Dimiristoylphosphatidylcholine/genistein molecular interactions: A physico-chemical approach to anti-glioma drug delivery systems

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ABSTRACT

Regarding free genistein small delivery to the central nervous system, physico-chemical parameters of dimiristoylphosphatidylcholine liposome-loaded genistein were investigated, as well as its *in vitro* activity against the DPPH radical and glioma cells. Data obtained by UV–vis spectroscopy, Fourier Transform Infrared Spectroscopy, Nuclear Magnetic Resonance, Differential Scanning Calorimetry and Dynamic Light Scattering were used to characterize the liposomal system with respect to motion restriction, hydration degree, trans-gauche isomerization and phase state. *In vitro* antitumoral effects were monitored through conting and viability assays. Genistein hydroxyl group and lipid hydrogen bonds may have important role in dimiristoylphosphatidylcholine phosphate and choline motion restriction. Genistein-induced choline restriction may be also related to a decrease in the group rotation rate. Genistein: dimiristoylphosphatidylcholine system showed higher molecular package at the acyl chains region compaired to empty liposomes, and it may be related to a decrease in gauche bonds quantity and system size. Lipid acyl chain length seems to influence different genistein effects on membranes, due to the presence of gauche conformers. Genistein: dimiristoylphosphatidylcholine liposome was more efficient as DPPH reducting system than the free-Gen. Liposomal system, at genistein 100 μM, was so efficient as the correspondent free-form genistein, probably showing higher stability to cross the blood-brain barrier. Genistein and the lipid did not show an additive activity against glioma cells. Antioxidant and anti-glioma genistein-loaded liposome potential may be related to the isoflavone location and its restriction effect in the lipid molecular motion. Anti-glioma activity may also be related to a decrease of system size and trans-gauche isomerization.

1. Introduction

Genistein (5,7,4′-trihydroxyisoflavone, Gen, Fig. 1), one of the main soy isoflavones, has important antioxidant and antitumoral activities. These biological effects are highly influenced by Gen chemical structure and molecular interactions (Fritsche and Steinhart, 1999; Villares et al., 2011). The presence of hydroxyl group at position 4′ and/ or 5, the oxo substitution at C4, the conjugated 2,3-double bond, as well as the catechol moiety at B-ring favor Gen radical-scavenging and metal chelation mechanisms (Ho et al., 2003; Qian and Shen, 2001; Tsao et al., 2003). Both hydroxyl groups on C-5 and C-7 positions at ring A are

important for tumoral cell growth inhibition (Xiong et al., 2015). Gen has significant inhibitory effects in almost all cancer cell lines (Li et al., 2012). In brain cancer context, Gen promoted cell cycle arrest and apoptosis in human glioma cell lines, inhibiting the activities of epidermal and platelet-derived growth factors, as well as the activity of the topoisomerase II (Weernink et al., 1996; Schmidt et al., 2008). For glioblastoma multiforme processes, it is known that there is a moderate relationship between the anti-proliferative effect and antioxidant properties of trihydroxyflavones (Grigalius and Petrikaite, 2017). Indeed, polyphenols have an important effect on cancer intracellular redox state, supporting anti and pro oxidant activities (Lastra and
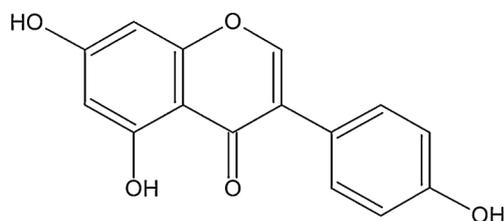
Fig. 1. Structure of genistein (Gen).

Villegas, 2007). However, Gen efficacy against brain tumors, such as gliomas and glioblastoma multiforme is limited and still a challenge, since its availability in the blood-brain barrier (BBB) and delivery to the central nervous system are small and poor (Tsai, 2005; Yang et al., 2014).

Gen is sensitive to oxidation and its solubility in water is lower than 1 μg/mL (Cohen et al., 2011; Aditya et al., 2013). Being a poorly-water solubilizing isoflavone, Gen may be encapsulated and solubilized into amphipathic carriers. Different nanocarriers has been used for Gen delivery in cancer treatment, such as polymeric nanoparticles, micelles emulsion and lipid-based nanoemulsions (Aditya et al., 2013; Pham et al., 2013; Zhang et al., 2015; Wu et al., 2016; Tyagi et al., 2018). Nowadays, liposomes are the most investigated and promising system to brain drug delivery applications, due to their biocompatibility (Vieira and Gamarra, 2016). The design of a liposomal system which may cross completely the BBB is still under investigation, although liposome- loaded drugs were yet approved for clinical trial use against brain tumors (Legge et al., 2011; Ananda et al., 2011). One example is the Myocet®, consisting of doxorubicin encapsulated into phosphatidylcholine/cholesterol-based liposomes, which were being tested against glioblastoma multiforme (Chastagner et al., 2015). Thus, considering the Gen efficacy as antitumoral substance, as well as phosphatidylcholine-based liposomes perspectives to cross BBB, it is very promising to characterize physico-chemical properties of Gen/phosphatidycholine based-liposomes complexes, which may influence the system pharmacodynamics and pharmacokinetics in the BBB. Indeed, there are strong evidences that Gen-loaded liposomes have the BBB crossing facilitated by their enhanced lipophilicity compared to free-form Gen (Azambuja et al., 2015; Garg et al., 2015). Some studies reported the physico-chemical characterization of Gen-phosphatidylcholine based liposomes (Pawlikowska-Pawlega et al., 2012, 2014; Raghunatan et al., 2012), but few works considered an approach for anti-glioma or glioblastoma treatment (Azambuja et al., 2015). The supracited studies may contribute to purpose a design of an efficient Gen-based drug delivery system to be applied in glioma therapy. For the liposome design, material choice, lamellarity and size define properties such as system permeability, stability, drug encapsulation efficiency and release kinetics (Ali et al., 2013). Previously, our research group characterized the physico-chemical properties of unsaturated soybean asolectin liposomes containing Gen, correlating them with *in vitro* antioxidant and cytotoxicity activities. These systems consisted of multilamellar large vesicles (MLV), showed higher molecular package than pure liposomes (those without Gen) and enhanced cytotoxic activity against glioma cell lines than Gen-free form (Azambuja et al., 2015). However, it is known that the presence of unsaturated lipids compromise the system stability. Furthermore, in the organism, reticulo endothelial absorption rate is higher for lower melting lipids, such as the natural-sourced ones, than for the higher melting lipids (Gabizon and Papahadjopoulos, 1988). On the other hand, saturated and symmetrical lipids may provide a higher molecular package to a liposomal system, enhancing the system stability (Ali et al., 2013). Liposome stability is also influenced by the lamellarity, since this one has an important role on liposome mean volume diameter. In this context, phosphatidylcholine-based unillamelar large vesicles (LUV) showed more physical stability than MLV, considering variances of mean volume diameter, zeta

potential values and pH maintenance (Plessis et al., 1996). However, it is important to note that, among unilamellar vesicles, the system size may compromise their stability. For example, unillamelar liposome which have core radii higher than a few hundred nm are more stable than those which show radii lower than 100 nm (Tayebi et al., 2012). This may be associated to the liposome size influence in the system surface curvature and structure asymmetry. For example, small unilamellar vesicles (SUV) have higher curvature than LUV or MLV and, consequently, higher assymetry related to their internal structure (Nakamura and Shinoda, 2013). Structure symmetry and surface curvature also affect the drug distribution and the extent of its effects into the membrane.

Previously, DMPC SUV were used to study location and interactions of Gen with lipid membranes by fluorescence and ESR spectroscopies (Kudzal et al., 2011). Considering the supracited lamellarity and size influence on stability and drug distribution, as well as the fact that LUV seems to be more resistant to deformation by osmotic stress and, consequently, are more suitable for drug delivery application (Elsayed et al., 2018), it is important to characterize the physicochemical properties of DMPC LUV containing Gen. For this, it is also important to assume the Gen solubilization in the lipid membranes.

Thus, in this work, Gen saturation concentration and its influence on hydration degree, rotational lipid motions, phase state and order of DMPC LUV specific groups was studied by Horizontal Attenuated Total Reflection Fourier Transform Infrared Spectroscopy (HATR-FTIR), ^{31}P Nuclear Magnetic Resonance (^{31}P NMR), ^1H Nuclear Magnetic Resonance (^1H NMR), Differential Scanning Calorimetry (DSC), Ultraviolet-visible spectroscopy (UV–vis) and Dynamic Light Scattering (DLS) techniques. To correlate the molecular interactions data to system biological properties focused on glioma treatment, *in vitro* antioxidant and antitumoral assays were also performed. The system reducting potential was tested against diphenyl-picril-hydrazyl radical (DPPH), and its cytotoxic activity was evaluated against murine glioma cells (C6) by MTT assay.

2. Materials and methods

2.1. Chemicals

Dimyristoylphosphatidylcholine was purchased from Avanti Polar Lipids, and genistein, 2,2-diphenyl-1-pycril-hydrazil (DPPH), deuterated water, sodium 3-(trimethylsilyl)-[2,2,3,3-2H4]-1-propionate (TSP, 0.05%) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) were obtained from Sigma-Aldrich (St.Louis, MO, USA). Fetal bovine serum (FBS, Gibco BRL) and Dulbecco's modified Eagle's medium (DMEM) were purchased from Invitrogen Co. (Carlsbad, CA, USA).Lipids were used without further purification whereas all other chemicals were of analytical grade.

2.2. Experimental section

The experimental section reports Gen-loaded DMPC liposome preparation and Gen quantification on it, its physico-chemical properties (considering the Gen-induced changes of specific lipid regions motion), as well as its biological activities (considering its antioxidant/antitumoral potentials).

2.2.1. Liposome preparation and Gen saturation concentration in the system

Unilamellar large vesicles were prepared by the reverse phase evaporation method (Szoka and Papahadjopoulos, 1978; Mertins et al., 2005). DMPC (in the concentration range from 30 mg/mL to 150 mg/mL) was co-solubilized with 1000 μL of chloroform and 20 μL of distilled water. The resulting emulsion was sonicated for 2 min, to generate a homogeneous dispersion of reverse micelles. The solvent was evaporated using a rotary evaporator, under vacuum. The resulting organogel, or lipid film, was placed under a dessicator, to evaporate

solvent traces, and then it was hydrated with 1000 μL of distilled water. After this, the organogel was resuspended by vortex in order to obtain the liposomes. Gen was incorporated into the liposome in the chloroform/lipid co-solubilization step, in initial Gen: DMPC ratios from 0.0 to 0.20 (corresponding to 0.0 mg/mL to 6.00 mg/mL of Gen). All liposomes were submitted to three freeze-thaw cycles. Gen saturation concentration into liposomes (or the maximum liposome-loaded Gen concentration) was spectrophotometrically determined through Gen efflux after surfactant-induced membrane solubilization (Ruiz et al., 1988). Briefly, liposomes were prepared adding different the initial Gen concentrations (0.0 to 6.00 mg/mL), expressed as Gen: DMPC molar ratio. After liposome preparation, the non-encapsulated (free-form) Gen was removed by washing (done three times with distilled water) and centrifugation procedures. Afterwards, liposome pellets were treated with Triton X-100 surfactant (0.6%, v/v) to dissolve the membrane. The Gen efflux from the liposomes was quantified at 262 nm by a UV-2550 Shimadzu spectrophotometer (Kyoto, Honshu, Japan). The Gen molar absorptivity corresponding to 35,842 M^{-1} cm^{-1} was considered to calculate liposome-loaded Gen concentration by Beer´s Law (Franke et al., 2009). Control assays without Triton X-100 and without Gen were also performed. For each Gen: DMPC ratios, triplicates from five independent experiments were performed.

2.2.2. Study of Gen-induced changes in physico-chemical properties of DMPC liposome regions

2.2.2.1. HATR-FTIR measurements.
HATR-FTIR technique was used to investigate the Gen influence on hydration degree, vibrational, rotational and translational parameters of polar, interfacial and non-polar liposome groups. HATR-FTIR experiments were performed with DMPC liposomes (150 mg/mL), either alone or containing Gen, in the maximum liposome-loaded concentration (see Section 2.2.1.) using Shimadzu IR Prestige-21 equipment (Kyoto, Japan) at 23 °C. Interferograms were averaged for 50 scans, recorded in the frequency range from 400 to 4000 cm^{-1}, with a resolution of 2 cm^{-1}.The spectra were analyzed by Shimadzu IR solution software 1.5. From the obtained spectra, it were detected axial stretching peaks of specific lipid groups, which assigments are listed in Table 1 (Manrique-Moreno et al., 2010).

HATR-FTIR spectra were analyzed considering the Gen-induced changes in frequency and bandwidth of the lipid stretching peaks. Bandwidth measurements were related to a straight baseline at 3/4 of the peak height position (Bilge et al., 2014; Casal and Mantsch, 1984).

2.2.2.2. NMR assays.
^{31}P and ^{1}H NMR were used to investigate rotational motion of DMPC liposome phosphate and choline groups (located at lipid polar region), as well as of the methylene ones (located at hydrophobic acyl chains). The ^{31}P-NMR measurements of liposomes (150 mg/mL) that were alone or contained Gen (at maximum liposome-loaded concentration, according to Section 2.2.1.) were performed using a NMR Bruker Avance 400 MHz spectrometer (Ettlingen, Germany) at 162 MHz, using deuterated water as external reference. Spectra were acquired in the following conditions: pulse time 15.05 μs, recycle delay 4 s, proton decoupling, 2048 scans. ^{1}H longitudinal relaxation time (T_1) measurements were performed at 400 MHz, at 20 °C. Inversion recovery pulse sequence (π- τ -π/2 acquisition) was performed with a time delay (τ) ranging from 0.2 to 102.4 s, time pulse

Table 1
HATR-FTIR peaks related to the axial stretching (ν) of DMPC groups.

DMPC group	HATR-FTIR stretching vibration	Frequency (cm^{-1})
phosphate	ν_{as} PO_2^{-}	1260–1220
choline	ν_{as} $N^{+}(CH_3)_3$	≈ 970
carbonyl	ν C=O	1740–1725
acyl chains methylenes	ν_s CH_2	≈2850
	ν_{as} CH_2	≈2920

ν_{as}: antisymmetric stretching vibration. ν_s: symmetric stretching vibration.

of 14 μs, 8 scans, using water: deuterated water (70:30, v/v) as solvent. Chemical shifts were referenced to TSP signal at 0 ppm (Lima et al., 2010; Azambuja et al., 2015).

2.2.2.3. DSC measurements.
DSC measurements were performed in order to study the Gen effects in thermodynamic parameters related to DMPC liposome acyl chains (located at hydrophobic region). DSC assays of DMPC liposomes (50 mg/mL), alone or containing Gen (at maximum liposome-loaded concentration, according to Section 2.3), were performed by Shimadzu DSC-60 equipment (Tokyo, Japan). The equipment was previously calibrated using Indium (melting temperature equivalent to 156.7 °C). The heating rate was set to 5 °C/min in temperatures ranging from 5 °C to 60 °C, under nitrogen flow (50/50 mL. min^{-1}) (Koynova and Caffrey, 1998; Lynch and Steponkus, 1989; Ulrich et al., 1994). In these conditions, the error in the analyses was estimated in 0.5 °C. An empty aluminum cell was used as a reference (Lima et al., 2010; Zhao et al., 2007). Phase transition temperatures (Tm) were obtained from the curve midpoint. The enthalpy variation (ΔH) values were obtained from the integration of the area under the DSC peaks.

2.2.2.4. Zeta potential (ζ) and size measurements.
Zeta potential (ζ) and size measurements were performed in order to investigate Gen influence on polar regions orientation and particle size of DMPC liposome, respectively. Thus, DLS measurements were performed using DMPC liposomes at 50 mg/mL, alone or loaded with Gen, in the highest isoflavone concentration loaded into liposomes (see Section 2.2.1.). Assays were performed by Zetasizer Nano Series of Malvern Instruments (Worcestershire, UK), at 25 °C, with an optical path of 1.0 cm, a detection angle of 90˚, dispersion medium viscosity of 0.894 m P and refractive index of 1.33. Milli-Q water was used in the sample dilutions.

2.2.2.5. Turbidity assays.
Gen-promoted effect on DMPC liposomes turbidity was evaluated in liposome at 30 mg/mL of lipid, either pure or loaded with different Gen concentrations (0.0 to 3.6 mg/mL, to reach the initial Gen: DMPC ratio range from 0.0 to 0.13 m/m). The experiment was performed at 400 nm wavelength, 20 °C, using a Shimadzu UV-2550 UV–vis spectrophotometer (Kyoto, Japan) (Sousa et al., 2013; Azambuja et al., 2015). Quartz cells were used with a 1.0 cm optical path. For each sample containing different Gen: DMPC ratios (m/m), three independent samples were prepared and analyzed.

2.2.3. Study of liposome systems containing Gen biological activities

2.2.3.1. Antioxidant activity assays (DPPH).
The antioxidant activity of free-form and Gen-loaded liposome was evaluated by the colorimetric test 2.2-diphenyl-1-picryl hydrazil (DPPH). The liposomes concentration was equivalent to 30 mg/mL and Gen concentrations (free or liposome-loaded) ranged from 0.0 mg/mL to 3.6 mg/mL. Thus, free and Gen-loaded liposomes were incubated with 1 mM methanol-solubilized DPPH for 40 min. After incubation, the optical density of the samples was detected at 515 nm in a Shimadzu UV-2550 spectrophotometer (Kyoto, Honshu, Japan) (Brand-Williams et al., 1995). For each sample, at least three independent experiments were prepared and analyzed.

2.2.3.2. Antitumoral assays

2.2.3.2.1. Cell culture procedures.
Rat malignant GBM cell line (C6) was obtained from American Type Culture Collection (Rockville, MD, USA). The cells were grown and maintained in low-glucose Dulbecco's Modified Eagle's Medium (DMEM) containing 0.1% Fungizone and 100 U/L penicillin/streptomycin, supplemented with10% FBS. Cells were kept at 37 °C in a humidified atmosphere with 5% CO_2 (Silveira et al., 2013).

2.2.3.2.2. In vitro treatment.
Glioma cells were seeded at 5 × 10^3 cells/well in DMEM/ 10% FBS in 96-well plates 24 h previously to the

treatment. Cultures were exposed for 48 h to DMPC (10, 20 and 100 μM). Control cells were treated only with the vehicle (1% of DMSO). All experiments were performed in triplicates.

2.2.3.2.3. Cell counting. At the end of 48 h after treatment, cell counting was performed, making it possible to estimate the number of viable cells per well. After dissociating cells with tripsin/EDTA, 100 μL aliquots were taken from each well for counting in a Neubauer chamber. Observations were made by the same investigator under a stereoscope microscope.

2.2.3.2.4. MTT cell viability assay. Cytotoxicity of Gen-loaded liposome was detected by MTT assay after the treatment described in Section 2.10.2. The MTT method estimates cell viability based on the ability of the mitochondria to reduce MTT to formazan as a product. MTT solution (sterile stock solution at 5 mg/mL) was added to the incubation medium (DMEM supplemented with 10% FBS) at a final concentration of 0.5 mg /mL. Cells were left for 60 min at 37 °C in a humidified 5% CO_2 atmosphere. The medium was then removed and plates were shaken with DMSO for 30 min. The optical density of each well was measured at 492 nm and the results were expressed as absorbance (Mosmann, 1983; Silveira et al., 2013).

2.2.4. Statistical analysis

In all experiments, except the assay related to Section 2.2.1, results were shown as the mean of triplicates from three independent experiments. Data related to the antioxidant and antitumoral assays were subjected to one-way analysis of variance (ANOVA), followed by Dunnett's (for antioxidant assays) and Tukey–Kramer (for antitumoral assays) post-hoc tests in order to have multiple comparisons. Differences among mean values were considered significant when $p < 0.05$.

3. Results and discussion

3.1. Gen saturation concentration in DMPC liposomes

Fig. 2 shows liposome-loaded Gen concentrations in function of the Gen: DMPC initial ratios used in liposome preparation (see Section 2.3.). The saturation concentration of an active substance into liposomes is detected from the relationship between its initial concentration added in the liposome preparation and its concentration loaded into the system. It can be defined as the last concentration before that the studied variables have an invertional relationship (Dimitrios and Sophia, 2002). As can be seen in Fig. 2, after reaching the saturation point, the Gen concentration loaded in the liposomes decreased. This behavior was observed in the higher tested Gen: lipid ratios (considering Gen
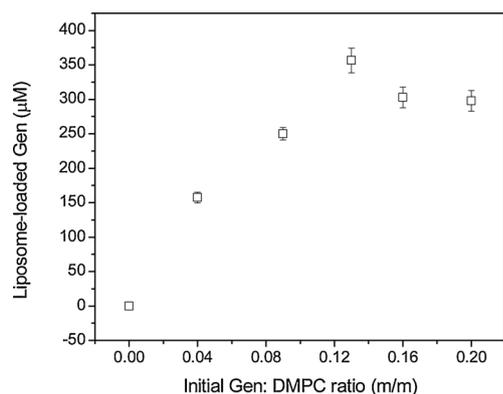
Fig. 2. DMPC liposome-loaded genistein as a function of the initial Gen concentration added in liposome preparation (0.0 to 6.00 mg/mL), expressed as Gen: DMPC molar ratio (Gen: DMPC). The graphic was obtained from Gen efflux from liposomes after solubilization with Triton X-100, monitored by UV–vis at 262 nm. Data are represented as mean ± S.D. of triplicates from five independent experiments. From the curve, Gen saturation concentration in DMPC liposomes was determined.

added in liposome preparation), equivalent to 0.16 and 0.20. Thus, loaded liposome- Gen saturation concentration corresponded to 357.00 ± 17.8 μM, related to the initial Gen: lipid ratio of 0.13 (m/m). In soybean asolectin-based MLV, the Gen saturation concentration was 26% higher than in DMPC LUV in the same Gen: lipid saturation ratio (Azambuja et al., 2015). It is known that MLV are capable of encapsulating a higher concentration of active substances than LUV and SUV (Kulkarni et al., 1995). However, as discussed before, there is a risk for less rigid membranes, such as those composed by soybean asolectin, to allow the escape of encapsulated substances during storage. In this work, the Gen saturation concentration (357.00 μM) was used for all subsequent physicochemical characterizations of the DMPC system.

3.2. Study of Gen-induced changes in physico-chemical properties of DMPC liposome regions

As cited previously, hydration degree and motional freedom of all DMPC regions in liposome (polar, interfacial and non-polar) were investigated by HATR-FTIR. The HATR-FTIR data related to groups of lipid polar region, such as phosphate and choline ones, were complemented and compared to ^{31}P and ^{1}H NMR results, as well as to ζ potential system values. For lipid hydrophobic region, HATR-FTIR results obtained to DMPC methylenes were complemented with ^{1}H NMR and DSC data. Global effects promoted by Gen in liposome were discussed from turbidity and size (DLS) assays.

3.2.1. HATR-FTIR assays

HATR-FTIR technique was used in order to obtain primarily information concerning to Gen location in the membrane and its influence on the dynamics of DMPC specific regions. HATR-FTIR spectra of liposomes empty and those containing Gen at 357.00 μM (the Gen saturation concentration into DMPC liposomes, see Section 3.1) were obtained and shown in Fig. 3A.

The HATR-FTIR spectrum of DMPC liposomes without Gen showed the following stretching vibrations: (a) ν_{as} PO_2^- at 1229.83 cm^{-1}, (b) ν_{as} $N^+(CH_3)_3$ at 970.48 cm^{-1}, (c) ν C=O at 1735.90 cm^{-1}, (d) ν_s CH_2 at 2851.12 cm^{-1} and (e) ν_{as} CH_2 at 2918.36 cm^{-1}. By the FTIR difference spectrum of Gen-loaded liposomes and empty liposomes (Fig. 3B), it was possible to assign some Gen peaks (showed as positive peaks in the spectrum) as: ν C=O at 1646. 80 cm^{-1}, δ 5-OH and ν C=C at 1514.20 cm^{-1}, ν C−O−C at 1270.30 cm^{-1} and ν C−O−C at 1012.00 cm^{-1}. These assignments are in agreement to the data reported by Cieslik-Boczula and co-workers (Cieslik-Boczula et al., 2012). Considering Gen FTIR peaks, there seems to not be peak overlaps between Gen and DMPC spectra. Spectra were interpreted with regard to Gen effects in each liposomal region: polar, interfacial and hydrophobic. In order to obtain more details concerning the influence of isoflavone on DMPC dynamics, HATR-FTIR results were complemented with data obtained by NMR, DSC and UV–vis techniques.

3.2.1.1. Gen effect in the DMPC polar region. -Phosphate group: The DMPC liposomes ν_{as} PO_2^- peak, (see Fig. 3), demonstrated a two-bands splitting, with a shoulder at lower frequency (1204 cm^{-1}). This peak splitting is related to the presence of hydrated and non-hydrated lipid phosphate groups in the membrane (Cieslik-Boczula et al., 2009; Banerjee et al., 2012). It is known that the assemblage of neighboring phospholipid polar heads occurs by dipolar/electrostatic interactions between phosphate and choline portions, which confer a high polarity to the liposomal medium. The lower-frequency shoulder indicates the presence of hydrated phosphate groups that is a consequence of weak phosphate-choline interactions (Basu et al., 2001). After interaction with Gen, ν_{as} PO_2^- peak splitted in three components, indicating that the isoflavone promotes different hydrogen bonds magnitudes in the lipid polar region and, consequently, affecting the intensity of neighboring lipids polar head interactions (Bradenburg, 1993; Jastrzebski et al., 1979). Probably, these different hydrogen bonds
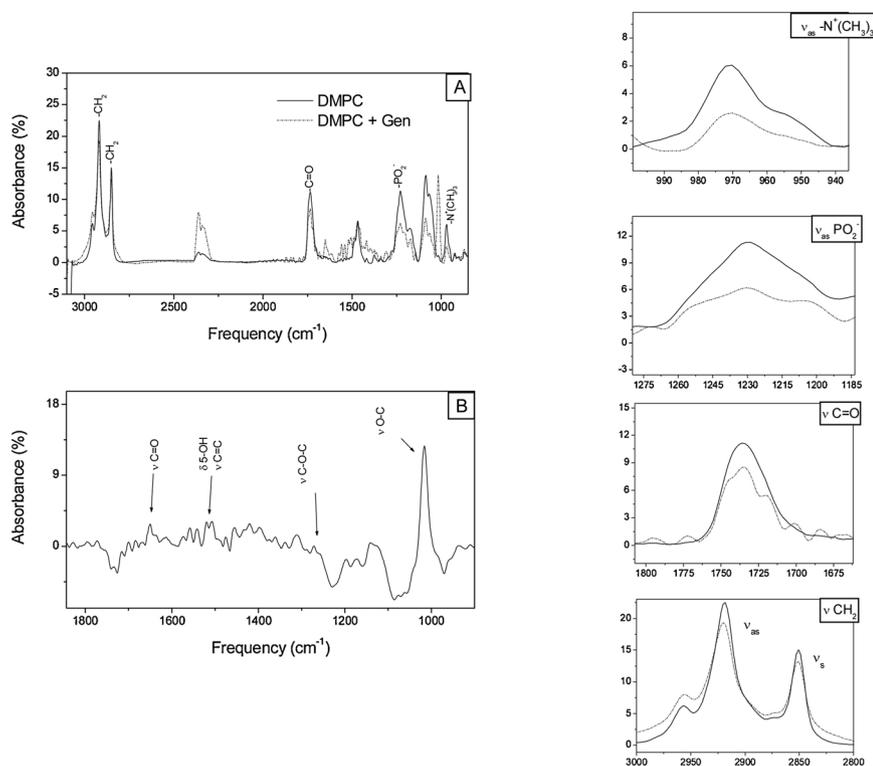
Fig. 3. (A) HATR-FTIR spectra of DMPC liposomes, empty and loaded with Gen (357.00 μM), (B) FTIR difference spectra of Gen-loaded DMPC liposomes and empty DMPC liposomes and at the right column, zooms of these spectra in the different lipid regions. Interferograms were averaged for 50 scans, recorded in the frequency range from 400 to 4000 cm^{-1}, with a resolution of 2 cm^{-1}.

intensities are related to interactions between lipid phosphate and Gen hydroxyl groups, as well as between phosphate groups and water.

The isoflavone also increased the ν_{as} PO$_2^-$ peak frequency by 4.42 cm^{-1}, ranging from 1222.35 cm^{-1} (empty liposomes) to 1226.77 cm^{-1} (Gen-loaded liposome). The ν_{as} PO$_2^-$ is sensitive to moisture, and its interaction with an exogenous molecule may reflect a change in orientation and hydration degree (Chen and Tripp, 2008; Herec et al., 2007; López-García et al., 1993; Manrique-Moreno et al., 2009, 2010). The Gen-induced increase in ν_{as} PO2- frequency indicated that the isoflavone caused a decrease in the amount of hydrogen bonds between lipid phosphate groups and water hydroxyl atoms. Therefore, Gen reduced the hydration degree of the lipid phosphate region. This behavior was also observed in soybean asolectin liposomes (Azambuja et al., 2015). Either DMPC and asolectin liposomes, containing or not Gen were analysed as aqueous suspensions. An opposite Gen-induced effect in HATR-FTIR ν_{as} PO$_2^-$ peak was observed by Cieslik-Boczula and co-workers (Cieslik-Boczula et al., 2012) in DPPC dehydrated films, in which water hydroxyl-promoted hydrogen bonds were not possible, showing direct hydrogen bonds between Gen and the lipid film. Thus, in DMPC liposomes, it seems to have a competition between Gen and water hydroxyl groups to interact with lipid phosphate region. Formation of hydrogen bonds requires an adequate alignment and distance between donors and acceptor groups. Thus, changes in the hydrogen bonds molecular quantities have direct influences on lipid behavior (Pink et al., 1998). The formation of flavonoids hydrogen bonds with lipid polar head groups is associated to changes in the lipid spatial arrangements and reduction of molecules packing (Grdadolnik and Hadzi, 1998).

To confirm this hypothesis, bandwidth analyses related to the HATR-FTIR ν_{as} PO$_2^-$ were performed. DMPC HATR-FTIR bandwidth values of the studied lipid polar groups are described in Fig. 3 and Table 2. Bandwidth changes reflect rotational, translational and/or collisional motions, as well as amplitudes and rates related to lipid groups (Casal et al., 1980). In membrane studies, the increase of a lipid group FTIR bandwidth is proportional to its motion freedom (Toyran and Severcan, 2003; Manrique-Moreno et al., 2009). Gen reduced the DMPC ν_{as} PO$_2^-$ bandwidth by 5.93 cm^{-1} (from 31.00 cm^{-1}, related to

Table 2

Influence of Gen (357.00 μM, liposome-loaded) on the DMPC HATR-FTIR bandwidth.

DMPC stretching bands (ν)	Bandwidth (cm^{-1})		Variation (cm^{-1})
	Without Gen	with Gen	
ν_{as} PO$_2^-$	31.00	25.07	−5.93
ν_{as} N$^+$(CH$_3$)$_3$	10.83	15.57	+4.74
ν C=O	22.28	20.35	+1.93

pure DMPC, to 25.07 cm^{-1}). HATR-FTIR bandwidth results showed that Gen was responsible for a restriction in the mobility of the DMPC phosphate group. Since Gen reduced the quantities of DMPC phosphate hydrogen bonds, it may be associated with an improvement of the membrane packing and restricts the motion at lipid polar region.

To reinforce this hypothesis, DMPC phosphorus phase state and possible fluidity of lipid polar head were investigated by ^{31}P NMR line width analyses. Fig. 4 shows ^{31}P NMR spectra related to DMPC liposomes alone and those loaded with Gen. ^{31}P NMR peak shape may indicate the membrane phase state (Villasmil-Sánchez et al., 2013). Also, peak line width measurements provide information about the nucleus chemical shift anisotropy (CSA, ppm), defined as the distance between the chemical shielding tensors components σ$_{II}$ and σ$\perp$ (Moreau et al., 1999). Thus, CSA is directly related to the fluidity and rotation at the polar head level. Indeed, NMR linewidth is associated to molecular mobility or conformational change (Cornell et al., 1982). A motion-restricted phosphorus group reflects in a broad ^{31}P NMR resonance, while a nucleus with a higher freedom of motion gives a narrow one (Debouzy et al., 2002). ^{31}P NMR peaks shown in Fig. 4 have a broad shoulder at the low field (corresponding to the σ$_{II}$ component) and a narrow one at the high field (corresponding to the σ$\perp$ component), which is typical of membranes in lamellar phase, organized in lipid bilayers (Villasmil-Sánchez et al., 2013; Lasic, 1998). Thus, the presence of Gen in liposomes did not change the system phase state. The ^{31}P NMR line width related to phosphorus in DMPC liposomes alone was 44.44 ppm (σ$_{II}$ correspondent to 27.30 ppm and σ$\perp$ correspondent
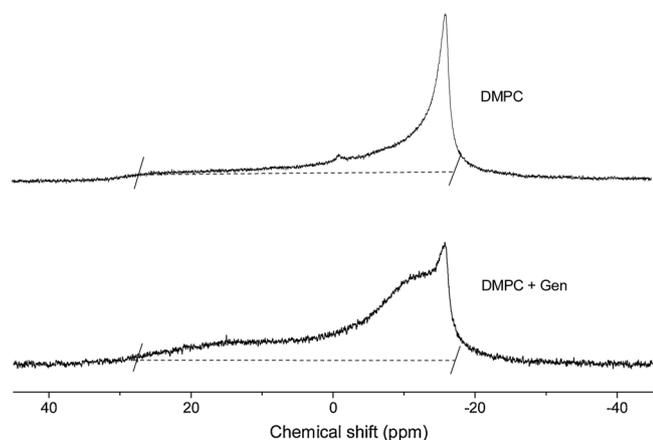
Fig. 4. ^{31}P NMR spectra of DMPC liposomes, empty and in the presence of Gen 357.00 μM (liposome-loaded). ^{31}P NMR measurements were recorded at 162 MHz.

to -17.14 ppm). The insertion of Gen (357.00 μM) into the liposomes did not change significantly the ^{31}P CSA (σ_{II} correspondent to 27.54 ppm and $\sigma_\perp$ correspondent to -17.40 ppm).

Thus, the Gen-induced change in DMPC phosphate motional freedom, seems to be related to the hydrogen bonds decrease and not to the group phase state or rotation rate.

Choline group- With respect to the liposome choline region, no HATR-FTIR frequency change was observed based on the influence of Gen. However, Table 2 shows that Gen increased the FTIR ν_{as} N$^+$(CH$_3$)$_3$ bandwidth by 4.74 cm^{-1} (from 10.83 cm^{-1}, related to empty DMPC liposomes, to 15.57 cm^{-1}, related to Gen- loaded liposomes). This indicates that Gen affected the behavior of this lipid polar region. In order to better understand Gen influence in lipid choline motion, ^{1}H NMR T_1 measurements at the choline peak (3.2 ppm) were performed. The FID signal recoveries are shown in Fig. 5, and from these curves, T_1 values were calculated.

Thus, for empty DMPC liposomes, the choline [1]H T_1 corresponded to 2.239 s. When Gen was loaded into liposomes, this value increased to 3.114 s. The [1]H T_1 difference reflects changes in lipid group motions, such as rotation (Bloom and Thewalt, 1994; Brown, 1984; Kroon et al., 1976). Indeed, for LUV, T_1 has a proportional relationship with correlation time, which is the time required to a nucleus rotate 1 rad/s (Dufourc, 2006; Ghosh, 1988). The T_1 increase of 39% indicates a restriction in the choline rotation motion.

To evaluate Gen effects in lipid polar orientation, zeta potential data were obtained. These data corresponded to -38.2 ± 0.4 mV for DMPC and -37.1 ± 0.5 mV for DMPC in the presence of Gen. Changes to more

negative and positive values of lipid potential zeta indicate a reorientation upper of the bilayer plane, related to lipid phosphate and choline group, respectively (Dimitrios and Sophia, 2002; Legrand et al., 1999). The isoflavone did not change the DMPC potential zeta values and, consequently, did not change the phosphate or choline group orientation. Thus, Gen seems to restrict the motion of DMPC phosphate and choline regions, by interaction of its hydroxyl groups. As no changes in the lipid polar head orientation were detected, it is probable that the restriction in DMPC phosphate groups is related to a Gen-promoted decrease of hydrogen bonds with water, and the reduction of choline rotation rate is induced by lipid-Gen dipolar interaction.

3.2.1.2. Gen effect in DMPC interfacial region. Fig. 1 shows no Gen-induced change in the ν C=O frequency. As in the case of ν_{as} PO$_2^-$, changes in ν C=O vibration reflect alterations in the carbonyl hydration degree (Arrondo and Goñi, 1998). Thus, Gen did not provoke changes in the quantities of DMPC carbonyl hydrogen bonds. Gen provoked no significant variation in the FTIR ν C=O band with (Table 2), which indicates no influence of the isoflavone in the DMPC interfacial region mobility (Lewis et al., 1990; Lewis and McElhaney, 2002).

3.2.1.3. Gen effect in the DMPC hydrophobic region. HATR-FTIR results related to the behavior of DMPC acyl chain methylenes after interaction with Gen are shown in Fig. 1. The isoflavone promoted a decrease of 4.51 cm^{-1} in the DMPC ν_s CH$_2$ (from 2853.51 cm^{-1} to 2849.00 cm^{-1}) and did not change ν_{as} CH$_2$ frequency values. It is known that the decrease of frequency values of CH$_2$ stretching modes is directly related to a conformational restriction in the lipid acyl chain, due to decrease of gauche bonds quantity (Chen and Tripp, 2008; Pawlikowska-Pawlega et al., 2012). Then, Gen restricted the motion of DMPC hydrophobic region, decreasing the number of gauche bonds. No Gen-induced changes in ν CH$_2$ bandwidth were observed. To obtain more information concerning to DMPC acyl chain methylenes motion, ^{1}H T_1 NMR analyses were performed. The ^{1}H FID signal recoveries related to the DMPC methylene groups, for both empty liposomes and those loaded with Gen, are shown in Fig. 6.

DMPC methylene [1]H T_1 values were then calculated from the curves described in Fig. 6 and corresponded to 1.839 s and 2.883 s for DMPC liposomes empty and loaded with Gen, respectively. This increase of 56% in DMPC [1]H T_1 values reinforces the Gen-induced motion restriction in the DMPC hydrophobic region, suggested by HATR-FTIR data. NMR results indicated that the restriction also occurs in the fast motion, such as rotation.

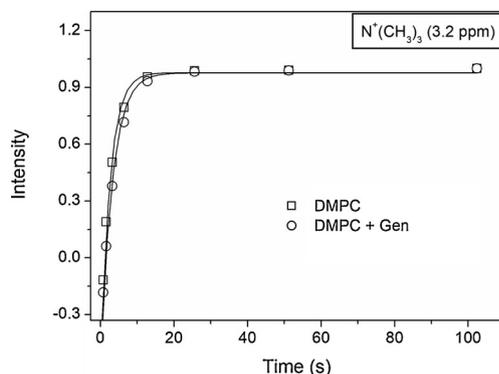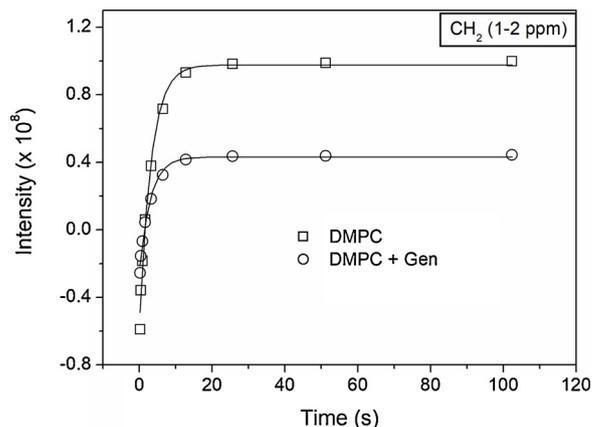Gen effects on Tm and ΔH of DMPC hydrophobic chains were also

Fig. 5. Gen (357.00 μM) effect on the recovery of DMPC liposome choline ^{1}H FID signal, after several inversion pulses (open circles curve). Empty DMPC liposomes were used as control (open squares curve). From these curves the T_1 values were calculated.

Fig. 6. Gen (357.00 μM) effect on the recovery of DMPC liposome acyl chain methylenes ^{1}H FID signal after several inversion pulses (open circles curve). DMPC pure liposomes were used as control (open squares curve). From these curves the T_1 values were calculated.
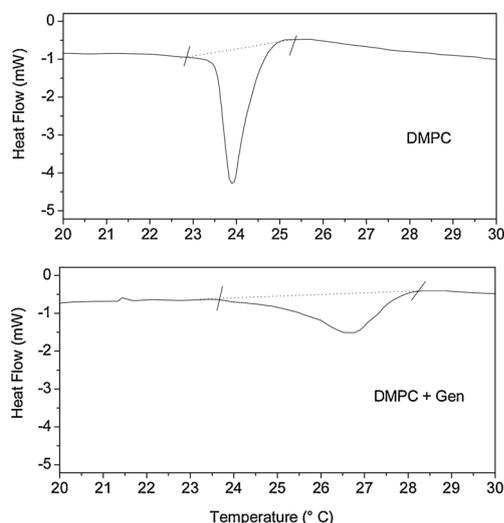
Fig. 7. DSC curves of pure DMPC liposomes and DMPC liposomes in the presence of Gen 357.00 μM (liposome-loaded). To DSC experiments, the heating rate was set to 5 °C/min in the temperature range from 5 to 60 °C, under N_2 flow (50 mL. min^{-1}).

Fig. 8. Turbidity changes for DMPC liposomes after interaction with different initial concentrations of Gen (0.0 mg/mL to 3.6 mg/mL), expressed as Gen: DMPC molar ratio (m/m). The optical density values were obtained at 400 nm by a UV–vis spectrophotometer.

evaluated by DSC. These values were calculated from the endothermic DSC curves shown in Fig. 7, related to empty liposomes and Gen-loaded ones. Gen induced an increase of 2.8 °C in the DMPC liposome Tm value (raising it from 23.9 °C to 26.7 °C). It is known that external agents can promote structural changes in lipid bilayers, leading to shifts in the phase transition temperature of the lipids. An increase of overall molecular package decreases the entropy difference between two system phases leading to a shift towards higher values in the typical Tm (Kiss et al., 2003). Thus, the observed Tm variation confirmed that Gen improves discreetly molecular package in DMPC liposomes, being in agreement with FTIR and NMR results. In previous studies concerning to DPPC membrane acyl chains, Gen promoted the opposite effect to the observed in DMPC liposomes. A Gen-induced fluidization effect (Cieslik-Boczula et al., 2012) was attributed to an enhancement of gauche conformers in DPPC hydrophobic region. It is known that the quantity and type of gauche bonds determine the membrane conformational order. As higher the carbon number in the lipid acyl chain, the quantity of kink conformers (gauche-trans-gauche sequences) increases (Casal and McElhaney, 1990). DPPC (C 16:0) differs from DMPC (14:0) by two methylenes in the hydrophobic chain, having more kink defects. In our work, the DMPC HATR-FTIR data showed a decrease of methylene gauche bonds quantity, after interaction with Gen, which enhances the van de Walls forces in this region. Thus, it is possible that the Gen-induced effects in the phosphatidylcholine molecular motion rate are strongly influenced by the quantity of lipid gauche conformers, which depends of lipid hydrophobic chain length. A molecular reorganization to a more restricted state in the lipid acyl chain is also suggested by the Gen-promoted variation in DMPC ΔH values (ΔΔH). The ΔH values of empty DMPC liposomes, as well as those containing Gen, were detected as 1.019 J/g and 1.482 J/g, respectively. The Gen-promoted increase in DMPC ΔH, was then equivalent to 0.463 J/g, which corresponds to 45.43%. This variation of ΔH showed that Gen is not inserted deep into the lipid bilayer. The discreete Gen-induced variation of Tm and ΔH in DMPC acyl chains reinforces the isoflavone preferential location in polar region and probably in the first methylenes of hydrophobic chains.

3.2.1.4. Turbidity and DLS measurements. Turbidity assays were performed in order to obtain information concerning the global influence of Gen in DMPC liposomes. The DMPC turbidity curve in the presence of several initial Gen concentrations (0.0 mg/mL to 3.60 mg/mL) is shown in Fig. 8.
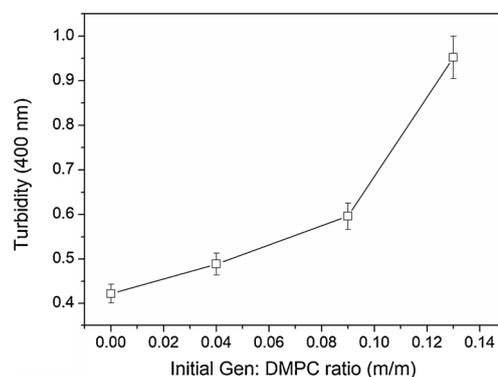
From Fig. 8, a Gen concentration-dependent increase of DMPC turbidity can be observed. At 3.6 mg/mL of Gen-added liposomes, which corresponds to a Gen-loaded liposome concentration of 357.00 μM, Gen promoted an increase of 55.67% in DMPC turbidity. Increase in membranes turbidity values may reflect two behaviors: 1) lipid system agglomeration process, which results in an increase in particle size (Elsayed and Cevc, 2011); 2) since the increase in lipid turbidity can be observed in fluid-gel phase transitions, due to alterations in the lipid refractive index (Yi and MacDonald, 1973), it may indicate a phase rearrangement to a more ordered state.

Thus, to investigate if the Gen-induced increase in the turbidity values was related to a possible liposome aggregation, DLS measurements were performed. From these assays, a diameter of 331.00 nm, related to empty DMPC liposomes, was detected. Gen encapsulation in liposomes reduced the system size to 278.50 nm. Polydispersity index values (PDI) corresponded to 0.48 and 0.45 for liposomes empty and Gen-loaded, respectively. PDI values below 0.5 are considered as having a good size distribution (Byun et al., 2011). Since Gen reduced the DMPC liposome size by 52.50 nm, it is suggested that the isoflavone-induced increase in DMPC liposome turbidity was not associated to an agglomeration process. The liposome size reduction may be then attributed to greater cohesion, intermolecular interactions and molecular package between the apolar chains, as the one induced by Gen (Sherry et al., 2013; Valenti et al., 2001). Thus, turbidity results indicate a Gen-induced ordering effect in liposomes, which is in agreement with the Gen-loaded DMPC liposome behavior observed by HATR-FTIR, NMR and DSC techniques. For unsaturated membranes, a particle size decrease is related to an increase of double-bond trans content (Giacometti et al., 2017). Thus, it is possible to extrapolate that the observed decrease in DMPC liposome size, after interaction with Gen, may be related to the decrease of gauche conformations.

In summary, dipole interactions between Gen hydroxyl groups and the phosphate and choline DMPC groups seems to be responsible to a motion restriction in this lipid region, although no changes in phase state or orientation were observed. Dipolar interactions are theorically stronger between Gen hydroxyl groups and lipid choline than phosphate, and it may affect the rotation rate restriction observed in this group, and not in the phosphate one. A Gen-induced enhancement of DMPC molecular package was detected in methylene region, probably related to a decrease in gauche bonds quantity. This increase in DMPC molecular package seems to reflect in the enhancement of lipid turbidity as well as in the reduction of liposome size. Due to the presence of gauche conformers, it is also possible that the hydrophobic chain length influences Gen effects in liposome composed by different phosphatidylcholines.
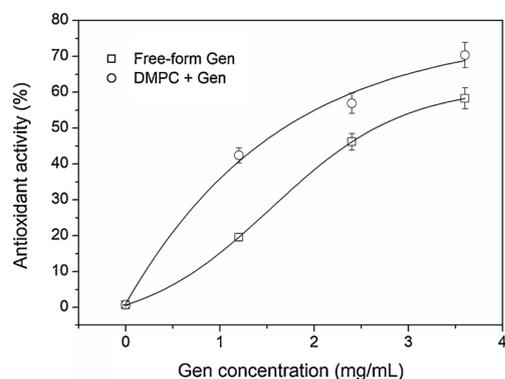
Fig. 9. Antioxidant activity (%) against DPPH radical of different initial concentrations of Gen (0.0–3.6 mg/mL), in free-form and incorporated into DMPC liposomes (DMPC + Gen).

3.3. Biological activities of DMPC liposomes-loaded Gen

3.3.1. Antioxidant activity

In vitro DMPC liposomes-loaded Gen antioxidant activity was evaluated by the DPPH method, at initial concentrations ranging from 0.00 to 3.60 mg/mL. Results were compared to the free-form Gen activity in the same reactional conditions and are shown in Fig. 9.

From Fig. 9, one can see that, at the reactional conditions, both free-form and Gen-loaded liposome showed a concentration-dependent reductant potential. However, Gen-loaded liposome was more efficient as antioxidant than the free-Gen. At the higher initial Gen concentration (added into liposome) of 3.60 mg/mL (which corresponds to 357.00 μM of liposome-loaded Gen), the isoflavone showed 70.32% antioxidant activity, while its free-form showed 58.26%. Hydroxyl substituents at positions 5, 7 and 4′ favor the Gen antioxidant activity compared to the isoflavones which do not contain the 5-OH (Tsao et al., 2003). The 12.06% higher efficiency of Gen-loaded liposome may be attributed to the isoflavone effect on the membrane, restricting the motion at polar and hydrophobic regions, leading to a reduction in the diffusion of free radicals in the system. This behavior was also proposed by Arora and co-workers (Arora et al., 2000). For other side, it is known that phosphatidylcholine may interact and induce the bleaching of DPPH radical absorption, which indicates that the lipid may scavenge this radical. The presence of the phospholipid was responsible for an increase of *Buddleja globosa's* extract DPPH scavenging, but not in an additive way (Letelier et al., 2008). This is maybe related to a hydrophobicity increase in the medium, allowing the interaction between DPPH and Gen.

3.4. Gen antitumoral activity in DMPC liposomes

Fig. 10 shows the counting of rat glioma (C6) cells in the presence of free-form Gen, DMPC liposomes empty (CD) and Gen-loaded liposome (DMPC + GEN). A control experiment was performed containing the cells and the medium without liposomes (CV). Free-form Gen at 10 μM, 20 μM and 100 μM reduced the cell numbers in 77%, 89% and 95%, respectively. Compared to the CV counting, empty DMPC liposomes reduced the number of C6 cells by 46%. The cell treatment of DMPC loaded with Gen concentrations of 10 and 20 μM did not show a significant reduction in the number of cells. However, DMPC loaded with Gen at the concentration of 100 μM reduced C6 cells by 79%. Compared to free-form Gen, Gen-loaded liposome at Gen 100 μM promoted a number of C6 cells 16% higher than the first at the same concentration. Furthermore, the effect of free-Gen 10 μM is very similar to the one caused by liposome-loaded Gen 100 μM.

Cell viability results are shown in Fig. 11. Free-Gen at 10 μM, 20 μM and 100 μM reduced the cell viability in 88%, 87% and 86%, respectively. It seems that at 10 μM, free-Gen reached a saturation effect in the cells. MTT reduction data shows that the CD groups are statistically
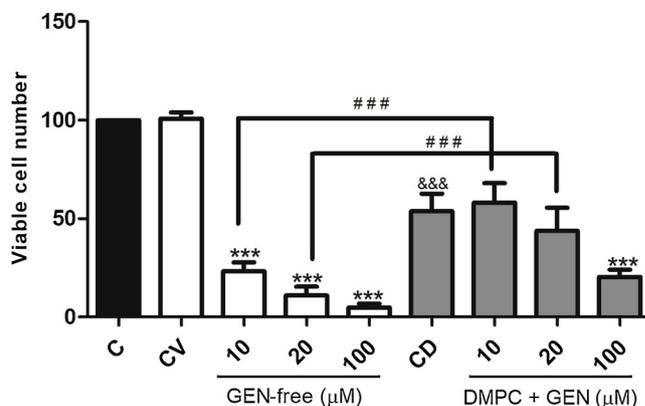

Fig. 10. Cell count in the C6 line after 48 h of treatment with free Gen and incorporated into DMPC liposomes at concentrations of 10, 20 and 100 μM. n = 6 wells, ANOVA followed by Tukey-Kramer. [***]$P < 0.001$ (difference compared to control of each treatment) [&&&] $p < 0.001$ (difference in relation to DMEM control) [###] $p < 0.001$ (difference comparing free Gen and incorporated into DMPC liposomes). C: DMEM control; CV vehicle control (DMSO); CD: DMPC control.
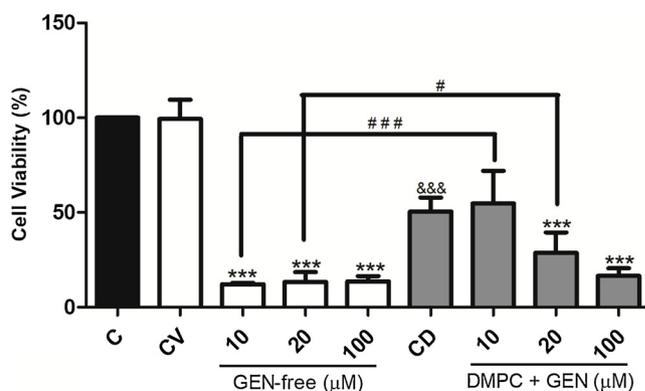

Fig. 11. Cell viability assay by MTT in the C6 line after 48 h treatment with free Gen and incorporated into DMPC liposomes at concentrations of 10, 20 and 100 μM. n = 8 wells, ANOVA followed by Tukey-Kramer. [***]$p < 0.001$ (difference in relation to the control of each treatment). [&&&] $p < 0.001$ (difference compared to DMEM control) [#] $p < 0.05$ and [###] $p < 0.001$ (difference comparing free Gen and incorporated into DMPC liposomes). C: DMEM control; CV: vehicle control (DMSO); CD: DMPC control.

different from the CV ones, confirming the action of DMPC by reducing cell viability. Empty DMPC liposomes reduced C6 viability by almost 50%. Nagami and co-workers (Nagami et al., 2006) tested, *in vitro,* the effect of pure DMPC and hybrid DMPC liposomes on the growth of tumor cell lines and observed a similar effect on the inhibitory concentration of 50% of Raji cell growth, confirming the antitumor effect of DMPC. Phosphatidylcholine-based empty liposomes may induce apoptotoc effects associated to skin tumors associated macrophage functions and phenotype, mediated by the tumor microenvironment (Konig et al., 2013). It was also previously reported that phosphatidylcholine may induces apoptosis in different cell lines such as colon and vascular endothelial cancer cells (Fukunaga et al., 2008; Cheng et al., 2006). Thus, it is possible that phosphatidylcholine affects the macrophages related to glioma cells. However, further investigations are necessaries to confirm this hypothesis.

Cell treatment with Gen-loaded liposome concentrations of 20 and 100 μM have significantly decreased C6 viability by 71% and 83%, respectively, when compared to the CV. It was not observed an additive antitumoral effect using a DMPC/Gen system. Indeed, Gen-loaded liposome at 20 μM of Gen was lower effective than the isoflavone free form by 16%. At higher concentration (100 μM) liposome-loaded Gen

had reached the effect described for its free-form. In comparison to CD, these Gen concentrations (20 and 100 μM, into liposomes) reduced cell viability by 22% and 34%, respectively. It is important to note that, in a Gen cytotoxicity study in testicular cells, the isoflavone showed a concentration-dependent dual effect. In concentration lower than 10 μg/mL (equivalent to 37.24 μM Gen), which is the case of Gen concentration used in this study, the isoflavone stimulated the cell growth. The growth and proliferation cell inhibition was only reached at Gen higher concentrations (superior to 37.24–370.24 μM) (Kumi-Diaka et al., 1999). However, astrocyte viability assays did not showed cytotoxicity for Gen at the tested concentration of 10 μM, 20 μM and 100 μM (Azambuja et al., 2015).

In general, below 100μM, cell counting and MTT data showed that free-form Gen was more effective against C6 than the Gen-loaded liposome. It is possible that the Gen-induced DMPC molecular order had reduced the isoflavone diffusion rate through the liposome. This may decrease the isoflavone *in vitro* availability to C6 cells. However, 100 μM of Gen loaded into liposomes may have compensated the slow isoflavone diffusion, reaching the Gen free form effect (this one obtained at lower Gen concentrations). Besides that, it must be considered that the free-Gen has low bioavailability in BBB, and that the incorporation of Gen into liposome membranes may enable oral administration, increasing intestinal absorbance and serum levels after ingestion and promoting the entry of the active substance in the BBB. Thus, compared to CV, Gen-loaded liposome (at 100μM) promoted reduction (around 80%) in C6 cell number and viability, suggesting that DMPC liposomes are interesting systems of controlled release of Gen to brain pathologies, including glioma treatment.

4. Conclusion

In this study, Gen-loaded liposome (357.00 mM) reduced the mobility of polar and hydrophobic liposome regions. Gen-induced restriction in DMPC molecular package may have reduced the diffusion of DPPH radical in the lipid bilayer, as well as have facilitated the interaction between the radical and Gen, enhancing the system antioxidant activity. Indeed, peroxidation processes are influenced by reactive species diffusion rates and distribution into hydrophilic and hydrophobic membrane regions (Khairutdinov et al., 2000). Thus, Gen location in DMPC polar head and at the first methylenes of hydrophobic chains, as well as its effect on the system molecular motion may reinforce or promote a lower system susceptibility to DPPH oxidation and other degradative reactions, enhancing its half-life time in the organism (Ali et al., 2013) than more disordered systems, such as asolectin-based liposomes. Also, it may prevent the oxidative stress related to the cancer process. Thus, Gen-loaded DMPC antioxidant potential may be very helpful to maintain the system integrity until the Gen delivery, in hypothetic *in vivo* tests, as well to contribute to antitumoral activity (Grigalius and Petrikaite, 2017).

Gen is a hydrophobic isoflavone, with a molecular weight of 270.24 daltons, which can cross BBB and reach glioma cells, but with low availability (Coward et al., 1993; Tsai, 2005; Yang et al., 2014). Considering the *in vitro* antiglioma assays, the efficiency of Gen, at free-form or liposome-loaded, seems not be concentration-dependent. Gen liposomal systems containing 20 μM of the isoflavone were less efficient against C6 than its free form. Gen superficial location in the DMPC membrane and the isoflavone-promoted reinforcement of liposomes van der Walls forces may have retarded the availability of the isoflavone content of Gen-loaded liposomes to *in vitro* C6 cells. Also, it is possible that the dipolar interaction between Gen hydroxyl groups and DMPC choline and phosphate groups have reduced the antitumoral role of the isoflavone C-5 and C-7 hydroxyl groups (for more information, see Xiong et al., 2015). It may justify the fact that, although the DMPC empty liposomes showed significant antitumor effect, the Gen insertion on them did not promoted a additive activity against C6 cells.

Compairing the liposome type and composition roles in the Gen

efficacy against C6 viability, at 100 μM, Gen-loaded DMPC LUV were 45% more efficient than soybean asolectin MLV containing the isoflavone (Azambuja et al., 2015). As a saturated phospholipid, DMPC bonds show typically more trans conformation than soybean asolectin ones. Furthermore, the restriction in DMPC molecular motion, decrease of hydration degree and gauche bonds number, promoted by Gen, favor the increase of trans bond conformation in the system. Studies focusing the influence of cis-trans double bond conversion in molecular properties of drug delivery liposomes have reported the importance of the number of trans bond configuration in size, stability, and capacity of encapsulation and release of antitumoral systems, improving their activity. Indeed, the synergic strategy between the increase of trans bond conformation (or the decrease of trans-gauche isomerization) in a phospholipid-based system and the active substance potential is considered as an innovative antitumoral strategy (Giacometti et al., 2017).

Also, it is important to note that the DMPC LUV are smallest in size than asolectin MLVs, and Gen promoted the size reduction of DMPC liposomes in approximately 50 nm. Tumour capillaries have pore diameters varyng from 100 to 700 nm. Gen reduced the DMPC liposome size to approximately 280 nm. Smaller liposomes sizes enhance the permeability and retention effect (EPR), favoring the passive targeting, and allow their accumulation in tumours (Yuan et al., 1995; Giacometti et al., 2017). These factors may be resposible to improve the antitumoral effect of liposomal Gen systems containing DMPC, compared to soybean asolectin composition.

Thus, at isoflavone concentration equals or superior than 100 μM, liposome-loaded Gen seems to be as efficient as Gen free-form to reduce C6 cells viability, and may be a more stable system to cross the BBB, being promising as an effective drug delivery system in antitumor therapy for the treatment of glioma.

Transparency document

The Transparency document associated with this article can be found in the online version.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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