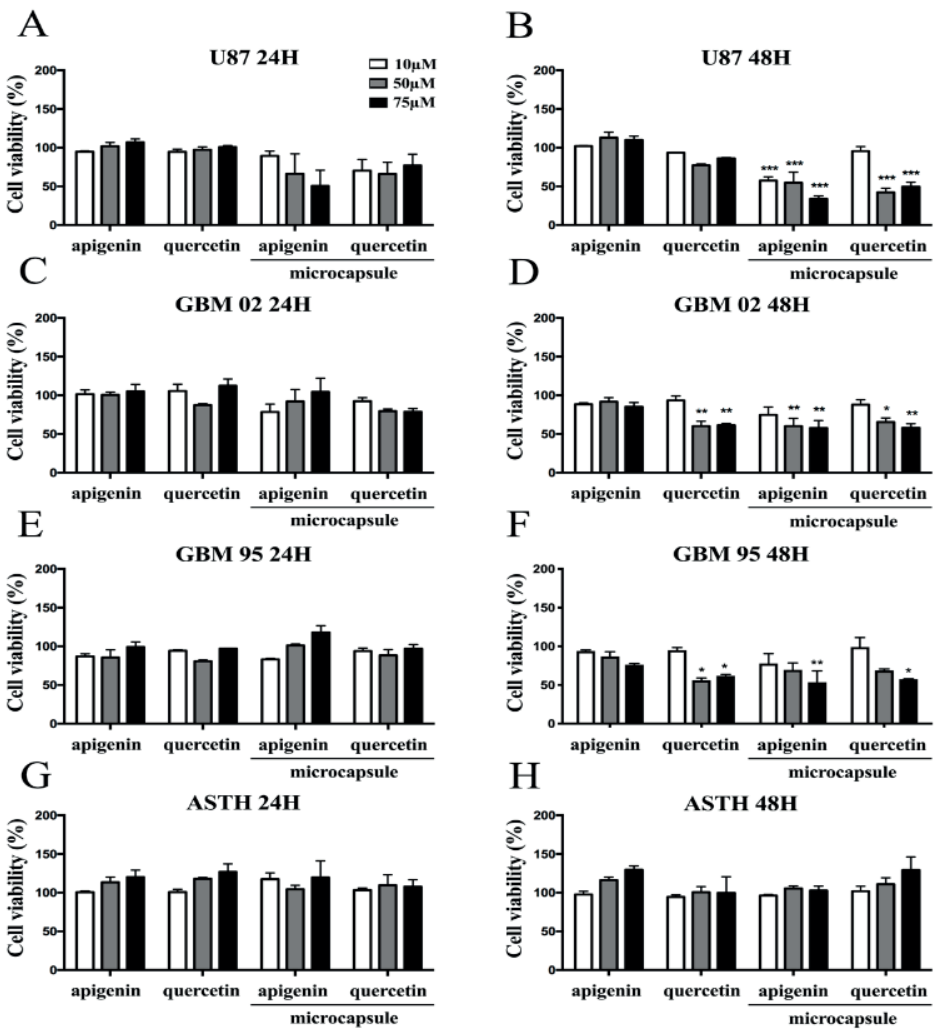

Fig-5: Graphs show the percentage of viable cells after treatment with 10 μM, 50 μM and 75 μM of microcapsules of apigenin (**3**), microcapsule of quercetin (**4**), apigenin (**5**) and quercetin (**6**) in the glioblastoma cell lines U87 (A and B), GBM 95 (C and D) and GBM 02 (E and F) and in human astrocyte cell culture (ASTH - G and H) for 24 and 48 h determined by MTT assay. The values (A-H) were normalized by the control (DMSO) and represent the mean and standard error of three independent experiments in triplicate. * $p<0.05$; ** $p<0,01$ *** $p<0,001$.

To determine if the reduction of cell viability was a reflection of a decrease in the number of cells in culture, we stained the cells with Hoechst in order to quantify the number of cells in each treatment condition. GBM 95 treated with 50 and 75μM apigenin microcapsules (**2**) showed a decrease of 62 and 71% in the number of cells in culture. In GBM 02, 50 and 75 μM apigenin microcapsules (**2**) reduced the cell number by 39 and 42%. Quercetin microcapsule (**4**) treatment reduced the number of GBM 95 cells by 57 and 75% at the concentration of 50 and 75 µM, respectively. Treatment with 50 and 75 μM quercetin microcapsules (**4**) promoted a decrease of 68 and 74% in the number of GBM 02, respectively (Fig. 6). These data show that microencapsulated apigenin and quercetin have an antitumor effect against GBM, decreasing cell viability and the number of cells in culture, and are not cytotoxic to healthy astrocytes.

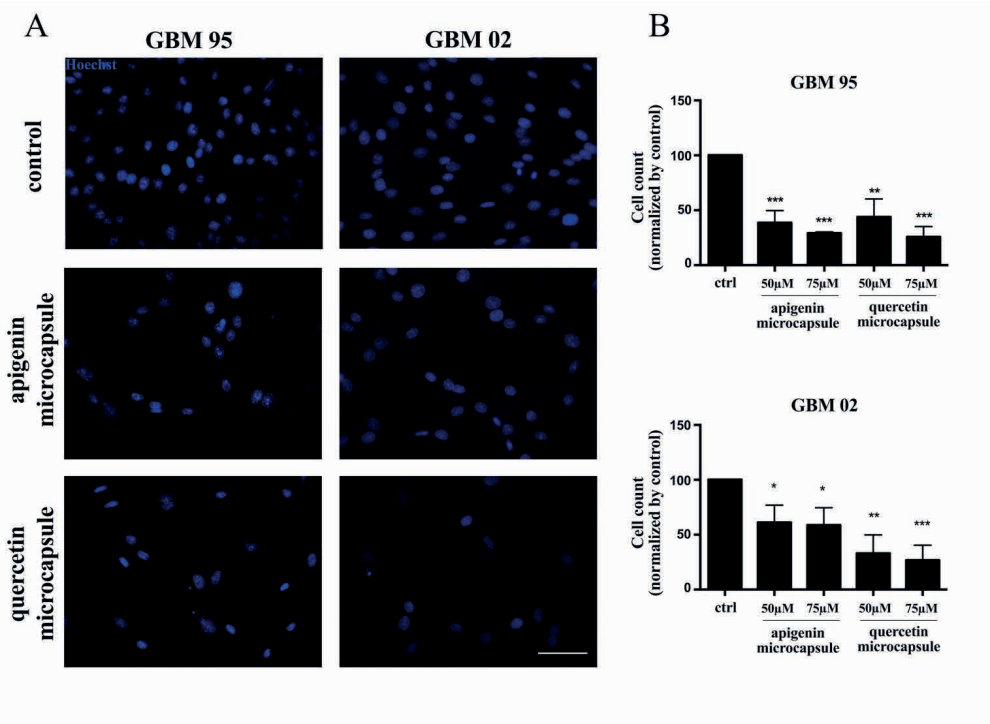

Fig-6: Hoechst nuclear staining of GBM 95 and GBM 02 treated for 48 h with 50 and 75 µM of microcapsules of apigenin (**2**) and quercetin (**4**). (A) Representative images of Hoechst staining. (B) Quantification of Hoechst staining in each treatment normalized by the control (DMSO) value. The values represent the mean and standard error of three independent experiments carried out in duplicate. * $p<0.05$; ** $p<0.01$ *** $p<0.001$. Scale bar 50µm.

The Blood Brain Barrier (BBB) separates the brain from the rest of the body, tightly regulating the exchange between peripheral blood circulation and cerebrospinal fluid. Due to the presence of the BBB, it is very difficult to ensure long-term drug administration to the brain. Nanocapsules and microcapsules constitute a new wave of therapies for the

prevention and treatment of neuronal diseases since they have can cross the BBB and release their content to target sites (33). Quercetin-loaded nanoliposomes enhanced the cytotoxic effect on C-6 glioma cells by necrosis, rather than apoptosis, during tumor cell death (34). PEG2000-DPSE-coated encapsulated quercetin enhanced induced programmed cell death of C6 glioma cells (16). Quercetin and apigenin encapsulated with PLGA have recently been described in the literature (26, 35) but the present assay is the first to report their cytotoxic action against gliomas cell lines. This is also the first citation of 7-apyosil-scutellarein nanocapsules and its action against gliomas.

Our study showed the importance of the encapsulation of glycosylate flavonoids into nanocapsules for the enhancement of the activity just described for other aglycones on different glioma cell lines. These studies showed that the sugar moiety is relevant for activity and the glycosylated form can reduce diffusion across the cell membrane, indicating a false inactive substance. Further studies are needed concerning the mechanisms of entry of these compounds into glioma cells.

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